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Histone deacetylase inhibitor AR42 regulates telomerase activity in human glioma cells via an Akt-dependent mechanism

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ABSTRACT

Epigenetic regulation via abnormal activation of histone deacetylases (HDACs) is a mechanism that leads to cancer initiation and promotion. Activation of HDACs results in transcriptional upregulation of human telomerase reverse transcriptase (hTERT) and increases telomerase activity during cellular immortalization and tumorigenesis. However, the effects of HDAC inhibitors on the transcription of *hTERT* vary in different cancer cells. Here, we studied the effects of a novel HDAC inhibitor, AR42, on telomerase activity in a PTEN-null U87MG glioma cell line. AR42 increased *hTERT* mRNA in U87MG glioma cells, but suppressed total telomerase activity in a dose-dependent manner. Further analyses suggested that AR42 decreases the phosphorylation of hTERT via an Akt-dependent mechanism. Suppression of Akt phosphorylation and telomerase activity was also observed with PI3K inhibitor LY294002 further supporting the hypothesis that Akt signaling is involved in suppression of AR42-induced inhibition of telomerase activity. Finally, ectopic expression of a constitutive active form of Akt restored telomerase activity in AR42-treated cells. Taken together, our results demonstrate that the novel HDAC inhibitor AR42 can suppress telomerase activity by inhibiting Akt-mediated hTERT phosphorylation, indicating that the PI3K/Akt pathway plays an important role in the regulation of telomerase activity in response to this HDAC inhibitor.

1. Introduction

Telomeres are repetitive TTAGGG nucleotide sequences located at the ends of chromosomes that protect chromosome ends from degradation and end-to-end fusions [1]. As DNA polymerases are incapable of replicating to the very end of the telomere, the length of telomeres is constitutively shortened in most somatic cells during each round of chromosomal DNA replication [2]. Once telomeres are shortened to a critical length, growth arrest or replicative senescence is induced, thereby limiting the replication potential of cells. To overcome this finite proliferative potential, a specialized ribonucleoprotein polymerase, telomerase, with *de novo* telomeric repeat synthesis activity is involved in repairing the chromosome ends and preventing cells from senescing. Because of its role in sustaining continuous cell division and preventing replicative senescence, telomerase is considered to be a potential factor in the acquisition of cellular immortality as well as in carcinogenesis [3]. Increasing evidence demonstrates that telomerase activity is increased in the majority of human cancers relative to non-malignant tissues.

Telomerase is a ribonucleoprotein complex composed of a catalytic subunit, human telomerase reverse transcriptase (hTERT), an RNA molecule human telomerase RNA component (hTERC), and other telomerase-associated proteins [4,5]. The RNA component hTERC serves as template for the synthesis of telomeric repeats. The protein subunit hTERT catalyzes the reaction and is the key determinant of telomerase activity [6]. As expression of *hTERT* has been shown to be characteristically higher in most malignant tumors, *hTERT* is suggested to be involved in tumorigenesis [7,8]. In normal cells, the transcription of *hTERT* can be regulated by chromatin remodeling through the reversible acetylation of histone tails [9]. However, the effects of histone-deacetylase

Abbreviations: TRAP, telomeric repeat amplification protocol; HDAC, histone deacetylase; PI3K, phosphatidylinositol 3-kinase; RT-PCR, reverse transcriptase-polymerase chain reaction.

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(HDAC) inhibitors on *hTERT* transcription vary in different cancer cell lines [10]. While telomerase activity was significantly reduced by treatment with sodium butyrate and trichostatin A (TSA), the level of *hTERT* mRNA was not always altered by these HDAC inhibitors [11]. These findings imply that another post-transcriptional mechanism may also be involved in the regulation of telomerase activity by HDAC inhibitors.

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway plays an important role in the regulation of cell growth, viability, motility, and metabolism [12]. Aberrant activation of this pathway via somatic mutation and gene amplification of its key components has been observed in many human cancers. Reports have indicated that protein kinase B (PKB/Akt) and other kinases can regulate telomerase activity at the post-translational level through phosphorylating hTERT [13]. HDAC inhibitors can also suppress phosphorylation of Akt, decrease Akt activity and limit the growth of tumor cells [14–17]. However, whether HDAC inhibitors regulate the phosphorylation of hTERT through their effect on the PI3K/Akt pathway is still unclear.

In this study, the effect of a novel HDAC inhibitor, AR42, on telomerase activity in a PTEN-null U87MG glioma cell line was evaluated. Our results demonstrated that AR42 facilitated dephosphorylation of hTERT via the PI3K/Akt pathway while phosphatase inhibitors increased activated Akt level and restored telomerase activity in AR42-treated cells. Furthermore, ectopic expression of constitutively active Akt also rescued telomerase activity from AR42-mediated inhibition. Together, the data indicate that the dephosphorylation of Akt kinase and hTERT underlies the diverse functions of HDAC inhibitors in mediating antineoplastic activities at different cellular levels.

2. Materials and methods

2.1. Cell culture and reagents

PTEN-null human glioblastoma U87MG cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS) containing 100 units/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Grand Island, NY). The HDAC inhibitor AR42 was synthesized as previously described [18]. PP1 phosphatase inhibitors calyculin A and tautomycin were purchased from Sigma–Aldrich (St. Louis, MO) and Calbiochem (La Jolla, CA), respectively. Rabbit antibodies against Akt, p-Ser^{473}-Akt, and phospho-Akt substrate (RXRXXS*/T*) were obtained from Cell Signaling Technology (Beverly, MA). Rabbit antibody against acetyl-histone H3 and hTERT were purchased from Upstate Biotechnology (Lake Placid, NY) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Mouse monoclonal anti-β-actin antibody was from ICN Biomedicals California (Costa Mesa, CA).

2.2. Immunoprecipitation and immunoblotting

Experiments were performed as described previously [14].

2.3. Ectopic expression of constitutively active Akt

Transfection of constitutively active Akt (HA-Akt$^{T308D/S473D}$, CA-Akt) in U87MG cells was performed as described previously [19].

2.4. Telomerase activity assay

Telomerase activity of cells was evaluated using a TRAPeze ELISA Telomerase Detection Kit (Chemicon International, Inc.) according to the manufacturer's instructions. Briefly, 1.5 μg of protein extracted from 1×10^6 cells was used in each telomeric repeat amplification protocol (TRAP) reaction, and reactions were performed in a thermocycler (GeneAmp PCR system 2400, Perkin Elmer). CHAPS cell lysis buffer was used as a negative control. The PCR products were subjected to ELISA to determine the telomerase activity in each sample. All experiments were performed in triplicate.

2.5. RNA preparation and RT-PCR

Total RNA was freshly isolated using RNeasy Mini Kit (Qiagen, Inc.) and DNase I to remove contaminated DNAs. One microgram of RNA was reverse-transcribed to cDNA using Omniscript RT (Qiagen) and oligo(dT) primers according to the manufacturer's instructions. RT-PCR was performed to a minimum number of PCR cycles within the linear amplification phase. Sequence information about the primer pairs used in this study was as previously described [20] and is listed below:

hTERT, 5′-CGGAAGAGTGTCTGGAGCAA-3′ and 5′-GGATGAAGCGGAGTCTGGA-3′;
hTERC, 5′-TCTAACCCTAACTGAGAAGGGCGTAG-3′ and 5′-GTTTGCTCTAGAATGAACGGTGGAAG-3′; β-Actin, 5′-CACTGTGTTGGCGTACAGGT-3′ and 5′-TCATCACCATTGGCAATGAG-3′

2.6. Data analysis

All data were collected from three independent experiments. Statistical analysis between two values was performed by paired *t*-test. For three or more values, data was analyzed by one-way ANOVA with Dunnett adjustment. All data analysis was performed using SPSS Statistics 17.0 (SPSS, Inc.).

3. Results

3.1. Total telomerase activity is suppressed by AR42 in U87MG glioma cells

Our previous report showed that the novel HDAC inhibitor AR42 (also known as HDAC-42) inhibited the survival and proliferation of U87MG glioma cells through regulating Akt kinase activity via a histone acetylation-independent mechanism [14]. As mentioned above, HDAC inhibitors have been shown to modulate telomerase activity which can also be regulated by Akt mediated hTERT phosphorylation. To investigate whether AR42 also regulates telomerase activity, the telomerase activity of U87MG cells treated with various doses of AR42 was assessed by TRAP assay. After treatment for 48 h, the telomerase activity of AR42 treated cells was significantly inhibited in a dose-dependent manner (Fig. 1). Telomerase activity was reduced approximately 25% and 45% in cells treated with 1.0 and 2.5 μM of AR42, respectively, in comparison to control cells. These results suggest that the telomerase activity of hTERT was strongly suppressed by AR42 in U87MG glioma cells.

3.2. AR42 upregulates the transcriptional activity of hTERT in U87MG cells

To determine whether the AR42-induced suppression of telomerase activity is through a reduction in *hTERT* gene transcription, semi-quantitative RT-PCR was performed to assess changes in *hTERT* mRNA inAR42-treated U87MG cells. As shown in Fig. 2A, *hTERT* mRNA levels showed dose-dependent increases after AR42 treatment for 48 h, while levels of *hTERC* mRNA were not affected. Western blotting analysis revealed that hTERT protein levels in AR42-treated U87MG cells also increased in a dose-dependent

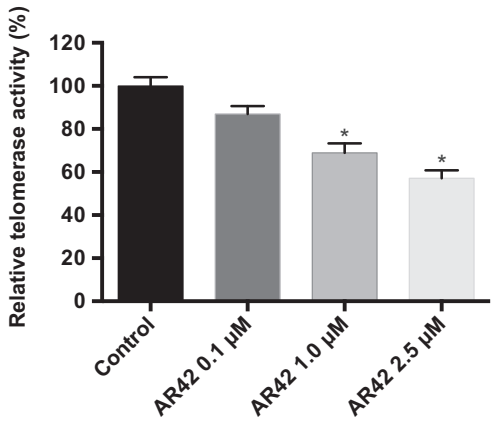

Fig. 1. AR42 reduces telomerase activity in U87MG glioblastoma cells. U87MG cells were treated with 0–2.5 μM of AR42 for 48 h and then telomerase activity was measured by TRAP assay. Telomerase activity is presented as a percentage of that of the control. Data are presented as means ± SD. Statistical analysis was performed using student *t*-tests. $^{*}P < 0.01$ versus control.

manner, in parallel to *hTERT* mRNA levels (Fig. 2B). These results strongly suggest that the reduction of telomerase activity by AR42 is unlikely to be mediated by suppression of *hTERT* transcription. This finding also suggests that HDAC inhibitors, such as AR42, may regulate the activity of hTERT at the post-transcriptional level, a result that agrees with our previous findings on the anticancer activity of HDAC inhibitors [14,21].

3.3. AR42 downregulates hTERT phosphorylation via the PI3K/Akt pathway

AR42 has been demonstrated to inhibit cell proliferation, induce cell cycle arrest, and induce apoptosis in many malignant cell types, including U87MG cells [14]. As previous reports demonstrated that AR42 can inhibit PI3K/AKT signaling in glioma, vestibular schwannoma and meningioma, hepatocellular carcinoma and prostate cancer cells [14–17,22], we hypothesized that AR42 may suppress hTERT activity through its inhibition of the PI3K/Akt pathway. To investigate whether AR42 inhibits hTERT phosphorylation in U87MG cells, hTERT of cells treated with AR42 at 2.5 μM for 48 h was immunoprecipitated with anti-hTERT antibody followed by immunoblotting with antibody specific to phospho-Akt substrate. As shown in Fig. 2C, AR42 significantly suppressed the Akt-mediated phosphorylation of hTERT in comparison to that of the control. To further validate this finding, a PI3K inhibitor, LY294002, was used to investigate the role of the PI3K/Akt signaling axis in regulating hTERT phosphorylation. As shown in Fig. 2C, treatment with LY294002 also reduced the level of phosphorylation of hTERT in a similar manner to AR42 treatment. Furthermore, histone H3 acetylation was dose-dependently increased in AR42-treated cells while the phosphorylation of Akt at Ser473 was decreased (Fig. 2D). These data suggest that the downregulation of TERT activity by AR42 is mainly through PI3K/Akt pathway-mediated hTERT phosphorylation, not by its effect on histone acetylation.

3.4. AR42-induced suppression of telomerase activity and phospho-AKT can be restored by phosphatase inhibitors

Protein phosphorylation plays a critical role in regulating a variety of cellular processes via the antagonistic actions of protein kinases and protein phosphatases. Protein phosphatase 1 (PP1) is a serine/threonine phosphatase that can dephosphorylate and decrease the activity of Akt kinase [14]. To examine whether inhibition of PP1 can modulate the effect of AR42 on Akt phosphorylation, two potent PP1 inhibitors, tautomycin and calyculin A, were used to pretreat U87MG cells for 1 h and 5 h at concentrations of 5 μM and 50 nM, respectively. Cells were then treated with 2.5 μM of AR42 for another 48 h. As shown in Fig. 3A, immunoblot data indicated that both calyculin A and tautomycin completely abolished the suppression of Akt phosphorylation by AR42. The telomerase activities of cells under the same treatment conditions were also measured with the TRAP assay. As shown in Fig. 3B,

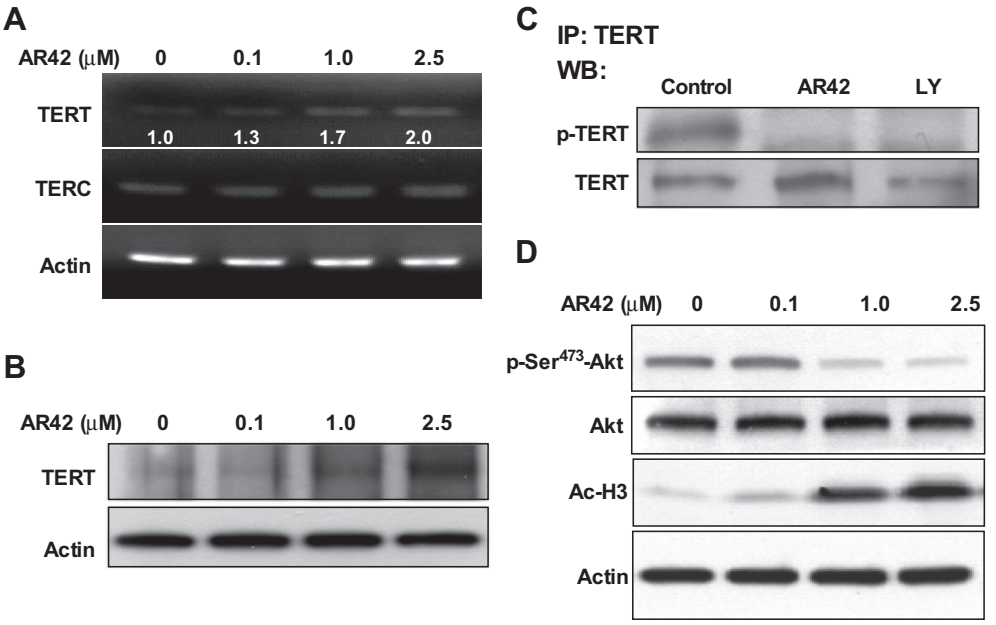

Fig. 2. AR42 increases *hTERT* mRNA and protein levels and decreases phosphorylation of Akt and hTERT in U87MG cells. U87MG cells were treated with AR42 at concentrations of 0–2.5 μM for 48 h. The levels of *hTERT* mRNA (A) and total protein (B) were determined by RT-PCR and Western blotting, respectively. Values below the TERT bands in panel A indicate relative expression levels compared to the control. (C) U87MG cells were treated with 2.5 μM of AR42 or 20 μM of LY294002 (LY) for 48 h. TERT-containing protein complexes were immunoprecipitated (IP) with anti-TERT antibody, and then immunoblotted (WB) for total (TERT) and the phospho-Akt substrate (RXRXXS*/T*) antibodies, respectively. (D) U87MG cells were treated with AR42 at the indicated concentrations for 24 h followed by immunoblotting to determine the levels of p-Ser^{473}-Akt, Akt, histone H3 acetylation (Ac-H3), and β-actin (Actin).

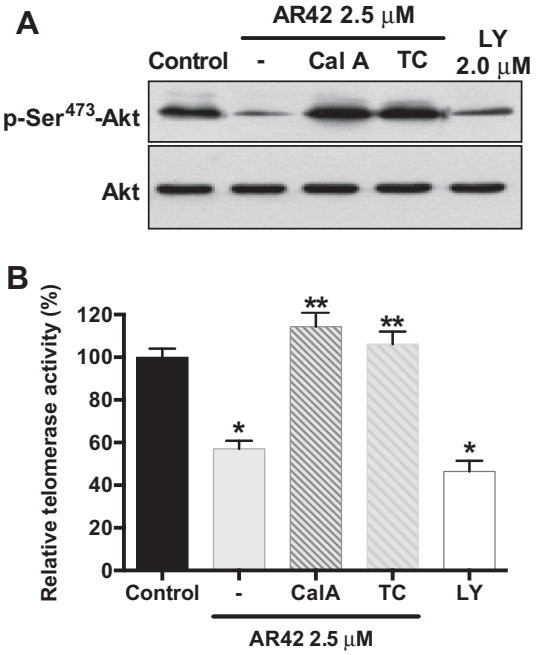

Fig. 3. PP1 inhibitors reverse AR42-induced Akt dephosphorylation and telomerase inhibition. (A) U87MG cells were pretreated with calyculin A (50 nM) or tautomycin (5.0 μM) individually for 1 h and 5 h, respectively, and then treated with 2.5 μM of AR42. After incubation for 48 h, the cell lysates were immunoblotted for p-Ser^{473}-Akt and Akt. CalA, calyculin A; TC, tautomycin; LY, LY294002. (B) Histograms representing the telomerase activities in U87MG cells treated as described in (A). Telomerase activities are presented as percentages of that of the control. Data are presented as means ± SD. Statistical analysis was performed using student *t*-tests. *indicates significant difference versus control ($P < 0.01$), **indicates significant difference versus AR42 2.5 μM ($P < 0.01$).

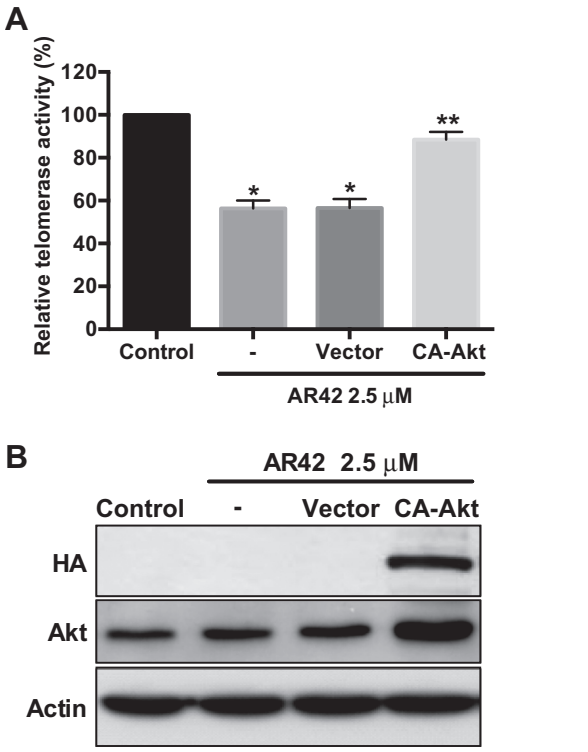

Fig. 4. Validation of the role of Akt in AR42-induced telomerase inhibition. (A) Histograms representing the telomerase activities in transfected U87MG cells treated with AR42 (2.5 μM, 48 h). Cells were transfected with plasmid encoding constitutively active Akt (CA-Akt) or the empty vector. Telomerase activities are presented as percentages of that of the vehicle-treated control. Data are presented as means ± SD. Statistical analysis was performed using student *t* tests. *indicates significant difference versus control ($P < 0.01$), **indicates significant difference versus AR42 2.5 μM ($P < 0.01$). (B) Western blot analysis of HA, Akt and β-actin in U87MG cells transfected with empty vector (Vector) or plasmids encoding HA-tagged constitutively active Akt mutant (T308D/S473D) (CA-Akt) and treated with AR42 (2.5 μM, 48 h) or vehicle.

the PP1 inhibitors restored telomerase activity in AR42-treated U87MG cells. Consistent with our previous report [14], these data suggest that the inhibitory effects of AR42 on both phospho-Akt and telomerase activity are mediated by PP1 activity.

To further confirm the role of Akt in AR42-mediated inhibition of telomerase activity, U87MG cells were transfected with a plasmid carrying hemagglutinin (HA)-tagged constitutively active mutant of Akt (CA-Akt$^{T308D/S473D}$), or with the empty vector control. Transfectants were then treated with AR42 (2.5 μM) for 48 h, and telomerase activity was measured with the TRAP assay. A marked increase in the levels of total Akt protein and HA signal in CA-Akt$^{T308D/S473D}$-transfected cells was observed indicating that the transfection was successful (Fig. 4B). The telomerase activity in control cells was inhibited by AR42 treatment; however, this drug-induced suppression of telomerase activity was significantly rescued by the ectopic expression of CA-Akt$^{T308D/S473D}$ (Fig 4A). Together, these data indicated that the suppressive effect AR42 on hTERT activity is through PP1-mediated regulation of Akt kinase activity.

4. Discussion

The effects of HDAC inhibitors on *hTERT* transcription vary according to cancer cell line. While HDAC inhibitors apparently inhibit *hTERT* transcription in some cancer cell lines such as LNCaP and PC-3 prostate cancer cells, and U937 leukemia cells [20,23], *hTERT* transcription is increased in some cervical cancer cell lines such as SiHa and HeLa, and is unchanged in others such as C33A cervical cancer cells and four different non-small cell lung cancer cells [10,24,25]. In human liver cancer cells, HDAC inhibitors have also been reported to significantly reduce telomerase activity, but the changes do not always correlate with *hTERT* mRNA level [11].

HDAC inhibitors have been shown to control the transcriptional activity of a defined set of genes in a histone acetylation-dependent manner, and also modulate signaling transducers through histone acetylation-independent mechanisms [21,26–28]. In addition, several reports have demonstrated that HDAC inhibitors induce dephosphorylation of Akt in different cancer cell lines [29,30]. Our previous study also showed that HDAC inhibitor-induced Akt dephosphorylation is partly through disruption of HDAC-PP1 complexes and reshuffling of PP1 between HDACs and Akt in U87MG cells [14].

In light of the evidence that HDAC inhibitors modulate telomerase activity, *hTERT* expression and Akt kinase activity in cancer cells, we examined whether the novel HDAC inhibitor AR42 exerts the same activity on these targets in U87MG glioma cells. We demonstrated that AR42 upregulates *hTERT* expression at the transcriptional level, but that the net telomerase activity was suppressed. Further analyses indicated that the suppression of telomerase activity is through reduction of Akt-mediated hTERT phosphorylation. The widely differing results regarding the effect of HDAC inhibition on *hTERT* transcription among different cancer cell lines suggests a complicated cellular context-dependent regulatory process. While the current study does not examine the mechanistic basis for this transcriptional regulation, different proximal promoter components of *hTERT*, such as Sp1 sites and/or E-boxes, in different cell lines may underlie these differing observations [10].

The catalytic activity of telomerase can be regulated at multiple levels. Whereas all human somatic cells constitutively express the human telomerase RNA component (hTERC), the cellular activity of telomerase is mainly determined by the presence or absence of the catalytic subunit, hTERT [31]. In addition to transcriptional regulation of *hTERT*, nuclear translocation and post-translational modification of hTERT are also involved in regulating telomerase activity. One of the post-translational modifications is phosphorylation of hTERT by kinases, such as Akt [32], Bcr-Abl [33], and PKC [34]. Our findings indicate that post-translational suppression by AR42-induced Akt inhibition is able to counteract the effects of the increase in *hTERT* transcription on telomerase activity. Moreover, our results also show that PP1 plays an important role in the inhibition of phosphorylation of Akt and hTERT by AR42. This finding is consistent with our previous work showing that PP1-mediated dephosphorylation of Akt is a histone acetylation-independent antitumor mechanism of HDAC inhibitors [14].

Telomerase activation through dysregulated *hTERT* gene expression is considered to be a critical step during cellular immortalization and malignant transformation, and thus represents a viable target for anticancer drug discovery. Our findings show that, despite *hTERT* transcription being upregulated by AR42 in U87MG glioma cells, overall telomerase activity is still suppressed. This is likely the result of reduction of hTERT phosphorylation by another histone-acetylation independent activity of AR42. In summary, this study provides new insight into the mechanisms by which HDAC inhibitors elicit coordinate changes in cellular protein phosphorylation and acetylation, and suggests that these protein modifications may contribute to the well-documented suppressive effects of HDAC inhibitors on cell growth and transformation. Moreover, we have demonstrated that AR42 is a novel HDAC inhibitor that reduces telomerase activity and hTERT phosphorylation through a PI3K/Akt-dependent pathway. The evidence presented herein suggesting that AR42 inactivates Akt through the reorganization of protein phosphatase complexes leading to inhibition of telomerase activity highlights the complexity of the pharmacological functions of HDAC inhibitors. In light of the potential clinical applications of HDAC inhibitors, a better understanding of this novel histone-independent mechanism will allow the design of more effective strategies to optimize the use of these agents in cancer treatment and/or prevention.

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