A Defect in the p53 Response Pathway Induced by de Novo Purine Synthesis Inhibition*

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The mammalian tumor suppressor protein p53 triggers cytoprotective growth arrests prior to S phase and mitosis in response to genotoxic stresses, induced by ultraviolet (1) or ionizing radiation (2) or treatment with any of several DNA-modifying agents (3). When cellular DNA damage is detected, p53 turnover is prevented, and accumulating p53 activates the transcription of a series of downstream targets, including p21cip1/waf1 (4), a key inhibitor of cyclin D-cdk 4/cdk 6, cyclin E-cdk 2, and cyclin B-cdk1 kinases (5, 6), with subsequent growth arrest in G1 and/or G2 phase of the cell cycle.

It is currently thought that either ribonucleotide depletion per se and/or a slowing of the progression of replication forks can also be detected independently of direct DNA damage, resulting in signaling to p53 and initiation of a p53-dependent cell cycle arrest (7, 8). Some of the strongest support for this concept comes from the effects of n-phosphonacetyl-L-aspartate (PALA),1 an inhibitor of an early step of de novo pyrimidine synthesis which causes pyrimidine ribonucleotide depletion but, surprisingly, not DNA strand breaks (8). PALA is an unusual case among the cancer chemotherapeutic agents, for even those drugs which inhibit enzyme targets directly and exclusively usually result in downstream DNA strand breaks.

Another rare cytotoxic compound that does not induce DNA strand breaks is the antimetabolite 5,10-dideazatetrahydrofolate (DDATHF) (9), the prototypical inhibitor of the first folate-dependent enzyme in de novo purine synthesis, glycinamide ribonucleotide formyltransferase (GART) (10–12). DDATHF and its analogs cause a rapid decline in ATP and GTP pools (11, 13, 14) and, concomitantly, potent inhibition of cell growth (11, 15–18). Second and third generation GART inhibitors have been developed, and have been or currently are in early phase clinical trials as anticancer agents (19–25). GART inhibitors are unique in that they are inactive against the classical enzymatic targets for antifolates, i.e. dihydrofolate reductase and thymidylate synthase, but cause a potent inhibition of purine synthesis and consequent cytotoxicity (26) without detectable DNA damage (16, 27). Others have reported (16) that GART inhibitors are cytotoxic to carcinoma cells that do not have p53 function but only cytostatic to carcinoma cells that retain wild-type p53 function; this suggestion would be quite compatible with the role of p53 as a direct sensor of nucleotide pools. However, we have found GART inhibitor-induced cytotoxicity to be unaffected by p53 status (28), and, moreover, despite a substantial accumulation of p53 following DDATHF, there was no G1 arrest, as shown below. This paradox begg explanation.

In this study, we report that human tumor cells respond to de novo purine synthesis blockade by GART inhibitors with p53 stabilization, but that the downstream transcriptional activation events causative of a G1 arrest are defective. The mechanism of inactivation of the p53 response involves interference with the posttranslational phosphorylation and acetylation of p53 and with histone acetylation at the promoters of p53-sensitive downstream targets, even though nuclear translocation and sequence-specific binding of this unmodified p53 are unaffected in vivo. This inactivation of the p53 pathway ex-

DDATHF, (6F7,5,10-dideazatetrahydrofolic acid; AG2034, 4-[2-(2-amino-3-oxo-4-oxo-4,6,7,8-tetrahydro-3H-pyrimidin-5-yl)-[1,4]thiazin-6-yl]-(3,4-dihydro-2-methyl-4-oxoquinazoline-6-yl-methyl)-N-methylaminel-2-thienyl)-L-glutamic acid; BSA, bovine serum albumin; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin immunoprecipitation; PIPES, 1,4-piperazinedithanesulfonic acid; RIPA, ribonuclease protection assay; ChIP, chromatin imm
plans why p53 wild-type and null function carcinoma cells are quiescent to the toxicity of the GART inhibitors.

**EXPERIMENTAL PROCEDURES**

**Drug Solutions**—Solutions of DDATHF, LY309887, D1694 (Eli Lilly), AG2204, AG2037 (Aptogen), PALA (National Cancer Institute), methotrexate (Sigma), and cycloheximide (Sigma) were dissolved in phosphate-buffered saline (PBS). Etosine (VP16)/Sigman) was dissolved in Me2SO, and 5-bromo-2′-deoxyuridine (BrDU) (Sigma) and inosine (Sigma), in PBS.

**Cell Culture**—Human colon carcinoma HCT116 p53+/+ and p53−/− cells were generously provided by Dr. Bert Vogelstein (Johns Hopkins University), MCF7 (human breast carcinoma) and A549 (human lung carcinoma) cell lines were from the American Type Culture Collection (ATCC). All cell lines were grown in RPMI 1640 (Invitrogen/Life Technologies) supplemented with 10% dialyzed fetal calf serum (dFCS) in a humidified atmosphere at 5% CO2 at 37 °C. Cells were determined free of mycoplasma using a ribosomal RNA PCR kit from ATCC. Irradiation was performed at a dose rate of 4.47 Gy/min using a Mark 137-I Cesium source.

**Flow Cytometry—**G0/G1 synchronization was achieved by holding HCT116 p53+/+ and p53−/− cells at confluence for 48 h, then releasing from postconfluent synchrony by replating to a lower density. For dual parameter western blotting, cell extracts were harvested from 3.5 cm2 of BDGy/Syn in media containing 10% dFCS, 65 mM BrDU (to continuously label cells actively synthesizing DNA) and either PBS, 10 μM DDATHF, or 400 μM PALA. Forty-eight hours later, cells were harvested by trypsinization (0.1% trypsin/0.04% EDTA), fixed in cold 70% ethanol, and stored at 4 °C until flow cytometry. Cells were washed in 0.5% BSA in PBS, and DNA was denatured with 2 N HCl for 20 min at room temperature. The cell suspensions were neutralized by the addition of 0.1 N sodium borate, pH 8.5, and again washed. Cell pellets were incubated for 1 h with mouse monoclonal anti-BrDu FITC-conjugated antibody (BD PharMingen), diluted 1:6 in 0.5% BSA/0.5% Tween 20/ PBS, washed, and resuspended in 50 μg/ml propidium iodide (Sigma) and 1% Triton X-100, and 7 Kunitz units/ml RNase B in 3.2 mM sodium citrate buffer. Flow cytometric analysis was performed on an EPICS XL-MCL flow cytometer (Beckman Coulter).

**Western Blotting—**Cells were lysed in buffer containing 62.5 mM Tris, pH 6.8, 5% glycerol, 2% SDS, 5% mercaptoethanol, 50 mM NaF, 0.05 mM Na2VO4, and 1× protease inhibitor complete mixture (Roche Applied Science). Protein concentrations were determined using a Bio-Rad assay, according to a standard of BSA. Total cellular proteins (20 μg) were resolved by electrophoresis on 7.5–8% SDS-polyacrylamide gels, and transferred to an Immobilon-P polyvinylidene fluoride membrane (Millipore) or nitrocellulose (Bio-Rad). For single pulse western blotting or for western flow cytometry studies, p53+/+ cells were released from synchrony into media containing either PBS or 10 μM DDATHF. Cells were fixed and stained in 50 μg/ml propidium iodide, 0.1% Triton X-100, and 7 Kunitz units/ml RNase B in 3.2 mM sodium citrate buffer. Flow cytometric analysis was performed on an EPICS XL-MCL flow cytometer (Beckman Coulter).

**Immunofluorescence—**Cells were stained in 50 μg/ml propidium iodide, 0.1% Triton X-100, and 7 Kunitz units/ml RNase B in 3.2 mM sodium citrate buffer. Flow cytometric analysis was performed on an EPICS XL-MCL flow cytometer (Beckman Coulter).

**Northern Blotting—**Total RNA was isolated using Trizol (Invitrogen/Life Technologies) and poly(A) RNA was selected using an Oligotex mRNA Kit (Qiagen). Poly(A) RNA was denatured by glyoxal/Mel/SE treatment at 55 °C and 2 μg of poly(A) RNA were separated on a 1% agarose gel. RNA was transferred to ICN Biotrans membrane and probed with random-primed cDNA (ATCC) of 10 μg/ml. After exposure the blot was developed with a 1:100 dilution of Ab-6 antibody to p53, and then subjected to Western blotting as described above.

**Ribonuclease Protection Assay—**Ribonuclease protection assay (RPA) reactions containing 5.8 × 105 cpm probe synthesized from the hStress-1 Ribo-Quant Multiprobe Template Set (BD Pharmingen), and 20 μg of total RNA were hybridized in 50% deionized formamide, 1 mM EDTA, pH 8.0, 400 mM NaCl, and 40 mM PIPEPS pH 6.7 overnight at 55 °C in the presence of RNAseH (100 units/ml) at 50 μg/ml ribonuclease (Sigma). After digestion with RNase A mixture (10 μg/ml TrisCl, pH 7.4, 1.4 mM EDTA, 200 mM NaCl, 100 mM LiCl) continued for 30 min at 30 °C, and was terminated with the addition of 10 μg proteinase K and 0.5% SDS. Protected fragments were ethanol-purified, fractionated on a 5% polyacrylamide-urea gel, the gel was dried and exposed to film, and fragments were quantitated by PhosphorImage (Molecular Dynamics) analysis.

**Immunofluorescence—**Following release from G0/G1 synchrony, HCT116 cells were seeded at densities between 8 × 104 and 4.5 × 105 cells/well on 18 mm glass coverslips in RPMI 1640 + 10% FCS with or without 10 μM DDATHF. Cells were washed, fixed in 100% MeOH at pH 7.5, and stored at −20 °C until samples were collected. Non-specific binding was blocked with 5% BSA in Tris-buffered saline containing Tween 20 and sodium azide (TBS-TA), pH 7.5 (0.14 M NaCl, 2.7 mM KCl, 25 mM Tris base) for 3 min, rinsed, and stored in PBS at 4 °C until samples from all time points were collected. Nonspecific binding was blocked with 5% BSA in TBS-TA, pH 7.5 (0.14 M NaCl, 2.7 mM KCl, 25 mM Tris base, 0.05% Tween 20, 0.05% sodium azide). All incubations were carried out at 37 °C for 30 min. All cells were incubated in mouse monoclonal anti-p53 Ab-6 (Oncogene) at a 1:20 dilution in 2% BSA in TBS-TA, and labeled with goat anti-mouse Texas Red antibody (Molecular Probes) at a 1:200 dilution in 2% BSA in TBS-TA. Cell staining was imaged using an Olympus P3000 200-bvit digital camera on an Olympus IX70 inverted fluorescent microscope (Olympus America) and analyzed using Adobe Photoshop software.

**Electrophoretic Mobility Shift Assay—**The procedures for nuclear extract preparation and EMSA were adapted from Siliciano et al. (29). For each condition, −5 × 106 HCT116 p53+/+ cells were washed twice with ice-cold PBS, scraped, pelleted, and lysed on ice for 3–5 min in buffer containing 20 mM HEPEPS pH 7.6, 25% glycerol, 1.5 mM MgCl2, 10 mM NaCl, 0.2 mM EDTA, 2 mM dithiothreitol, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, 20 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM Na3VO4, and 1 mM NaF. 25 ng of nuclear lysates (500 μg) were preheated for 1 h with a 50% slurry of protein A-Sepharose beads (Amersham Biosciences), and incubated overnight with 2 μg of anti-p53 (Ab-6, Oncogene). All incubations were carried out at 4 °C. Protein-antibody complexes were collected through the addition of 30 μl of 50% protein A-Sepharose bead slurry for 1 h. Beads were washed four times in PBS containing 10 μg/ml RIPA buffer, then resuspended in 50 μl of denaturing sample buffer (62.5 mM Tris, pH 6.8, 5% glycerol, 2% SDS, 5% 2-mercaptoethanol) and boiled for 5 min. After brief centrifugation to pellet the beads, 15-μl of immunoprecipitate were loaded onto a 7% SDS-polyacrylamide gel. Western blotting was performed, and ubiquitinated p53 was detected by probing the blot with the Ab-6 p53 antibody as described above.

**Northern Blotting—**Total RNA was isolated using Trizol (Invitrogen/Life Technologies) and poly(A) RNA was selected using an Oligotex mRNA Kit (Qiagen). Poly(A) RNA was denatured by glyoxal/Mel/SE treatment at 55 °C and 2 μg of poly(A) RNA were separated on a 1% agarose gel. RNA was transferred to ICN Biotrans membrane and probed with random-primed cDNA (ATCC) of 10 μg/ml at 50 μg/ml. After exposure the blot was developed with a 1:100 dilution of Ab-6 antibody to p53, and then subjected to Western blotting as described above.
DDATHF-induced Defect in the p53 Response

GAT-3') was added, and incubation continued for another 20 min. For supershift analysis, nuclear extract or recombinant p53 was preincubated with 0.5 µg of Ab-6 p53 antibody (Oncogene) for 15 min prior to the addition of binding buffer. For competition studies, 20 or 40-fold molar excess cold wild-type p21 or cold mutant p21 (5'-ATC AAT TCT CGA GGA AAC GTC CCT CCC AAA CGT TTG CTC GAG GAT-3') oligonucleotide was added simultaneously with the wild-type 32P-labeled p21 probe. Samples were loaded onto a pre-run 4% non-denaturing polyacrylamide gel and DNA:protein complexes were resolved by electrophoresis in 0.5 X Tris borate/EDTA buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). Gels were dried and visualized by autoradiography.

Chromatin Immunoprecipitation (ChIP)—The ChIP assay was based on the protocol described by Kuo and Allis (30). Approximately 1 x 10^7 cells per condition were incubated in 1% formaldehyde for 10 min at room temperature, after which cross-linking was stopped by the addition of glycine to a final concentration of 0.125 M for 5 min. Cells were washed twice in ice-cold PBS, scraped, washed in buffer I (10 mM HEPES pH 7.5, 0.5 mM EGTA, pH 7.5, 10 mM EDTA, pH 8.0, 0.25% Triton X-100), then washed in buffer II (10 mM HEPES pH 7.5, 0.5 mM EGTA, pH 7.5, 1 mM EDTA, pH 8.0, 200 mM NaCl). All ChIP buffers contained 1 µg/ml pepstatin, 1 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM Na3VO4, and 10 mM NaF. Cells were lysed in buffer containing 5 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, and sonicated (Misonix) using conditions found to yield DNA fragments less than 1000 bp in size. Lysates corresponding to 2.5 x 10^6 cells were precleared for 1 h at 4°C in a 50% protein A-Sepharose (Amersham Biosciences) bead slurry blocked with 0.2 mg/ml sonicated salmon sperm DNA and 8 µg BSA, then incubated with antibody overnight. Antibodies used were 2 µg of p53 Ab-6 (Oncogene), 1 µg of di-acetyl lysine Histone H3 (Upstate), 4 µg of RNA polymerase II (Santa Cruz Biotechnology), 5 µg of p300 N-15 (Santa Cruz Biotechnology), and 4.5 µg of CBP A-22 (Santa Cruz Biotechnology). Antibody–protein–DNA complexes were captured by the addition of 30 µl of blocked 50% protein A-Sepharose bead slurry for 1 h at 4°C. Beads were pelleted, the supernatant from the controls containing no antibody was collected, and the DNA contained within was referred to as input DNA. Beads were washed extensively in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40), high salt buffer (500 mM NaCl, 50 mM Tris, pH 8.0, 0.1% SDS, 1% Nonidet P-40), LiCl buffer (250 mM LiCl, 50 mM Tris, pH 8.0, 0.5% sodium deoxycholate, 1% Nonidet P-40) and TE (10 mM Tris, 1 mM EDTA, pH 8.0) for 10 min at 4°C with end-over-end rocking. Protein–DNA complexes were eluted by 2% SDS, 10 mM dithiothreitol, 0.1 mM NaHCO3, and cross-links were reversed by the addition of 0.2 M NaCl and incubation at 65°C for 6 h. DNA was phenol-chloroform extracted, ethanol-precipitated, and dissolved in TE (total input DNA in 100 µl immunoprecipitated DNA in 20 µl).

Semi-quantitative and Real Time PCR—Semi-quantitative PCR was performed with 10–20 ng of input DNA and 0.5 or 1 µl of immunoprecipitated DNA in reactions containing 1 mM MgCl2, 0.2 mM dNTPs, and 0.6 µM primers. For the upstream p53 consensus binding site on the p21 promoter, the primers were 5’-CTT TCC ACC TTT CAC CAT TCC-3’ (sense), 5’-AAG GAC AAA ATA GCC ACC AGC-3’ (antisense), and the product size was 234 bp. Amplification conditions were: 94°C 30 s, 53°C 30 s, 72°C 30 s. For the downstream p53 binding site on the p21 promoter, the primers first described by Barlev et al. (31) were 5’-CCA GCC TCT TGG ATG TTT TG-3’ (sense) and 5’-GCC TCC TTT CTC TGC A-3’ (antisense), and the product size was 420 bp. Reactions contained 1 mM MgCl2, 0.1 mM dNTPs, and 0.6 µM primers, and amplification conditions were: 94°C 1 min, 58.5°C 1 min, 72°C 1 min. PCR cycle number was varied from 25–30 cycles to keep the reaction within the linear range. Quantitative real time PCR was performed using the Quantitect SYBR Green PCR Master Mix (Qiagen). The primers flanking the 5’ p53 binding site were those described above, whereas the primers flanking the 3’ p53 binding site were 5’-GAG GTC AGC TGC TGT AGA GG-3’ (sense), and 5’-TGG AGA GGA TGC ATT GTC-3’ (antisense), and this primer generated a 154 bp fragment. Real-time PCR analysis was performed with three-step amplification plus melt curve, and the amplification conditions were: 94°C 15 s, 60°C 30 s, 72°C 30 s, with Tm values of 53°C and 58.5°C for the upstream and downstream p53 binding sites, respectively. ChiP real-time PCR experiments were performed twice, each with triplicate samples.

RESULTS

Inactivation of the p53-dependent G1 Checkpoint by DDATHF—Normal embryonic skin fibroblasts have been reported to arrest in G1 and G2 after induction of a nucleotide deficiency with the de novo pyrimidine synthesis inhibitor PALA, whereas fibroblasts in which the p53 pathway had been inactivated by adenoviral E6 expression advance into and accumulate in S phase (8). We found some striking differences when we compared the effects of p53 on cell cycle traverse of carcinoma cells treated with the de novo purine synthesis inhibitor DDATHF and the de novo pyrimidine inhibitor PALA. HCT116 cells with wild-type (+/+ ) p53 function and isogenic HCT116 cells bearing two null alleles of p53 were synchronized in G0/G1 and released into medium containing BrdU and either 10 µM DDATHF (C) or 400 µM PALA (D). After 48 h in drug and BrdU, cells were fixed and stained with propidium iodide and anti-BrdU FITC, and cell cycle progression was monitored via flow cytometry, gating out any sub-G1 debris.

FIG. 1. Regardless of p53 status, G1/G1 synchronized HCT116 cells traverse into and accumulate in S phase in response to de novo purine synthesis inhibition by DDATHF. Human colon carcinoma HCT116 cells with p53 wild-type (p53+/+ ) or null (p53−/−) status were synchronized in G0/G1, and analyzed by two parameter flow cytometry prior to release from synchronization (A) into media containing 65 µM BrdU and either PBS (B), 10 µM DDATHF (C) or 400 µM PALA (D). After 48 h in drug and BrdU, cells were fixed and stained with propidium iodide and anti-BrdU FITC, and cell cycle progression was monitored via flow cytometry, gating out any sub-G1 debris.

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2 Previous dose-response curves indicated that this concentration of DDATHF resulted in maximal growth inhibition and cell kill (12, 26, 28).
unsynchronized cells (28) treated with DDATHF demonstrated an equally facile exit from G2 of HCT116 cells with p53 function or without functional p53 alleles. Thus, the p53 signaling pathway detected the PALA-induced changes in pyrimidine ribonucleotide pools, but the p53-dependent G1 and G2 checkpoints appeared to be inactivated following a treatment with DDATHF (data not shown). Both MCF7 and A549 cells accumulated substantial levels of p53 following DDATHF treatment but failed to induce p21 as a result (Fig. 2D). We concluded that the block between p53 accumulation and induction of p21 was a general phenomenon.

**Fig. 2.** Despite the increase in p53 expression induced by pyrimidine nucleotide depletion, the immediate downstream transcriptional target p21 fails to accumulate in human carcinoma cells. A, Western blot analysis of p53 and p21 expression in HCT116 p53+/+ and p53−/− cells exposed to 10 μM DDATHF for the indicated time. Cell lysate protein (20 μg) was subjected to SDS-PAGE and immunoblotting with antibodies against p53 or p21. The first lane of each panel was loaded with protein from HCT116 p53+/+ cells exposed to 10 μM etoposide (VP16) for 24 h. Western blots were stripped and reprobed with an actin antibody. B, HCT116 p53+/+ cells were treated with either 10 μM VP16 for 24 h, or exposed to 12 or 15 Gy irradiation and harvested after 3.5 h, and Western blotting was performed. C, Western blot analysis of p53 protein levels in HCT116 p53+/+ cells upon treatment with 10 μM DDATHF in the presence or absence of the purine salvage metabolite inosine (50 μM). The first two lanes were loaded with lysates from p53+/+ cells treated with 10 μM VP16, or untreated. D, cell lysates of human breast carcinoma cells (MCF7) and human lung carcinoma cells (A549) were prepared after treatment with 10 μM DDATHF for the indicated periods, and Western blot analysis was conducted. Actin levels were equal in D.

When a source of preformed purines, such as hypoxanthine or inosine, was added to cell cultures treated with DDATHF, the purine salvage pathway prevented the decline in ATP and GTP pools caused by GART inhibition and rescued the growth inhibitory effects of DDATHF on tumor cells (11). Likewise, addition of 50 μM inosine to HCT116 p53+/+ cultures prevented the increase in p53 protein levels induced by DDATHF (Fig. 2C). Hence, the effect of DDATHF on p53 levels can be linked to purine nucleotide pool decreases caused by GART inhibition.

To determine whether the unexpected response of HCT116 cells to DDATHF was general for dividing carcinoma cells or was cell-context specific, a human breast carcinoma cell line, MCF7, and a human lung carcinoma cell line, A549, were also treated with DDATHF. These cell lines express wild-type p53 and respond to ionizing irradiation with a strong G1 block (32); yet, these cell lines also progressed into S phase upon treatment with DDATHF (data not shown). Both MCF7 and A549 cells accumulated substantial levels of p53 following DDATHF treatment but failed to induce p21 as a result (Fig. 2D). We concluded that the block between p53 accumulation and induction of p21 was a general phenomenon.

**Impairment of Transcriptional Activation or Repression by the p53 Accumulating in GART Inhibitor-treated Cells—**We questioned whether the p53 accumulating after DDATHF treatment in these cells was active as a transcriptional activator by examining the steady-state mRNA levels for p21 and several other transcriptional targets of p53. In the HCT116 p53+/+ cells, there was a barely detectable increase of p21 mRNA following treatment with DDATHF (Fig. 3A), measurable only by RPA (Fig. 3B and Table I). Likewise, mRNA levels of mdm2, whose transcription is normally regulated by p53 (33), remained constant after GART inhibition when normalized by gapdh levels (Fig. 3A). Average values of mdm2/gapdh in two experiments were 122 and 108% of untreated controls after 12 and 24 h of DDATHF exposure, respectively. In contrast, there was a marked induction of both p21 and mdm2 (9-fold and 6-fold, respectively) in HCT116 cells treated with VP16 as a control (Fig. 3A). Interestingly, a reproducible, but transient increase in Mdm2 protein was observed after 6 and 12 h of GART inhibition, apparently due to post-transcriptional mechanisms that were dependent on p53 (compare Fig. 3, C with A). The role of this increased Mdm2 in limiting the transcriptional activation of the accumulating p53 is unclear (see “Discussion”).

RPAs were used to analyze the effect of DDATHF on the transcription of several p53-regulated stress-inducible genes (Fig. 3B and Table I). The impairment of DDATHF-stabilized p53 to regulate transcription was reflected in several other downstream genes, in addition to p21 and mdm2. The pro-apoptotic p53-regulated gene bax was induced 3.4-fold upon VP16 treatment, but following GART inhibition, neither bax nor the anti-apoptotic gene mcl1 were induced (Fig. 3B); this agreed with a constant level of Bax protein expression in DDATHF-treated cells (Fig. 3C). However, there was an increase in Gadd45 protein found after both VP16 and DDATHF exposure, but Western blots suggest this increase is not a p53-dependent change, as HCT116 p53−/− cells also demonstrated an elevation in Gadd45 protein (Fig. 3C). Quite interestingly, the anti-apoptotic gene bclx, whose promoter has been shown to be repressed by p53 (34) was induced up to 3.6-fold after 24 h of purine synthesis inhibition. These data collectively suggested that the p53 accumulating after GART inhibition is ineffective at activating (p21, mdm2, bax) or repressing (bclx) downstream p53 target genes.
Mechanism of Increase in p53 Levels Following DDATHF Treatment—The p53 elevation following DDATHF treatment was not due to enhanced p53 transcription (Fig. 3B). However, upon treatment with DDATHF followed by addition of the protein synthesis inhibitor cycloheximide, p53 protein turned over with a half-life greater than 2 h, roughly equivalent to the stability of p53 accumulating in cells after ionizing radiation (Fig. 4A). p53 in untreated cells decayed with a half-time of

| Condition  | bclx  | p53   | p21   | bax   | gadd45   | mcl 1 |
|------------|-------|-------|-------|-------|----------|-------|
| VP16, 24 hr. | 1.9 ± 1.0 | 1.4 ± 0.37 | 8.9 ± 2.5 | 3.4 ± 1.0 | 2.3 ± 1.5 | 2.1 ± 0.85 |
| 0 h. control | 1     | 1     | 1     | 1     | 1        | 1     |
| DDATHF, 6 h. | 2.0 ± 0.36 | 0.83 ± 0.19 | 0.60 ± 0.13 | 1.1 ± 0.38 | 0.90 ± 0.40 | 1.2 ± 0.58 |
| DDATHF, 12 h. | 1.5 ± 0.44 | 1.1 ± 0.73 | 2.6 ± 0.60 | 1.9 ± 0.90 | 3.2 ± 1.1 | 1.1 ± 0.60 |
| DDATHF, 24 h. | 3.6 ± 0.94 | 1.7 ± 0.38 | 1.9 ± 0.44 | 1.4 ± 0.38 | 2.9 ± 1.3 | 0.69 ± 0.29 |

**Table I**

**Quantitation of p53-regulated transcripts following de novo purine synthesis inhibition by DDATHF**

The degree of induction or reduction of HCT116 p53+/+ mRNA was quantitated by PhosphorImager analysis of RPA experiments, utilizing the ribosomal housekeeping gene, L32, values to normalize for differences in RNA loading. The average change in transcript level, relative to that found at 0 h., with S.D. (n = 7) is indicated. Values greater than one indicate gene induction, and those less than one indicate gene repression.

**Fig. 3.** The transcriptional activating and repressing functions of p53 are impaired after inhibition of de novo purine synthesis by DDATHF. A, Northern blot analysis of p21 and mdm2 induction following treatment of HCT116 p53+/+ cells with 10 μM DDATHF. Total RNA was isolated after the indicated times of DDATHF exposure and 2 μg poly(A) RNA subjected to Northern blotting. The sample in the first lane was 2 μg of mRNA from HCT116 p53+/+ cells treated with 10 μM VP16 for 24 h. The blot was stripped and reprobed for gapdh. B, RPA of p53-regulated genes following exposure of p53+/+ cells to DDATHF. Twenty micrograms of total RNA were hybridized to a probe synthesized from a stress-inducible multiprobe template. Shown in the first lane are the migration positions of the unhybridized probe and, in the last lane, yeast rRNA hybridized with probe as a test of specificity of hybridization. For details, see “Experimental Procedures.” C, Western blot analysis of p53 downstream transcriptional targets Mdm2, Gadd45, and Bax was performed on lysates of HCT116 p53+/+ and p53−/− cells exposed to 10 μM DDATHF for the indicated times. Stripping and reprobing of the blots with actin was used to assess protein loading.
Subcellular Localization of p53 Following DDATHF Treatment—The ability of p53 to translocate to the nucleus is a prerequisite for transcriptional activation; yet this active process might well be compromised by diminished levels of purine nucleotides. To investigate this possibility, G1/G0-synchronized HCT116 p53+/+ cells were released from synchrony into media containing either PBS or 10 μM DDATHF, and cells were analyzed by flow cytometry, by Western blotting for p53 and p21 expression, and by immunofluorescence to determine the subcellular localization of p53 (Fig. 5). There was a clear increase in p53 nuclear staining concomitant with the accumulation of cells in S phase and with p53 expression levels, which peaked after 24 h of DDATHF exposure and remained elevated after 48 h (Fig. 5C). This elevation in p53 nuclear staining was similar to that seen in p53+/+ cells treated with VP16 for 24 h (Fig. 5D). Nuclear p53 staining was not seen in control p53−/− cells, even at high magnification (Fig. 5E). We concluded that, as carcinoma cells reach the G1/S border in the presence of DDATHF, p53 becomes stabilized, and there is no impediment to the cytoplasmic-nuclear translocation step in the activation of p53.

Disruption of p53 Signaling to p21 Is Unique for GART Inhibitors Among the Various Classes of Antifolates—Other folate analogs inhibitory to purine synthesis were studied as well as agents inhibitory to other steps in folate metabolism. Both the second generation GART inhibitors LY309887 and AG2034 and the third generation GART inhibitor AG2037 had effects identical to DDATHF, causing robust p53 accumulation, but no p21 induction (Fig. 6A). In contrast, treatment of HCT116 cells with the dihydrofolate reductase inhibitor methotrexate resulted in p53 accumulation and marked p21 transcription, as did the antifolate thymidylate synthase inhibitor D1694 (tomudex, raltitrexed) (Fig. 6B). The induction of p21 was clear within 6 h of methotrexate treatment, and was quite striking after 24 h. Hence, the observed defect in the p53 response pathway is a general and distinctive characteristic of any GART inhibitor, but this response is not shared by other antifolates. This specificity is very interesting, given that methotrexate and all of the other known inhibitors of mammalian dihydrofolate reductase are thought to result in an indirect inhibition of de novo purine synthesis as well as of thymidylate synthesis.

Post-translational Modifications of the p53 That Accumulate After GART Inhibition—Several serine residues in the N-terminal domain of p53 are known to be phosphorylated in response to genotoxic insults such as ionizing radiation and ultraviolet light, and these events are thought to contribute to the stabilization and dissociation of p53 from human Mdm2 (36) and the nuclear translocation of the accumulating p53 (37). Similarly, acetylation of lysines in the C terminus of p53 has been implicated in the binding of nuclear p53 to motifs in p53-sensitive promoters (38, 39). In distinct contrast, upon DDATHF treatment, phosphorylation could not be detected using an antibody specific for p53 phosphorylated at serine 15 (compare Fig. 7A with Fig. 2A). Serine 15 was strongly phosphorylated in response to VP16, methotrexate, and D1694, with a time course similar to that of p21 induction (Fig. 7A). When equivalent amounts of p53 were loaded onto a gel from each lysate to allow assessment of the ratio of phosphate groups per p53 molecule, the level of serine 15 phosphorylation of DDATHF-stabilized p53 was very low, in contrast to the strong phosphorylation levels in response to VP16 or ionizing radiation (Fig. 7B). Similarly, serines 6 and 20 of p53 remained hypophosphorylated in response to DDATHF (Fig. 7B).

The acetylation of lysines 373 and 382 of p53 was assessed by immunoprecipitating with a pantropic p53 antibody, followed

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**Fig. 4. DDATHF-induced stabilization of p53.** A. Western blot displaying p53 protein levels following inhibition of protein synthesis. HCT116 p53+/+ cells were treated with 10 μM DDATHF for 8 h, or were exposed to 15 Gy ionizing radiation and incubated for 2.5 h, at which time 20 μg/ml cycloheximide was added. Lysates were prepared at the indicated times and Western blotting was performed with the p53 antibody Ab-6. B. in vivo ubiquitination assay showing laddering of ubiquitinated p53 conjugates. HCT116 p53+/+ or p53−/− cells were either untreated or exposed to 10 μM DDATHF for 6 or 12 h, or to 10 μM VP16 for 24 h. p53 was immunoprecipitated from whole cell lysates and ubiquitination was assessed by Western blotting with a p53 antibody, Ab-6. The heavy (H) and light (L) chains of the immunoprecipitating antibody are indicated.

About 10 min (Fig. 4A), as expected from previous studies. Hence, de novo purine synthesis inhibition leads to p53 protein stabilization.

Normal turnover of the p53 protein is modulated by the ubiquitin/proteasomal pathway whereby the activity of a ubiquitin ligase, such as Mdm2, results in progressive addition of several residues of ubiquitin, a 9 kDa protein. Because ubiquitination and subsequent proteosomal degradation of the tagged p53 requires ATP, we reasoned that the limited purine pools might be affecting turnover of p53 in DDATHF-treated cells. The marking of p53 for degradation by ubiquitination was studied in HCT116 p53+/+ cells treated with VP16 or DDATHF. As can be seen in Fig. 2A, the levels of p53 protein rose within 6 h and peaked after 12 h of GART inhibition in HCT116 cells; p53 was immunoprecipitated over this time course and p53 ubiquitination was analyzed by Western blotting. Ubiquitinated p53 conjugates were detected after treatment with VP16, as evidenced by a ladder of species ranging from 53 to 90 kDa, that were immunoreactive with a p53 antibody (Fig. 4B). This increase was expected and appears to reflect the ubiquitin ligase activity of Mdm2, based upon previous literature (35). However, when p53+/+ HCT116 cells were exposed to DDATHF, p53 ubiquitination was not detectable over this time period, despite the increased steady state levels of p53 and Mdm2 protein. Thus, inhibition of de novo purine synthesis results in the inability to properly ubiquitinate p53, resulting in stabilization of this protein against proteosomal degradation.
by Western blotting. In order to compare the level of acetylation present on p53 before and after drug treatment, p53 levels in immunoprecipitates were first quantitated, and then equivalent amounts of immunoprecipitated p53 were loaded onto a second gel. The gel was subjected to Western blotting with an antibody against acetylated lysines 373 and 382. Acetylation of these residues was not stimulated after DDATHF or VP16 treatment, and, in fact, the number of acetyl groups per p53 molecule dropped substantially below untreated levels by 12 and 24 h of GART inhibition as p53 accumulated, implying that the p53 being synthesized after DDATHF exposure was not being acetylated (Fig. 7C). Thus, although p53 stabilization occurs upon DDATHF treatment, both N-terminal phosphorylation and C-terminal acetylation were severely compromised on the accumulating p53 protein.

In Vitro Binding of DDATHF-stabilized p53 to a p53 Consensus Sequence in the Human p21 Promoter—One of the reported consequences of p53 N-terminal phosphorylation, particularly at serine 15, is enhanced recruitment, by p53, of the histone acetyltransferase CBP/p300, and enhanced acetylation of p53 (40, 41). CBP/p300 is capable of transferring acetyl groups to both the p53 C terminus and to the chromatin itself, thereby enabling transcription. Therefore, to determine whether the largely unmodified p53 that accumulates in the nucleus following GART inhibition was able to bind to the p21 promoter, electrophoretic mobility shift assays (EMSA) were employed. Nuclear extracts from p53+/+ cells treated with 10 μM VP16 exhibit binding to an oligonucleotide containing the upstream p53 binding site of the p21 promoter previously studied by EMSAs (29)(Fig. 8A). Surprisingly, after only 6 h of purine synthesis inhibition by DDATHF, nuclear extracts from p53 wild-type cells were capable of p21 probe binding, and binding continued for 24 h. Furthermore, the specificity of binding to the p21 oligonucleotide was demonstrated upon the addition of excess cold wild-type and mutant oligonucleotides to the binding reactions (Fig. 8B). Twenty-fold excess cold wild-type oligonucleotide interfered with binding of the 32P-labeled probe, while up to 40-fold excess of a related oligonucleotide with the p53 binding site mutated failed to compete out the wild-type probe (Fig. 8B). The specificity of this binding for nuclear extracts from either VP16 or DDATHF-treated carcinoma cells was confirmed by supershift analysis with the p53 antibody Ab-6 (Fig. 8B). Hence, the largely unmodified species of p53 that accumulated in DDATHF-treated cells was capable of binding to a p53 binding site of the p21 promoter despite the absence of acetyl groups on the C-terminal domain, in agreement with the conclusions of recent in vitro studies on this promoter (42).

Binding of p53 to the p21 Promoter in Intact Cells Treated with DDATHF—Chromatin immunoprecipitation (ChIP) experiments were performed to study the events of transcriptional activation of the p21 promoter in DDATHF-treated cells...
DDATHF-induced Defect in the p53 Response

Fig. 6. GART inhibitors, as a class, induce p53 accumulation without concomitant p21 induction, but this phenomenon is not common to all antifolates. A, Western blot analysis of p53 and p21 expression following exposure of HCT116 p53+/+ cells to the second generation GART inhibitors LY309887 (10 μM) and AG2034 (10 μM), and the third generation GART inhibitor, AG2037 (10 μM). B, Western blot analysis of p53 and p21 protein levels after treatment of HCT116 p53+/+ cells with the thymidylate synthase inhibitor, D1694 (10 μM), or the dihydrofolate reductase inhibitor, methotrexate (10 μM).

Fig. 7. Analysis of the post-translational modifications of the p53 that accumulates following GART inhibition indicates lack of serine phosphorylation and lack of lysine acetylation. A, HCT116 p53+/+ cells were exposed to either 10 μM DDATHF, 10 μM VP16, 10 μM D1694, or 10 μM methotrexate for the indicated times. SDS-PAGE and immunoblotting were performed on 25 μg of protein, phosphorylation of p53 was determined by probing with an antibody specific to phosphorylated p53 at serine residue 15, and the membranes were stripped and reprobed for actin. B, cells were treated with 10 μM DDATHF (12 h), 10 μM VP16 (24 h), or irradiated at 15 Gy and harvested after 3.5 h. A preliminary Western blot was performed to quantitate total p53 levels, and equivalent amounts of p53-containing lysate were subsequently loaded and subjected to immunoblotting with antibodies specific for p53 phosphorylated at serines 6, 15, or 20. C, immunoprecipitation with an antibody to p53 was performed on HCT116 p53+/+ cell lysates prior to immunoblotting. p53 protein levels were determined, and equal amounts of p53-containing immunoprecipitate were subjected to Western blotting. Acetylation of C-terminal residues of p53 was examined utilizing an acetylation-specific antibody to lysines 373/382. Blots were stripped and reprobed with an antibody to p53 to confirm p53 loading.

in vivo. Two functional p53 binding sites have been identified on the human p21 promoter (4, 43). In our experiments, genomic DNA was sheared to fragments predominantly on the distribution range of 200–1000 bp to allow distinction of p53 binding to the upstream and downstream p53 binding sites located at approximately –2300 and –1300 bp, respectively (Fig. 9A). When chromatin from VP16-treated p53+/+ cells was immunoprecipitated with a p53 antibody, a strong 234 bp product could be amplified from this immunoprecipitate using PCR primers flanking the 5′ p53 binding site of the p21 promoter (Fig. 9, A and B), and a second DNA fragment centered on the 3′ p53 binding site was, likewise, present in the immunoprecipitate (Fig. 9B). The same 5′ and 3′ PCR products of similar intensity to those seen in cells bearing DNA damage by VP16 were also seen in the chromatin extracted from DDATHF-treated p53+/+ cells, indicating that the p53 accumulating upon GART inhibition is, indeed, capable of binding to the p21 promoter at both the upstream and downstream p53 binding sites in intact cells (Fig. 9B). Virtually identical results were obtained for VP16 (Fig. 9B) or IR-treated cells (data not shown). When real-time PCR was used to quantitate p53 bound to these immunoprecipitated DNAs, the increase of p53 residency at each binding site in the p21 promoter induced by DDATHF treatment was as extensive as that seen with VP16-treated cells, with p53 binding at least 4-fold higher than untreated controls (Fig. 9B). Hence, the defect in the p53 pathway responsible for a failure to transcriptionally activate p21 was downstream of nuclear accumulation of p53 and binding to the previously defined p53 sites in the p21 promoter.

Recruitment of the Elements of the Transcriptional Initiation Complex to the p21 Promoter in DDATHF-treated Cells—The residency of the most common universal coactivator of transcription, CBP/p300 (44), by p53 bound to the upstream and downstream p53-binding sites was studied by ChIP experiments. Interestingly, this coactivator was recruited to the 5′ and 3′ p53 binding sites in HCT116 cells poorly, if at all, by either the fully functional p53 that accumulated in response to the DNA damage or the p53 lacking N-terminal phosphorylation and C-terminal acetylation modifications after DDATHF treatment (Fig. 9C). This result was obtained with each of two antibodies raised against CBP/p300. The recruitment of RNA polymerase II to these regions of the p21 promoter was also analyzed and was found to reflect the binding of p53 (Fig. 9C).
Thus, genomic DNA fragments corresponding to both 5' and 3' p53 binding sites were enriched in complexes immunoprecipitated by an antibody against RNA polymerase II in cells containing VP16-induced DNA damage or in DDATHF-treated cells to an equivalent extent (2-fold over control cells). Hence, the recruitment of RNA polymerase II by p53 binding was independent of the post-translational modifications of p53 studied.

Acetylation of Chromatin Decorating the p53 Binding Sites of the p21 Promoter—When the acetylation of histone H3 at lysine residues 9 and 14 was examined over the p53 binding sites in the p21 promoter by ChIP, the DNA from VP16-treated cells exhibited strong histone H3 acetylation over the DNA fragments surrounding the 5'- and the 3'-p53 binding sites, but this was not seen with DDATHF-treated cells (Fig. 9C). Ionizing radiation induced histone H3 acetylation to levels similar to those seen with VP16 treatment (data not shown). Hence, upon GART inhibition, we find p53 nuclear translocation and p21 promoter binding, despite the lack of key post-translational modifications; yet, there remains insufficient chromatin acetylation and remodeling to facilitate transcriptional activation.

**DISCUSSION**

We have found a major difference in the response of the p53 pathway to inhibition of de novo purine synthesis compared with the response previously reported for inhibition of de novo pyrimidine synthesis. Upon inhibition of de novo pyrimidine synthesis with PALA, p53 protein accumulates in the absence of measurable DNA damage, an observation that led to the concept that p53 can accumulate as a direct result of nucleotide pool imbalances (8). Our results provide additional support for this concept: following de novo purine synthesis blockade by the GART inhibitors studied, p53 levels also markedly increase in tumor cells (Figs. 2 and 6). However, the responses of the p53 pathway for PALA and DDATHF diverged immediately past the point of p53 accumulation. PALA-initiated p53 accumulation strongly induces p21 (8, 45). In contrast, the p53 accumulating after GART inhibition is almost inactive as a transcriptional activator at several downstream p53 target genes, including p21 (Figs. 2 and 3). As a result, PALA-inhibited normal human diploid fibroblasts (8) or human colon carcinoma HCT116 p53+/- cells (Fig. 1) respond to accumulating p53 by the induction of G1 and G2 cell cycle blocks; after exposure to DDATHF, p53 wild-type carcinoma cells progress into and accumulate in S phase (Fig. 1).

We believe that GART inhibitor-induced p53 stabilization results, not from DNA strand breaks, but rather, from purine ribonucleotide depletion. Restoration of ATP and GTP pools via the salvage pathway using extracellular inosine prevented elevation of p53 levels (Fig. 2C). Moreover, it is known that DDATHF does not induce detectable DNA strand breaks at early time points when analyzed by filter elution studies (27); only after 3 days of treatment with AG2034 do DNA strand breaks become detectable by the much more sensitive TUNEL assay (16), that is, well after the induction of p53 by the GART inhibitors. Easily detectable DNA strand breaks are induced early in the course of action of antifolates inhibitory to thymidylate synthase (46, 47) or dihydrofolate reductase (48, 49), as a result of DNA synthesis in the absence of TTP but anomalous levels of dUTP, with subsequent patch excision of dUMP in DNA by DNA-uracil glycosylase. Presumably, stabilization of p53 in these latter cases is a response to DNA damage, although it may have a component that is initiated by depletion of TTP per se.

Although the classic view of p53 stabilization involves stress-induced phosphorylation of p53 which releases Mdm2 binding, this view has been challenged by more recent studies. It has been shown that p53 phosphorylation is not essential for DNA damage-induced stabilization of p53, and that p53 mutants incapable of phosphorylation are still capable of stabilization following UV irradiation or adriamycin (50, 51). Our results furnish support for the concept that phosphorylation in the p53 peptide involved in binding Mdm2 is not essential for the accumulation of p53. Another mechanism for p53 accumulation seems operative after GART inhibition: the limited purine pools appear to decrease p53 ubiquitination and proteasomal degradation, allowing a prolonged stability of the protein (Fig. 4). It was initially surprising to note that the level of Mdm2 transiently increases after DDATHF treatment (Fig. 3C), without a measurable increase in mdm2 mRNA (Fig. 3A). However, we note that the decreased p53 ubiquitination after purine nucleotide depletion would most likely also reflect a decreased ubiquitination of Mdm2, explaining the increased stability of this protein. Whatever role this increased Mdm2 plays in limiting the effects of p53 would appear to be minor, based on the facts that nuclear p53 accumulates in DDATHF-treated cells

**FIG. 8.** The p53 accumulating in the nucleus after GART inhibition is capable of binding an oligonucleotide containing the p53 binding element of the p21 promoter. A, EMSA examining the in vitro binding of p53 to its 5' consensus element on the p21 promoter. The first three lanes show binding of recombinant p53, while the remaining lanes show binding of p53 extracted from nuclei of DDATHF or VP16-treated HCT116 p53+/- cells exposed to drug as in Fig. 2. The bottom of the film, which had bands indicating free probe and nonspecific binding, is not shown. B, EMSA displaying the specificity of binding to the p21 oligonucleotide. The top gel shows data from VP16-treated nuclei, and the bottom gel, from DDATHF-treated nuclei. Either 20- or 40-fold molar excess of cold wild-type or mutant p21 oligonucleotide were added simultaneously with 32P labeled wild-type p21 probe. Specific p53 binding is supershifted by the addition of the p53 antibody Ab-6. For details, see "Experimental Procedures."
and that the nuclear p53 binds to the p21 promoter equally well as is seen with IR or VP16-treated cells (Figs. 8 and 9).

Our data support a model whereby DDATHF-induced purine ribonucleotide depletion is sensed, either directly or indirectly by p53, which then is stabilized and accumulates (Fig. 10). However, the limiting purine pools render the kinases responsible for p53 phosphorylation inactive, resulting in a hypophosphorylated and hypoacetylated p53 species. It has been suggested that phosphorylation of p53 at serine 15 is a critical event for p53 transcriptional activation (40), and is the primary signal that is then amplified by phosphorylation of residues 6, 9, and 18 (52). These phosphoserines, in turn, promote first acetylation of p53 itself, and then the recruitment of HATs to target promoters and the activation of localized histone acetylation (52). Despite the lack of post-translational modifications of p53 in DDATHF-treated cells, our ChIP experiments (Fig. 9) provide direct in vivo evidence that this unmodified p53 is capable of nuclear retention and binding to the p21 promoter, notably of histone H3, and a failure to stimulate transcription.

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and the promoter transcriptionally inactive. The response of the p53 pathway to GART inhibitors appears to represent a "non-genotoxic stress" and, hence, invites comparison with those initiated by hypoxia or hydroxyurea treatment. Recent studies have reported that hypoxic stress or cell cycle arrest with hydroxyurea can also result in the accumulation of a p53 protein without transcriptional activation of downstream genes, but the intermediate steps in the mechanisms involved differed from that observed with GART inhibitors. Hypoxia, a classic "non-genotoxic" stress, leads to stabilization of a transcriptionally latent p53 that is not acetylated at the C terminus but is phosphorylated at residues in the N-terminal peptide (53). Hydroxyurea (HU) was reported to induce the accumulation and nuclear localization of a p53 species incapable of target gene induction despite the modification of both N-terminal phosphorylation sites and C-terminal acetylation sites (7). The mechanism of the inactivity of p53 as a transcriptional activator after HU has not yet been solved, but it is very interesting to note that cells treated with HU failed to mount an effective p53 response to a subsequent challenge with IR (7). To our knowledge, the mechanisms whereby p53 accumulates within the nucleus following treatment with other non-genotoxic stimuli such as hypoxia or PALA remain unclear.

We conclude that there are multiple factors needed for a transcriptionally productive p53 response and that binding of p53 to its cognate sites in the promoters of downstream target genes is necessary but not sufficient for transcriptional activation. Although p53 is activated upon de novo purine synthesis inhibition by DDATHF, insufficient recruitment of chromatin-remodeling complexes prevents transcriptional activation of the p21 gene and allows for S phase entrance of tumor cells. This explains why tumor cells with wild-type or null/mutant p53 alleles are equally sensitive to cell growth inhibition and cell kill by DDATHF or AG2034 (28): p53 wild-type cells become functionally p53 null after treatment with GART inhibitors.

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A Defect in the p53 Response Pathway Induced by \textit{de Novo} Purine Synthesis Inhibition

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