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Inhibition of histone acetyltransferase activity by anacardic acid sensitizes tumor cells to ionizing radiation

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Abstract Histone acetyltransferases (HATs) regulate transcription, chromatin structure and DNA repair. Here, we utilized a novel HAT inhibitor, anacardic acid, to examine the role of HATs in the DNA damage response. Anacardic acid inhibits the Tip60 HAT in vitro, and blocks the Tip60-dependent activation of the ATM and DNA–PKcs protein kinases by DNA damage in vivo. Further, anacardic acid sensitizes human tumor cells to the cytotoxic effects of ionizing radiation. These results demonstrate a central role for HATs such as Tip60 in regulating the DNA damage response. HAT inhibitors provide a novel therapeutic approach for increasing the sensitivity of tumors to radiation therapy.

Keywords: ATM; Anacardic acid; Tip60; HAT; Radiosensitivity; DNA repair

1. Introduction

The acetylation of proteins is a dynamic event involving acetylation by histone acetyltransferases (HATs) [1,2] and deacetylation by histone deacetylases (HDACs) [3]. HATs primarily acetylate the N-terminus of histones, and this acetylation is associated with transcriptionally activate chromatin [4]. These acetylated lysine residues can function as docking sites for bromodomains, protein domains which bind acetylated lysine residues [5]. Protein acetylation therefore represents a cellular regulatory system for controlling protein function, and disruption of protein acetylation can have significant impact on cell function. For example, HDAC inhibitors have a range of effects on cells, including altered gene expression, growth arrest, differentiation and cell death [3]. These effects are attributed to the accumulation of hyper-acetylated histones and proteins within the cells. HDAC inhibitors are currently undergoing clinical evaluation for efficacy in the treatment of human tumors. Disruption of protein acetylation pathways is therefore a promising target for therapeutic development.

In addition to HDAC inhibitors, HAT inhibitors may also posses therapeutic potential. Several distinct families of HAT proteins have been identified, including the GCN5/PCAF, p300/CBP and MYST families [2]. The Tip60 HAT, a MYST family member, is involved in regulating the cells response to genotoxic events. Tip60 is activated by ionizing radiation and regulates the activation of the ATM protein kinase [6], apoptotic responses [7], the p53 protein [8], and the acetylation of histones [9,10]. Loss of Tip60 function inhibits the effective repair of DNA damage [6]. Inhibitors of Tip60 should therefore sensitize cells to genotoxic agents such as ionizing radiation. A limited number of HAT inhibitors have been described. Peptide-CoA conjugates can inhibit purified PCAF, but are not cell permeable [11]. Isothiazolone derivatives also inhibit PCAF, but they exhibit significant off target activity in vivo due to their high chemical reactivity with thiol groups [12]. Previous studies demonstrated that anacardic acid inhibits the p300 and PCAF HATs in vitro [13], but its in vivo effects have not been evaluated. Here, we demonstrate that anacardic acid is an effective inhibitor of HAT activity in vivo and that it sensitizes cells to ionizing radiation.

2. Materials and methods

2.1. Cell culture and irradiation

HeLa and 293T cells were grown in Dulbecco's modified Eagle's medium/10% fetal calf serum. SQ20B and SCC35 squamous cell carcinoma cell lines were cultured in modified eagles medium/10% fetal calf serum. For cell survival experiments, cells were plated at the appropriate dilution, irradiated, and surviving colonies were stained with crystal violet 10 days later [6]. For anacardic acid (EMD Biosciences, CA) exposure, cells were preincubated with anacardic acid for 40 min, irradiated, then allowed to recover for 4 h. Cells were then switched to fresh media and allowed to grow for 10 days.

2.2. Western blot and HAT assays

Cells were lyzed in lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 0.2% Tween 20, 1.5 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 50 mM NaF, 500 μM Na_3VO_4, 1 mM PMSF, 3 μg/ml aprotinin, 3 μg/ml leupeptin). ATM was detected with antibodies 5C2 (Genetex, TX), anti-phosphoserine 1981 (Rockland Biochemicals, PA) or anti-acetyl lysine antibody (Upstate Biotech, NY). DNA–PKcs antibody (clone 25-4), was purchased from Neomarkers, CA and pS2056 DNA–PKcs antibody was kindly provided by Dr Benjamin Chen.

HAT assay. Cell extracts were immunoprecipitated with Tip60 antibody (Upstate Biotech, NY), washed three times in lysis buffer and twice in HAT assay buffer (50 mM Tris, pH 8/10% glycerol/0.1 mM EDTA/1 mM DTT). Immunoprecipitates were incubated in 60 μl of HAT assay buffer supplemented with acetyl-CoA (100 μM), and biotinylated histone H4 peptide (0.5 μg) for 30 min at 30 °C. An aliquot of the reaction was immobilized onto streptavidin plates, and acetylation detected using a HAT ELISA as described by us [14].

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Abbreviations: HAT, histone acetyltransferase; HDAC, histone deacetylase; SER, sensitizer enhancement ratio

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3. Results

Tip60 is required for many cellular responses to DNA damage, including activation of the ATM protein kinase [6], repair of DNA-strand breaks [7,9], and both histone acetylation [10] and chromatin remodeling at sites of DNA damage [15]. To determine if anacardic acid could inhibit Tip60, Tip60 was immunoprecipitated from cell extracts and incubated with a peptide derived from the N-terminus of histone H4, which is efficiently acetylated by Tip60 [6]. Cell extracts immunoprecipitated with IgG (Fig. 1A, inset), had minimal HAT activity, as did extracts immunoprecipitated with Tip60 antibody and then incubated without substrate peptide. Immunoprecipitations with Tip60 in the presence of the peptide yielded robust Tip60 HAT activity. Immunoprecipitated Tip60 was then incubated with the increasing concentrations of anacardic acid and the associated HAT activity measured. Anacardic acid inhibited Tip60 with an IC_{50} of 9 μM (Fig. 1A, graph), with maximal inhibition above 30 μM. Anacardic acid is therefore an efficient inhibitor of Tip60 in vitro.

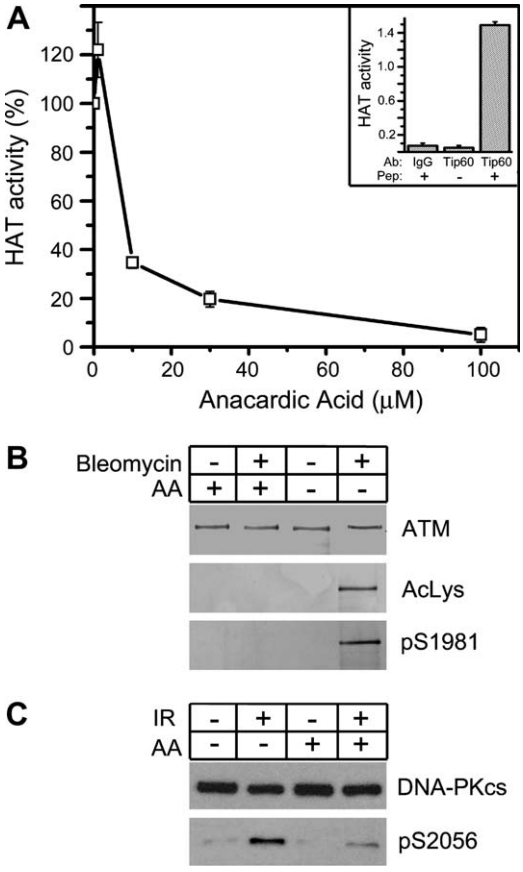

Fig. 1. Anacardic acid inhibits Tip60 and blocks activation of ATM and DNA–PKcs. (A) *Inset*. HeLa cell extracts were immunoprecipitated (Ab) with IgG or Tip60 antibody. HAT assays were carried out on washed immunoprecipitates in the absence (−) or presence (+) of the substrate peptide derived from the N-terminal of histone H4. *Graph*. Immunoprecipitated Tip60 was incubated with either solvent (DMSO) or anacardic acid for 10 min. Histone H4 substrate peptide was added, and Tip60 activity expressed as the percent decrease in activity compared to solvent controls. (B and C). 293T cells were preincubated in either DMSO (−) or anacardic acid (30 μM: +) for 30mins. Cells were then treated with bleomycin (5 μM) or ionizing radiation (IR: 2 Gy) as indicated. Reactions were terminated 40 min later and cell extracts analyzed by western blot for ATM protein levels, ATM acetylation (AcLys), ATM autophosphorylation (pS1981), DNA–PKcs and DNA–PKcs autophosphorylation of serine 2056 (pS2056).

Several previously described HAT inhibitors are not cell permeable, and therefore cannot be utilized for cell based studies [11]. Therefore, we examined the ability of anacardic acid to inhibit Tip60 functions in vivo. Tip60's HAT activity is required for the activation of 2 key regulators of the cells response to DNA damage – the ATM and DNA–PKcs protein kinases [6,14]. Tip60 acetylates ATM, activating ATM's kinase activity [6,14], and increasing the autophosphorylation of ATM on serine 1981. ATM activation can, therefore, be monitored with antibodies which detect the acetylation and autophosphorylation of the ATM protein. In Fig. 1B, cells were expose to the radiomimetic agent bleomycin, which generates DNA-strand breaks. ATM was both acetylated and autophosphorylated when cells were exposed to bleomycin. Further, prior treatment with anacardic acid inhibited both the Tip60-dependent acetylation and activation of the ATM protein kinase. DNA–PKcs is also activated in a Tip60-dependent manner [14], and this activation can be monitored through autophosphorylation of serine 2056 [16,17]. Fig. 1C demonstrates rapid autophosphorylation of serine 2056 of DNA–PKcs in response to ionizing radiation. Anacardic acid significantly reduced the autophosphorylation of serine 2056, consistent with previous reports that activation of DNA–PKcs by DNA damage is partly dependent on the Tip60 HAT [14]. Fig. 1 therefore demonstrates that anacardic acid inhibits both Tip60 HAT activity in vitro, and Tip60 dependent signaling pathways in vivo.

The Tip60-dependent activation of ATM is required for cells to survive exposure to ionizing radiation [6]. This implies that inhibition of the Tip60-ATM pathway by anacardic acid should increase cellular sensitivity to ionizing radiation. Initially, potential cytotoxic effects of anacardic acid treatment were examined. HeLa cells were exposed to anacardic acid for 4hr (Fig. 2A), and cell viability examined using a colony forming assay. Anacardic acid at up to 10 μM did not significantly affect cell viability (Fig. 2A: control), although at higher concentrations there was a small decrease in cell survival. In Fig. 2B, cells were irradiated in the absence or presence of increasing concentrations of anacardic acid. In the absence of anacardic acid, irradiation decreased cell viability to 0.16 (Fig. 2B). When cells were irradiated in the presence of anacardic acid, a significant decrease in cell survival was seen, with maximal radiosensitization between 30 and 100 μM. The higher concentrations of anacardic acid required to increase radiosensitivity in vivo (30–100 μM: Fig. 2B) compared to the inhibition of Tip60 HAT activity in vitro (10–30 μM: Fig. 1A) presumably reflect the uptake and distribution of anacardic acid within the cell. The HAT inhibitor anacardic acid is therefore an effective radiosensitizer of cells which displays minimal cytotoxicity. Note that the surviving fractions in Fig. 2B are corrected for the small decrease in viability caused by exposure to anacardic acid alone (described in the legend to Fig. 2B).

To further characterize the ability of anacardic acid to sensitize cells to ionizing radiation, several tumor cell lines, including HeLa cells (cervical adenocarcinoma) and 2 relatively radioresistant squamous carcinoma cell lines, SQ20B and SCC35 [18] were analyzed. HeLa cells exhibited a 3-fold

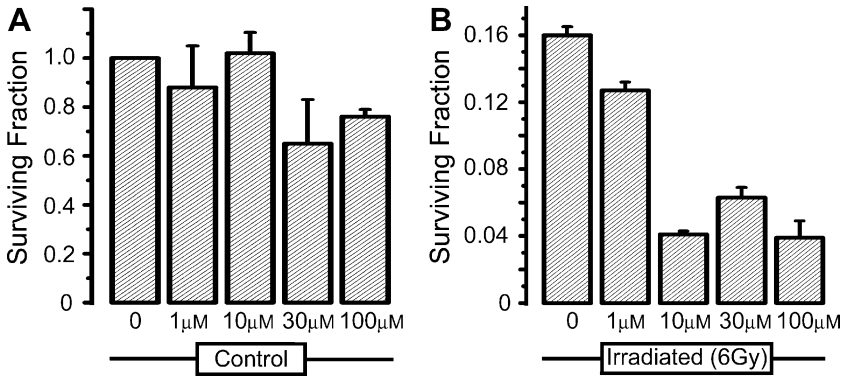

Fig. 2. Anacardic acid sensitizes HeLa cells to radiation. (A). HeLa cells were incubated in DMSO (0.1%) or anacardic acid for 4 h. The cells were then switched to fresh media, allowed to grow for 10 days, and surviving cells assessed using a colony formation assay. (B). HeLa cells were incubated in DMSO (0.1%) or anacardic acid for 30 min and then irradiated (6 Gy). 3.5 h post-irradiation, cells were switched to fresh media, and surviving cells measured using a colony formation assay. To control for the low levels of toxicity exhibited by anacardic acid at higher concentrations (control), the surviving fraction of irradiated cells was calculated using the following formula. Surviving fraction = [number of colonies with AA + IR]/[number of colonies with AA], where AA = anacardic acid, IR = ionizing radiation. Results ± S.D. ($n = 4$).

increase in radiosensitivity when exposed to anacardic acid at all doses of ionizing radiation tested (Fig. 3A). In contrast, slightly lower levels of radiosensitization by anacardic acid were detected in the SQ20B and SCC35 cell lines, with an average of 2-fold increase in radiosensitivity. The sensitizer enhancement ration (SER) for each cell line is shown in Table 1. Both HeLa and SQ20B cells showed significant sensitization at both low (2 Gy) and high (6 Gy) doses, whereas SCC35 showed a small effect at 2 Gy (SER = 1.5 ± 0.2) compared to 6 Gy (SER = 8.0 ± 2.0). The radiosensitization observed with anacardic acid is less than that achieved through genetic inactivation of Tip60 [6]. This difference may reflect the transient suppression of the ATM/DNA–PKcs DNA repair pathways by anacardic acid compared to the complete inhibition achieved by genetic methods. Further, anacardic acid treated cells retain residual DNA–PKcs and Tip60 activity (Fig. 1), which may contribute to cell survival. Overall, the HAT inhibitor anacardic acid was an effective sensitizer of cells to the cytotoxic effects of ionizing radiation.

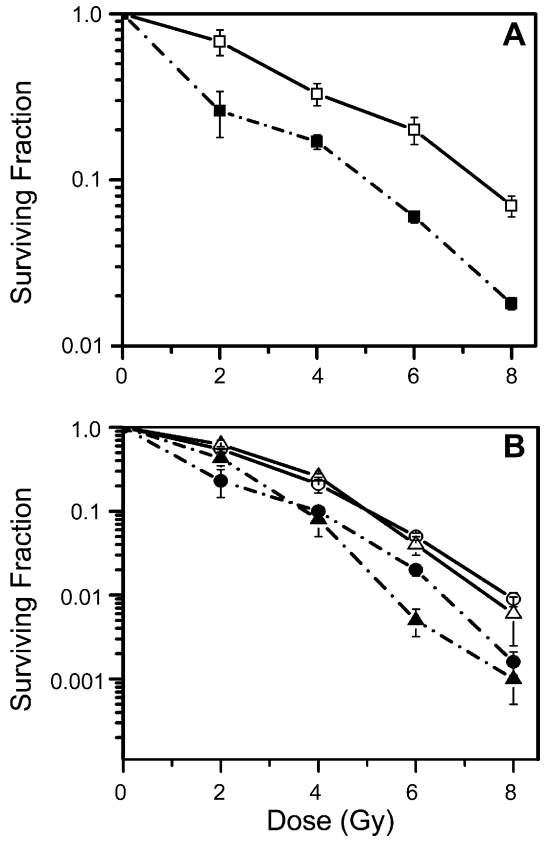

Fig. 3. Anacardic acid sensitizes tumor cells to the cytotoxic effects of ionizing radiation. (A) HeLa cells (squares) or (B) SCC35 (circles) or SQ20B (triangles) cells were incubated with DMSO (0.1%: open symbols) or anacardic acid (30 μM: filled symbols) and then irradiated. 4 h later cells were switched to fresh medium, and the surviving fraction measured 10 days later by colony formation assay. Results ± S.D. ($n = 4$).

4. Discussion

Anacardic acid inhibited the Tip60-dependent acetylation and activation of the ATM protein kinase in HeLa cells, and sensitized the cells to the cytotoxic effects of radiation. This is consistent with the observation that genetic inactivation of either Tip60 [6] or ATM [19] increases the sensitivity of cells to DNA damage. Further, the ability of anacardic acid to inhibit Tip60 signaling pathways in vivo demonstrates that anacardic acid can cross the cell membrane. This is in contrast to other HAT inhibitors, including peptide-CoA conjugates [11], which are not cell permeable. These results therefore demonstrate the utility of anacardic acid as an in vivo HAT inhibitor.

Table 1
SER for anacardic acid

Cell line	SER*	
	2 Gy	6 Gy
HeLa	2.6 ± 0.4	3.0 ± 0.6
SQ20B	2.4 ± 0.2	2.5 ± 0.3
SCC35	1.5 ± 0.2	8.0 ± 2.0

*SER = sensitizer enhancement ratio at 30 μM anacardic acid. Results ± S.D. ($n = 4$).

Previous studies have shown that anacardic acid inhibits the PCAF and p300 HATs in vitro, with maximal inhibition at 15 μM anacardic acid [13]. Here, we demonstrate that anacardic acid is also an effective inhibitor of the Tip60 HAT in vitro, with maximal Tip60 inhibition occurring between 10-30 μM anacardic acid (Fig. 1). PCAF, p300 and Tip60 belong to distinct HAT sub-families [1,2], with distinct catalytic mechanisms [20]. In addition, anacardic acid has structural similarities to acetyl-CoA (the acetyl donor for HATs), and may inhibit binding of acetyl-CoA to the active site of HATs. Anacardic acid is therefore a relatively non-specific HAT inhibitor, with the potential to inhibit multiple HATs. Exposure to anacardic acid is likely to block global cellular HAT activity, resulting in decreased acetylation of both histones and other cellular proteins. Since the acetylation of the N-terminal tails of histones is associated with an open chromatin structure and active gene transcription [4], global inhibition of cellular HAT activity by anacardic acid will lead to decreased histone acetylation, a more compacted chromatin structure and reduced transcriptional activity. The combination of these processes may account for the toxicity of anacardic acid seen at higher concentrations.

It has been clearly established that activation of the ATM protein kinase is required for cells to repair and survive exposure to ionizing radiation [21]. The ability of anacardic acid to inhibit Tip60 activity and the subsequent activation of ATM may therefore make a significant contribution to the radiosensitizing effects of anacardic. However, given the non-specific nature of anacardic acid, including its ability to inhibit PCAF and p300 [13], a significant contribution from altered histone (or other protein) acetylation patterns to the observed radiosensitizing effects of anacardic acid cannot be excluded.

In conclusion, these experiments provide proof of principle that inhibition of cellular HAT activity can lead to increased sensitivity to the cytotoxic effects of ionizing radiation. Similar to the use of HDAC inhibitors, the disruption of the cellular protein/histone acetylation code by HAT inhibitors can be exploited to develop new anti-cancer strategies. These results have important implications for the development of compounds which can enhance the efficacy of radiation therapy in a clinical setting. Further, HAT inhibitors can be used to probe the role of global histone and protein acetylation in cellular functions, and provide starting point for the development of more specific inhibitors of individual HATs.

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