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The Role of Thymidylate Synthase Induction in Modulating p53-regulated Gene Expression in Response to 5-Fluorouracil and Antifolates[1]

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ABSTRACT

Thymidylate synthase (TS) is a critical target for chemotherapeutic agents such as 5-fluorouracil (5-FU) and antifolates such as tomudex (TDX), multitargeted antifolate, and ZD9331. Using the MCF-7 breast cancer line, we have developed p53 wild-type (M7TS90) and null (M7TS90-E6) isogenic lines with inducible TS expression (~6-fold induction compared with control after 48 h). In the M7TS90 line, inducible TS expression resulted in a moderate ~3-fold increase in 5-FU IC-50^{72h} dose and a dramatic >20-fold increase in the IC-50^{72h} doses of TDX, multitargeted antifolate, and ZD9331. S-phase cell cycle arrest and apoptosis induced by the antifolates were abrogated by TS induction. In contrast, cell cycle arrest and apoptosis induced by 5-FU was unaffected by TS expression levels. Inactivation of p53 significantly increased resistance to 5-FU and the antifolates with IC-50^{72h} doses for 5-FU and TDX of >100 and >10 μM, respectively, in the M7TS90-E6 cell line. Furthermore, p53 inactivation completely abrogated the cell cycle arrest and apoptosis induced by 5-FU. The antifolates induced S-phase arrest in the p53 null cell line; however, the induction of apoptosis by these agents was significantly reduced compared with p53 wild-type cells. Both inducible TS expression and the addition of exogenous thymidine (10 μM) blocked p53 and p21 induction by the antifolates but not by 5-FU in the M7TS90 cell line. Similarly, inducible TS expression and exogenous thymidine abrogated antifolate but not 5-FU-mediated up-regulation of Fas/CD95 in M7TS90 cells. Our results indicate that in M7TS90 cells, inducible TS expression modulates p53 and p53 target gene expression in response to TS-targeted antifolate therapies but not to 5-FU.

INTRODUCTION

TS[4] catalyzes the reductive methylation of dUMP by 5,10-methylene tetrahydrofolate to produce dTMP (1). This reaction provides the sole *de novo* intracellular source of dTMP, which is essential for DNA replication and repair. TS is also a critical target for chemotherapeutic agents such as 5-FU (2, 3) and antifolates such as TDX, MTA, and ZD9331 (4–6). *In vitro* and *in vivo* studies have demonstrated that increased TS expression correlates with increased resistance to 5-FU and that TS is a key determinant of resistance to TDX (7, 8). Furthermore, several studies have demonstrated that treatment of cancer cells with 5-FU, TDX, MTA, and ZD9331 acutely up-regulates TS synthesis (9). The molecular basis for this acute up-regulation appears to be inhibition of a TS autoregulatory feedback loop, in which TS binds its own mRNA and inhibits translation (10). TS is also known to form ribonucleotide complexes *in vitro* with a number of other mRNAs including that of the *p53* tumor suppressor gene (11, 12). Binding of TS to p53 mRNA has been reported to down-regulate p53 translation, and in addition, p53 has been shown to inhibit transcription from the mouse TS promoter (13, 14).

The *p53* gene is frequently mutated in human cancers (15) and has been described as a "molecular policeman" that acts to maintain DNA integrity after DNA damage (16). The mechanism by which p53 maintains DNA integrity is related to its activity as a transcriptional transactivator (17), although transcription-independent functions may also be important (18, 19). p53 transcriptionally up-regulates the G1 cyclin-dependent kinase inhibitor p21^{WAF1}, which inhibits entry into S-phase (20). p53 also contributes to G_2 arrest by transcriptionally up-regulating the G_2-M checkpoint genes such as *14-3-3σ* and *GADD45* (21, 22). Furthermore, p53 is involved in the induction of proapoptotic genes such as *Bax* (23) and *Fas/CD95* (24) and repression of antiapoptotic genes such as *bcl-2* (25). Expression of wild-type p53 has been shown to be required for 5-FU-induced apoptosis *in vitro* and to greatly potentiate 5-FU cytotoxicity (26, 27). p53 mutations are very common in solid tumors, and several reports have suggested that in patients with colorectal cancer, p53 status may be an important determinant of response to 5-FU-based chemotherapy (28, 29). However, other studies have failed to demonstrate any relationship between p53 expression and response to 5-FU-based therapy (30, 31).

In the present study, we used a TS-inducible expression system to examine the role of TS induction in modulating p53 and p53 target gene expression in MCF-7 breast cancer cells. In the absence of drug treatment, p53 expression was unaffected by inducible TS expression. Surprisingly, inducible TS expression had no effect on p53 up-regulation in response to 5-FU treatment. In contrast, antifolate mediated up-regulation of p53 and its downstream transcriptional target Fas/CD95 were almost completely abrogated by TS induction. These observations provide important insights into the relationship between TS and p53 expression during the cellular response to TS-targeted chemotherapies.

MATERIALS AND METHODS

Generation of a TS-inducible Construct. The TS coding region was PCR amplified from cDNA with the introduction of *Sac*II restriction sites at the 5′- and 3′-ends. The TS coding region was ligated into the pUHD10-3 tet-inducible expression vector (Ref. 32; Clontech Laboratories, Palo Alto, CA) to generate a TS-inducible construct.

Generation of TS-inducible and p53 null Cell Lines. The MCF-7 founder cell line was generated by the stable transfection of the human MCF-7 breast cancer cell line with the pUHD15-1 tet transactivator construct (Clontech Laboratories) under selection with geneticin (G418) as described previously (33). Ten μg of the TS-inducible construct and 1 μg of a plasmid that expresses the puromycin resistance gene were cotransfected into the MCF-7 founder cell line using the lipofectin reagent according to the manufacturer's instructions (Life Technologies, Inc. Paisley, Scotland). Transfected cells were selected in 1 μg/ml puromycin, and resistant colonies were isolated. Inducible expression of exogenous TS was assessed by Northern blotting, and the M7TS90 clone was selected. The M7TS90 cell line was further transfected with 10 μg of a plasmid expressing the human papilloma virus E6 protein from a cytomegalovirus promoter and 1 μg of a plasmid that expresses the hygromycin resistance gene. Transfected M7TS90 cells were selected in 200 μg/ml

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[4] The abbreviations used are: TS, thymidylate synthase; 5-FU, 5-fluorouracil; TDX, Tomudex; MTA, multitargeted antifolate; FdUMP, fluorodeoxyuridine monophosphate; tet, tetracycline; IC-50^{72h}, 50% inhibitory concentration at 72 h; FasL, Fas ligand.

hygromycin, and resistant colonies were selected. Inactivation of p53 was assessed by Western blotting, and the M7TS90-E6 cell line was selected.

Cell Culture. M7TS90 cells were maintained in 5%CO_2 at 37°C in DMEM with 10% dialyzed bovine calf serum supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μg/ml penicillin/streptomycin, 100 μg/ml G418 (all from Life Technologies, Inc.), 1 μg/ml puromycin, and 1 μg/ml tet (both from Sigma, Poole, Dorset, United Kingdom). M7TS90-E6 cells were maintained in M7TS90 growth medium supplemented with 200 μg/ml hygromycin (Life Technologies, Inc.). To induce expression of exogenous TS, cells were washed three times with 1× PBS and incubated in culture medium lacking tet.

Western Blotting. Cells were washed twice in ice-cold 1× PBS, harvested, and resuspended in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, and 0.1% SDS. Cells were then lysed by sonication using three 2–3-s bursts and centrifuged at 10,000 × *g* for 15 min to remove cell debris. Protein concentrations were determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL). Thirty to 50 μg of protein lysates were resolved by SDS-polyacrylamide gel (12%). The gels were electroblotted onto nitrocellulose membranes (Hybond-P, Amersham). Antibody staining was performed with a chemiluminescence detection system (Supersignal; Pierce), using the TS mouse monoclonal primary antibody (Rockland, Gilbertsville, PA) and the p53, p21, and Fas/CD95 mouse monoclonal and Fas Ligand rabbit polyclonal primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), in conjunction with horseradish peroxidase-conjugated sheep antimouse or donkey antirabbit secondary antibodies (Amersham). Equal lane loading was assessed using a β-tubulin mouse monoclonal primary antibody (Sigma). Membranes were stripped at 65°C for 10 min in 62.5 mM Tris (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol.

TS Biochemical Assay. Cells were harvested from T175 tissue culture flasks, washed three times in 1× PBS, and resuspended in 230 μl of 80 mM KH_2PO_4, 20 mM K_2HPO_4 before being lysed by sonication as described above. TS catalytic and FdUMP binding activities were determined as described previously (3).

Drug Sensitivity Assays. Cells were seeded onto 24-well tissue culture plates at 2×10^4 cells/well. After 24 h, cells were washed three times in 1× PBS and incubated in either +tet or −tet culture medium, supplemented with the described concentrations of 5-FU, TDX, ZD9331, and MTA. After 72 h of continuous drug exposure, cells were detached and counted using a Z2 Particle and Size Analyser (Coulter, Miami, FL).

Flow Cytometry Analysis. Cells were seeded at 1×10^5 per well of six-well tissue culture plates. After 24 h, cells were washed three times in 1× PBS and incubated in either +tet or −tet culture medium, supplemented with 10 μM 5-FU or 100 nM TDX. Samples (including detached cells) were harvested 72 h after drug addition, and their DNA content was evaluated after propidium iodide staining of cells. Fluorescence-activated cell sorting analysis was carried out using an EPICS XL flow cytometer (Coulter). Two-tailed *t* tests were performed to establish whether changes in cell cycle distributions were statistically significant.

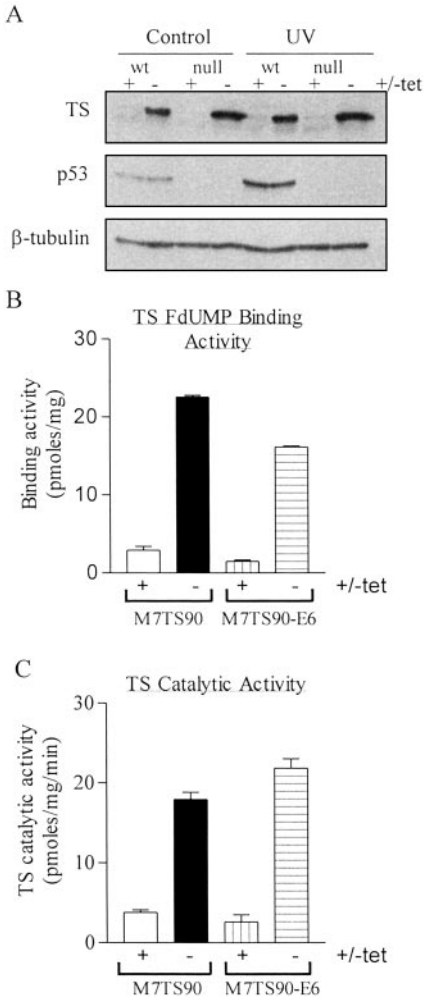

Fig. 1. *A*, Western blot analysis of TS and p53 expression in p53 wild-type M7TS90 (*wt*) and p53 null M7TS90-E6 (*null*) cells after 48-h incubation in the presence (+tet) and absence (−tet) of tetracycline. TS and p53 expression were also assessed 6 h after exposure of cells to 20 J/m^2 UV irradiation. β-Tubulin expression was used to assess equal lane loading. TS FdUMP binding (*B*) and TS catalytic activity (*C*) in the M7TS90 and M7TS90-E6 cell lines incubated for 48 h in the presence (+*tet*) and absence (−tet) of tet are shown. *Bars*, SD.

RESULTS

Development of tet-inducible Cell Lines. A tet-inducible TS expression system termed M7TS90 was established in the p53 wild-type MCF-7 breast cancer cell line as described previously (34). A subline, M7TS90-E6, was generated from this cell line by stable transfection of a construct that expresses the human papillomavirus E6 protein from a cytomegalovirus promoter. Inducible TS expression was confirmed in both these cell lines by Northern and Western blot analysis, and exogenous TS protein levels were found to be maximal 48 h after tet withdrawal (Fig. 1*A*). Consistent with these data, biochemical analysis demonstrated a 6-fold increase in both TS FdUMP binding and TS catalytic activity in both the p53 wild-type M7TS90 and the p53 null M7TS90-E6 cell lines, indicating that in both cases TS was biologically active (Fig. 1*B*). To confirm that constitutive expression of E6 functionally inactivated p53 in M7TS90-E6 cells, we carried out Western blot analysis after UV irradiation. Treatment of M7TS90 and M7TS90-E6 cells with 20 J/m^2 of UV resulted in stabilization of p53 protein levels in the M7TS90 cells; as expected, however, similar treatment of the M7TS90-E6 cells failed to activate p53, indicating that it was successfully targeted for degradation by E6 (Fig. 1*A*). In contrast to previous reports that TS can inhibit p53 translation (13), inducible expression of TS in the M7TS90 cells had no obvious effect on p53 protein levels before or after UV irradiation (Fig. 1*A*).

Impact of p53 and TS Expression on Chemosensitivity to 5-FU and Antifolates. To determine the relationship between TS and p53 in mediating resistance to TS-directed inhibitors, we evaluated the cytotoxic effects of a range of compounds including 5-FU, TDX, MTA, and ZD9331 on the p53 wild-type M7TS90 and p53 null M7TS90-E6 cell lines. Inducible expression of TS was found to increase the $IC\text{-}50^{72h}$ dose of 5-FU in M7TS90 cells from 4.2 to 11.2 μM (Fig. 2*A*; Table 1). In contrast, inactivation of p53 had a much more dramatic effect on 5-FU resistance, resulting in an $IC\text{-}50^{72h}$ dose in M7TS90-E6 cells of >100 μM. Moreover, inducible TS expression in the p53 null M7TS90-E6 line had no additional effect on 5-FU sensitivity. In the case of TDX, inducible TS expression increased the $IC\text{-}50^{72h}$ dose by ~23-fold in the p53 wild-type M7TS90 cell line (Fig. 2*B*; Table 1). Similar to that observed after 5-FU treatment, loss of endogenous p53 expression resulted in a marked increase in resist-

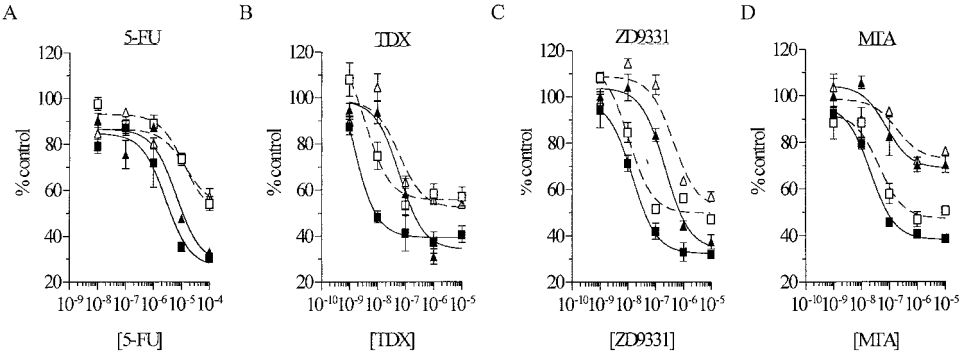

Fig. 2. Growth inhibition of TS-induced (−*tet*) and TS-uninduced (+*tet*) p53 wild-type M7TS90 and p53 null M7TS90-E6 cells after 72 h of continuous exposure to 5-FU (*A*), TDX (*B*), ZD9331 (*C*), and MTA (*D*). ■, M7TS90 +tet; ▲, M7TS90 −tet; □, M7TS90-E6 +tet; △, M7TS90-E6 −tet. Triplicate samples were counted for each treatment using a Z2 Particle and Size Analyzer (Coulter). The IC-50^{72h} doses for each cell line in the presence and absence of TS induction are given in Table 1. Results are representative of those obtained in three separate experiments. *Bars*, SD.

Table 1 *IC-50^{72h} doses for 5-FU and antifolates in the p53 wild-type M7TS90 and p53 null M7TS90-E6 cell lines*[a]

	M7TS90 (p53 wild type)		M7TS90-E6 (p53 null)	
Drug	+tet	−tet	+tet	−tet
5-FU	4.2 μM	11.2 μM	>100 μM	>100 μM
TDX	8 nM	189 nM	>10 μM	>10 μM
ZD9331	41 nM	769 nM	2.9 μM	>10 μM
MTA	83 nM	>10 μM	637 nM	>10 μM

[a] Results are representative of those obtained in three separate experiments.

ance to TDX, indeed an IC-50^{72h} dose was not achieved for TDX in the M7TS90-E6 cell line (Fig. 2*B*; Table 1). In the p53 null cell line, exogenous TS expression further increased TDX resistance; however, the magnitude of this increase was significantly less than that observed in the parental p53 wild-type line (Fig. 2*B*). A similar pattern of resistance to that of TDX was observed with ZD9331 in the presence of TS induction and loss of p53 function (Fig. 2*C*; Table 1). MTA toxicity was extremely sensitive to TS expression levels in the M7TS90 cells, with the IC-50^{72h} dose increasing from 83 nM to >10 μM (at least a 120-fold increase) after induction of exogenous TS (Fig. 2*D*; Table 1). p53 inactivation had a smaller impact on MTA sensitivity (8-fold increase in the IC-50^{72h} dose) than that observed after treatment with 5-FU, TDX, and ZD9331 (Fig. 2*D*; Table 1). Inducible TS expression in the p53 null setting also had a marked effect on MTA toxicity, increasing the IC-50^{72h} by >16-fold.

Impact of TS and p53 Expression on Cell Cycle Progression. To examine the cell cycle events underlying these observed growth-inhibitory effects, we assessed the effects of exogenous TS expression and p53 status on the cell cycle progression of cells treated with 10 μM 5-FU and 10 nM TDX (these concentrations correspond approximately to IC-50^{72h} doses in the MCF-7 parental line). In the absence of drug, expression of exogenous TS had no effect on the cell cycle distribution of either the p53 wild-type or null cell line (Fig. 3*A*). Treatment of p53 wild-type M7TS90 cells with 10 μM 5-FU or 10 nM TDX for 72 h resulted in a significant increase ($P < 0.05$, *t* test) in the number of cells arrested in S-phase (55 ± 2% and 46 ± 3%, respectively) compared with untreated cells (17 ± 1%; Fig. 3, +tet). Furthermore, a significant increase ($P < 0.05$, *t* test) in the sub-G_0-G_1 population, indicative of apoptotic cell death, was observed in cells treated with 5-FU or TDX (25 ± 4% and 32 ± 4%, respectively, compared with 8 ± 2% of control cells; Fig. 3, +tet). Inducible TS expression abrogated the S-phase arrest and the sub-G_0-G_1 apoptotic population induced by TDX in these cells from 46 ± 2% to 21 ± 1% and from 32 ± 3% to 15 ± 1%, respectively (Fig. 3*C*). Of note, inducible expression of TS failed to modulate the S-phase arrest or sub-G_0-G_1 peaks induced by 5-FU, suggesting differential sensitivity to TS expression levels for 5-FU and TDX (Fig. 3*B*).

Treatment of the p53 null M7TS90-E6 cells with 5-FU failed to induce S-phase arrest or a sub-G_0-G_1 apoptotic peak, consistent with a central role for p53 in mediating these effects (Fig. 3*B*). In contrast, treatment of M7TS90-E6 cells with TDX still induced a substantial S-phase arrest (35 ± 3%) relative to untreated controls (9 ± 1%; Fig. 3*C*). Although TDX treatment induced 18 ± 3% of the p53 null M7TS90-E6 cells to accumulate in sub-G_0-G_1 [unlike 5-FU treatment (10 ± 1%) or untreated controls (10 ± 1%)], this was significantly reduced ($P < 0.05$, *t* test) compared with p53 wild-type cells (32 ± 2%). Furthermore, inducible expression of TS abrogated the TDX-mediated S-phase arrest and sub-G_0-G_1 apoptotic peak in the M7TS90-E6 cells. A similar pattern to that observed for TDX was also observed in the M7TS90 and M7TS90-E6 cell lines treated with MTA and ZD9331 (data not shown).

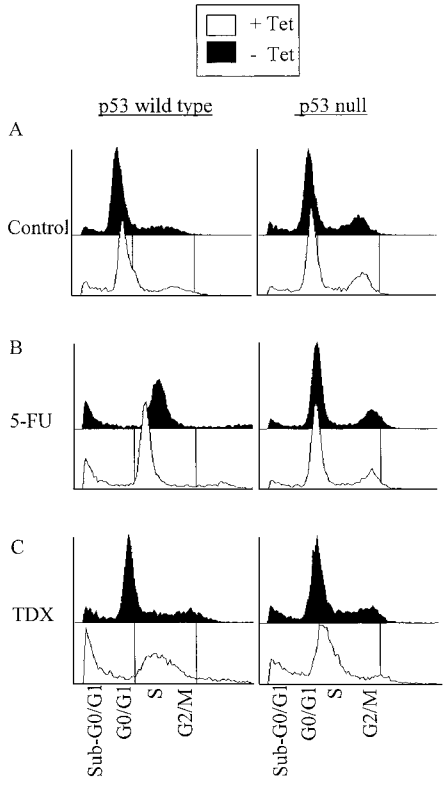

Fig. 3. Fluorescence activated cell sorting analyses of propidium iodide-stained, TS-induced (−*tet*, *black histograms*) and TS-uninduced (+*tet*, *white histograms*) M7TS90 (*p53 wild type*) and M7TS90-E6 (*p53 null*) cells. Triplicate samples were harvested after 72-h exposure to no drug (control; *A*), 10 μM 5-FU (*B*), or 10 nM TDX (these drug concentrations correspond to IC-50^{72h} doses in the parental MCF-7 cell line; *C*). The fractions corresponding to sub-G_0-G_1, G_0-G_1, S, and G_2-M phases are indicated. The analyses were carried out on an EPICS XL flow cytometer (Coulter). Results are representative of those obtained in three separate experiments.

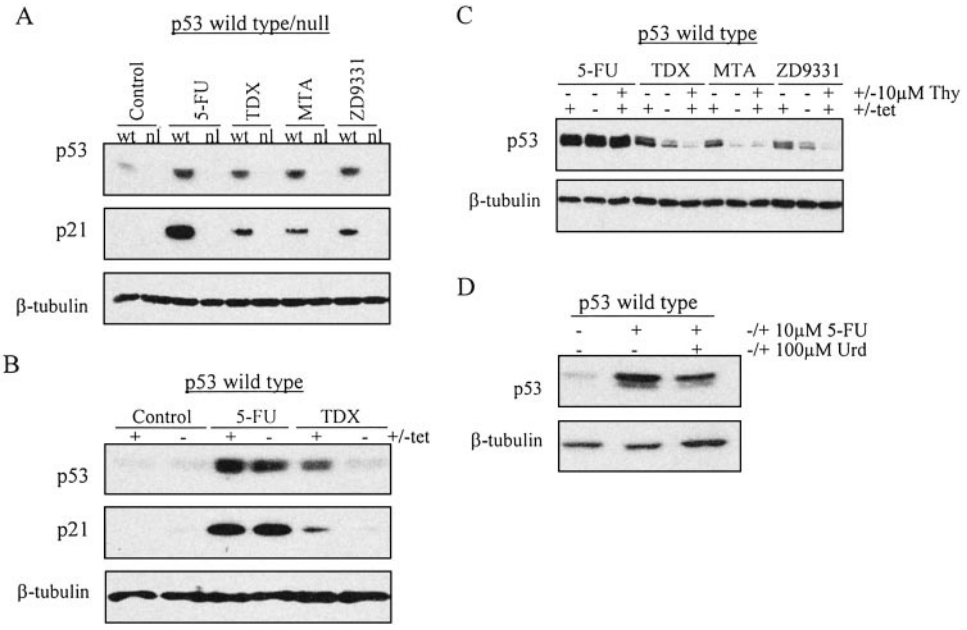

Fig. 4. *A*, Western blot analysis of p53 and p21 expression in p53 wild-type M7TS90 (*wt*) and p53 null M7TS90-E6 (*nl*) cells treated for 48 h with no drug (*Control*), 10 μM 5-FU, 10 nM TDX, 100 nM MTA, or 100 nM ZD9331 (these drug concentrations correspond to IC-50^{72h} doses in the parental MCF-7 cell line). Cells were incubated in the presence of tet (exogenous TS not induced). *B*, Western blot analysis of p53 and p21 expression in p53 wild-type M7TS90 cells treated for 48 h with no drug (*Control*), 10 μM 5-FU, or 10 nM TDX in the presence (−tet) or absence (+tet) of inducible TS expression. *C*, Western blot analysis of p53 expression in p53 wild-type M7TS90 cells treated for 48 h with no drug (*Control*), 10 μM 5-FU, 10 nM TDX, 100 nM MTA, or 100 nM ZD9331 in the presence (−*tet*) or absence (+*tet*) of inducible TS expression or in the presence of 10 μM thymidine (*Thy*). *D*, Western blot analysis of p53 expression in p53 wild-type M7TS90 cells treated for 48 h with no drug (*Lane 1*), 10 μM 5-FU (*Lane 2*), or 10 μM 5-FU plus 100 μM uridine (*Lane 3*). For each Western blot, β-tubulin expression was used to assess equal lane loading.

Modulation of p53 and p53 Target Gene Expression by Inducible TS. We carried out Western blot analysis to evaluate the effect of TS induction on p53 and p21 expression levels after treatment with 5-FU and antifolates. p53 and p21 expression levels increased approximately 5- and 11-fold, respectively, in the M7TS90 cells, 48 h after treatment with 5-FU. Similarly, treatment with TDX, MTA, and ZD9331 each resulted in 3–6-fold increases of both p53 and p21 expression levels (Fig. 4*A*). p53 and p21 expression were not detected in the M7TS90-E6 cells after treatment with 5-FU or the antifolates (Fig. 4*A*). We next examined the effect of exogenous TS expression on p53 and p21 expression in the M7TS90 cell line. Inducible TS expression had no effect on p53 or p21 expression levels after treatment with 10 μM 5-FU (Fig. 4*B*). In contrast, inducible expression of TS completely abrogated TDX-mediated induction of p53 and p21 in these cells (Fig. 4*B*). To determine whether the failure of TDX to activate p53 after TS induction was directly or indirectly related to inducible TS expression, we investigated the effect on p53 expression levels of adding exogenous thymidine (10 μM) after treatment with 5-FU and TDX. 5-FU-mediated induction of p53 was unaffected by exogenous thymidine treatment, similar to that observed after inducible expression of TS (Fig. 4*C*). However, as was the case after TS induction, treatment with exogenous thymidine abrogated p53 induction in cells treated with 10 nM TDX, suggesting an indirect effect of TS induction on p53 expression (Fig. 4*C*). A similar observation was made in M7TS90 cells treated with 100 nM MTA and 100 nM ZD9331 (Fig. 4*C*). To examine whether incorporation of the 5-FU metabolite fluorouridine triphosphate into RNA contributed to 5-FU-induced p53 up-regulation, we analyzed the effect of adding exogenous uridine. We found that cotreatment with 100 μM uridine partially reduced p53 induction by ~2-fold in M7TS90 cells treated with 10 μM 5-FU. No further reduction was observed when the concentration of uridine was increased to 1000 μM (data not shown).

We next decided to examine the effect of TS expression on the p53-regulated Fas/CD95 apoptotic pathway after treatment with 5-FU and TDX. Expression of the Fas/CD95 death receptor and its ligand (FasL) were examined in both untreated and treated M7TS90 and M7TS90-E6 cell lines. Treatment with either 10 μM 5-FU or 10 nM TDX resulted in >20-fold induction of Fas/CD95 in the p53 wild-type M7TS90 line after 72 h (Fig. 5*A*). However, Fas/CD95 induction was not observed in the p53 null M7TS90-E6 cells after identical treatments, indicating a p53-dependent pathway (Fig. 5*A*). Similar to the observations for p53 and p21, inducible TS expression abolished Fas/CD95 induction mediated by TDX but not by 5-FU (Fig. 5*B*). Furthermore, cotreatment with 10 μM thymidine in the absence of TS induction was found to prevent Fas/CD95 induction by TDX but not by 5-FU (Fig. 5*C*). FasL expression was found to be unaffected by 5-FU and TDX treatment and by both p53 inactivation and TS overexpression (Fig. 5, *A* and *B*).

DISCUSSION

Our analysis of the M7TS90 and M7TS90-E6 TS-inducible cell lines has confirmed their usefulness as physiologically relevant reagents with which to study the relationship between elevated TS expression and p53 status on the sensitivity of malignant cells to TS-directed chemotherapies. The extent of TS induction in these cell lines was ~6-fold, which is similar to the magnitude of the acute increases in TS expression that have been observed in tumor cells treated with 5-FU- and TS-targeted antifolates (9). Inducible TS expression in the p53 wild-type M7TS90 line resulted in dramatically increased resistance to the TS-targeted antifolate therapies TDX, MTA, and ZD9331 but had a smaller protective effect against 5-FU toxicity, although a 2–3-fold increase in 5-FU IC-50^{72h} dose was consistently observed after TS induction. Furthermore, inducible TS expression in the p53 wild-type line was found to dramatically diminish the S-phase arrest and induction of apoptosis by TDX (and MTA and ZD9331; data not shown), but significantly, not that induced by 5-FU.

Inactivation of p53 resulted in dramatic increases in the IC-50^{72h} doses of 5-FU, TDX, and ZD9331 and a significant increase in the IC-50^{72h} dose of MTA. Exogenous TS expression in the p53 null M7TS90-E6 cell line further increased resistance to the antifolates (most notably to MTA) but had no further effect on sensitivity to 5-FU. p53 null cells underwent neither cell cycle arrest nor apoptosis in response to 5-FU, demonstrating that p53 plays a key role in mediating these effects. The p53 downstream effector gene *p21* (*CIP1, WAF1*) plays a key role in mediating cell cycle arrest in response to DNA damage (20). Treatment of p53 null cells with TDX

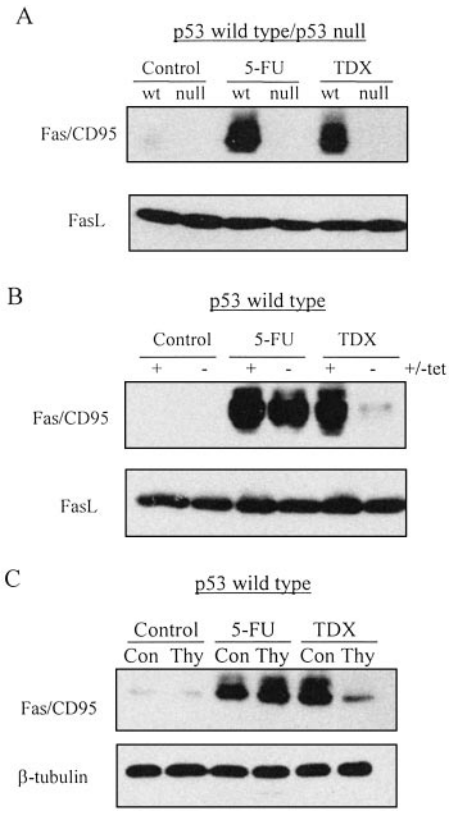

Fig. 5. *A*, Western blot analysis of Fas/CD95 and FasL expression in p53 wild-type M7TS90 (*wt*) and p53 null M7TS90-E6 (*null*) cells treated for 72 h with no drug (*Control*), 10 μM 5-FU, or 10 nM TDX (these drug concentrations correspond to IC-50^{72h} doses in the parental MCF-7 cell line). Cells were incubated in the presence of tet (TS not induced). *B*, Western blot analysis of Fas/CD95 and FasL in p53 wild-type M7TS90 cells treated for 48 h with no drug (*Control*), 10 μM 5-FU, or 10 nM TDX in the presence (*+tet*) or absence (*−tet*) of inducible TS expression. *C*, Western blot analysis of Fas/CD95 expression in p53 wild-type M7TS90 cells treated for 72 h with no drug (*Control*), 10 μM 5-FU, or 10 nM TDX in the absence (*Con*) or presence (*Thy*) of 10 μM thymidine. β-Tubulin expression was used to assess equal lane loading.

(and MTA and ZD9331; data not shown) resulted in significant S-phase arrest in the absence of p21 induction, indicating that neither p53 nor p21 are required for cell cycle arrest by this agent. This finding is in agreement with those of others (27, 35) and is consistent with the model proposed by Matsui *et al.* (36) in which the S-phase arrest induced by antifolates results from a block in DNA synthesis during replication caused by a lack of thymidine that renders the DNA replicating machinery functionally dormant. The extent of apoptosis induced by TDX was significantly reduced in the p53 null cell line compared with the p53 wild-type cell line and correlated with a lack of induction of the Fas/CD95 death receptor (similar effects were observed for MTA and ZD9331; data not shown).

Examination of p53 expression in M7TS90 cells indicated that, in the absence of drug, inducible TS expression had no effect on p53 levels. This was surprising in light of previous studies that have reported that TS protein can bind to p53 mRNA and down-regulate its translation (12, 13). In an earlier study, we observed no down-regulation of mutant p53 in MDA435 breast cancer cells after inducible TS expression (34). Furthermore, similar levels of p53 expression were detected in parental MCF-7 cells and a TDX-resistant daughter cell line (MCF-7$_{TDX}$) that overexpresses TS by >30-fold attributable to TS gene amplification (data not shown). Our results are in agreement with those of Kastanos *et al.* (37), who also found that p53 expression in MCF-7 cells was unaffected by TS induction. We conclude that direct modulation of p53 expression by TS through formation of a p53 mRNA/TS protein ribonucleotide complex does not appear to be an important regulatory mechanism in every cell line.

Exposure of M7TS90 cells to 10 μM 5-FU resulted in dramatic p53 induction in accordance with the findings of others (27). Treatment with 10 nM TDX, 100 nM MTA, and 100 nM ZD9331 also caused significant p53 induction. Most strikingly, inducible TS expression abrogated the up-regulation of p53 by the antifolates but not that mediated by 5-FU. As expected, we found a close correlation between p53 induction and p21 expression after 5-FU and antifolate treatments in the M7TS90 cell line. Notably, we found that p21 up-regulation after TDX treatment (and MTA and ZD9331; data not shown) was completely abrogated in cells expressing exogenous TS; however, 5-FU-induced up-regulation of p21 was unaffected by TS expression levels. The p53-regulated Fas/CD95 apoptotic pathway has been identified by Houghton *et al.* (38) as playing a role in mediating apoptosis after thymidine depletion. We found that Fas/CD95 expression was dramatically up-regulated in p53 wild-type cells treated with 5-FU and TDX. Significantly, Fas/CD95 induction in M7TS90 cells by TDX treatment was almost completely abrogated by inducible TS expression, whereas 5-FU-induced up-regulation of Fas/CD95 was unaffected.

These results indicate that induction of p53-mediated downstream events after 5-FU treatment in this cell line were not dependent on TS inhibition. Non-TS-directed mechanisms of cytotoxicity have been described for 5-FU, such as incorporation of its metabolites fluorouridine triphosphate and fluorodeoxyuridine triphosphate into RNA and DNA, respectively (39, 40). However, cotreatment with uridine only reduced 5-FU-mediated p53 induction by ~2-fold and had only a small protective effect against 5-FU toxicity in growth sensitivity assays (data not shown). In contrast, activation of *p53* target genes in response to the antifolate drugs was found to be completely dependent on TS inhibition. Furthermore, the fact that exogenous thymidine could prevent the antifolate-mediated induction of p53 suggests that it was an imbalance in nucleotide pools caused by TS inhibition that resulted in p53 up-regulation. It is thought that depletion of dTMP and concomitant increase in dUMP after TS inhibition leads to misincorporation of dUTP residues into DNA, thereby activating excision repair mechanisms. Futile cycles of repair in which dUTP is reinserted and re-excised then ensue, eventually leading to DNA strand breaks (41). Such DNA damage presumably activates p53, although nucleotide pools imbalances have also been reported to directly up-regulate p53 (42).

Immunohistochemical and reverse transcription-PCR quantification of TS expression in clinical tumor specimens have demonstrated that high TS expression predicts for resistance to 5-FU-based chemotherapy (43). Some clinical studies have reported that disrupted p53 function is predictive for resistance to 5-FU-based chemotherapy (28, 29), although there have been other conflicting reports (30, 31). Interestingly, we found that resistance to 5-FU was relatively insensitive to TS expression levels in MCF-7 cells, suggesting that in certain tumors TS levels may not predict for response to 5-FU. The profound effect of p53 status on the cytostatic and cytotoxic effects of 5-FU in our preclinical model system suggests that p53 may be a good predictive marker of response for this agent. Furthermore, our findings indicate that TS expression and p53 status may be key determinants of response to TS-targeted antifolate therapies because high TS expression and/or lack of functional p53 dramatically increased resistance to these agents. Finally, our observation that induction of TS expression inhibits antifolate-mediated activation of p53 and its downstream target genes further suggests that TS expression levels are likely to play a significant role in predicting response to these chemotherapies *in vivo*.

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