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Torin2 Suppresses Ionizing Radiation-Induced DNA Damage Repair

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Abstract

Several classes of inhibitors of the mammalian target of rapamycin (mTOR) have been developed based on its central role in sensing growth factor and nutrient levels to regulate cellular metabolism. However, its ATP-binding site closely resembles other phosphatidylinositol 3-kinase-related kinase (PIKK) family members, resulting in reactivity with these targets that may also be therapeutically useful. The ATP-competitive mTOR inhibitor, Torin2, shows biochemical activity against the DNA repair-associated proteins ATM, ATR and DNA-PK, which raises the possibility that Torin2 and related compounds might radiosensitize cancerous tumors. In this study Torin2 was also found to enhance ionizing radiation-induced cell killing in conditions where ATM was dispensable, confirming the requirement for multiple PIKK targets. Moreover, Torin2 did not influence the initial appearance of γ -H2AX foci after irradiation but significantly delayed the disappearance of radiation-induced γ -H2AX foci, indicating a DNA repair defect. Torin2 increased the number of radiation-induced S-phase specific chromosome aberrations and reduced the frequency of radiation-induced CtIP and Rad51 foci formation, suggesting that Torin2 works by blocking homologous recombination (HR)-mediated DNA repair resulting in an S-phase specific DNA repair defect. Accordingly, Torin2 reduced HR-mediated repair of I-Sce1-induced DNA damage and contributed to replication fork stalling. We conclude that radiosensitization of tumor cells by Torin2 is associated with disrupting ATR- and ATM-dependent DNA damage responses.

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Our findings support the concept of developing combination cancer therapies that incorporate ionizing radiation therapy and Torin2 or compounds with similar properties.

INTRODUCTION

Tumor cells have dysfunctional DNA damage responses making them more susceptible than most normal tissues to DNA damaging agents and providing a rationale for radiation therapy and some classes of chemotherapeutic compounds (1–3). Several agents have been successfully combined with radiation therapy to enhance therapeutic efficacy by increasing the level of residual, unrepaired DNA double-strand breaks (DSBs) (4–8). Incorporation of targeted compounds that further suppress DNA repair, thus enhancing DNA damage in tumor cells, could provide a therapeutic advantage, especially in tumors where conventional therapy is less effective (7, 8).

Torin2 is a small molecule kinase inhibitor with activity against several members of the phosphatidylinositol 3-kinase-related kinases (PIKK) family, including Ataxia telangiectasia mutated protein (ATM), ATM and Rad3-related protein (ATR) and DNA-dependent protein kinase (DNA-PK) all of which have been explored as radiosensitization targets (9–12). As the name suggests, Torin2 was originally developed as a catalytic inhibitor of the mammalian target of rapamycin (mTOR), a member of the highly conserved PIKK family (9, 13) that regulates cellular proliferation, growth and metabolism in response to exogenous nutrients, growth factors and metabolic stress (14–17). In many cases catalytic mTOR inhibitors exhibit more potent antitumor activity than rapalogues, likely due to more complete inhibition of mTORC1 and concomitant inhibition of mTORC2 (13, 18). Interestingly, comprehensive kinase profiling revealed that Torin2 has other related targets belonging to the PIKK family members with DNA repair functions, including ATM, ATR and DNA-PK [50% growth inhibition (IC_{50}), 2–250 nM] (19). Based on this profiling data and other reports on BEZ235, a phosphoinositide 3-kinase (PI3K)/mTOR dual inhibitor also exhibiting activity against ATM, ATR and DNA-PK (20), we hypothesized that Torin2 would be synergistic with agents or biological processes that cause DNA damage, especially in cases where high levels of replicative stress and nononcogenic addiction to DNA repair factors are present (21, 22). We characterized the role of Torin2 as an effector of DNA damage responses in human tumor-derived cell lines and found that Torin2 enhances both radiation-induced cell killing and residual chromosome aberrations at metaphase. Torin2 slowed the kinetics of DNA repair, impaired homologous recombination (HR) processes and destabilized replication fork progression, thus increasing vulnerability to replication stress.

MATERIALS AND METHODS

Cell Culture, Compound Treatment and Irradiation

Cell lines used in this study include A549, H1299, 634T (ATM wild-type) and T2 (ATM null). All cell lines used were cultured in DMEM containing 5% fetal bovine serum and penicillin/streptomycin in a humidified 37°C incubator with 5% CO₂. Torin2 was dissolved in dimethyl sulfoxide (DMSO), and 10 mM stocks were aliquoted, then stored at –20°C. The working concentration of 100 μM was prepared immediately before use. Cells were

treated with compounds for 2 h before gamma irradiation from a ^{137}Cs source (JL Shepherd, San Fernando, CA) at the indicated doses (dose rate of 3.89 Gy/min).

Western Blot Analyses and Immunofluorescence Staining

For Western blot analysis of phospho-p70S6 kinase, p70S6 kinase, phospho-pS1981 ATM and total ATM, whole-cell extracts were prepared with RIPA buffer containing 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS. The Bradford method was used to estimate protein concentrations, and gels were run with 10–25 µg of protein. Immunofluorescence staining of cells was performed as previously described, with modifications (23). Antibodies were as follows: anti-phospho-γ-H2AX, anti-rad51 and anti-CtIP for primary antibodies; Rhodamine Red™-X and Alexa Fluor® 488/568 were used for secondary antibodies (Molecular Probes®, Grand Island, NY). The cells were fixed, permeabilized and blocked according to the recommended protocol from the supplier and incubated with primary antibodies for either 2 h or overnight at 4°C. Secondary antibodies were incubated for 1 h at room temperature for immunofluorescence. The slides were mounted with Vectashield® hardset mounting media containing DAPI. The images were acquired and recorded using the Zeiss Axio Imager at 63× magnification (Carl Zeiss MicroImaging Inc., Thornwood, NY).

Colony Formation Assays

Colony formation assays were performed as previously described, with some modifications (24). Cell plating efficiency was predetermined by plating 100–10,000 cells in 60 mm dishes in triplicate and irradiating at 0, 2, 4, 6 and 8 Gy doses. Cells were plated for different radiation doses in triplicate onto 60 mm dishes, allowed to attach for 4 h, treated with the Torin2 for 2 h, then irradiated with graded radiation doses. Surviving colonies were stained with crystal violet approximately 10–14 days later. The colonies were counted and survival fractions were calculated. Colonies were defined as clusters of >50 cells. Cell survival measurements were fitted by the linear-quadratic equation, $SF = \exp(-\alpha d - \beta d^2)$, SF is the surviving fraction and d is the dose in Gy. Calculations were performed with GraphPad Prism software (GraphPad Software Inc., LaJolla, CA). The radiation enhancement ratio (RER) was calculated with ratio of area under the curve (mean inhibition dose) for irradiation/area under the curve for irradiation + compound treatment.

Cell Cycle Analysis

The A549 cells were asynchronously cultured for 48 h, and changed into fresh media with or without Torin2 treatment for 2 h followed by irradiation. The cells were collected by trypsinization at the indicated times and fixed with ethanol overnight. Cells were then stained with propidium iodide. Fluorescence-activated cell sorting (FACS) cell cycle analysis was performed with a FACSCaliber™ instrument and the data were analyzed using FlowJo software (Ashland, OR).

Assay for Chromosome Aberrations at Metaphase

Stage-specific chromosome aberrations were analyzed at metaphase after irradiation, as previously described (25). G₁-phase chromosome aberrations were assessed in cells exposed

to 3 Gy then incubated for 12 h, after which the metaphase cells were collected. S-phase specific chromosome aberrations were assessed after exponentially growing cells (pulse labeled with BrdU) were 2 Gy irradiated. Metaphases were harvested 3 h after irradiation and S-phase type chromosome aberrations scored. For G₂-phase specific aberrations, cells were 1 Gy irradiated and those in metaphase were collected 1 h after treatment. Chromosome spreads were prepared after hypotonic treatment of cells, fixed in methanol acetic acid and stained with Giemsa. The categories of G₁-phase asymmetrical chromosome aberrations that were scored include dicentrics, centric rings, interstitial deletions/acentric rings and terminal deletions. S-phase chromosome aberrations were measured by counting both the chromosome and chromatid aberrations, including triradial and quadriradial exchanges per metaphase. G₂-phase chromosome aberrations were measured by counting chromatid breaks and gaps per metaphase, as previously described. Fifty metaphase sets were scored for each postirradiation time point.

DNA Fiber Assay

We performed DNA fiber spreads using previously described procedures (26) with the following minor modifications. Exponentially growing cells were pretreated with Torin2 then irradiated under the same conditions used for the global replication assay. Sites undergoing DNA replication were labeled with 5-iododeoxyuridine (IdU; 50 μ M) for 20 min before exposure to hydroxyurea (HU; 2 mM). The HU was removed by washing cells four times with PBS before labeling with media containing 5-chlorodeoxyuridine (CldU; 50 μ M) to mark the replication recovery sites. Cells labeled with IdU and CldU were mixed with unlabeled cells in a ratio of 1:10, and 2 μ l cell suspensions were dropped onto a glass slide and then mixed with a 20 μ l hypotonic lysis solution [10 mM Tris-HCl, pH 7.4, 2.5 mM MgCl₂, 1 mM phenyl-methylsulfonyl fluoride (PMSF) and 0.5% NonidetTM P-40] for 8 min. Air-dried slides were fixed, washed with 1 $\times$ PBS, blocked with 5% bovine serum albumin (BSA) for 15 min and incubated with primary antibodies against IdU and CldU [rat monoclonal antibody anti-IdU (1:150 dilution; Abcam[®], Cambridge, MA) and mouse anti-CldU (1:150 dilution; BD Biosciences, Franklin Lakes, NJ)] and secondary antibodies [anti-rat Alexa Fluor 488-conjugated (1:150 dilution) and anti-mouse Alexa Fluor 568-conjugated (1:200 dilution) antibodies] for 1 h each. Slides were washed with 1 $\times$ PBS with 0.1% Triton-X-100 and mounted with Vectashield mounting media without 4',6-diamidino-2-phenylindole (DAPI). DNA fibers were analyzed with ImageJ software (National Institutes of Health, Bethesda, MD).

Homologous Recombination and Non-Homologous End Joining Reporter Assays

The HR reporter-based assay was performed as described with minor modifications (27, 28). The assay utilizes a SceGFP construct consisting of GFP with the introduction of an I-SceI restriction site in-frame with the termination codons and is expressed under the control of a hCMV enhancer/promoter. The DR-GFP recombination substrate construct was kindly given by M. Jasin (Memorial Sloan-Kettering Cancer Center, New York, NY). For the non-homologous end joining (NHEJ) reporter assay, we obtained an IRES_TK_EGFP reporter gene plasmid, a gift from T. Kohno (National Cancer Research Institute, Tokyo, Japan), and the assay was performed as previously described, with minor modifications (29). For both assays the substrate constructs were stably transfected into H1299 cells, and an I-SceI

expression construct was transiently co-transfected into cells pretreated with DMSO, Torin2 and siRNAs. The GFP-positive cells were detected and percentage positivity was determined using FACS 48 h after transfection.

RESULTS

Torin2 Enhances Ionizing Radiation-Induced Cell Killing

In situ kinome profiling for Torin2 using chemical proteomics revealed that Torin2 binds to several PIKK family members involved in DNA repair processes, including mTOR, ATM, ATR and DNA-PK with IC₅₀ in the range of 2–250 nM (19). We therefore hypothesized that Torin2 would also inhibit the DNA repair function of these proteins, thereby enhancing radiation-induced cell death (Fig. 1). Given that radiosensitizers will likely be used clinically in the context of radioresistant tumor types, we chose to study radioresistant cell lines A549 and H1299 (30). After treatment with Torin2 for 2 h, cells were irradiated with 8 Gy, a dose commonly used clinically in hypofractionated treatments. Short-term cell viability was assessed after 96 h in the presence and absence of radiation. The concentration of Torin2 at IC₅₀ and confidence interval statistics were calculated using GraphPad Prism. We observed a two- to fourfold inhibitory shift in the IC₅₀ values in the radiation alone compared to radiation + Torin2 treatment groups (using nM for measuring) (Fig. 1A) indicating a statistically significant radiosensitizing effect. To confirm this, a clonogenic survival assay was performed. Cells were pretreated with a sublethal 10 nM dose of Torin2 (Fig. 1A) to limit compound-induced anti-proliferative activity while maintaining the anti-enzymatic activity of the inhibitor (Fig. 1C), and cells were then irradiated in the presence of Torin2. Torin2 significantly reduced the number of surviving colonies (Fig. 1B). The RERs were 1.41 and 1.44 for A549 and H1299, respectively, confirming that the drug enhances radiosensitization (31). Torin2 has an 800-fold greater selectivity for cellular mTOR than PI3K, and has potent biochemical and cellular activity against PIKK family members (19). Consistent with prior studies, we also observed that mTOR inhibitors with little activity against PI3K or other PIKK targets, such as Torin1 and INK128, are not as potent as Torin2 for inducing radiosensitivity (Fig. 1C and D), suggesting that the radiosensitizing effect of Torin2 in this setting is most likely dependent on PI3KK targets rather than PI3K.

Torin2 Enhances Radiation-Induced S- and G₂-Phase Specific, but not G₁-Specific Chromosome Aberrations

Chromosome aberrations, including chromosome breaks, can be observed soon after irradiation and are dependent on radiation dose (32, 33). To evaluate whether Torin2 pretreatment alters the levels of residual chromosomal DNA damage in cells after repair of radiation-induced DNA damage (25, 34) we examined chromosome aberration frequencies in A549 cells. Chromosome and chromatid-type aberrations were evaluated in mitotic cells after radiation doses were optimized to enable quantitation of residual aberrations, as previously reported (35). G₁-phase aberrations are usually chromosomal types (dicentric, with acentric fragments) with very few chromatid-type aberrations. S-phase aberrations are of both chromosomal and chromatid type, and G₂-phase aberrations are predominantly of chromatid type. To evaluate G₁-phase aberrations, cells were 3 Gy irradiated and aberrations analyzed 12 h later. For S-phase aberrations, cells were 2 Gy irradiated and aberrations

analyzed 6 h later. And for G₂-phase aberrations, cells were 1 Gy irradiated and aberrations analyzed at 1 h later. Aberrations were scored according to the number of asymmetric chromosomes including dicentrics, centric rings, interstitial and terminal deletions, and number of chromatid aberrations such as breaks and gaps. We did not observe any increase in G₁-phase chromosome aberrations, but a significant increase in S- and G₂-phase chromosome aberrations was observed (Fig. 2A – D). Cell cycle analysis (Fig. 2E) was performed, and no significant difference in the cell cycle distribution was detected among cells in the presence or absence of Torin2. Thus, we conclude that the increase in residual S/G₂-phase chromosome aberrations is due to direct effects of Torin2 on DNA damage repair processes instead of a cell cycle checkpoint defect. This increase in S/G₂-phase chromosome aberrations is suggestive of defects in HR-dependent repair since this is the favored mode of DNA repair in S/G₂ where resection is initiated and commitment to HR is coordinated with DNA replication (36–38).

Torin2 Alters the Kinetics of Radiation-Induced γ -H2AX Foci Appearance, Disappearance and Recruitment of HR-Associated DSB Repair Proteins

In mammals, the phosphorylation of H2AX (γ -H2AX) at Ser-139 by ATM plays a critical role in recruiting other signaling factors to DNA damage sites (39, 40). To characterize the effect of Torin2 on H2AX-phosphorylation by immunofluorescence, cells were pretreated with Torin2, then 2 Gy irradiated to facilitate distinct and scorable γ -H2AX foci and to estimate overall γ -H2AX intensity as phosphorylation readouts. Torin2 treatment alone did not significantly alter the H2AX phosphorylation or formation of γ -H2AX foci (Fig. 3A and D). However, when combined with radiation, Torin2 treatment delayed H2AX phosphorylation and foci formation and also delayed their resolution (Fig. 3A – D). Indeed, in control cells the majority of γ -H2AX foci disappeared within 6 h, whereas in Torin2-treated cells γ -H2AX foci persisted even at 12 h postirradiation (Fig. 3B – D). Since dephosphorylation of γ -H2AX is known to control the release of cells from cell cycle checkpoints and restore the epigenome to predamage status (41, 42), the delay in foci resolution (Fig. 3A – D) further points to a defect in DNA damage processing including possible defects in either dephosphorylation of γ -H2AX or recruitment of factors to DNA damage sites.

Because γ -H2AX foci formation is regulated by ATM kinase activity (40), we next investigated how Torin2 modulates radiation-induced ATM signaling by performing a time course analysis of ATM activation, as measured by Western blot analysis of pATM-Ser1981 formation (Fig. 4A). Basal levels of pATM-Ser1981 were unaffected by Torin2 treatment in the absence of radiation, suggesting that Torin2 by itself does not contribute to ATM activation *vis a vis* the baseline genomic stress inherent in tumor cells (Fig. 4A). After irradiation, phosphorylated ATM was immediately observed in DMSO-treated cells but largely absent from Torin2-treated cells until 30 min postirradiation (Fig. 4A). Dephosphorylation of ATM began by 4 h in recovering control cells and was complete by 24 h, whereas there was no decrease in pATM-Ser1981 levels in Torin2-treated cells even by 24 h. The delayed activation and inactivation is mirrored in the kinetics of Chk2 phosphorylation (Thr68), the preferred ATM phosphorylation site (43).

To test if ATM is the only component of the PI3KK family that contributes to DNA damage-associated radiosensitivity, we analyzed Torin2 and radiation-induced cytotoxicity in wild-type and ATM-null tumor cells derived from murine lung. If ATM inhibition is the only determinant of increased residual DNA damage after cell irradiation, Torin2 treatment would not be expected to enhance radiosensitivity in ATM-null cells at the doses we used. As expected, ATM-null cells were radiosensitive, however, radiosensitivity at 2 Gy was further enhanced by the addition of Torin2 (Fig. 4B), suggesting that ATM is not the sole target of Torin2 contributing to the impaired DNA damage responses.

Torin2 Reduces HR Efficiency

Because the chromosome aberration studies showed defects in S and G₂, which suggested defects in HR-mediated DNA repair, we investigated the effect of Torin2 on factors related to HR-mediated repair of DNA damage. Compared to other DNA repair processes such as NHEJ, HR has high fidelity and is less likely to result in mutations. Early events in HR involve resection of DNA ends to yield 3'-ssDNA overhangs, followed by recombinase-mediated homologous DNA pairing and strand exchange (44). Recruitment of CtIP to DNA damage sites is a key step in the resection stage of the HR DNA repair process. CtIP is also a target for BRCA1-dependent phosphorylation by ATM after DNA double-strand breakage and plays a role in DNA-damage-induced cell cycle checkpoint control at the G₂/M transition. After irradiation, the immunofluorescence analysis of CtIP in control cells, formation of distinct foci was detected, however, in Torin2-treated cells the foci were less intense and fewer in number (Fig. 5A and C), suggesting defects in steps upstream of resection during HR. Next, we analyzed for foci related to Rad51, a recombinase that plays a crucial role in DNA homology search during HR (45). Radiation exposure induced a dramatic increase in the number and size of Rad51 foci in control cells at 5 h, whereas Torin2-treated cells displayed a significant decrease in the number and intensity of Rad51-positive cells (Fig. 5B and D). These results indicate that Torin2 affects both damage recognition (ATM and γ -H2AX) and recruitment of HR repair factors (CtIP and RAD51) to DNA damage sites, thus significantly reducing the efficiency of HR repair.

To directly confirm that Torin2 impairs DSB repair by HR, we used a reporter-based HR assay that reconstitutes a cellular GFP signal if HR is functional (27, 28). The HR-GFP reporter construct was stably expressed in H1299 cells to create a reporter cell line. When an I-SceI plasmid is transiently transfected into the reporter cell line to induce DSBs, GFP conversion takes place, depending on the efficiency of HR. Cells transfected with Rad51 siRNA were used as a positive control for suppression of HR (Fig. 5E). Torin2 treatment effectively downregulated HR repair of the I-SceI-induced DNA DSBs (Fig. 5A – E), consistent with our other evidence that Torin2 impairs HR-mediated DSB repair processes. Finally, to exclude the possibility that NHEJ is effected by Torin2, we used a reporter assay developed previously (29, 46) and found that Torin2 does not significantly suppress NHEJ at the doses used in this study (10 vs. 50 nM), although higher Torin2 doses (100 nM) can impair NHEJ efficiency.

Torin2 Induces Replication Stress and Slows Replicative Fork Progression

Homologous recombination is a key mechanism for maintenance of sequence fidelity during DNA replication (47). DNA replication can only start at defined sites of initiation, termed replication origins, which are “fired” during each round of replication, and also regulated by DNA damage responses. Origin activation is controlled by the ATR checkpoint kinase and its downstream effector kinase Chk1, which suppresses origin firing in response to replication blocks and also during normal S phase of the cell cycle (48–49). In unperturbed cells, Chk1 depletion causes reduced replication fork progression and also leads to unregulated origin firings (48). Since biochemical studies have shown that Torin2 affects ATR kinase activity (19) and Western blot analysis indicated suppression of radiation-induced Chk1 phosphorylation, a substrate of ATR in the presence of Torin2 (Fig. 6A), we evaluated the role of Torin2 in regulating replication fork progression by DNA fiber assay (25–50). Cells were pulse labeled with IdU for 30 min, then washed and pulse labeled with CIdU for 60 min, as previously described (25). The stalled replication fork progression was calculated by comparing the total number of fibers labeled with IdU alone against all fibers incorporating IdU (Fig. 6B and C). New origin firings were calculated by comparing the total number of DNA fibers labeled with CIdU alone to all fibers incorporating CIdU (Fig. 6B and D). Torin2 treatment decreased CIdU labeling consistent with retardation of replicative fork progression (Fig. 6C). Interestingly, we observed an increase in the number of new origins with Torin2 treatment (Fig. 6D). Both of these findings are consistent with a previously reported Chk1 defective phenotype showing increased origin activation and reduced rates of replication fork progression (48). We further confirmed this effect by calculating the replication fork speed (Kb/min). We observed that the Torin2-treated cells exhibited a reduced fork speed distribution compared to control DMSO-treated cells (Fig. 6E). These results together suggest that downregulation of Chk1 activity through Torin2 treatment destabilizes replicative fork progression and promotes uncontrolled origin firings.

DISCUSSION

We have used multiple approaches to show that Torin2 can radiosensitize cancer cell lines and demonstrated that radiosensitization is associated with suppression of HR-dependent DSB repair. Additionally, we provide evidence that this effect is dependent on multiple PI3KK family members, not just ATM. Because Torin2 inhibits several of these critical associations, we speculate that it could potentially affect multiple stages of the repair process including sensing DNA damage or transducing signals required for repair. Torin2 could also cause indirect effects through mTOR inhibition that impact DNA repair.

The kinase activities of ATM, ATR and DNA-PK have varied roles in sensing DNA damage, which may be inhibited by Torin2. In unirradiated cells ATM exists as a dimer but after irradiation, ATM undergoes autophosphorylation, thereby stimulating release of catalytically active monomers and activation of ATM signaling pathways (51). Kinase activity has also proven important for the physical interactions between ATM and DNA: autophosphorylation of ATM at S1981 (52) and S2996 (53) stabilizes ATM on DNA damage sites, and without this activity ATM association with DSBs is attenuated. The relationship between ATR kinase activity and interaction with DNA or other components of the DNA damage sensing

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machinery is less well characterized but there are indications that many characteristics are conserved between ATR and the other PIKK family members (54). Although more active in NHEJ, which was not extensively explored as a target of Torin2 in our study, the kinase activity of DNA-PK is essential for rejoining DNA DSBs (55) and there is also evidence that DNA-PK phosphorylation and autophosphorylation activity stabilizes its interaction with DNA (56). Torin2-mediated inhibition of kinase-mediated damage sensing is compatible with the altered DNA damage-response kinetics observed in our study.

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Torin2 may also promote radiosensitivity by impairing signal transduction downstream of DNA damage sensing. After exposure to radiation many proteins become phosphorylated; in a seminal study 1,099 cellular proteins were phosphorylated after irradiation and over 60% of these modifications were ATM dependent. Substrates included such key proteins as p53, Mdm2, BRCA1, Chk2 and Nbs1 (53). Gamma-H2AX is another major substrate of ATM and DNA-PK (57). In published genetic and biochemical studies, it has been suggested that phosphorylation of H2AX plays a key role in the recruitment and retention of DNA repair factors to the DNA break sites (58, 59). Similarly, ATR transmits DNA damage signals through phosphorylation of proteins such as p53, Chk1 and BRCA1 (60, 61). Torin2 could conceivably act by impairing transmission of these repair signals and this effect would be consistent with the radiosensitization we detected.

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The Torin2-dependent delays seen in our DNA replication fork experiments raise the possibility that Torin2 treatment may also exacerbate replicative stress. DNA replication starts at many replication origins, which are fired during each S phase. This activation is controlled by the ATR checkpoint kinase and its downstream effector kinase Chk1, which suppresses uncontrolled origin firing in response to replication blocks and during normal S phase by inhibiting the cyclin-dependent kinase Cdk2 (48). Thus, defective Chk1 activation leads to an increase in the number of firing replicative origins and reduced rates of replication fork progression, similar to what we saw in our fiber assays. Inhibitors of Chk1 are currently being evaluated as chemotherapeutic agents based on the premise that, in contrast to normal tissues, most tumors have a defective G₁-DNA damage checkpoint and therefore rely on the Chk1-dependent S and G₂ checkpoints. If true, selective inhibition of Chk1 or regulators of Chk1 such as ATR would selectively target tumor tissues when used in combination with radiation, and Torin2 may be acting in this way to enhance radiosensitivity (62). Additional studies are needed to directly evaluate the relative activity of Torin2 in ATR versus ATM inhibition, since the degree of inhibition on the respective pathways appears to be distinct (for comparison, see Figs. 4A and 6A).

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Although there is little evidence for a direct interaction between mTOR and components of the DNA repair machinery, mTOR may exert effects indirectly by virtue of its role as a critical regulator of gene translation. In several examples, ATP-competitive mTOR inhibitors have impaired radiation-induced translation of mRNA coding for proteins involved in DNA replication, recombination and repair (63, 64). Although we did not examine this aspect specifically, it is possible that Torin2 may also promote radiosensitivity through similar mechanisms. Nevertheless, it is clear from our data that direct mTOR inhibition alone is not responsible for the radiosensitization we observed (Fig. 1C and D). This supports the

hypothesis that simultaneous targeting of multiple PI3KK family members is a key determinant of the radiosensitizing activity of Torin2.

From a translational perspective, Torin2 has properties that may provide significant clinical advantages compared to other mTOR inhibitors currently in clinical development. Most ATP-competitive mTOR inhibitors such as SF1126, GSK1059615 and BEZ235 were developed based on PI3 kinase inhibitors as the starting point and therefore have dual specificity for PI3K and mTOR pathways (65–67). Incidentally, some of these inhibitors are also being developed as radiosensitizers in preclinical settings (20, 68). Dissimilarly, Torin2 shows little activity on PI3K, and an enhanced selectivity for the mTOR/PIKK pathway. We hypothesize that if the radiosensitizing effects of Torin2 are more related to its anti-PIKK activity than the PI3K activity this may be a distinct advantage for Torin2 with respect to developing radiosensitizing activity. It is worth noting that in this study we specifically tested the lower nanomolar range of Torin2 (10–50 nM) activity, which is expected to target ATM and ATR, but not DNA-PK. This decision allowed us to distinguish effects related to DNA repair from autophagic responses (19). Moreover, a recently reported study demonstrated that at sublethal doses, Torin2 synergizes with cisplatin to inhibit the growth of ovarian carcinoma cell lines (69), suggesting that low doses of Torin2 are capable of enhancing the DNA damage response when combined with existing radiotherapeutic agents routinely used in clinical settings. Importantly, Torin2 was also reported to have a good safety profile in mouse models, suggesting that the drug may not have deleterious effects on normal tissues (16).

Another potential advantage of Torin2 is that it causes accumulation of residual radiation-induced DNA aberrations in the S/G₂, but not the G₁, suggesting that Torin2 could cause repair defects selectively in actively replicating cells such as tumors. Additionally, in mouse models Torin2 appears rapidly in plasma after oral administration, but also has a short half-life (0.72 h) by classical pharmacology standards. This might have practical clinical advantages for a radiosensitizer that could be given immediately before radiation therapy. Rapid clearance would decrease exposure to the compounds and the resulting risk of deleterious side effects, such as secondary malignancies, which are theoretically possible with agents that potentially contribute to DNA damage in nondiseased tissues.

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FIG. 1.

Tumor cell killing is enhanced by combined Torin2 and radiation treatment. Panel A: Viability of Torin2-treated A549 and H1299 cell lines 96 h after 8 Gy irradiation. Error bars represent SEM; viability was normalized to DMSO control. Statistics derived with nonlinear fit in GraphPad Prism. IC₅₀ values in nM are as follows. A549: 27 vs. 11 (95% CI: 21–36 vs. 8–14; H1299: 7 vs. 2 (95% CI: 5.6–9 vs. 0.9–4). Panel B: Clonogenic cell survival after irradiation in the presence of 10 nM Torin2 or DMSO. Error bars represent SEM. Cell survival measurements were fitted by a linear-quadratic equation [$SF = \exp(-\alpha d - \beta d^2)$].

The RER was calculated with ratio of area under the curve (mean inhibition dose) for irradiation/area under the curve for irradiation + compound treatment as indicated in the figure insets. Panel C: Clonogenic survival assay comparing Torin1, and INK128 (both specific mTOR inhibitors) versus Torin2 (mTOR/PI3KK inhibitor) at the indicated inhibitor concentrations and at the absorbed radiation dose. Panel D: Fold decrease in survival fraction of A549 cells obtained from clonogenic survival assay at 6 Gy irradiation in the presence of inhibitors as indicated.

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FIG. 2.

Torin2 enhances residual chromosome aberrations in radiation-treated cells. Panels A–C: Scoring of chromosome aberrations in A549 cells per metaphase in G₁, G₂ and S phase of the cell cycle. Error bars represent SD from replicates (**P* 0.05). Panel D: Representative chromosomal spreads from A549 cells treated as indicated. Arrows highlight abnormalities. Panel E: Propidium iodide staining and cell cycle analysis of the A549 cells. The cell cycle distribution after 6 h was evaluated in the presence of DMSO or Torin2 as indicated after 6

Gy dose. The percentage of cells in the G_1 , S and G_2 phase of the cell cycle with various treatment conditions are indicated in the bottom panel.

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FIG. 3.

Torin2 delays radiation-induced γ -H2AX foci formation/dissolution. Panel A: Immunofluorescence staining of γ -H2AX foci (red) with and without Torin2 treatment at indicated radiation doses and time points in A549 cells. The nucleus is counterstained with DAPI (blue). Panels B and C: Quantitation of cells with foci number (panel B) and intensities (panel C). Error bars represent SEM from replicates (* $P < 0.05$). Panel D: Western blot analysis of γ -H2AX foci formation time course in A549 cells after irradiation with and without Torin2 treatment.

FIG. 4.

Torin2 modulates the activation kinetics of ATM. Panel A: Western blot analysis of A549 cells pretreated with Torin2 or DMSO showed delays in activation and resolution of ATM pathways in the presence of Torin2. Panels B and C: Cell viability for wild-type (panel B) and ATM-null (panel C) in mouse lung tumor-derived cell lines shows further enhancement of the radiosensitivity of ATM-null cells. Error bars represent SEM from replicates (* P 0.05).

FIG. 5. Torin2 decreases HR efficiency. Panels A and B: Immunofluorescence staining of CtIP foci (green, panel A) and Rad51 foci (red, panel B) in A549 cells, treated as indicated. Panels C and D: Bar graphs show the percentage of cells positive for CtIP (panel C) and Rad51 (panel D) foci. Error bars represent SEM (* P 0.05, ** P 0.01). Panel E: Quantitation of Torin2 effect on HR with an I-Sce1 HR reporter assay. Rad51 siRNA was used as a positive control. Error bars represent SEM (** P 0.02). Panel F: Quantification of Torin2 effect on NHEJ.

NU4771, a DNA-PKCs, inhibitor was used as a positive control. Error bars represent SEM (** $P < 0.02$); ns = not statistically significant.

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FIG. 6.

Torin2 increases replication fork stalling and reduces replication fork progression. Panel A: Western blot analysis of Chk1 phosphorylation at Ser 345 in A549 cells show a decrease in response to Torin2 treatment. Panel B: DNA fiber time course. Treatment time points with different nucleotide analogs are indicated (left side) and representative images are shown (right side). Panel C: Quantitation of the stalled replication forks. Panel D: Quantitation of the forks with new origins. Panel E. Quantitation of the average fork speed in the DMSO- and Torin2-treated A549 cells. X-axis represents the fork speed measured in Kb/min. Error bars represent SEM (* $P < 0.05$).