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Biochimica et Biophysica Acta 1587 (2002) 194–205

BIOCHIMICA ET BIOPHYSICA ACTA BBA

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Review

Induction of thymidylate synthase as a 5-fluorouracil resistance mechanism

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Received 24 January 2002; accepted 24 January 2002

Abstract

Thymidylate synthase (TS) is a key enzyme in the de novo synthesis of 2′-deoxythymidine-5′-monophosphate (dTMP) from 2′-deoxyuridine-5′-monophosphate (dUMP), for which 5,10-methylene-tetrahydrofolate (CH_2-THF) is the methyl donor. TS is an important target for chemotherapy; it is inhibited by folate and nucleotide analogs, such as by 5-fluoro-dUMP (FdUMP), the active metabolite of 5-fluorouracil (5FU). FdUMP forms a relatively stable ternary complex with TS and CH_2THF, which is further stabilized by leucovorin (LV). 5FU treatment can induce TS expression, which might bypass dTMP depletion. An improved efficacy of 5FU might be achieved by increasing and prolonging TS inhibition, a prevention of dissociation of the ternary complex, and prevention of TS induction. In a panel of 17 colon cancer cells, including several variants with acquired resistance to 5FU, sensitivity was related to TS levels, but exclusion of the resistant variants abolished this relation. For antifolates, polyglutamylation was more important than the intrinsic TS level. Cells with low p53 levels were more sensitive to 5FU and the antifolate raltitrexed (RTX) than cells with high, mutated p53. Free TS protein down-regulates its own translation, but its transcription is regulated by E2F, a cell cycle checkpoint regulator. Together, this results in low TS levels in stationary phase cells. Although cells with a low TS might theoretically be more sensitive to 5FU, the low proliferation rate prevents induction of DNA damage and 5FU toxicity. TS levels were not related to polymorphisms of the TS promoter. Treatment with 5FU or RTX rapidly induced TS levels two- to five-fold. In animal models, 5FU treatment resulted in TS inhibition followed by a two- to three-fold TS induction. Both LV and a high dose of 5FU not only enhanced TS inhibition, but also prevented TS induction and increased the antitumor effect. In patients, TS levels as determined by enzyme activity assays, immunohistochemistry and mRNA expression, were related to a response to 5FU. 5FU treatment initially decreased TS levels, but this was followed by an induction, as seen with an increased ratio of TS protein over TS-mRNA. The clear retrospective relation between TS levels and response now forms the basis for a prospective study, in which TS levels are measured before treatment in order to determine the treatment protocol. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Thymidylate synthase; p53; E2F; 5-fluorouracil; Antifolate

Abbreviations: 5FU, 5-fluorouracil; FUdR, 5-fluoro-2′-deoxyuridine; TS, thymidylate synthase; TSER, TS enhancer regions; FPGS, folylpolyglutamate synthetase; dUMP, 2′-deoxyuridine-5′-monophosphate; FdUMP, 5-fluoro-dUMP; dTMP, 2′-deoxythymidine-5′-monophosphate; CH_2-H_4-folate, N^5,N^{10}-methylene-5,6,7,8-tetrahydrofolate; LV, leucovorin; RTX, raltitrexed, *N*-[5-(*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-yl-methyl)-amino)-2-thenyl)]-L-glutamic acid (Tomudex, ZD1694); ALIMTA, multitargeted antifolate, *N*-(4-(2-(2-amino-4,7-dihydro-4-oxo-3*H*-pyrrolo[2,3-D]pyrimidin-5-yl)-ethyl)-benzoyl]-L-glutamic acid (LY231514); GW1843U89, (*S*)-2-(5-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)-methyl)-amino)-1-oxo-2-isoindolinyl)-glutaric acid; AG337, nolatrexed, 3,4-dihydro-2-amino-6-methyl-4-oxo-5-(4-pyridylthio)-quinazoline (Thymitaq); SRB, sulforhodamine B; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; CDK, cyclin-dependent kinase; DPD, dihydropyrimidine dehydrogenase; wt-p53, wild type p53; mt-p53, mutant p53

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PII: S0925-4439(02)00082-0

1. Introduction

Thymidylate synthase (TS) is a key enzyme in the de novo synthesis of 2′-deoxythymidine-5′-monophosphate (dTMP). TS catalyzes the methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) to dTMP for which 5,10-methylene-tetrahydrofolate (CH_2-THF) is the methyl donor. Detailed characteristics of the TS enzyme have been described by others [1,2]. TS is an important target for chemotherapy. It is inhibited by analogs of the folate cofactor, such as raltitrexed (RTX) and ALIMTA, and the nucleotide co-factor, such as 5-fluoro-dUMP (FdUMP), which is the active metabolite of 5-fluorouracil (5FU). FdUMP is a competitive inhibitor with a K_i in the low nanomolar range. Inhibition of TS by FdUMP is considered to be the main mechanism for the action of 5FU (Fig. 1). The inhibition by FdUMP is mediated by the formation of a covalent ternary complex between FdUMP, TS and CH_2-THF, while the retention of inhibition is also dependent on the ratio between free dUMP and FdUMP levels [3,4]. A low sensitivity to 5FU has been related to a rapid disappearance of FdUMP. A high dUMP concentration or a limited FdUMP binding to TS may reduce retention of TS inhibition.

Several mechanisms of resistance to 5FU have been attributed to TS (Table 1) [5]. The stability of the ternary complex is highly dependent on the availability of CH_2-THF or one of its polyglutamates [4]. LV can increase the availability of CH_2-THF (Fig. 1). After transfer across the membrane, mediated by the reduced folate carrier [6], LV will be metabolized to CH_2-THF [7], which will be polyglutamylated leading to enhanced inhibition of TS [8]. A decreased activity of folylpolyglutamate synthetase (FPGS) [9] and altered binding of FdUMP to TS [10–12] have been associated with acquired 5FU resistance. In the absence of CH_2-THF or one of its polyglutamates [5,12–14], FdUMP forms an unstable binary complex, which results in poor inhibition. Disturbed folate pools [14] (usually decreased) and a high level of enzyme before treatment lead to intrinsic resistance [5,11,12]. Gene amplification of TS and mutations in the gene lead to acquired resistance [12,13,15]. This paper focuses on the role of intrinsic TS levels and that of TS induction in 5FU resistance and on their regulation in various systems.

Table 1
Mechanisms of resistance to 5FU

(A) Decreased accumulation of activated metabolites
(a) Decreased activation
(b) Increased inactivation
(c) Increased inactivation of 5FU nucleotides
(B) Target-associated resistance
(a) Decreased RNA effect
(b) Altered effect on thymidylate synthase
• Aberrant enzyme kinetics
• Increased dUMP levels
• Decreased FdUMP accumulation
• Decreased stability of ternary complex
• Depletion of intracellular folates
• Decreased polyglutamylation of folates
• Recovery and enhanced enzyme synthesis
• Gene amplification
• Enzyme induction
(C) Pharmacokinetic resistance
(a) The drug does not reach the tumor
(b) Disease state affects drug distribution
(c) Increased elimination

Modified from Ref. [5].

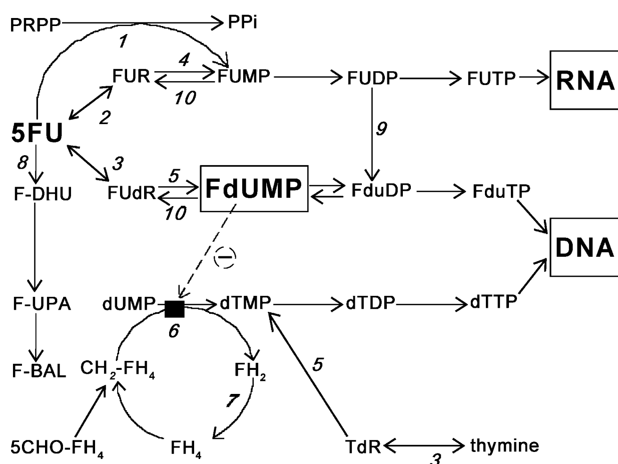

Fig. 1. Metabolic conversions of 5FU. The enzymes involved in these reactions are: (1) orotate phosphoribosyltransferase (OPRT); (2) uridine phosphorylase (UP); (3) thymidine phosphorylase (TP); (4) uridine kinase (UK); (5) thymidine kinase 1 (TK1); (6) thymidylate synthase (TS); (7) dihydrofolate reductase; (8) dihydropyrimidine dehydrogenase (DPD); (9) ribonucleotide reductase; (10) 5′-nucleotidases and phosphatases. Metabolic pathways are depicted as solid lines. The inhibition of TS by FdUMP is depicted as a broken line with a minus sign.

2. Experimental procedures

2.1. Cell lines and tissues

The sources of the cell lines have been described previously [16,17]. Sensitivity to 5FU and antifolates was determined in 96-wells plates using either the sulforhodamine B (SRB) or 3-[4,5-yl]-2,5-diphenyl tetrazolium bromide (MTT) assays as described previously [16,18,19]. In some cell lines, antifolates cause swelling of the cells leading to an increased protein content, which interferes with the protein-based SRB assay. In case this swelling was observed, we used the MTT assay for antifolates. 5FU did not cause swelling. For all cell lines, we added drugs 24 h after plating of the cells. Cells were exposed for 72 h and linearity of growth was assured for untreated cells during this 72-h period.

For enzyme assays, immunoblotting, RNA and DNA extraction, all cell lines were cultured in 75-cm^2 flasks and harvested in the logarithmic growth phase by trypsinization, then washed and counted. Thereafter, the cell pellets were frozen in liquid nitrogen.

Tumors from animals were removed before and at different time points after drug treatment and immediately frozen

in liquid nitrogen. Tumors (primary tumors and liver metastases) from patients were removed during surgery (after administration of 5FU) and immediately frozen in liquid nitrogen as described previously [20]. Frozen tissues were pulverized [21] and the still frozen powder was further processed in appropriate buffer either for enzyme assays or RNA/DNA extraction.

2.2. TS enzyme assays

TS assays in cell lines were performed as described previously [16]. We used two assays. One assay measured the number of free FdUMP binding sites of TS by estimation of the binding capacity of ^{3}H-FdUMP. The other determined the catalytic activity of TS by means of [^{3}H]-H_2O-release during the TS catalyzed conversion of ^{3}H-dUMP into dTMP. Measurement of TS levels in tissues was slightly different from that in cell lines, which has been previously described [20,22,23]. In tissues from 5FU-treated patients and animals, we measured TS levels in the extracts containing the non-dissociated ternary complex, which enabled TS inhibition to be expressed as the residual TS catalytic activity (TS-residual), and as the number of free binding sites for FdUMP (TS-free). We also dissociated the ternary complex, which enabled the measurement of total catalytic activity (TS-total) and all available FdUMP binding sites (TS-tot). The ratios between TS-residual/TS-total and TS-free/TS-tot (× 100%) was considered as the percentage inhibition.

2.3. TS immunoblotting

Protein expression was estimated using Western blotting as described earlier [24,25]. Proteins were separated by size through a 10% SDS-polyacrylamide gel and were electrophoretically transferred to a nitrocellulose membrane, on which they were detected using horseradish peroxidase (HRP)-conjugated secondary antibodies directed against primary antibodies. The HRP reaction was visualized by means of chemiluminescence and autoradiography.

2.4. TS immunohistochemistry

Sources of primary and secondary antibodies, staining procedures and evaluation of staining have been described previously [26,27]. A pathologist and two investigators investigated all slides. Statistical evaluation was performed using the nonparametric Kruskal–Wallis and Mann–Whitney *U* ranking test. All values were based on two-tailed statistical analysis. Survival was evaluated using Kaplan–Meier curves. All statistical procedures were carried out with SPSS 9.0 (SPSS, Chicago, IL, USA).

2.5. TS-mRNA expression

Total RNA was isolated from pulverized tissues using the RNAzole method and purity was checked by UV absorbance and electrophoresis of 1 μg RNA on a neutral 1.2% agarose gel. cDNA was synthesized from 5 μg of total RNA with 6 μg of random primers using M-MLV reverse transcriptase as described previously [28]. PCR amplifications were carried out in a Perkin/Elmer/Cetus DNA thermal cycler. A total of 25 cycles was used, in which each cycle consisted of 1 min primer denaturation at 94 °C, 1 min of primer annealing at 55 °C, and 1 min of primer extension at 72 °C. The cDNA samples were diluted depending on transcript abundance, while three cDNA dilutions were used for each primer in order to verify that measurements were taken in the linear phase of the reaction. A detailed description of the procedure as well as the sequences of the primers for the target gene TS and the reference genes β-actin and 18S are described previously [28].

2.6. TS enhancer region (TSER)

TSER was amplified [29,30] from genomic DNA with the sense primer (5′-GTGGCTCCTGCGTTTCCCCC-3′ and the antisense primer: 5′-GCTCCGAGCCGGCCACAGGCATGGCGCGG-3′). PCR was carried out in 1 × PCR buffer (50 mM KCl, 10 mM Tris pH 9, 0.1% Triton X-100), containing 1.25 mM magnesium chloride, 10% DMSO, 0.2 mM dNTPs, 0.2 ng each primer, 50–100 ng genomic DNA and 2.5 units of Taq in a 50 μl reaction. Samples were overlaid with mineral oil and amplified in a thermal cycler for 30 cycles. Each cycle was 1 min at 94 °C, 1 min at 60 °C and 2 min at 72 °C, and the final cycle was maintained at 72 °C for 5 min. PCR products were separated by electrophoresis on a 4% agarose gel and visualized with ethidium bromide.

3. TS and p53 levels in relation to sensitivity to 5FU and antifolates

3.1. Physiological regulation of TS levels

Expression of TS under physiological conditions is related to the cell cycle, and has a high activity during the S-phase [31], but decreases when the cells do not proliferate [32]. Various transcription factors and cell cycle-dependent kinases (CDK) control the increase in TS levels in the cell cycle. Cell cycle progress through the G1/S checkpoint is tightly regulated by cyclin/CDK complexes, which are activated by phosphorylation. Phosphorylation of various cyclins-CDKs can subsequently hyperphosphorylate the Rb and E2F complex, which results in the release of E2F from phosphorylated Rb. The free transcription factor E2F can subsequently activate the transcription of several DNA synthesis-dependent proteins such as TS.

TS levels are also controlled at the level of translation. The translation of the TS-mRNA appears to be controlled by its end product, the TS protein, in an autoregulatory manner. This phenomenon possibly contributes to the large variation

of TS levels not only in normal tissues, but also between tumors and within tumors. TS protein cannot only regulate its own translation but also that of other proteins, such as p53 [33]. In addition, wild-type p53 (wt-p53) protein can also inhibit TS promoter activity [34]. Thus, regulation of TS expression is a very complicated process, which may even be more disrupted (more induction) in cells with mutated p53 (mt-p53) than with wt-p53 (low induction). Although many nucleotide synthesizing enzymes are increased in tumors compared to their normal counterparts [35], the increase of TS levels in, e.g. colon tumors compared to normal colon mucosa, varies from negligible to many fold higher [36,37], and is even higher in metastases compared to primary colon tumors [38,39]. This increase was associated with an increase in p53 and cell cycle genes. Consequently, in colon cancer cell lines and tumors, a large variation in TS levels has been observed [4,12,16–18,24,25,39–41].

3.2. Intrinsic and amplified TS levels in relation to sensitivity to 5FU and antifolates

Since TS is the target for 5FU, it was hypothesized that the large variation in TS levels would be related to 5FU sensitivity. Indeed, in a small panel of cell lines consisting of several 5FU-resistant cells and some nonselected cells, Johnston et al. [17] found a relation between TS levels and 5FU sensitivity, but omission of the resistant lines weakened this relation considerably. In a limited number of cell lines with a different histological origin, Beck et al. [40] found a weak correlation ($r^2 = 0.22$, $P = 0.042$) between 5FU sensitivity and TS levels, but in the subpanel of colon cancer cells this relation was not present.

Recently, we reported TS levels in a panel of 13 unselected colon cancer cell lines, not including cells with induced resistance. The TS catalytic activity varied from 62 to 777 pmol/h/10^6 cells, while the number of FdUMP binding sites varied from 203 to 2758 fmol/mg protein [16]. This panel has now been extended with several cell lines with intrinsic TS levels in the same range and with cell lines with induced resistance to 5FU described by Johnston et al. [17]. In this panel, levels of the TS catalytic activity were in a range up to 51,000 pmol/h/10^6 cells, and the number of FdUMP binding sites in a range up to 6800 fmol/mg protein (Fig. 2). In this extended panel including the resistant cells, the number of FdUMP binding sites correlated positively with the sensitivity to 5FU ($r = 0.864$; $P < 0.0001$). However, when the resistant cell lines were excluded (FdUMP binding sites varying from 203–2758 fmol/mg protein; 15 cell lines), no relation between TS levels and 5FU sensitivity could be found. As shown in our previous results, sensitivity to the antifolates RTX, ALIMTA, AG337 and GW1843 was not related to the TS levels [16]. However, the activity of FPGS was still positively correlated to sensitivity to both RTX and ALIMTA. TS catalytic activity was significantly correlated to both

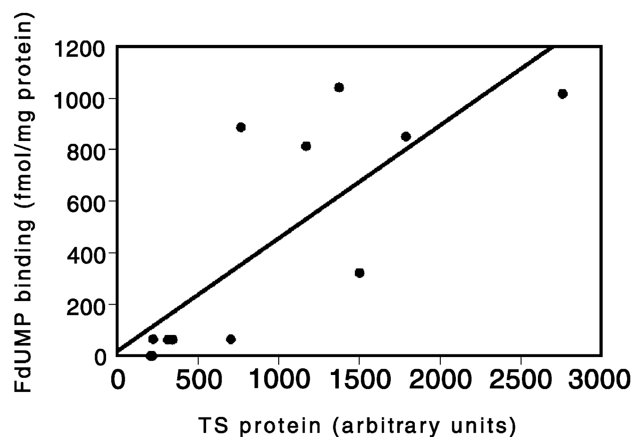

Fig. 2. Relationship between FdUMP binding (fmol/mg protein) and relative TS protein content as determined by Western blotting (Pearson correlation 0.786; $P = 0.002$). TS protein was quantified using purified TS as a standard. Updated from Ref. [16]. FdUMP binding was also correlated with TS catalytic activity (expressed as pmol/h/10^6 cells) measured at saturating substrate concentrations ($r = 0.810$, $P = 0.0001$) and at half-maximal substrate concentration ($r = 0.732$, $P = 0.003$). TS catalytic activity correlated with TS-mRNA expression ($r = 0.733$, $P = 0.04$).

FdUMP binding (Fig. 2) and TS protein measured by Western blotting as described previously [16,17]. TS levels were also related to TS-mRNA expression. Recently, Grem et al. [41] also observed a lack of correlation between 5FU sensitivity and TS levels in the National Cancer Institute 60-cell line panel, either evaluated as TS protein or TS-mRNA expression. This lack of correlation might have several reasons, such as the relatively short drug exposure time (48 h), after which growth inhibition was determined. This continuous presence of 5FU possibly leads to a complete inhibition of TS independently of the endogenous TS levels as was observed in cells with induced TS [32]. Thus, growth inhibition might be dependent on additional parameters, such as the extent of induction of DNA damage [42]. Alternatively, sensitivity to 5FU might also be dependent on other parameters in addition to TS levels, such as toxicity mediated by incorporation of 5FU into RNA. Recently, Scherf et al. [43] investigated a large panel of mechanistically different drugs using cluster analysis after running microarrays. 5FU clustered with drugs with an RNA-directed effect, rather than with other TS inhibitors.

It seems that very high amplified TS levels as found in cells with acquired resistance to 5FU are only related to 5FU sensitivity. In other cells, the mechanism of 5FU might not be TS directed (e.g. RNA-mediated). Alternatively, the continuous exposure to 5FU (varying from 48 to 96 h) may produce sufficient FdUMP to inhibit TS intracellularly to undetectable levels even in cells with a 10-fold difference in TS levels. In an in situ TS assay, 5FU at IC_{50} levels inhibited intracellular TS more than 50% within only 4 h in cells with varying levels of TS [18]. With RTX, we observed using in situ TS inhibition that a partial inhibition after 4 h exposure increased to a complete inhibition after 24 h exposure [32]. Therefore, it seems that the usual in

vitro cell culture conditions (continuous exposure) are not representative for evaluation of a relation between TS levels and 5FU sensitivity, and certainly not for an evaluation of antifolate sensitivity [43]. The latter is due to the fact that intracellular folate homeostasis will rescue cells from antifolate toxicity [44,45], especially when FPGS levels are high, since this would enable trapping of normal folates as polyglutamates in the cell.

3.3. Sensitivity to 5FU and RTX in relation to p53 expression

Besides the well-known and reasonably well-characterized resistance mechanisms of 5FU (Table 1), recently more evidence for other potential mechanisms has been provided. Ample evidence is now available that cytotoxic drug treatment of cells will result in an induction of p53 in cells with wt-p53 (reviewed in Ref. [42]). This will lead to an increase of p21, which can inhibit cell cycle progress by inhibition of CDK2, resulting in a cell cycle arrest and enabling the cells to repair drug-induced DNA damage. Alternatively, p53 induction can also transactivate bax levels, which promotes cell death. However, in cells with mt-p53, the expression of bax also increased [24,25], and cells died, but without the appearance of apoptotic features. In contrast, cells became necrotic. The difference in p53 status indeed led to a significantly different sensitivity for 5FU and RTX sensitivity in wt-p53 and mt-p53 cells (Fig. 3). For 5FU, these results are comparable to the increased 5FU sensitivity in wt-p53 cells from the National Cancer Institute 60-cell line panel compared to the cells with a mt-p53 expression [41,46]. This might be related to higher susceptibility of wt-p53 cells to enter apoptosis.

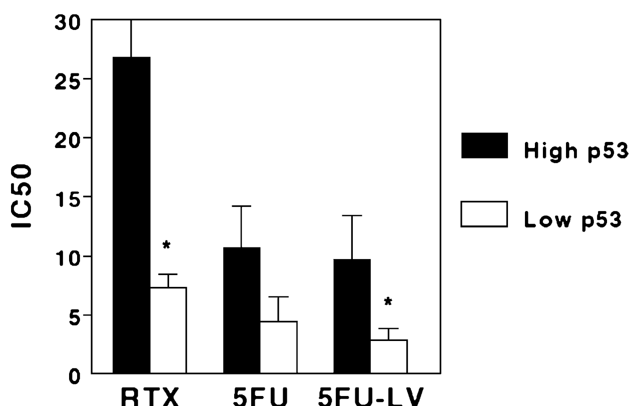

Fig. 3. Relation between p53 expression and sensitivity to 5FU and RTX. Sensitivity was determined by means of a 72-h exposure to the drugs; values are means ± S.E. of IC_{50} values (nM for RTX, μM for 5FU and 5FU-LV; LV was added at 10 μM). IC_{50} values for RTX and LV-5FU were significantly lower (★) in cells with a wt-p53 (4 cell lines with a low p53; $P=0.042$ and 0.019, respectively) than in cells with a mt-p53 phenotype (10 cell lines with high p53). For 5FU, this difference tended to be significant ($P=0.060$). From Refs. [16,24].

Table 2
Expression of TSER and p53 in colon cancer cells

Cell line	p53		TSER
SW620	mut	7.29	2/2
SNU-C4	wild	0.27	2/2
SW1116	mut	8.31	2/2
WiDr	mut	7.86	2/2
WiDr/F	mut	5.06	2/2
Colo320	mut	6.99	2/2
SNU-C1	wild	0.37	3/3
Lovo	wild	0.09	2/2
LS174T	wild	0.11	2/3
SW948	mut	0.08	2/2
HT29	mut	6.07	2/2
SW1398	mut	7.24	2/2
Colo205	mut	7.31	2/2
Colo201	mut	5.3	2/3
H630	mut	n.d.	3/3

Blots were scanned for p53; actin was used to control blots for loading of protein. The p53 status was based on mutation analysis reported; p53 expression were normalized on a reference amount of p53 protein. TSER 2/2 means that the cells were homogenous for the double repeat, 2/3 that they were heterogeneous for the double and triple repeat, and 3/3 that they were homogenous for the triple repeat. n.d., not done.

3.4. TS levels in relation to TS gene promoter polymorphisms

Evidence has been provided by Marsh et al. [29], that polymorphism of TS gene promoter regions may be related to TS-mRNA and protein expression. Tandem repeat sequences near the initiation start site in the 5′-untranslated region (5′ UTR) of the human TS promoter act as a *cis*-acting enhancer element in the TS gene and have been shown to influence TS expression. These TSERs are polymorphic and contain either two or three 28-bp tandem repeats. In vitro the triple tandem repeat (TSER*3) increased the TS expression compared to the double tandem repeat (TSER*2). Therefore, we also analyzed the tandem repeats in our panel of cell lines (Table 2). Only two cell lines (H630 and SNU-C1) showed a homogenous TSER*3, and two other cell lines a heterogeneous double/triple repeat. Interestingly, SNU-C1 and SNU-C4, which are cell lines derived from the same patients, showed a different TSER genotype. When the TS levels (activity, protein, mRNA) in these lines (TSER*3 and TSER*2/3) were compared with the other cell lines (all TSER*2), no significant differences were found. In addition, the 5FU resistant variants H630-R1 and H630-R10 also showed a homogenous TSER*3, although their TS levels were markedly increased compared to H630 cells. Apparently, in this cell line panel, the tandem repeats do not influence TS levels, or the number of cell lines was too small to draw such conclusions.

Also in patients, the occurrence of tandem repeats has been investigated. In DNA from 121 patients with colorectal cancer, 29% of the patients were homogenous for TSER*3, 16% were homogenous for TSER*2 and 55% were heterogeneous [29]. In 44/45 paired samples, the TSER was

similar in colon tumors and normal mucosa. Patients homogenous for TSER*2 had a longer survival than those with a homogenous TSER*3. Also, Villafranca et al. [47] observed that patients with homogenous TSER*2 and heterogeneous TSER*2/3 tended to have a longer survival than patients with a homogenous TSER*3 (81% vs. 41%, $P = 0.17$). Iacopetta et al. [48] observed that from 221 patients with Dukes' C colon cancer, 26% were homogenous for TSER*3 and did not benefit from 5FU-based chemotherapy. However, those patients with a heterogeneous TSER*2/3 or homogenous TSER*2 genotype showed significant gain in survival ($P = 0.005$) from treatment. The value of the genomic polymorphisms in the TS gene promoter region should be investigated prospectively.

4. TS induction in cell lines

The regulation of TS levels in the cell cycle can be disrupted by various TS inhibitors [49]. When FdUMP is bound to TS in the ternary complex, the protein can no longer autoregulate its own synthesis, leading to a derepression and increase in TS protein. Thus, inhibition of TS in vitro either by the formation of the ternary complex between FdUMP, the enzyme and 5,10-CH_2-THF [50,51], or by specific TS inhibitors such as RTX [49], disrupt the regulation of enzyme synthesis. This is shown as an increase in TS protein expression, which is not accompanied by an increase in TS-mRNA. The increase in TS protein, however, may also be due to stabilization of the protein due to decreased degradation of the ternary complex [52]. The 5FU induced increase could be prevented by interferon-γ [53]. Increase in TS levels in cell lines after exposure to 5FU or antifolate TS inhibitors seems to be a universal finding, but it is not clear from the in vitro studies whether this increase is related to sensitivity to 5FU or antifolate TS inhibitors. The increase is usually between two- and five-fold, but is concentration and time-dependent [24,25,54]. Exposure to 5FU leads to an initial decrease in free TS and an increase in the ternary complex (Fig. 4); however, the total amount of TS protein exceeded that of untreated cells [24,25,55]. In cells treated with RTX and other antifolate TS inhibitors, free TS protein accumulated in time [24,25,32,54]. In a panel of six colon cancer cell lines, the extent of TS protein induction was not related to sensitivity to 5FU or RTX [24]. Actually, in WiDr cells, induction of TS protein by RTX was not sufficient to prevent complete inhibition of TS in the in situ TS inhibition assay [32]. Apparently, the continuous presence of the inhibitor will lead to TS protein induction, but will also be sufficient to allow complete inhibition of TS catalytic activity. As mentioned above, this possibly also explains the lack of correlation between TS protein levels and 5FU and antifolate sensitivity.

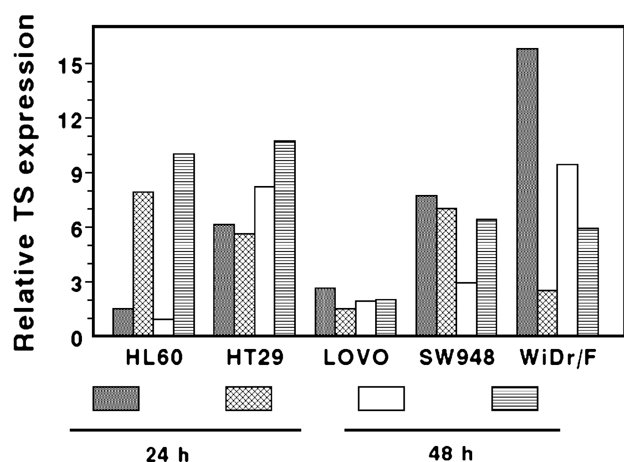

Fig. 4. Effect of 24- and 48-h treatment with 50 μM 5FU on TS levels in the colon cancer cell lines, HT29, SW948, WiDr/F (all mt-p53) and LoVo (wt-p53) and the p53 null myeloid leukemia cell line HL60. Data were expressed as the ratio of $(OD \times mm^2)_{treated}/(OD \times mm^2)_{control}$. Specific protein levels in drug-treated cells were expressed relatively to the specific protein levels in the untreated control cells (at similar total protein loads; set at 1). Data are from one representative experiment as described previously [24]. ▩, □ free TS (36-kDa band) after 24 and 48 h, respectively; ▨, ▤ ternary complex (38-kDa band) after 24 and 48 h, respectively.

5. TS levels and induction in solid experimental tumors

Evidence for a lack of relation (or at most a poor relation) between TS levels and in vitro sensitivity patterns for 5FU and antifolates is accumulating. However, this does not mean that such a relationship would not exist in vivo. Spears et al. [56] have already observed that experimental colon tumors with a high TS activity were less sensitive to 5FU. Although the reported panels are not as large as for the in vitro studies, all together there seems to be a reasonably good relationship between TS levels and 5FU sensitivity, despite the fact that in vivo pharmacokinetics determine 5FU concentrations. Actually, the similarity of 5FU plasma pharmacokinetics between humans and mice might be in favor for extrapolation of murine data to humans [21]. Also, in our panel of tumors, we found that 5FU-sensitive tumors had a lower TS activity than 5FU-resistant tumors [22,23,57]. Treatment with a therapeutic dose of 5FU resulted in a more pronounced inhibition in the sensitive tumors compared to the resistant tumors. In addition, 5FU treatment resulted in a two- to three-fold induction of TS in the resistant tumors, which already had a high activity [22,23,57]. This TS induction was already found 7 days after treatment with 5FU at 100 mg/kg and was more pronounced after three treatment cycles (Fig. 5). However, in the sensitive tumors, this induction was not observed, and TS levels were further reduced following the second and third injection. In the resistant tumors, the induction could also be prevented by repeated administration, but only by the use of a higher dose of 5FU (150 mg/kg) (Fig. 6). The latter is only possible when toxicity of

5FU to mice is selectively protected by uridine (or a uridine prodrug) administration [58]. Uridine administration enabled a 1.5-fold increase in the 5FU dose, which was also associated with an increased antitumor activity. Also, treatment with 5-fluorodeoxyuridine (FUdR) resulted in a two-fold induction of TS; however, for FUdR the onset of TS induction was later (10 days) than for 5FU (7 days). FUdR, when given as a bolus, was a more effective treatment than 5FU and is thought to be entirely TS directed [57].

The most relevant clinically important finding was the observation that the enhanced antitumor activity of the 5FU/LV combination compared to 5FU alone, was associated with the prevention of TS induction by LV (Fig. 5). The combination of 5FU with LV and uridine provided a double modulation: LV increased the antitumor effect of 5FU, while uridine enabled to use a higher 5FU dose by protection of gastrointestinal and myeloid toxicity. Uridine selectively protects normal tissues since the toxicity is mediated by incorporation into RNA [59,60], which is protected by uridine. Uridine also protected 5FU-induced apoptosis in the gut [61]. The double modulation resulted in the best therapeutic efficacy compared to 5FU alone, since both LV and an increased 5FU dose apparently provide an enhanced and prolonged TS inhibition in tumors.

TS induction was not only observed in tumors, but also in normal tissues. Local administration either by hepatic artery infusion or isolated liver perfusion of 5FU are more effective treatments than systemic administration, but may result in local hepatotoxicty. Local administration of 5FU not only exposes the liver metastases but also the normal liver tissue to the high concentrations of 5FU. Local administration of 5FU resulted in a pronounced inhibition of TS in liver metastases (> 70%) [62]. In contrast, local 5FU administration resulted in a six- to seven-fold TS induction

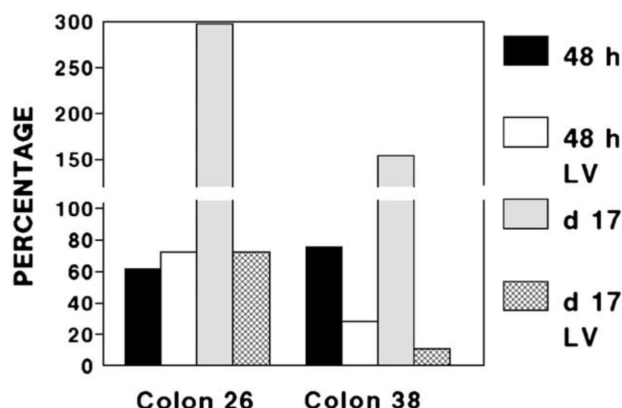

Fig. 5. Effect of 5FU treatment on TS levels (TS inhibition and induction) in experimental tumors. Mice bearing Colon 26 (5FU resistant) or Colon 38 (5FU sensitive with complete remissions) were treated weekly (days 0, 7, 14) at the therapeutic dose of 5FU (100 mg/kg). LV was given as a double dose of each 50 mg/kg, the first dose at 1 h before 5FU and one dose together with 5FU [22]. TS catalytic activity in untreated tumors was 2.32 ± 0.72 and 0.80 ± 0.23 nmol/h/mg protein, respectively (means ± S.D.), and was set at 100%. Values are means; S.D. were less than 15%.

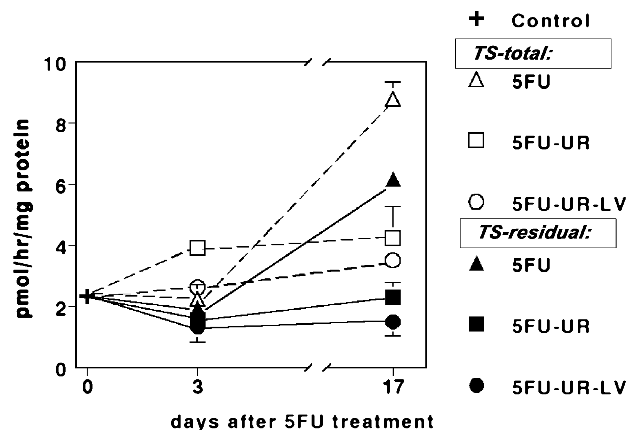

Fig. 6. Effect of uridine (UR) and LV on 5FU-induced TS inhibition and induction in Colon 26 tumors. Mice were treated weekly (days 0, 7, 14) with 5FU alone at 100 mg/kg and in combination with uridine (3500 mg/kg, 2 and 18 h after each 5FU dose) at 150 mg/kg. LV was given as described in the legend to Fig. 5. This 5FU dose was well tolerated because normal tissue toxicity could be protected by providing uridine as a salvage treatment. Values are means ± S.D. After 17 days, TS-total and TS-residual were significantly higher after 5FU treatment compared to 5FU-UR and 5FU-UR-LV ($P<0.02$); after 3 days, TS-total, and after 17 days, TS-residual were significantly higher after 5FU-UR compared to 5FU-UR-LV ($P<0.05$). From Ref. [58].

in normal liver. Since no liver toxicity was seen with local 5FU administration, the induction of TS apparently provides an additional protection of the normal tissues. Interestingly, TS was also induced in normal gastrointestinal mucosa, adding to a selective effect. Similarly, Welsh et al. [54] observed a larger induction of TS in normal tissues compared to tumor tissues, although this was after treatment with an analog of RTX, ZD9331.

6. TS inhibition and induction in human disease

6.1. TS levels and inhibition in relation to response and survival

Although one of the most widely used drugs, 5FU is only moderately active in patients. Since modulation of 5FU with LV enhanced in vitro cytotoxicity of 5FU and the in vivo antitumor effect of 5FU (reviewed in Refs. [63,64]), the combination was entered into the clinic and proved to give a better therapeutic efficacy than 5FU alone [65], both in terms of survival and response. In order to determine whether tumoral TS levels and 5FU-induced inhibition would be related to the response to 5FU, we gave patients a 500 mg/m^2 i.v. dose before surgery and removed the tumors between 1 and 72 h after drug administration. Most patients were subsequently entered in a clinical study in which they were treated with a hepatic artery infusion of 5FU at a dose of 1000 mg/m^2/day for 6 days. The study aimed to determine what extent of TS inhibition in primary human colon tumors and in liver metastases would be

sufficient to result in a response to 5FU. TS inhibition in tumors is retained for at least 48–72 h after a bolus injection of 500 mg/m^2 5FU [20,66]. In those patients in whom the TS activity in tumors remained low after 45 h, we observed a significantly higher response compared to patients, in which the activity was high. In 19 patients responding to 5FU hepatic artery infusion, TS levels in the tumor were significantly lower than in the tumors from 21 non-responding patients (Fig. 7). In breast cancer patients, the number of FdUMP binding sites increased and the effect of CH_2-THF decreased during development of resistance [67].

TS levels were also evaluated using immunohistochemistry, which essentially resulted in a similar pattern [26,27]; untreated patients had a relatively high TS expression in the tumor, while 5FU administration decreased this level. After 45 h, most patients displayed a normal level of TS expression. In addition, patients with a low TS expression had a longer survival than patients with a high TS staining (Fig. 8).

This study also evaluated the effect of LV on TS levels. The TS assays not only enabled us to evaluate TS inhibition, but by dissociation of the ternary complex also to evaluate total TS levels. 5FU administration increased total TS levels two-fold at 24 h (Table 3); LV not only increased TS inhibition, but also reduced the TS induction. These clinical data support the biochemical basis for LV modulation and also demonstrate the validity of the translation of preclinical data to the clinic.

6.2. Regulation of TS induction

Although immunohistochemistry gives a good insight in the morphological distribution of TS, its discriminative potential is rather poor, while the cytosolic staining of TS is more difficult to quantify than an enzyme assay and mRNA expression. Since TS-mRNA expression in colon tumors is relatively low, Northern blots could not be used and TS expression was quantified using RT-PCR with β-actin and 18S as the reference genes. TS-mRNA expression varied 17.7-fold relative to β-actin-mRNA and 41.7-fold relative to 18S-rRNA (Fig. 9). TS-mRNA was correlated with TS catalytic activity in these samples; both when expressed as TS/β-actin ($P = 0.8243$, $P = < 0.0001$) and TS/18S ($r = 0.6226$, $P = 0.0034$); also for FdUMP binding capacity, a good correlation was found with TS-mRNA

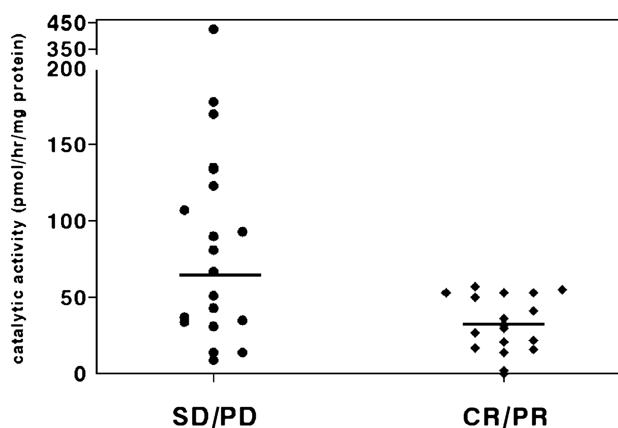

Fig. 7. Relation between TS levels and response to 5FU treatment. Tumors were removed from the patients at the indicated time points and immediately stored in liquid nitrogen. TS-total levels (measured at 1 μM dUMP) are given. The difference in TS levels between both groups is significant at the level $P < 0.01$. Modified from Refs. [20,66].

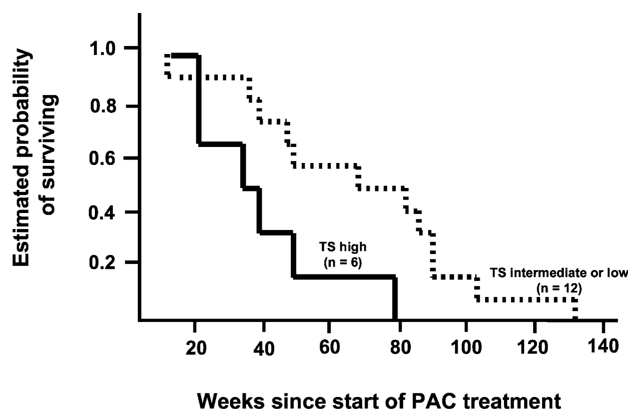

Fig. 8. Relation between TS expression as determined by immunohistochemistry and survival after 5FU treatment via hepatic artery infusion given with a Porth-a-Cath (PAC). The Kaplan–Meier curves show the probability of survival of 18 patients with liver metastases. Patients with a low expression had a significantly better survival than those with a high expression ($P = 0.025$, log-rank test). From Ref. [27].

Table 3
TS levels in patients after 5FU administration

TS assay	Drug/time			
	5FU (2 h)	5FU (23 h)	5FU (45 h)	5FU/LV (45 h)
TS catalytic activity (pmol/h/mg protein)				
TS-total	21	53*	37	31
TS-residual	11	32*	30	13
FdUMP binding (fmol/mg protein)				
TS-tot	58	128**	72	92
TS-free	0	22**	23	22
TS-mRNA expression				
TS/β-actin ($\times 10^{-3}$)	4.2 ± 1.1	8.0 ± 1.5*	3.2 ± 1.0	n.a.
TS/18S ($\times 10^{-6}$)	1.5 ± 3.1	15.2 ± 5.0**	5.7 ± 1.5	n.a.

Values for TS catalytic activity and FdUMP binding are medians of 11–15 patients. TS-total is the total catalytic activity, and TS-tot the total number of FdUMP binding sites, as measured after dissociation of the ternary complex. TS inhibition is given as TS-residual, which is the catalytic activity of TS, and TS-free, which is the number of free FdUMP binding sites, as measured before the dissociation of the ternary complex was performed [20]. The TS-mRNA expression (means ± SD) is a relative TS-mRNA expression calculated as the ratio between TS-mRNA expression and that of either β-actin or 18S [28]. Significantly different from the 2-h values at the level: *, $P < 0.05$ or **, $P < 0.02$ using either Mann–Whitney test (TS activity) or a Student's *t*-test (TS-mRNA). Time points indicate medians of the sample times. There was no significant difference in the β-actin/18S expression ratio between the various time points.

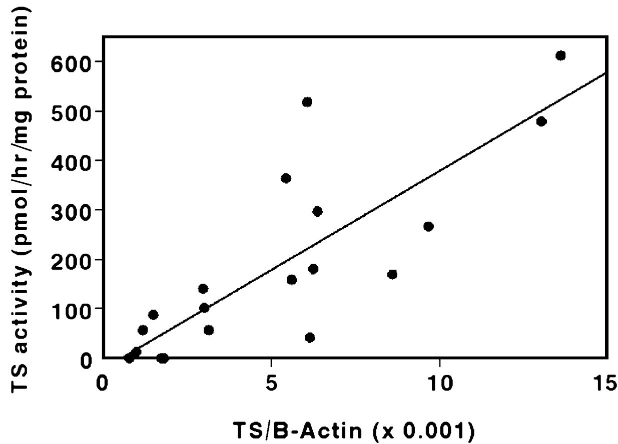

Fig. 9. Relation between TS-mRNA expression (normalized to β-actin) and TS catalytic activity (pmol/h/mg protein measured at 10 μM dUMP) in colon tumors. The linear regression coefficient was 0.8243 with a $P<0.001$.

expression. Following 5FU treatment, the TS/β-actin ratio significantly ($P=0.0493$) increased two-fold from the 2 h samples to the 23 h samples followed by a significant ($P=0.0212$) decrease from the 23 to 45 h samples (Table 3). Induction of TS was also found when comparing enzyme activities at these time points.

In order to determine whether the amount of TS enzyme per expression unit of TS-mRNA changed, we calculated the ratio of enzyme levels relative to TS/β-actin. Interestingly, the ratio catalytic activity compared to either TS/βactin or TS/18S gradually increased (Fig. 10). However, the ratio FdUMP binding compared to TS/β-actin or TS/18S was induced at 23 h compared to 4 h, but decreased again at 45 h. For TS/18S, these data were significant. These data indicate a greater induction of TS protein (as seen for the FdUMP binding) relative to TS-mRNA. This is in line with a TS autoregulation as proposed by Chu et al. [33,53]. According to this model, TS protein binds to TS-mRNA inhibiting its own translation, TS bound by FdUMP will not be not able to inhibit its translation, and the translation is derepressed, leading to more enzyme synthesis. Since in 2-h samples, the major part of TS is bound by FdUMP, the autoregulation is disrupted and TS protein is synthesized more compared to TS-mRNA. The TS protein then reaches a plateau at 23 h when evaluated as the ratio TS protein/TS-mRNA (measured as FdUMP binding capacity), but when expressed as TS catalytic activity/TS-mRNA the ratio increased. This indicates that more functional protein is present after 45 h.

Altogether, the pattern of changes in TS-mRNA and TS protein is quite complicated, and possibly due to a dual or even triple effect in these tumors. Initially, TS bound by FdUMP derepresses the autoregulation of TS-mRNA translation, leading to more synthesis of TS protein. Subsequently, the amount of E2F [26] increases, which induces transcription leading to more TS-mRNA, encoding for even more TS protein. Next to these effects, formation of the ternary complex also leads to a stabilization of TS, with as an ultimate effect an increase in total TS protein. Fortunately, although TS protein is induced, FdUMP at sufficiently high concentrations can still almost completely inhibit TS. This was associated with a better response to 5FU therapy when the residual TS and free TS were below certain thresholds [20].

7. Conclusions and perspectives

The expression of TS is controlled at several levels, which may all affect the final enzyme catalytic activity in the cell and the ability of drugs to inhibit the functional activity of the enzyme. Since TS is the target for 5FU and several folate-based TS inhibitors, it was expected that TS expression would be related to the sensitivity to 5FU and the antifolates. Acquired resistance to 5FU or antifolates has indeed been associated with an increased TS expression (both at the mRNA and protein level) [5,11,15]. When such cell lines are included in panels to evaluate a potential

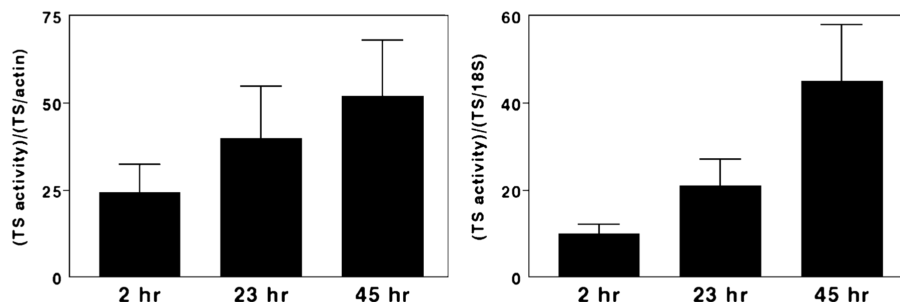

Fig. 10. Effect of 5FU treatment on TS enzyme levels in relation to TS-mRNA expression. The ratio TS catalytic activity divided by the relative TS-mRNA expression was calculated for each patient. Values are means ± S.D. The increase was significant ($P<0.05$) at 45 h for the TS/β-actin data and at 23 and 45 h for the TS/18S data. Interestingly, for the ratio FdUMP binding/TS-mRNA, a peak (3-fold for TS/β-actin $P=0.0136$; and 4.5-fold for TS/18S, $P=0.0115$) was observed at 23 h, while after 45 h, the ratio was back to that observed at 2 h.

relationship between TS levels and 5FU sensitivity, a significant correlation has been found repeatedly [5,16,17]. When including resistant cell lines in our cell line panel, 5FU sensitivity was related to TS levels. Although we previously reported a poor relation between TS levels and 5FU sensitivity [16], this relation did not exist when we extended the cell line panel with more unselected cells. Statistically, this unexpected finding might be explained by inclusion of more data, which will correct for an intrinsically poor relationship. Mechanistically, the lack of correlation may be explained by the fact that the cell lines are exposed continuously to 5FU. This leads to a continuing synthesis of FdUMP, which enables an almost complete inhibition of TS. This has been seen in the in situ TS inhibition assay with intact cells, in which TS activity was still inhibited although TS protein was induced [32]. In addition, a more pronounced effect may be found in rapidly proliferating cells that tend to have a higher TS activity [41]. The continuous exposure does not reflect most commonly used schedules to treat either animals or patients [5,21,65]. Currently, most treatment protocols consist of bolus injections or short infusion up to 24 or 48 h, which will result in high drug levels for a relatively short period followed by a rapid plasma clearance. Although the drug is retained longer in tissues [21], continuous exposure in vitro in cell lines does not reflect the in vivo conditions in animals and patients. In cell lines, the effect is quantified at the end of the exposure, while in animals and patients, the response is evaluated after a period of at least several weeks. Therefore, extrapolation of experiments with cell lines should be done carefully, taking into account these variations. In contrast, cell lines can very well be used to study single parameters, such as the potential mechanism of TS induction and the mechanisms by which 5FU and antifolates can cause cell death. In cell lines, it has also been shown that phosphorylation of the protein [68,69] may affect TS activity, which might explain some of the discrepancies between TS catalytic activity and FdUMP binding. In addition, the presence of antisense TS-mRNA [70] may affect expression of the normal TS-mRNA.

Protein expression (either with immunohistochemistry or Western blots) and TS-mRNA expression may reflect the catalytic activity of TS in the cell. However, when cells are treated, TS protein can be induced without a corresponding increase in TS-mRNA levels. Several studies now report a consistent relationship between intrinsic TS levels and response and survival to 5FU-based chemotherapy (reviewed in Ref. [71]). This relationship was found for various types of cancer, when TS levels were evaluated either by classical TS activity assays (e.g. Refs. [20,67]), TS immunohistochemistry (e.g. Refs. [27,72]) or TS-mRNA expression [73,74]. However, when the role of TS expression in adjuvant treatment of primary disease is evaluated, the role of TS expression is less clear. For example, Johnston et al. [72] reported that patients with a high TS expression had a longer survival when treated with adjuvant chemotherapy than patients with a low TS expression. Apparently in primary disease other factors, such as proliferation markers and repair enzymes may play another important role [73,75]. Before using TS expression to select patients for a specific type of adjuvant therapy, these factors should be characterized in large patient populations. However, in advanced disease, the correlation between TS levels and effect of 5FU is consistent throughout a variety of studies [71]. Therefore, the type of treatment of advanced colorectal cancer can be based on the TS expression in a prospective manner. Patients with low TS enzyme levels (TS activity, immunohistochemistry or mRNA) are expected to respond better to a 5FU-based therapy than patients with a high TS expression. When TS expression was combined with that of dihydropyrimidine dehydrogenase (DPD), none of the patients with high TS and DPD expression responded to 5FU-based therapy [76]. These patients should be selected for a non-5FU-based therapy such as with the topoisomerase I inhibitor irinotecan or the platinum analog oxaliplatin.

Acknowledgements

This research was supported by various grants from the Dutch Cancer Society (IKA 92-88, VU 93-627, VU 96-1240). We also thank M. Koudijs, M. Van der Ent and P. Noordhuis for technical assistance. Drs. G.W. Aherne and S. Welsh (Institute for Cancer Research, Sutton, Surrey, England) are acknowledged for critical reading of the paper.

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