

P21^{Cip1} Is a Critical Mediator of the Cytotoxic Action of Thymidylate Synthase Inhibitors in Colorectal Carcinoma Cells

James I. Geller,^{1,4} Kinga Szekely-Szucs,¹ Istvan Petak,² Belinda Doyle,¹ and Janet A. Houghton^{1,3}

¹Division of Molecular Therapeutics, Department of Hematology-Oncology, St. Jude Children's Research Hospital, Memphis, Tennessee; ²Semmelweis University and ^{1st} Institute of Pathology and Experimental Cancer Research, Budapest, Hungary

ABSTRACT

We have demonstrated previously that interferon (IFN)- γ sensitizes human colon carcinoma cell lines to the cytotoxic effects of 5-fluorouracil combined with leucovorin and to the thymidylate synthase inhibitor, ZD9331, dependent on thymineless stress-induced DNA damage, independent of p53. Here we demonstrate that the cyclin-dependent kinase (CDK) inhibitor p21^{Cip1} regulates thymineless stress-induced cytotoxicity in these cells. HCT116 wild-type (wt) and p53^{-/-} cells underwent apoptosis and loss in clonogenic survival when exposed to ZD9331, whereas p21^{Cip1}^{-/-} cells were resistant. In contrast, IFN- γ induced marked cytotoxicity in p21^{Cip1}^{-/-} cells only. ZD9331 induced p21^{Cip1} up-regulation in all of the cell lines examined, as did thymidine deprivation in thymidylate synthase-deficient (thymidylate synthase⁻) cells. Furthermore, selective induction of p21^{Cip1} in RKO was sufficient to induce apoptosis. P21^{Cip1}, cdk1, cdk2, and cyclin E mRNA expression increased coincident with S-phase accumulation in HT29 cells treated with ZD9331 or 5-fluorouracil/leucovorin, as demonstrated by cDNA microarray analyses. Cell cycle analyses revealed that HCT116 wt and p21^{Cip1}^{-/-} cells accumulated in S phase within 24 h of ZD9331 exposure; however, wt cells exited S-phase more rapidly, where apoptosis occurred before mitosis, either in late S or G₂. Finally, the CDK inhibitor roscovitine potentiated the cytotoxic activity of ZD9331 in both wt and p21^{Cip1}^{-/-} cells, strongly suggesting a role for p21^{Cip1}-dependent CDK inhibition in cytotoxicity induced by thymidylate synthase inhibition. In summary, p21^{Cip1} positively regulates the cytotoxic action of thymidylate synthase inhibitors, negatively regulates the cytotoxic action of IFN- γ , and enhances S-phase exit after thymineless stress, possibly via interaction with CDK-cyclin complexes.

INTRODUCTION

Colorectal cancer is a leading cause of cancer-related morbidity and mortality each year in the United States. Thymidylate synthase inhibitors, such as 5-fluorouracil, continue to maintain their role as first-line chemotherapy for the treatment of colorectal cancer. Efforts to maximize efficacy of thymidylate synthase inhibitor-based regimens have addressed ways to selectively modulate the cellular response to thymidylate synthase inhibition (1), as well as to develop more specific thymidylate synthase inhibitors including ZD9331 and ZD1694 (tomudex and raltitrexed). These folate-based inhibitors of thymidylate synthase hold promise for greater therapeutic selectivity compared with 5-fluorouracil/leucovorin (2). At this time, however, our understanding of key effectors downstream of DNA damage required for thymidylate synthase inhibitor-induced cytotoxicity remains limited.

Previous work in our laboratory has demonstrated synergism between 5-fluorouracil/leucovorin combined with interferon (IFN)- γ in

tumor cell killing in human colorectal cancer, with promising preclinical (3) and clinical (4) activity. The interaction mechanism requires 5-fluorouracil/leucovorin-induced DNA damage and does not affect cells that demonstrate 5-fluorouracil-induced RNA-mediated cytotoxicity that occurs in normal gastrointestinal tissues (1, 5, 6) or certain human colorectal cancer cell lines (e.g., HCT8 and HCT116; ref. 3). Thymineless stress-induced apoptosis in colorectal cancer cells devoid of thymidylate synthase activity (thymidylate synthase⁻ cells; refs. 1, 7), 5-fluorouracil/leucovorin-induced cytotoxicity (3), and the synergistic interaction between 5-fluorouracil/leucovorin and IFN- γ (3, 4) all involve the Fas death receptor and its signaling pathway. More recently, we have demonstrated that IFN- γ is also synergistic in potentiating the cytotoxic effect of ZD9331 (2). In contrast to 5-fluorouracil/leucovorin + IFN- γ , ZD9331 +/- IFN- γ displays a broader range of cytotoxicity that occurs via DNA damage-induced cell death. In expanded studies with ZD9331, we demonstrated additional "apoptosis-related" sites of interaction between thymidylate synthase inhibitors and IFN- γ , independent of Fas, and downstream of the mitochondria, involving up-regulation and activation of specific caspases (2). All of the studies confirm that thymidylate synthase inhibition, either via conventional agents (5-fluorouracil/leucovorin) or via the newer thymidylate synthase inhibitors (ZD9331), induce apoptosis and cytotoxicity in human colorectal cancer cells independent of the cell-cycle/DNA-damage response protein p53. However, the role of the multifunctional cell cycle-related protein p21^{Cip1} in these models is less well characterized.

P21^{Cip1} functions at multiple levels (8), and although knowledge regarding the multifunctionality of this protein continues to increase, many of its functions remain to be determined. P21^{Cip1} is well known as a p53 response gene (9) capable of inhibiting multiple cyclin-dependent kinases (CDKs), resulting in the induction of G₁ or G₂ cell cycle arrest, assuring the normal progression of cells through the cell cycle (10). CDKs are protein kinases that, when complexed to the appropriate cyclin, enhance cell cycle progression (reviewed in ref. 10). P21^{Cip1} null cells, paradoxically, appear more susceptible to accumulation in an arrested phase of the cell cycle, including S phase (11). Additionally, deletion of p21^{Cip1} has been shown to induce uncoupling of S phase and mitosis in cells treated with anticancer agents (12), and p21^{Cip1}-induced G₁ arrest itself can lead to subsequent depletion of mitosis-control proteins and abnormal mitosis with endoreduplication in recovering cells (13). These cell cycle phenomena are presumed to be important in determining whether or not a cell will recover from a given form of DNA damage.

As an important mediator of such cell cycle phenomena, which are now known to play a critical role in apoptotic responses (14), it is not surprising that p21^{Cip1} has been reported to have both pro- and antiapoptotic roles. Overexpression of p21^{Cip1} in human cancer cell lines has been reported to induce apoptosis (15), implying a proapoptotic role, whereas overexpression of p21^{Cip1}, leading to G₁ arrest, can protect against p53-mediated apoptosis in other human cancer models (16). P21^{Cip1} may also be involved in the regulation of apoptosis induced by tumor necrosis factor α (17), tumor necrosis factor-related apoptosis-inducing ligand (18, 19), and Fas (20), as well as cytotoxic agents (11, 19, 21).

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Requests for reprints: Janet A. Houghton, Division of Molecular Therapeutics, Department of Hematology-Oncology, St. Jude Children's Research Hospital, 332 North Lauderdale, Memphis, TN 38105. Phone: 901-495-3456; Fax: 901-495-3966; E-mail: janet.houghton@stjude.org.

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In the current study we have demonstrated, using HCT116 isogenic cell lines, that p21^{Cip1} ^{-/-} cells are highly resistant to the cytotoxic effects of thymidylate synthase inhibition mediated by ZD9331 and are highly sensitive to IFN- γ . In contrast, wild-type (wt) and p53 ^{-/-} cells are sensitive to ZD9331 and resistant to IFN- γ treatment alone, whereas the cytotoxic action of ZD9331 is potentiated by IFN- γ (2). All of the HCT116 isogenic cell lines were equally sensitive to 5-fluorouracil/leucovorin, mediated by RNA-dependent cytotoxicity and also to etoposide and doxorubicin. Taken together, the data suggest that p21^{Cip1} plays a critical role in the positive regulation of apoptosis and in the cytotoxic response to thymidylate synthase inhibitors, specific to thymineless stress-induced DNA damage, independent of p53 (2), whereas p21^{Cip1} is an important negative regulator of cytotoxicity induced by IFN- γ in human colorectal cancer cells. Additional investigations into cell cycle-related events are presented.

MATERIALS AND METHODS

Cell Lines. HT29 was obtained from American Tissue Type Culture Collection (Manassas, VA). The thymidylate synthase⁻ cell line (GC₃/clthymidylate synthase⁻c3/c3) is a doubly cloned mutant isolated from wt GC₃/cl (7). HCT 116 wt, p53^{-/-}, and p21^{Cip1}^{-/-} cells were provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD), and RKO was obtained from Dr. Michael Kastan (St Jude Children's Research Hospital, Memphis, TN). RKO cells with the ecdysone-inducible expression of p21^{Cip1} or p27^{Kip1}, referred to as RKO p21 IND or RKO p27 IND, respectively, were obtained from Drs. Zhen Fan and Mathias Schmidt (The University of Texas M.D. Anderson Cancer Center, Houston, TX). Cell cultures were maintained in low folate-containing, thymidine (dThd)-free media composed of RPMI 1640 containing 10% dialyzed fetal bovine serum, 80 nM [6RS]5-methyltetrahydrofolate, and 712 μ M Ca²⁺ (2, 3).

Clonogenic Survival Assays. Cell lines were plated at a density of 1,500 cells/well in six-well plates (Costar). After overnight attachment, cells were treated, in triplicate, with the various treatment conditions for 72 hours. Subsequently the media were aspirated and replaced with media supplemented with dThd (20 μ M). Cells were harvested 5–7 days after dThd rescue (equivalent to seven doubling times), and clonogenic survival was evaluated as described previously (2, 3, 7, 22). ZD9331 was a generous gift from Astra-Zeneca.

Apoptosis Assays. Cell lines were plated at a density of 100,000–200,000 cells/well in six-well plates. After overnight attachment, HCT116 isogenic cell lines were each treated, in duplicate, with ZD9331 (50 nM) for 72 hours. RKO, RKO p21 IND, and RKO p27 IND cells were treated with ZD9331 (50 nM), Muristerone A (3 μ M; Invitrogen, Carlsbad, CA), alone or in combination, for total exposures of 48 hours or 72 hours. All of the cells, both floating and attached, were combined after trypsinization. After fixation with 70% ethanol, samples were stored at -20° C. Before analysis, samples were prepared in 1 ml of Na₂HPO₄ buffer (pH 7.8) containing 50 μ g/ml RNase and filtered. Apoptotic cells were detected as a sub-G₁ fraction after propidium iodide staining and analyzed using a Becton Dickinson FACScan (2, 22).

Fas Expression. HCT116 isogenic cell lines were plated at a density of 200,000 cells/well in 12-well plates. After overnight attachment, cells were either untreated or treated with ZD9331 (100 nM), in duplicate, for 24 hours. Cell surface Fas expression was determined by fluorescence-activated cell sorter analysis using standard procedures, as described previously (2, 22).

Cell Cycle Analysis. HCT116 wt and p21^{Cip1}^{-/-} cells were plated at a density of 200,000–400,000 cells/well in six-well plates. After overnight attachment, cells were either untreated or treated with ZD9331 (50 nM) for 24, 48, or 72 hours, in duplicate. At collection, the media (and floating cells) were discarded, and the remaining attached cells were washed with Hank's solution, trypsinized, suspended in PBS solution and centrifuged at 1400 rpm \times 10 min at 4 $^{\circ}$ C. The liquid was aspirated and replaced with 1 ml of cell cycle buffer [10 mmol/L 1,4-piperazinediethanesulfonic acid (pH 6.8), 0.1 mol/L NaCl, 2 mmol/L MgCl₂, and 0.1% Triton X-100] mixed with RNase (100 ng/ml). The cells were suspended by repetitive pipetting and then incubated at 37 $^{\circ}$ C for 30 min. Propidium iodide (20 μ L; 1 mg/ml) was added and cell cycle distribution determined by fluorescence-activated cell sorter analysis. G₁-, S-, and G₂M-

phase cells were quantitated using the Modfit software program (Verity Software House, Topsham, ME). M-phase analysis was conducted using immunofluorescent detection of phosphorylated histone H3 (23). HCT116 wt and p21^{Cip1}^{-/-} cells were either untreated, or treated with ZD9331 (50 nM) for 24, 48, 72, and 96 hours. Harvested cells (attached and floating) were washed in PBS, fixed in 70% ethanol, and stored at -20° C for up to 72 hours. After fixation, cells were washed twice with PBS, resuspended in 1 ml of PBS containing 0.25% Triton X-100, incubated at room temperature for 15 min, and then resuspended in 100 μ L of primary antibody solution [0.75 μ g rabbit polyclonal antiphosphohistone 3 antibody (Upstate Biotechnology, Lake Placid, NY) in 100 μ L of antibody buffer (sterile PBS containing 0.5% Tween and 5% fetal bovine serum)]. After 3-hour incubation at room temperature, the cells were rinsed with 1 mL of antibody buffer and resuspended in 100 μ L of secondary fluorescein isothiocyanate-conjugated goat antirabbit antibody (Sigma-Aldrich, St. Louis, MO) diluted 1:30 in antibody buffer. After 1 hour at room temperature, cells were rinsed with antibody buffer and resuspended in 1 mL of 0.033 mg propidium iodide/1 mL PBS solution. After 1 hour of incubation at room temperature in the dark, cells were analyzed using a Becton Dickinson FACScan.

cDNA Microarrays. HT29 cells, cultured in a T75 flask, were treated when 50–70% confluent with ZD9331 (50 nM) or 5-fluorouracil/leucovorin (3 μ M/1 μ M) for 24 hours. RNA was collected using standard techniques using RNazolB (TEL-TEST Inc., Friendswood, TX), and subjected to quality control as described previously (2). The human cDNA microarray, constructed in the St Jude Children's Research Hospital Hartwell Center for Bioinformatics, contains \sim 9,400 PCR products corresponding to human expressed sequence tags (the Human Unigene 1 collection) obtained from Incyte Genomics (Palo Alto, CA) designated as the H1 chip. Data files generated from the chip experiments were imported to Spotfire Decisionsite for Functional Genomics (Spotfire Inc., Somerville, MA) for additional analysis. Outliers were identified using Cy5/Cy3 ratios after background subtraction, filtering of inappropriate spots (improper morphology, small size, and intensity/background ratio $<$ 1.4), and global normalization. Dye flip replicates were performed for every experiment. Average fold-changes and SDs were generated from analysis of four experiments for each treatment condition.

RNase Protection Assay. HT29 cells were either untreated or treated with various agents [IFN- γ (100 units/ml), 5-fluorouracil/leucovorin (3 μ M/1 μ M), or ZD9331 (50 nM)] for 24 hours, and RNA was extracted as described above. The RiboQuant Multi-Probe RNase protection assay was performed according to the manufacturer's specifications (PharMingen). The hCC-2 multiprobe template panel was used (p130, Rb, p107, p53, p57, p27^{Kip1}, p21^{Cip1}, p19, p18, p16, p14/15, L32, and GAPDH). Thymidylate synthase⁻ cells were grown in dThd-replete or -depleted media for 72 hours, and RNA was extracted as described above. The RNase protection assay was performed using the hStress-1 multiprobe template panel (p53, GADD45, c-Fos, p21, Bax, Mcl-1, L32, and GAPDH).

Western Analysis. HT29, RKO, RKO p21 IND, RKO p27 IND, and HCT116wt were either untreated or treated with ZD9331 (100 nM) or muristerone (3 μ M) for either 48 hours only or for 2, 8, 24, 48, and 96 hours, followed by determination of the level of expression of p21^{Cip1} (Oncogene/Calbiochem), p27^{Kip1} (BD PharMingen), or actin (Sigma) by Western analysis, as described previously (2, 24).

RESULTS

p21^{Cip1} Regulates the Cytotoxic Response to Thymidylate Synthase Inhibitors and IFN- γ in Colorectal Cancer. In clonogenic survival assays, ZD9331 demonstrated cytotoxicity in the nM range (IC₅₀ approximately 30–50 nM) in HCT116wt and HCT116p53^{-/-} cell lines (Fig. 1A; 2). In each case ZD9331-induced cytotoxicity was reversible by dThd, consistent with thymineless stress (and subsequent DNA damage) induced by thymidylate synthase inhibition as the predominant mechanism of cell death. IFN- γ potentiated the cytotoxic activity of ZD9331 in a synergistic manner in these cell lines, an effect also reversed by dThd, demonstrating the requirement for ZD9331-induced DNA damage in the mechanism of sensitization by IFN- γ (Fig. 1A; 2). In contrast, p21^{Cip1}^{-/-} cells were highly resistant to the effects of thymidylate synthase inhibition by ZD9331

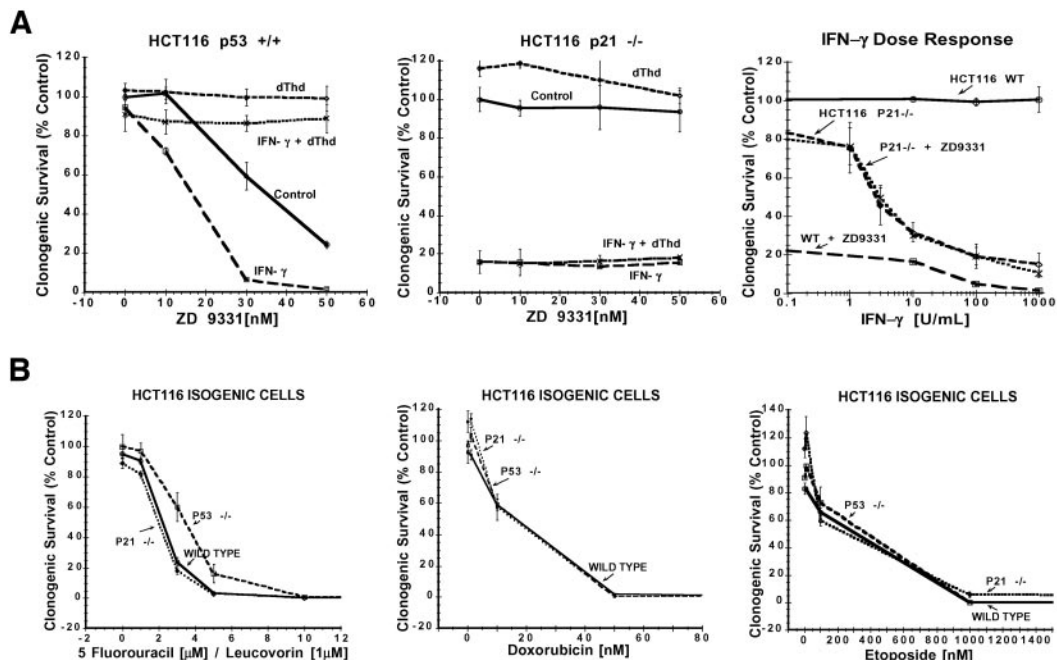


Fig. 1. P21^{Cip1} is required for ZD9331-induced loss in clonogenic survival and such requirement is specific to thymineless stress-induced cell death, whereas p21^{Cip1} mediates resistance to IFN-γ. HCT116 isogenic cell lines were treated with various concentrations of ZD9331 (0–50 nM) either in the absence or presence of IFN-γ (100 units/ml) or dThd (20 μM) for 72 hours, or IFN-γ (0–1000 units/ml) in the absence or presence of ZD9331 (50 nM) for 72 hours (A). HCT116 isogenic cells lines (wt, p53^{-/-}, p21^{Cip1}^{-/-}) were treated with various concentrations of 5-fluorouracil/leucovorin (0–10 μM 5-fluorouracil/1 μM leucovorin), doxorubicin (0–50 nM), or etoposide (0–1000 nM) for 72 hours (B). Clonogenic survival is reported as percentage of control (untreated). Data (% of control) represent the mean of three determinations at each concentration of ZD9331; bars, ±SD.

(Fig. 1A; IC₅₀ approximately 1 μM; data not shown) but were exquisitely sensitive to IFN-γ (Fig. 1, A and B). Treatment of HCT116wt and p21^{Cip1}^{-/-} cells with another pure thymidylate synthase inhibitor, Tomudex, yielded similar results (data not shown). Of note, whereas HCT116wt cells were completely resistant to IFN-γ alone (Fig. 1A), pharmacologically relevant IFN-γ concentrations (10 units/ml; 4) were sufficient to sensitize HCT116wt to ZD9331 (28% versus 16% survival) as well as to sensitize HT29 to 5-fluorouracil/leucovorin treatment (data not shown). Extremely low concentrations of IFN-γ alone were sufficient to induce a cytotoxic response in HCT116 p21^{-/-} cells (25% loss in clonogenic survival at 1 units/ml; IC₅₀ < 3 units/ml; Fig. 1A).

p21^{Cip1} Does Not Regulate the Cytotoxic Response to Other Investigated Agents. Agents that induce uridine-reversible RNA-mediated damage in HCT116 cells (5-fluorouracil/leucovorin), alkylation or topoisomerase I inhibition (doxorubicin), or topoisomerase II inhibition (etoposide) did not demonstrate preferential cytotoxicity in HCT116wt, p53^{-/-}, or p21^{Cip1}^{-/-} isogenic cell lines, with the exception that p53^{-/-} cells maintained a slight increased resistance to 5-fluorouracil/leucovorin in survival assays when compared with

wt or p21^{-/-} cells (Fig. 1B). Data demonstrate that p21^{Cip1} is required for thymineless-stress-induced cell death in HCT116, and the requirement for p21^{Cip1} is specific for cytotoxicity induced by this mode of cellular stress.

p21^{Cip1} Is Required for Thymineless Stress-Induced Apoptosis, Independent of Induced Elevation of Fas Cell-Surface Expression. HCT116wt and p53^{-/-} cells readily underwent an equivalent degree of apoptosis when exposed to ZD9331 (50 nM, 72 hours), whereas p21^{Cip1}^{-/-} cells demonstrated minimal apoptosis above baseline after such exposure (Fig. 2A). Because the Fas signaling pathway is important in thymineless stress-induced apoptosis (3, 4, 7), we explored the possible role of deregulated Fas expression in resistance of p21^{Cip1}^{-/-} cells to the consequences of thymidylate synthase inhibition. Cell surface Fas expression was determined in HCT116 isogenic cell lines both before and after treatment with ZD9331 (50 nM, 24 hours). Data demonstrated that Fas expression was reduced in p53^{-/-} cells but increased in p21^{Cip1}^{-/-} cells, compared with wt controls, and all of the cell lines demonstrated an ~2.5-fold increase in Fas expression after 24-hour exposure to ZD9331 (Fig. 2B).

Fig. 2. ZD9331 induces apoptosis in HCT116 wt and p53^{-/-} cells, dependent on p21^{Cip1}, independent of Fas cell-surface expression. A, HCT116 isogenic cell lines were treated with ZD9331 (50 nM) for 72 hours and the sub-G₁ (apoptotic) fraction quantified via fluorescence-activated cell sorter analysis. B, fluorescence-activated cell sorter analysis of cell surface Fas expression in HCT116 isogenic cell lines cells after 24-hour exposure to ZD9331 (100 nM).

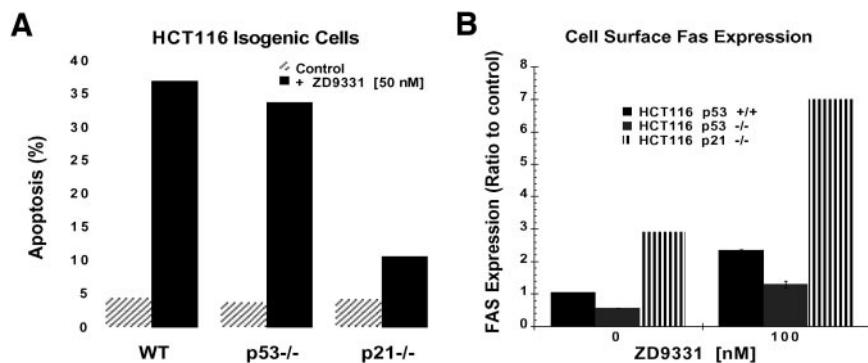
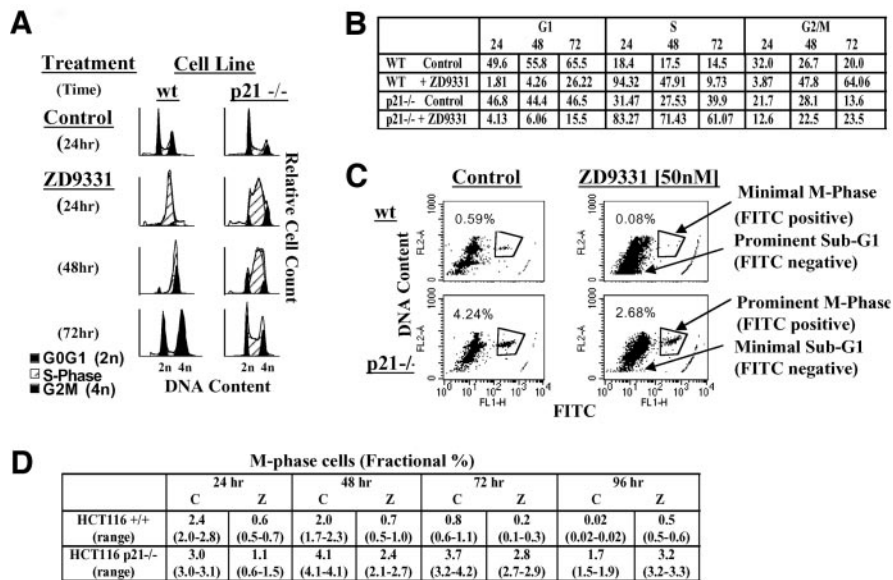


Fig. 3. P21^{Cip1} is required for rapid S-phase exit and for the induction of apoptosis, before M phase, after treatment with ZD9331. HCT116 wt and p21^{Cip1}−/− cells were treated with ZD9331 (50 nM) for 24, 48, or 72 hours, and cellular DNA content was assayed (A) via fluorescence-activated cell sorter analysis presented as DNA histograms demonstrating G₁, S, and G₂ peaks, and (B) additionally quantitated with the Modfit cell cycle software. One representative experiment is presented. HCT116 wt and p21^{Cip1}−/− cells were treated with ZD9331 for 72 hours after labeling with an M phase-specific fluorescein isothiocyanate-conjugate antiphosphohistone 3 antibody, DNA content and cell cycle distribution were determined via fluorescence-activated cell sorter analysis. C, HCT116 wt or p21^{Cip1}−/− cells either untreated or treated with ZD9331 for 72 hours are depicted graphically as examples. Fluorescein isothiocyanate-positive (M-phase positive) cells are gated, and the percentage of cells gated (*i.e.*, the M-phase fractional percentage) is provided. D, tabulated M-phase fractional percent data for control and treated samples at the various time points are presented and represent the mean of duplicate experiments. C, control; Z, ZD9331.



P21^{Cip1} Promotes Exit from S Phase and Accumulation of Cells in Sub-G₁ after Thymidylate Synthase Inhibition. After treatment with ZD9331 (50 nM), HCT116wt and p21^{Cip1}−/− cells each accumulated in S phase by 24 hours (Fig. 3, A and B). By 48 hours, wt cells were exiting S phase and progressing into G₂M, with evidence of a sub-G₁ apoptotic fraction (data not shown), in contrast to p21^{Cip1}−/− cells, which remained in S phase. At 72 hours, the S-phase fraction in wt cells approximated control levels, and the sub-G₁ fraction was >35% (Fig. 2A). In contrast, S-phase arrest in p21^{Cip1}−/− cells persisted, and no significant sub-G₁ fraction was detected. Data support a functional role for p21^{Cip1} in facilitating S-phase exit in HCT116wt cells after thymidylate synthase inhibition.

HCT116wt Cells Undergo Apoptosis before M-Phase Entry after Thymidylate Synthase Inhibition. HCT116 wt and p21^{Cip1}−/− cells were either untreated or treated with ZD9331 (50 nM) for up to 96 hours, and their M-phase fraction was analyzed. Representative graphic analyses at 72 hours are shown for each condition (Fig. 3C), and the average of duplicate independent experiments for all of the conditions and time points is tabulated (Fig. 3D). At 24 hours, the M-phase fraction was greatly reduced in wt and p21^{Cip1}−/− cells, consistent with S-phase accumulation in both cell lines after treatment with ZD9331. As wt cells progressed through S and into G₂M phases, the low M-phase fraction persisted, in contrast to p21^{Cip1}−/− cells, which demonstrated an increased M-phase fraction at 48 hours. By 72 hours, dramatic differences were observed in control *versus* ZD9331-treated wt cells. Despite a decrease in M-phase in control cells due to increasing confluence (observed by confocal microscopy), treated cells demonstrated an almost nonexistent M-phase fraction at this time. At 96 hours, the M-phase fraction had returned to control levels in p21^{Cip1}−/−-treated cells. The sub-G₁ fraction, detected in ZD9331-treated wt cells, did not label positive for phosphohistone-H3, indicating that cells were actively undergoing apoptosis before entry into M-phase (Fig. 3C). Therefore, data are consistent with entry of wt cells into the sub-G₁ compartment after thymidylate synthase inhibition either late in S phase or in the G₂ phase of the cell cycle.

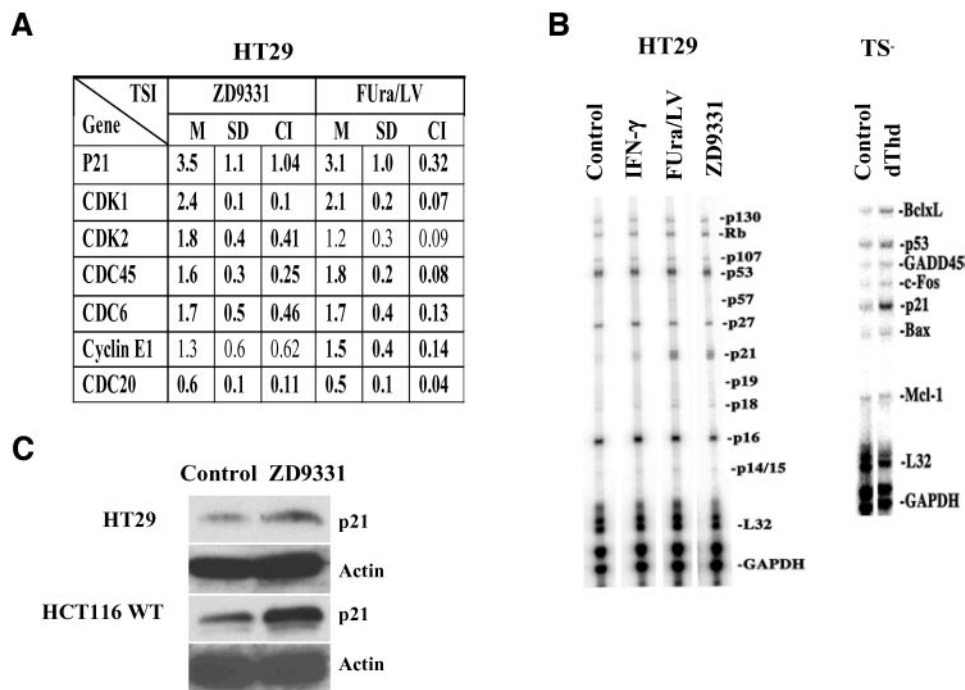
ZD9331-Induced S-Phase Arrest Leads to Increased Expression of Specific Cell Cycle-Related Genes. Treatment with ZD9331 (data not shown) or 5-fluorouracil/leucovorin (22) led to S-phase accumulation in various colorectal carcinoma cell lines, including HT29, by 24 hours. HT29 cells expressing mp53 were exposed to either ZD9331

(50 nM) or 5-fluorouracil/leucovorin (3 μM/1 μM) for 24 hours, and both treated and untreated cells were harvested for RNA isolation and genomic comparison via cDNA microarray analysis. Genes involved in cell cycle regulation were specifically assessed for fold changes after S-phase arrest. (The microarray data were mined via Spotfire software using the search phrases “cyclin” or “cell cycle.”) Genes that were either up-regulated or down-regulated by >50% are shown in Fig. 4A. Of the ~70 genes examined, only 6 genes demonstrated significant up-regulation by either ZD9331 or 5-fluorouracil/leucovorin (p21^{Cip1}, CDK1, CDK2, CDC45, CDC6, and cyclin E1), and 4 (p21, CDK1, CDC45, and CDC6) were up-regulated by both drugs. (Complete analyses of the array data are available at www.stjudere-search.org.) P21^{Cip1} mRNA increased to the greatest degree (>3-fold) followed by CDK1 (>2-fold). CDC20 was the only cell cycle-related gene assessed in our search to be down-regulated by either treatment. Verification of these findings was provided by RNase protection assay that focused on CDK inhibitors (p16, p18, p19, p21^{Cip1}, p27^{Kip1}, and p57), including p21^{Cip1}. Only p21^{Cip1} was up-regulated by either ZD9331 or 5-fluorouracil/leucovorin treatment in HT29 cells (Fig. 4B). Optical densitometry determinations demonstrated a selective 4–5-fold increase in p21^{Cip1} mRNA expression in HT29 after thymidylate synthase inhibition (data not shown), closely correlating with the >3-fold up-regulation of this gene from cDNA microarray analyses. Furthermore, both assays were concordant with isolating p21^{Cip1} as the only CDK inhibitor induced by thymineless stress in HT29 cells. In GC₃/c1 thymidylate synthase⁻ cells, also expressing mp53, p21^{Cip1} mRNA was up-regulated by 72 hours after dThd deprivation (Fig. 4B), at the time the cells were undergoing apoptosis and 50% loss in clonogenic survival (1, 7). Thymineless-stress induced up-regulation of p21^{Cip1} was confirmed at the protein level in all of the cell lines tested (HT29, HCT116wt, and RKO; Fig. 4C and Fig. 5A).

Selective Overexpression of p21^{Cip1} Is Toxic to RKO But Does Not Enhance ZD9331-Induced Cytotoxicity. Induction of p21^{Cip1} or p27^{Kip1} in isogenic edysone-inducible RKO cell lines demonstrated that ZD9331 induced expression of p21^{Cip1} protein but not p27^{Kip1} in all of the RKO isogenic cell lines (Fig. 5A). Furthermore muristerone induced p21^{Cip1} in RKO p21 IND by 8 hours and p27^{Kip1} in RKO p27 IND by 2 hours (Fig. 5B). Significant G₁ arrest was evident by either induction by 24 hours (data not shown). A time-dependent increase in apoptosis was observed when p21^{Cip1} was selectively induced, without any other intervention (Fig. 5C). P27^{Kip1}

Fig. 4. Expression of p21^{Cip1} and cell cycle-related genes by cDNA microarray analysis after treatment with ZD9331 (50 nM) or 5-fluorouracil/leucovorin (3 μM/1 μM) for 24 hours in HT29 cells.

A, relative mRNA levels, of which the average gene expression was calculated from four independent cDNA microarray assessments for each treatment condition, and reflected a >50% up- or down-regulation compared with control were tabulated (mean; bars, ±SD). B, RNA extracted from HT29 cells after treatment with IFN-γ (100 units/ml), 5-fluorouracil/leucovorin (3 μM/1 μM), or ZD9331 (50 nM) for 24 hours was assayed for relative expression of various cell cycle-related genes by RNase protection assay as described in Materials and Methods. Additionally, RNA extracted from thymidylate synthase⁻ cells maintained in either dThd-replete or dThd-depleted medium for 72 hours was assayed for relative expression of various cell-cycle- and apoptosis-related genes by RNase protection assay as described in Materials and Methods. C, protein expression of p21^{Cip1} examined in HCT116wt and HT29 cells either untreated or treated with ZD9331 (50 nM) for 48 hours via Western analysis.



induction was also capable of inducing apoptosis at later time points (72 hours) in these cells but to a much lesser extent compared with p21^{Cip1} induction and serves as a vector-transfected control (Fig. 5C). Selective induction and overexpression of p21^{Cip1} or p27^{Kip1} simultaneously with ZD9331 treatment led to attenuated cytotoxicity compared with ZD9331 alone. Delayed induction of p21^{Cip1} or p27^{Kip1}, 24 hours after ZD9331 exposure, did not attenuate or enhance ZD9331-induced cytotoxicity (Fig. 5, D [SCAP] and E). These data suggest that whereas induction of p21^{Cip1} is independently sufficient to induce apoptosis in this colorectal carcinoma cell line, such abnormally elevated p21^{Cip1} (or p27^{Kip1}) expression causes the cells to undergo a G₁ arrest that mitigates the effects of S phase-specific drugs. Similarly, pharmacological induction of p21^{Cip1} in various cell lines (HCT116wt and HT29) with sodium butyrate demonstrated similar sequence-dependent attenuation of the effects of thymidylate synthase inhibition (data not shown).

CDK Inhibition by Roscovitine Potentiates ZD9331-Induced Cytotoxicity in HCT116wt and p21^{Cip1}-/- Cells. Exposure of HCT116 wt and p21^{Cip1}-/- cell lines to the CDK inhibitor roscovitine (72 hours) demonstrated a dose-dependent reduction in clonogenic survival in both cell lines, with p21^{Cip1}-/- cells demonstrating slightly increased sensitivity to this agent (IC₅₀: wt, 13 μM; p21^{Cip1}-/-, 7 μM; data not shown). Coexposure with roscovitine (3 μM) and ZD9331 (50 nM and 100 nM) for 72 hours demonstrated potentiation of the cytotoxic activity of ZD9331 in wt and p21^{Cip1}-/- cells. Roscovitine (3 μM) initiated ~10% loss in clonogenic survival in both cell lines. ZD9331 (100 nM) alone yielded ~90% loss in clonogenic survival in wt cells and ~20% in p21^{Cip1}-/- cells, which are considerably more resistant to ZD9331. However, roscovitine (3 μM) + ZD9331 (100 nM) yielded 65% loss in clonogenic survival in p21^{Cip1}-/- cells, more than double the anticipated additive effect. Similar potentiation was evident in wt cells (Fig. 6) and p53^{-/-} cells (data not shown). Data suggest that inhibition of CDK function, normally performed endogenously by p21^{Cip1}, may be important in the mechanism of thymineless stress-induced cytotoxicity.

DISCUSSION

The role of p21^{Cip1} in influencing chemosensitivity in colorectal cancer remains to be clearly defined. The isogenic HCT116 wt and p21^{Cip1}-/- cell lines have been used by several investigators to explore the role of p21^{Cip1} in chemotherapeutic response *in vitro*. Studies have demonstrated increased sensitivity of p21^{Cip1}-/- cells to anticancer agents compared with wt cells, supporting the contention that p21^{Cip1} functions as an antiapoptotic protein (11, 25, 26). It has been reported that p21^{Cip1}-/- cells are more sensitive to the apoptosis-inducing effects of doxorubicin (27); however, we have demonstrated that neither doxorubicin nor etoposide induce preferential cytotoxicity in p21^{Cip1}-/- cells, determined in clonogenic survival assays, in contrast to apoptosis assays (27). More importantly, we have demonstrated that p21^{Cip1} is required for maximal cytotoxicity induced by thymineless stress in colorectal carcinoma, suggesting a proapoptotic role for p21^{Cip1} in this setting.

The endogenous CDK inhibitor, p21^{Cip1}, has been reported to play a role in the regulation and promotion of apoptosis, in contrast to its antiapoptotic role, in other settings. One report demonstrated 50% reduction in sensitivity of HCT116 p21^{Cip1}-/- cells to apoptosis induced by manumycin and paclitaxel, with concomitant decrease in caspase-3 activity (28). Induced p21^{Cip1} expression in human carcinoma cell lines also induced apoptosis, similar to our findings in RKO (14, 29). Li *et al.* (30) demonstrated that flavopiridol-induced p21^{Cip1} expression in Rb-deficient osteosarcoma cells, with increased cdk2 inhibition, yielded enhanced doxorubicin sensitivity, placing p21^{Cip1} and cdk2 interactions in the center of the apoptotic pathway in this model. The CDK inhibitor olomoucine, p21^{Cip1} antisense, or cdk1 antisense have each inhibited the induction of apoptosis in human Hep3 cells, confirming an important role for p21^{Cip1} and cdk1 in the induction of retinoic acid-induced apoptosis (31). Importantly, in addition to colorectal cancer cell lines HCT116 and RKO, p21^{Cip1} has been shown to enhance cytotoxicity from platinum drugs in the colorectal cancer cell line DLD-1 (32). Thus, p21^{Cip1} (14, 29, 30, 32) and its CDK substrates (21, 30, 31, 33–35) appear to be able to promote apoptosis, with or without concomitant chemotherapeutic

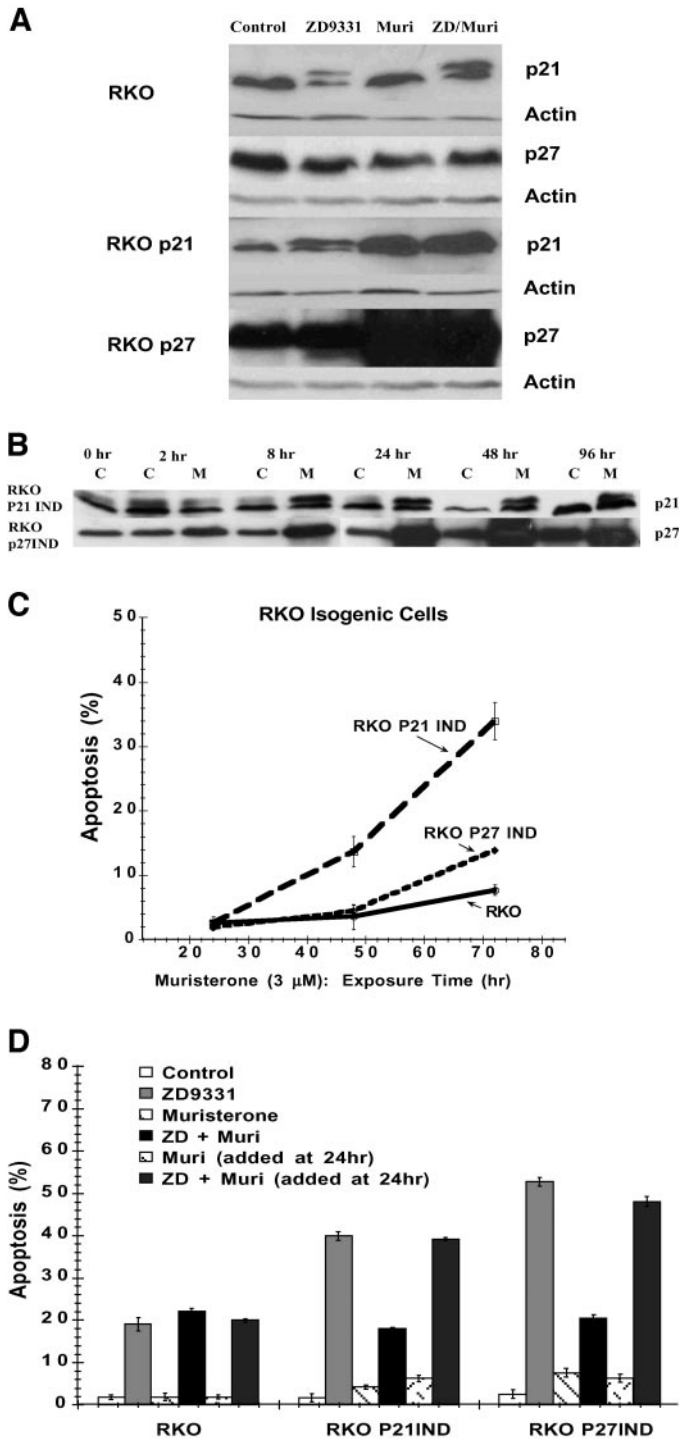


Fig. 5. Induction and overexpression of p21^{Cip1} induces apoptosis in RKO and is antagonistic to ZD9331-induced cytotoxicity. A, RKO, RKO p21 IND, or RKO p27 IND untreated or treated with ZD9331 (50 nM), muristerone (3 μM), or ZD9331 + muristerone for 48 hours. Expression of p21^{Cip1} or p27^{Kip1} was determined by Western analysis. B, RKO p21 IND or RKO p27 IND untreated or treated with muristerone (3 μM) for 2, 8, 24, 48, or 96 hours; cells were collected and assayed for expression of p21^{Cip1} or p27^{Kip1} by Western analysis. C, RKO, RKO p21 IND, or RKO p27 IND treated with muristerone (3 μM) for 24, 48, or 72 hours, and assayed for apoptosis via fluorescence-activated cell sorter analysis. Data represent the mean of duplicate experiments. D, RKO, RKO p21 IND, and RKO p27 IND were untreated or treated with ZD9331 (50 nM), muristerone (3 μM), or ZD9331 + muristerone for 48 hours, and the extent of apoptosis assayed by fluorescence-activated cell sorter analysis. Data represent the mean of duplicate experiments.

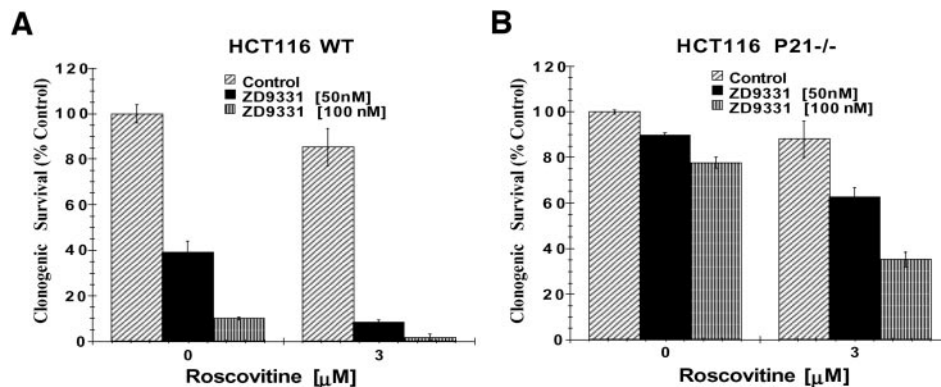
agents, and the consistent enhancing effect of p21^{Cip1} in colorectal cancer is noteworthy given the prognostic role of p21^{Cip1} in such patient tumor samples.

This report and others (25) also demonstrate that cells lacking p21^{Cip1} are intrinsically sensitive to the cytotoxic effects of IFN-γ in contrast to wt cells that demonstrate resistance to IFN-γ. Therefore, within the same isogenic cellular system (HCT116), p21^{Cip1} appears to promote cytotoxicity from thymineless stress (proapoptotic) as well as to antagonize cytotoxicity from IFN-γ (antiapoptotic). Several hypotheses have been proposed to explain the antiapoptotic role of p21^{Cip1}. Firstly, p21^{Cip1} can induce cell cycle arrest in G₁-phase thereby protecting cells from DNA damage-inducing agents. Secondly, p21^{Cip1} can bind to procaspase-3, thereby preventing cleavage to active caspase-3. Thirdly, either direct inhibition of stress-activated protein kinases by p21^{Cip1} or association between cytoplasmic p21^{Cip1} and ASK1 can suppress ASK1 activity and mitogen-activated protein kinase cascade activation (8). With regard to the ability of p21^{Cip1} to induce G₁ arrest, we have demonstrated that induction and overexpression of p21^{Cip1} alone in RKO (RKO p21 IND) induces apoptosis not attributable to the inducing agent muristerone. However, this overexpression of p21^{Cip1} also promotes an initial G₁ arrest in RKO (data not shown), thereby attenuating the cytotoxic effect of the S phase-specific thymidylate synthase inhibitor, ZD9331. This is similar to a reported role for p21^{Cip1} in protection of RKO cells when exposed to cytotoxic agents (36). Consistent with this hypothesis is the finding by Rau *et al.* (37) who demonstrated that induction of p21^{Cip1} over basal expression levels led to radiochemotherapeutic resistance *in vivo* in patients with rectal carcinoma. Thus, the cell cycle modulating effect of p21^{Cip1}, specifically G₁ arrest, is likely to be antagonistic to the cytotoxic effects of various chemotherapeutic agents, as is overexpression of p21^{Cip1}. It is likely that the endogenous level of p21^{Cip1} expression, its subcellular localization, and other factors regulating its function each play a role in determining cellular fate after drug exposure.

CDK activity, controlled by cyclins, p21^{Cip1}, and other CDK inhibitor-related proteins and processes, in the context of thymidylate synthase inhibition, has received limited investigation. Yin *et al.* (38) demonstrated in head and neck carcinoma cell lines deficient in both p53 and p21^{Cip1} that thymidylate synthase inhibition by tomudex led to increased cyclin E, cdk2, and cyclin A expression. Increased cyclinE-cdk2 activity correlated with effective thymidylate synthase inhibition, decreased E2F1, decreased DNA synthesis, and S-phase arrest. Furthermore, they showed that thymidylate synthase inhibition by tomudex in HCT-8 led to induction of p21^{Cip1} (38). Although no direct correlation of increased CDK activity to cytotoxicity was reported, these findings are consistent with our findings of thymidylate synthase inhibitor-induced expression of p21^{Cip1}, cdk2, and cyclin E in HT29 arrested in S phase after ZD9331-induced thymidylate synthase inhibition. In other p21^{+/+} cellular systems, human breast cancer cell lines forced to arrest in S phase after thymidylate synthase inhibition by tomudex demonstrated increased levels of E2F-1, cyclin A, and cdk2 levels that appeared to correlate with nonspecific cell cycle changes rather than to cytotoxicity. They also showed that induction of p21^{Cip1} reversed tomudex resistance in MDA-bcl7 cells (bcl-2 overexpressing clones), consistent with our findings of the requirement for p21^{Cip1} in the apoptosis-promoting effect of thymineless stress in colorectal carcinoma (39). Li *et al.* (40) also demonstrated increased sensitivity to tomudex after induction of p21^{Cip1} in SaOS-2 osteosarcoma cells, extending the connection between p21^{Cip1} and thymidylate synthase inhibition to sarcomas. Interestingly, thymidylate synthase itself has been found to down-regulate p21^{Cip1} expression in cancer models (41).

The role of p21^{Cip1} in mediating S-phase exit and the consequences of S-M phase asynchrony are particularly intriguing. Prolonged S-phase accumulation in p21^{Cip1}−/− cells compared with wt cells, supporting a role for p21^{Cip1}-mediated exit from S phase after induc-

Fig. 6. The CDKI inhibitor, roscovitine, potentiates ZD9331-induced cytotoxicity in HCT116 wt and p21^{Cip1}^{-/-} cells. A, HCT116 wt and (B) HCT116p21^{Cip1}^{-/-} cells treated with ZD9331 (50 nM or 100 nM) either in the absence or presence of roscovitine (3 μ M) for 72 hours, and clonogenic survival was determined. Data represent the mean of three determinations for each condition; bars, \pm SD.



tion of thymineless stress, as demonstrated herein, is similar to the effect reported after exposure of these cells to nitrogen mustard (11). Additionally we demonstrate that pure thymineless stress (thymidylate synthase⁻ cells) or drug-induced thymineless stress (ZD9331) each strongly induce p21^{Cip1} expression, independent of p53. Reports have suggested that at low expression levels, p21^{Cip1} may enhance cyclin-CDK activity, whereas at high expression levels, p21^{Cip1} inhibits this function (42). Whether p21^{Cip1} is required to activate cyclin A-cdk2 required for S-phase exit (as opposed to the normal CDK inhibitory function of p21^{Cip1}), or cyclinA-cdk2 is dysfunctional after thymidylate synthase inhibition in the p21^{Cip1}^{-/-} cell line, remains to be determined. Data also demonstrate that HCT116 wt cells undergo apoptosis before mitotic entry and that inhibition of thymidylate synthase induces up-regulation of both p21^{Cip1} and cdk1 as well as other components of the DNA replication origin of replication complex (cdk2, cdc45, and cdc6; 10), concomitant with down-regulation of CDC20, while the cells are arrested in S phase. CDC20 is critical for promoting anaphase (mitotic) onset, and therefore must be closely controlled in S phase to prevent premature mitosis and resultant aneuploidy (43). Exactly how the various S phase- and M phase-specific proteins interact to avoid S-M-phase asynchrony or aneuploidy, and perhaps apoptosis after cellular stress, is an area of active investigation, and we hypothesize that such cellular events require tight coordination of p21 and cyclin/CDK function.

Of clinical and biological importance is the finding that roscovitine, an inhibitor of CDK1, CDK2, and CDK5 (44), was shown to be both toxic in HCT116 isogenic cell lines, as well as capable of potentiating ZD9331-induced cytotoxicity in these lines. Inhibition of CDKs, as opposed to activation of CDKs as discussed previously (21, 30, 31, 33–35), has been shown by others to be cytotoxic in various models including colorectal carcinoma cells (45, 46). The finding that potentiation is evident in p21^{Cip1}^{-/-} cells, resistant to ZD9331 treatment alone, strongly suggests that the function of p21^{Cip1} as a CDK inhibitor may be required for thymidylate synthase inhibitor-induced apoptosis and cytotoxicity in colorectal cancer.

We, and others have demonstrated that the cytotoxic effects of thymidylate synthase inhibition in colorectal carcinoma cells are consistently independent of p53 (2, 4, 47). Furthermore, the prognostic value of p53 cannot be substantiated in patients with colorectal cancer, as there appears to be an equal number of reports attesting to its value as a poor, nonsignificant, or favorable prognostic indicator in patients with colorectal cancer undergoing thymidylate synthase inhibitor-based therapy (48). In contrast, the majority of the published literature is consistent with the notion that greater p21^{Cip1} expression imparts a favorable outcome in such patients (49). Our review of the published literature has included 1809 colorectal tumor samples, of which ~45% lack detectable p21^{Cip1} protein as measured via immunohistochemistry (37, 49–60). Overall, data support the association

among p21^{Cip1} expression, enhanced responsiveness, and overall survival in patients exposed to thymidylate synthase inhibitor-based therapy. More specifically, P21^{Cip1} protein expression has been shown to significantly predict response to 5-fluorouracil/leucovorin-based therapy (50), and numerous trials using various therapies have demonstrated a favorable prognostic role for positive p21^{Cip1} staining in overall survival as demonstrated in multivariate analysis (51–53) or univariate analysis (51–56).

In conclusion, we have demonstrated that p21^{Cip1} is requisite for effective thymidylate synthase inhibitor-induced cytotoxicity that occurs via DNA damage from thymineless stress, whereas IFN- γ is toxic to human colorectal carcinoma cells devoid of functional p21^{Cip1} protein. These data are of importance clinically for the ~45% of patients with colorectal carcinoma who demonstrate underexpression or deletion of p21^{Cip1}, and strongly support the rationale for combining IFN- γ (inducing cytotoxicity in p21^{Cip1}^{-/-} tumors but resistance in p21^{Cip1}^{+/+} tumors) with thymidylate synthase inhibitors (cytotoxic in p21^{Cip1}^{+/+} tumors but not in p21^{Cip1}^{-/-} tumors), a combination that has the additional advantage of synergistic interaction in multiple colorectal cancer cell lines as presented here and elsewhere (2–4). Induction or overexpression of p21^{Cip1} via genetic or pharmacological means, which would promote G₁ arrest, is not likely to be successful in exploiting this finding, and it is likely that endogenous levels of p21^{Cip1}, its subcellular distribution, as well as cell type- and cell cycle- specific effects are important in determining the ultimate outcome from manipulation of this protein pharmacologically. Clarification of how the key downstream effectors of cytotoxicity from these agents play their part in the induction of apoptosis requires additional investigation.

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