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Modulating Radiation Resistance by Inhibiting Ribonucleotide Reductase in Cancers with Virally or Mutationally Silenced p53 Protein

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Abstract

Therapeutic ionizing radiation damages DNA, increasing p53-regulated ribonucleotide reductase (RNR) activity required for *de novo* synthesis of the deoxyribonucleotide triphosphates used during DNA repair. This study investigated the pharmacological inhibition of RNR in cells of virally or mutationally silenced p53 cancer cell lines using 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP, Triapine[®] NSC #663249), a chemotherapeutic radiosensitizer that equally inhibits RNR M2 and p53R2 small subunits. The effects of 3-AP on RNR inhibition and resulting radiosensitization were evaluated in cervical (CaSki, HeLa and C33-a) and colon (RKO, RKO-E6) cancer cells. 3-AP treatment significantly enhanced radiation-related cytotoxicity in cervical and colon cancer cells. 3-AP treatment significantly decreased RNR activity, caused prolonged radiation-induced DNA damage, and resulted in an extended G₁/S-phase cell cycle arrest in all cell lines. Similar effects were observed in both RKO and RKO-E6 cells, suggesting a p53-independent mechanism of radiosensitization. We conclude that inhibition of ribonucleotide reductase by 3-AP enhances radiation-mediated cytotoxicity independent of p53 regulation by impairing repair processes that rely on deoxyribonucleotide production, thereby substantially increasing the radiation sensitivity of human cancers.

INTRODUCTION

Therapeutic ionizing radiation damages DNA, which must be efficiently repaired for cell survival. The rate-limiting step in *de novo* synthesis of deoxyribonucleotide triphosphates required for DNA repair is the exchange of ribose sugar's 2'-hydroxyl moiety for a proton to create the corresponding 2'-deoxyribonucleotide, a reaction catalyzed by the enzyme ribonucleotide reductase (1,2). Mammalian ribonucleotide reductase functions as a heterotetrameric enzyme, having two homodimeric active-site subunits (RNR-M1), and two homodimeric small subunits (RNR-M2), carrying diferric iron centers stabilizing a tyrosyl free radical critical for catalytic function (1,2). Human ribonucleotide reductase has at least two small subunit isoforms, designated RNR-M2 and p53R2 (or RNR-M2b) (3–5). The RNR-M1

protein has a long half-life (≥ 20 h) and is therefore present in excess throughout the cell cycle (2), while RNR-M2 and p53R2 proteins have relatively short (3-h) half-lives (6,7). In quiescent (G_0) cells, RNR-M2 and p53R2 protein levels are constitutively low (2,8). RNR-M2 and p53R2 ribonucleotide reductase activity appears to be regulated by p53 protein-protein binding, such that DNA damage releases bound p53 from cytosolic RNR-M2 and p53R2 to allow RNR-M1 subunit co-association and functional enzyme activity (4,5,8). It has been speculated that DNA damage-induced ribonucleotide reductase activity increases initially through release of p53R2 (3,8,9) and then through complementary RNR-M2 induction (10). Over-expression of RNR-M2 increases radiation resistance (11).

In human cancers with unchecked ribonucleotide reductase activity due to virally or mutationally silenced p53, chemotherapeutic inhibition of RNR-M2 and p53R2 after irradiation may lead to impaired supply of *de novo* deoxyribonucleotides needed for radiation-induced DNA repair, enhancing radiosensitivity and perhaps improving cancer control. The radiation-sensitizing effect of ribonucleotide reductase inhibition may be particularly important in cervical cancer, where 90% of worldwide cervical cancers contain high-risk HPV-16 or HPV-18 viral DNA (12) and therefore express viral proteins E6 and E7, which inactivate p53 and pRb. Inhibition of these two critical cell cycle control proteins causes abrogation of the G_1 restriction checkpoint, allowing viral replication (13,14). We previously showed that human CaSki cervical cancer cells demonstrated a 17-fold rise in RNR-M2 protein and a fourfold rise in ribonucleotide reductase activity 18 to 24 h after irradiation (10). Thus it is not surprising that the ribonucleotide reductase inhibitor hydroxyurea sensitizes human cervical cancers to radiation (15,16).

The investigational chemotherapeutic drug 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP, Triapine[®], NSC#663249) is a more potent inhibitor of the RNR-M2 and p53R2 subunits than hydroxyurea (17–19). *In vitro* and *in vivo* radiosensitization by 3-AP has been shown in glioma, pancreas and prostate cancer cell lines and in athymic mice with human tumor xenografts (20). Here we tested the hypothesis that 3-AP-targeted inhibition of the RNR-M2 and p53R2 subunits of ribonucleotide reductase would enhance radiation-related cytotoxicity through a p53-independent mechanism involving sustained radiation-induced DNA damage.

MATERIALS AND METHODS

Cell Cultures and Chemicals

Two human colon cancer cell lines were used: RKO (parental) cells with wild-type p53 and isogenic RKO-E6 transfected cells with a stably integrated human papillomavirus (HPV) E6 oncogene under control of the cytomegalovirus promoter (13). Additionally, three cervical cancer cell lines were used: HPV-16 positive, wild-type p53 CaSki cells (21), HPV-18 positive, wild-type p53 HeLa cells, (22) and HPV-naïve, mutated p53 (codon 273 Arg-Cys) C33-a cells (23). All human tumor cell lines were obtained from the American Type Culture Collection (Rockville, MD).

Caski cells were cultured in RPMI 1640 medium (Grand Island, NY), supplemented with 10% fetal bovine serum, L-glutamine and 1% penicillin/streptomycin. RKO, RKO-E6, HeLa and C33-a cells were propagated in Eagle's minimum essential medium (Grand Island, NY) supplemented with 10% fetal bovine serum, sodium bicarbonate, 1 mM sodium pyruvate, and 1% non-essential amino acids. All cells were maintained at 37°C in a humidified 95% air/5% CO₂ atmosphere. Chemicals used were purchased from Sigma (St. Louis, MO) unless otherwise specified. 3-AP (NSC #663249) is an investigational agent supplied under a Material Transfer Agreement involving Case Western Reserve University (Cleveland, OH), Vion Pharmaceuticals, Inc. (New Haven, CT), and the National Cancer Institute Cancer Therapy

Evaluation Program (Bethesda, MD). Radiation (0–10 Gy) was delivered using a ^{137}Cs γ irradiator (J. L. Shepherd and Associates, San Fernando, CA) at a dose rate of 36 Gy/min.

Ribonucleotide Reductase Assays

Adapting the methods of Gao *et al.* (24) and Lin *et al.* (25), intracellular deoxycytidine triphosphate (dCTP) pools were quantified after radiation (4 Gy) and 3-AP (5 μM) treatments using a DNA polymerase extension assay with modification of the template sequence. Exponentially growing cells were plated on 100-mm dishes to yield 1.0×10^6 cells per dish. Six hours after treatment, cells were collected by trypsinization and washed once with phosphate-buffered saline (PBS), and intracellular dCTP was extracted using ice-cold 60% methanol (50 $\mu\text{l}/10^6$ cells). Cells suspended in methanol were kept overnight at -20°C . The methanol extract was vortexed vigorously, heated at 95°C for 5 min, and centrifuged briefly to remove cell debris. The clear supernatant was stored at -80°C until analysis.

The nucleotide sequence for the DNA polymerase extension assay template was 5'-AAA GAA AGA AAG AAA GAA AGG GCG GTG GAG GCG G-3' and the primer was 5'-CCG CCT CCA CCG CC-3' (Integrated DNA Technologies, Coralville, IA). For this assay, 5 μl of the tumor cell extract was added to 45 μl DNA polymerase extension assay reaction mixture, consisting of 50 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 , 5 mM dithiothreitol, 10 μg BSA, 0.25 mM template primer, 0.1 U Klenow fragment of DNA polymerase, and 1 μM ^3H -dTTP [specific activity 15 Ci (0.555 TBq)/mmole, Perkin Elmer, Waltham, MA]. This reaction mixture was incubated at 37°C for 30 min and then spotted onto Whatman DE81 filter paper, which was then dried under an infrared lamp, washed three times with 5% Na_2HPO_4 , rinsed with water and ethanol, and dried. A liquid scintillation counter was used to quantify radioactivity on the filter paper. The amount of ^3H -dTTP radioactivity incorporated was linearly proportional to the concentration of dCTP up to 2.0 pmol.

Flow Cytometry for Cell Cycle Analysis and γ -H2AX Staining

Exponentially growing cells were plated on 100-mm dishes to yield $0.6\text{--}2.0 \times 10^6$ colonies per dish. For cell cycle analysis, cells were harvested by trypsinization, washed with cold PBS, and fixed in cold 70% ethanol. The cells were then incubated in 33 $\mu\text{g}/\text{ml}$ propidium iodide, 1 mg/ml RNase, 0.5 mmol/liter EDTA, and 0.2% NP40 overnight at 4°C until analysis. For γ -H2AX staining analysis, cells were harvested by trypsinization, washed with cold PBS, fixed in cold 0.5% formaldehyde at 37°C for 10 min, and stored in ice-cold methanol overnight at -20°C until analysis. The day before analysis, fixed cells were washed once with PBS, incubated in BSA/PBS solution (2% BSA in PBS containing 0.1% Triton) for 30 min on ice, and stained with an FITC-conjugated antibody to γ -H2AX (Millipore, Billerica, MA) in BSA/PBS solution (1:500 dilution or 4 $\mu\text{g}/\text{ml}$) for 1 h on ice. Flow cytometry analyses were done using a Coulter EPICS XL-MCL flow cytometer (Coulter Co., Miami, FL). At least 20,000 events were analyzed using ModFit LT 3.0 (Verity, Inc., Topsham, ME) and WinMDI 2.9 (The Scripps Research Institute, San Diego, CA). Cells stained for propidium iodide having 2C DNA content represented G_1 -phase cells and those having 4C DNA content were G_2/M -phase cells. Cells with greater than 2C but less than 4C DNA content cells were identified as S-phase cells. One-half of the S-phase cell proportion was added to the proportion of G_1 -phase cells to determine a G_1/S -phase proportion. Cells stained for γ -H2AX were scored as positive after the indicated treatment if the recorded intensity was greater than the intensity of the control cells and expressed as a percentage of total number of events analyzed.

Clonogenic Survival Assay and Analysis of Radiation-Drug Interactions

Exponentially growing cells were plated on 60-mm dishes to yield 300 to 600 cells per dish. Cells received radiation (0–10 Gy) and/or a 6-h exposure to 3-AP (1–10 μM) as indicated. Fourteen days after plating, colonies were fixed in 70% ethanol and stained with 0.5% crystal

violet. Surviving colonies (>50 cells) were counted, and results were expressed as the surviving fraction normalized to the plating efficiency of nontreated controls.

Western Immunoblot Analyses

Exponentially growing CaSki and C33-a cervical cancer cells (1.0×10^6) were treated with 3-AP and radiation as indicated. Cells were harvested by trypsinization, washed with cool PBS, and lysed in 0.65 ml of ice-cold cell lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40 and 1% SDS) with freshly added protease inhibitors (Sigma, St. Louis, MO). Cells were lysed by sonication, debris was removed by centrifugation (13,000 rpm, 10 min, 4°C), and the total protein concentration in the supernatant was measured using a commercial protein assay (Bio-Rad, Hercules, CA). Proteins (60 µg) were fractionated by 14% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were kept for 2 h in blocking buffer (5% nonfat dried milk, 2% Tween in 20 mM TBS, pH 7.6). Afterward, membranes were probed with mouse rabbit anti-human p53 and p21 polyclonal antibodies (1:1000 dilution, 1 h at room temperature; Novus Biologicals, Littleton, CO). Membranes were then incubated for 1 h with alkaline phosphatase-conjugated anti-rabbit secondary antibodies with blocking buffer. β-Actin was used as a protein loading control. Immunoreactive bands were visualized by chemiluminescence.

Statistical Methods

Using a balanced complete block factorial design, a two-stage method of survival analysis that includes multivariate analyses of variance (MANOVA) statistics analyzed radiation-drug interactions at all treatment levels (i.e. combinations) with all levels of every other treatment, as described previously by our group for clonogenic survival assays (26,27). As a generalization of ANOVA and *t* tests appropriate for multiple responses, MANOVA statistical analyses provide a unified alternative to testing differences among fitted response vectors one parameter at a time. A joint test of the parameters serves as a global test for differences between dose responses. Simply, MANOVA techniques statistically compare overall “shapes” of different survival curves. MANOVA statistics ($\alpha = 0.05$) were computed using statistical software (SPSS 12.0, Chicago, IL). For flow cytometry, median cell proportions in the G₁/S and G₂/M phase of the cell cycle were determined with SPSS 12.0 statistical software. For ribonucleotide reductase activity and γ-H2AX assays, analysis of variance (ANOVA) or *t* tests of significance ($\alpha = 0.05$) were done using SPSS 12.0 statistical software.

RESULTS

Enhanced Radiosensitization of Cervical Cancer Cells to Ionizing Radiation and 3-AP Treatment

Single-agent dose–response curves for radiation or 6-h 3-AP treatment are presented in Fig. 1a. Radiation cytotoxicity (2–6 Gy) was similar among CaSki, HeLa and C33-a cervical cancer cells. Only minor cytotoxicity was observed for 3-AP treatment alone, with a median cytotoxicity of 19% among the three cervical cancer cell lines after the 6-h 3-AP exposure.

Combination dose–response curves for 3-AP-radiation cytotoxicity are presented in Fig. 1b. Significant positive interactions of increasing 3-AP concentration on CaSki, HeLa and C33-a cell radiation toxicity were observed ($P < 0.001$). At the lowest 3-AP concentration (1 mM), clonogenic survival of radiation + 3-AP-treated cells was not significantly different from that of cells treated with radiation only (Fig. 1b). For 5 µM and 10 µM 3-AP + radiation treatments, cytotoxicity was greater than that of radiation alone (Fig. 1b). These findings support the hypothesis that inhibition of ribonucleotide reductase, the rate-limiting enzyme in the on-demand *de novo* supply of dNTPs, sensitizes cells to radiation and enhances cell death.

Enhanced G₁/S Cell Cycle Arrest in Cervical Cancer Cells after Inhibition of Ribonucleotide Reductase

To explore the mechanism underlying the role of inhibition of ribonucleotide reductase on enhanced radiation cytotoxicity in cervical cancer cells, ribonucleotide reductase activity was assayed in CaSki, HeLa and C33-a cells after treatment with radiation plus 3-AP. The effects of the combination on cell cycle distribution were also assessed (Fig. 2). In untreated cells, intracellular dCTP levels were higher in HPV-positive CaSki and HeLa cells than in HPV-naïve C33-a cells (Fig. 2a). Intracellular dCTP levels increased after radiation treatment, confirming elevated ribonucleotide reductase activity after radiation-induced DNA damage, as we have shown previously (10). In contrast, 3-AP treatment alone or after irradiation resulted in a reduction in dCTP levels in all cervical cancer cell lines (Fig. 2a), indicating that the DNA damage-induced elevation of dNTP levels needed for immediate that the DNA repair is prevented when cells are treated with the ribonucleotide reductase inhibitor 3-AP.

To relate the findings of suppressed dNTP levels to alterations in cell cycle distribution, the DNA content of cells treated with radiation and/or 3-AP were analyzed by flow cytometry. Ribonucleotide reductase plays an important role in DNA synthesis and DNA damage repair, with inhibition of its activity stalling DNA replication and promoting a delay in cell cycle progression (28,29). 3-AP treatment leads to a G₁/S-phase cell cycle arrest (Fig. 2b). When assessed 6 h after initiation of 3-AP treatment, 3-AP-treated cells showed a cell cycle distribution similar to that of untreated cells (median G₁/S: 57% for 5 μ M 3-AP compared to 58% for untreated), but at 18 h after initiation of treatment (12 h after a 6-h 3-AP exposure) a significant G₁/S cell cycle arrest was observed in the treated cells (median G₁/S: 69% for 5 μ M 3-AP compared to 44% for untreated). In cells treated with radiation, the cell cycle response was similar 6 h after irradiation with or without 3-AP (median G₁/S: 56% for radiation plus 5 μ M 3-AP compared to 60% for radiation). In contrast to radiation-treated cells, which arrested in G₂/M 18 h after treatment, radiation + 3-AP-treated cells retained a more prominent G₁/S cell cycle arrest at this later time (median G₁/S: 79% for radiation plus 5 μ M 3-AP compared to 13% for radiation). Use of 10 μ M 3-AP did not result in further enhancement of the G₁/S arrest compared with 5 μ M 3-AP regardless of the radiation treatment (Fig. 2b).

To further investigate the temporal relationship between ribonucleotide reductase inhibition and cell cycle alteration, CaSki, HeLa and C33-a cells treated with radiation and 3-AP were analyzed for DNA content by flow cytometry (Fig. 3a). An 18-h end point was chosen because substantial G₁/S arrest was observed at this time in cells treated with 5 μ M or 10 μ M 3-AP (Fig. 2), and previously published data indicated a significant increase in RNR-M2 protein steady-state levels 18 h after irradiation (10). All three cervical cancer cell lines showed a G₁/S cell cycle block that was prominent at 18 h (12 h after radiation + 3-AP treatment). At 24 and 36 h after exposure (18 and 30 h after radiation + 3-AP treatment), these cells progressed through G₂/M and returned to a near-normal log-phase cell cycle distribution (Fig. 3a, Table 1). In cells treated with radiation alone, a substantial fraction halted in G₂/M phase 18 h after treatment (Fig. 3a, Table 1). By 36 h, radiation-treated cells were progressing to G₁. radiation + 3-AP-treated cells showed an 18-h G₁/S cell cycle block, a slow transition to G₂/M phase, and an increased sub-G₁ population suggestive of radiation + 3-AP cytotoxicity (Fig. 3a).

To illustrate important changes in cell regulatory proteins at the G₁/S checkpoint at 18 h after initiation of treatment, we analyzed the levels of two related DNA damage proteins (p53/p21) in HPV-positive, wild-type p53 CaSki and HPV-naïve, mutant-p53 C33-a cervical cancer cells. CaSki cells contain HPV-E6 protein, facilitating rapid turnover of wild-type p53 through coupling to a ubiquitin ligase (30–32), resulting in low steady-state levels of p53 protein (Fig. 3b). Conversely, C33-a cells express a mutant p53 protein (23), altering its ability to interact with its downstream effectors. C33-a mutant p53 protein can be detected but is unchanged by 3-AP or radiation treatment (Fig. 3b). The protein p21, one known downstream effector of p53,

was increased in CaSki cells treated with radiation or radiation + 3-AP but was not detected in C33-a cells due to the presence of a mutant p53 (Fig. 3b). These findings suggest that the p53-independent induction of p21, which has been described in a number of model systems (33), may play a role in the observed G₁/S-phase block after radiation plus 3-AP treatment.

Ribonucleotide Reductase Inhibition Inhibits or Delays Radiation-Mediated DNA Damage Repair

To further ascertain whether ribonucleotide reductase inhibition hampered repair of DNA damage, cells were treated with radiation and/or 3-AP as indicated and allowed to recover for up to 6 h after treatment (Fig. 4). The effect of 3-AP on radiation-mediated DNA repair was investigated through analysis of H2AX phosphorylation at Ser-139 (γ -H2AX) as a marker of DNA double-strand breaks. The loss of γ -H2AX signal in irradiated cells is an indicator of DNA damage repair and is typically observed 3 to 4 h after irradiation (34,35) (Fig. 4a, b). The cervical cancer cells treated with radiation alone or in combination with 3-AP showed a significant increase in γ -H2AX at 1 h (Fig. 4b, $P < 0.001$). Four hours after treatment, cells treated with radiation alone showed a loss of γ -H2AX signal while those cells treated with radiation plus 3-AP showed substantially higher levels of γ -H2AX signal, suggesting sustained DNA damage (Fig 4b). The findings confirm the hypothesis that inhibition of p53R2 and RNR-M2 by 3-AP significantly impairs repair of radiation-induced lesions, thereby delaying recovery from DNA damage. While there are likely more molecular mechanisms governing radiosensitization after 3-AP treatment, it is clear that protracted inhibition of ribonucleotide reductase renders cells more susceptible to DNA-damaging agents and therefore exacerbates radiation-mediated cytotoxicity.

3-AP Treatment Enhances Radiosensitivity of RKO and RKO-E6 Cells

Single-agent and combination treatments with radiation and 3-AP were also examined in RKO and RKO-E6 cells to more directly evaluate the effect of p53 on changes in ribonucleotide reductase activity response after radiation or 3-AP treatment (Fig. 5a). There was a significant increase in dCTP pools in both RKO and RKO-E6 cells 1 h after irradiation, suggesting increased ribonucleotide reductase activity in both cell lines ($P < 0.001$ for both). For RKO cells, 3-AP treatment alone and in combination with radiation resulted in significant reductions in dCTP pools compared to untreated cells. The reduction in dCTP pool in 3-AP-treated RKO cells observed after irradiation was also statistically significant ($P < 0.001$). For RKO-E6 cells, 3-AP treatment alone and in combination with radiation resulted in a significant reduction in dCTP pools 6 h after treatment, and 3-AP + radiation treatment also resulted in a 33% reduction in dCTP pools in RKO-E6 cells. These findings suggest that the level of functional ribonucleotide reductase activity is significantly reduced by 3-AP in cells with wild-type p53 (RKO) and those without functional p53 (RKO-E6).

To evaluate the role of p53 in 3-AP-mediated effects on survival, cell cycle progression, and DNA damage, RKO and RKO-E6 cells were examined after treatment with 3-AP alone or in combination with radiation, as described above. Given the presence of sustained high levels of γ -H2AX foci in the cervical cancer cells after 4 h (Fig. 4), cell cycle distribution and γ -H2AX foci were analyzed in RKO and RKO-E6 cells at this time. RKO cells showed a pronounced G₁/S-phase cell cycle arrest at 4 h after 3-AP treatment alone (median G₁/S: 56% for untreated compared to 75% for 5 μ M 3-AP) or in combination with radiation (39% for radiation compared to 79% for 5 μ M 3-AP and radiation; Fig. 5b). RKO-E6 cells showed a pronounced G₁/S-phase cell cycle arrest at 4 h after 3-AP treatment without radiation (median G₁/S: 60% for untreated compared to 84% for 5 μ M 3-AP) or with radiation (41% for radiation compared to 79% for 5 μ M 3-AP and radiation; Fig. 5b). In addition, RKO and RKO-E6 cells also showed a significant increase in γ -H2AX at 1 h after treatment with radiation alone or radiation + 3-AP (Fig 5c). At 4 h after treatment, radiation + 3-AP-treated cells maintained a

significantly increased number of γ -H2AX foci compared to cells treated with radiation alone. Clonogenic survival after treatment with radiation and 3-AP illustrated enhanced the radiation dose-dependent cytotoxicity in RKO and RKO-E6 cells (Fig. 5d). Collectively, these results suggest that inhibition of ribonucleotide reductase after irradiation significantly enhances cytotoxicity through a p53-independent G₁/S-phase cell cycle arrest.

DISCUSSION

Ribonucleotide reductase activity varies during the cell cycle and in response to DNA damage (6,10,28,36,37). Our findings in CaSki/HeLa/C33-a cervical cancer cells and RKO/RKO-E6 colon cancer cells support the concept that ribonucleotide reductase inhibitors such as 3-AP induce a protracted G₁/S arrest, perpetuate radiation-related DNA damage when given after irradiation, and thereby enhance radiation-related cytotoxicity. Our observations are consistent with a mechanism by which 3-AP inhibits ribonucleotide reductase activity, resulting in a p53-independent G₁/S cell cycle arrest.

DNA in mammalian cells becomes damaged when exposed to radiation, activating specific cellular responses to repair the various types of damage. In response to radiation-mediated DNA damage, proteins in the nucleotide excision and base excision repair pathways excise damaged nucleotides along with a limited number of adjacent undamaged nucleotides (38). DNA repair of radiation damage occurs rapidly, often within 3 h, using DNA repair complexes stabilized by γ -H2AX (34,35). Deoxyribonucleotide triphosphates (dNTPs) used in the repair of DNA damage can be either drawn from stored intracellular pools or synthesized *de novo*, with a rate-limiting step catalyzed by ribonucleotide reductase (3–5,8,10). To optimally repair DNA damage, a cell benefits from coordinated pathways that recognize the need for DNA repair and halt or slow cell cycle progression until the damage has been repaired.

It is clear that deficient or unbalanced dNTP pools cause genotoxic abnormalities and cell death (39). Since balanced dNTP pools are required for proper DNA replication and repair, large fluctuations in dNTP pools are best avoided. To maintain this balance, ribonucleotide reductase activity is tightly regulated by S-phase-specific transcription of the RNR-M1 and RNR-M2 genes (6,40), binding of nucleoside triphosphate allosteric effectors to the R1 protein (39), and anaphase-promoting complex-Cdh1-mediated degradation of the RNR-M2 protein in late mitosis (6). However, cells respond to radiation by altering dNTP levels, by extracting and quickly replenishing dNTPs from intracellular pools, or by synthesizing dNTPs *de novo* for integration into DNA through processes of repair. Transcriptional induction and synthesis of ribonucleotide reductase small subunit protein is time consuming (5,8,10), often taking 18 to 24 h, well beyond the 3-h time frame of radiation-related DNA repair. To facilitate more rapid ribonucleotide reductase activity, cells use the sequence-specific DNA binding and protein-protein stabilization properties of the well-characterized cell cycle regulator p53 to recruit and to sequester reductase subunits (4,5). From this vantage, we hypothesize that chemotherapeutic inhibitors targeting RNR-M2 and p53R2 should provide meaningful radiation-sensitizing effects.

The ribonucleotide reductase inhibitor 3-AP is an efficient iron chelator and disrupts the diferric iron center that stabilizes the tyrosyl free radical critical for catalytic function of ribonucleotide reductase small subunits (15,16). Through this effect, 3-AP potentially interferes with ribonucleotide reductase function and ultimately impedes DNA repair (Fig. 2 and Fig 4). Although 3-AP inactivates ribonucleotide reductase, our data for cervical cancer cells show minimal cytotoxicity after 3-AP alone for 6 h at a reportedly maximal (10 μ M) therapeutic drug exposure; however, significant cytotoxicity was observed when 3-AP was used in combination with radiation (Fig. 1). To determine whether inhibition of ribonucleotide reductase delayed or blocked the repair of radiation-induced DNA damage, the accumulation

of γ -H2AX foci in cells exposed to radiation and to 3-AP was measured. While there was an immediate (i.e. 1-h) increase in γ -H2AX foci indicative of DNA double-strand breaks, there was a significant delay (i.e. greater than 4 h) in the resolution of these foci in the presence of 3-AP (Fig. 4). In that delayed resolution of radiation-mediated γ -H2AX foci indicates impaired DNA damage repair and hence radiation sensitivity (34,35), accumulation of and persistence of radiation-related DNA damage after 3-AP treatment identifies at least one mechanism increasing cell cytotoxicity. However, as yet unexplored molecular mechanisms governing 3-AP-mediated enhancement of radiation sensitivity other than retardation of DNA damage repair are also likely to be active.

3-AP induces a G₁/S-phase cell cycle checkpoint arrest either alone or in combination with radiation as a result of inhibition of ribonucleotide reductase small subunits (Fig. 2). It is proposed that 3-AP-impaired ribonucleotide reductase function (due to titration of available RNR-M2 or p53R2 tyrosyl radicals and inhibition of functional enzyme) delays timely repair of radiation-mediated DNA damage and consequently enhances cellular radiosensitivity. The dominant checkpoint response to DNA damage in mammalian cells traversing G₁ is the ATM (ATR)/CHK2(CHK1)-p53/p21 pathway, which is capable of inducing sustained and sometimes even permanent G₁ arrest (41). ATM/ATR directly phosphorylates p53 and thereby inhibits binding to its ubiquitin ligase, MDM2, resulting in a net accumulation of p53 (42). The key transcriptional target of p53 is p21CIP1/WAF1, an inhibitor of cyclin-dependent kinases (41), which silences the G₁/S-promoting cyclin E/Cdk2 kinase. This preserves the Rb/E2F pathway in its active, growth-suppressing mode and prevents DNA synthesis, thereby sustaining the G₁ blockade. By contrast, exposure to radiation leads to a predominant G₂/M-phase cell cycle checkpoint arrest so that un-repaired DNA damage inflicted during previous G₁ or S phases is not propagated through mitosis (Fig. 2 and Fig 3). G₂/M checkpoint restriction follows inhibition of cyclinB/CDK1 kinase, which may be inactivated by the ATM(ATR)/CHK2(CHK1)-p38 sequestration pathway or by inhibiting CDC25 phosphatases that normally activate CDK1 at the G₂/M boundary (43,44). To begin to understand the interaction of 3-AP and important G₁/S-phase regulatory proteins, we evaluated cervical and colon cancer cells with wild-type, virally inhibited or mutated p53.

CaSki cells express wild-type p53 that is abrogated by the HPV-16 E6 protein, interfering with p53-mediated regulation of the cell cycle (13,45–47). The HPV E6 protein binds to p53 and facilitates turnover by forming a trimeric complex with the cellular ubiquitin ligase E6AP (30–32). This rapid p53 turnover results in low steady-state levels (45), thus mitigating cell cycle checkpoint restrictions on DNA synthesis. The ability of p53 to bind and attenuate RNR-M2 or p53R2 association with RNR-M1 is lost in the presence of HPV E6 protein. Lembo *et al.* have recently shown that normal fibroblasts transfected with HPV E6 and exposed to oxidative stress have elevated intracellular levels of p53R2 (47). Ectopic expression of E6 in fibroblasts or inherent expression in three HPV-positive cervical cancer cell lines impaired p53 and p53R2 but not RNR-M2 response to oxidative DNA damage (47). In HPV-positive cervical cancer cells, RNR-M2 has been shown to rise to maximal levels 18 to 24 h after irradiation (10). In our studies, untreated HPV-16-positive, wild-type p53 CaSki cells show very low levels of p53 and p21 (Fig. 3). Eighteen hours after radiation or 3-AP + radiation treatment (with corresponding G₁/S-phase arrest), there was no detectable p53 protein as expected for E6-expressing cells and a slight increase in p21 steady-state protein levels. The p53-independent induction of p21 has been reported previously, and the role of this mode of p21 induction remains an active area of investigation (33). The observation that intranuclear p53R2 and p21 protein-protein dissociation occurs after exposure to ultraviolet radiation during DNA repair at G₁/S-phase arrest is consistent with the slight increase in detected p21 steady-state level observed after irradiation (48). In summary, the HPV E6-mediated wild-type-p53 degradation in CaSki cells supports the hypothesis that the protracted G₁/S-phase arrest we

observed after 3-AP enhanced radiation damage was not regulated solely by a p53-dependent mechanism.

In the case of C33-a cervical cancer cells, a point mutation (CGT-TGT) at amino acid 273 renders p53 unable to regulate target proteins. In our studies, mutant-p53 protein levels were unchanged after radiation or 3-AP treatment and p21 protein levels were low and increased only minimally after radiation treatment, because mutant-p53 was unable to induce transcription of this G₁/S-phase regulatory protein (Fig. 3). These observations are consistent with the hypothesis that the radiation-induced DNA damage response of ribonucleotide reductase is deregulated due to a mutated p53 protein, again indicating a p53-independent mechanism.

Enhanced cytotoxicity was expected and observed in both clonogenic survival and flow cytometry assays in cells treated with radiation and 3-AP due to the inhibition of RNR-M2 by 3-AP at the time of DNA damage. Given that HPV E6 shuttles wild-type p53 toward ubiquitin-controlled degradation in CaSki cells, the protracted G₁/S-phase arrest we observed was unlikely to be regulated primarily by a p53-dependent mechanism. We also observed a protracted G₁/S-phase arrest in C33-a cells where the radiation-induced DNA damage response is deregulated due to the presence of a mutated p53 protein, again indicating a p53-independent mechanism of G₁/S cell cycle arrest. These findings were confirmed in RKO cells, which have wild-type p53, and RKO-E6 cells, where p53 is degraded by viral E6 protein. Both RKO and RKO-E6 cells show reduced ribonucleotide reductase activity in the presence of 3-AP, accompanying G₁/S cell cycle arrest, accumulation of DNA damage, and reduced cell survival after irradiation. The maintenance of DNA damage after treatment with 3-AP + radiation in both RKO and RKO-E6 cells supports a p53-independent mechanism of G₁/S cell cycle arrest.

The tightly controlled regulation of ribonucleotide reductase small subunit steady-state levels in quiescent cells but relative overexpression in tumor-forming and metastatic cells (49,50) makes 3-AP an exciting new chemotherapeutic drug. 3-AP enhances radiosensitivity in glioma, pancreas and prostate cancer cell lines and human tumor xenograft models (20). 3-AP has been shown to be well tolerated when used as a single agent in Phase I solid tumor clinical trials (51–53) and is currently being evaluated in Phase I and II studies in combination with cisplatin and radiation therapy in gynecological malignancies at the Case Comprehensive Cancer Center (NCI CTEP protocols 7336 and 8327; CASE protocols 1805 and 11808). Through effects on ribonucleotide reductase activity in cervical and colon cancer cells, the radiosensitizing effect of 3-AP described here offers evidence of enhanced cytotoxicity mediated through protracted G₁/S-phase cell cycle arrest and delays or inhibits the repair of DNA damage. This therapeutic strategy holds promise for chemoradiation treatment of cancer, especially for cervical cancer, which is a leading cause worldwide of female cancer-related mortality.

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FIG. 1.

3-AP was an effective radiosensitizer in CaSki, HeLa and C33-a cervical cancer cells. Panel A: Cells were treated with radiation or 3-AP for 6 h. Radiation-related ($P = 0.83$) and 3-AP-related ($P = 1.00$) cytotoxicities in the three cervical cancer cell lines were not significantly different. Panel B: Cells were treated with radiation followed by escalating doses of 3-AP for 6 h. A significant positive radiation + 3-AP interaction was observed for all three cervical cancer cell lines ($P < 0.001$). For 1 μM 3-AP-radiation, CaSki ($P = 0.97$), HeLa ($P = 0.69$), and C33-a ($P = 0.39$) cytotoxicity was similar to radiation alone. Significant enhancement of cytotoxicity was observed for 5 μM 3-AP + radiation ($P < 0.001$) and 10 μM 3-AP + radiation ($P < 0.001$) compared to radiation alone. Error bars are $\pm\text{SD}$.

FIG. 2.

Treatment with 3-AP significantly lowered dCTP pools and caused elongation of the G₁/S cell cycle arrest. Panel A: Cells were treated with radiation (4 Gy), 3-AP (5 μM × 6 h), or radiation-3-AP (4 Gy, 5 μM × 6 h). 3-AP treatment alone led to a significant reduction in dCTP levels at 6 h after treatment in CaSki ($P < 0.001$), HeLa ($P < 0.001$), and C33-a ($P < 0.01$) cells compared to untreated cells. Radiation + 3-AP also led to a significant reduction in dCTP levels at 6 h in CaSki ($P < 0.001$), HeLa ($P < 0.001$), and C33-a ($P < 0.001$) cells compared to radiation alone. Panel B: Cells were untreated or treated with radiation (6 Gy) and/or 3-AP (5, 10 μM × 6 h) and harvested at the indicated times. DNA content was analyzed by propidium staining and flow cytometry in triplicate. Using CaSki cells as an illustrative example, 3-AP resulted

in protracted G₁/S-phase arrest 18 h after 3-AP or radiation + 3-AP treatment (*P* values are comparisons of treatment groups for each time indicated). Cell cycle G₁/S arrest was similar after 5 μ M 3-AP or 10 μ M 3-AP without (6 h: *P* = 1.00, 18 h: *P* = 0.98) or with (6 h: *P* = 0.12, 18 h: *P* = 1.00) radiation.

FIG. 3.

Panel A: Treatment with 3-AP prolonged G₁/S-phase cell cycle arrest. Cells were treated with radiation (10 Gy), 3-AP (5 μ M $\times$ 6 h), or radiation + 3-AP (10 Gy, 5 μ M $\times$ 6 h) and harvested at 18, 24 and 36 h for DNA content analysis by flow cytometry in triplicate. While radiation-treated cells showed a G₂/M arrest, cells treated with radiation + 3-AP showed a greater G₁/S-phase cell cycle arrest. Panel B: Cells were untreated or treated with radiation (10 Gy) and/or exposed to 6 h of 3-AP (5 μ M) and harvested at 18 h after treatment. At 18 h, CaSki cell p53 was not detected due to the presence of the HPV-16 E6. C33-a cells expressed a mutant p53, detected by antibody, that was unchanged after radiation or 3-AP. The downstream

effector protein p21 showed a moderate increase after irradiation in CaSki cells but was not detected in C33-a cells.

A HeLa cells 4 hours after treatment

B

FIG. 4.

3-AP treatment led to an accumulation of DNA damage after irradiation. Panel A: HeLa cells were treated as indicated with 3-AP ($5 \mu\text{M} \times 6 \text{ h}$) and/or radiation (6 Gy) and harvested 4 h later for $\gamma\text{-H2AX}$ analysis by microscopy. Radiation + 3-AP-treated cells demonstrated an increased number of $\gamma\text{-H2AX}$ foci compared to radiation alone, indicative of DNA double-strand breaks. Panel B: Cells were untreated (closed circles) or were treated with 3-AP ($5 \mu\text{M} \times 6 \text{ h}$, open circles), radiation (6 Gy, closed triangles), or radiation + 3-AP (6 Gy, $5 \mu\text{M} \times 6 \text{ h}$, open triangles) and harvested 1 and 4 h afterward for $\gamma\text{-H2AX}$ analysis by flow cytometry. At 4 h after treatment, there were significant sustained $\gamma\text{-H2AX}$ foci in radiation + 3-AP-treated cells where ribonucleotide reductase activity had been inhibited compared to radiation alone ($P < 0.004$, each cell line). Error bars are $\pm\text{SD}$.

FIG. 5.

RKO and RKO-E6 cells demonstrated enhanced radiosensitivity when treated with the 3-AP. Panel A: Cells were treated with 3-AP and/or radiation and assessed at 6 h after treatment. 3-AP treatment led to a significant reduction in dCTP levels ($P < 0.01$ for RKO and RKO-E6 cells with 3-AP alone; $P < 0.001$ for RKO and RKO-E6 cells with radiation + 3-AP). Panel B: Cells were treated with 3-AP and radiation as indicated and harvested 0 and 4 h afterward for analysis of DNA content by flow cytometry (P values are comparisons of treatment groups for each time indicated). After 3-AP treatment, a G₁/S arrest was apparent at 4 h in RKO and RKO-E6 cells. Panel C: After radiation + 3-AP compared to radiation alone, there was significant sustained DNA damage as indicated by persistent γ -H2AX foci ($P < 0.001$, each cell line). Panel D: After radiation + 3-AP treatment, there was a significant reduction in the clonogenic survival of RKO and RKO-E6 cells ($P < 0.001$). Error bars are $\pm$ SD.

TABLE 1
Median Cell Cycle Proportions of Cervical Cancer Cells after 3-AP and/or Ionizing Radiation Treatment

Cell line	Time (h)	Control			3-AP (5 μ M)			Radiation (10 Gy)			Radiation + 3-AP		
		G ₁	S	G ₂ /M	G ₁	S	G ₂ /M	G ₁	S	G ₂ /M	G ₁	S	G ₂ /M
CaSki	0	63	30	7	64	30	6	63	28	9	60	28	12
	18	44	42	14	69	27	14	12	8	80	81	10	9
	24	45	42	13	63	7	30	52	3	45	87	3	10
HeLa	36	68	22	10	61	25	14	40	10	50	63	15	22
	0	56	32	12	53	30	17	63	20	17	60	25	15
	18	46	43	11	69	27	4	13	8	79	75	16	9
C33-a	24	46	46	8	61	30	9	20	12	68	86	5	9
	36	50	41	9	56	8	36	37	15	48	29	14	57
	0	68	23	9	69	25	6	68	22	10	67	24	9
	18	66	20	14	79	19	2	1	19	80	78	17	5
	24	60	24	16	72	2	26	42	17	41	93	5	2
	36	68	24	8	44	12	44	60	12	28	3	21	76