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Biology Contribution

Identification of ATIC as a Novel Target for Chemoradiosensitization

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Summary

Detection of a frameshift mutation in the gene encoding the purine biosynthetic enzyme ATIC in a panel of radiosensitive individuals prompted studies to elucidate the role of ATIC in DNA damage responses and ascertain whether inhibition of ATIC might be an effective chemoradiosensitization strategy. Knockdown of ATIC or inhibition of its enzymatic activity compromised DNA double-strand break repair and reduced cell survival after irradiation.

Purpose: Mutations in the gene encoding 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (ATIC), a bifunctional enzyme that catalyzes the final 2 steps of the purine de novo biosynthetic pathway, were identified in a subject referred for radiation sensitivity testing. Functional studies were performed to determine whether ATIC inhibition was radiosensitizing and, if so, to elucidate the mechanism of this effect and determine whether small molecule inhibitors of ATIC could act as effective radiosensitizing agents.

Methods and Materials: Both small interfering RNA knockdown and small molecule inhibitors were used to inactivate ATIC in cell culture. Clonogenic survival assays, the neutral comet assay, and γ H2AX staining were used to assess the effects of ATIC inhibition or depletion on cellular DNA damage responses.

Results: Depletion of ATIC or inhibition of its transformylase activity significantly reduced the surviving fraction of cells in clonogenic survival assays in multiple cancer cell lines. In the absence of ionizing radiation exposure, ATIC knockdown or chemical inhibition activated cell cycle checkpoints, shifting cells to the more radiosensitive G2/ M phase of the cell cycle, and depleted cellular adenosine triphosphate but did not result in detectable DNA damage. Cells in which ATIC was knocked down or inhibited and then treated with ionizing radiation displayed increased numbers of DNA double-strand breaks and a delay in the repair of those breaks relative to irradiated, but otherwise untreated, controls. Supplementation of culture media with exogenous adenosine triphosphate ameliorated the DNA repair phenotypes.

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Conclusions: These findings implicate ATIC as an effective, and previously unrecognized, target for chemoradiosensitization and, more broadly, suggest that purine levels in cells might have an underappreciated role in modulating the efficiency of DNA damage responses that could be exploited in radiosensitizing strategies. © 2017 Elsevier Inc. All rights reserved.

Introduction

In humans, hypersensitivity to ionizing radiation frequently co-occurs with a constellation of clinical and laboratory features that include an increased cancer incidence, immunodeficiency, neurologic abnormalities, and DNA breakage. This constellation has been termed the XCIND syndrome (x-ray sensitivity, cancer predisposition, immunodeficiency, neurologic involvement, and DNA double-strand break [DSB] repair deficiency) (1, 2). Several well-defined human autosomal recessive disorders with these clinical features arise from mutations in key DNA damage response molecules (3). Although these inherited disorders are individually rare, identification of the causative genes has provided broadly applicable insights into the mechanisms and components of cellular DNA damage responses. These include the identification of the ataxiatelangiectasia mutated (ATM) gene, the primary regulator of cellular responses to DNA DSBs (4, 5) that is mutated in ataxia-telangiectasia (A-T); Nibrin (NBN), a regulator of the S-phase checkpoint, which is mutated in Nijmegen breakage syndrome (6-8); and the delineation of a novel role for ubiquitination in regulating the cellular response to ionizing radiation through the study of ring finger protein 168 (RNF168) deficiency (9, 10). However, not all cases of apparently genetic hypersensitivity to ionizing radiation can be accounted for by these established disorders (11-14). The identification of mutations in subjects with unexplained radiation hypersensitivity could point to genes or cellular pathways not previously implicated in DNA damage responses, providing novel mechanistic insights and potential drug targets for chemoradiosensitization.

We previously described a panel of subjects referred for diagnostic testing for A-T or NBS (13). The subjects shared the clinical features characteristic of these disorders, and, where available, the cell lines established from them display radiation hypersensitivity in clonogenic survival assays. However, sequencing of the ATM and NBN genes in these subjects failed to reveal credible causative mutations. Applying exome sequencing to this panel, we previously identified 1 subject with a homozygous missense mutation in the gene mitochondrial poly(A) polymerase (MTPAP), which had not been previously implicated in DNA damage responses. We demonstrated that this mutation was responsible for the radiation hypersensitivity by complementation (15). We characterized the product of a second gene mutated in 1 subject in this panel, ATIC, which encodes the bifunctional purine biosynthetic enzyme 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase. We found that depletion of *ATIC* by small interfering RNA (siRNA) treatment or chemical inhibition of its enzymatic activity impairs the ability of cells to repair DNA DSBs, reduces cell survival after irradiation, and perturbs intracellular adenosine triphosphate (ATP) pools.

Methods and Materials

Cell lines and reagents

GM00637, an SV40-transformed human fibroblast line obtained from the National Institute of General Medical Sciences repository, was maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin-glutamine. HCT116, a human colorectal carcinoma line, was provided by Dr Robert Hromas. SW48, a colorectal adenocarcinoma line, and U2OS, an osteosarcoma line, were purchased from American Type Culture Collection (Manassas, VA). The HCT116, SW48, and U2OS cell lines were maintained in McCoy's 5A medium supplemented with 10% FBS and 1% penicillin-streptomycin-glutamine at 37°C with 5% carbon dioxide, 95% humidity, and a pH of 7.4. The cells were harvested under log growth phase conditions, and irradiation was performed using a GammaCell 40 Exactor Cs-137 sealed source irradiator at a dose rate of ~ 1 Gy/min.

The chemical inhibitor of ATIC homodimerization, Cpd14 (16-18), was purchased from EMD Millipore (Billerica, MA), and the ATIC active site inhibitors NSC30171 and NSC326203 were obtained from the National Cancer Institute's Developmental Therapeutics repository.

RNA interference knockdown and cell viability assay

siRNA sequences (Table E1; available online at www .redjournal.org) directed against 3 different sites in each of 4 genes, *ATIC*, *ATM*, *NBN*, and *MTPAP*, were printed onto a 96-well plate (Qiagen, Valencia, CA). The negative control siRNA, a nonsilencing RNA with no homology to any known mammalian gene, was also purchased from Qiagen (catalogue no. 1027310). Cationic lipid-based transfection reagents were diluted in Opti-MEM (Invitrogen, Carlsbad, CA) and added to the assay plates, and 6000 GM00637 cells were added per well. At 48 hours after transfection, the cells were irradiated with 0 or 4 Gy of γ -radiation. After an additional 72 hours, cell viability was measured using an MTT assay (Promega, Madison, WI) in accordance with the supplier's instructions. Each knockdown was performed in triplicate.

Quantitative polymerase chain reaction

Total cellular RNA was extracted from GM00637 cells 48 hours after siRNA transfection. Complementary DNA (cDNA) was prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). All primer pairs used were prefabricated mixtures of forward and reverse primers (Quantitect Primer Assay; Qiagen). Relative quantification was performed with SYBR Green (Invitrogen) using glyceraldehyde 3-phosphate dehydrogenase as an internal control for normalization. Target and reference samples were tested in triplicate for each experiment. PCR efficiency was determined by constructing a standard curve with cDNA containing the target template.

Western blot analysis

The cells were seeded at 3×10^5 cells per well in 6-well plates, and duplicate wells were treated with siRNA at the indicated concentration for 48 hours. Cell lysates were prepared and total protein concentration determined using the BCA protein assay (ThermoFisher, Waltham, MA). Next, 20-µg aliquots of each sample were separated on 5% to 12% sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose blotting membrane (Bio-Rad Laboratories, Hercules, CA) and immunoblotted with antibodies directed against ATM, ATIC (Abnova, Taipei City, Taiwan), NBN (Cell Signaling, Danvers, MA), MTPAP (EMD Millipore), p53 (Santa Cruz Biotechnology, Dallas, TX), p21 (Cip1/WAF1), cyclin B, tubulin, adenosine monophosphate (AMP) kinase (AMPK), or phosphorylated T172 AMPK (Cell Signaling), all at a dilution of 1:1000, and incubated at 4°C overnight. The membranes were washed and incubated with horseradish peroxidaseconjugated secondary antibodies for 1 hour at room temperature, washed and developed using enhanced chemiluminescence protocols (GE Health Care Life Science, Piscataway Township, NJ), and quantified using ImageQuant software (GE Health Care Life Science).

Colony survival assay

HCT116, SW48, and U2OS cells were treated separately with 1 of 3 different *ATIC* siRNAs (siATICa, siATICb, or siATICc; Table E1; available online at www.redjournal.org) or chemical inhibitors at the indicated concentrations for 48 hours. Cells, 250 to 2000/well in a 6-well plate, were plated in triplicate with McCoy's 5A medium and sham treated or irradiated. After 3 weeks, the colonies were fixed with 10% formaldehyde in phosphate-buffered saline, stained with 0.1% crystal violet in phosphate-buffered saline, and counted. The results were compared with those from the sham-treated plates to determine the survival fraction percentage, and survival curves were generated using SigmaPlot software (Systat Software Inc, Chicago, IL). Radiation dose-modifying factors were calculated at 10% clonogenic survival from linear-quadratic curve equations used to plot the curves shown in the figures. The dose-modifying factors reported are the root values for control curves divided by the values for treated curves.

Cell cycle analysis

HCT116, SW48, and U2OS cells were treated with siA-TICa or Cpd14 at the indicated concentration for 24 or 48 hours, collected and fixed in 70% ethanol at -20° C, stained with propidium iodide/RNAase buffer for DNA content, and analyzed using an LSR II flow cytometer (BD Biosciences, San Jose, CA). ModFit software (Verity Software House, Topsham, ME) was used to assess the cell cycle distribution.

Measurement of intracellular ATP

HCT116 colon cancer cells were treated with siATICa or Cpd14 at the indicated concentration. At 12, 24, 36, and 48 hours, the ATP levels were measured using the luciferase/ luciferin-based ENLITEN ATP assay system (Promega, Madison, WI). All samples were tested in triplicate.

Irradiation-induced focus assay

HCT116 colon cancer cells were treated with siATICa or Cpd14 for 48 hours, followed by radiation exposure at 2 Gy. The cells were collected at 1, 2, 6, 24, and 48 hours, plated on coverslips, fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, blocked in 10% FBS, and incubated with anti-y-H2AX (1:300; Santa Cruz Biotechnology) for 1 hour at room temperature. The coverslips were washed, blocked with 10% FBS, and incubated with an Alexa Fluor-488 secondary antibody (1:400; Invitrogen) for 45 minutes at room temperature. The coverslips were washed a final time and mounted on slides using Prolong Gold antifade reagent containing DAPI (Applied Biosystems). The foci were imaged using a Zeiss fluorescent microscope equipped with an AxioVision camera and software. The cells were scored as positive if they contained \geq 4 foci/nuclei. To measure the initial induction of DSB after radiation, the cells were collected at 1 hour after radiation exposure, and the foci/nuclei were scored. To measure DSB repair, the cells were collected at 1, 2, 6, 24, and 48 hours after radiation exposure and scored for the reduction of foci relative to the 1-hour measurement point.

Neutral comet assay

HCT116 cells were treated with siATICa or Cpd14 for 48 hours. The cells were then sham treated or irradiated with 2 Gy, collected at 1 hour after radiation, resuspended in 1% low melting point agarose, and plated on 2-well comet assay slides. Once the agarose had solidified, the slides were immersed in cell lysis solution (Trevigen Inc, Gaithersburg, MD) overnight at 4°C. The slides were electrophoresed and stained using SYBR Green (Invitrogen). Comets were visualized using a Zeiss fluorescent microscope equipped with an AxioVision camera and software. The tail moments (TMs) of the comets were scored using CometScore software (TriTek, Sumerduck, VA). The percentage of repair was determined by monitoring the return to the baseline TM levels. The cells were sham treated or irradiated with 2 Gy, collected at 1 and 12 hours after radiation, resuspended in 1% low melting point agarose, and assayed as described in the previous paragraphs. The initial induction of DSBs was measured 1 hour after 2 Gy of γ -ray exposure. The residual DSBs after repair were measured 12 hours after exposure. The TMs were normalized to those of the unirradiated cells. The percentage of repair was calculated as the difference in the TM between the 1- and 12-hour points relative to the 1-hour point (TM_{1hr} - TM_{12hr})/TM_{1hr}.



Fig. 1. Small interfering RNA (siRNA) knockdown of ATIC is radiosensitizing. (A) GM00637 cells were transfected with pooled siRNAs targeting *ATM* (siATM), *NBN* (siNBS1), *MTPAP* (siMTPAP), or *ATIC* (siATIC). Plot showing the mean percentage \pm standard error of the mean of viable cells in the irradiated (4 Gy) samples relative to the mock-irradiated samples (**P*<.05; ***P*<.01, compared with the negative control siRNA [siControl]). (B) Plot showing the fraction of transcripts in cells treated with gene-specific siRNAs compared with siControl. (C) Cells treated with either siControl or the pools of siRNAs directed against the indicated genes were immunoblotted for the products of the targeted genes. (D) Protein levels from (C) were quantified and expressed as percentages relative to siControl.



Fig. 2. Effect of ATIC inhibition or depletion on radiation survival. (A) HCT116, (B) SW48, and (C) U2OS cells were treated with the indicated concentrations of small interfering (si)ATICa or the ATIC inhibitor, Cpd14, for 48 hours before irradiation at 0, 1, 2, and 4 Gy. Plot showing the surviving fraction relative to 0 Gy of treatment. *Abbreviation:* DMSO = dimethyl sulfoxide.



Statistical analysis

All data are presented as the mean \pm standard error of the mean. Statistical significance (*P*<.05) was determined using the *t* test with GraphPad Prism, version 5.0, software.

Results

ATIC is required for normal cell survival after irradiation

Exome sequencing of 1 subject referred for testing for A-T or NBS revealed heterozygosity for a complex frameshift mutation, c.130_131insG;131_132insA, and a missense substitution of unknown consequence, c.347C>G, p.Thr116Ser, in the *ATIC* gene. Although no clinical information was available for this subject, we noted a previous report that had described a single patient with severe neurologic and developmental features who was heterozygous for the same frameshift mutation in the *ATIC* gene but had a different and potentially more damaging missense substitution affecting a critical residue on the ATIC transformylase domain (19).

Given the shared genetic features in these 2 subjects, we tested whether reducing the cellular level of ATIC might affect the DNA damage responses. A cell line with normal radiation sensitivity, GM00637, was treated with a pool of 3 siRNAs targeting ATIC and irradiated at 4 Gy. The viable cells were counted after 72 hours (Fig. 1A). For comparison purposes, GM00637 cells were treated in parallel with similarly designed pools of siRNAs that target genes with established roles in cellular responses to ionizing radiation, ATM, NBN, and MTPAP. The effectiveness of the siRNA knockdown of the genes was confirmed for messenger RNA and protein using quantitative PCR and immunoblotting, respectively (Fig. 1B-D). Knockdown of ATIC significantly reduced the cell counts relative to the mock-treated controls. Despite comparable levels of knockdown (Fig. 1D), siRNA depletion of ATIC had a less significant effect on cell viability than for the well-established DNA damage response genes ATM and NBN (Fig. 1A).

A small molecule inhibitor, Cpd14, that interferes with the homodimerization of ATIC has been identified and characterized. Dimerization is essential to form the active site for ATIC's transformylase (16-18). Treatment of HCT116, SW48, or U2OS cells with siRNAs directed against *ATIC* or with Cpd14 48 hours before irradiation resulted in a dosage-dependent reduction in cell survival relative to the untreated, but irradiated, controls in a clonogenic survival assay (Fig. 2 and Fig. E1; available online at www.redjournal.org). The efficiency of the knockdown was confirmed by immunoblotting (Fig. E2; available online at www.redjournal.org). The radiation dose-modifying factors for siATICa treatment of HCT116 cells at 10 nM and 30 nM were 1.17 and 1.30 and for Cpd14 at 800 μ M and 1000 μ M were 1.2 and 1.4, respectively. The radiation dose-modifying factors for siATICa treatment of SW48 cells at 10 nM and 30 nM were 1.22 and 1.48 and for Cpd14 at 800 μ M and 1000 μ M were 1.17 and 1.47, respectively. The radiation dose-modifying factors for siATICa treatment of U2OS cells at 10 nM and 30 nM were 1.40 and 1.61 and for Cpd14 at 800 μ M and 1000 μ M were 1.28 and 1.52, respectively.

In addition to the ATIC dimerization inhibitor Cpd14 (17), a virtual ligand screen identified a number of active site inhibitors that are specific for the transformylase activity of ATIC (20, 21). We tested 2 of the compounds (NSC30171 and NSC326203) identified in this screen for their effects on cell survival in irradiated HCT116 cells. Both compounds provided comparable radiosensitization to that obtained with Cpd14 but at 10-fold and 2-fold lower concentrations, respectively (Fig. E3; available online at www.redjournal.org).

ATIC deficiency or inhibition activates cell cycle checkpoints

Treatment of HCT116 cells with either siATICa or Cpd14 resulted in modest cell cycle redistribution in asynchronous cultures with decreased numbers of cells in S phase and increased numbers of cells in the more radiosensitive G2/M (Fig. 3A). Relative to the mock-treated controls, the treated cells displayed increased levels of p53 and the cyclin-dependent kinase inhibitor p21 and decreased levels of cyclin B1, consistent with activation of the G1/S and G2/M checkpoints, respectively, and the observed cell cycle distribution (Figs. 3B and 3C). Comparable results were observed in 2 SW48 and U2OS cells (Figs. E4 and E5; available online at www.redjournal.org).

Increased radiation induced DNA DSBs in cells deficient in ATIC

The effect of ATIC depletion or inhibition on the repair of DNA DSBs was assessed in HCT116 cells by γ H2AX focus formation and the neutral comet assay. Perturbation of ATIC alone did not result in any significant increase in

Fig. 3. ATIC inhibition or depletion activates cell cycle checkpoints. (A) DNA content was assessed by flow cytometry in untreated cells and cells treated with small interfering (si)ATICa or Cpd14. Cell cycle distributions are indicated as follows: gray for G1 and G2/M and cross-hatched for S phase. HCT116 cells treated with either (B) siATICa or (C) Cpd14 at indicated doses were immunoblotted for checkpoint proteins. β -Actin served as a loading control. (D) Relative protein levels of Western blots in (B) and (C) were quantified using ImageQuant (**P*<.05, compared with controls). *Abbreviation:* DMSO = dimethyl sulfoxide.



Fig. 4. ATIC inhibition or depletion increases the incidence of DNA double-strand breaks in HCT116 colon cancer cells after irradiation. (A) γ H2AX staining at 0 and 1 hour after irradiation (2 Gy). Cells were sham treated or treated with small interfering (si)ATICa or Cpd14 at the indicated concentration for 48 hours before radiation exposure (IR). (B) Percentage of γ H2AX-positive cells in treated and control groups (**P*<.05). (C) Neutral comet assay performed at 0 and 1 hour after irradiation (2 Gy). Cells were sham treated or treated with siATIC or Cpd14 at the indicated concentration for 48 hours before radiation for 48 hours before radiation (2 Gy). Cells were sham treated or treated with siATIC or Cpd14 at the indicated concentration for 48 hours before radiation exposure. (D) Quantitation of tail moments in treated and control groups (**P*<.05).

 γ H2AX foci (Fig. 4A; 0-hour point). However, when combined with ionizing radiation, increased numbers of γ H2AX foci per cell were detected (Fig. 4A) and a significant increase occurred in the percentage of cells containing γ H2AX foci (Fig. 4B). Similar results were obtained with comparably treated cells in the neutral comet assay. The TMs at 1 hour after irradiation were increased relative to those of the mock-treated irradiated controls (Figs. 4C and 4D). Thus, the results from the γ H2AX focus assay and neutral comet assay indicated that depletion of ATIC or inhibition of its transformylase activity results in increased numbers of DNA DSBs specifically in irradiated cells.

Effects of ATIC inhibition ATP levels and DNA damage responses

Treatment of cells with Cpd14 has been shown to result in accumulation of 5-aminoimidazole-4-carboxamide ribotide (ZMP), the substrate for the ATIC transformylase (16), which, because of its structural similarity to AMP, can

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Fig. 5. ATIC inhibition or depletion reduces intracellular adenosine triphosphate (ATP) levels and activates adenosine monophosphate kinase (AMPK) ($^*P < .05$, $^{**}P < .01$, compared with control). (A) HCT116 cells were treated with small interfering (si)ATICa (30 nM) or Cpd14 (1000 μ M), as indicated. At 12, 24, 36, and 48 hours, ATP levels were measured. (B) HCT116 cells were treated with siATIC or a chemical ATIC inhibitor at the concentration indicated. After 48 hours of treatment, total cell lysates were analyzed by Western blot for ATIC, total AMPK, and phosphorylated AMPK expression levels.

activate AMPK (22). Marie et al (19) reported increased levels of ZMP, ZDP, and ZTP in red blood cells from their patient with biallelic ATIC mutations but also a significant decline in the ATP/AMP ratio. They attributed that to the phosphorylation of ZMP by adenosine kinase, which occurs in Lesch-Nyhan syndrome (23, 24). We observed that treatment of HCT116 cells with either siATICa or Cpd14 also resulted in a significant and sustained reduction in intracellular ATP levels (Fig. 5A) and confirmed that this perturbation of ATP levels was associated with activation of AMPK by phosphorylation on T172 of its catalytic α -subunit (Fig. 5B).

To explore the relationship between radiation sensitivity and alterations in cellular ZMP and ATP levels, the effect of ATP supplementation on the induction and resolution of DNA DSBs by ionizing radiation in cells in which ATIC was depleted or inhibited was assessed. The specific phenotype of DNA DSB repair was assayed, because ATP supplementation might be expected to have general effects on broader phenotypes, such as cell viability, unrelated to the inhibition or depletion of ATIC. The percentage of γ H2AX-positive cells in cultures treated with siATICa (Fig. 6A) or Cpd14 (Fig. 6B) was increased, relative to the untreated controls, at 2 hours after irradiation and remained elevated throughout the 48 hours of the assay. The addition of exogenous ATP to the culture media 1 hour before irradiation did not prevent the initial increase in γH2AX foci in cells treated with siATICa or Cpd14 but did reduce the percentage of positive cells at later time points. In the neutral comet assay, ATP supplementation significantly decreased DNA fragmentation in cells treated with siATICa or Cpd14 at later time points relative to that observed at 1 hour after irradiation (Fig. 6C). This effect was observed across a range of supplementary ATP concentrations from 1 to 4.5 mM using siATICa (Fig. E6; available online at www.redjournal.org). Supplementation with GTP, CTP, or TTP at 2.5 mM did not ameliorate the effect of siATICa on DNA repair in the neutral comet assay (Fig. E7; available online at www.redjournal.org).

Discussion

In the present study, we explored the effect of depleting ATIC or inhibiting its transformylase activity on cellular radiation survival and DSB repair, motivated by the detection of a loss of function mutation in the *ATIC* gene during exome sequencing of a panel of radiosensitive





Fig. 6. Adenosine triphosphate (ATP) supplementation reverses the effects of ATIC inhibition or depletion ($^*P < .05$, $^{**}P < .01$, compared with control). (A) HCT116 cells were left untreated (small interfering [si]Control) or pretreated for 48 hours with 30 nM siATICa and either not supplemented (siATICa) or supplemented with 2.5 mM ATP (siATICa+ATP) 1 hour before irradiation (2 Gy). Double-strand break repair was measured using the γ H2AX staining assay as described in the Methods and Materials section. (B) HCT116 cells were left untreated (dimethyl sulfoxide [DMSO]) or pretreated for 48 hours with 1000 μ M Cpd14 and either not supplemented (Cpd14) or supplemented (Cpd14+ATP) with 2.5 mM ATP 1 hour before irradiation (2 Gy). Double-strand break repairs were measured using the γ H2AX staining assay, as described in the Methods and Materials section. (C) Cells were treated as in (A) and (B), and DSB repair was measured via the neutral comet assay as described in the Methods and Materials section. (C) Cells were treated as in (A) and (B), and DSB repair was determined by monitoring the return of the tail moments (TM) of comets to baseline levels [(TM_{1hr} – TM_{12hr})/TM_{1hr}] ($^*P < .05$).

individuals. A bifunctional enzyme, ATIC possesses separate aminoimidazole carboxamide ribonucleotide transformylase and inosine monophosphate cyclohydrolase activities, which catalyze the penultimate and final steps in de novo purine synthesis, respectively (23, 24). Vertebrates rely primarily on the purine salvage pathway, rather than this synthesis pathway, to provide purines for nucleotide and nucleic acid synthesis. Consistent with this, we observed only a modest effect of ATIC inhibition in human cells on cell cycle progression. When the purine salvage pathway is disrupted, such as in the genetic disorder Lesch-Nyhan syndrome (23, 24), the transformylase activity of ATIC becomes rate-limiting for purine synthesis and its substrate, ZMP, accumulates. This effect can be mimicked by treatment with the cell-permeable precursor 5-aminoimidazole-4-carboxamide riboside, which is converted

to ZMP in cells by phosphorylation. Antimetabolites that target folate metabolism, such as methotrexate, can also affect ATIC's transformylase activity by depleting 10formyltetrahydrofolate, the 1-carbon donor necessary for the formylation of ZMP. The ATIC c.347C>G variant detected in the subject we studied has been reported to be associated with adverse responses to methotrexate, suggesting a possible deleterious effect on ATIC (25). Both 5aminoimidazole-4-carboxamide riboside and methotrexate have been reported to radiosensitize, although their mechanisms are unresolved (26-28). We have demonstrated that ATIC knockdown, or treatment with any of 3 different small molecule inhibitors of ATIC's transformylase activity, acting by 2 different mechanisms, dimerization inhibition or active site competition, results in radiosensitization. We have further demonstrated that this radiosensitization

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arises, in part, because of deficient DNA DSB repair in cells in which ATIC's transformylase activity is inhibited. Considered together, these previous reports and the present findings strongly suggest that the transformylase activity of ATIC plays an important role in radioprotection.

The treatment of cells in culture with the ATIC dimerization inhibitor Cpd14 has been shown to increase ZMP levels (16). Also, the red blood cells from a previously reported patient with biallelic deleterious mutations in ATIC displayed increased ZMP levels and a decline in the ratio of ATP to AMP (19). AMPK is activated by either of these conditions, but also by DNA damaging agents, including ionizing radiation (29). We observed that ATIC inhibition activated AMPK, resulting in phosphorylation on the same residue, T172, of the regulatory α -subunit, which is also phosphorylated in response to irradiation. This raises the possibility that AMPK could serve as an integrator of independent stress signals arising from combined treatment of cells with ionizing radiation and an ATIC inhibitor. A role for AMPK as an integrator of cellular stress signals has been previously proposed (30). On activation, AMPK phosphorylates multiple substrates that act to balance the requirements of cell growth with the available energy supply through acute effects on metabolism, longer term transcriptional adaptations, and regulation of cell cycle progression. It is unclear whether AMPK acts directly on the components of DNA DSB repair pathways. However, we did observe that ATP supplementation could ameliorate the DNA repair defect caused by ATIC inhibition. Also, a long history of reports highlighting the ATP dependence of repair of ultraviolet or ionizing radiation damage to DNA exists (31-37). In addition, previous studies have shown that inhibition of AMPK activity can result in radioresistance (38, 39), and AMPK activators can augment cell killing by ionizing radiation (40-44).

Conclusions

Depletion of ATIC by siRNA knockdown impairs DNA DSB repair, reducing cellular radiation survival. Small molecule inhibitors specific for the transformylase activity of ATIC have comparable effects, suggesting that ATIC might be an effective target for chemoradiosensitization strategies.

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