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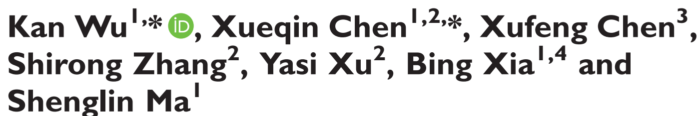
Suberoylanilide hydroxamic acid enhances the radiosensitivity of lung cancer cells through acetylated wild-type and mutant p53-dependent modulation of mitochondrial apoptosis



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Abstract

Objective: Suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor, has shown potential as a candidate radiosensitizer for many types of cancers. This study aimed to explore the radiosensitization mechanism of SAHA in lung cancer cells.

Methods: Mutations in p53 were generated by site-directed mutagenesis using polymerase chain reaction. Transfection was performed to generate H1299 cells carrying wild-type or mutant p53. The radiosensitizing enhancement ratio was determined by clonogenic assays. Mitochondrial apoptosis was detected using JC-1 staining and flow cytometry analysis.

Results: Our results showed that SAHA induced radiosensitization in H1299 cells expressing wild-type p53, $p53^{R175H}$ or $p53^{P223L}$, but this enhanced clonogenic cell death was not observed in

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parental H1299 (p53-null) cells or H1299 cells expressing p53 with K120R, A161T and V274R mutations. In SAHA-sensitized cells, mitochondrial apoptosis was induced following exposure to irradiation. Additionally, we observed that a secondary mutation at K120 (K120R) could eliminate p53-mediated radiosensitization and mitochondrial apoptosis.
Conclusions: The results of this study suggest that wild-type and specific mutant forms of p53 mediate SAHA-induced radiosensitization by regulating mitochondrial apoptosis, and the stabilization of K120 acetylation by SAHA is the molecular basis contributing to radiosensitization in lung cancer cells.

Keywords
Non-small cell lung cancer, radiosensitization, suberoylanilide hydroxamic acid, p53, apoptosis, acetylation

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Introduction

Lung cancer is the leading cause of cancer-related death worldwide.[1] Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all cases, and up to one-third of these patients have unresectable locally advanced disease at diagnosis (stage IIIA–IIIB). For these patients, radical radiotherapy is the primary treatment modality. However, because of the intrinsic radiation resistance of lung cancer cells, the disease control and survival rates of patients who receive radiotherapy remain unsatisfactory. Therefore, the identification of new therapeutic targets and/or drugs to enhance the effects of radiation is crucial.

The tumor suppressor protein p53 regulates radiosensitivity by activating apoptosis.[2] Furthermore, wild-type (wt) p53 can increase the sensitivity of cancer cells to radiation, whereas the results for p53 mutants remain controversial.[3] p53 acts as a transcription factor and controls a series of downstream targets through a variety of post-translational modifications, including acetylation. Several acetylation sites in p53 were found to play a positive role in regulating the function of p53. Histone acetylation is a reversible process. Histone acetyltransferases transfer the acetyl moiety from acetyl coenzyme A to lysine, and histone deacetylases remove the acetyl groups, thereby re-establishing a positive charge in the histones.[4] Recent studies have shown that NSCLC patients may benefit from epigenetic drugs,[5,6] and histone deacetylase inhibitors (HDIs) have shown promise as candidate radiosensitizers for many types of cancers, including lung cancer.[7–9] However, the sensitization mechanism is not well understood. Suberoylanilide hydroxamic acid (SAHA), a safe and broad-spectrum HDI, has been demonstrated to restore the native conformation of wt p53.[10–12] The aim of the study was to elucidate the p53 (wt or mutant)-based mechanism involved in the radiosensitization of H1299 cells treated with SAHA.

Material and methods

Cell culture and reagents

Human H1299 cells were obtained from the American Type Culture Collection and

maintained in RPMI-1640 medium (Gibco, Waltham, MA, USA) containing 10% fetal bovine serum (Gibco, Waltham, MA, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). SAHA was obtained from Sigma-Aldrich (St. Louis, MO, USA). Lipofectamine 2000 and the MitoProbe™ JC-1 Assay Kit were purchased from Invitrogen (Carlsbad, CA, USA). The Q5® Site-Directed Mutagenesis Kit was purchased from New England Biolabs (Ipswich, MA, USA). Ethical approval was not applied for because this study did not involve patients or animals.

Site-directed mutagenesis and transfections

Full length wt p53 cDNA was inserted into pcDNA3.1-C at BamHI and EcoR V sites, and this plasmid was used as a template for generating p53 mutations ($p53^{K120R}$, $p53^{A161T}$, $p53^{A161T+K120R}$, $p53^{R175H}$, $p53^{R175H+K120R}$, $p53^{V274P}$, $p53^{V274P+K120R}$, $p53^{P223L}$, $p53^{P223L+K120R}$ $p53^{R273H}$ and $p53^{R273H+K120R}$) by site-directed mutagenesis. All p53 plasmid constructs were then confirmed by sequencing. Transfection was performed with Lipofectamine 2000 following the manufacturer's instruction. Stable transfectants were then selected by G418 *in vitro*.

Clonogenic assay

To evaluate radiosensitivity, cells grown as monolayers in standard 6-well plates or 60-mm tissue culture plates were treated with 500 nM SAHA [final dimethylsulfoxide (DMSO) concentration $<0.1\%$) or 0.1% DMSO as a control. Twelve hours later, cells were exposed to irradiation (0, 1, 2, 4, 6 and 8 Gy). Irradiated cells were maintained in SAHA-containing medium for 10 to 14 days. The cells were fixed with 70% ethanol and stained with 0.5% crystal violet (Sigma, St. Louis, MO, USA). Colonies containing >50 cells were counted as surviving colonies, and the number of colonies was normalized to that observed for unirradiated controls. The survival curves were fitted using the single-hit multi-target model in GraphPad Prism 8.0 (GraphPad Software Inc., La Jolla, CA, USA). Then, the mean lethal dose (D0), quai−threshold (Dq) and survival fraction at 4 Gy (SF4) were obtained based on the clonogenic assay. Mean inactivation doses were determined using the method reported by Fertil et al.,[13] and the sensitizer enhancement ratio (SER) for SAHA treatment was calculated as the ratio of the mean inactivation $dose_{control}$/mean inactivation $dose_{SAHA\text{-}treated}$.

Mitochondrial membrane potential (MMP) assay

Cells were treated with 500 nM SAHA or 0.1% DMSO as a control and exposed to irradiation (0 and 2 Gy) 12 hours later. The MMP was analyzed by JC-1 staining in accordance with the manufacturer's instruction. Briefly, 1×10^6 cells were collected, suspended in fresh medium and stained with 2 μM of JC-1 for 15 minutes. Fluorescence was assessed using flow cytometry by measuring both the monomer (527-nm emission; green) and J-aggregate (590-nm emission; red) forms of JC-1 following 488-nm excitation. The percentage of the monomeric form (indicated by green fluorescence) was then quantified as the MMP.

Bioinformatics model

We developed a bioinformatics model using computational protein-protein docking (HawkDock server) to illustrate p53 acetylation and p53-Bax interactions in accordance with previously published methods.[14]

Statistical analysis

GraphPad Prism 8.0 software was used for statistical analyses. Data were presented as the mean ± standard deviation. Statistical analyses were performed using Student's t-test, and a P value <0.05 was considered significant.

Results

SAHA enhances the radiosensitivity of H1299 cells expressing wt and specific p53 mutants

The results from clonogenic survival assays showed that SAHA enhanced radiosensitization in wt p53 H1299 cells (Figure 1a). We calculated survival parameters with the single-hit multi-target statistical model SF = 1–(1exp(–D/Do))^N using GraphPad Prism 8.0 and found that SF4, D_0 and Dq were significantly lower in the SAHA-treated group than that in the control group (P <0.001), and the SER was 1.789. Similar results were observed for the colony forming ability of the SAHA-treated group versus the control group for H1299 cells expressing $p53^{P223L}$ or $p53^{R175H}$ mutants, with the SER equal to 1.391 and 1.420, respectively. However, no enhancement effects were detected by clonogenic cell death analysis in parental H1299 (p53-null) cells or H1299 cells expressing K120R, A161T or V274R p53 mutations (Table 1, Figure 1b).

The mutation spectrum of p53 for the p53-mediated enhancement of radiation-induced mitochondrial apoptosis by SAHA in H1299 cells

We detected significantly increased MMP in H1299 cells that were treated with SAHA and exposed to irradiation when cells expressed wt p53, $p53^{R175H}$ or $p53^{P223L}$ (P <0.05). However, no enhancement was observed in parental H1299 (p53-null) cells or H1299 cells with A161T, V274R or R273H p53 mutations (Figure 2). Interestingly, we found that a secondary mutation at K120 (K120R) could abolish the effects of SAHA on p53-mediated radiosensitization and MMP induction in irradiated cells expressing wt p53 or $p53^{R175H}$ (P <0.001) (Figure 3). Using computational protein-protein docking, we generated a bioinformatics model of p53 acetylation and p53-Bax interactions for the spectrum of p53 mutations (Figure 4).

Discussion

Radiation therapy is commonly used for lung cancer patients with either curative or palliative disease. However, the intrinsic radioresistance of cancer cells limits the efficacy of radiotherapy. Hence, elucidating the mechanism of radioresistance in lung cancer cells is urgently needed.

p53 plays crucial roles in regulating the therapeutic sensitivity of cancer cells. p53 can activate/deactivate gene expression at the transcriptional level or regulate protein-protein interactions, subsequently modifying the signaling pathways involved in cell growth arrest or apoptosis at the post-transcriptional level. However, these regulatory functions of p53 are often compromised when p53 is mutated.[15]

Post-translational protein modifications, such as phosphorylation and acetylation, have been demonstrated to be indispensable for the ability of p53 to regulate gene expression and downstream signaling pathways.[16,17] Interestingly, studies have shown that acetylation restored the native conformation of wt p53 protein and activated transcription-independent apoptotic signaling, partially through enhanced p53-Bax interactions and mitochondrial pathway activation. However, the downstream apoptotic signaling of mutant p53 after

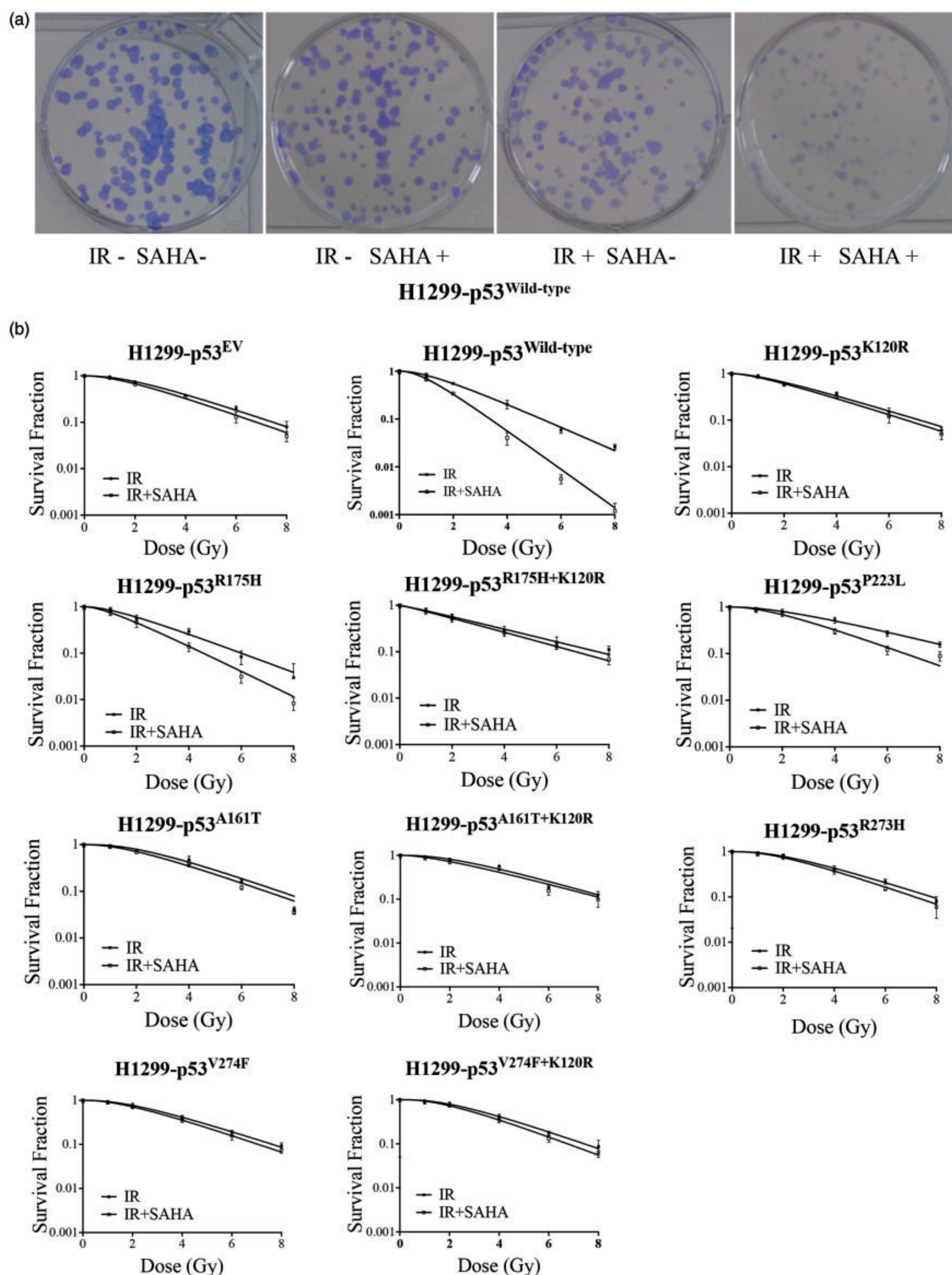

Figure 1. Effect of SAHA on the clonogenic survival of irradiated H1299 cells expressing wt and mutant p53. (a) Representative images showing the colony formation of irradiated (2 Gy) H1299 cells expressing wt p53 following pretreatment with 0.1% DMSO or 500 nM SAHA. (b) Clonogenic cell survival curves of irradiated H1299 cells expressing wt or mutant forms of p53 following pretreatment with 500 nM SAHA or 0.1% DMSO as the control. Data represent the average of three experiments. Error bars indicate the standard deviation.
wt, wild-type; EV, empty vector; IR, ionization radiation; SAHA, suberoylanilide hydroxamic acid; DMSO, dimethylsulfoxide.

Table 1. Radiosensitive effects of SAHA on H1299 cells carrying wild-type or different p53 mutants.

Cell line	Treatment	D0	Dq	α	β	SF4	SER
H1299-EV	DMSO (0.1%)	2.346	2.139	0.125	0.024	0.380 ± 0.012	
	SAHA (500 nM)	2.257	1.679	0.161	0.027	0.350 ± 0.022	1.145
H1299-p53$^{Wild\text{-}type}$	DMSO (0.1%)	1.763	1.265	0.331	0.017	0.203 ± 0.040	
	SAHA (500 nM)	1.076	0.922	0.660	0.026	0.040 ± 0.012**	1.789
H1299-p53^{K120R}	DMSO (0.1%)	2.535	1.385	0.174	0.022	0.370 ± 0.030	
	SAHA (500 nM)	2.402	1.171	0.221	0.020	0.320 ± 0.022	1.095
H1299-p53^{A161T}	DMSO (0.1%)	2.198	2.472	0.004	0.049	0.471 ± 0.090	
	SAHA (500 nM)	2.198	1.935	0.085	0.042	0.395 ± 0.062	1.138
H1299-p53$^{A161T+K120R}$	DMSO (0.1%)	2.670	2.562	0.098	0.022	0.566 ± 0.022	
	SAHA (500 nM)	2.889	1.749	0.145	0.020	0.497 ± 0.047	1.112
H1299-p53^{R175H}	DMSO (0.1%)	2.058	1.314	0.216	0.029	0.294 ± 0.048	
	SAHA (500 nM)	1.588	0.927	0.382	0.028	0.137 ± 0.030**	1.391
H1299-p53$^{R175H+120R}$	DMSO (0.1%)	3.152	0.329	0.327	-0.006	0.295 ± 0.058	
	SAHA (500 nM)	2.803	0.300	0.330	0.001	0.256 ± 0.040	1.179
H1299-p53^{V274F}	DMSO (0.1%)	2.366	2.266	0.130	0.022	0.410 ± 0.042	
	SAHA (500 nM)	2.262	1.927	0.176	0.019	0.353 ± 0.037	1.104
H1299-p53$^{V274F+K120R}$	DMSO (0.1%)	2.203	2.426	0.121	0.024	0.414 ± 0.052	
	SAHA (500 nM)	2.061	2.075	0.177	0.022	0.341 ± 0.040	1.135
H1299-p53^{R273H}	DMSO (0.1%)	2.408	2.358	0.078	0.030	0.423 ± 0.063	
	SAHA (500 nM)	2.232	2.086	0.123	0.029	0.378 ± 0.060	1.121
H1299-p53^{P223L}	DMSO (0.1%)	3.110	2.395	0.098	0.017	0.523 ± 0.073	
	SAHA (500 nM)	2.154	1.780	0.269	0.006	0.300 ± 0.040*	1.420

*$P < 0.05$, **$P < 0.01$ (Student's t-test). EV; empty vector; SAHA, suberoylanilide hydroxamic acid; D0, mean lethal dose; Dq, quai-threshold; SF4, survival fraction at 4 Gy; SER, sensitizer enhancement ratio.

HDI-induced acetylation varied between different p53 non-lysine mutations.[10–12] Moreover, although the acetylation sites on p53 appear to be redundant as the loss of some of these sites can be largely compensated for by the acetylation of other sites, a previous study demonstrated that the combined loss of major acetylation sites in p53 partially rendered it transcriptionally inactive.[18] Therefore, not only are the mutation sites of p53 lysine residues crucial for acetylation and its protein conformation important for its interaction with other proteins, but the mutation of non-lysine residues may also affect the conformation of mutant p53 itself and subsequently alter the downstream signaling of acetylated p53.

In this study, we aimed to investigate the potential mutation spectrum of p53 that could be used for screening patients to identify those that might benefit from treatment with a combination of HDIs. The p53 mutations used in this study were selected based on the mutation frequency of the *p53* gene according to the National Center for Biotechnology Information website (http: //p53.free.fr/Database/p53_cancer/p53_Lung.html), and these mutations are hotspot p53 mutations detected with high frequency in lung cancer patients. The results presented here clearly showed that SAHA could induce radiosensitization in lung cancer cells expressing wt or p53$^{R175H/P223L}$ mutants, but no radiosensitization effects were detected in cells expressing p53 with A161T, V274R or R273H mutations. Most importantly, we found that SAHA pretreatment did not

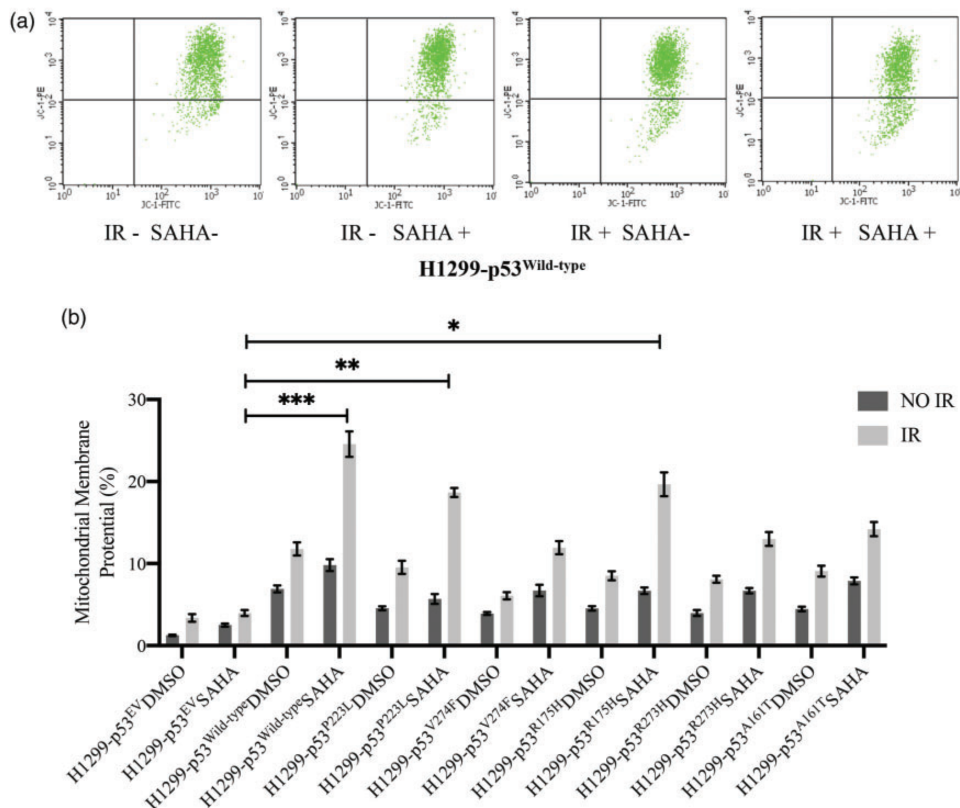

Figure 2. Effect of SAHA on the mitochondrial apoptotic response to irradiation in H1299 cells bearing wt and mutant p53. (a) Representative images showing the mitochondrial apoptotic response of H1299 cells expressing wt p53 that were pretreated with 0.1% DMSO or 500 nM SAHA and then exposed to radiation (2 Gy). (b) Mitochondrial apoptosis rate of H1299 cells expressing wt or single p53 point mutations that were pretreated with 0.1% DMSO or 500 nM SAHA and then exposed to radiation (2 Gy). Data represent the average of three experiments. Error bars indicate the standard deviation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Student's t-test).
wt, wild-type; EV, empty vector; IR, ionization radiation; SAHA, suberoylanilide hydroxamic acid; DMSO, dimethylsulfoxide.

induce radiosensitization in p53-deficient control lung cancer cells. Thus, these results indicate that the radiosensitization effects of SAHA are medicated by wt p53 or specific p53 mutants.

p53 acetylation is primarily controlled by acyltransferases (CBP/p300/PCAF/Tip60) and deacetylases (HDAC1/SiRT1). For example, Tip60 and MOZ are involved in the acetylation of lysine residues at 120 (K120R), which was found to be important for p53 regulation of cell survival processes, such as DNA damage and cellular senescence.[18–20] Of interest, our data showed that mutagenesis at K120 in $p53^{R175H/P223L}$ abolished the radiosensitization effects of SAHA, suggesting a role of acetyltransferases in the SAHA-mediated radiosensitization in cells bearing mutant p53.

Based on these findings and observations, we presented a novel bioinformatics

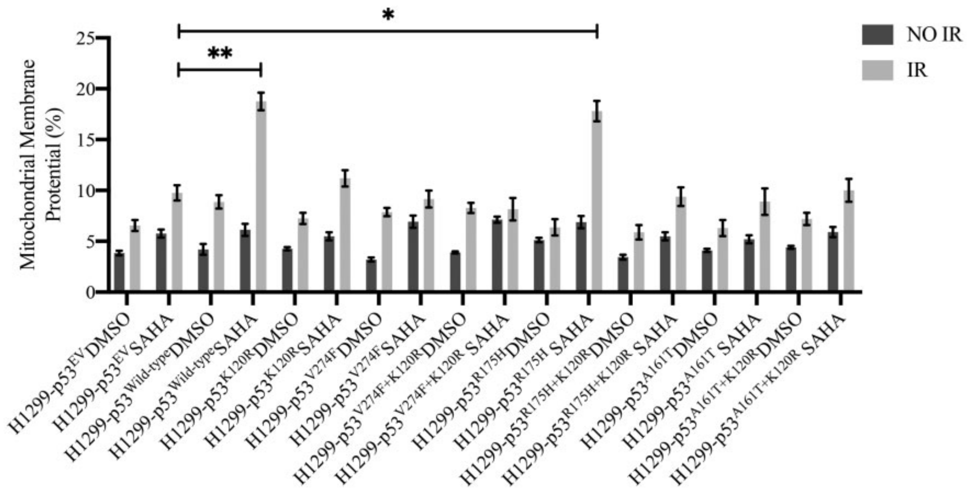

Figure 3. Effect of SAHA on the mitochondrial apoptotic response in H1299 cells expressing p53 with or without K120R. Mitochondrial apoptosis rate of H1299 cells expressing wt or mutated p53 with or without K120R that were pretreated with 0.1% DMSO or 500 nM SAHA and then exposed to radiation (2 Gy). Data represent the average of three experiments. Error bars indicate the standard deviation. * $p < 0.05$,** $p < 0.01$ (Student's t-test).
wt, wild-type; EV, empty vector; IR, ionization radiation; SAHA, suberoylanilide hydroxamic acid; DMSO, dimethylsulfoxide.

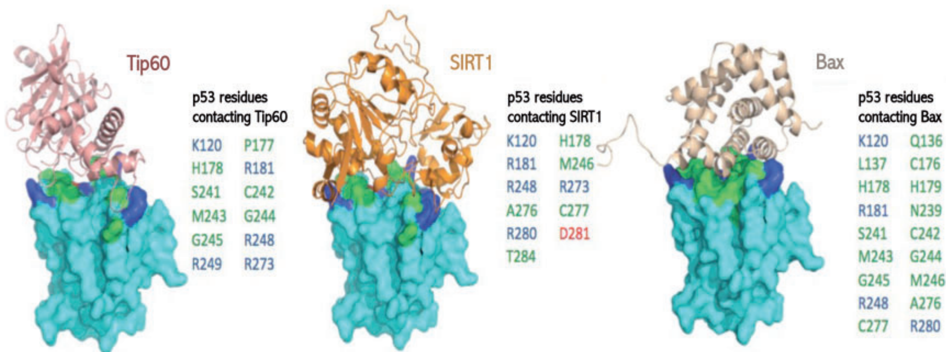

Figure 4. A p53 acetylation and p53-Bax interaction bioinformatics model. A bioinformatics model for the potential interactions of p53 mutants with acetyltransferases and Bax in human lung cancer cells following SAHA-induced radiosensitization. The p53 mutation sites shown on the right side of each interaction are predicted residues that may affect the protein-protein interactions.
Tip60, TAT-interactive protein 60 kDa; SIRT1, sirtuin 1; Bax, Bcl-2 associated X-protein.

model generated with computational protein-protein docking to illustrate p53 acetylation and p53-Bax interactions for the spectrum of p53 mutations that may mediate the mutant p53-enhanced therapeutic response of lung cancer cells to irradiation. In this model, we also included the potential p53-SIRT1 complex, an

interaction that may control p53 nuclear signaling involved in transcriptional regulation or mitochondrial signaling required for p53-Bax complex formation.[21,22] In the future, we plan to use this bioinformatics model to identify and validate the p53 mutations as therapeutic targets for radiotherapy combined with SAHA for lung cancer patients.

In conclusion, we demonstrated that the HDI inhibitor SAHA could induce p53-dependent radiosensitization in lung cancer cells. SAHA also exhibited the ability to restore the functions of mutant p53 in the regulation of mitochondrial apoptotic signaling in cancer cells in response to irradiation treatment. However, this restoration is dependent on the mutation site of p53, and K120 is an important lysine residue that may control the fate of p53 signaling pathways in cells treated with the combination of SAHA and irradiation. The bioinformatics model established in this study may provide a tool for identifying the mutation spectrum of p53 and screening patients that might benefit from a potential novel radiotherapeutic strategy. The limitation of the current study is that the *in vitro* environment only partially mimics the *in vivo* environment. Thus, further studies are required to confirm our results.

Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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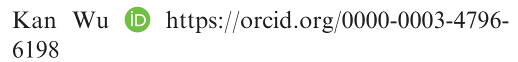
Kan Wu  https://orcid.org/0000-0003-4796-6198

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