p14<sup>ARF</sup> Expression Increases Dihydrofolate Reductase Degradation and Paradoxically Results in Resistance to Folate Antagonists in Cells with Nonfunctional p53

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ABSTRACT

The p14<sup>ARF</sup> protein, the product of an alternate reading frame of the INK4A/ARF locus on human chromosome 9p21, disrupts the ability of MDM2 to target p53 for proteosomal degradation and causes an increase in steady-state p53 levels, leading to G<sub>1</sub> and G<sub>2</sub> arrest of cells in the cell cycle. Although much is known about the function of p14<sup>ARF</sup> in the p53 pathway, not as much is known about its function in human tumor growth and chemosensitivity independently of up-regulation of p53 protein levels. To learn more about its effect on cellular proliferation and chemoresistance independent of p53 up-regulation, human HT-1080 fibrosarcoma cells null for p14<sup>ARF</sup> and harboring a defective p53 pathway were stably transfected with p14<sup>ARF</sup> cDNA under the tight control of a doxycycline-inducible promoter. Induction of p14<sup>ARF</sup> caused a decrease in cell proliferation rate and colony formation and a marked decrease in the level of dihydrofolate reductase (DHFR) protein. The effect of p14<sup>ARF</sup> on DHFR protein levels was specific, because thymidylate kinase and thymidylate synthase protein levels were not decreased nor were p53 or p21WAF1 protein levels increased. The decrease in DHFR protein was abolished when the cells were treated with the proteasome inhibitor MG132, demonstrating that p14<sup>ARF</sup> augments proteosomal degradation of the protein. Surprisingly, induction of p14<sup>ARF</sup> increased resistance to the folate antagonists methotrexate, trimetrexate, and raltitrexed. Depletion of thymidine in the medium reversed this resistance, indicating that p14<sup>ARF</sup> induction increases the reliance of these cells on thymidine salvage.

INTRODUCTION

The INK4A/ARF locus on human chromosome 9p21 codes for the p16INK4A and the p14<sup>ARF</sup> protein. The p16INK4A protein is the product of alternatively spliced exons 1α, 2, and 3 and functions as a cyclin-dependent kinase inhibitor, regulating the G<sub>1</sub>-phase exit by inhibiting the phosphorylation of the retinoblastoma protein (1–3). The p14<sup>ARF</sup> protein (p19<sup>ARF</sup> in mouse) is transcribed from distinct exon 1β and shared exons 2, and 3 and has been shown to bind the MDM2 protein, alleviating MDM2-driven degradation of p53, which results in elevated p53 levels that lead to cell cycle arrest in the G<sub>1</sub>-S and G<sub>2</sub>-M boundaries of the cell cycle (4, 5). Induction of ARF has been shown to occur in response to proliferative signals such as myc, E1A, Ras, and E2F-1 (6–9). Thus, induction of ARF provides a link between retinoblastoma protein and p53 tumor-suppressive pathways. However, p19<sup>ARF</sup> also causes a G<sub>1</sub>-phase cell cycle arrest independent of p53 (10). This finding is not surprising, because tumors exist in which p53 and p14<sup>ARF</sup> are inactivated, indicating that p14<sup>ARF</sup> may have other functions (10–12). It has been shown recently that p14<sup>ARF</sup> and p19<sup>ARF</sup> are able to bind to E2Fα and alter their transcriptional ability and stability, possibly explaining why ARF is able to inhibit cell cycle progression independent of p53 (13–15).

Although much is known about the function of ARF in the p53 pathway, less is known about its functions in human tumor growth and chemosensitivity independently of its regulation of p53 via binding to MDM2. Mouse embryonic fibroblasts expressing E1A and exogenous ARF are more sensitive to killing by doxorubicin than their normal counterparts that only express E1A (7). Alterations in the INK4A/ARF locus reduce p53 induction and cytotoxicity of cyclophosphamide in mouse lymphomas (16). Also, recombiant adenovirus-mediated p14<sup>ARF</sup> overexpression sensitizes MCF-7 breast cancer cells to cisplatin (17). Thus, expression of ARF appears to increase sensitivity to chemotherapeutic drugs. However, this increase in sensitivity has been associated with up-regulation of p53. The question of whether p14<sup>ARF</sup> can also increase sensitivity to chemotherapeutics independently of p53 up-regulation has remained unanswered. To investigate the effect of p14<sup>ARF</sup> on growth and sensitivity to chemotherapeutic agents independent of p53 up-regulation, we transfected the HT-1080 human fibrosarcoma cell line that lacks p14<sup>ARF</sup> and harbors a non-functional p53 pathway (18–20) with the pTET-ON, pTRE2 vector containing p14<sup>ARF</sup> cDNA, in which expression is tightly regulated by doxycycline. Herein, we report that restoration of p14<sup>ARF</sup> causes a marked decrease in dihydrofolate reductase (DHFR) protein levels by altering DHFR protein stability; decreases the proliferation rate of these cells by reducing colony formation; and selectively confers resistance to the antifolates methotrexate, trimetrexate, and raltitrexed by increasing the reliance of these cells on thymidine salvage.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The HT-1080 human fibrosarcoma cell line and p14JM p14<sup>ARF</sup>-inducible clone were grown in RPMI 1640 supplemented with penicillin/streptomycin and 10% Tet approved fetal bovine serum (FBS) from Clontech (Palo Alto, CA).

Cloning and Establishment of p14<sup>ARF</sup>-Inducible Expression in HT-1080 Cells. The p14<sup>ARF</sup> cDNA cloned between BamHI and EcoRI in the Bluescript KS+ vector was a kind gift from Dr. G. Peters (Imperial Cancer Research Fund Laboratories, London, United Kingdom). The plasmid was digested with BamHI and HindIII, and the p14<sup>ARF</sup> cDNA was isolated and cloned into the pTRE-2 vector that was also digested with BamHI and HindIII. The pTRE-2 vector containing the p14<sup>ARF</sup> cDNA was then cotransfected with the pTet-On vector in a ratio of 2:1 in cells that were approximately 30% confluent in a 6-well plate with or without 1 μg/ml doxycycline for 24 h, lysed, and then analyzed by Western blot analysis.

Western Blotting. Actively growing cells were harvested from culture, and protein lysates were prepared by lysing cells in the presence of lysis buffer [50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.5% sodium deoxycholate, and 0.5% NP40] containing Protease Inhibitor mixture from Pharmingen (San Diego, CA; 10 μg/ml phenanthrene, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride).

The lysates were kept on ice for 15 min, vortexed twice, and centrifuged at 4°C for 10 min at 10,000 × g in an Eppendorf microcentrifuge; and clear supernatant recovered. The protein concentration was determined by the Bio-

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Rad assay. Fifty µg of protein were analyzed on SDS-PAGE (varying between 12% and 14% depending on protein analyzed). The proteins were electroblotted onto nitrocellulose membrane and probed with the appropriate primary and secondary antibodies. The protein bands were visualized on X-ray film using the enhanced chemiluminescence reagent from Amersham Biosciences (Arlington Heights, IL). The p21 (F-5) and p53 (Bp53-12) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal thymidine kinase (TK) antibody was a gift from Dr. T. Kelly (Memorial Sloan Kettering Cancer Center, New York). The polyclonal thymidylate synthase (TS) antibody was a gift from Dr. F. Maley (Wadsworth Center, New York State Department of Health, Albany, NY).

The α-tubulin antibody (B-5-1-2) was purchased from Sigma (St. Louis, MO), and the p14ARF antibody (NB 200-111) was purchased from Novus Biologicals (Littleton, CO).

mRNA Analysis. Cells were grown for various times in the presence or absence of 1 µg/ml doxycycline, collected, and lysed; mRNA isolation was performed using the Invitrogen mRNA isolation kit, and reverse transcriptase reactions were performed using the Invitrogen cDNA synthesis kit. Primers were designed to amplify sequences on the p14ARF and DHFR genes, which were used as controls for RNA integrity. The primers were 5'-TCCTGGGACAC. cTTGGTTCGCTAAACTG-3' (p14ARF) and 5'-GAACACTGAGATGTTCTTGAA-3' (DHFR). Amplification was performed using TaqMan 1,000 RXN Gold with buffer A kit and the ABIPrism 7700 thermocycler (Applied Biosystems). The temperature profile was 95°C, 10 min (1 cycle); and T1, 95°C, 15 s; T2, 60°C, 60 s (42 cycles). The primer and probe sequences are as follows:

A) (TTGTTGTGCTAAACTGCGC) and (GTTCCCTCCCCTGTTCTC); and B) (ATGCCCATATGTCTGGGACAC).

Growth Assays and Fluorescence-Activated Cell Sorter Analysis. The short-term growth assay was carried out by plating 50,000 cells in each well of a six-well plate in growth medium, with or without doxycycline. Every 24 h, starting on the day of plating, cells exposed or not exposed to 1 µg/ml doxycycline were detached from the wells by treatment with 0.25% trypsin/0.25% EDTA, resuspended in 1 ml of growth medium, and counted. Cells were grown for various times in the presence or absence of 1 µg/ml doxycycline; and 14 days later, the medium was removed, and the cells were washed with PBS and then stained with 2% crystal violet solution. For thymidine deplete media studies, cells were incubated with thymidine for 15 min to deplete the thymidine pool. For the above experiments for the various time points, the data were plotted and analyzed by Image-Pro Plus software.

Cytotoxicity Assay. For cytotoxicity studies, 1,000 cells were plated in each well of 96-well microtiter plates (plating density approximately the same as the one used in short-term growth assay) in replicates of six, with or without 1 µg/ml doxycycline in regular growth medium or in medium in which thymidine was depleted using TP. The following drugs were added at the indicated concentrations 24 h later and left in the medium for 96 h. Methotrexate, trimetrexate, raltitrexed, and cytarabine were added at concentrations ranging from 256 to 100 µM. Doxorubicin was added at concentrations of 51 µM to 10 µM, and hydroxyurea was added at concentrations of 26 µM to 2 mM. After 96 h, a solution containing 1 mg/ml 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide sodium salt (XTT) and 25 µM phenazine methosulfate was added to achieve a final concentration of 0.2 mg/ml XTT and 5 µM phenazine methosulfate and incubated for 2–4 h at 37°C, and absorbance was measured at 450 nm with a microplate reader, as described previously (23). The above experiments were repeated at least three times.

TK Activity Assay. This is a modification to a previously shown method (24, 25). In brief, cells treated or not treated with 1 µg/ml doxycycline were incubated at 37°C for 72 h, then lysed in 50 mM Tris-HCl (pH 7.9), containing 1 mM β-mercaptoethanol, by three repeated freeze-thaw cycles, centrifuged, and the supernatant recovered. Four µg of whole-cell protein lysate were added to a reaction mix consisting of 10 µM thymidine, 50 mM MgCl2, 5 mM ATP, 4 mM DTT, 10 mM NaF, and 1 µCi tritiated thymidine (25 µl reaction). The mixture was incubated at 37°C for 20, 40, and 60 min. At each time point, 20 µl were withdrawn and spotted on DE-81 filter paper. The filter paper was allowed to dry, washed three times in 5 mM ammonium formate, and placed in a scintillation vial containing 10 ml of Scintiverse BD scintillation fluid; and radioactivity was determined.

In Situ (Whole-Cell) Thymidylate Synthesis Activity Assay. The method has been described previously (26, 27). In brief, p14JM cells were resuspended in growth medium at concentration of 2 × 106 cells/ml and placed in 1.5-ml Eppendorf tubes. The cells were incubated for 3 h at 37°C. After the 3 h incubation, [5-3H]deoxyuridine solution was added in 40-µl volume to cells to achieve a final concentration of 2 µCi/ml. At time points of 0, 15, 30, and 45 min, a 100-µl cell suspension from the induced or uninduced cells was aliquoted to a separate tube containing 200 µl of charcoal suspension and vortexed. The tubes were then centrifuged for 5 min at 15,000 rpm. The supernatant (100 µl) was then added to 5 ml of scintillation mixture in 20-ml scintillation vials and counted in a scintillation counter. Samples were counted in the order of 0, 15, 30, and 45 min. A set of four samples plus the corresponding time points were used to calculate slope. Thymidylate synthesis activity is expressed as cpm/min/106 cells and calculated as follows: Activity (cpm/min/106 cells) = [slope/center number] × 106.

Analysis of Intracellular Triterium-Labeled Thymidine Metabolites. Cells were plated in 100-mm plates. One batch of cells was treated with 1 µg/ml doxycycline. After 72 h, the doxycycline-treated and untreated groups were incubated for an extra 4 h in the presence of 25 µM thymidine containing 10 µCi [methyl-3H]thymidine. The cells were then trypsinized and collected by centrifugation. After washing with cold PBS, resuspended cells were transferred to a 1.5-ml tube and centrifuged for 15 s at 12,000 × g at 4°C. The pellet was immediately resuspended in 0.3 ml of 0.6 M trichloroacetic acid (4°C) and incubated at 4°C for 10 min followed by centrifugation. The acid supernatant was recovered and added to an equal volume of cold freon containing 0.5 m tri-n-octylamine. The mixture was vortexed for 15 s and centrifuged for 30 s at 12,000 × g. The aqueous upper phase was recovered and analyzed by high performance liquid chromatography, as described previously (28). Thymidine metabolite peaks from cell extracts were identified by comparison of retention times using thymidine mono- di-, and triphosphates as standards.

Proteasome Inhibitor MG-132 Assay. Cells treated or not treated with 1 µg/ml doxycycline for 48 h were treated or not treated with 100 nM MG-132 (Calbiochem, La Jolla, CA) or 0.1% DMSO for 12 h. Cells were lysed, and proteins were analyzed by Western blotting.

Protein Synthesis Inhibition Assay. Cells treated or not treated with 1 µg/ml doxycycline for 24 h were treated for various times with 10 µM cycloheximide. Cells were then harvested, whole-cell lysate was recovered, and proteins were analyzed by Western blotting. The approximate half-life of DHFR was determined by averaging band intensity of at least three separate experiments for the various time points by use of Image-Pro Plus software.

RESULTS

Selection of a p14ARF-pTRE2-Inducible Clone. After screening several clones, one clone designated p14JM was isolated that expressed p14ARF in the presence of 1 µg/ml doxycycline. Expression of p14ARF was detected by the appearance of a band that comigrated with another band detected in BT-549 cells that are known to express p14ARF (Fig. 1A). Induction of p14ARF could be detected 8 h after exposure to 1 µg/ml doxycycline, with maximal expression occurring at 24 h (data not shown). Induction of p14ARF was detected with concentrations as low as 10 ng/ml doxycycline, with maximal induction occurring at 1 µg/ml doxycycline (Fig. 1B).

Induction of p14ARF in HT-1080 Cells Inhibits Cellular Proliferation and Colony Formation. ARF has been shown to induce cell cycle arrest in a p53-dependent and -independent manner (4, 5, 10). To examine whether p14ARF restoration had any effect on growth rate in the HT-1080 cells, the effect of expressing p14ARF on p14JM cell survival was examined using a clonogenic assay. Colony formation in

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the cells induced to express p14ARF was reduced by more than 50% compared with cells that were not induced (Fig. 2A). This was not due to a doxycycline-induced increase in p53 or p21 levels (Fig. 2B). The effect of p14ARF on the short term growth of p14JM cells was also examined by seeding cells with or without doxycycline (1 μg/ml) for 5 days. The doxycycline-treated cells expressing p14ARF had a slower growth rate than that of the untreated cells not expressing p14ARF (Fig. 2C). One reason for the reduced colony formation and growth rate of the p14ARF-expressing cells might be the ability of p14ARF to arrest cells in G1 (4, 6). When cells were stained with propidium iodide and analyzed by fluorescence-activated cell sorting and crystal violet solution, there was a modest increase in the amount of cells in G1 versus S phase in the induced cells (Fig. 2D), with no change in the amount of cells in sub-G1 (data not shown). Therefore, the increase in the amount of cells in G1 observed indicated that p14ARF is affecting the progression of cells from G0 to S, rather than due to an increase in cell death. In addition, no difference was observed in the percentage of viable cells between the induced and uninduced cells, as measured by trypan blue staining after 72 h of doxycycline induction.

**p14ARF Expression Decreases DHFR Stability in a Proteosome-Dependent Manner.** DHFR is a key enzyme in the de novo pathway for thymidylate synthesis, allowing for the recycling of dihydrofolate, the product of this reaction, to tetrahydrofolate. One of the expected consequences of inhibiting this enzyme would be the decreased production of thymidylate and therefore a reduction in DNA replication, leading to a decrease in growth rate. Induced expression of p14ARF resulted in a greater than 9-fold decrease in DHFR protein levels beginning at 48 h, with no significant effect on the levels of TK or TS (Fig. 3A, I and J). There was no significant decrease of DHFR protein levels seen in the first 24 h of p14ARF induction (data not shown). Addition of the same concentration of doxycycline for 72 h did not affect DHFR levels in HT-1080 parental cells (Fig. 3A, 2). Since there was a small difference in DHFR mRNA levels between the induced and uninduced cells, as shown by quantitative PCR (Fig. 3B), indicating that this decrease was primarily posttranscriptional. This decrease, which may be related to the known effect of p14ARF on E2F1 transcription, would not explain the large difference in DHFR protein levels. Addition of cycloheximide to inhibit protein translation revealed a decreased stability of DHFR in the presence of p14ARF expression; the T1/2 of DHFR changed from over 20 to 8 h (Fig. 3C). When p14JM cells were exposed to 100 nM proteasome inhibitor MG-132, upon induction with p14ARF, there was complete restoration of DHFR protein levels (Fig. 3D). These results indicate that p14ARF affects DHFR protein stability via increased proteosomal degradation. The decreased stability of DHFR upon p14ARF induction appears to be indirect, because efforts to detect p14ARF-DHFR binding were unsuccessful (data not shown).
**p14arf Expression Conveys Selective Resistance to the Antifolate DHFR Inhibitors Methotrexate, Trimetrexate, and the Antifolate Raltitrexed, a TS Inhibitor.** The role of p14arf in p14JM sensitivity to chemotherapeutics was examined by inducing cells to express p14arf and adding various concentrations of methotrexate, trimetrexate, and raltitrexed to separate cells in different plates. After continuous doxycycline induction and drug exposure for 4 days, cell viability was assessed by the XTT colorimetric assay (23). Expression of p14arf conferred resistance to all three drugs (Fig. 4A). In particular, compared with uninduced cells, the expression of p14arf was associated with marked resistance to methotrexate and raltitrexed, even at very high concentrations of these drugs. In contrast, when cytotoxicity experiments were carried out using hydroxyurea, doxorubicin, or cytarabine, also S-phase inhibitors with different mechanisms of action, there were no differences in cytotoxicity observed in the absence or presence of p14arf expression (Fig. 4B). Therefore, p14arf expression selectively altered resistance to the antifolates methotrexate, trimetrexate, and raltitrexed. Because FBS contains high levels of thymidine (29–31), the possibility that increased thymidine salvage explained the resistance to the antifolates was examined. Cytotoxicity studies were conducted in the presence of 10% FBS depleted of thymidine, by pretreating serum with TP (32). In the presence of thymidine-depleted FBS, the resistance conferred by p14arf expression to methotrexate, trimetrexate, and raltitrexed was completely abolished (Fig. 4C). Although depletion of thymidine did not affect the sensitivity profile of doxorubicin or cytarabine (data not shown), to rule out an effect of doxycycline on antifolate sensitivity, parental HT-1080 cells were separately treated with methotrexate, in the presence or absence of 1 μg/ml doxycycline, and allowed to grow for various amounts of time. Average values for three experiments of DHFR/ACTIN ratios are shown with their respective SD. C, p14JM cells incubated with (+DOX) or without (−DOX) 1 μg/ml doxycycline for 72 h were also subjected to Western analysis using DHFR and tubulin antibodies as described above. The p14JM whole-cell lysates from cells incubated with (+DOX) or without (−DOX) 1 μg/ml doxycycline for 72 h for the TS and TK Western blots were electrophoresed on a separate polyacrylamide gel and blotted simultaneously with the TK and TS primary antibody. B, relative DHFR mRNA levels using TaqMan quantitative reverse transcription-PCR analysis (probe and primers specific for DHFR cDNA and normalized by actin message levels) of p14JM cells that were allowed to reach confluency, replated, incubated in the presence (+DOX) or in the absence (−DOX) of 1 μg/ml doxycycline, and allowed to grow for various amount of time. Average values for three experiments of DHFR/ACTIN ratios are shown with their respective SD. C, p14JM cells incubated with (+DOX) or without (−DOX) 1 μg/ml doxycycline for 24 h were treated with 10 μg/ml cycloheximide. Right panel, at the indicated times, cells were lysed and Western blot was performed using DHFR and α-tubulin antibodies. Intensity of protein bands at different time points after cycloheximide addition relative to band intensity at the 0-h time point was plotted. Left panel, graph represents average and SDs of at least three separate experiments. D, cells were incubated with (+) or without (−) 1 μg/ml doxycycline for 48 h, and 100 nM MG-132 or 0.1% DMSO was added for 12 h. The cells were then lysed, and Western blots were performed using the antibodies as described above.

Fig. 3. p14arf inhibition of DHFR protein by proteasomal degradation. A, 1, Western blot with each respective antibody of p14JM lysates of cells that were allowed to reach confluency, detached, replated, and incubated with (+DOX) or without (−DOX) 1 μg/ml doxycycline (DOX) for various amounts of time. 2, as a control, HT-1080 parental cells treated (+DOX) or not treated (−DOX) with 1 μg/ml doxycycline for 72 h were also subjected to Western analysis using DHFR and tubulin antibodies as described above. 3, the p14JM whole-cell lysates from cells incubated with (+DOX) or without (−DOX) 1 μg/ml doxycycline for 72 h for the TS and TK Western blots were electrophoresed on a separate polyacrylamide gel and blotted simultaneously with the TK and TS primary antibody. B, relative DHFR mRNA levels using TaqMan quantitative reverse transcription-PCR analysis (probe and primers specific for DHFR cDNA and normalized by actin message levels) of p14JM cells that were allowed to reach confluency, replated, incubated in the presence (+DOX) or in the absence (−DOX) of 1 μg/ml doxycycline, and allowed to grow for various amount of time. Average values for three experiments of DHFR/ACTIN ratios are shown with their respective SD. C, p14JM cells incubated with (+DOX) or without (−DOX) 1 μg/ml doxycycline for 24 h were treated with 10 μg/ml cycloheximide. Right panel, at the indicated times, cells were lysed and Western blot was performed using DHFR and α-tubulin antibodies. Intensity of protein bands at different time points after cycloheximide addition relative to band intensity at the 0-h time point was plotted. Left panel, graph represents average and SDs of at least three separate experiments. D, cells were incubated with (+) or without (−) 1 μg/ml doxycycline for 48 h, and 100 nM MG-132 or 0.1% DMSO was added for 12 h. The cells were then lysed, and Western blots were performed using the antibodies as described above.
were restored, and a difference in sensitivity to methotrexate was still observed (Fig. 4D).

**p14ARF Confers Resistance to Antifolates by Altering the Balance of Thymidine Supply.** The fact that resistance to antifolates was abolished upon depleting the serum of thymidine indicated that expression of p14ARF may increase thymidine salvage by either increasing thymidine uptake and/or increasing TK activity. Cells were collected 72 h post induction with 1 μg/ml doxycycline and lysed, and TK activity was measured. There was no difference in TK activity between the cells uninduced and induced to express p14ARF (Fig. 5A).

In another experiment, radiolabeled thymidine-treated cells were also collected at 72 h and analyzed for thymidine mono-, di-, and triphosphates by high performance liquid chromatography. There were no differences found in the thymidine metabolite peak profiles between doxycycline-treated and untreated cells (Fig. 5B). Thus neither TK activity nor thymidine uptake or anabolism explained the thymidine reversal of antifolate resistance. Other mechanisms of known antifolate resistance include decreased influx of drug, decreased polyglutamate formation, and increased DHFR expression (33). The fact that resistance is seen also with trimetrexate, which does not use the same active transport mechanism as methotrexate, renders impaired uptake an unlikely mechanism of resistance. Confirming this observation, uptake of tritiated methotrexate was similar in the p14ARF-expressing and non-p14ARF-expressing cells (data not shown). Decreased polyglutamylation and retention as a mechanism of resistance was ruled out by the fact that these cells were incubated continuously in the presence of drugs and therefore polyglutamylation would not affect cytotoxicity, due to the continuous availability of the extracellular level of the drug (34) and by the fact that trimetrexate does not undergo polyglutamylation. Paradoxically, resistance of p14JM cells to antifolates occurred in a situation in which DHFR protein levels were not increased but rather were drastically decreased. An explanation for this finding lies in the understanding of the balance between *de novo* thymidylate synthesis and the salvage pathway for thymidylate. DHFR is an important enzyme in the *de novo* pathway in that it provides the necessary reduced folate cofactor, 5,10 methylene tetrahydrofolate, for the conversion of dUMP to dTMP via reduction of dihydrofolate to tetrahydrofolate and subsequent conversion to 5,10-methylene tetrahydrofolate via serine hydroxymethyl transferase. However, dTMP can also be generated via thymidine in the medium. As a consequence of the marked decrease in DHFR activity, p14JM cells that are induced to express p14ARF would be depleted of 5,10-methylene tetrahydrofolate. This in turn would cause a decrease in TS activity and therefore a reduction in the amount of *de novo* dTMP being produced. Fig. 5C shows that indeed, whole-cell thymidylate synthesis activity is decreased. Because the supply of thymidine is not reduced, as seen by the unaltered TK activity and thymidine pools (Fig. 5, A and B) in the p14ARF-expressing...
**DISCUSSION**

The p53-dependent effects of ARF have been widely studied (35, 36). However, p53-independent effects of ARF have just recently been described (10, 13–15). An important question left unanswered has been the effect of p14ARF on chemosensitivity. Although a few studies have attempted to answer this question (7, 16, 17, 37), in most cases, tumors with an intact p53 response pathway have been used as the model. In this study, we used a human HT-1080 fibrosarcoma cell line, which is null for p14ARF and harbors a nonfunctional p53 pathway (18–20). Although p53 is expressed in this cell line, it is nonfunctional, as demonstrated by the lack of a G1-S block after treatment with γ radiation or N-(phosphonacetyl)-L-aspartic acid (18).

The reasons for its lack of function are unclear. MDM2 is also expressed in HT-1080 cells, and the expression of relatively high levels of p53 levels suggests that perhaps MDM2 in this cell line is mutated or nonfunctional. p14ARF protein expression was induced using a doxycycline-inducible system, thus eliminating clonal variation. Upon induction of p14ARF, we observed a decreased growth rate, decreased colony formation, selective resistance to antifolates, and increased dependence of the p14JM p14ARF-expressing cells on thymidine salvage, in the face of a marked decrease in DHFR protein levels. The decrease in growth rate observed upon induction of p14ARF demonstrates that p14ARF is able to inhibit growth, independent of its ability to increase p53 protein levels. The decrease in cell growth may be explained by a delay in S-phase entry. It has been shown that p19ARF, the mouse homologue of p14ARF, can arrest cells at G1 independently of p53, but the arrest occurs in the absence of MDM2 (10). Because HT-1080 cells express high levels of MDM2 (20), this may explain the lack of a more pronounced G1-S cell cycle arrest after p14ARF induction. However, the decrease in cellular proliferation rate was delayed until 48 h after induction of p14ARF, at which time a decrease in DHFR protein levels and an increase in the amount of cells in G1 were seen, all consistent with a more delayed p53-independent response to ARF expression (10). TK and TS levels were not altered after p14ARF expression. Therefore, the decrease in cell growth after restoration of p14ARF expression is not due to a general decrease in expression of cell cycle genes but rather to a more targeted event. Induction of p14ARF is associated with a decrease in DHFR protein levels in p14JM cells, and therefore a slowdown in proliferation may be due in part to the lack of generation of reduced folate pools, i.e., 5,10-methylene tetrahydrofolate, needed in for conversion of dUMP to dTMP by TS. A decrease in whole-cell TS activity was observed correlating with decreased DHFR activity.

p14ARF and p19ARF are able to bind to E2F-1 and alter its transcriptional activity and stability (13–15). Although neither a decrease in E2F-1 protein levels, nor a significant change in DHFR mRNA levels was found after p14ARF induction in p14JM cells, inhibition of E2F-1 transcription for other E2F-1 targets cannot be ruled out. We also cannot rule out the involvement of other downstream targets of p14ARF that have not yet been discovered.

An explanation for the decrease in DHFR levels after p14ARF
restoration is a decrease in DHFR stability, reversed in the presence of the proteasome inhibitor MG-132. Therefore, p14ARF expression may augment proteasomal degradation of DHFR. Although Martelli et al. (14) has found that p19ARF is capable of targeting E2Fs for proteasomal degradation through direct binding of ARF to E2Fs, we were not able to show p14ARF binding to DHFR. Although there are studies that have provided information on DHFR stability in other cell lines (38), there is no information dealing specifically with the mechanisms of endogenous DHFR degradation. One possible mechanism of action is one in which p14ARF interferes with the basal DHFR degradation machinery in increasing the activity of proteins that normally augment its degradation, decreasing the activity of proteins that stabilize it, or through a combination of both, possibly involving ubiquitination.

Another important finding in our study is the observation that expression of p14ARF renders p14JM cells resistant to antifolates. This finding may seem counterintuitive due to the fact that a known cause of methotrexate or trimetrexate antifolate resistance is increased levels of DHFR. An explanation for this result is that thymidine is known to rescue cells from methotrexate inhibition, and antifolate sensitivity depends on the balance between de novo dTMP synthesis and thymidine salvage (39). In the de novo pathway, dTMP is synthesized by TS from dUMP, using the reduced folate 5,10-methylene tetrahydrofolate as a cofactor. In thymidine salvage, thymidine is taken up by the cell and converted to dTMP via TK. In p14JM cells, induction of p14ARF causes a marked decrease in DHFR protein levels, which in turn causes an inhibition of de novo dTMP synthesis. However, because there is no decrease in TK activity or in thymidine levels, p14ARF expression creates a situation in which the salvage to de novo dTMP synthesis balance is shifted to salvage. Although growth is suboptimal, addition of drugs that inhibit the de novo pathway such as trimetrexate, methotrexate, or raltitrexed in a p14ARF-expressing cell will be less cytotoxic in the presence of thymidine, due to the fact that these cells are more reliant on extracellular thymidine for growth; in the absence of thymidine, cell growth is markedly slowed. Hence, depletion of thymidine via TP treatment of FBS from the medium in p14ARF-expressing cells restores the sensitivity of these cells to methotrexate, trimetrexate, and raltitrexed. Because incubation of p14ARF-expressing p14JM cells with $1 \times 10^{-7}$ M concentration of thymidine, levels that are physiological in humans, also resulted in these cells being resistant to the antifolates, these results are not an artifact of the very high thymidine levels found in FBS (29–31).

p14ARF expression in p14JM cells, through mechanisms still to be determined, increases DHFR proteasomal degradation and in so doing provides an explanation for the decrease in cell growth in the absence of an active p53 pathway. This decrease in cell growth is accentuated in the absence of thymidine in the medium. Although earlier studies have demonstrated that ARF expression contributes to drug sensitivity, we show that in a situation in which the p53 pathway is not active, p14ARF causes resistance to antifolates.

Antifolates are used for the treatment of leukemias, lymphomas, breast cancer, and other tumors (33). This study has relevance to human cancer in that many tumors harbor p53 mutations and also express p14ARF (5) and would be expected to be less sensitive to antifolates.

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p14ARF Expression Increases Dihydrofolate Reductase Degradation and Paradoxically Results in Resistance to Folate Antagonists in Cells with Nonfunctional p53

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