The Roles of Thymidylate Synthase and p53 in Regulating Fas-Mediated Apoptosis in Response to Antimetabolites

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ABSTRACT

Fas (CD95/Apo-1) is a member of the tumor necrosis factor receptor family. Receptor binding results in activation of caspase 8, leading to activation of proapoptotic downstream molecules. We found that expression of Fas was up-regulated >10-fold in MCF-7 breast and HCT116 and RKO colon cancer cell lines after treatment with IC_{60} doses of 5-fluorouracil (5-FU) and raltitrexed (RTX). Combined treatment with the agonistic Fas antibody CH-11 and either 5-FU or RTX resulted in a highly synergistic induction of apoptosis in these cell lines. Similar results were obtained for another antifolate, Alimta. Induction of thymidylate synthase expression inhibited Fas induction in response to RTX and Alimta, but not in response to 5-FU. Furthermore, thymidylate synthase induction abrogated the synergy between CH-11 and both antifolates but had no effect on the synergistic interaction between 5-FU and CH-11. Inactivation of p53 in MCF-7 and HCT116 cell lines blocked 5-FU- and antifolate-mediated up-regulation of Fas. Furthermore, Fas was not up-regulated in response to 5-FU or antifolates in the p53-mutant H630 colon cancer cell line. Lack of Fas up-regulation in the p53-null and -mutant lines abolished the synergistic interaction between 5-FU and CH-11. Interestingly, synergy was still observed between the antifolates and CH-11 in the p53-null HCT116 and p53-mutant H630 cell lines, although this was significantly reduced compared with the p53 wild-type cell lines. Our results indicate that Fas is an important mediator of apoptosis in response to both 5-FU and antifolates.

INTRODUCTION

5-Fluorouracil (5-FU) is widely used in the treatment of a range of cancers including colorectal and breast cancers and cancers of the aerodigestive tract. The mechanism of cytotoxicity of 5-FU has been ascribed to the misincorporation of fluoronucleotides into RNA and DNA and to the inhibition of the nucleotide synthetic enzyme thymidylate synthase (TS; Ref. 1). TS catalyzes the conversion of dUMP to dTMP with 5,10-methylene tetrahydrofolate as the methyl donor. This reaction provides the sole intracellular source of thymidylate, which is essential for DNA synthesis and repair. The 5-FU metabolite fluorodeoxyuridine monophosphate forms a stable complex with TS and 5,10-methylene tetrahydrofolate, resulting in enzyme inhibition (1). Recently, more specific folate-based inhibitors of TS have been developed such as raltitrexed (RTX) and Alimta (MTA), which form a stable complex with TS and dUMP that inhibits binding of 5,10-methylene tetrahydrofolate to the enzyme (2, 3). TS inhibition causes nucleotide pool imbalances that result in S-phase cell cycle arrest and apoptosis (4–6).

Drug resistance is a major factor limiting the effectiveness of TS-targeted chemotherapies. DNA microarray profiling can be used to identify genes that play key roles in determining the response of cancer cells to chemotherapy. Using such an approach, we previously identified a number of 5-FU-inducible target genes in MCF-7 breast cancer cells that may play a role in 5-FU-induced cell death (7). One of the target genes identified was the *Fas* (*CD95/Apo-1*) death receptor. Coupling of the cell surface receptor Fas by Fas ligand (FasL) or agonistic antibodies targeted against the extracellular domain of the receptor can trigger apoptosis in various cell types (8). Fas ligation leads to death-inducing signaling complex formation and activation of the "initiator" caspase, caspase 8/FADD-like interleukin 1β-converting enzyme, which in turn activates downstream caspases such as caspases 3, 6, and 7 (9). These so-called "executioner" caspases cleave a cassette of substrates resulting in changes in cellular and nuclear morphology and, ultimately, apoptosis (10). Caspase 8 can also activate mitochondria-mediated apoptosis by activating BID, which is a proapoptotic Bcl-2 family member (11). The purpose of this study was to examine the role of Fas-mediated apoptosis in mediating cell death in response to the antimetabolite drugs 5-FU, RTX, and MTA and to assess the effect of TS induction and p53 status on drug-induced activation of this pathway.

MATERIALS AND METHODS

Cell Culture. All cells were maintained in 5% CO_2 at 37°C. MCF-7, H630, and RKO cells were maintained in DMEM with 10% dialyzed bovine calf serum supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μg/ml penicillin/streptomycin (Life Technologies, Inc., Paisley, United Kingdom). M7TS90 cells (6) were maintained in MCF-7 medium

supplemented with 1 μg/ml puromycin, 1 μg/ml tetracycline (Sigma, Poole, Dorset, United Kingdom), and 100 μg/ml G418 (Life Technologies, Inc.). M7TS90-E6 cells (6) were maintained in M7TS90 medium supplemented with 200 μg/ml hygromycin (Life Technologies, Inc.). To induce expression of exogenous TS, cells were washed three times in $1\times$ PBS and incubated in growth medium lacking tetracycline. HCT116 $p53^{+/+}$ and $p53^{-/-}$ isogenic human colon cancer cells were kindly provided by Prof. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). HCT116 cells were grown in McCoy's 5A medium (GIBCO) supplemented with 10% dialyzed FCS, 50 μg/ml penicillin-streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate.

Northern Blot Analysis. Northern blots were performed as described previously using a cDNA probe complementary to the *Fas* coding region (7). Equal loading was assessed using a 18S cDNA probe.

Western Blotting. Western blots were performed as described previously (6). The Fas/CD95, Bcl-2, and BID (Santa Cruz Biotechnology, Santa Cruz, CA), caspase 8 (Oncogene Research Products, Darmstadt, Germany), and poly(ADP-ribose) polymerase (PARP; PharMingen, BD Biosciences, Oxford, United Kingdom) mouse monoclonal antibodies were used in conjunction with a horseradish peroxidase-conjugated sheep antimouse secondary antibody (Amersham, Little Chalfont, Buckinghamshire, United Kingdom). FasL rabbit polyclonal antibody (Santa Cruz Biotechnology) was used in conjunction with an horseradish peroxidase-conjugated donkey antirabbit secondary antibody (Amersham). TS sheep monoclonal primary antibody (Rockland, Gilbertsville, PA) was used in conjunction with a horseradish peroxidase-conjugated donkey antisheep secondary antibody (Serotech, Oxford, United Kingdom). Equal loading was assessed using a β-tubulin mouse monoclonal primary antibody (Sigma).

Cell Viability Assays. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay (12). Cells were seeded at 2500 cells/well on 96-well plates 24 h before drug treatment and then treated with a range of concentrations of 5-FU, RTX, and MTA for 72 h, after which time the agonistic Fas monoclonal antibody CH-11 (MBL, Watertown, MA) was added (10–250 ng/ml) for an additional 24 h. MTT (0.5 mg/ml) was then added to each well, and the cells were incubated at 37°C for an additional 3 h. The culture medium was removed, and formazan crystals were reabsorbed in 200 μl of DMSO. Cell viability was determined by reading the absorbance of each well at 570 nm using a 96-well microplate reader (Molecular Devices, Wokingham, United Kingdom).

Flow Cytometric Analysis. Cells were seeded at 1×10^5 cells/well of a 6-well tissue culture plate. After 24 h, 5-FU, RTX, or MTA was added to the medium, and the cells were cultured for an additional 72 h, after which time 250 ng/ml CH-11 was added for 24 h. DNA content of harvested cells was evaluated after propidium iodide staining of cells using an EPICS XL Flow Cytometer (Coulter, Miami, FL).

Statistical Analyses. The nature of the interaction between the chemotherapeutic drugs and CH-11 was determined by calculating the R index (RI), which was initially described by Kern *et al.* (13) and later modified by Romaneli *et al.* (14). The RI is calculated as the ratio of expected cell survival (S_{exp}, defined as the product of the survival observed with drug A alone and the survival observed with drug B alone) to the observed cell survival (S_{obs}) for the combination of A and B (RI = S_{exp}/S_{obs}). Synergism is then defined as an RI of greater than unity. Romanelli *et al.* (14) suggest that a synergistic interaction may be of pharmacological interest when RI values are around 2.0. This method was selected because treatment with CH-11 alone had little effect on cell viability, which meant that other methods such as the median effect principle (15) and isobologram methods were not suitable (16). To further assess the statistical significance of the interactions, we designed a univariate ANOVA analysis using the SPSS software package. This was an additive model based on the null hypothesis that there was no interaction between the drugs.

RESULTS

Fas Is Highly Up-Regulated in Response to 5-FU and RTX. Using DNA microarray profiling, we previously identified the Fas death receptor as being highly up-regulated in response to 5-FU in MCF-7 cells (7). Northern blot analyses confirmed that Fas mRNA was up-regulated in MCF-7 cells 48 h after treatment with an IC_{60} dose (5 μM) of 5-FU (Fig. 1*A*). Analysis of Fas protein expression in MCF-7 cells revealed that it was up-regulated by ~12-fold 72 h after treatment with 5-FU (Fig. 1*B*). Fas was also highly up-regulated (by ~7-fold) in response to treatment with an IC_{60} dose (25 nM) of RTX (Fig. 1*B*).

The Agonistic Fas Monoclonal Antibody CH-11 Synergistically Activates Apoptosis in Response to 5-FU and RTX. To examine the role of the Fas signaling pathway in mediating the response of MCF-7 cells to 5-FU and RTX, we used the agonistic Fas monoclonal antibody CH-11. Cells were treated with IC_{60} doses of each drug for 72 h, after which time they were treated with 250 ng/ml CH-11 for an additional 24 h. Treatment with 5 μM 5-FU alone resulted in an ~60% reduction in cell viability compared with control (Fig. 1*C*). Treatment with CH-11 alone without prior incubation with 5-FU caused a modest ~6% decrease in cell viability. However, treatment with 5-FU followed by CH-11 was found to result in an ~84% decrease in cell viability. The combined treatment had an RI value of 2.40, indicating that the interaction was highly synergistic. This was further confirmed by ANOVA analysis, which indicated that the synergistic interaction between the drugs was highly statistically significant ($P < 0.0005$). Similarly, treatment with 25 nM RTX for 72 h followed by CH-11 for 24 h produced a highly synergistic decrease in cell viability (RI = 2.22; $P < 0.0005$; Fig. 1*D*). An IgM isotype control antibody had no effect on the cell viability of drug-treated cells (data not shown).

To assess the degree of apoptosis in MCF-7 cells treated with 5-FU and RTX individually or in combination with CH-11, we carried out flow cytometry of propidium iodide-stained cells and analyzed the sub-G_1-G_0 apoptotic fraction. Cells were treated with either 5-FU or RTX for 72 h, followed by treatment with 250 ng/ml CH-11 for 24 h. We found that CH-11 alone had little effect on apoptosis (Fig. 1, *E* and *F*). Treatment with 5-FU alone for 96 h resulted in a modest ~2-fold induction of apoptosis in response to 5 μM 5-FU (Fig. 1*E*). However, addition of
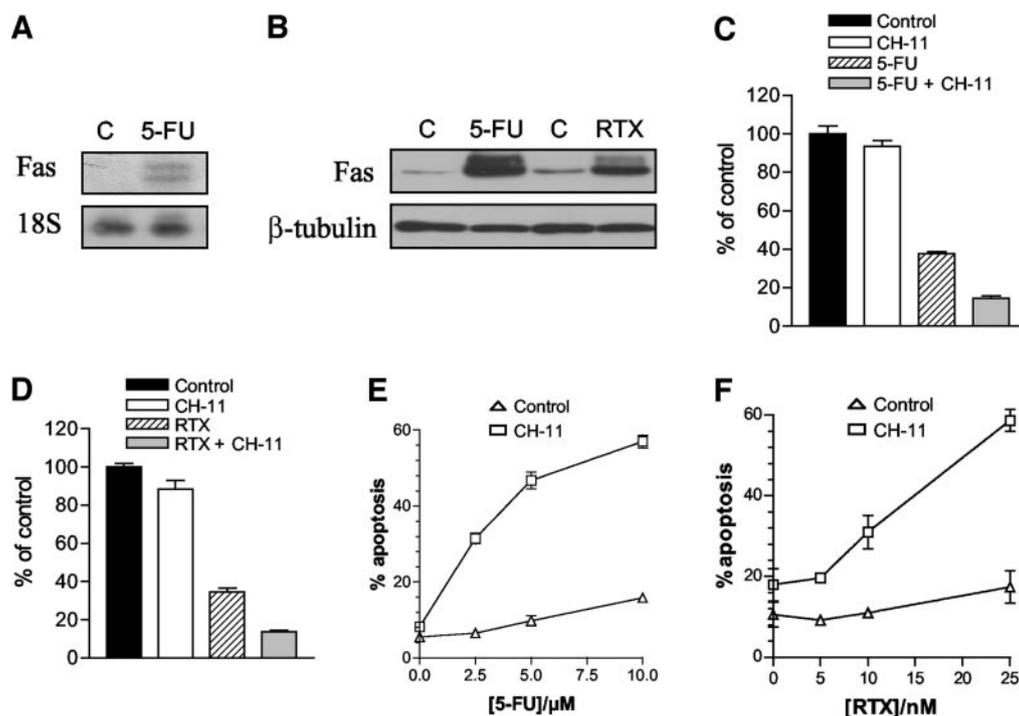
Fig. 1 A, Northern blot analysis of Fas mRNA expression in MCF-7 cells 48 h after treatment with no drug (*C*) or 5 μM 5-fluorouracil (5-FU). Equal loading was assessed by analyzing 18S rRNA expression. *B,* Western blot analysis of Fas expression in MCF-7 cells 72 h after treatment with no drug (*C*), 5 μM 5-FU, or 25 nM raltitrexed (RTX). Equal loading was assessed by analyzing β-tubulin expression. *C,* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assays in MCF-7 cells treated with no drug (control), CH-11 alone (250 ng/ml), or 5-FU alone (5 μM) or cotreated with 5-FU and CH-11. The decrease in cell viability for the combined treatment was highly synergistic (RI = 2.40; $P < 0.0005$). *D,* MTT cell viability assays in MCF-7 cells treated with no drug (control), CH-11 alone (250 ng/ml), or RTX alone (25 nM) or cotreated with RTX and CH-11. The decrease in cell viability for the combined treatment was highly synergistic (RI = 2.22; $P < 0.0005$). *E,* analysis of apoptosis in 5-FU + CH-11-cotreated MCF-7 cells. *F,* analysis of apoptosis in RTX + CH-11-cotreated MCF-7 cells. Apoptosis was assessed by analyzing the sub-G_1-G_0 fraction of propidium iodide-stained cells by flow cytometry. For both the MTT and flow cytometric analyses, the cells were pretreated with each chemotherapeutic drug for 72 h followed by CH-11 for an additional 24 h.

CH-11 to 5-FU-treated cells resulted in a dramatic increase in apoptosis, with an ~12-fold induction of apoptosis after cotreatment with 5 μM 5-FU and CH-11. Similarly, the combination of RTX with CH-11 resulted in dramatic activation of apoptosis, with ~60% of cells in the sub-G_1-G_0 apoptotic phase after combined treatment with 25 nM RTX and CH-11 compared with ~11% in untreated control cells, ~16% in cells treated with RTX alone, and ~18% in cells treated with CH-11 alone (Fig. 1*F*). The activation of apoptosis by CH-11 in 5-FU- and RTX-treated cultures was observed across a range of concentrations of each drug (Fig. 1, *E* and *F*), indicating that the synergistic interaction between CH-11 and both drugs was due to activation of apoptosis.

We next examined the ability of CH-11 to activate apoptosis in other cell lines. Treatment of HCT116 $p53^{+/+}$ colon cancer cells with 5-FU resulted in potent up-regulation (>10-fold) of Fas expression after 48 h (Fig. 2*A*). Furthermore, treatment with 5 μM 5-FU followed by 250 ng/ml CH-11 synergistically decreased cell viability in this line with an RI value of 1.92 ($P < 0.005$; Fig. 2*B*). Similarly, RTX treatment dramatically increased Fas expression after 72 h (Fig. 2*C*), whereas treatment with RTX followed by CH-11 resulted in a highly synergistic decrease in cell viability (Fig. 2*D*; RI = 3.44;

$P < 0.0005$). We also examined another p53 wild-type colon cancer cell line, RKO. As was the case with both MCF-7 and HCT116 $p53^{+/+}$ cells, both 5-FU and RTX treatments resulted in dramatic Fas up-regulation 48 h posttreatment (Fig. 2, *E* and *G*). Furthermore, treatment of RKO cells with 5-FU or RTX followed by CH-11 synergistically decreased cell viability with RI values of 1.74 ($P < 0.0005$) and 2.31 ($P < 0.0005$), respectively (Fig. 2, *F* and *H*). These results indicate that CH-11 not only activates apoptosis of 5-FU- and RTX-treated MCF-7 breast cancer cells, but also of HCT116 $p53^{+/+}$ and RKO colon cancer cells. We also found that treatment with the antifolate MTA up-regulated Fas expression and synergistically interacted with CH-11 to decrease cell viability in all three cell lines (data not shown).

Effect of 5-FU and RTX on Fas Signal Transduction. We next examined drug-induced activation of the Fas signaling pathway in response to 5-FU and RTX. Although Fas was highly up-regulated (>10-fold) in MCF-7 cells in response to IC_{60} doses of either drug, FasL expression was unaffected (Fig. 3*A*). Surprisingly, neither caspase 8 nor its substrate, BID, was activated in 5-FU- or RTX-treated cells as indicated by a lack of down-regulation of the levels of procaspase 8 or full-length BID (Fig. 3*A*). We subsequently analyzed activation of the Fas
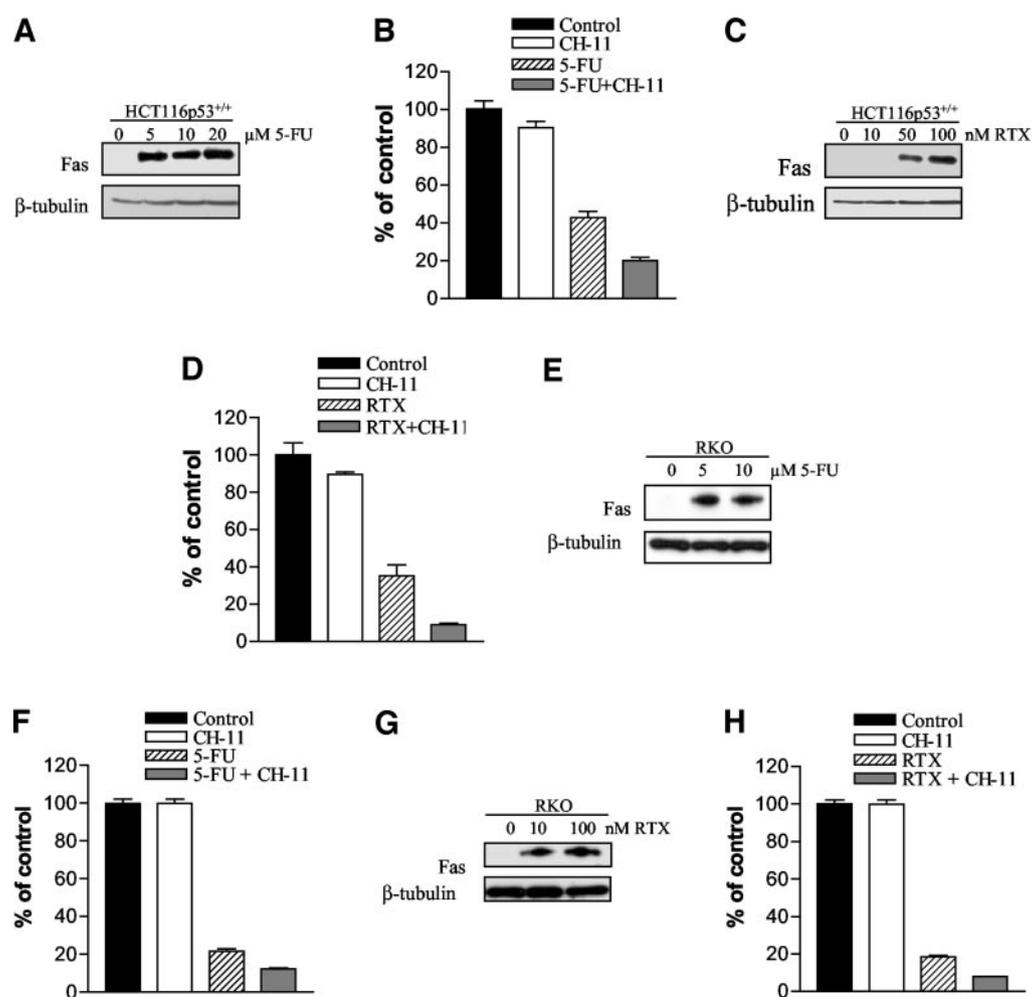
Fig. 2 *A,* Western blot analysis of Fas expression in HCT116 *p53*^{+/+} cells treated with a range of concentrations of 5-fluorouracil (5-FU) for 48 h. *B,* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assays in HCT116 *p53*^{+/+} cells treated with no drug (control), CH-11 alone (250 ng/ml), or 5-FU alone (5 μM) or cotreated with 5-FU and CH-11. The decrease in cell viability for the combined treatment was synergistic (RI = 1.92; *P* < 0.005). *C,* Western blot analysis of Fas expression in HCT116 *p53*^{+/+} cells treated with a range of concentrations of raltitrexed (RTX) for 48 h. *D,* MTT cell viability assays in HCT116 *p53*^{+/+} cells treated with no drug (control), CH-11 alone (250 ng/ml), or RTX alone (50 nM) or cotreated with RTX and CH-11. The decrease in cell viability for the combined treatment was highly synergistic (RI = 3.44; *P* < 0.0005). *E,* Western blot analysis of Fas expression in RKO cells treated with a range of concentrations of 5-FU for 48 h. *F,* MTT cell viability assays in RKO cells treated with no drug (control), CH-11 alone (250 ng/ml), or 5-FU alone (5 μM) or cotreated with 5-FU and CH-11. The decrease in cell viability for the combined treatment was synergistic (RI = 1.74; *P* < 0.005). *G,* Western blot analysis of Fas expression in RKO cells treated with a range of concentrations of RTX for 48 h. *H,* MTT cell viability assays in RKO cells treated with no drug (control), CH-11 alone (250 ng/ml), or RTX alone (5 nM) or cotreated with RTX and CH-11. The decrease in cell viability for the combined treatment was highly synergistic (RI = 2.31; *P* < 0.0005). Equal loading of Western blots was assessed by analyzing β-tubulin expression. For each combined treatment, the cells were pretreated with chemotherapeutic drug for 72 h followed by CH-11 for an additional 24 h.

pathway in MCF-7 cells after cotreatment with 5-FU and CH-11. Fas, procaspase 8, and BID expression levels were determined in cells treated with 5 μM 5-FU for 72 h followed by 250 ng/ml CH-11 for 24 h and compared with cells treated with 5-FU alone or CH-11 alone for the appropriate time periods (Fig. 3*B*). Treatment with CH-11 alone had no effect on Fas, procaspase 8, or BID expression (Fig. 3*B, Lane 2*). As already noted, treatment with 5-FU alone resulted in dramatic up-regulation of Fas but had no effect on procaspase 8 or BID expression, indicating that neither molecule was activated (Fig. 3*B, Lane 3*). However, treatment of MCF-7 cells with 5-FU and CH-11 resulted in a dramatic activation of both caspase 8 and

BID, as indicated by complete loss of procaspase 8 and full-length BID expression in these cells (Fig. 3*B, Lane 4*). Similarly, in HCT116 *p53*^{+/+} cells, activation of caspase 8 was only observed after cotreatment with either 5-FU and CH-11 or RTX and CH-11 (Fig. 3*C*). Furthermore, cleavage of PARP, a hallmark of apoptosis, was only observed in HCT116 *p53*^{+/+} cells cotreated with each drug and CH-11.

We next compared the kinetics of caspase 8 activation with cleavage of PARP. Six h after addition of CH-11 to MCF-7 cells pretreated for 72 h with 5 μM 5-FU, procaspase 8 levels were reduced by ~3-fold compared with time 0 (Fig. 3*D*). This coincided with PARP cleavage, which is indicative of cells
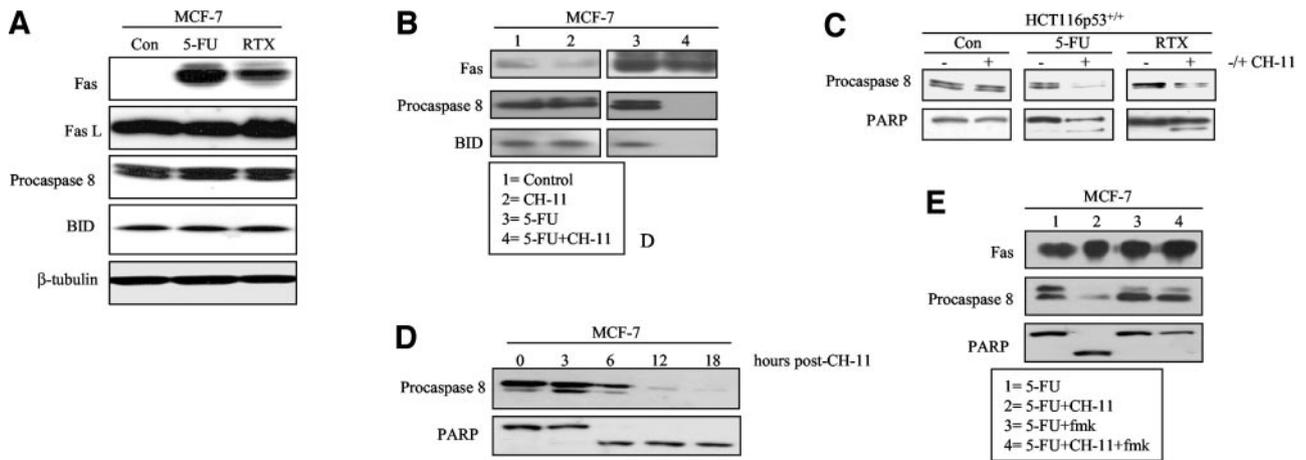
Fig. 3 A, Western blot analysis of Fas, Fas ligand, procaspase 8, and BID expression in MCF-7 cells treated with IC$_{60}$ doses of 5-fluorouracil (5-FU; 5 μM) and raltitrexed (RTX; 25 nM) for 72 h. Equal loading was assessed using a β-tubulin antibody. B, Western blot analysis of Fas, procaspase 8, and BID expression in MCF-7 cells treated no drug (control), CH-11 alone (250 ng/ml), or 5-FU alone (5 μM) for 96 h or cotreated with 5-FU for 72 h followed by CH-11 for an additional 24 h. Cotreatment with 5-FU and CH-11 resulted in activation of caspase 8 and BID, as indicated by processing of procaspase 8 and full-length BID (Lane 4). C, Western blot analysis of procaspase 8 and poly(ADP-ribose) polymerase (PARP) expression in HCT116 p53$^{+/+}$ cells treated with no drug (control), 5 μM 5-FU, or 50 nM RTX, alone or in combination with 250 ng/ml CH-11. D, Western blot analysis examining the kinetics of caspase 8 activation and PARP cleavage in MCF-7 cells treated for 72 h with 5 μM 5-FU followed by 250 ng/ml CH-11 for the indicated times. E, Western blot analysis of Fas, procaspase 8, and PARP expression in MCF-7 cells treated with 5 μM 5-FU for 72 h followed by 250 ng/ml CH-11, 10 μM IETD-fluoromethyl ketone, or a combination of CH-11 and IETD-fluoromethyl ketone for 24 h.

undergoing apoptosis. Thus, activation of caspase 8 coincided with the onset of apoptosis. Twelve and 18 h after CH-11 treatment, the levels of procaspase 8 had fallen to <5% of that observed at time 0, indicating potent activation of caspase 8. We further examined the relationship between caspase 8 activation and apoptosis using the specific caspase 8 inhibitor IETD-fluoromethyl ketone. Cells were pretreated with 5 μM 5-FU for 72 h followed by 250 ng/ml CH-11, 10 μM IETD-fluoromethyl ketone, or a combination of CH-11 and IETD-fluoromethyl ketone for 24 h. Fas was highly up-regulated in all treatment groups (Fig. 3E). As noted above, the combination of 5-FU and CH-11 resulted in a dramatic activation of caspase 8 and PARP cleavage (Fig. 3E, Lane 2). Addition of the caspase 8 inhibitor had no effect on protein expression in cells treated with 5-FU alone (Fig. 3E, Lane 3). However, IETD-fluoromethyl ketone blocked processing of procaspase 8 in cells cotreated with 5-FU and CH-11 (Fig. 3E, Lane 4). This result indicates that caspase 8 activity is necessary for procaspase 8 processing at the death-inducing signaling complex and is consistent with the induced proximity model proposed for caspase 8 activation (17). Significantly, blocking caspase 8 activation also inhibited PARP cleavage in 5-FU + CH-11-cotreated cells, indicating that apoptosis of these cells is dependent on caspase 8 activation.

Effect of TS Induction on the Synergy between CH-11 and 5-FU, RTX, and MTA. Treatment with 5-FU and TS-targeted antifolates has been shown to acutely increase TS expression, most likely through disruption of a negative feedback mechanism in which TS binds to and inhibits translation of its own mRNA (18). This constitutes a potential mechanism of resistance because TS induction would facilitate recovery of enzymatic activity. We therefore examined the effect of inducible TS expression on 5-FU and antifolate-mediated up-regulation of Fas and the synergistic interaction between CH-11 and

each drug. To do this, we used the MCF-7-derived M7TS90 cell line (6), in which transcription of a TS trans-gene is activated after withdrawal of tetracycline (tet) from the culture medium (Fig. 4A). In agreement with our previous findings, TS induction in the M7TS90 cell line abrogated RTX- and MTA-mediated up-regulation of Fas, but not 5-FU-mediated up-regulation of Fas (Fig. 4B; Ref. 6). Furthermore, induction of the TS trans-gene had little effect on the synergistic interaction between 5-FU and CH-11 (Fig. 4C). However, TS induction completely abolished the synergistic decrease in cell viability caused by the combination of both 100 nM RTX and CH-11 and 1 μM MTA and CH-11 (Fig. 4C).

We next assessed the effect of inducible TS on caspase 8 activation. We found that TS induction abrogated caspase 8 activation in response to cotreatment with both antifolates and CH-11 but had no effect on caspase 8 activation in response to cotreatment with 5-FU and CH-11 (Fig. 4D). Similarly, TS induction abrogated processing of PARP in cells cotreated with the antifolates and CH-11, but not in cells cotreated with 5-FU and CH-11 (Fig. 4D). The differential effects of TS induction on apoptosis of 5-FU- and antifolate-treated M7TS90 cells were further analyzed by flow cytometry by assessment of the sub-G$_0$-G$_1$ fraction in cells cotreated with drug and CH-11. Cotreatment with 5-FU and CH-11 resulted in a dramatic ~20-fold induction of apoptosis in M7TS90 cells that was only modestly reduced to ~17-fold when TS was induced (Fig. 4E). In contrast, RTX and CH-11 cotreatment resulted in an ~15-fold increase in the apoptotic fraction, which was reduced to ~5-fold by TS induction (Fig. 4E). Similarly, combined treatment with MTA and CH-11 resulted in a dramatic ~26-fold induction of apoptosis that was almost completely abolished by inducible TS expression (Fig. 4E). These results indicate that the activation of Fas-
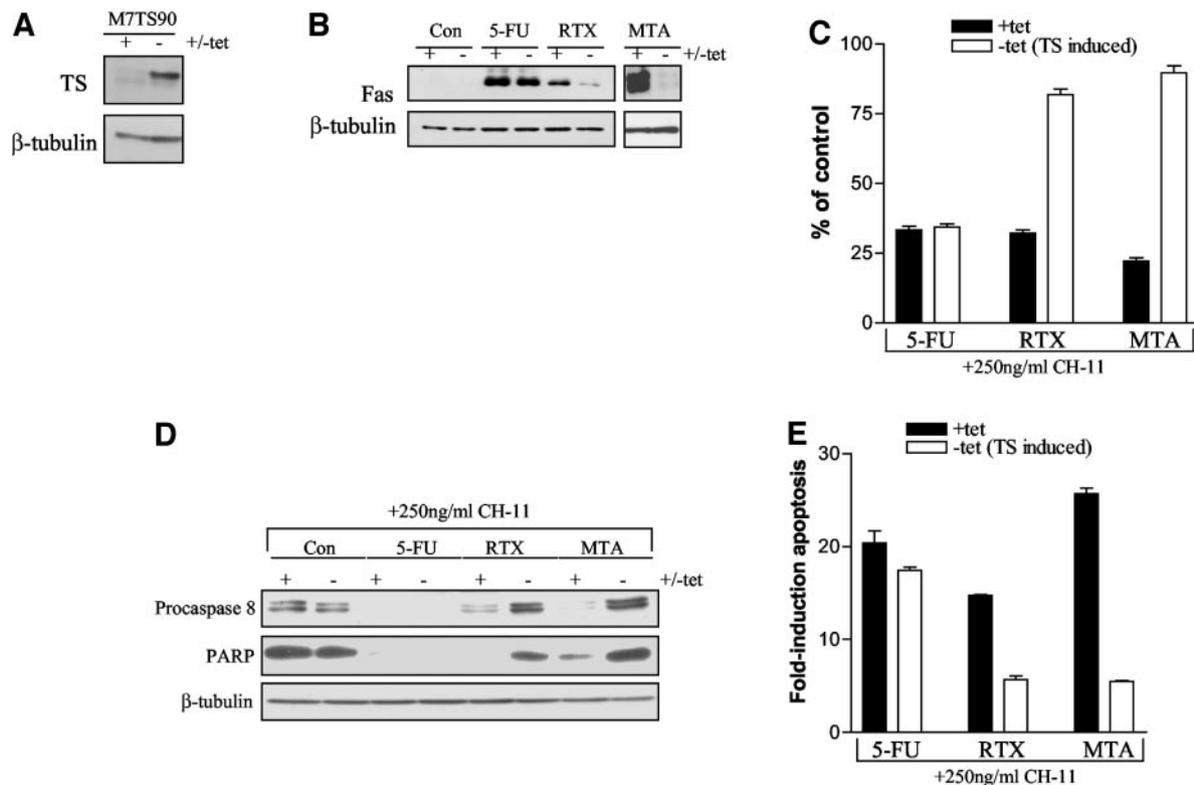
Fig. 4 *A,* tetracycline (*tet*)-regulated expression of a *TS trans*-gene in M7TS90 cells. *B,* Western blot analyzing the effect of TS induction (−*tet*) on Fas up-regulation in M7TS90 cells in response to treatment with 10 μM 5-fluorouracil (5-FU), 100 nM raltitrexed (RTX), or 1 μM MTA for 72 h. *C,* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay showing the impact of TS induction (−*tet*) on viability of M7TS90 cells after treatment with 5-FU (10 μM), RTX (100 nM), or MTA (1 μM) in the presence of cotreatment with 250 ng/ml CH-11. *D,* impact of TS induction on caspase 8 activation and processing of full-length (118-kDa) poly(ADP-ribose) polymerase in M7TS90 cells treated with 5-FU (10 μM), RTX (100 nM), or MTA (1 μM) followed by 250 ng/ml CH-11. *E,* effect of exogenous TS expression on the induction of apoptosis in M7TS90 cells treated with 5-FU (10 μM), RTX (100 nM), or MTA (1 μM) in the presence of cotreatment with 250 ng/ml CH-11. Apoptosis was assessed by analyzing the sub-G_1-G_0 fraction of propidium iodide-stained cells by flow cytometry. Equal loading of Western blots was assessed by analyzing β-tubulin expression. For each combined treatment, the cells were pretreated with chemotherapeutic drug for 72 h followed by CH-11 for an additional 24 h.

mediated apoptosis in antifolate-treated cells was highly dependent on TS expression levels. In contrast, the 5-FU/CH-11 interaction was relatively insensitive to TS induction in this cell line, suggesting that non-TS-directed effects were primarily responsible for 5-FU cytotoxicity in these cells.

Effect of p53 Inactivation on the Synergy between CH-11 and 5-FU, RTX, and MTA. We next examined the role of p53 in the observed synergy between CH-11 and each drug. p53 has been reported to be an important regulator of Fas expression, both transcriptionally (19) and posttranscriptionally (20). We previously described the generation of p53-null M7TS90-E6 cells by transfection of M7TS90 cells with human papilloma virus E6 (6). Treatment of these p53-null M7TS90-E6 cells with 10 μM 5-FU, 100 nM RTX, or 1 μM MTA did not result in Fas up-regulation (Fig. 5*A*). Furthermore, in contrast to the parental line, the combination of 5-FU and CH-11 did not synergistically decrease cell viability (RI = 0.97; Fig. 5*B*). Similarly, inactivation of p53 also abolished the synergy between RTX and CH-11 and between MTA and CH-11 (RI = 0.85 and 1.02, respectively; Fig. 5*B*).

We further examined the effects of p53 inactivation on drug sensitivity by comparing caspase 8 activation in the p53

wild-type and p53-null isogenic M7TS90 lines. Activation of caspase 8 was not observed in the p53-null M7TS90-E6 cells cotreated with each drug and CH-11 (Fig. 5*C*). In contrast, caspase 8 was potently activated in the parental p53 wild-type cell line in response to each cotreatment (Fig. 5*C*). Inactivation of p53 also completely attenuated PARP cleavage in response to cotreatment with 5-FU and CH-11 (Fig. 5*C*). However, processing of PARP was evident in p53-null cells treated with both the RTX/CH-11 and MTA/CH-11 combinations, although to a lesser extent than that seen in the p53 wild-type line (Fig. 5*C*). Because caspase 8 was not activated, this suggests that antifolate-mediated PARP cleavage in the p53-null cells was not due to activation of Fas-mediated apoptosis by CH-11. Indeed, we found that PARP was also processed in the p53-null cell line in response to treatment with either RTX alone or MTA alone (data not shown). These results indicate that treatment with the antifolates activated p53-and Fas-independent apoptosis in M7TS90-E6 cells. This was further confirmed by flow cytometric analysis of apoptosis in the p53-null cell line. RTX (100 nM) and MTA (1 μM) significantly induced apoptosis of M7TS90-E6 cells by ~8-fold and ~6-fold, respectively, 96 h after drug treatment (Fig. 5*D*). In contrast, little apoptosis was
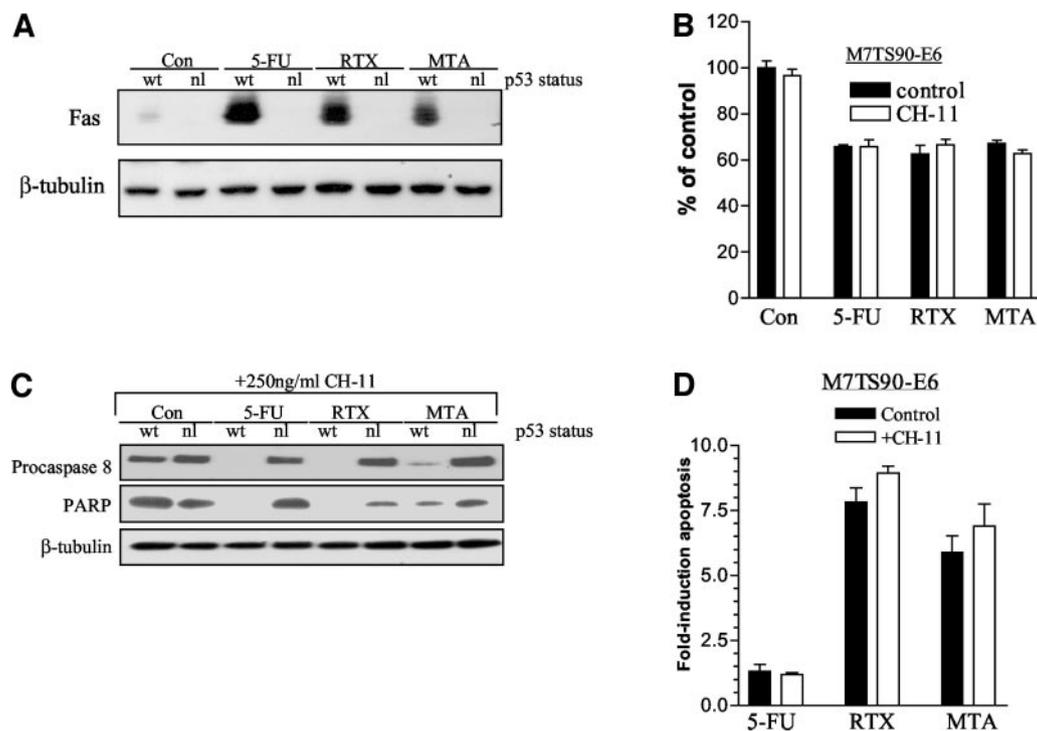
Fig. 5 *A,* Western blot analysis of Fas expression in p53 wild-type (*wt*) M7TS90 and p53-null (*nl*) M7TS90-E6 cells 72 h after treatment with no drug (*Con*), 10 μM 5-fluorouracil (5-FU), 100 nM raltitrexed (RTX), or 1 μM MTA. *B,* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell viability assays in p53-null M7TS90-E6 cells treated with 10 μM 5-FU, 100 nM RTX, or 1 μM MTA in combination with 250 ng/ml CH-11. *C,* Western blot analysis of procaspase 8 and full-length (118-kDa) poly(ADP-ribose) polymerase expression in M7TS90 (*wt*) and M7TS90-E6 (*nl*) cells treated with 5-FU (10 μM), RTX (100 nM), or MTA (1 μM) followed by 250 ng/ml CH-11. *D,* effect of CH-11 (250 ng/ml) on the induction of apoptosis in M7TS90-E6 cells treated with 5-FU (10 μM) RTX (100 nM), or MTA (1 μM). Apoptosis was assessed by analyzing the sub-G_1-G_0 fraction of propidium iodide-stained cells by flow cytometry. Equal loading of Western blots was assessed by analyzing β-tubulin expression. For each combined treatment, the cells were pretreated with chemotherapeutic drug for 72 h followed by CH-11 for a additional 24 h.

observed in M7TS90-E6 cells after treatment with 10 μM 5-FU (Fig. 5*D*). Importantly, CH-11 had no significant effect on apoptosis induced by any of the drugs in the p53-null cell line.

We extended our studies into the role of p53 in regulating antimetabolite-induced Fas-mediated apoptosis by examining the interaction between these drugs and CH-11 in the p53-null HCT116 $p53^{-/-}$ cell line. This cell line was derived from the HCT116 $p53^{+/+}$ cell line by somatic knockout of both *p53* alleles (21). Compared with the p53 wild-type cell line, there was very little Fas induction in response to 5-FU (Fig. 6*A*) and RTX (Fig. 6*C*) in the HCT116 $p53^{-/-}$ cell line, with an approximately 2–3-fold induction of Fas expression observed in response to 10 μM 5-FU and 50 nM RTX. Furthermore, no synergistic interaction was observed between 5-FU and CH-11 in the p53-null cell line (RI = 1.01; Fig. 6*B*). Interestingly, a significant synergistic interaction was still observed between RTX and CH-11 in HCT116 $p53^{-/-}$ cells (RI = 1.62; $P = 0.01$; Fig. 6*D*), although this was significantly less synergistic than the interaction observed in the p53 wild-type parental line (Fig. 2*D*; RI = 3.44; $P < 0.0005$). This suggests that RTX-mediated sensitization of HCT116 cells to CH-11 is not wholly p53 dependent.

The role of p53 in mediating Fas-mediated apoptosis was further examined in the p53-mutant H630 colon cancer cell line. Similar to the p53-null cell lines, Fas expression was not sig-

nificantly altered in H630 cells in response to 5-FU (Fig. 6*E*) or RTX (Fig. 6*G*). No synergistic decrease in cell viability was observed between 5-FU and CH-11 (Fig. 6*F*; RI = 0.99); however, a statistically significant synergistic interaction was observed between RTX and CH-11 (Fig. 6*H*; RI = 1.64; $P < 0.0005$). This interaction was observed despite the lack of any apparent up-regulation of Fas in response to this agent, suggesting that Fas expression is not the sole determinant of sensitivity to CH-11 in this cell line.

DISCUSSION

We have found that the Fas death receptor is highly up-regulated in response to 5-FU and the TS-targeted antifolates RTX and MTA in MCF-7 breast cancer and HCT116 $p53^{+/+}$ and RKO colon cancer cells; however, this, in itself, was not sufficient to activate caspase 8. Expression of FasL by activated T cells and natural killer cells induces apoptosis of Fas-expressing target cells *in vivo* (22). To mimic the effects of these immune effector cells in our *in vitro* model, we used the agonistic Fas monoclonal antibody CH-11. We found that CH-11 potently activated Fas-mediated cell death in 5-FU- and antifolate-treated cells. Furthermore, the interaction between CH-11 and each drug was highly synergistic. These findings agree with those of Tillman *et al.* (23), who found that the cytotoxicity of
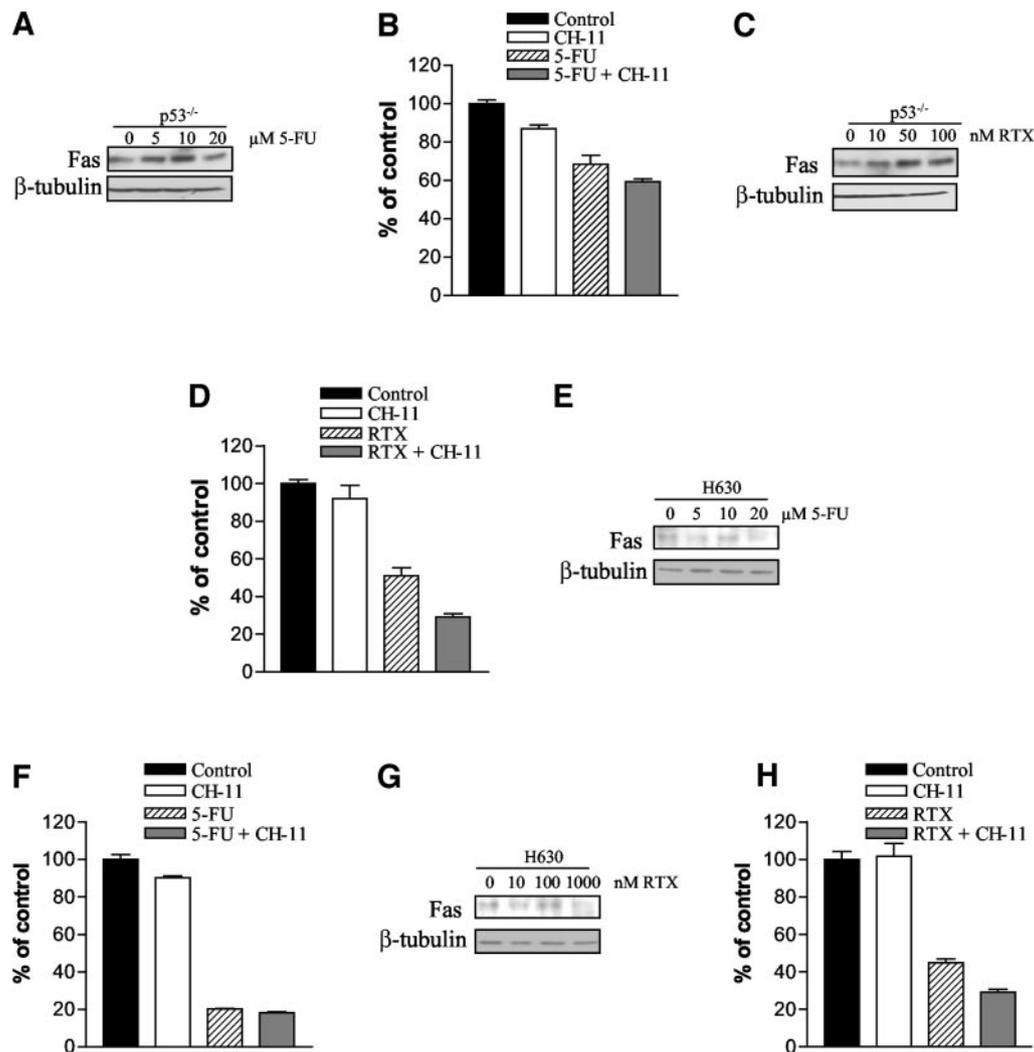
Fig. 6 *A,* Western blot analysis of Fas expression in HCT116 *p53*$^{-/-}$ cells treated with a range of concentrations of 5-fluorouracil (5-FU) for 48 h. *B,* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assays in HCT116 *p53*$^{-/-}$ cells treated with no drug (control), CH-11 alone (250 ng/ml), or 5-FU alone (10 μM) or cotreated with 5-FU and CH-11. The decrease in cell viability for the combined treatment was not synergistic (RI = 1.01). *C,* Western blot analysis of Fas expression in HCT116 *p53*$^{-/-}$ cells treated with a range of concentrations of raltitrexed (RTX) for 48 h. *D,* MTT cell viability assays in HCT116 *p53*$^{-/-}$ cells treated with no drug (control), CH-11 alone (250 ng/ml), or RTX alone (50 nM) or cotreated with RTX and CH-11. The decrease in cell viability for the combined treatment was synergistic (RI = 1.62; *P* = 0.01). *E,* Western blot analysis of Fas expression in H630 cells treated with a range of concentrations of 5-FU for 48 h. *F,* MTT cell viability assays in H630 cells treated with no drug (control), CH-11 alone (250 ng/ml), or 5-FU alone (10 μM) or cotreated with 5-FU and CH-11. The decrease in cell viability for the combined treatment was not synergistic (RI = 0.99). *G,* Western blot analysis of H630 cells treated with a range of concentrations of RTX for 48 h. *H,* MTT cell viability assays in H630 cells treated with no drug (control), CH-11 alone (250 ng/ml), or RTX alone (50 nM) or cotreated with 5-FU and CH-11. The decrease in cell viability for the combined treatment was synergistic (RI = 1.41; *P* < 0.005). Equal loading of Western blots was assessed by analyzing β-tubulin expression. For each combined treatment, the cells were pretreated with chemotherapeutic drug for 72 h followed by CH-11 for an additional 24 h.

5-FU in HT29 colon cancer cells was reversed on coincubation with antagonistic anti-FasL antibodies, indicating the involvement of the Fas pathway in mediating the effects of 5-FU in this cell line. Our results suggest that the Fas signaling pathway is an important mediator not only of 5-FU-induced cell death, but also of antifolate-induced cell death.

We found that although FasL was not induced after drug treatment, it was highly expressed in MCF-7 cells. Many tumor cells overexpress FasL, and it has been postulated that tumor

FasL induces apoptosis of Fas-sensitive immune effector cells, thereby inhibiting the antitumor immune response (22). This hypothesis has been supported by both *in vitro* and *in vivo* studies (24, 25). The strategy of overexpressing FasL requires that the tumor cells develop resistance to Fas-mediated apoptosis to prevent autocrine and paracrine induction of tumor cell death. Fas signaling may be inhibited by a Fas splice variant soluble Fas (sFas), which is a secreted protein that lacks the transmembrane domain of full-length Fas and may inhibit bind-

ing of FasL to Fas (26). Similarly, the Fas decoy receptor DcR3 is another secreted protein that binds to FasL with high affinity, inhibiting its interaction with Fas (27). Downstream of Fas ligation, FADD-like interleukin-1β-converting enzyme-inhibitory protein (c-FLIP) and Fas-associated phosphatase-1 (FAP-1) can inhibit caspase 8 recruitment and activation at the Fas death-inducing signaling complex (28, 29). The lack of caspase 8 activation in response to treatment with 5-FU and the antifolates suggests that Fas-mediated apoptosis may be inhibited in MCF-7, HCT116, and RKO cancer cells. However, cotreatment with CH-11 was sufficient to overcome this resistance and activate Fas-mediated apoptosis.

Our findings raise the possibility of using antimetabolite drugs in combination with anti-Fas antibodies as a novel anticancer strategy. Furthermore, several other studies have also shown increased sensitivity to Fas-targeted antibodies in tumor cells treated with a range of chemotherapeutic agents including cisplatin, methotrexate, and doxorubicin (19, 30). Targeting Fas may be particularly useful against tumor cells that overexpress FasL and Fas pathway inhibitors and thereby evade Fas-mediated elimination by immune cells. However, systemic treatment with Fas antibodies or recombinant FasL in mouse models has been shown to cause severe damage to liver and other organs (31). Some recent studies have focused on local administration of recombinant FasL or the use of FasL-expressing vectors as gene therapy to overcome systemic toxicity (31). In addition, a novel agonistic Fastargeted antibody, HFE7A, has been developed recently that was not hepatotoxic in murine models, suggesting that it may be possible to develop less toxic Fas-targeted antibodies (32). However, strategies targeted against molecules that inhibit Fas-mediated apoptosis such as DcR3 and c-FLIP may prove to be a more viable approach for activating Fas-mediated apoptosis in drug-treated cancer cells.

Treatment with TS inhibitors has been shown to acutely induce TS expression in cell lines and tumors (18, 33). Furthermore, preclinical and clinical studies have found that TS is a key determinant of sensitivity to 5-FU, with high TS expression correlating with increased resistance (1, 34). We therefore examined the effect of elevated TS expression on activation of Fas-mediated apoptosis in 5-FU- and antifolate-treated cells using a tetracycline-regulated TS expression system (M7TS90). Interestingly, we found that activation of apoptosis by CH-11 in response to 5-FU was not affected by increased TS expression. In contrast, TS induction completely abrogated the synergistic interaction between both RTX and CH-11 and MTA and CH-11. These findings correlated with Fas expression, the up-regulation of which was almost completely abrogated by TS induction in RTX- and MTA-treated cells, but not 5-FU-treated cells. These results indicate that the primary locus of 5-FU cytotoxicity in this cell line was not TS inhibition. Indeed, our previous studies have suggested that misincorporation of fluoronucleotides into RNA was the primary cytotoxic effect of 5-FU in this line (6). Thus, despite expressing high levels of TS, certain tumors may still be sensitized to Fas-mediated apoptosis by 5-FU. However, high TS expression is likely to inhibit Fas-mediated apoptosis in response to folate-based TS inhibitors.

Several preclinical studies have demonstrated that loss of p53 function reduces cellular sensitivity to 5-FU (6, 21). Furthermore, a number of clinical studies have found that p53

mutations correlated with resistance to 5-FU, although other studies found no such association (34). We assessed the effect of p53 inactivation on drug-induced Fas-mediated apoptosis in two p53 wild-type and p53-null isogenic cell line pairs: the MCF-7-derived M7TS90 and M7TS90-E6 cell lines; and the HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cell lines. p53 inactivation attenuated Fas up-regulation in response to both drugs in both the MCF-7 and HCT116 cell lines and inhibited the activation of apoptosis by CH-11 in 5-FU- and antifolate-treated cells, indicating that p53 is an important determinant of Fas-mediated apoptosis in response to these agents. Interestingly, some synergy was still observed between RTX and CH-11 in the HCT116 $p53^{-/-}$ cell line, although it was significantly reduced compared with that seen in the p53 wild-type cell line. We also examined activation of Fas-mediated apoptosis in response to the antimetabolites in the p53-mutant H630 colon cancer cell line. Similar to the HCT116 $p53^{-/-}$ cell line, little Fas induction was observed after drug treatment, and no synergy was observed between 5-FU and CH-11. However, a statistically significant synergistic interaction was again observed between RTX and CH-11. Our results suggest that RTX (but not 5-FU) can sensitize at least some cancer cell lines with nonfunctional p53 to Fas-mediated apoptosis. Furthermore, this effect appears to be independent of Fas up-regulation, suggesting that factors other than increased Fas expression contribute to the sensitization of tumor cells to Fas-mediated apoptosis in response to this agent.

Our results agree with those of Muller *et al.* (19), who found that p53-null Hep3B hepatoma cells failed to up-regulate Fas in response to anticancer drugs; however, transfection of these cells with wild-type p53 restored their ability to up-regulate Fas in response to drug treatment and sensitized them to Fas-mediated apoptosis (19). This study also reported that p53 up-regulates Fas expression at the transcriptional level. Furthermore, p53 has been reported to be involved in transport of Fas from the Golgi apparatus to the cell membrane (20). Our data suggest that tumors with mutated p53 would be more resistant to Fas-mediated apoptosis in response to antimetabolites, in particular 5-FU. However, the discriminatory p53 mutants Pro-175 and Ala-143 have been shown to transcriptionally up-regulate Fas expression (35), suggesting that certain p53-mutant tumors may be sensitized to Fas-mediated cell death by chemotherapy.

In conclusion, we have found that the agonistic Fas monoclonal antibody CH-11 dramatically increases the apoptotic response to 5-FU and TS-targeted antifolates in MCF-7, HCT116 $p53^{+/+}$, and RKO cells. Induction of exogenous TS abrogated this synergistic interaction for the antifolates, but not 5-FU; however, the extent of the interaction was highly p53 dependent for each drug. Our findings suggest that the Fas signaling pathway is an important regulator of 5-FU- and antifolate-mediated cell death and that targeting the Fas pathway in conjunction with either 5-FU or antifolates may have therapeutic potential.

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The Roles of Thymidylate Synthase and p53 in Regulating Fas-Mediated Apoptosis in Response to Antimetabolites

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