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Original Article

The radioprotective agent WR1065 protects cells from radiation damage by regulating the activity of the Tip60 acetyltransferase

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Abstract: Background: The aminothiol WR1065 is a highly effective free radical scavenger which can protect cells from the cytotoxic effects of ionizing radiation. Currently, WR1065 is used clinically to protect patients from radiation injury occurring during radiation therapy protocols. However, it is becoming increasingly clear that WR1065 can alter radiosensitivity through a mechanism which is independent of its ability to function as a free radical scavenger. Here, we examined the ability of WR1065 to directly regulate signaling pathways involved in the DNA damage response. Methodology: The ability of WR1065 to enhance the survival of irradiated bone marrow cells and primary cultures was established. DNA damage signaling was monitored by measuring activation of the ATM kinase by western blot analysis and activation of Tip60 using an in vitro acetylation assay. Tip60 function was abrogated by expression of a catalytically inactive Tip60, and the effect on radiosensitivity evaluated. Principal findings: Treatment of cells with WR1065 led to a small but significant increase in the kinase activity of ATM. Further, WR1065 robustly activated the Tip60 acetyltransferase, which is a key upstream regulator of the ATM kinase. In addition, WR1065 directly activated the acetyltransferase activity of purified Tip60 in vitro, indicating a direct interaction between WR1065 and Tip60. Finally, cells with reduced levels of Tip60 activity exhibited a significant reduction in radioprotection by WR1065. Conclusions: Direct regulation of Tip60's acetyltransferase activity by WR1065 makes a significant contribution to the radioprotective effects of WR1065. Activators of Tip60 may therefore make effective clinical radioprotectors.

Keywords: Aminothiol WR1065, ionizing radiation, radiation injury, prevention, Tip60 acetyltransferase

Introduction

Amifostine is an aminothiol prodrug which protects normal tissues from the cytotoxic effects of radiation [1]. The major biological activity of amifostine is mediated by the thiol group, which functions as a highly effective free radical scavenging agent [2-5]. Amifostine is preferentially taken up by normal tissue, but is only poorly taken up by tumor cells [6-8], indicating that amifostine should be effective at protecting normal tissue from the cytotoxic effects of radiotherapy and chemotherapy. Accordingly, amifostine is deployed in the clinic as a protective agent for use in treatment of head and neck cancer [1], and is undergoing trials in other types of cancer.

The biologically active form of amifostine, WR1065, is generated in tissues through the dephosphorylation of amifostine by alkaline phosphatases. In addition to direct antioxidant effects, WR1065 has a significant impact on DNA damage signaling pathways which protect normal tissue from ionizing radiation. WR1065 increases p53 protein levels in cells [9-11], leading to upregulation of p21 and other p53 regulated genes [12, 13]. WR1065 also activates the NFκB transcription factor [14], leading to increased levels of the antioxidant enzyme MnSOD [15]. The activation of p53 by WR1065 does not involve generation of DNA damage by WR1065 [10]. Instead, WR1065 can interact directly with both p53 and NFκB [14], and this interaction may mediate the observed stabilization and activation of p53 and NFκB in cells. The ability of WR1065 to regulate p53 and NFκB may therefore make a significant contribution to the ability of WR1065 to protect cells from the cytotoxic effects of radiation.

The cells response to DNA damage involves multiple proteins, many of which are regulated through redox state or by reactive oxygen species. Among these pathways, the product of the ataxia telangiectasia gene, the ATM protein kinase, is a critical factor. The ATM protein kinase is activated in response to DNA double-strand Breaks (DSBs) [16-18] and phosphorylates multiple DNA damage response proteins, including nbs1, p53, Chk2 and SMC1 (reviewed in [17]). Cells lacking functional ATM protein exhibit defects in DNA repair, loss of cell cycle checkpoints and increased radiosensitivity [16, 17, 19], resulting in increased sensitivity to ionizing radiation. Activation of ATM's kinase activity requires contributions from 2 protein complexes – the Mrell-Rad50-Nbs1 (MRN) DNA binding complex [20-23] and the Tip60 acetyltransferase [24-26]. MRN recruits ATM to DSBs, and mutations in the individual protein components of MRN reduce or abolish activation of ATM's kinase activity by DNA damage [20-23]. The Tip60 acetyltransferase then acetylates ATM, leading to the acetylation and activation of ATM's kinase activity [24, 25, 27]. Tip60 is a ubiquitously expressed acetyltransferase involved in several aspects of the cells response to DNA damage. Tip60 can acetylate histones adjacent to DSBs, and this acetylation plays a key role in regulating chromatin structure during DNA repair [28, 29]. Tip60 also acetylates p53 [30], and this modification is critical for the ability of p53 to regulate apoptosis. Tip60 is therefore a key component of the cells response to DSBs, and is essential for regulating ATM activation [25, 27], p53 function [30] and chromatin structure [31]. Because WR1065 exerts significant effects on DNA damage signaling, we investigated if WR1065 regulated the acetyltransferase activity of the Tip60 protein.

Materials and methods

Cell lines

HeLaS3 cells and GM5849 A-T cells (Coriel Institute, NJ) were cultured in 10% fetal bovine serum in Minimal Eagles Medium. A-T cells expressing either vector (A-T) or complemented with a full-length ATM protein (A-T^{ATM}) are described in [25]. HeLa cells expressing either Tip60 vector ($Tip60^{WT}$) or a catalytically inactive Tip60 ($Tip60^{HD}$) and clonogenic cell survival assays are as described by us [25]. WR1065 was obtained from the Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD. Bone marrow cells were isolated from femurs and tibiae of 16 week-old C57BL/6J mice and resuspended in DMEM containing 10% fetal bovine serum as previously described [32]. Ethics statement: Mouse experiments were reviewed and approved by the Dana -Farber Cancer Institute Animal Care and Use Committee (protocol #04-105). All veterinary care was consistent with the recommendations of the American Veterinary Medical Association. The Dana-Farber Cancer Institute is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

The total bone marrow cells were treated with WR1065 for 30 min and exposed to ionizing radiation. The cells were allowed to recover for 30 min at 37°C, and washed twice with Dulbecco's Modified Eagles Medium containing 10% fetal bovine serum to remove the WR1065. Cells were then seeded in 12 well plates at a density of 70,000 mononuclear cells per well in methylcellulose medium containing growth factors (Methocult GF M3434, Stem Cell Technologies, Vancouver, BC, Canada). Cultures were incubated at 37°C in 5% CO_2 for 10 days and hematopoietic colonies (CFU-Cs) were counted.

Western blot analysis

Cell lysis buffers, immunoprecipitation and western blot analysis are as described by us [24, 25]. Cells ($1x10^7$) were lyzed in ATM lysis buffer (20mM Hepes pH7.4; 150mM NaCl; 0.2% Tween 20; 1.5mM $MgCl_2$; 1mM EGTA; 2mM DTT; 50mM NaF; 500μM Na_3VO_4; 1mM PMSF; 1μg/ml aprotinin; 1μg/ml leupeptin) and cleared by centrifugation. Western blot analysis, using antibodies to ATM (2C1: Genetex, TX) or phospho-Ser 1981 ATM (Rockland Biochemicals, PA) was then carried out.

HAT assays

For HAT assays, cell lysates (prepared as described above) were immunoprecipitated with Tip60 antibody (Upstate Biotechnology, NY), washed twice in HAT assay buffer (50mM Tris pH8.0; 10% Glycerol; 0.1mM EDTA; 1mM DTT), and incubated in HAT assay buffer (60μl) supplemented with acetyl-CoA (100μM), and biotinylated Histone H4 peptide (0.5μg) for 30min at 30°C. An aliquot of the reaction was immobi-

lized onto 96-well streptavidin plates, and acetylation of the H4 peptide detected using a HAT ELISA according to the manufacturer's instructions (Upstate Biotechnology, NY). HAT activity is expressed as the change in absorbance relative to the reference wavelength (450nm-540nm). Validation of the acetyltransferase assay, and details of procedures, are described in [24, 25, 27].

Results

To examine how WR1065 regulates Tip60, we first sought to establish the toxicity profile of the compound. This is important because several reports have indicated that WR1065 can be cytotoxic in some cell lines [1, 33], and cytotoxicity may lead to activation of DNA damage response pathways. To establish conditions under which WR1065 exerted maximal efficacy with minimal toxicity, we determined the ability of WR1065 to protect bone marrow hematopoietic progenitor cells from ionizing radiation. Hematopoietic progenitor cells are particularly sensitive to both cytotoxic and genotoxic agents. Therefore, we first investigated if WR1065 was an effective protector of these primary cells. Bone marrow cells were isolated from mice and incubated with WR1065 prior to irradiation. Hematopoietic progenitor cells were very sensitive to both 4 and 6Gy IR (**Figure 1A**). Treatment with WR1065 at either 0.8mM or 4mM also had a significant impact on overall survival of the hematopoietic progenitor cells, reducing survival to approximately 70% (71.7 ± 9.7% standard error) compared to untreated cells (**Figure 1A**). WR1065 therefore has significant toxicity in the hematopoietic progenitors. However, when WR1065 was combined with ionizing radiation, WR1065 provided a 6-10 fold radioprotection of the cells, with 4mM WR1065 providing the highest radioprotection. Further, although this concentration of WR1065 exhibited significant toxicity in the hematopoietic progenitor cells, WR1065 exhibited minimal toxicity towards either human fibroblast cells or HeLaS3 cells (**Figure 1B**). This is consistent with the known sensitivity of hematopoietic progenitor cells to cytotoxic and genotoxic agents. **Figure 1** therefore demonstrates that, although WR1065 has some toxicity towards hematopoietic progenitor cells, it can, when combined with radiation, effectively protect bone marrow hematopoietic progenitor cells from the cytotoxic effects of radiation.

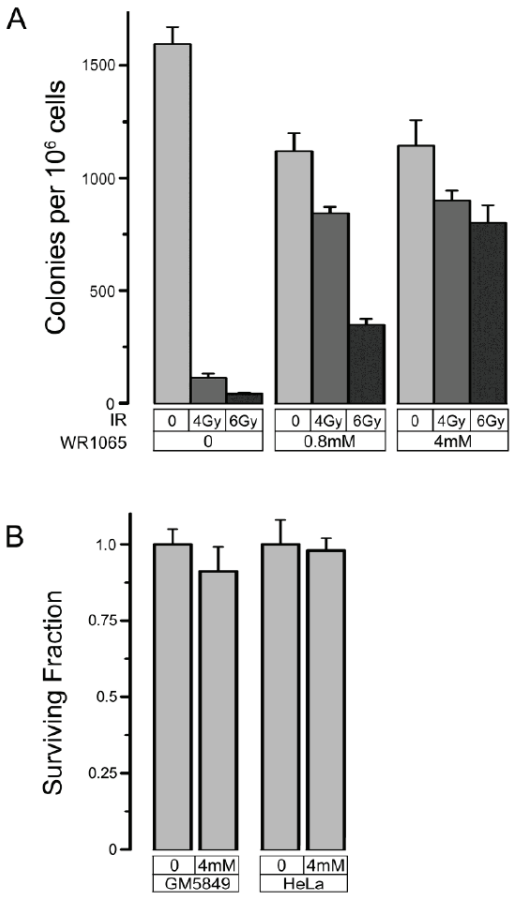

Figure 1. WR1065 pre-treatment protects bone marrow hematopoietic progenitors from radiation damage. (A) Isolated mouse bone marrow cells were pretreated with WR1065 and then irradiated at the indicated dose. WR1065 was removed by washing, and the cells plated in methyl cellulose medium containing growth factors. The cultures were grown for 10 days at 37^{0}C and hematopoietic colonies (CFU-Cs) scored. Results ± SE (n = 3). (B) GM5849 human fibroblast cells or HeLaS3 cells were incubated with WR1065 (4mM) for 8hr. Following washing, cells were allowed to recover for 12 days, before clonogenic cell survival assays were carried out. The surviving fraction was calculated relative to the untreated control cells. Results ± SE (n = 3).

Although the primary mechanism of action of WR1065 is likely to be related to its anti-oxidant properties, several lines of evidence have demonstrated that WR1065 can also activate DNA damage signaling pathways, including the p53 and NFκB pathways [9-11, 14]. The ATM kinase is a key regulator of both p53 and NFκB, and, in turn, activation of ATM by DNA damage involves

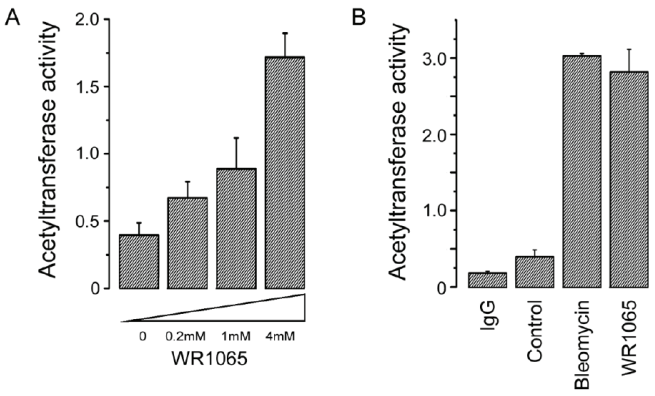

Figure 2. WR1065 activates Tip60's acetyltransferase activity. (A) Tip60 was immunopurified from HeLa cells and then incubated, in vitro, with increasing concentrations of WR1065 for exactly 15 minutes at 4°C. Following this preincubation, acetylCoA and substrate histone H4 peptide were added, and the levels of Tip60 acetyltransferase activity measured at 30°C. Results ± SE (n = 3). (B) HeLa cells were pretreated with either solvent (control), WR1065 (4mM) or bleomycin (5μM) for 30 minutes. Tip60 was immunopurified from HeLa cells, and the intrinsic acetyltransferase activity of the Tip60 measured. Mock purification with IgG is shown for comparison. Results ± SE (n = 3).

the direct acetylation and activation of ATM's kinase activity by the Tip60 acetyltransferase [25]. In **Figure 2**, we examined if WR1065 could directly activate Tip60's acetyltransferase activity. Purified Tip60 was incubated with increasing concentrations of WR1065, and the ability of Tip60 to acetylate a substrate peptide derived from histone H4 was examined. WR1065 increased Tip60's acetyltransferase activity in a dose dependent manner (**Figure 2A**), indicating that WR1065 can regulate Tip60 activity in vitro. To determine if WR1065 could also regulate Tip60's in vivo acetyltransferase activity, HeLa cells were either untreated, exposed to the radiomimetic agent bleomycin (which generates DSBs) or WR1065. Tip60 was then immunopurified from the cells, and the associated acetyltransferase activity measured. **Figure 2B** demonstrates that untreated cells had minimal Tip60 acetyltransferase activity, whereas bleomycin treatment significantly increased Tip60's acetyltransferase activity, as we have previously shown [25]. Mock immunopurification with IgG antibody is shown for comparison. Significantly, WR1065 treatment activated Tip60's acetyltransferase activity to the same extent as seen with the DNA-damaging agent bleomycin. WR1065 can therefore activate Tip60's acetyltransferase activity both in vivo and in in vitro assays.

To determine if activation of Tip60 by WR1065 led to activation of the ATM signaling pathway, the activation of ATM's kinase after WR1065 exposure was monitored. ATM activation leads to the autophosphorylation of ATM on serine 1981 [34] and provides a convenient marker for measuring ATM kinase activity. In **Figure 3A**, exposure of cells to ionizing radiation led to a rapid increase in ATM autophosphorylation.

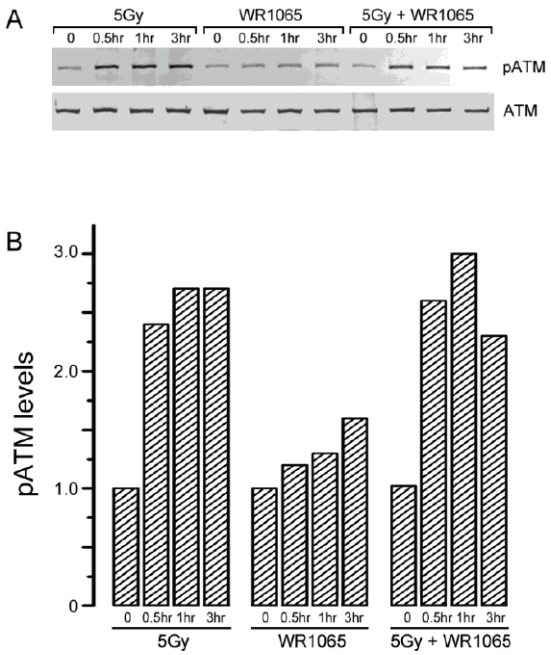

Figure 3. WR1065 activates the ATM kinase. (A) HeLa cells were treated with either WR1065 (4mM), ionizing radiation (IR: 5Gy) or both WR1065 and ionizing radiation. At the indicated time points, cell extracts were examined by western blot analysis for ATM protein levels and for autophosphorylation of ATM on serine 1981 (pATM). (B) Western blots in (A) were scanned and the levels of ATM phosphorylation quantitated. Signal levels were normalized to the level of total ATM protein in each lane of the blot. Unirradiated samples were assigned a value of 1.

However, WR1065 treatment revealed minimal increases in ATM activation (**Figure 3A**), despite the robust activation of Tip60's acetyltransferase activity (**Figure 2B**). Quantitation of the western blot in figure 3A confirms a minimal

accumulation of activated, autophosphorylated ATM in cells incubated with WR1065 (**Figure 3B**) compared to ionizing radiation alone. This indicates that activation of Tip60 by WR1065 alone is insufficient to lead to ATM activation, and implies that additional factors contribute to maintaining ATM in the active state. Further, exposure of irradiated cells to WR1065 did not lead to any significant increase in ATM activation compared to ionizing radiation alone. Instead, the accumulation of phosphorylated ATM was reduced at 3hr post-irradiation in the presence of WR1065 (**Figures 3A** and **B**).

Next, the contribution of Tip60 and ATM pathway to the radioprotective effects of WR1065 was quantified. For this, the ability of WR1065 to radioprotect cells lacking either ATM (**Figure 4A**) or Tip60 (**Figure 4B**) was measured. In **Figure 4A**, A-T cells were significantly more sensitive to IR than A-T cells complemented with exogenously expressed ATM (A-T^{ATM}), and WR1065 protected both cell types from radiation. To quantify the effectiveness of WR1065 in protecting the cells, the Dose Modifying Factor (DMF) was calculated. DMF was calculated by comparing the dose of radiation required to yield 10% survival in the absence and presence of WR1065. The DMF for WR1065 in A-T and A-T^{ATM} cells was 1.9 and 1.8 respectively (**Figure 4A**), indicating that ATM status does not affect the ability of WR1065 to protect the cells from ionizing radiation exposure. In **Figure 4B**, cells expressing wild type Tip60 (Tip60^{wt}) or a catalytically inactive Tip60 (Tip60^{HD}), which does not posses any significant acetyltransferase activity [25], were irradiated in the absence and presence of WR1065. Tip60^{HD} cells were significantly more sensitive to IR than Tip60^{wt} cells and WR1065 protected both the Tip60^{wt} and Tip60^{HD} cells from IR. However, WR1065 was significantly more effective in protecting Tip60^{wt} cells from ionizing radiation when compared to the cells expressing Tip60^{HD}. The DMF for WR1065 in the Tip60^{wt} cells was 3.3 compared to 1.9 for the Tip60^{HD} cells. Tip60's acetyltransferase activity therefore makes a significant contribution to the radioprotective protective of WR1065.

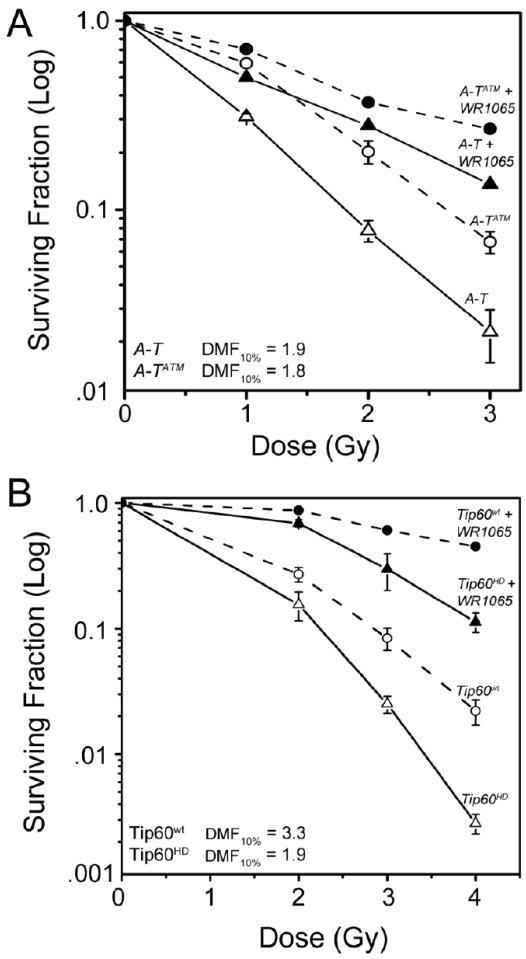

Figure 4. WR1065 protects ATM and Tip60 negative cells from ionizing radiation induced cell death. (A) GM5849 cells derived from A-T patients expressing either vector (A-T; Δ, ▲) or complemented with ATM (A-T^{ATM}; ○, ●) were incubated for 30 minutes in WR1065 (4mM; closed symbols). Cells were irradiated at the indicated dose, and clonogenic cell survival assays carried out as described in methods. Results ± SE (n = 3). (B) HeLa cells expressing either wild type (Tip60^{WT}; Δ, ▲) or a catalytically inactive Tip60 (Tip60^{HD}; ○, ●) were incubated for 30 minutes in WR1065 (4mM; closed symbols). Cells were irradiated at the indicated dose, and clonogenic cell survival assays carried out as described in methods. Results ± SE (n = 3). Dose Modifcation Factor ($DMF_{10\%}$) = [Dose with WR1065]/[Dose without WR1065], where dose equals level of radiation required to yield a surviving fraction of 10%.

Discussion

These results demonstrate that the radioprotective compound WR1065 can increase the acetyltransferase activity of the Tip60 protein. Further, WR1065 activates Tip60 both in vitro and in vivo, implying a direct action of WR1065 on Tip60. Previous work has clearly shown

WR1065 can also interact directly with the NFκB and Jun/AP1 transcription factors [14] and that WR1065 can activate the DNA-binding activity of p53 in vitro [9-11]. Interestingly, NFκB, p53 and Jun/AP1 are all regulated by changes in cellular redox state. WR1065's antioxidant activity can alter cellular redox state, including increasing overall levels of GSH in the cell [10, 11]. Further, there is evidence indicating that WR1065 may activate p53 in vitro by direct reduction of key cysteine residues on p53 [9, 10, 35]. The ability of WR1065 to activate Tip60's acetyltransferase activity in vitro could therefore be due to direct redox modulation of essential cysteine residues on Tip60, or through direct binding of WR1065 to a key domain of Tip60. Activation of Tip60 could also trigger NFκB activation and protect the cells from radiation, since the radioprotector flagellin causes robust radioprotection in mice through NFκB activation [36]. In either case, the activation of Tip60's acetyltransferase activity by WR1065 demonstrates that WR1065 has multiple effects on the cell in addition to its ability to scavenge free radicals.

Tip60 functions in multiple aspects of the cells response to DNA damage, including acetylation and activation of p53 [30], acetylation and activation of the ATM kinase [25, 27], and in acetylating histones adjacent to sites of DNA damage [28, 29]. However, although WR1065 fully activated Tip60 (**Figure 2**), this resulted in only minimal activation of ATM's kinase activity. Activation of Tip60 by WR1065 alone is therefore insufficient to evoke robust ATM activation, and implies that additional factors, such as concentrating ATM at sites of DNA damage, contribute to maintaining ATM in the active state following DNA damage. Further, WR1065 protected both normal cells and cells lacking ATM expression (**Figure 4**) equally from ionizing radiation. WR1065 does not, therefore, exert its radioprotective effects through the Tip60-dependent modulation of ATM. WR1065 can activate the p53 protein [9-11], and at least some of WR1065's effects maybe mediated through p53 [37, 38]. Although Tip60 can acetylate and activate p53 [30], WR1065 efficiently protected HeLaS3 cells, which lack detectable p53 expression. Tip60-dependent regulation of p53 is therefore also unlikely to contribute to the radioprotective effects on WR1065.

A key finding from this study was the demonstration that the radioprotective effects of WR1065 were dependent on the intrinsic acetyltransferase activity of Tip60. Cells expressing an acetyltransferase defective Tip60 protein had higher radiosensitivity than paired wild type cells. Further, the radioprotective properties of WR1065 were significantly reduced in the Tip60 -defective cells. This data clearly demonstrates that the acetyltransferase activity of Tip60 is essential for the ability of WR1065 to protect cells from ionizing radiation induced DNA damage. The ability of WR1065 to activate Tip60's acetyltransferase activity implies that acetylation of key target proteins by Tip60 is important for this effect. We have already excluded 2 key targets of Tip60, p53 and ATM, suggesting that some other, unknown protein may be acetylated by Tip60 following WR1065 treatment. One potential target is the chromatin. Tip60 can acetylate histones H3 and H4 [28, 29], and this histone acetylation is associated with open, relaxed chromatin structures. WR1065 may therefore stimulate the Tip60-dependent acetylation of histones, leading to the formation of more open, relaxed chromatin structures in the cell. This, in turn, may facilitate DNA repair by increasing access to sites of DNA damage.

In conclusion, we have identified a novel pathway for WR1065-mediated radioprotection which involves the direct activation of Tip60's acetyltransferase activity by WR1065. Tip60 is a crucial component of the cells DNA damage response, and is required for cells to repair and survive exposure to ionizing radiation [24, 25, 27]. The ability of WR1065 to fully activate Tip60 indicates modulation of Tip60's acetyltransferase activity can have profound effects on radiosensitivity. The observation that WR1065 mediates radioprotection through Tip60-dependent acetylation therefore provides a new target to identify novel radioprotectors based on their ability to activate Tip60's acetyltransferase activity.

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