Limits to Thymidylate Synthase and TP53 Genes as Predictive Determinants for Fluoropyrimidine Sensitivity and Further Evidence for RNA-Based Toxicity as a Major Influence

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Abstract
The major determinants of 5-flourouracil (5-FU) response would seem, based on accumulated literature, to be thymidylate synthase (TYMS, TS) expression levels, TS gene modifications, and TP53 status. We tested 5-FU sensitivity in yeast and human cancer cell models in which TS or TP53 alleles and expression were varied. Polymorphic TS tandem repeat status, TS expression levels reported, TS intragenic mutations, and TP53 status in outbred and experimental cancer cell lines did not predict 5-FU sensitivity or resistance. Novel observations included a dose-resistant persistence of unbound TS protein in many cancers and, upon 5-FU treatment of the colon cancer cell line, HCT116, evidence of allelic switch favoring transcripts of the mutant TS allele. The reported alleles having an intragenic mutation could not be causally associated with major degrees of 5-FU sensitivity. In yeast, TS protein was altered upon treatment with FdUMP, but 5-FU toxicity seemed to be largely RNA-based, being rescued by uridine rather than thymidine. Cancer cell lines were also rescued from 5-FU toxicity with uridine rather than thymidine. Cancer cell lines were also rescued from 5-FU toxicity with uridine rather than thymidine. Additionally, a TS (CDC21) knockout yeast strain, obviating any potential role for TS protein as a target, was hypersensitive to 5-FU. When denatured proteins from cancer cells treated with radiolabeled 5-FU were labeled, species with alternative molecular weights other than TS were visualized, providing further evidence for alternative 5-FU protein targets. These data emphasize that TS and TP53 status do not consistently explain the variance in responses of fluoropyrimidine-treated cancer cells, in part due to RNA-based toxicity. [Cancer Res 2009;69(3):984–91]

Introduction
5-Flourouracil (5-FU) is one of the most widely used chemotherapeutics against gastrointestinal malignancies. Heidelberger and colleagues created 5-FU over 50 years ago by substituting a fluorine atom for a hydrogen in the pyrimidine ring to create an antagonist of uracil (1). Administered primarily intravenously either by bolus or prolonged continuous infusion schedules, 5-FU is metabolized both in the liver and then intracellularly by a number of enzymes. The prevalent view in the literature represents 5-FU’s primary mode of action as being through its metabolite, FdUMP, which covalently binds to thymidylate synthase (TS or TYMS; ref. 2). Because cancer cells are highly replicating cells compared with normal cells, inhibition of TS is believed to cause thymidylate depletion and eventual “thymidineless death” of tumor cells (2).

Even though 5-FU has multiple metabolites that have been previously shown to induce cell death by the incorporation of fluorinated nucleotides into DNA and RNA, investigators have focused primarily on TS as the major target (3). Santi and others first showed that TS forms a stable complex (termed the ternary complex or classic complex) from the 5-FU metabolite, FdUMP, and 5,10-methylenetetrahydrofolate (4–6). Since these early biochemical experiments exploring the FdUMP relationship with TS, a number of investigations focused on 5-FU’s relationship with TS expression levels in clinical settings (7). Johnston and colleagues pioneered this field, describing a direct relationship between increased TS expression levels (mRNA and protein) and decreased 5-FU response (8, 9). Recently, Johnston and colleagues have modified their initial thesis (10). These investigators now propose TS expression in the metastatic lesions to be more predictive of 5-FU response than TS levels in the primary tumor (7).

Others tried to use the classic complex formation to obtain informative clinical information. Peters and colleagues showed an inhibition of thymidylate synthase enzymatic activity upon the formation of classic complexes in cancer cells and proposed this to be more predictive of response compared with TS expression levels (11). Brody and colleagues advanced a technique put forth by Drake and colleagues (12) in detecting classic complexes in vivo, thus promoting the idea that the tissue detection of classic complexes may be predictive, potentially allowing more appropriate dosing for 5-FU cancer cell targeting and monitoring of cellular effects of 5-FU in patients (6).

Other molecular determinants evolved from the TS/5-FU connection. TS tandem repeat (TS-TR) genotyping was proposed as a means to predict levels of TS expression in patients. Three main TS genotypes are prevalent in the population: (a) homozygous for two tandem repeats (2R/2R), (b) homozygous for three tandem repeats (3R/3R), and (c) heterozygous (2R/3R; refs. 13, 14). The molecular basis for using the TS repeat sequence as a marker for protein expression, however, is ambiguous. These investigations presumably concern the efficiency of ribosome translation, which relies on initiation at the Kozak consensus sequence. The studies performed by Yawata and Kawakami used the Kozak sequence of the firefly luciferase gene, and not the exact Kozak sequence of the human TS gene (13, 15). Thus, the laboratory experiments did not completely establish that the tandem repeat sequence is a clinically relevant marker for TS protein expression and the related 5-FU...
TS artificially acquired a homozygous mutation of study, the colorectal cancer cell line HCT C was mutagenized by change (tyrosine to histidine at residue 33; refs. 16, 17). In another this mutation, the mutation affected the binding capacity of laboratory investigations exploring the functional significance of the studies did not cite nor take into account this prior work. In utilization of this cell line by investigators studying 5-FU, yet most of the studies did not cite nor take into account this prior work. In laboratory investigations exploring the functional significance of this mutation, the mutation affected the binding capacity ofFdUMP to the TS protein, which harbored the mutated amino acid change (tyrosine to histidine at residue 33; refs. 16, 17). In another study, the colorectal cancer cell line HCT C was mutagenized by EMS and a subclone, C18, was identified by selecting for cells having reduced TS enzymatic activity. The C18 subline had artificially acquired a homozygous mutation of TS; a new point mutation (C → T) at nucleotide 647 converted Ser316 to leucine, yielding a nonfunctional allele as tested in autotrophic TS-null Escherichia coli (18). This type of intragenic null TS mutation is not reported to exist naturally in human cancers. The authors determined the effect of these mutations when TS protein was expressed in hamster cells or prokaryotes, yet did not directly determine whether they altered sensitivity towards 5-FU in cancer cells.

Besides TS, other variables have been proposed to affect 5-FU response. Bunz and colleagues showed that TP53 disruption in a clone of cancer cells was associated with resistance to high concentrations of 5-FU (19). A number of studies followed this report (more than 450 citations to date). Other predictors have also been proposed for 5-FU response and sensitivity. These are either integral parts of the 5-FU metabolic pathway that produce FdUMP or are thought to regulate TS expression, including the YES gene, thymidine kinase, thymidine phosphorylase, dihydroprymidine dehydrogenase, orotate phosphoribosyl-transfase, or 5,10 methylene tetrahydrofolate reductase (20–26). Ribic and colleagues found that patients with colorectal cancer whose tumors had the classic form of microsatellite instability (MSI) did not benefit from 5-FU therapy, whereas other patients did (27). This finding was significant because roughly 15% of colorectal tumors have the MSI form of genetic instability. MSI cancers harbor a greater number of subtle nucleotide sequence changes than the majority of colorectal cancers; usually, these cancer types have gross chromosomal changes (known as chromosomal instability) without high levels of sequence instability (28).

In addition to DNA-based toxicity, 5-FU-induced RNA-based toxicity has been described. For example, Cory and colleagues reversed the toxicity of 5-FU in Novikoff hepatoma cells by adding uridine to the medium (29). Pritchard and colleagues showed that in TP53-deficient mice, 5-FU-induced apoptosis was due to a disruption in RNA rather than in DNA metabolism (30), and Klubes and colleagues found uridine to rescue 5-FU toxicity in vivo (31). Bunz and colleagues showed that 400 μmol/L of uridine, but not 400 μmol/L of thymidine, rescued TP53 wild-type cancer cells from apoptosis otherwise induced by exposure to 5-FU (19).

Non–cancer cell models were also used to study the mechanism of 5-FU toxicity. Previously, we used yeast to study the TS/S-FU relationship, finding that yeast TS is covalently modified upon 5-FU exposure (6). Yeast models can be used to distinguish between DNA-based and RNA-based mechanisms of toxicity (32). Recently, Gustavsson and Ronne showed that tRNA-modifying enzymes can be targeted by 5-FU in yeast cells (33). In the current study, we evaluated a number of cancer cell lines and yeast experimental models to explore 5-FU sensitivity and resistance.

Materials and Methods

Cancer cell lines and yeast strains obtained or generated. Colorectal cancer cell lines RKO, HCT116 cells, the TP53 knockout cell line (derived from HCT116), and DLD-1 were either collected from the American Type Culture Collection or donated by Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD). We generated a heterozygous TS knockout clone in RKO cells and a derivative of the cell line ("add-back") in which exogenous TS expression was introduced, as previously described (Table 1; ref. 14). Here, we further generated stable cell lines of the RKO parental, TS+/−, and HCT116 cell lines that included overexpression of wild-type TS and of a reported HCT116-derived mutant allele changing tyrosine at amino acid 33 to a histidine (ref. 16; see description and names of cell lines in Table 1). The HCT C parental line and a subclone (HCT C18) containing the TS homozygous mutation was donated by Dr. S. Berger (18). The parental and genetically engineered yeast TS knockout strains were provided to us by Dr. J.F. Diffley and maintained as previously described (34) except as indicated otherwise. We generated drug-resistant HCT116 cell lines by continuouslypassaging parental clones in increasing amounts of 5-FU (i.e., 1 μmol/L for 1 month, followed by 1.5, up to 3 or 10 μmol/L). Cells were maintained in culture with ≥3 μmol/L of 5-FU for >1 year, with fresh medium and 5-FU provided twice weekly.

Cell culture assays and survival curves. We plated roughly 1 × 10⁶ cells per well in a 96-well plate. The next day, cells were treated with 5-FU without a change in the medium (Sigma). Five to 7 days after a single treatment, cells were washed, lysed in water, and the DNA labeled with 0.5% Picogreen (Molecular Probes), whereby fluorescence was subsequently measured to assess changes in the surviving cell population.

DNA sequencing. PCR-amplified products from genomic DNA templates were produced from the indicated cell lines and samples. The primers used for the amplification of serine hydroxymethyltransferase (SHMT) and TS are available upon request. PCR products were purified and analyzed by automated capillary sequencing. The mutations in cell lines HCT C18, HCT116, and TS+/−/RKO were confirmed by sequencing.

Detection and TA cloning of the HCT116 mutant allele. HCT116 has a heterozygous mutation of TS, Y33H (16). We treated HCT116 cells at various concentrations overnight. RNA of individual cultures were converted to bulk cDNA for sequencing. Additionally, we inserted PCR products into a TA vector (pCR 2.1) to examine the sequences of a consecutive series of plasmid clones to enumerate the relative expression of the two alleles. A mutated clone was subcloned into the pCDNA3.1 Zeo vector (Invitrogen) for follow-up functional studies (14).

Radiolabeling cell culture experiments. We treated two cancer cell lines with [C] labeled 5-FU for >48 h and then made protein lysates, which were electrophoretically separated on a 10% Bis-Tris gel (Invitrogen). The gel was fixed and dried. Autoradiography exposure was performed after more than 2 months at −80°C. Protein lysates were plated and then lysed. Protein lysates were separated by electrophoresis on 10% Bis-Tris gels (Invitrogen) and TS immunoblotting performed as previously published (6).

TS-TR detection PCR-based assay. We performed PCR with previously published primer sets (35) using genomic DNA of cell lines and tumor
**Table 1. Engineered cancer cell lines and gastrointestinal cancer lines characterized by their manipulated TS expression, TP53 and TS-TR status, and in vitro 5-FU IC<sub>50</sub>**

<table>
<thead>
<tr>
<th>Parental line*</th>
<th>TS-TR status</th>
<th>TS expression</th>
<th>TP53 status</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (μmol/L)</th>
<th>Derived line</th>
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<td>WT</td>
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* RKO, HCT116, DLD-1, and PL5 are MSI cell lines.
† The TS-TR status was not previously reported (14).
‡ Has allelic imbalance at this locus.

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Samples. PCR-amplified products were separated using 3% LB agarose gels (Faster Better Media, LLC; ref. 36).

**Real-time PCR assay for the analysis of TS mRNA levels.** RNA was extracted using the RNeasy mini kit (Qiagen). RNA (1 μg) was converted to cDNA using SuperScript II Reverse Transcriptase (Invitrogen). Real-time PCR was performed on the iCycler (Bio-Rad) with TS- and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)—specific primer sets (available upon request). The relative fold change was normalized using GAPDH as a control, and fold change was calculated by comparing TS expression of treated and untreated cultures. For each primer set, a standard curve, control, and fold change was calculated by comparing TS expression of the samples. PCR-amplified products were separated using 3% LB agarose gels (Faster Better Media, LLC; ref. 36).

**Results**

**Characterization of cell culture models.** Using RKO cells engineered to have a heterozygous knockout of the TS gene (14) and parental RKO cells, we further manipulated TS expression in cancer cell lines using exogenous overexpression of wild-type and mutant TS gene sequences. Figure 1A shows manipulated TS protein overexpression in the RKO.TS, 116.HCTmut, TS+/-—add back (14), TS +/-—HCTmut, and TS+/— (see Fig. 1A and Table 1 for labels and descriptions of the generated cell lines).

Selected cell lines were treated with 5-FU for 48 hours with a concentration 10 times the IC<sub>50</sub> for each cell line (Fig. 1B). On immunoblots, bound and unbound TS bands (both an upper and a lower band) were present, the distinction between these two bands have been previously described (refs. 6, 12; Fig. 1B). In brief, the upper band represents the formation of the classic complex (a ternary complex of drug, thymidylate synthase, and a methylene group), and the lower band is unmodified TS protein (6, 12). TS protein expression was similar among these cell lines, even though they had differing TS-TR status (Table 1; Fig. 1B and C). We extended and verified previous work identifying the TS-TR status in these cancer cell lines (Table 1; Fig. 1C; ref. 14).

**TS and TP53 status do not associate robustly with 5-FU sensitivity.** Examining expression levels, TS protein modification, TS-TR status, or mutational status of TS or TP53 genes, we did not find a notable association with 5-FU sensitivity (Table 1; Fig. 2A). Figure 2A shows the 5-FU dose-response of the majority of publicly available pancreatic cancer cell lines. As indicated from Table 1 and Fig. 1C, these cell lines have different TS-TR status and TP53 status, yet all have broadly similar sensitivity to 5-FU.

**DLD-1 cells are more resistant to 5-FU than to other lines.** The survival data of three MSI cancer cell lines (RKO, HCT116, and DLD-1 cells) were determined (Fig. 2A; Table 1). RKO and HCT116 have similar sensitivity, whereas DLD-1 cells are resistant to 5-FU. We further sequenced the coding sequence of the TS gene in a number of colon MSI cell lines (RKO, DLD-1, HT 29, and SW48). Functionally, SHMT catalyzes the reversible interconversion of serine and THF to methyleneTHF. Because the MSI line, DLD-1, was an outlier, resistant cell line in our experiments, we thus sequenced the repetitive nucleotide tracts in the coding sequence of SHMT in DLD-1 and the other lines. No mutations were found in SHMT or TS in the above listed cell lines (other than the reported mutations of the TS gene in HCT116 and HCT C18; data not shown).

**The TS mutant allele in HCT116 cells.** The IC<sub>50</sub> of parental HCT116 cells was <5 μmol/L. We generated two drug-resistant HCT116 cell lines (116.R1 and 116.R2). These cultures were passaged in 10 μmol/L (116.R1) or 3 μmol/L 5-FU (116.R2), for...
Disruption of TP53 did not affect 5-FU sensitivity at standard 5-FU concentrations. Figure 2C shows the consequences of disrupting p53 in a previously developed model (116.–/–p53; ref. 19). Although reported to have relative resistance at 400 μmol/L 5-FU (ref. 19; confirmed in Fig. 2C) in the cell survival assay, we did not find differences in sensitivity between the isogenic p53 wild-type and the 116.–/–p53 cells when testing lower, clinically relevant 5-FU concentrations (19). In another experiment, HCT116 derivative clones showed no resistance irrespective of whether p53 was disrupted or the HCT116 mutant allele was stably overexpressed (Fig. 2C). Furthermore, we did not find any systematic differences in 5-FU sensitivity in unrelated cell lines known to be TP53 wild-type or TP53-mutated (Fig. 1A; Table 1).

Clone C18, having a homozygous mutation in TS, resembles its parental cell line HCT C in 5-FU sensitivity. We confirmed the homozygous nature of the HCT C mutation by sequencing (data not shown). The TS-mutated protein was not readily detected by the TS-106 antibody, perhaps due to mutational disruption of the epitope or by protein instability (Fig. 1D; ref. 18). Figure 2C shows that the HCT C isogenic paired cell lines, differing in TS status, did not differ in 5-FU sensitivity.

The HCT116 cell line has differential expression of the wild-type and mutant TS alleles upon exposure to 5-fluoropyrimidines. We confirmed the heterozygous mutation in HCT116 cells (T>C mutation in the coding region of genomic DNA and cDNA; data not shown; Fig. 3A; ref. 16). We extracted RNA at different time points from HCT116 cell cultures exposed to various 5-FU concentrations, predominantly detecting the mutated TS allele in 25 of 26 separate cultures by sequencing of cDNA (Fig. 3A; Table 2). As a control, 4 out of 5 untreated cultures predominantly expressed wild-type TS cDNA (P ≤ 0.001; Table 2). cDNA cloning confirmed a similar pattern of TS allele-specific expression. All 12 cloned PCR products from an untreated culture were wild-type in sequence, whereas four of five clones from a 5-FU–treated culture expressed the mutant allele (P ≤ 0.002; Fig. 3A; Table 2).

Real-time PCR analysis of TS mRNA expression in HCT116 cells revealed an up-regulation of TS expression in 5-FU-treated cells versus untreated cells (Fig. 3B). This induction in HCT116 cells occurred at 5 to 20 μmol/L of 5-FU, whereas RKO cells did not induce TS expression as strongly (Fig. 3B).

Drug-resistant HCT116 lines (116.R1 and 116.R2) were evolved through constant drug exposure. They had a predominant expression of the mutated TS allele upon the sequencing of bulk cDNA (Table 2), yet the termination of 5-FU exposure reduced the TS expression (indicated as 116.R2, comparing 10 μmol/L of 5-FU and no 5-FU; Fig. 3B). HCT116 is homozygous for the TS tandem-repeat polymorphism (2R/2R; Table 1; Fig. 1C), thus an allelic difference at the polymorphic site could not explain our findings. Also, in vitro studies showed that the mutated TS allele in this cell line was not directly responsible for 5-FU resistance (Fig. 2D), and thus, a different survival of cells selectively overexpressing the mutant allele seems implausible. Thus, the observed induction of TS mRNA expression, which seems to be allele-specific in HCT116, indicates autoregulation through a transcriptional activation of the TS gene most likely exerted at the level of genomic DNA. This is distinct from reports supporting or detracting from a proposed autoregulation system in which TS protein binds the TS mRNA to control mRNA translation (37). Such mechanisms of negative feedback autoregulation would tend to mitigate the role of TS as a major determinant of 5-FU toxicity, enhancing interest in alternative targets of the drug.

Figure 1. TS status and detection of classic complexes in cell culture. A and B, we determined the TS protein levels of the cell lines generated for survival assays. Nonspecific bands (NS) reflect equal loading of protein per lane. Cell lines were treated as indicated with 5-FU (+) or untreated (–) before cell lysates were made for immunoblot analysis. C, the TS-TR assay distinguishes between the upper band (3R) and lower band (2R) as separated on an agarose gel. D, minimal immunodetectable TS protein exists in the mutated HCT C18 knockout cells had striking differences in 5-FU sensitivity compared with the parental cell line HCT C in 5-FU sensitivity. This isogenic paired cell lines, differing in TS status, did not differ in 5-FU sensitivity.

Figure 2. Drug-resistant cell lines (116.R1 and 116.R2) were evolved through constant drug exposure. These HD116 lines (116.R1 and 116.R2) were evolved through constant drug exposure. They had a predominant expression of the mutated TS allele upon the sequencing of bulk cDNA (Table 2; ref. 16). We extracted RNA at different time points from HCT116 cell cultures exposed to various 5-FU concentrations, predominantly detecting the mutated TS allele in 25 of 26 separate cultures by sequencing of cDNA (Fig. 3A; Table 2). As a control, 4 out of 5 untreated cultures predominantly expressed wild-type TS cDNA (P ≤ 0.001; Table 2). cDNA cloning confirmed a similar pattern of TS allele-specific expression. All 12 cloned PCR products from an untreated culture were wild-type in sequence, whereas four of five clones from a 5-FU–treated culture expressed the mutant allele (P ≤ 0.002; Fig. 3A; Table 2).

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Uridine and not thymidine rescues cancer cells from 5-FU toxicity. In RKO cells having different TS levels (Fig. 1), uridine (100 μmol/L) rescued 5-FU toxicity whereas thymidine did not (Fig. 2D). Even when treated at high doses of 5-FU (200 μmol/L), uridine (100 μmol/L) rather than thymidine (100 μmol/L) rescued RKO parental cultures (Fig. 2D). This confirms many prior reports in which mammalian and human cancer cells were rescued from 5-FU toxicity preferentially by uridine (19, 29, 38–41).

**TS-null yeast are more sensitive to 5-FU than wild-type yeast.** We previously showed that TS was altered upon FdUMP

Figure 2. Survival assays of cancer cell lines treated with 5-FU. Each panel is a representative single experiment, one of at least three independent experiments. Points, mean of three replicate wells from the experiment; bars, SE. A, unrelated pancreatic cancer cell lines (left), MSI colorectal cancer lines (middle), HCT116 lines evolved to be drug-resistant as compared with parental HCT116 and DLD-1 cells (right). B, manipulated TS status in isogenic RKO cell lines (left), manipulated TS status in the background of the TS+/− cell line (middle), cells having stable overexpression of the HCT116 mutation as compared with other HCT116 cell lines (right). C, isogenic p53-null and wild-type p53 cell lines (left), manipulated TS status in isogenic HCT116 cell lines, HCT1162 represents a second flask of HCT116 parental cells (middle), HCT C isogenic cell lines (right). D, uridine (open column), but not thymidine (filled column), rescued cancer cells from 5-FU toxicity. Cell line clones having different genetically manipulated TS levels were derived from the same parental line (RKO) and tested for uridine and thymidine rescue from 5-FU toxicity (25–100 μmol/L). A, an RKO parental clone; B, the TS+− add back clone; C, TS+/− cells. The parental RKO line was tested at an extended range of 5-FU (up to 200 μmol/L). Bars, individual determinations obtained in a single experiment.
treatment of yeast cells (6). TS-null yeast were more sensitive to 5-FU (Fig. 4A; ref. 34). TS-null yeast grown in the presence of uridine, but not those in thymidine, avoided toxicity (Fig. 4B). Figure 4C confirms the absence of TS protein in lysates of the TS knockout strain. Uridine rescue of yeast treated with 5-FU further supports the importance of RNA-related 5-FU toxicity, and use of a TS-null strain removes the possibility of an effect from having targeted the TS protein.

Potential for other proteins to be covalently modified upon treatment with 5-FU. C14-labeled 5-FU (10 μmol/L) was added to overnight cultures. Proteins were then electrophoretically separated. Three sizes of proteins were detected (50, 36, and 20 kDa) by autoradiography. One protein was presumably TS (at 36 kDa), whereas the others were unknown (Fig. 4D).

Discussion

TS is known to be targeted by FdUMP, and hence, the field has been largely focused on the TS/5-FU relationship (7). However, when analyzing both experimental models and the literature, the time-honored connection between TS and 5-FU is ambiguous (7). Logically, TS, an essential enzyme for replicating cells, could serve as a good therapeutic target in cancer cells. Perhaps in the future, more specific, novel or yet-to-be developed TS inhibitors (42) could use TS expression levels as a predictive marker for drug response.

We conclude that at clinically relevant concentrations of 5-FU, we observed no relation between TP53 status and 5-FU sensitivity among either outbred cell lines or the paired parental and TP53 knockout cell line (19). From the time of the TP53 knockout publication, more than 400 reports have investigated a link between TP53 and 5-FU (19). Before this seminal publication, fewer than 80 publications studied this connection.

Although TP53 and TS status have enjoyed a dominant literature position in relation to 5-FU sensitivity and resistance, other investigators have explored alternative molecular determinants of 5-FU sensitivity (43–45). These studies, including the results of gene expression profiling (46), further warn of the complexity that is obscured when attempting to correlate a single molecular variable to 5-FU response. Optimistically, however, the search for alternative 5-FU targets could help better define practical clinical predictors for fluoropyrimidine-based therapy (Fig. 4D). RNA-based toxicity has been described recently, and in the past, as a plausible target, but many investigators chose to ignore it (19, 29–32). Plausible candidate 5-FU targets related to RNA-mediated toxicity would include tRNA (33) and DNA primase (Fig. 4D; ref. 47).

An unsuspected finding was that the variant and wild-type TS alleles had differential induction of transcripts upon 5-FU treatment in HCT116 cells (Fig. 4A; ref. 34). TS-null yeast grown in the presence of uridine, but not those in thymidine, avoided toxicity (Fig. 4B). Figure 4C confirms the absence of TS protein in lysates of the TS knockout strain. Uridine rescue of yeast treated with 5-FU further supports the importance of RNA-related 5-FU toxicity, and use of a TS-null strain removes the possibility of an effect from having targeted the TS protein.

### Table 2. Evidence for allelic switching upon treatment of HCT116 cells with 5-FU

<table>
<thead>
<tr>
<th></th>
<th>Wild-type*</th>
<th>Mutant*</th>
<th>Wild-type †</th>
<th>Mutant †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>4</td>
<td>1</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>5-FU treated</td>
<td>1</td>
<td>25</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

*TS genotypes were derived from sequencing of bulk cDNA of cultures. Results of separate flasks treated or untreated (P = 0.0008).
† Allelic switching quantified using alternate allelic assay. Results are of individual flasks cultured in parallel, treated or untreated. TS genotypes were tallied from cloning the PCR products derived from multiple cultures and analyzing each clone by restriction enzyme digestion (RsaI; P = 0.0021).
on TS protein as the major determinant of toxicity, or in which the use of antifolates largely removes the possibility of a competing RNA-based mechanism of toxicity.

In summary, our work shows that variables other than TS or TP53, some being novel and others being known but often overlooked, serve as major factors altering the experimental and clinical sensitivity of cancer cells to 5-FU exposure. Therefore, many proposed biomarkers cannot be expected to readily serve as predictors of treatment response in real-life clinical settings in which many salient variables, including those that have not yet been discovered, operate simultaneously. The effectiveness of 5-FU may lie in the fact that multiple metabolites and targets exist (2), which also makes predicting a patient’s response to this drug potentially impossible. Nevertheless, a genetic understanding of cancer and the molecular aspects that drive tumorigenesis prepares us for a new era of modern drug design that can produce agents that target limited numbers of target molecules (including TS) in cancer cells without off-target effects (48, 49), allowing clinicians to stratify patients based on a limited number of variables. An improved understanding of the targets of 5-FU may permit the further development of derivative drugs that inactivate only a select subset of the 5-FU “target universe.” Folate antagonists are but one example of this line of reasoning.

Disclosure of Potential Conflicts of Interest

The authors (J.R. Brody and S.E. Kern) are governed by the Conflict of Interest policies of the Johns Hopkins University School of Medicine. The authors’ conflict of interest derives from rights they may have regarding intellectual property concerning new electrophoretic conductive media and as owners of Faster Better Media LLC, to which these rights have been assigned.

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References


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Figure 4. Evidence for alternative 5-FU targets from yeast and cancer cells. A, the yeast TS knockout strain is sensitive to 5-FU as compared with the wild-type parental strain. B, uridine can rescue yeast TS knockout strains from 5-FU toxicity. C, model validation: no detectable TS protein exists in the yeast knockout strain. Equal amounts of protein were loaded in each lane. D, two cancer cell lines (HCT116 and RKO) were treated with C14-labeled 5-FU, lysed, and proteins separated on an SDS-polyacrylamide gel. Three species were detected by autoradiography at approximately 50, 36 (TS), and 20 kDa.


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Limits to Thymidylate Synthase and *TP53* Genes as Predictive Determinants for Fluoropyrimidine Sensitivity and Further Evidence for RNA-Based Toxicity as a Major Influence

Jonathan R. Brody, Tomas Hucl, Christina L. Costantino, et al.

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