Interferon-γ–Induced Sensitization of Colon Carcinomas to ZD9331 Targets Caspases, Downstream of Fas, Independent of Mitochondrial Signaling and the Inhibitor of Apoptosis Survivin

James Geller, Istvan Petak, Kinga Szekely Szucs, Katalin Nagy, David M. Tillman, and Janet A. Houghton
Division of Molecular Therapeutics, Department of Hematology-Oncology, St. Jude Children’s Research Hospital, Memphis, Tennessee

ABSTRACT
We have demonstrated previously a Fas-dependent component in thymineless death of human colon carcinoma cells. Importantly, the cytotoxic effects of thymidine deprivation induced by 5-fluorouracil (FUra) combined with leucovorin (LV) was enhanced by IFN-γ, and the synergism was shown to be dependent on Fas, FUra-induced DNA damage, and independent of p53. Subsequently we examined the potential for synergistic interactions between IFN-γ and the specific thymidylate synthase inhibitor, ZD9331. IFN-γ sensitized colon carcinomas to ZD9331-induced apoptosis and loss in clonogenic survival, also dependent on ZD9331-induced DNA damage, independent of p53. Synergism occurred in HCT116, demonstrating previously RNA-mediated FUra/LV cytotoxicity that could not be potentiated by IFN-γ. Manipulation of the Fas death receptor pathway from the level of the receptor (Nok1/Nok2, Fas overexpression, and DN-FADD) to the mitochondria (Bcl-xL and Bcl-2) did not modulate ZD9331 ± IFN-γ-induced cytotoxicity in HT29, with the exception that Nok1/Nok2-blocking antibodies partially protected HT29 from the cytotoxic activity of ZD9331 alone. However, IFN-γ alone (but not ZD9331) up-regulated the expression of caspases -3, -4, -7, and -8, and in combination with ZD9331 demonstrated enhanced caspase activation and cleavage of poly(ADP-ribose) polymerase that was not prevented by overexpression of Bcl-2. Additionally, IFN-γ increased the activity of the proteasome in HT29, leading to selective down-regulation of the anti-apoptotic protein survivin, whereas simultaneously increasing Fas expression. However, reduction in the survivin:Fas ratio by transfection of survivin small interfering RNA and/or overexpression of Fas did not affect sensitivity of HT29 to ZD9331 ± IFN-γ. Data demonstrate that IFN-γ combined with ZD9331 is synergistic in additional cell lines that demonstrate RNA-mediated FUra/LV cytotoxicity, and that a major target of interaction is at the level of caspases, downstream of Fas, and independent of involvement of either the mitochondria or survivin.

INTRODUCTION
Thymidylate synthase (TS) inhibitors continue to maintain their role as first line chemotherapy for the treatment of colorectal cancer. Efforts to maximize efficacy of TS inhibitor-based regimens have addressed ways to selectively modulate the cellular response to TS inhibition in tumor cells in vivo (1, 2), as well as to develop newer, more specific TS inhibitors (3). Effective modulation in enhancing the therapeutic selectivity of 5-fluorouracil (FUra) has been achieved with the reduced folate leucovorin (LV), which potentiates the anti-TS effects of FUra, resulting in more extensive induction of thymineless stress and cellular cytotoxicity (1). For these reasons, combinations of FUra with LV have proven superior to FUra alone (4). Other efforts have focused on antisense approaches (5, 6) to down-regulate important determinants of the cellular response to TS inhibitors including TS itself, or alternatively, dUTPase, an enzyme involved in maintaining intracellular nucleotide homeostasis after TS inhibition (7). Additional modulation of colorectal carcinoma tumor biology, with promising preclinical (8) and clinical (9) activity, is evident from our previous reports demonstrating that IFN-γ is synergistic in potentiating the cytotoxic effect of FUra/LV in human colon carcinoma cell lines, in part by modulation of the Fas death receptor and its signaling pathway (8). IFN-γ in combination with FUra/LV has already demonstrated activity in a Phase I trial (9), and currently this regimen is entering Phase II clinical investigation. The interaction mechanism requires FUra/LV-induced DNA damage, does not affect tumor cells that demonstrate FUra-induced RNA-mediated cytotoxicity, and is independent of p53 (8).

IFN-γ is a type II IFN known for its critical role in promotion of both the innate and adaptive immune responses, essential for host defense against infection (10). Promotion of the host response in immune surveillance of human cancers is also well documented (10). The mechanism(s) by which this cytokine modulates gene expression in cancer cells are unclear; however, >200 genes are known to be transcriptionally regulated by IFN-γ (11, 12).

More specific TS inhibitors including ZD1694 or ZD9331...
factors of the inhibitor of apoptosis (IAP) family or nuclear Similar to Bcl-2 and Bcl-xL, no role was identified for survival signaling complex, and is independent of the mitochondria. occurs downstream of Fas and its associated death-inducing signaling upstream of the mitochondria by dominant negative inhibition downstream of the mitochondria is critical to synergy ob-

On the basis of previous work in our laboratory demonstrat-
ing synergism between FUra/LV and IFN-γ in tumor cell killing in human colon carcinoma cell lines, we now demonstrate that IFN-γ and the specific TS inhibitor, ZD9331, also exert a synergistic cytotoxic effect in these model systems. In contrast to FUra combined with LV, ZD9331 ± IFN-γ displays a broader range of cytotoxicity that occurs via a thymineless stress-induced cell death mechanism. We also demonstrate that Fas receptor modulation via the use of blocking antibodies (Nok1/Nok2) partially inhibits cytotoxicity induced by ZD9331 but not ZD9331 + IFN-γ, whereas overexpression of Fas partially promotes Fas-mediated cytotoxicity, but does not increase the cytotoxic action of ZD9331 ± IFN-γ. In addition, blocking signaling upstream of the mitochondria by dominant negative fas-associated death domain (DN-FADD) or at the level of the mitochondria by overexpression of Bcl-xL or Bcl-2, did not inhibit the cytotoxic activity of ZD9331 ± IFN-γ, suggesting that a major target of interaction between ZD9331 and IFN-γ occurs downstream of Fas and its associated death-inducing signaling complex, and is independent of the mitochondria. Similar to Bcl-2 and Bcl-xL, no role was identified for survival factors of the inhibitor of apoptosis (IAP) family or nuclear factor κB (NFκB). Noteworthy is that survivin was downregulated after IFN-γ treatment due to IFN-γ-induced proteosomal degradation of this IAP member. Whereas the overall effect of IFN-γ was to decrease the survivin:Fas ratio, dramatic alteration of this ratio using combined isogenic Fas overexpression and small interfering RNA (siRNA) suppression of survivin did not affect the cytotoxic response of ZD9331/IFN-γ combinations. Although a dominant role for either the Fas death recep-
tor, signaling upstream of the mitochondria, or survivin could not be definitively demonstrated in the synergistic interaction between ZD9331 and IFN-γ, it is evident that IFN-γ increased the expression of vital caspases and, in combination with ZD9331, resulted in increased caspase activation that could not be inhibited by overexpression of Bcl-2, confirming that signaling downstream of the mitochondria is critical to synergy observed when ZD9331 and IFN-γ are combined.

**MATERIALS AND METHODS**

**Cell Lines.** The HT29 human colon carcinoma cell line was obtained from American Tissue Type Culture Collection, and VRC-4/c1 was established as reported previously (21, 22). HCT116 and HCT116 p53−/− were obtained from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). Cell cultures were maintained in low folate-containing, thymidine (dThd)-free media composed of RPMI 1640 containing 10% diazoy fetal bovine serum, 80 nM [6RS]5-methyltetrahydrofolate, and 712 μM Ca²⁺ (8).

**Retroviral Expression Vectors.** The retroviral expression vectors pMSCV-I-GFP [expressing green fluorescent protein (GFP)], pMSCV-Fas, (expressing human Fas and GFP separated by an internal ribosomal entry site sequence), and pMSCV-DN-FADD (expressing the death domain only of FADD) were kindly gifts from Drs. Jill M. Lahti and Vincent J. Kidd (St. Jude Children’s Research Hospital) and have been described previously (23). The retroviral expression vector pMSCV-Bcl-xL (expressing human Bcl-xL and GFP, separated by an internal ribosomal entry site sequence) and pMSCV-Bcl-2 (expressing human Bcl-2 protein) were kindly provided by Dr. John Cleveland (St. Jude Children’s Research Hospital). Retroviral supernatants were prepared as described previously (24). HT29 cells were incubated overnight in a 50% mixture of RPMI 1640 and supernatant in the presence of Polybrene (8 μg/ml; Sigma). After replacement of this medium with fresh viral supernatants and culture medium, HT29 cells were incubated at 37°C for an additional 48 h. The viral-transferred cells were isolated according to GFP expression using a fluorescence-activated cell sorter (Becton Dickinson), and stable GFP-positive cells were selected. The expression of Bcl-xL and Bcl-2 were confirmed by Western blotting. The expression of Fas was analyzed as in “Materials and Methods” describing Fas expression.

**Immunoblotting.** HT29, HT29GFP, HT29Bcl-xL, or HT29Bcl-2 cells were treated with ZD9331 (100 nM) ± IFN-γ (100 units/ml) for 24–72 h, followed by determination of the level of expression of procaspases-3 (Alexis Corporation), -4, -6, -7, -8, and -9 (MBL International Corp., Woburn, MA), as well as determination of the cleavage of procaspase-3, -7, -8, and poly(ADP-ribose) polymerase (Transduction Laboratories) by Western analysis. HT29 cells were also treated with IFN-γ (100 units/ml) ± MG132 (1 μM) for 48 h, and the levels of survivin (R&D Systems), Bcl-xL (Transduction Laboratories), XIAP (MBL), cellular FLICE inhibitory protein (c-FLIP) (Upstate), and cellular IAP-1 (c-IAP-1) (Alexis Corporation) were assessed by Western analysis. The fold reduction in survivin expression after treatment of HT29Fas cells with siRNA (400 nm) directed to the survivin gene was determined by absorbance measurements using NIH Image Software Version 1.61. Antibodies used for confirmation of successful retroviral transduction also include FADD and Bcl-2 (Transduction Laboratories). ZD9331 was a generous gift from AstraZeneca.

**Clonogenic Survival Assays.** Cell lines were plated at a density of 1500 cells/well in six-well plates (Costar). After overnight attachment, cells were treated, in triplicate, with the various treatment conditions for 72 h. Subsequently the media were aspirated and replaced with media supplemented with
were identified using Cy5: Cy3 ratios after background subtraction, filtering of bad spots (improper morphology, small size, intensity to background ratio < 1.4 and so forth), and global normalization. Dye flip replicates were performed for every experiment. Average fold-changes and 95% confidence intervals were generated from analysis of eight experiments.

**Proteasome Kinetics Assay.** HT29 cells were treated with IFN-γ (100 units/ml) ± MG132 (1 μM) for 48 h. Cells (5 × 10^4) were collected and washed twice in PBS. PBS (100 μl) containing 5 mM EGTA was added, and the cells were subsequently plated in a 96-well plate. Assay buffer [100 μl; PBS-5 mM EGTA containing 20 mM DTT and 50 μM suc-leu-leu-val-tyr (LL Vy)-amino-coumarin (AMC)] was added last to yield a final concentration in each well of 5 mM EGTA, 10 mM DTT, and 25 μM LL Vy-AMC. Cells were incubated at 37°C for 10 min. LL Vy-AMC is a specific substrate of the chymotryptic component of the proteasome complex. The active proteasome cleaves off the AMC-end of the substrate yielding free AMC that is fluorogenic, and measurable at an extinction wavelength of 390 nm and at an emission wavelength of 460 nm with the CytoFluor 2300 System. Proteasome activity was measured over a period of 30 min.

**siRNA Transfections.** HT29GFP and HT29Fas cells were plated at a density of 50,000 cells/well in six-well plates (Costar). After overnight fixation, transfection with 21-mer siRNA oligonucleotides directed against the survivin gene was performed using oligofectamine according to the manufacturer’s instructions (Invitrogen Life Technologies, Inc., Carlsbad, CA). The survivin siRNA sequence used was AAGCAUCUCGG-CGUUGCcGU, and a Duplex siRNA sequence (Scramble II Duplex), used as a control, was GCGCGCUUUGUAGGAU-GUUGCGCU. Each siRNA was 2'-deprotected, desalted, annealed, and purified by the manufacturer. For clonogenic assays, after a 6-h transfection period, cells were collected, counted using a Coulter Counter, and replated in six-well plates at a density of 1,000 cells/well. Nontransfected HT29GFP and HT29Fas cells from the same passage were also plated as such at this time and used as additional controls. After overnight fixation, the cells were treated, in triplicate, with ZD9331 (0–100 nM) ± IFN-γ (100 units/ml) and ± dThd (20 μM) for 72 h, and subsequently analyzed for clonogenic survival. Western analysis confirming survivin protein down-regulation at 24 h after transfection, as described previously, was performed each time siRNA was used. Additionally, survivin expression in HT29, with or without siRNA transfection, was assayed via Western analysis at multiple time points to determine the expected duration of protein suppression.

**RESULTS**

**ZD9331 and IFN-γ Are Synergistic in Human Colon Carcinoma Cell Lines Independent of p53.** In clonogenic survival assays, ZD9331 demonstrated cytotoxicity in the nM range (IC_{50} ~ 50 nM or less) in all of the cell lines examined (HT29, VRC5/c1, HCT116, and HCT116p53−/−). In all of the cases ZD9331-induced cytotoxicity was reversible by dThd, consistent with thymineless stress (and subsequent DNA damage) induced by TS inhibition as the predominant mechanism of
cell death. IFN-γ potentiated the cytotoxic activity of ZD9331 in a synergistic manner in all of the cell lines, an effect that was reversed by dThd supplementation in all of the cell lines tested, thereby demonstrating the requirement for ZD9331-induced DNA damage in the mechanism of sensitization by IFN-γ (Fig. 1). HT29, VRC5/c1, and HCT116p53−/− maintain a mutant p53 phenotype, whereas HCT116 is wild-type for p53, demonstrating the p53 independence of the interaction between ZD9331 and IFN-γ, similar to the that observed between FUra/LV and IFN-γ (26).

**ZD9331 ± IFN-γ Induce Apoptosis.** After 72-h exposure, quantitation of the sub-G1 compartment via fluorescence-activated cell sorter analysis demonstrated ZD9331-induced apoptosis in HT29GFP cells, potentiated in the presence of IFN-γ, and reversible by dThd (Fig. 2A), consistent with data derived in clonogenic survival assays (Fig. 1). Coincubation with the pan-caspase inhibitor ZVAD-FMK (50 μM) for 72 h was inhibitory to the induction of apoptosis induced by ZD9331 (0–100 nM) ± IFN-γ (100 units/ml) by ~50% in comparison with N-benzyloxy carbonyl Phe-Ala fluoromethylketone (ZFA) (20 μM; control; Fig. 2B). Clonogenic survival assays also demonstrated partial protection from ZD9331 ± IFN-γ-induced cytotoxicity by ZVAD-FMK (Fig. 2C).

**p53 Influences the Induction of Fas Expression by ZD9331.** We demonstrated previously that IFN-γ up-regulates Fas expression in human colon carcinoma cell lines independent of p53 (8). In contrast, FUra/LV-induced elevation in Fas expression is influenced by the p53 status, the increase being considerably greater in the presence of wild-type p53 (26). After 24-h exposure to ZD9331 (50–100 nM), Fas was elevated by 0.5–1-fold in HT29 (mp53) and HCT116p53−/− (p53 null), similar to results obtained with FUra/LV (26), in contrast to HCT116 (wild-type p53), which demonstrated 2.5-fold elevation in Fas expression (Fig. 3A). These results demonstrate a role for p53 in enhancing up-regulation of Fas in response to ZD9331. Of note in HT29, Fas up-regulation by IFN-γ (3.5-fold) was not enhanced by coexposure to ZD9331 (data not shown).

**Overexpression of Fas Sensitizes HT29 to Fas-Mediated Cytotoxicity.** HT29Fas was derived to critically evaluate the role of up-regulation of Fas expression alone in Fas-mediated or
Fig. 2  ZD9331 induces apoptosis. A, HT29 cells were treated for 72 h with ZD9331 (100 nM) ± IFN-γ (100 units/ml) ± thymidine (dThd) (20 μM); B, HT29 cells, 2 h after pretreatment with either the pan-caspase inhibitor ZVAD-FMK (50 μM) or control (ZFA; 20 μM) were treated with ZD9331 (0–100 nM) ± IFN-γ (100 units/ml) for 72 h and apoptosis (B) or clonogenic survival (C) determined. Results are the means of two (B) or three (C) determinations per point; bars, ±SD.

Fig. 3  In HT29 ZD9331 induces increased Fas expression, and over-expression of Fas partially sensitizes cells to Fas ligand (sFasL)-induced loss in clonogenic survival. A, fluorescence-activated cell sorter analysis of cell surface Fas expression in HT29, HCT116wt, and HCT116p53−/− cells after 24-h exposure to ZD9331 (50–100 nM); B, fluorescence-activated cell sorter analysis of cell surface Fas expression in HT29 cells retrovirally transduced with vector alone (HT29GFP) or vector containing a human Fas cDNA (HT29Fas); C, HT29GFP or HT29Fas were treated with sFasL (50 ng/ml) for 72 h, and the effect on clonogenic survival determined. Results in A and B constitute duplicate determinations, and in C the mean of three determinations; bars, ±SD.
ZD9331-induced cytotoxicity. Retroviral transduction of Fas (HT29Fas) in comparison with vector control (HT29GFP), yielded a 28.7-fold increase in the expression level of Fas (Fig. 3B). Clonogenic survival assays after 72-h exposure to soluble Fas ligand (sFasL) (50 ng/ml) demonstrated enhanced sensitivity to ligation of the Fas death receptor in HT29Fas, where clonogenic survival was decreased by 70% (Fig. 3C).

**ZD9331 + IFN-γ Demonstrate Interaction Sites Downstream of Fas.** In HT29 cells after 72-h exposure to varied concentrations of ZD9331 (0–100 nm), ZD9331-induced loss in clonogenic survival was completely reversed by dThd and partially inhibited in the presence of Nok1/Nok2 antibodies that block Fas/Fas ligand (FasL) interactions (500 ng/ml each), similar to data derived for FUra/LV (8). However, no protection after coincubation with Nok1/Nok2 antibodies was demonstrated when the cytotoxic activity of ZD9331 was potentiated in the presence of IFN-γ (100 units/ml; Fig. 4A). The cytotoxic activity of ZD9331 ± IFN-γ was examined subsequently in HT29GFP in comparison with HT29Fas after 72-h drug exposures (Fig. 4B). Loss in clonogenic survival was similar for ZD9331 alone or in combination with IFN-γ in both HT29GFP and HT29Fas. Data suggest that although sensitivity to TS inhibitors may be influenced by Fas, other targets downstream of the Fas death receptor are involved in the synergistic interaction between IFN-γ and ZD9331.

**Influence of DN-FADD or Bcl-xL Overexpression on Cytotoxicity Induced by ZD9331 ± IFN-γ in HT29 Isogenic Cell Lines.** Clonogenic survival assays were undertaken in HT29 isogenic cell lines to determine the role of signaling upstream of the mitochondria (DN-FADD), or involvement at the level of the mitochondria (Bcl-xL overexpression), in ZD9331-induced cytotoxicity either alone or in combination with IFN-γ. DN-FADD did not protect HT29 cells from ZD9331 (0–100 nm) either in the absence or presence of IFN-γ 100 units/ml (Fig. 5A). Similarly, transfection of DN-caspase-8 (23) did not protect these cells from the cytotoxic activity of ZD9331 ± IFN-γ (data not shown). Furthermore, at the level of the mitochondria, Bcl-xL overexpression did not protect cells from ZD9331-induced cytotoxicity alone or in combination with IFN-γ (Fig. 5B), neither did Bcl-2 overexpression afford protection (data not shown). Data suggest that target(s) downstream or independent of the mitochondria are involved in the interaction between ZD9331 and IFN-γ.

**Expression and Activation of Caspases.** After 24-h exposure of HT29 cells to IFN-γ (100 units/ml), mRNA for caspases-1, -3, -4, -5, -7, and -8 but not caspases -2, -6, or -9 were elevated by >2-fold as analyzed by cDNA microarray (Table 1). In contrast, mRNA for the caspases was not significantly elevated after 24-h exposure of HT29 to ZD9331 (50 nm; Table 1). Expression of procaspases -3, -4, -7, and -8, but not procaspases -6 or -9, determined by Western analysis, were elevated by 24 h, and consistently by 48 h, after exposure to IFN-γ (100 units/ml; Fig. 6A). No elevation in caspase expression after treatment with ZD9331 (50 nm) was evident by Western analysis (Fig. 6A), consistent with data obtained from cDNA microarray analysis. When ZD9331 and IFN-γ were combined, activation of caspases -3 and -7, and cleavage of poly(ADP-ribose) polymerase were potentiated at 48 h (Fig. 6A) and caspase-8 at 72 h (data not shown). IFN-γ-induced up-regulation of caspases -3, -4, -7, and -8, and enhanced caspase activation after treatment with ZD9331 + IFN-γ were also found in the presence of Bcl-2 overexpression (Fig. 6B), indicating no Bcl-2 inhibitory effect on caspase activation, consistent with the lack of protection of Bcl-xL or Bcl-2 seen in clonogenic survival assays.

**IFN-γ Up-Regulates Proteasomal Activity and Down-Regulates Expression of Survivin in HT29.** Proteasome kinetic assays demonstrated a marked increase in proteasomal chymotryptic activity after 48-h exposure to IFN-γ (100 units/ml), inhibited after simultaneous exposure of HT29 cells to the
proteasome inhibitor MG132 (1 μM; Fig. 7A). These data are in concordance with cDNA microarray analyses of IFN-γ-treated HT29 cells, which demonstrated significant elevation in expression of 34 of 35 cDNA oligonucleotides designated on the microarray to represent the genes that encode proteasomal subunits after IFN-γ (100 units/ml) exposure for 24 h (data not shown). Expression of survival factors known to be degraded by proteasomal cleavage and to be expressed in HT29 (XIAP, c-FLIP, c-IAP1, Bcl-xL, and survivin) were examined after treatment with IFN-γ (100 units/ml) for 48 h (Fig. 7B). Survivin expression was decreased by 48 h, whereas expression of other antiapoptotic proteins was unaffected by IFN-γ treatment. IFN-γ-induced down-regulation of survivin expression was attenuated by inhibition of proteasomal activity (MG132, 1 μM; Fig. 7C). Activation of the proteasome can also increase the activation of NFκB. However, treatment of HT29 cells with IFN-γ (100 units/ml) did not activate NFκB as determined by electrophoretic mobility shift analysis (data not shown).

No Influence of the Survivin:Fas Ratio in Sensitivity to ZD9331 ± IFN-γ. To explore the simultaneous biological modulation of increased Fas and decreased survivin expression on the cytotoxic activity of ZD9331 ± IFN-γ. HT29GFP and HT29Fas cell lines were used in conjunction with siRNA directed toward the survivin gene. Transfection of survivin siRNA (400 nM) decreased survivin protein expression by 24 h in HT29 (Fig. 8A), which was maintained for up to 120 h (data not shown). Survivin expression was also down-regulated 19.4-fold after siRNA exposure in HT29Fas by 24 h (Fig. 8A). After survivin siRNA transfection, thereby decreasing the survivin:Fas ratio by >500-fold in HT29Fas (19.4-fold decrease in survivin and 28.7-fold increase in Fas) in comparison with HT29, no increase in the cytotoxic activity of ZD9331 (0–100 nM) alone or in combination with IFN-γ (100 units/ml), either in HT29GFP (Fig. 8B) or HT29Fas (Fig. 8C), was demonstrated.

DISCUSSION

The development of newer and more selective folate-based inhibitors of TS has the potential for greater therapeutic selectivity in comparison with FUra/LV (27–29). In a study of 13 colon cancer cell lines, TS levels maintained predictive value with respect to response to FUra, but did not correlate with

Table 1  C-DNA microarray analysis of caspase expression

<table>
<thead>
<tr>
<th>Caspase</th>
<th>IFN-γ (100 units/ml)</th>
<th>ZD9331 (50 nM)</th>
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<tbody>
<tr>
<td>Caspase-1</td>
<td>3.8 (0.4)</td>
<td>1.3 (0.1)</td>
</tr>
<tr>
<td>Caspase-2</td>
<td>1.5 (0.3)</td>
<td>1.0 (0.03)</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>2.3 (1.3)</td>
<td>1.1 (0.1)</td>
</tr>
<tr>
<td>Caspase-4</td>
<td>2.8 (1.1)</td>
<td>1.1 (0.1)</td>
</tr>
<tr>
<td>Caspase-5</td>
<td>2.4 (0.9)</td>
<td>1.1 (0.07)</td>
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<tr>
<td>Caspase-6</td>
<td>1.6 (0.6)</td>
<td>1.1 (0.07)</td>
</tr>
<tr>
<td>Caspase-7</td>
<td>2.6 (0.5)</td>
<td>1.2 (0.1)</td>
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<tr>
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<tr>
<td>Caspase-10</td>
<td>1.6 (0.3)</td>
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resistance to the newer folate-based TS inhibitors (30). After exposure to pure TS inhibitors including ZD1694 and ZD9331, TS levels within colorectal tumor cell lines, including HT29, increased to a lesser degree than the increase observed in normal colonic epithelial cells after similar exposure (31), suggesting that toxicity from ZD9331 may be mitigated by the differential expression of TS levels in normal versus neoplastic colonic tissue.

We have demonstrated that the combination of IFN-γ/H9253 and ZD9331 displays synergistic cytotoxicity in human colon carcinoma cell lines, similar to the synergistic interaction between FURA/LV and IFN-γ, and requires ZD9331-induced DNA damage (TS inhibitory effect). This synergism does not depend on functional wild-type p53, as evidenced by synergistic cytotoxicity in cell lines expressing mp53 (HT29 and VRC5/c1), and in HCT116p53/H11002 cells. This is important in the setting of metastatic colorectal carcinoma where the majority of tumors display a mutant p53 phenotype (32). In addition, whereas FURA/LV is cytotoxic in HCT-116 via RNA-mediated damage (8), the current studies demonstrate that ZD9331 and IFN-γ, unlike FURA/LV and IFN-γ, are synergistic in HCT116 cells via an anti-TS effect (non-RNA mediated), suggesting that this combination may have a broader range of activity in colon carcinomas.

Mechanistically, the role of Fas/FasL interactions as mediators of anticancer therapy has been variable, dependent on the cell type and agent under investigation (33, 34). In colon carcinoma cell lines, the interaction between cytotoxic agents and Fas signaling pathways may be specific for inhibitors of TS, such as FURA, and not for other agents that damage DNA, including doxorubicin, topotecan, or VP-16 (25). Fas is a type I transmembrane protein of Mr 48,000 belonging to the tumor necrosis factor receptor family. FasL is a cytokine belonging to the tumor necrosis factor family, synthesized as a type II membrane protein of Mr 40,000 (34). After FasL-induced formation of Fas receptor microaggregates, recruitment of FADD and procaspase-8 occur to form the death-inducing signaling complex. Subsequent downstream events proceed by either a type I mechanism involving high levels of caspase-8 activation at the death-inducing signaling complex followed by direct activation of executioner caspases or by a Type II mechanism involving mitochondrial activation and initiation of an amplification loop required to ultimately yield downstream caspase activation (35, 36). Human colon carcinoma cell lines used in the current study represent both type I (GC3/c1) and type II cells (HT29, VRC5/c1, and HCT116) for death receptor signaling (37), and in both cell types IFN-γ and TS inhibitor combinations demonstrate synergistic cytotoxicity.

We demonstrated previously the importance of the Fas signaling pathway in thymineless stress-induced apoptosis in colon carcinoma cells (25, 26, 38–40) and as a component in the sensitivity of human colon carcinoma cell lines to FURA/LV-induced cytotoxicity, potentiated by IFN-γ (8). The purest manner in which to study thymineless stress-induced apoptosis is to use TS− cells, where thymineless death is induced solely by depriving the cells of preformed dThd, in contrast to TS inhibitors that are known to have targets in addition to the enzyme, TS. In TS− cells, blocking Fas/FasL interactions with the Nok1 antibody completely protected cells from thymineless death induced after dThd withdrawal (39), demonstrating the essential role of Fas, FasL, and Fas signaling in the mechanism of cell death induced by thymineless stress. Furthermore, Nok1 +

![Image](https://example.com/image.png)
Nok2 antibodies partially, but not completely, protected HT29 cells from the cytotoxic action of FUra/LV either in the absence or in the presence of IFN-γ, demonstrating a Fas-dependent component in FUra/LV-induced thymineless death and in sensitization by IFN-γ (8). The importance of Fas has also been demonstrated in the mechanism of FUra-induced apoptosis of hepatocellular carcinoma cells in vitro (41) and in mouse thy-
unit complex known to be responsible for degradation of numerous cellular protein substrates via ubiquitin targeting (57, 58). Functionally, the proteasome has known proapoptotic and antiapoptotic effects. Activation of the proteasome can be lethal to cells (e.g., thymocytes), where proteasome inhibition protects from glucocorticoid- or etoposide-induced cytotoxicity via prevention of c-IAP1 and XIAP degradation (59). The use of proteasome inhibitors to protect from the toxicity of chemotherapeutic agents has also been documented in human leukemic cells (60). In general proteasome inhibitors have reached clinical trial due to their effective induction of cytotoxicity in numerous cancers (61).

Substrates of the proteasome important in apoptosis include the antiapoptotic factors Bcl-2, Bcl-xL, c-IAP1, c-FLIP, XIAP, and survivin (59, 62–64), and activation of proteasomal activity can also elevate the expression of the survival factor NFκB (65). Because HT29 lacks Bcl-2 and c-IAP2, the influence of IFN-γ on levels of expression of XIAP, Bcl-xL, c-IAP1, c-FLIP, and survivin was determined. Whereas IFN-γ increases cell surface Fas expression, the expression of survivin was selectively decreased by IFN-γ treatment, dependent on enhanced proteasomal activity, and independent of NFκB. Survivin expression has been reported to influence colon tumorigenesis (66) as well as the prognosis for survival after curative resection of stage II colorectal carcinomas (67). In addition, the survivin:Fas ratio has been reported to predict for aggressive behavior and/or recurrent disease in neuroblastoma, as well as in pediatric renal tumors of multiple subtypes (68, 69).

Using HT29Fas and HT29GFP isogenic cell lines and siRNA oligonucleotides directed against survivin, reduction of the survivin:Fas ratio by >500-fold did not enhance the cellular sensitivity to ZD9331 ± IFN-γ. Thus, IFN-γ-induced down-regulation of survivin expression did not appear to be involved directly in the interaction mechanism between ZD9331 and IFN-γ in HT29 cells. The mechanisms behind the synergistic interaction between IFN-γ and TS inhibitors are complex. The interaction between FUra/LV and IFN-γ in inducing a cytotoxic response and the involvement of Fas and its signaling pathway determined in human colorectal carcinoma cell lines, will be additionally explored in Phase II investigation, based on results from the Phase I trial (9). The studies presented here also provide a rationale for combining IFN-γ with the new and specific TS inhibitor, ZD9331, that may induce a synergistic interaction with IFN-γ in a higher proportion of colorectal tumors. Furthermore, a major target of this interaction appears to be at the level of the caspases, by enhanced expression of caspases in the presence of IFN-γ, and enhanced activation when IFN-γ and ZD9331 are combined, downstream of Fas and independent of the mitochondria. The role of proteasomal activation by IFN-γ followed by degradation of survivin, together with IFN-γ-induced increase in Fas expression that results in a significant decrease in the survivin:Fas ratio, whereas an attractive target for potential interaction between IFN-γ and TS inhibitors demonstrated no influence on ZD9331/IFN-γ-induced cytotoxicity in HT29 in the current investigation. However, this would be worthy of more detailed exploration in an expanded panel of human colorectal carcinoma cell lines and in other cancer models.

Fig. 8 Decreasing the survivin:Fas ratio does not sensitize HT29 isogenic cell lines to the cytotoxic effects of ZD9331 ± IFN-γ. A, HT29 or HT29Fas cells were transfected with siRNA (400 nM) directed at the survivin gene or control siRNA (400 nM; Scramble II duplex; Dharmacon), and analyzed by Western analysis at 24 h. HT29GFP (B) or HT29Fas (C) were transfected with siRNA (400 nM) directed at the survivin gene (or control), and subsequently treated with ZD9331 (0–100 nM) ± IFN-γ (100 units/ml) ± thymidine (20 μM) for 72 h. Clonogenic survival was assessed as described previously (mean of three determinations per point); bars, ±SD.
REFERENCES


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