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Deoxynucleoside Salvage Facilitates DNA Repair During Ribonucleotide Reductase Blockade in Human Cervical Cancers

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Abstract

Cells generate 2'-deoxyribonucleoside triphosphates (dNTPs) for both replication and repair of damaged DNA predominantly through *de novo* reduction of intracellular ribonucleotides by ribonucleotide reductase (RNR). Cells can also salvage deoxynucleosides by deoxycytidine kinase/thymidine kinase 1 in the cytosol or by deoxyguanosine kinase/thymidine kinase 2 in mitochondria. In this study we investigated whether the salvage dNTP supply pathway facilitates DNA damage repair, promoting cell survival, when pharmacological inhibition of RNR by 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP, NSC no. 663249) impairs the *de novo* pathway. Human cervical cancer cells were subjected to radiation with or without 3-AP under medium deoxynucleoside concentrations of 0, 0.05, 0.5 and 5.0 μ M. Efficacy of DNA damage repair was assessed by γ -H2AX flow cytometry and focus counts, by single cell electrophoresis (Comet assay), and by caspase 3 cleavage assay as a marker of treatment-induced apoptosis. Cell survival was assessed by colony formation. We found that deoxyribonucleotide salvage facilitates DNA repair during RNR inhibition by 3-AP and that salvage reduces the radiochemosensitivity of human cervical cancer cells.

INTRODUCTION

Two pathways generate 2'-deoxyribonucleoside triphosphates (dNTPs) used in DNA replication and repair. The predominant pathway involves *de novo* reduction of intracellular ribonucleoside diphosphates (NDPs) to their corresponding deoxyribonucleoside counterparts (dNDPs) by ribonucleotide reductase (RNR, Fig. 1A) (1–3). A complementary pathway salvages deoxynucleosides (dNs) through deoxynucleoside kinases (Fig. 1B) (1–3). Cells coordinate the *de novo* and salvage pathways such that dNTP supply matches dNTP demand for replication and repair, with minimal imbalances in dNTP pools to avoid deleterious genotoxic stress (4).

In the *de novo* ribonucleotide reduction pathway, RNR functions as a rate-limiting heterotetrameric enzyme containing two active site large subunits (M1) and two small catalytic subunits [M2 or p53R2 (M2b)] (5, 6). Protein M1, a long-lived protein, can be detected in all phases of the cell cycle (6). An M1–M2 protein complex has been shown to reduce ribonucleotides during S phase, and the complex is abruptly degraded in late mitosis due to a KEN-box sequence promoting proteasome-dependent breakdown of M2 (7–9). The M1-p53R2 protein complex has been detected in all phases of the cell cycle and has been suggested to act as a DNA damage response protein, with both its transcription and activity regulated by p53 (7–9). Sources of dNTPs after DNA-damaging insults such as ionizing radiation include ribonucleotide reduction through an M1-p53R2-mediated process first and subsequently through a complementary M1-M2 mechanism (10–12). This DNA damage response can be blocked substantially by administration of 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP), a 1000X more potent RNR inhibitor than hydroxyurea currently under investigation in clinical trials (12). Nevertheless, our hypothesis that the salvage pathway also supplies dNTPs for DNA damage repair and thus circumvents or replaces the *de novo* dNTP pathway when blocked is supported to the extent that salvage enzyme activation occurs after exposure to ionizing radiation (13, 14).

In the salvage dNTP pathway, deoxynucleoside kinases act as rate-limiting dNTP supply enzymes. These enzymes [TK1 and/or dCK in the cytosol and thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK) in mitochondria, Fig. 1B] phosphorylate deoxyribonucleosides to produce deoxyribonucleoside monophosphates (dNMPs) (15). TK1 is S-phase specific through a mechanism similar to that of M2; the other three deoxynucleoside kinases are constitutively active across the cell cycle. Deoxynucleoside kinases are variably expressed in human normal and cancer tissues, with TK1 being elevated in cervical cancers (16). The substrates of these salvage enzymes, deoxynucleosides, enter cells and mitochondria passively by plasma membrane equilibrative nucleoside transporters (ENTs, Fig. 1) (17).

In this study, we investigated whether dNTP salvage enables enough DNA damage repair to reduce radiation-mediated cytotoxicity when *de novo* ribonucleotide reduction is inhibited by 3-AP.

MATERIALS AND METHODS

Cell Culture and Chemicals

C33-a [human papillomavirus (HPV)-naïve, mutated p53 (codon 273 Arg-Cys)] (18) and CaSki (HPV-16 positive, wild-type p53) (19) cervical cancer cells were obtained from American Type Culture Collection (Rockville, MD) and cultured at 37°C in a humidified 95% air/5% CO₂ atmosphere. For culture, Eagle's minimum essential medium (Grand Island, NY) supplemented with 10% fetal bovine serum, 1% nonessential amino acids and 1% penicillin/streptomycin was used. However, for experiments, we used dialyzed medium consisting of Eagle's minimum essential medium (Grand Island, NY) supplemented with 1% nonessential amino acids, 1% penicillin/streptomycin, and 40-nm filtered 10% fetal bovine serum with “add back” concentrations of 0, 0.05, 0.5 or 5 μ M deoxynucleosides (i.e., 1 deoxyadenosine:1 deoxycytidine:1 deoxyguanosine:1 deoxyuridine:1 deoxythymidine). As indicated, either culture medium or dialyzed medium with 0, 0.05, 0.5 or 5 μ M deoxynucleosides was provided for the duration of 6-h assays done in triplicate. Chemicals used were purchased from Sigma (St. Louis, MO). 3-AP (NSC no. 663249) is an investigational agent provided to Case Western Reserve University (Cleveland, OH) under an agreement with the National Cancer Institute Cancer Therapy Evaluation Program (NCI-CTEP, Bethesda, MD). Radiation (0–10 Gy) was delivered using a ¹³⁷Cs γ irradiator (J. L. Shepherd and Associates, San Fernando, CA) at a dose rate of 3.27 Gy/min.

Clonogenic Survival Assays

Exponentially growing cells were plated on 24-well dishes to yield 300 (0–6 Gy) or 1000 cells (1 Gy) per well. Cells received radiation (0, 2, 4, 6 or 10 Gy) alone or in combination with a 6-h exposure to 3-AP (5 μ M), chosen because of prior pharmacokinetic data and experience (12, 20, 21). Surviving colonies (>50 cells) stained with 0.1% crystal violet were counted after fixation in 70% ethanol 7 days after plating in the indicated medium. Colony counts were normalized to the plating efficiency of nontreated control cells.

Deoxycytidine Triphosphate Assay

Deoxycytidine triphosphate (dCTP) intracellular pools were quantified using a DNA polymerase extension assay (12). The nucleotide 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). A liquid scintillation counter quantified 3 H-dTTP radioactivity, with incorporation linearly related to intracellular dCTP concentration (>2.0 pmol). All cells were collected by trypsinization 6 h after irradiation (6 Gy) in the indicated medium to assay radiation-mediated change in dCTP levels with active RNR (i.e., *de novo* pathway competent) compared to RNR blocked by 5 μ M 3-AP to isolate the salvage pathway.

DNA Damage (γ -H2AX) and Caspase 3 Assays

Exponentially growing cells (1×10^6 /100-mm dish) were exposed to radiation (0 or 6 Gy) alone or to radiation plus 3-AP (5 μ M, 6 h) in the indicated medium. Fixed cells were washed and immunostained with a primary antibody γ -H2AX [mouse anti-human fluorescein isothiocyanate (FITC)-conjugated anti- γ -H2AX antibody (Millipore, Billerica, MA) used at 1:500 dilution] or cleaved caspase 3 [caspase 3-Alexa Fluor 488 polyclonal antibody (Beckman Coulter Inc., Miami, FL) used at 1:300 dilution] for 90 min at 4°C in PBS with 2% BSA (12). DNA was stained with propidium iodide at 2 mg/ 10^6 cells or DAPI at 0.25 μ g/ 10^6 cells. Cells were measured with a Coulter EPICS XL-MCL flow cytometer (Beckman Coulter Inc.). Cells were excited with a 488 nm laser. Data were analyzed with ModFit LT 3.0 (Verity, Inc., Topsham, ME) and WinMDI 2.9 (The Scripps Research Institute, San Diego, CA). A model-independent slope for γ -H2AX resolution was calculated as the γ -H2AX proportion at 360 min minus that at 120 min divided by 240 min.

For manual γ -H2AX focus counts, cells were grown on cover slips and exposed to radiation and 3-AP as indicated. Cells were then fixed in 3% formaldehyde for 30 min on ice 6 h after irradiation and treated with 0.1% Triton X-100 in PBS for 10 min at room temperature, then IFA buffer (PBS containing 1% bovine serum albumin, 0.1% Tween 20). Cover slips were incubated with primary γ -H2AX antibody (Millipore, 1:500) in IFA buffer for 60 min at room temperature followed by secondary horse anti-mouse antibody conjugated to Texas red (Vector Laboratories, Burlingame, CA; 1:200) for 60 min at room temperature. γ -H2AX foci in each of 50 cells per treatment group were counted manually using an inverted Leitz fluorescence microscope. Images were photographed with a Spot RT digital camera.

Single Cell Electrophoresis

Aliquots of cell suspensions [volume (V) = 10 μ l] that had been irradiated with or without 3-AP were collected 6 h after irradiation with a sterile pipette for neutral single-cell gel electrophoresis (comet) assay. Single cells were loaded on agarose microgels (V = 90 μ l), lysed and electrophoresed (22 volts) for 60 min at 4°C in neutral buffer (Tris base/sodium acetate/dH₂O). Microgels were washed, fixed with 70% ethanol for 10 min, dried and stained with commercial dye (SYBR green, Trevigen). Samples were analyzed for DNA tail

moment on an inverted-type imaging cytometer (iCyte, Compucyte, Cambridge, MA) with computer-based Comet Assay IV software (Perceptive Instruments, United Kingdom).

Statistical Methods

MANOVA statistics ($\alpha = 0.05$) using a balanced complete block factorial design were calculated for a global test for differences between radiation-3-AP responses (12, 22, 23). dCTP and γ -H2AX assays were analyzed by analysis of variance (ANOVA) or *t* tests of significance ($\alpha = 0.05$) using SPSS Statistics 18.0. Means and standard deviations are reported for triplicate experiments.

RESULTS

Deoxynucleoside Supplementation of Standard Growth Medium

In this work, we set out to evaluate the impact of deoxynucleoside supplementation (0.05 μ M) of tissue culture medium containing 10% dialyzed serum on the growth and dCTP levels in cervical cancer cells treated with radiation (Fig. 2). Survival after irradiation was similar in cells cultured in control or supplemented medium (CaSki, $P = 0.56$; C33-a, $P = 0.32$; Fig. 2A). dCTP levels were also similar for both medium conditions (CaSki, $P = 0.91$; C33-a, $P = 0.28$; Fig. 2B). Medium containing deoxynucleoside-supplemented dialyzed serum had little effect on growth and nucleoside metabolism, suggesting that serum cofactors ≥ 40 nm in particle size missing from dialyzed medium did not appear to alter postirradiation growth or 6-h intracellular dCTP levels.

Effect of Deoxynucleoside Supplementation on Chemoradiosensitization in Cervical Cancer Cells

Passive diffusion of deoxynucleoside across cell plasma membranes renders serum and intracellular deoxynucleoside concentrations equal to one another (24, 25). Accordingly, salvage of deoxynucleosides provides at least one pathway for cells to create dNTPs to facilitate repair of damaged DNA (Fig. 1). We thus evaluated the effect of deoxynucleoside depletion and supplementation on cell survival after radiation-mediated DNA damage (Fig. 3A). Survival of cervical cancer cells cultured in deoxynucleoside-free (0 μ M) medium and treated with radiation was significantly reduced ($P < 0.001$) compared to irradiated cells cultured in standard medium (0.05 μ M). Supplementation with 10-fold more deoxynucleosides than present in standard serum-containing medium (0.50 μ M) enhanced cell survival after irradiation ($P < 0.001$). Supplementation with 100-fold more deoxynucleosides (5.00 μ M) resulted in significantly higher cell death compared to standard (0.05 μ M) medium ($P < 0.001$). The cytotoxicity in 100-fold excess dN resulted in cytoreduction similar to that observed in deoxynucleoside-free medium.

Similar effects were seen when survival was assessed in cells in which RNR was inhibited by 3-AP (5 μ M, Fig. 3B). As demonstrated in prior work with CaSki and C33-a cervical cancer cells (12, 20), radiation and 3-AP treatment caused greater cytoreduction than radiation alone ($P < 0.01$ in each case). A pattern of reduced survival with deoxynucleoside-free medium (0 μ M, $P < 0.001$) and enhanced survival with 10X-supplemented medium (0.50 μ M, $P < 0.001$) was found after irradiation and 3-AP treatment (Fig. 3B). Considerable reduction in cell survival ($P < 0.001$) was noted after irradiation and 3-AP with 100-fold deoxynucleoside (5 μ M) medium compared to standard (0.05 μ M) medium. With 10-fold deoxynucleoside supplementation promoting survival and the absence of deoxynucleosides reducing survival, it is clear that intracellular deoxynucleosides can be used to complement *de novo* synthesis of dNTPs for DNA damage repair. It is also reasonable to conclude that deoxynucleoside salvage provides a mechanism of dNTP supply for repair of DNA damage

in the face of inhibition of RNR and hence inactivation of the *de novo* dNTP supply pathway.

Pathways of Deoxynucleoside Triphosphate Generation after DNA Damage

Intracellular dNTPs drawn upon for DNA replication and repair arise chiefly via the tightly regulated pathway of ribonucleotide reduction by RNR, although cells are capable of dN salvage as well. Previous work has shown that radiation-mediated DNA damage induces a rise in intracellular dCTP levels (12, 20). Identification of the pathway(s) stimulated by radiation could provide important clues to the mechanism of control of DNA damage responses mediated by proteins involved in dNTP generation by *de novo* paths or regeneration by salvage paths. To explore this, CaSki and C33-a cervical cancer cells were incubated for 6 h after irradiation (6 Gy) or sham irradiation in deoxynucleoside-free (0 μ M) or supplemented (0.05, 0.5, 5 μ M) medium with or without 3-AP (5 μ M) with dCTP pools measured at the end of 6 h (Fig. 4). After radiation-induced DNA damage, cervical cancer cells exhibited an increase in dCTP concentration, consistent with prior work (12, 20). Levels of dCTP rose after irradiation in cells cultured in deoxynucleoside-free medium compared to irradiated cells in standard medium. One possible interpretation of this is that activation of the *de novo* RNR pathway is stronger when salvage substrates are absent (Fig. 4A, B). Under these same medium conditions, levels of dCTP fell after 3-AP alone or radiation plus 3-AP treatment, consistent with (1) a low contribution of salvage to regenerate consumed dNTPs when RNR is inactivated by 3-AP and (2) a predominance of RNR ribonucleotide reduction after radiation-mediated DNA damage (Fig. 4A, B). Addition of 10-fold (0.50 μ M) and 100-fold (5.00 μ M) deoxynucleoside medium suppressed dCTP levels in irradiated cells compared to their corresponding cells in standard medium ($P < 0.01$ in irradiated treated cells; Fig. 4A, B), perhaps suggestive of feedback allosteric inhibition of RNR by surplus dATP due to excess salvage of deoxyadenosines. When RNR is inactivated by 3-AP, deoxynucleoside supplementation provides increased dCTP levels through enhanced salvage pathway substrate whether a radiation-mediated demand for dNTPs is present or not (Fig. 4A, B). Together, treated cervical cancer cells demonstrated a complementary means of deoxynucleoside triphosphate supply through salvage deoxynucleoside pathways when the RNR *de novo* ribonucleotide reduction pathway is blocked.

Resolution of DNA Damage in Cervical Cancer Cells Conditioned by Deoxynucleoside Supplementation

The effect of deoxynucleoside depletion or supplementation on radiation-mediated DNA damage repair was investigated by resolution time course analysis of H2AX phosphorylation at Ser-139 (γ -H2AX) as a signal of DNA damage. Prior work has shown that the peak of the γ -H2AX signal occurs ~1 h after irradiation; resolution of the γ -H2AX signal indicates DNA damage repair, with usual return to baseline levels ~3 h after irradiation (26). Here, cells were treated with radiation (6 Gy), 3-AP (5 μ M), or a combination treatment and assayed for γ -H2AX at 0, 120, 240 and 360 min after irradiation. Compared to cells treated with standard medium (dN = 0.05 μ M), irradiated cells with medium purged of deoxynucleosides (dN = 0 μ M) had minimal delay in resolution of radiation-induced γ -H2AX signal when the *de novo* pathway was intact but had substantial protraction of radiation-induced γ -H2AX signal when RNR activity was blocked by 3-AP (Fig. 5). The decay slopes were $-0.07\%/min$ (0 μ M) and $-0.03\%/min$ (0.05 μ M) for irradiated CaSki cells and $-0.06\%/min$ (0 μ M), $-0.06\%/min$ (0.05 μ M) for irradiated plus 3-AP-treated CaSki cells. For irradiated C33-a cells, the decay slopes were $-0.07\%/min$ (0 μ M) and $-0.05\%/min$ (0.05 μ M) and $-0.07\%/min$ (0 μ M) and $-0.08\%/min$ (0.05 μ M) for irradiated and irradiated plus 3-AP-treated C33-a cells, respectively. Addition of 10-fold (0.5 μ M) and 100-fold (5 μ M) deoxynucleoside medium reduced the delayed effect on γ -H2AX signal decay relative to cells in standard

medium (Fig. 5C–H). Decay slopes were $-0.02\%/min$ after irradiation compared to $-0.10\%/min$ after irradiation and 3-AP for CaSki cells and $-0.04\%/min$ after irradiation compared to $-0.07\%/min$ after irradiation and 3-AP for C33-a cells with 10-fold ($0.50\ \mu M$) deoxynucleoside medium. Decay slopes were $-0.03\%/min$ after irradiation compared to $-0.08\%/min$ after irradiation and 3-AP for CaSki cells and $-0.02\%/min$ after irradiation compared to $-0.06\%/min$ after irradiation and 3-AP for C33-a cells in $5\ \mu M$ deoxynucleoside medium. As deoxynucleoside concentrations were increased above that in standard medium, resolution of γ -H2AX signal was not altered after irradiation alone or combined with and 3-AP (i.e., under conditions of inactivated RNR ribonucleotide reduction), perhaps suggesting saturation of DNA polymerase λ and μ of DNA double-strand break repair for dNTP substrate when excess deoxynucleosides are available for salvage.

It is unclear whether loss of γ -H2AX signal precisely correlated with DNA damage repair because cell sorting fluorescence techniques do not distinguish DNA damage from short-lived double-strand breaks resulting from replication forks or from cells fractionating DNA during apoptosis. As such, CaSki cervical cancer cells treated with radiation (6 Gy), 3-AP ($5\ \mu M$), or both in the presence of the indicated dN medium underwent manual γ -H2AX focus counts (Fig. 5), single gel (comet) electrophoresis for DNA tail moment (Fig. 5), and an assay for caspase 3 cleavage (Table 1). Unlike cell sorting results, which may register up to 2% of all cells positive for γ -H2AX signal (Fig. 5), manual counts indicate that untreated cervical cancer cells had 1 or less γ -H2AX-positive foci. Cells treated with radiation alone retained on average 5 γ -H2AX-positive foci 6 h after irradiation. Cells treated with radiation plus 3-AP had 20 or more γ -H2AX-positive foci on average 6 h after treatment. Unresolved γ -H2AX-positive foci could be explained by persistent DNA double-strand breaks if 3-AP impeded their repair. Neutral comet assays were able to detect more double-strand breaks 6 h after irradiation plus 3-AP (Fig. 5) than no treatment or radiation alone, with little difference in detected tail moment attributed to differences in the dN concentration in the medium. Analysis of cleaved caspase 3 in cells 6 h after irradiation with or without 3-AP treatment confirmed the absence of significant numbers of nuclei with apoptotic signals (Table 1). Therefore, unresolved γ -H2AX-positive foci in these cells do not appear to be explained by treatment-induced apoptosis, an expected outcome since mitotic cell death is associated with radiation exposure.

DISCUSSION

Our data demonstrate that deoxynucleoside salvage facilitates DNA repair during ribonucleotide reductase blockade in human cervical cancer cell lines. As expected, inhibition of RNR by 3-AP led to increased radiation-related cytotoxicity and protracted γ -H2AX signal resolution under depleted conditions or in excess deoxynucleoside medium. In contrast, withdrawal of deoxyribonucleosides from the medium when cells are actively repairing radiation-mediated DNA damage results in enhanced radiation cytotoxicity despite stimulation of *de novo* RNR ribonucleotide reduction to supply dNTPs to fix DNA damage.

In this study, an “add back” strategy was employed to control deoxynucleoside concentrations in an attempt to isolate the effect of salvage dNTP supply in DNA repair. Understanding that deoxynucleoside concentrations are equal on both sides of the plasma membrane, we first examined complete removal of deoxynucleosides through dialysis of serum. Once we established that crucial co-factors for growth and metabolism were not removed in the dialysis step (Fig. 2), we tested whether cells were capable of repairing radiation-mediated DNA damage under conditions where salvage pathways would be expected to produce a net efflux of deoxynucleosides to the extracellular milieu. We observed that DNA damage, here induced by radiation in deoxynucleoside-free medium,

stimulated the *de novo* RNR ribonucleotide reductase pathway to increase dCTP concentration (Fig. 4). We also observed that deoxynucleoside depletion modestly slowed γ -H2AX resolution (Fig. 5) but caused a substantial increase in radiation cytotoxicity (Fig. 3) compared to cells treated in standard medium (0.05 μ M). When 3-AP was used to block the ribonucleotide reduction pathway, the effect was more pronounced. Based on prior work where radiation-mediated DNA damage was associated with a rise in RNR activity (12, 20), it is therefore reasonable to conclude that elevated dCTP concentrations provided by RNR may not be exclusively incorporated into DNA because cells may leach a portion of newly synthesized dNTPs as dNs. This effect perhaps would account for the cytotoxicity seen after irradiation. When RNR is inhibited by 3-AP and the extracellular space is devoid of deoxynucleosides, it is expected that even fewer dNTPs would be incorporated into DNA (i.e., pronounced delay in γ -H2AX signal resolution) and cytotoxicity would be enhanced, as observed (Figs. 3–5). We are unable to resolve the debate regarding whether dNTPs are exclusively generated in the cytosol and diffuse into the nucleus (27) or whether RNR co-localizes with damaged DNA through a Tip60 protein-protein interaction for on-the-spot *de novo* supply of dNTPs (28).

Compared to cells treated with standard medium with excess dN concentrations, we observed that radiation-mediated DNA damage was associated with suppressed levels of dNTPs (Fig. 4), moderate increases in the speed of γ -H2AX resolution (Fig. 5), and reduced radiation cytotoxicity (Fig. 3). However, it appears that 100-fold higher dN concentrations flood intracellular dN pools, perhaps inhibiting RNR by dATP binding to its activity site to promote conditions of excess genotoxic stress. We also see that excess dN concentrations slightly speed γ -H2AX resolution but that the overall throughput of DNA polymerase λ and μ may not be sufficiently altered, perhaps due to dNTP substrate saturation and/or other steps being rate-limiting.

In this work, we chose to monitor resolution of DNA damage by an automated cytometry detection method for γ -H2AX immunofluorescence. Such a technique affords rapid and robust (i.e., >10,000 cells gated) detection of γ -H2AX immunofluorescence for cells sampled over a time course. Cells are deemed positive for γ -H2AX signal if fluorescence exceeds a threshold determined by analysis of untreated cells. The technique is unable to determine the number of γ -H2AX foci present. We overcame this limitation by reporting actual cell γ -H2AX focus counts, which better captures the true resolution kinetics of γ -H2AX foci. Future studies incorporating a rad51 marker may be able to discriminate double-strand breaks processed by homologous recombination from those processed by non-homologous end joining. Currently, our techniques are unable to monitor DNA damage and its resolution resulting from fluctuations in endogenous oxidative stress.

Cervical cancer cells showed that deoxynucleoside salvage pathways helps molecular mechanisms of DNA damage repair when ribonucleotide reductase is inhibited by 3-AP. Whether cervical cancer cell resistance to radiation, cisplatin and 3-AP treatment emerges secondary to deoxynucleoside salvage enzyme (and thus pathway flux) increases in patients is currently being monitored in a phase 2 clinical trial at the Case Comprehensive Cancer Center (NCI-CTEP protocol 8327).

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REFERENCES

1. Hakansson P, Hofer A, Thelander L. Regulation of mammalian ribonucleotide reduction and dNTP pools after DNA damage and in resting cells. *J Biol Chem.* 2006; 281:7834–41. [PubMed: 16436374]
2. Sandrini M, Piskur J. Deoxyribonucleoside kinases: two enzyme families catalyze the same reaction. *Trends Biochem Sci.* 2005; 30:225–8. [PubMed: 15896737]
3. Reichard P. Interactions between deoxyribonucleotide and DNA synthesis. *Annu Rev Biochem.* 1988; 57:349–74. [PubMed: 3052277]
4. Chang E, Shiu A, Mendel E, Mathews L, Mahajan A, Allen P, et al. Phase I/II study of stereotactic body radiotherapy for spinal metastasis and its pattern of failure. *J Neurosurg Spine.* 2007; 7:151–60. [PubMed: 17688054]
5. Eklund H, Uhlin U, Farnegardh M, Logan DT, Nordlund P. Structure and function of the radical enzyme ribonucleotide reductase. *Prog Biophys Mol Biol.* 2001; 77:177–268. [PubMed: 11796141]
6. Kolberg M, Strand KR, Graff P, Andersson KK. Structure, function, and mechanism of ribonucleotide reductases. *Biochim Biophys Acta.* 2004; 1699:1–34. [PubMed: 15158709]
7. Chabes A, Thelander L. Controlled protein degradation regulates ribonucleotide reductase activity in proliferating mammalian cells during the normal cell cycle and in response to DNA damage and replication blocks. *J Biol Chem.* 2000; 275:17747–53. [PubMed: 10747958]
8. Suzuki C, Jacobson H, Hatschek T, Torkzad M, Boden K, Eriksson-Alm Y, et al. Radiologic measurements of tumor response to treatment: Practical approaches and limitations. *Radiographics.* 2008; 28:329–44. [PubMed: 18349443]
9. Xue L, Zhou B, Liu X, Qiu W, Jin Z, Yen Y. Wild-type p53 regulates human ribonucleotide reductase by protein-protein interaction with p53R2 as well as hRRM2 subunits. *Cancer Res.* 2003; 63:980–6. [PubMed: 12615712]
10. Zhou B, Liu X, Mo X, Xue L, Darwish D, Qiu W, et al. The human ribonucleotide reductase subunit hRRM2 complements p53R2 in response to UV-induced DNA repair in cells with mutant p53. *Cancer Res.* 2003; 63:6583–94. [PubMed: 14583450]
11. Kuo ML, Kinsella T. Expression of ribonucleotide reductase after ionizing radiation in human cervical carcinoma cells. *Cancer Res.* 1998; 58:2245–52. [PubMed: 9605773]
12. Kunos C, Chiu S, Pink J, Kinsella T. Modulating radiation resistance by inhibiting ribonucleotide reductase in cancers with virally or mutationally silenced p53 protein. *Radiat Res.* 2009; 172:666–76. [PubMed: 19929413]
13. Csapo Z, Keszler G, Safrany G, Spasokoukotskaja T, Talianidis I, Staub M, et al. Activation of deoxycytidine kinase by gamma-irradiation and inactivation by hyperosmotic shock in human lymphocytes. *Biochem Pharmacol.* 2003; 65:2031–9. [PubMed: 12787883]
14. Boothman D, Davis T, Sahijdak W. Enhanced expression of thymidine kinase in human cells following ionizing radiation. *Int J Radiat Oncol Biol Phys.* 1994; 30:391–8. [PubMed: 7928466]
15. Eriksson S, Munch-Petersen B, Johansson K, Eklund H. Structure and function of cellular deoxyribonucleoside kinases. *Cell Mol Life Sci.* 2002; 59:1327–46. [PubMed: 12363036]
16. Fujiwaki R, Hata K, Moriyama M, Iwanari O, Katabuchi H, Okamura H, et al. Clinical value of thymidine kinase in patients with cervical carcinoma. *Oncology.* 2001; 61:47–54. [PubMed: 11474248]
17. Belt J, Marina N, Phelps C, Crawford C. Nucleoside transport in normal and neoplastic cells. *Adv Enzyme Regul.* 1993; 33:235–52. [PubMed: 8356910]
18. Crook T, Wrede D, Vousden K. p53 point mutation in HPV negative human cervical carcinoma cell lines. *Oncogene.* 1991; 6:873–5. [PubMed: 1646990]
19. Patillo R, Husa R, Story M, Ruckert A, Shalaby M, Mattingly R. Tumor antigen and human chorionic gonadotropin in CaSki cells: a new epidermoid cervical cancer cell line. *Science.* 1977; 196:1456–8. [PubMed: 867042]
20. Kunos C, Radivoyevitch T, Pink J, Chiu S, Stefan T, Jaccobberger J, et al. Ribonucleotide reductase inhibition enhances chemoradiosensitivity of human cervical cancers. *Radiat Res.* 2010; 174:574–81. [PubMed: 20954859]

21. Kunos C, Waggoner S, Von Gruenigen V, Eldermire E, Pink J, Dowlati A, et al. Phase I trial of intravenous 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP, NSC #663249) in combination with pelvic radiation therapy and weekly cisplatin chemotherapy for locally advanced cervical cancer. *Clin Cancer Res.* 2010; 16:1298–306. [PubMed: 20145183]
22. Radivoyevitch T, Taverna P, Schupp J, Kinsella TJ. The linear-quadratic log-survival radiation dose response model: Confidence ellipses, drug-drug interactions, and brachytherapeutic gains. *Med Hypotheses Res.* 2004; 1:23–8.
23. Lindstrom MJ, Kunugi KA, Kinsella TJ. Global comparison of radiation and chemotherapy dose–response curves with a test for interaction. *Radiat Res.* 1993; 135:269–77. [PubMed: 7690150]
24. Molina-Arcas M, Casado F, Pastor-Anglada M. Nucleoside transporter proteins. *Curr Vasc Pharmacol.* 2009; 7:426–34. [PubMed: 19485885]
25. Ritzel M, Ng A, Yao S, Graham K, Lowewen S, Smith K, et al. Recent molecular advances in studies of the concentrative NAz-dependent nucleoside transporter (CNT) family: identification and characterization of novel human and mouse proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (system cib). *Mol Membr Biol.* 2001; 18:65–72. [PubMed: 11396613]
26. Sternberg C, Whelan P, Hetherington J, Paluchowska B, Slee P, Vekemans K, et al. Phase III trial of satraplatin, an oral platinum plus prednisone vs. prednisone alone in patients with hormone-refractory prostate cancer. *Oncology.* 2005; 68:2–9. [PubMed: 15741753]
27. Pontarin G, Fijolek A, Pizzo P, Ferraro P, Rampazzo C, Pozzan T, et al. Ribonucleotide reduction is a cytosolic process in mammalian cells independently of DNA damage. *Proc Natl Acad Sci U S A.* 2008; 105:17801–6. [PubMed: 18997010]
28. Niida H, Katsuno Y, Sengoku M, Shimada M, Yukawa M, Ikura M, et al. Essential role of Tip60-dependent recruitment of ribonucleotide reductase at DNA damage sites in DNA repair during G1 phase. *Genes Dev.* 2010; 24:333–8. [PubMed: 20159953]

FIG. 1.

Schematic representation of ribonucleotide reduction (panel A) and deoxynucleoside salvage (panel B) pathways. Nucleoside diphosphate (NDP), ribonucleotide reductase M1, M2, and p53R2 subunits, deoxynucleoside monophosphate (dNMP), deoxynucleoside diphosphate (dNDP), deoxynucleoside triphosphate (dNTP), deoxynucleoside (dN), thymidine kinase 1 (TK1) and 2 (TK2), deoxycytidine kinase (dCK), deoxyguanine kinase (dGK), cytosolic deoxynucleotidase (cdN), mitochondrial deoxynucleotidase (mdN), deoxynucleotide carrier (DNC), equilibrative nucleoside transporter (ENT), thymidine phosphorylase (TP), purine nucleoside phosphorylase (PNP), and mitochondrial DNA (mtDNA).

FIG. 2.

Clonogenic survival (panel A) and [dCTP] (panel B) of CaSki and C33-a cervical cancer cells under conditions of standard medium (10% FBS) or dialyzed medium with deoxynucleosides (dN) “added back” (40-nm filtered 10% FBS with each [dN] = 0.05 μM). These data indicate that filtration followed by dN supplementation does not substantially impact survival or dCTP concentrations.

FIG. 3.

Clonogenic survival after irradiation (panel A) or irradiation and 5 μ M 3-AP (panel B) in CaSki and C33-a cervical cancer cells. As seen in panel A, deoxynucleoside-free medium (denoted FBS [0.0]) or medium with excess dN (FBS [5.0]) enhanced cytotoxicity of irradiated cells relative to standard medium (FBS [0.05]). Tenfold deoxynucleoside supplementation (FBS [0.5]) reduced cytotoxicity in irradiated cells compared to standard medium. The effects of dN concentration seen in panel A persisted when 3-AP was used (panel B).

FIG. 4.

dCTP concentration relative to dN concentration after 0 or 6 Gy irradiation combined with 0 or 5 μM 3-AP in CaSki (panel A) or C33-a (panel B) cells. Radiation-mediated DNA damage increased the dCTP concentration through elevation of ribonucleotide reductase activity. Supplementing the medium with deoxynucleosides, particularly deoxyadenosine, which becomes dATP, suppressed this response through allosteric inhibition of ribonucleotide reductase. When ribonucleotide reductase is blocked by 3-AP, an increase in deoxynucleoside salvage occurs. This is most pronounced when radiation-mediated DNA damage occurs.

FIG. 5.

γ -H2AX induction and resolution time course after 6 Gy irradiation without (●) and (○) with 5 μ M 3-AP at various dN concentrations in the medium in CaSki (panels A, C, E, G) and C33-a (panels B, D, F, H) cells. The peak radiation-induced γ -H2AX signal was similar for all conditions, consistent with the peak being determined by the radiation dose. Treatment with 3-AP delayed significantly γ -H2AX resolution, suggesting impaired supply of dNTPs by ribonucleotide reductase to fix DNA damage. Withdrawal of deoxynucleosides (dN) slightly slowed the repair of γ -H2AX foci (compare panels A and C, B and D). Supplementation of dNs beyond concentrations of 0.50 μ M suggests dNTP saturation of DNA polymerase λ and μ that are used in double-strand break repair (panels E–H). Photographs of γ -H2AX staining are shown for CaSki cells for each treatment condition at 6 h. Manual γ -H2AX focus counts are tabulated for untreated (a), irradiated (b), and irradiated plus 3-AP-treated (c) CaSki cells. Corresponding single cell electrophoresis (comet) assay tail moments are shown. Error bars are shown.

TABLE 1

Proportion of Cells with Caspase 3 Activation 6 h after Treatment

Treatment	dN concentration ^a			
	0	0.05	0.50	5.00
None	5.9 (2.9)	6.8 (3.5)	8.9 (3.1)	8.6 (4.4)
Radiation (6 Gy)	7.6 (4.8)	6.7 (4.3)	9.6 (4.2)	8.8 (3.4)
Radiation (6 Gy) + 3-AP (5 μ M)	10.5 (5.3)	11.0 (2.0)	10.3 (2.2)	11.6 (1.2)

^aMean (standard deviation).