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Antifolates Targeting Purine Synthesis Allow Entry of Tumor Cells into S Phase Regardless of p53 Function[1]

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ABSTRACT

The class of folate antimetabolites typified by (6R)-dideazatetrahydrofolate (lometrexol, DDATHF) are specific inhibitors of *de novo* purine synthesis because of potent inhibition of glycinamide ribonucleotide formyltransferase (GART) but do not induce detectable levels of DNA strand breaks. As such, they are a test case of the concept that ribonucleotide depletion can be sensed by p53, resulting in a G_1 cell cycle block. The GART inhibitors have been proposed previously to be cytotoxic in tumor cells lacking p53 function but only cytostatic in p53 wild-type tumor cells. We have investigated this concept. Cell cycle progression into and through S phase was slowed by DDATHF, but both p53 +/+ and −/− human colon carcinoma cells entered and completed one S phase in the presence of drug. This inability of p53 to initiate a G_1 arrest after DDATHF treatment was mirrored by an independence of the cytotoxicity of DDATHF on p53 function. We conclude that carcinoma cells are killed equally well by DDATHF and related compounds whether or not the p53 pathway is intact and that the utility of GART inhibitors would not be limited to p53-negative tumors.

INTRODUCTION

DDATHF (1)[3] was identified as a unique drug entity because of its selective inhibition of the first folate-dependent enzyme of the *de novo* purine synthesis pathway, GART. As an antifolate, DDATHF is not incorporated into DNA, in contrast with the purine nucleotide antimetabolites. DDATHF and its polyglutamate metabolites bind tightly to GART, resulting in a rapid and prolonged depletion of intracellular purine ribonucleotides (2, 3). During initial clinical trials, the 6-R diastereomer of DDATHF (lometrexol) induced several objective responses but was limited by sustained and poorly reversible thrombocytopenia (4, 5). It was subsequently found that supplementation with oral folic acid ameliorated the cumulative toxicities of this agent in second generation clinical trials (6, 7), and parallel animal studies demonstrated that coadministration of folic acid allowed preservation of antitumor activity (8). Second and third generation GART inhibitors were developed (9, 10), and the Lilly DDATHF analog LY309887, and Agouron compounds AG2034 and AG2037 also advanced to human trials (11–13).

Neither DDATHF nor its analogs cause DNA strand breaks (14–16), classically believed to be the main stimulus for initiating p53 stabilization and the downstream G_1 checkpoint. However, the p53 response pathway can also be activated by ribonucleotide deprivation (17). Thus, in normal human fibroblasts containing wild-type p53, *N*-phosphonacetyl-L-aspartate depletes pyrimidine ribonucleotide pools, and subsequently induces p53 accumulation and a pronounced G_1 arrest (17). Interestingly, previous work concluded that the second generation GART inhibitor AG2034 caused cytostasis in tumor cells with wild-type p53 function but pronounced cytotoxicity in p53 null or mutant tumor cells (16).

The hypothesis that GART inhibitors distinguish between tumor cells based on p53 function became the focus of this investigation. When we analyzed cell cycle traverse after GART inhibition in tumor cell lines that differed in p53 function, we found a transient accumulation of cells in S phase regardless of p53 status, followed by a distinctly slowed traverse through S phase. In agreement with this inability of p53-competent cells to induce a G_1 arrest in the face of GART inhibitors, cells containing wild-type p53 and those containing mutant, null function, or knock-out p53 alleles were equally sensitive to cytotoxicity by the GART inhibitors lometrexol and AG2304.

MATERIALS AND METHODS

Solutions. Stock solutions of 6R-DDATHF (Eli Lilly) and AG2034 (Agouron) were prepared in PBS [0.144 g/liter KH_2PO_4, 9 g/liter NaCl, and 0.795 g/liter $Na_2HPO_4 \cdot 7H_20$ (pH 7.4)], and concentrations were adjusted based on UV absorption at basic pH using mM extinction coefficients of 11.7 at λ 272 nm and 19.35 at λ 282 nm, respectively.

Cell Culture. Mouse leukemia L1210 cells were maintained as suspension cultures in RPMI 1640 supplemented with 10% dFCS. Human colon carcinoma HCT116 p53 +/+ and p53 −/− cells were a gift from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD) (18) and were maintained in RPMI 1640 with 10% dFCS. Human breast carcinoma cells (MCF7), lung carcinoma cells (A549), colon carcinoma cells (SW480), and cervical carcinoma cells (HeLa S3) were obtained from the American Type Culture Collection and were maintained and treated in folate-free RPMI 1640 supplemented with 50 nM folic acid and 10% dFCS. All of the cell lines were periodically determined to be free of *Mycoplasma* using a PCR-based *Mycoplasma* detection kit from American Type Culture Collection. HCT116, MCF7, and A549 cells have been shown to have wild-type p53 by direct DNA sequencing and also by the functional assay of imposition of a G_1 block after γ-irradiation (19, 20). SW480 has an inactivating point mutation in the *p53* gene at codon 273 (21), and in HeLa S3, p53 is disrupted by the expression of human papillomavirus 18 viral protein E6 (22).

Growth Inhibition Studies. L1210 cells were preincubated for 16 h in RPMI 1640 containing 10% dFCS, 100 μM hypoxanthine, and DDATHF. Cultures were split to 2–3 × 10^5 cells/ml, and culture densities were electronically determined with time thereafter. HCT116 cells were plated at 8 × 10^4 cells/60-mm dish. Beginning 24 h later, cells were exposed to DDATHF for 72 h in RPMI 1640 containing 10% dFCS. Cells were washed with PBS, harvested with 0.1% trypsin/0.4% EDTA, and counted.

Flow Cytometry. L1210 cells were pretreated with DDATHF and hypoxanthine, as described above, before flow cytometry. At the indicated times, cells were removed, washed in cold PBS, and resuspended in propidium iodide stain (50 μg/ml propidium iodide, 0.1% Triton X-100, and 7 Kunitz units per ml RNase B in 3.2 mM sodium citrate buffer) at a density of 1 × 10^6 cells/ml. For dual parameter flow cytometry experiments, L1210 cells were pulse labeled with 10 μM BrdUrd for 30 min to label all of the cells actively synthesizing DNA. Excess BrdUrd was washed from the cells, and cells were resuspended in medium containing either DDATHF and hypoxanthine or DDATHF alone. At various times thereafter, cells were washed with PBS and fixed in cold 70% ethanol. The following day, cells were washed in 0.5% bovine serum albumin in PBS then treated with 2 N HCl for 20 min to denature cellular DNA. The suspension was brought to neutral pH with 0.1 M sodium borate (pH 8.5) and incubated for 1 h in mouse monoclonal anti-BrdUrd

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[3] The abbreviations used are: DDATHF, (6R)-5,10-dideazatetrahydrofolate; GART, glycinamide ribonucleotide formyltransferase; BrdUrd, bromodeoxyuridine; dFCS, dialyzed fetal calf serum; AG2034, 4-[2-(2-amino-4-oxo-4,6,7,8-tetrahydro-3H-pyrimidino[5,4−6][1,4] thiazin-6-yl)-(S)-ethyl]-2,5-thienoyl-L-glutamic acid.

FITC-conjugated antibody (BD PharMingen) diluted 1:6 in 0.5% bovine serum albumin/0.5% Tween 20/PBS. Cells were then resuspended in propidium iodide stain without Triton X-100. In some experiments, cells were incubated with 0.2 μg/ml nocodazole and 1 μM (L1210 cells) or 10 μM DDATHF (HCT116 cells). Cells were washed with PBS and resuspended in propidium iodide stain. Samples were kept at 4°C until analysis on an EPICS XL-MCL flow cytometer (Beckman Coulter).

Clonogenic Assays. HCT116 cells were plated at densities of 300 cells/100-mm dish and allowed to attach for 4–6 h. Medium containing 0–100 μM DDATHF and 10% dFCS was added, and cells were treated continuously for 24 h. Medium was then aspirated, cells were washed with PBS, drug-free medium was added, and cultures were fed with medium containing dFCS every 2–3 days. After 14 days, colonies were fixed in methanol and stained with 1:10 diluted Wright-Giemsa reagent. Typical plating efficiencies were 60% for p53+/+ cells and 30% for p53−/− cells. In some experiments, 50 μM inosine was added to the plates 5 days after drug treatment to ensure that viable cells could recover from inhibition of purine synthesis and form colonies; thereafter, inosine was added to all of the medium used to feed the cells during colony formation. Clonogenic assays for the MCF7, A549, SW480, and HeLa S3 involved treatment with AG2034 for 4 h starting 4 h after plating, and propagation in medium containing 10% dFCS and 50 nM folic acid for 2 weeks before fixing and staining colonies. Plating efficiencies for these cell lines were: MCF7, 70%; A549, 50%; SW480, 90%; and HeLa S3, 90%.

RESULTS

Rapid Inhibition of Tumor Cell Growth by Effective GART Inhibition. Earlier studies with the L1210 murine leukemia cell line demonstrated that cellular ATP and GTP levels were reduced to <25% of control after 6 h of DDATHF exposure, and that the concentration range of DDATHF causing ribonucleotide depletion was identical to that for cell growth inhibition (2). To understand exactly how quickly *de novo* purine synthesis inhibition blocked the proliferation of tumor cells, L1210 were preincubated in medium containing DDATHF and 100 μM hypoxanthine for 16 h, and the effects of purine synthesis inhibition were precipitated by removal of hypoxanthine to minimize any delays due to drug transport or metabolism. At the concentration of 30 μM DDATHF, cell growth slowed after 2–4 h of GART inhibition (Fig. 1A).[4] When the dose of DDATHF was reduced to 1 μM [a concentration which completely (>95%) blocked the formylation of glycinamide ribonucleotide by GART *in vivo* in this cell line (2)], onset of growth inhibition was likewise observed after 4 h of DDATHF treatment (Fig. 1*A*). Within 22 h of *de novo* purine synthesis inhibition, cell density had increased only by 21% in 1 μM DDATHF, and, after 100 h, the treated cultures reached a peak density 1.63-fold higher than the original plating density, whereas control cultures proliferated 128-fold over this interval (data not shown). These experiments established that, although cell growth rates were affected by 4–6 h of purine synthesis inhibition, there was a slow increase in cell number, which continued at a greatly reduced rate. This slow emergence of cells from M phase did not appear to be due to an incomplete block of the purine synthesis pathway, but rather, to be the result of an insensitivity of cell cycle progression to *de novo* purine synthesis inhibition.

Effects of DDATHF on Cell Cycle Progression in L1210 Cells. Because blockade of *de novo* purine synthesis by DDATHF clearly permitted L1210 cell cycle progression but limited it to less than one cell cycle traverse, the position in the cell cycle at which blockade occurred was studied. Flow cytometric analysis of propidium iodide-stained cells indicated a rapid loss of the G_2/M phase population of cells after DDATHF exposure and, by 8 h, there was an early S phase

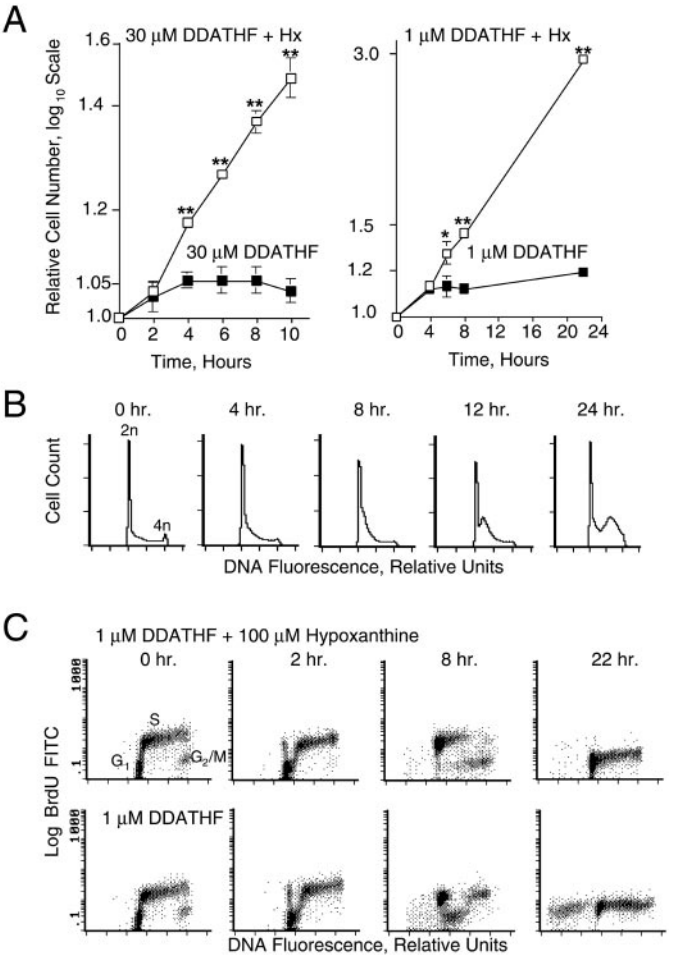

Fig. 1. DDATHF induces rapid and complete growth inhibition and cell cycle arrest in murine leukemia L1210 cells. Triplicate cultures of L1210 cells were maintained in RPMI 1640 containing DDATHF and 100 μM hypoxanthine for 16 h, then fresh medium containing either 30 μM (*A*) or 1 μM (*A–C*) DDATHF with or without hypoxanthine was added. Cell densities were determined electronically at the indicated times after this medium change. Statistically significant ($P < 0.05$ or 0.001) differences in growth of treated and control cultures are indicated by * or **, respectively. *B*, propidium iodide analysis of L1210 cells indicates S phase accumulation after exposure to a growth-inhibitory concentration of DDATHF. After the above treatment, cells were stained with propidium iodide at the indicated times, and DNA content was examined by flow cytometry. *C*, BrdUrd labeling demonstrates progression of cells through all phases of the cell cycle. L1210 cells were treated overnight in medium containing DDATHF and hypoxanthine, and then pulse-labeled with 10 μM BrdUrd for 30 min. Hypoxanthine was removed from half of the cultures, and cells were collected and fixed over time. Anti-BrdUrd FITC and propidium iodide were added, and two parameter flow cytometry was performed. For details, see "Materials and Methods;" *bars*, ±SD.

accumulation of cells (Fig. 1*B*). Cells continued to accumulate in early S phase after 12 h of GART inhibition but by 24 h the S phase population appeared to be slowly shifting to higher DNA content, and hence, from mid-to-late S phase.[5] There was maintenance of a steady G_1 population of cells throughout DDATHF treatment, but it was not clear whether this G_1 component represented cells blocked at G_1-S or cells emerging from S and M phases repopulating G_1. To separate the effects of purine synthesis inhibition on G_1 and G_2 cells from those in S phase, L1210 cells were pulsed with 10 μM BrdUrd for 30 min, effectively labeling the population of cells that were synthesizing DNA during the pulse. Untreated cells that were in G_1 upon BrdUrd pulsing traveled into G_2/M phase within 8 h, and the BrdUrd-positive S phase cells in the untreated cultures had completed almost one full

[4] For reference, L1210 cells were half-maximally inhibited at 0.023 ± 0.0085 μM ($n = 3$) DDATHF in a 72-h continuous exposure experiment, an IC_{50} equivalent to published values (2).

[5] The accumulation in S phase has been reported previously in CEM leukemia cells treated with DDATHF or LY309887 (2, 15).

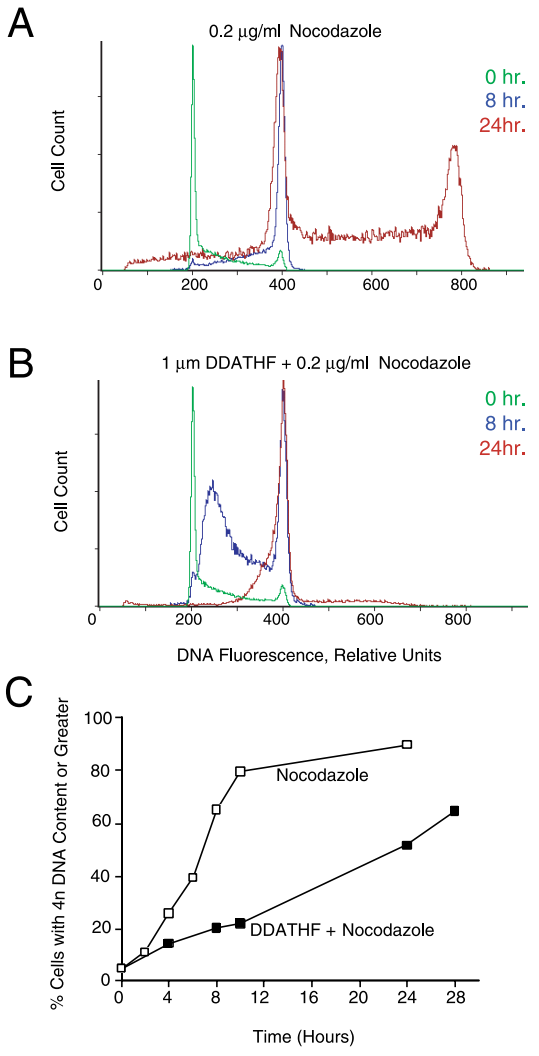

Fig. 2. Flow cytometric analysis of nocodazole-blocked L1210 cells. *A* and *B*, DDATHF-treated L1210 cells traverse S phase and exit into G_2/M, but at a greatly reduced rate. L1210 cells were treated with either 1 μM DDATHF and 0.2 μg/ml nocodazole, or nocodazole alone. Cells were collected at the various times thereafter and resuspended in propidium iodide stain. Cell cycle analysis was performed as described in "Materials and Methods." *C*, estimation of the rate of traverse of S phase by DDATHF-treated cells. After flow cytometry, the percentages of cell populations in G_2/M phase were calculated using ModFit LT (Verity) software.

cell cycle by 8 h, reappearing in G_1 and G_2 and mid-to-late S phase (Fig. 1*C*). In contrast, cells in G_1 at the time of pulsing in DDATHF-treated cultures were capable of entering S phase after DDATHF treatment, but at a much slower pace than their control counterparts. Drug-treated BrdUrd-positive cells labeled early in S phase cycled into G_2/M by 8 h, whereas those labeled late in S phase reappeared in G_1, but did not re-enter S phase after 8 h of GART inhibition. By 22 h of DDATHF treatment, cellular debris with a DNA content <2n was observed, apparently reflecting cell death in a fraction of the population.

Nocodazole trapping was used to monitor the rate at which G_1 cells exposed to DDATHF entered and exited S phase, without the complication of cells re-entering G_1 from M. The population of control L1210 cultures grown in medium containing DDATHF and hypoxanthine entered G_2/M phase behind a nocodazole block within one generation time (12 h; Fig. 2). After 24 h of a nocodazole block, a significant percentage of the control population (23%) had become octaploid, re-entering S phase and rereplicating their DNA without cell division. A strikingly different profile was evident for those L1210 cells treated with DDATHF and nocodazole in the absence of purines. By 8 h of nocodazole blockade in DDATHF-treated cells, the G_1 population had progressed into S phase but accumulated in mid-S, clearly as a result of slowed DNA synthesis. Cells with an 8n DNA content were not observed 24 h after drug treatment. It appeared that L1210 cells could slowly initiate and complete S phase in the face of complete GART inhibition, pass through G_2 and reach mitosis, but were subsequently incapable of further DNA synthesis. S phase traverse rate was estimated to be slowed by a factor of 5-fold by GART inhibition from the rate of accumulation of cells in G_2/M behind a nocodazole block (Fig. 2*C*).

Effects of *de Novo* Purine Synthesis Inhibition on Cell Cycle Traverse in Human Colon Carcinoma Cells Containing Wild-Type or Knockout p53 Alleles. L1210 cells have a point mutation in the DNA-binding domain of p53, resulting in the substitution of valine for glycine at position 151 (23). It became of importance to determine whether wild-type p53 would prevent cancer cells from entering S phase upon GART inhibition. Rather than making comparisons between cell lines of different genetic backgrounds, human colon carcinoma HCT116 cells with both alleles of the *p53* locus deleted by homologous recombination (kindly provided by Dr. Bert Vogelstein, Johns Hopkins University, Baltimore, MD) were compared with otherwise isogenic HCT116 cells with the p53 +/+ genotype. The sensitivity of p53+/+ and p53−/− cells to growth inhibition by DDATHF was identical in these cell lines (Fig. 3*A*; IC_{50} for the p53+/+ cells, 0.0405 ± 0.001 μM, and 0.0434 ± 0.002 μM for the p53 −/− cells),[6] indicating equivalent biochemical effects of DDATHF on purine synthesis. A nocodazole trapping experiment was performed in the HCT116 cells after exposure to 10 μM DDATHF, and cell cycle profiles were analyzed after 24 and 48 h, roughly the equivalents of one and two generation times for uninhibited HCT116 cells (Fig. 3, *B–E*). Interestingly, control cultures of HCT116 p53 +/+ and p53 −/− cells differed in their response to a prolonged nocodazole block. HCT116 cells lacking p53 function replicated their DNA a second time without mitosis, accumulating with an 8n DNA content (Fig. 3*E*). HCT116 cells with p53 function did not, indicating that the intact function of p53 prevents a new round of DNA synthesis without completion of mitosis (Fig. 3*B*). Much to our surprise, on DDATHF treatment both the p53 +/+ and the p53−/− cells failed to halt at the G_1-S checkpoint and entered S phase at similar rates (Fig. 3, *C* and *D*). Furthermore, the advancement of much of the initial population into G_2/M occurred by 48 h, with a small buildup of cells in late S phase in cultures of both genotypes. In the p53 −/− cells, the nocodazole-induced 8n DNA peak was prevented by DDATHF, indicating that a second initiation and/or transit of S phase was blocked. A sub-G_1 population of cell debris accumulated in both cell types after 48 h of DDATHF and nocodazole treatment. Thus, after *de novo* purine synthesis inhibition, p53 fails to block S phase entrance in colon carcinoma cells that otherwise have an intact p53 response pathway to agents such as 5-fluorouracil and Adriamycin (24). Based on these flow cytometry studies, GART inhibition produced cell death to some degree in both p53 +/+ and p53 −/− HCT116 cells. Cell kill by GART inhibition and its reliance on p53 status was further investigated.

Cytotoxicity of GART Inhibitors to Tumor Cells with Wild-Type and Mutant p53. Clonogenic assays were performed in which HCT116 p53+/+ and p53−/− cells were exposed to varying DDATHF concentrations for 24 h, then maintained in medium free of drug for 14 days (Fig. 4, *A* and *B*). Significant cytotoxicity was observed in cells of both genotypes at 1 μM DDATHF. Only 12–14%

[6] Equivalent sensitivity to lometrexol of HCT116 cells and HCT116 cells transfected with the E6 gene was also recently reported in Ref. 19.

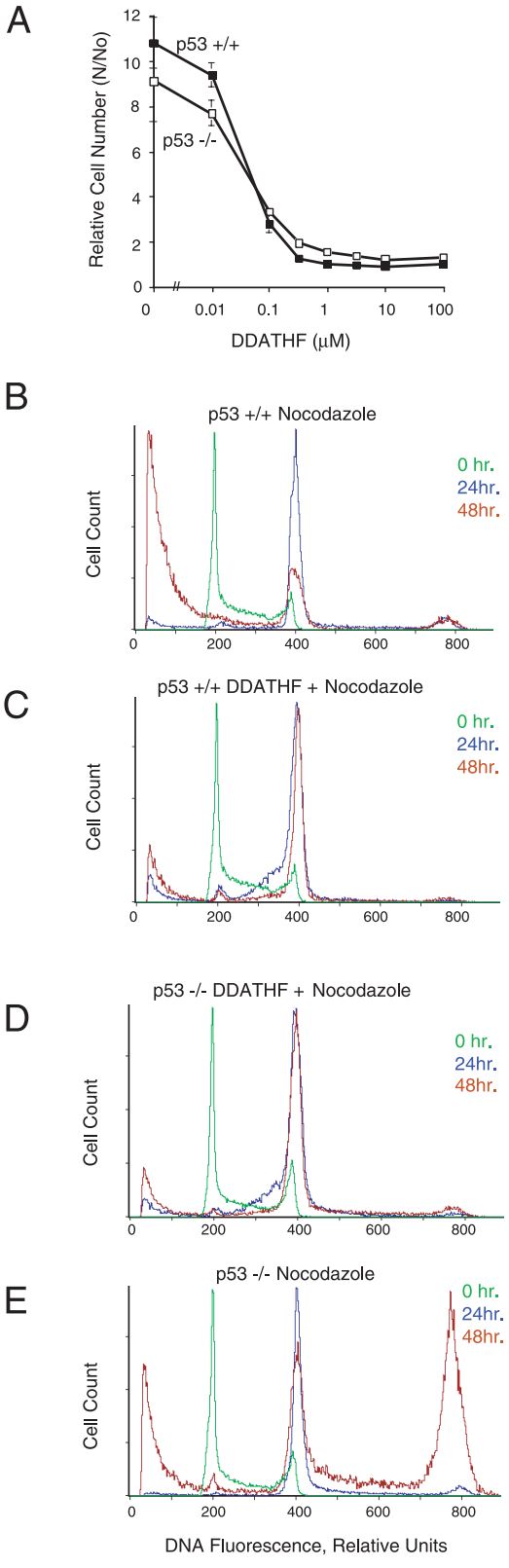

Fig. 3. p53 function does not prevent entry of human HCT116 colon carcinoma cells into and through S phase in the presence of a DDATHF-induced block of purine synthesis. *A*, growth inhibition of isogenic p53+/+ and p53−/− HCT116 cells exposed to DDATHF. Cells were exposed to the indicated concentrations of DDATHF beginning 24 h after plating. Cells were continuously exposed to DDATHF for 72 h, after which cells were trypsinized and culture densities determined. The ratio of final density to initial plating density (N/No) was calculated. *Points* represent the average values from three experiments each with two dishes per condition; *bars*, ±SE. *B–E*, entry of DDATHF-treated HCT116 cells into S phase in the presence of nocodazole. Cells were exposed to either 0.2 μg/ml nocodazole (*B* and *E*) or 0.2 μg/ml nocodazole and 10 μM DDATHF (*C* and *D*), collected at the indicated times, and resuspended in propidium iodide stain.

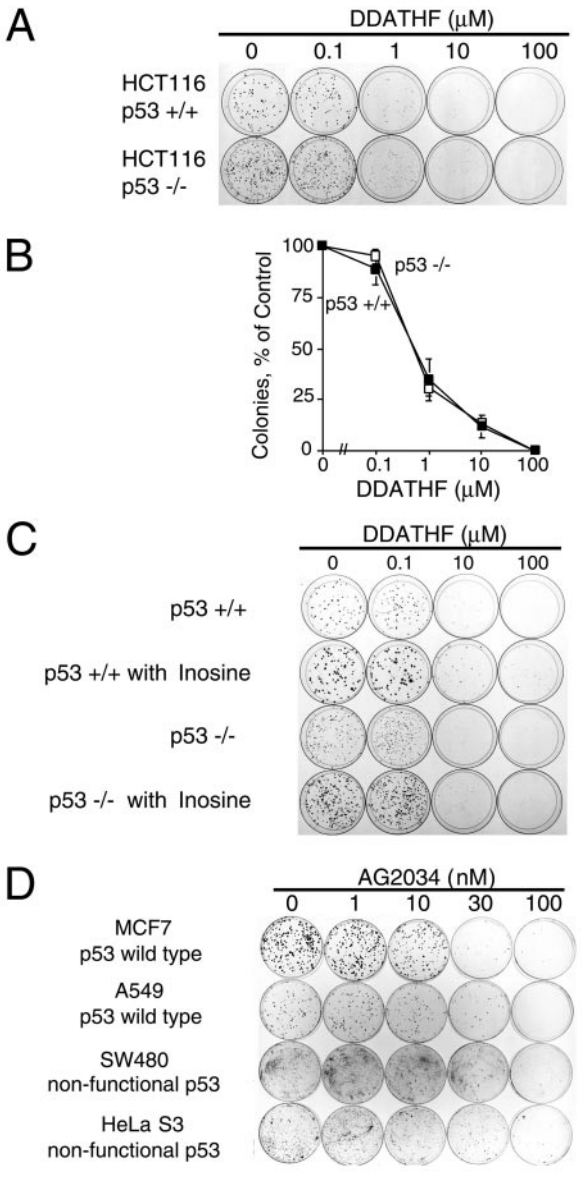

Fig. 4. GART inhibitors are cytotoxic to tumor cells with and without p53 function. *A*, clonogenic survival is equivalent for HCT116 p53+/+ and p53−/− cells treated with DDATHF. HCT116 cells were plated and permitted to adhere for 4–6 h. Medium containing DDATHF was added, and cells were exposed to drug for 24 h, after which drug was washed from the cells. Cells were subsequently maintained in DDATHF-free medium for 14 days, when colonies were fixed and stained. *B*, quantitation of colony formation after a 24-h exposure to DDATHF. The colony formation observed in two experiments such as that shown in *A* were counted by eye and the results were combined. *C*, DDATHF produces equivalent cytotoxicity in p53 +/+ and p53 −/− cells with the addition of the salvage metabolite inosine. The survival of HCT116 cells treated with DDATHF was estimated by a colony formation experiment in which 50 μM inosine was added to all medium changes starting 5 days after a 24-h DDATHF exposure. *D*, the second-generation GART inhibitor AG2034 is cytotoxic regardless of p53 function. Cells with wild-type p53 function (*MCF7* and *A549*) and cells with mutant p53 function (*SW480* and *HeLa S3*) were seeded in modified RPMI 1640 containing 10% dFCS and 50 nM folic acid and, after attachment, cells were exposed to the indicated concentrations of AG2034 for 4 h. Cells were washed and maintained in drug-free medium for 2–3 weeks, after which colonies were fixed and stained. The plates shown are representative of results from three independent experiments; *bars*, ±SE.

clonogenic survival was apparent at 10 μM DDATHF. By 100 μM DDATHF, clonogenic capacity dropped to less than one colony per plate for both HCT116 p53+/+ and p53−/− cells. Given that the inhibitory effect of DDATHF on *de novo* purine synthesis may be prolonged because of cellular retention of DDATHF polyglutamates, the protocol of these cytotoxicity assays was modified, with the addition of 50 μM inosine to the plates 5 days after DDATHF

treatment and thereafter, thus permitting any viable, growth-inhibited cells to form colonies. Even with inosine rescue, cell kill occurred to a similar degree in the p53+/+ and p53−/− cells (Fig. 4*C*). The hypothesis (16) that GART inhibitors are cytotoxic to cells containing mutated p53 and cytostatic to cells with wild-type p53 was given serious reconsideration. This proposal was drawn from a series of studies comparing the ability of two cell lines with wild-type p53 function (MCF7 and A549) and two cell lines with mutant p53 function (HeLa S3 and SW480) to form colonies in the face of continual *de novo* purine synthesis block by the second generation GART inhibitor AG2034. This concept was reinvestigated using clonogenic assays in which the MCF7, A549, HeLa S3, and SW480 cells were exposed to AG2034 for 4 h, after which drug was removed and cells were maintained in drug-free medium with dialyzed FCS for 2 weeks. Cell survival was not different between the cell lines with and without p53 function (Fig. 4*D*). Several variations of the clonogenic assay were performed in these cell lines, altering the drug concentration range and length of exposure, and adding preformed purines to the medium after drug exposure, but all of these experiments failed to demonstrate differences in clonogenicity based on p53 status (data not shown).

DISCUSSION

In recent years, it has become clear that the selectivity of many cytotoxic anticancer drugs lies not in the characteristics of the drugs, but in the molecular alterations introduced during carcinogenesis and tumor progression. One of the key genetic alterations introduced during the evolution of the fully developed neoplastic phenotype is mutation or loss of p53. A substantial and often contradictory literature (recently reviewed in Refs. 25, 26) has accumulated on whether loss of p53 function sensitizes or protects tumors from drug-induced cytotoxicity. Several studies have demonstrated that oncogene-transformed cells lacking p53 function are resistant to cell kill and/or apoptosis by 5-fluorouracil, etoposide, Adriamycin, and DNA alkylating agents (see 25, 26 and references therein). In addition, the requirement for p53 for efficient tumor cell kill has been demonstrated by several of these studies. On the other hand, the ability of tumor cells to respond to a drug by induction of a G_1 and/or a G_2 block would be predicted to allow time for recovery of DNA synthesis, repair of DNA breaks, or reversal of the effects of microtubule disrupting drugs, and hence, to ameliorate the cytotoxicity of many anticancer drugs. From this, one would expect that tumor cells with the mutant or null p53 phenotype would be more sensitive to many antiproliferative drugs than tumor cells which have not lost p53 function. This has been demonstrated for several classes of drugs, including Taxol, vincristine, cisplatin, Adriamycin, camptothecin, etoposide, and bleomycin (see 25, 26). In the face of this complexity, others have drawn the conclusion that the effect of p53 on commitment to apoptosis *versus* a protective G_1 arrest in the face of an initiating exposure to a chemotherapeutic drug is largely decided by "cell context" (26), that is, by the detailed genotype of the cell population and the mutations, gene activation events, and genetic deletions that have occurred during oncogenesis.

The other variable that has been clearly identified as important to the outcome of studies of p53 influence on drug toxicity has been the exact assay used to assess cell death or apoptosis. A striking study demonstrated that the result one obtains differs dramatically with the chosen end point of the assay (24). Thus, using the classical and generally reliable index of apoptosis, nuclear morphology, it was demonstrated that p53 +/+ HCT116 colon carcinoma cells were much more sensitive to 5-fluorouracil than were isogenic double knockout p53 −/− cells; however, the two cell lines were equisensitive to the cytotoxicity of 5-fluorouracil as judged by a colony-forming assay. Recent studies have also called into question several common short-term assays of phenomena associated with apoptosis on the basis that these parameters seem to measure more the rate of development of cytotoxicity rather than cytotoxicity *per se* (25). The ability of a drug to decrease the capability of a tumor cell population to form viable progeny in a clonogenic survival assay still remains the gold standard of cytotoxicity.

It is somewhat puzzling that GART inhibitors are cytotoxic. Any cell that is in interphase should not need *de novo* purine synthesis. That is, any cellular ATP or GTP consumed is regenerated from ADP or GDP in the course of carbohydrate metabolism. Likewise, in most cells, RNA metabolism is in a tight steady state, and any molecule of RNA made is exactly balanced by a molecule degraded, and the purine nucleotides released are stringently conserved. Hence, it would be expected that GART inhibitors would only be deleterious when a cell population attempted to double its DNA content during S phase. This concept would agree with the observation that mammalian cells exposed to DDATHF do not commit to cell death for approximately one generation time after inhibition of purine synthesis (27) and that DDATHF-treated HCT116 cells appear to die out of S phase (Fig. 3). However, it is also quite surprising that both L1210 cells (Figs. 1 and 2) and HCT116 cells (Fig. 3) can finish one S phase in the presence of sufficient DDATHF for complete inhibition of *de novo* purine synthesis. A 1 μM concentration of DDATHF would generate an intracellular DDATHF polyglutamate concentration of ~2 μM in excess of the cellular content of GART; this level of free drug would constitute ~4000 times the K_i determined previously for pure mouse GART (28). In addition, previous studies have demonstrated >95% inhibition of the GART reaction *in vivo* by 1 μM DDATHF, on the basis of experiments measuring the flux of glycinamide ribonucleotide to formylglycinamide ribonucleotide in intact L1210 (2). Yet, the data in Figs. 2 and 3 demonstrate a slow but sure duplication of the genome in the presence of 1 μM DDATHF, raising the question of where the purine bases required for DNA synthesis were originating. It can be calculated that the content of ATP and GTP in mammalian cells is the equivalent of about 15–20% of the number of purine bases incorporated into DNA during replication. Hence, to replicate the genome in the presence of a complete block of *de novo* purine synthesis, purine nucleotides from catabolized RNA must be shunted into DNA synthesis.

It has been suggested that the GART inhibitor, AG2034, distinguishes cells on the basis of p53 status, killing cells which have lost G_1 checkpoint function, but only causing cytostasis in cells which have an intact p53 pathway (16). This is a very appealing conclusion, given that it would predict selective cytotoxicity of GART inhibitors for cells that had lost p53 function, a phenomenon seen only in neoplastically transformed cells. Unfortunately, our results do not agree with this conclusion. The published conclusions (16) were drawn on the basis of the ability of four human carcinoma cell lines to form colonies when grown in the continuous presence of the GART inhibitor AG2034 for 2 weeks. When we repeated these experiments exactly as described in the literature with the same cell lines, both p53 wild-type and p53 mutant cell lines were equisensitive to AG2034 (data not shown). When we used experimental designs involving colony formation after removal of drug, to avoid confounding growth inhibition with cell kill, we again saw no difference in the cytotoxicity of GART inhibitors to p53-positive or -negative cell lines (Fig. 4*D*). In other experiments, we added purines to the medium during colony formation to take into account the fact that DDATHF and other GART inhibitors are extensively metabolized to polyglutamate forms, which persist in cells and which are, in fact, the cytotoxic metabolites of these drugs; again, cell kill was independent of p53 function. Finally,

studies on the truly isogenic HCT116 cell lines differing only in the function of the *p53* gene demonstrated equivalent cytotoxicity of the prototypical GART inhibitor DDATHF (Fig. 4, *A–C*). Yet, these somewhat surprising cytotoxicity results concur with the observation that the p53 +/+ genotype did not prevent the HCT116 cells from entering S phase after DDATHF exposure (Fig. 3). Nevertheless, the results reported in this manuscript concur with the observation from the Agouron group that GART inhibitors are effective *in vivo* against both tumors that retain p53 function and those that have lost p53 function (29).

Why do cells enter S phase in the face of a blockade of *de novo* purine synthesis but intact p53? This seems to contradict the concept that p53 can detect reduced purine ribonucleotide levels and prevent entry into S phase. This is not the case; in studies that will be reported separately, we have found that p53 accumulates in any of several human carcinoma cell lines with wild-type p53 after exposure to GART inhibitors. However, the downstream events causative of a G_1 block, which are normally initiated by p53 stabilization, are not functional in GART-inhibitor treated cells.

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