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Article

Nitric Oxide-Mediated Histone Hyperacetylation in Oral Cancer: Target for a Water-Soluble HAT Inhibitor, CTK7A

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SUMMARY

Altered histone acetylation is associated with several diseases, including cancer. We report here that, unlike in most cancers, histones are found to be highly hyperacetylated in oral squamous cell carcinoma (OSCC; oral cancer) patient samples. Mechanistically, overexpression, as well as enhanced autoacetylation, of p300 induced by nucleophosmin (NPM1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) causes the hyperacetylation, which is nitric oxide (NO) signal dependent. Inhibition of the histone acetyltransferase (HAT) activity of p300 by a water-soluble, small molecule inhibitor, Hydrazinocurcumin (CTK7A), substantially reduced the xenografted oral tumor growth in mice. These results, therefore, not only establish an epigenetic target for oral cancer, but also implicate a HAT inhibitor (HATi) as a potential therapeutic molecule.

INTRODUCTION

Chromatin is a dynamic entity that plays a critical role in nucleus-related phenomena such as transcription, replication, and repair (Carrozza et al., 2003). Posttranslational modifications of chromatin play an important role in maintaining chromatin structure-function and hence regulate gene expression, cell growth, and differentiation. Increasing evidence suggests that chromatin structure-function has a pivotal role in several disease manifestations (Thorne et al., 2009). This is evident from the fact that genetic alterations and/or a more diverse group of epigenetic changes may result in cancer development and other diseases (Thorne et al., 2009; Fraga et al., 2005; Varambally et al., 2002; Seligson et al., 2005; Das et al., 2009; Pfister et al., 2008; Esteller, 2007; Bhaumik et al., 2007).

Of the various histone acetyltransferases (HATs), the global transcriptional coactivator p300 (Debes et al., 2003) has been observed to have altered expression in a few tumors. For example, some tumors show higher levels of p300 (Debes et al., 2003; Ishihama et al., 2007), some primary tumors and cell lines exhibit mutations in p300 acetyltransferase (Gayther et al., 2000), and loss of heterozygocity at the p300 locus has been shown to be associated with colorectal, breast, and brain cancer (glioblastoma) (Gayther et al., 2000; Van Beekum and Kalkhoven, 2007). Although these data indicate the involvement of the p300 gene, the enzyme (acetyltransferase) activity has not been established as the cause of malignancy (Van Beekum and Kalkhoven, 2007).

Recently, alterations of histone modifications have been reported in different cancers. The loss of Lys 16 acetylation and Lys 20 methylation of H4 were found to be associated with primary tumors and tumor cell lines (Fraga et al., 2005). In another study, changes in bulk histone modifications of cancer cells were found to be predictive of the clinical outcome of prostate cancer (Seligson et al., 2005). However, a rare exception of histone hyperacetylation has been observed in hepatocellular carcinoma (Bai et al., 2008). Apart from cancer, dysfunction of lysine acetyltransferases has been implicated in other diseases such as inflammatory processes, Huntington's disease, cardiac disease, and diabetes (Davidson et al., 2005; Zhou et al., 2004; Rouaux et al., 2003). These observations suggest that specific and relatively nontoxic inhibitors of acetyltransferases could be considered as new generation therapeutic agents for cancer. Although several HAT inhibitors have been discovered (Selvi and Kundu, 2009; Cole, 2008) with a potential therapeutic importance in HIV and cardiac disease (Davidson et al., 2005; Mantelingu et al., 2007; Balasubramanyam et al., 2004; Morimoto et al., 2008), the effect of HAT inhibitors on cancer manifestation has not yet been tested.

The hallmark of cancer is hyperproliferative cells, which have evaded the cellular apoptotic machinery and hence, exhibit overexpressed antiapoptotic proteins. NPM1 (also known as B23) (Grisendi et al., 2006; Shandilya et al., 2009) and GAPDH (Altenberg and Greulich, 2004) are two such genes that are known to be frequently upregulated in many cancers, including oral cancer. Both of these proteins are suggested to be positive regulators of cell proliferation.

Here, we report that histone H3 is hyperacetylated in oral cancer patient samples and is positively correlated with upregulated NPM1 and GAPDH protein levels. We also present a novel mechanism to explain how hyperacetylation of H3 could be regulated by NPM1 and GAPDH in a NO-dependent manner involving p300 acetyltransferase. Furthermore, we show that a novel water-soluble HATi, CTK7A, inhibits oral tumor cell growth in nude mice.

RESULTS

Histone H3 Is Hyperacetylated in Oral Cancer

To investigate the histone acetylation status in different cancers, histones were isolated from different cell lines and subjected to immunoblotting analysis with antiacetylated histone H3 (anti-H3AcK9AcK14) antibodies. We observed that histones are predominantly hyperacetylated in oral (KB, OSCC cell) and liver (HepG2) cancer cell lines (Figure 1A). Histone hypoacetylation is a hallmark of cancer, except hepatocarcinoma (Bai et al., 2008). The enhanced H3 acetylation in the oral cancer cell line was paradoxical yet interesting. These results, therefore, led us to examine the acetylation levels of histone H3 in the tissues obtained from grade II OSCC (tumor samples from this grade were readily available) patients by immunohistochemistry (IHC) (Figure 1B; also see Table S1 available online) and immunoblotting (Figure 1C) analyses using specific antibodies. The immunostaining revealed that histone H3 (predominantly H3K14) is hyperacetylated in the cancerous tissues in comparison to the adjacent normal tissue (Figure 1B). As H3K14 is the predominant in vivo target of p300-mediated acetylation (Kouzarides, 2007), we next investigated the expression level of p300, which was found to be significantly overexpressed in the malignant tumor as compared to the adjacent normal tissue (Figure 1B). Overexpression of p300 was also confirmed by real-time RT-PCR analysis of RNA isolated from patient samples (Figure S1). Other HATs such as CBP and PCAF showed no significant change as monitored by IHC and RT-PCR analysis (Figure 1B; Figure S1). These results suggest that highly active acetylated p300 could be involved in the histone hyperacetylation in malignant oral tumors. Because autoacetylation of p300 enhances its acetyltransferase activity (Thompson et al., 2004), the autoacetylation status of p300 was also verified using a polyclonal antibody, which specifically recognizes acetylated p300 (ac-p300) molecules (Thompson et al., 2004). Interestingly, p300 was found to be hyperacetylated in oral cancer samples (Figure 1B).

Autoacetylation of p300 could be enhanced by several factors (Thompson et al., 2004; Sen et al., 2008; Hansson et al., 2009; Turnell et al., 2005; Huang and Chen, 2005), some of which are overexpressed in different cancers. We found that GAPDH, an enhancer of p300 autoacetylation (Sen et al., 2008), is significantly overexpressed in the oral tumor tissues as compared with the control (Figure 1B). As reported recently (Shandilya et al., 2009), a concomitant increase of NPM1 was also observed in these patient samples (Figure 1B). Subsequently, we took six different pairs of tissue samples (tumor and corresponding adjacent normal tissue) and determined the levels of protein overexpression by immunoblotting analyses. In all of the samples, histone H3 was hyperacetylated as probed by anti-H3AcK9AcK14 acetylation-specific antibody (Figures 1B and 1C). Interestingly, we also observed the hyperacetylation of H2AK5 and H3K56 (Figures 1B and 1C), other p300-specific sites which suggest further the role of p300-mediated acetylation. The levels of acH4K16 (Figures 1B and 1C) were found to be significantly low in the oral tumor samples, as observed in several cancers (Fraga et al., 2005). However, the H4K8 acetylation was minimally altered (Figures 1B and 1C). Furthermore, GAPDH and NPM1 were also found to be overexpressed in all of the tumor tissue samples (Figures 1B and 1C). Collectively, these data suggest that the overexpression of GAPDH and NPM1 are positively correlated to histone hyperacetylation in oral cancer. An interesting question raised at this juncture: is there any systematic molecular correlation of p300 autoacetylation and overexpression of these proteins that could be cell signal driven?

NO-Induced Histone Acetylation is Associated with NPM1 and GAPDH Overexpression via p300 Autoacetylation

The free radical gas NO is generated by the nitric oxide synthase (NOS) family of enzymes. NO is a pleiotropic signaling molecule that has been identified as a mediator for numerous physiological and pathophysiological conditions (Moncada et al., 1991). Because increased production of NO was noticed in oral cancer with a simultaneous upregulation of inflammatory (predominantly, NFk-B-responsive) genes (Gallo et al., 1998; Czesnikiewicz-Guzik et al., 2008), we hypothesized that NO signaling could be the link to overexpression of NPM1 and GAPDH, which in turn induces autoacetylation of p300, followed by hyperacetylation of histones. Immunohistochemical analysis revealed that indeed the inducible nitric oxide synthase (iNOS) levels were significantly enhanced in tumor tissue samples (Figure 2A). COX2 levels were also found to be higher in these tumor tissue samples (Figure 2A). A recent report implicates NO-dependent, nuclear localized GAPDH as an enhancer of p300 autoacetylation and thereby its catalytic activity (Sen et al., 2008). In the oral cancer patient samples analyzed, GAPDH was predominantly localized to the nucleus (Figure 2A). To investigate the causal relationship of iNOS and overexpression of NPM1 and GAPDH, KB cells were treated with the NO donor, S-nitrosoglutathione (GSNO). It was observed that the expression of both NPM1 and GAPDH was enhanced by GSNO in a concentration-dependent manner (Figure 2B; see also Figure S2). In agreement with the previous report (Sen et al., 2008), we also found that GAPDH is acetylated in a NO-dependent manner (Figure 2C). Interestingly, acetylation of NPM1 was also dramatically enhanced by GSNO treatment in KB cells (Figure 2C). Similar results were observed upon GSNO treatment in HeLa and KOSC-2 cells (see Figure S2).

In order to gain insight into signaling pathways, we investigated the effect of interferon-γ (IFN-γ) on NPM1 and GAPDH acetylation in KB cells, as it is known to activate iNOS gene expression to produce NO (Fukumura et al., 2006). We found that IFN-γ efficiently enhanced the NPM1 and GAPDH acetylation in a NO-dependent manner in KB (Figure 2D), HeLa, and KOSC-2 cells (Figure S2), which was abolished or reduced in the presence of a specific iNOS inhibitor, N-(3-(Aminomethyl) benzyl)acetamidine (1400 W). Furthermore, we found that IFN-γ treatment could induce the translocation of the cytosolic protein GAPDH to the nucleus of KB cells (Figure 2E) and HeLa and KOSC-2 cells (Figure S2). Taken together, these results suggest the involvement of NO signaling in the overexpression of NPM1 and GAPDH and their acetylation in KB cells.

GAPDH, which is positively regulated by NO signaling, enhances the autoacetylation of p300 (Sen et al., 2008) and presumably leads to histone hyperacetylation. These observations prompted us to investigate the role of NPM1 in the activation of p300 (autoacetylation). p300 autoacetylation reaction was performed in the presence of NPM1 and ^{3}H-acetyl-CoA. NPM1

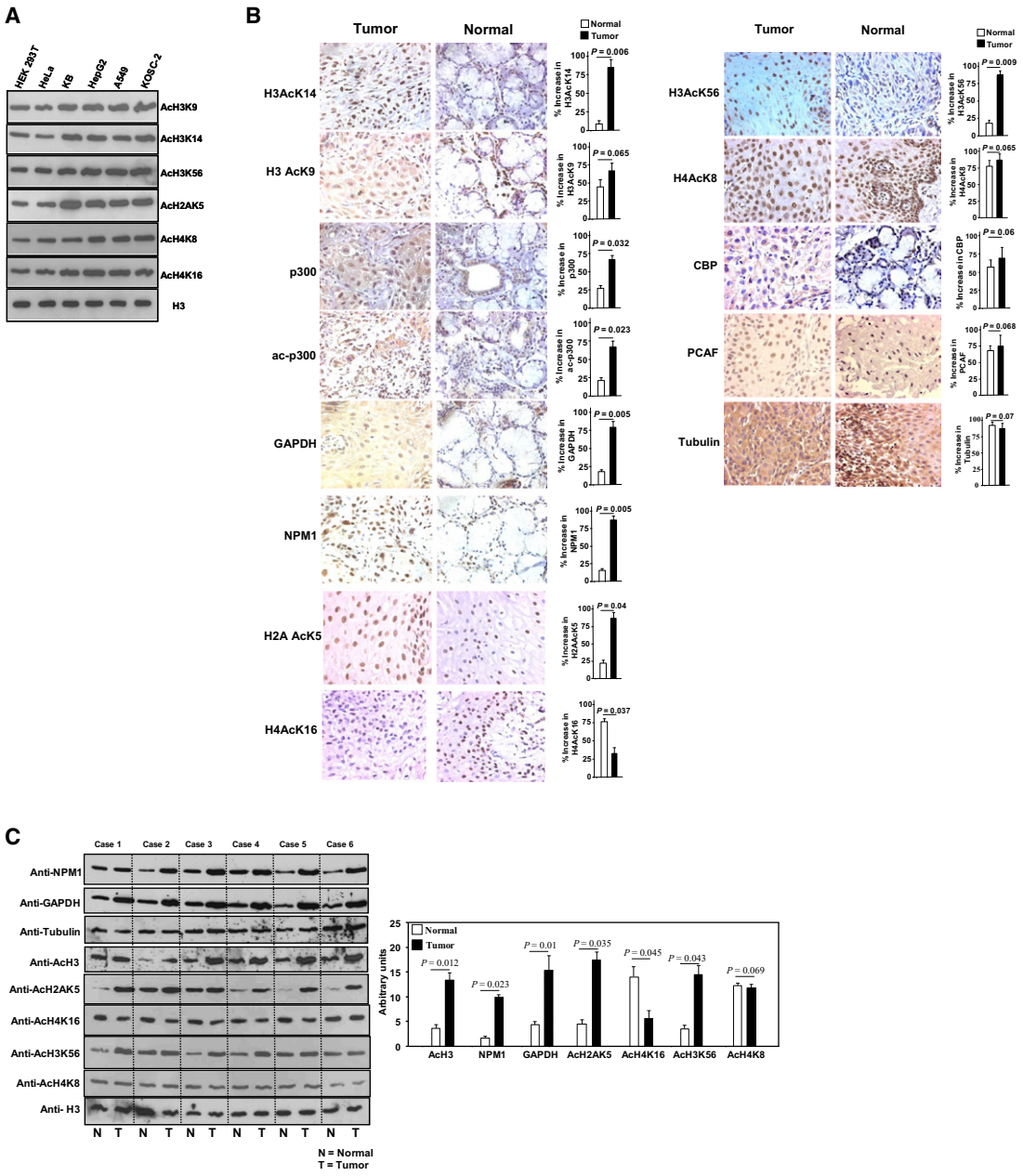

Figure 1. Histone Hyperacetylation Is Associated with Upregulation of NPM1 and GAPDH in Human Oral Cancer

(A) Histones are hyperacetylated in oral cancer cells. Histones were isolated from different cell lines as indicated and histone acetylation was analyzed by western blotting with the indicated antibodies. Anti-H3 was used as a loading control.

(B) Immunohistochemical detection of histone acetylation and expression of different proteins in oral cancer samples. Representative images (40× magnification) are shown. Respective P values are shown, sample size; n = 27, mean ± SD, one-way ANOVA.

(C) Immunoblotting analysis to compare the protein levels and acetylation status of histone H3 in the tumor (T) and the adjacent normal tissue (N) of the different patient samples (left). Tissue lysates were immunoblotted with anti-NPM1 and anti-GAPDH. Tubulin was used as a loading control. Histones isolated from the same patient tissue were used for the western blotting using the indicated antibodies and anti-H3 was used as a loading control. Densitometric analysis (C, right) of the data in (C, left) was done using phosphoimager (Fuji). Results are the mean ± SD, n = 6, one-way ANOVA. See also Figure S1 and Table S1.

was found to activate the autoacetylation of p300 in a dose-dependent manner (Figure 2F). GAPDH was used as a positive control (Sen et al., 2008). In order to validate the results in vivo, transient transfection of Flag-NPM1 was performed. NPMI was found to be predominantly localized to the nucleolus of the cells while a minor fraction was also found to be present in the nucleoplasm (Figure S3A) as reported earlier (Shandilya et al., 2009). Interestingly, we observed that the KB cells overexpressing NPM1 had higher levels of acetylated histone H3, as observed by coimmunofluorescence analysis (Figure S3A). Furthermore,

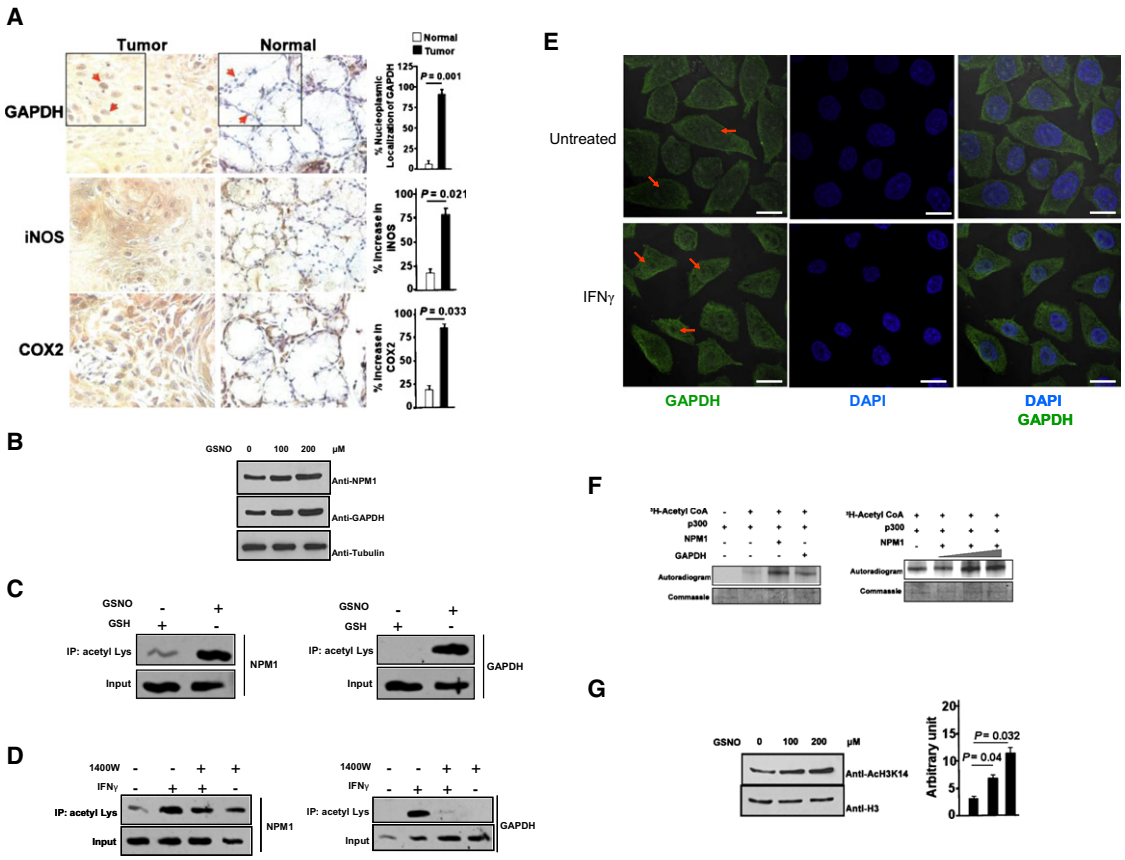

Figure 2. NO Enhances Acetylation of Histone and Nonhistone Proteins

(A) Immunohistochemical detection of GAPDH, iNOS, and COX2 expression in human oral cancer (as done in Figure 1A). Arrows indicate the nuclear localization of GAPDH in the tumor sample (inset; 100× magnification) as compared with adjacent normal cells. Representative images (40× magnification) are shown. Respective P values are shown, n = 27, mean ± SD, one-way ANOVA.

(B) GSNO induces expression of NPM1 and GAPDH. KB cells were treated with the indicated concentration of GSNO for 24 hr. Cell lysates were used to perform the western blotting using an anti-NPM1 and anti-GAPDH antibody. Tubulin was used as a loading control.

(C) NO induces the acetylation of NPM1 and GAPDH. KB cells were grown in the presence of 200 μM GSNO or GSH for 24 hr. Cell lysates were immunoprecipitated (IP) with anti-acetyl lysine antibody and the immunoprecipitates were analyzed by western blotting with anti-NPM1 (left panel) and anti-GAPDH (right) antibodies.

(D) IFN-γ treatment enhances acetylation of NPM1 (left) and GAPDH (right), which is abolished by the iNOS inhibitor 1400 W (100 μM). IP was performed as described above.

(E) GAPDH translocates to the nucleus of KB cells after exposure to IFN-γ (10 ng/ml) for 16 hr and stained with anti-GAPDH antibody (green) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; blue). Red arrows indicate the nuclear GAPDH. Experiment was performed two times. Representative figure is shown. Scale bars, 10 μm.

(F) NPM1 (600 ng) and GAPDH enhances the autoacetylation of full-length p300 (p300fl; 100 ng) in vitro (left) and in a concentration-dependent manner (300, 600, and 900 ng of NPM1) (right) as shown by autoradiography.

(G) NO causes the hyperacetylation of histone H3K14. In vivo isolated histones from the GSNO-treated KB cells were immunoblotted with anti-acetylated H3K14 and anti-H3 antibody. Densitometric analysis (right panel) of the data was done using phosphoimager (Fuji).

P values are shown, n = 2, mean ± SD, one-way ANOVA. See also Figures S2–S6.

western blotting analysis also shows that NPM1 overexpression could lead to concomitant increase in histone H3 and H4 acetylation, presumably due to enhanced autoacetylation of p300 mediated by NPM1(Figure S3B) .

In order to further confirm the role of NPM1 in the p300-mediated acetylation, we also performed small interfering RNA (siRNA)-mediated silencing of NPM1 for 48 hr in three different cell lines (KB, HeLa, and KOSC-2 cells) (see Experimental Procedures). The results show that indeed silencing of NPM1 significantly reduced histone H3 acetylation status (Figures S3C and S3D) which correlates with the reduction in the autoacetylated p300 (Figure S3C). The siRNA-mediated silencing of GAPDH (Figure S4) and iNOS (Figure S5) also abrogated the acetylation of p300 and histone H3 which suggest the involvement of GAPDH and iNOS in p300-mediated acetylation upon IFN-γ treatment. The link of IFN-γ treatment and p300-mediated acetylation was further shown by employing a p300 HAT-specific inhibitor plumbagin (RTK1) (Ravindra et al., 2009) (Figure S6). Because NO signaling induces the p300 autoacetylation, we next investigated the role of NO. GSNO treatment of KB cells

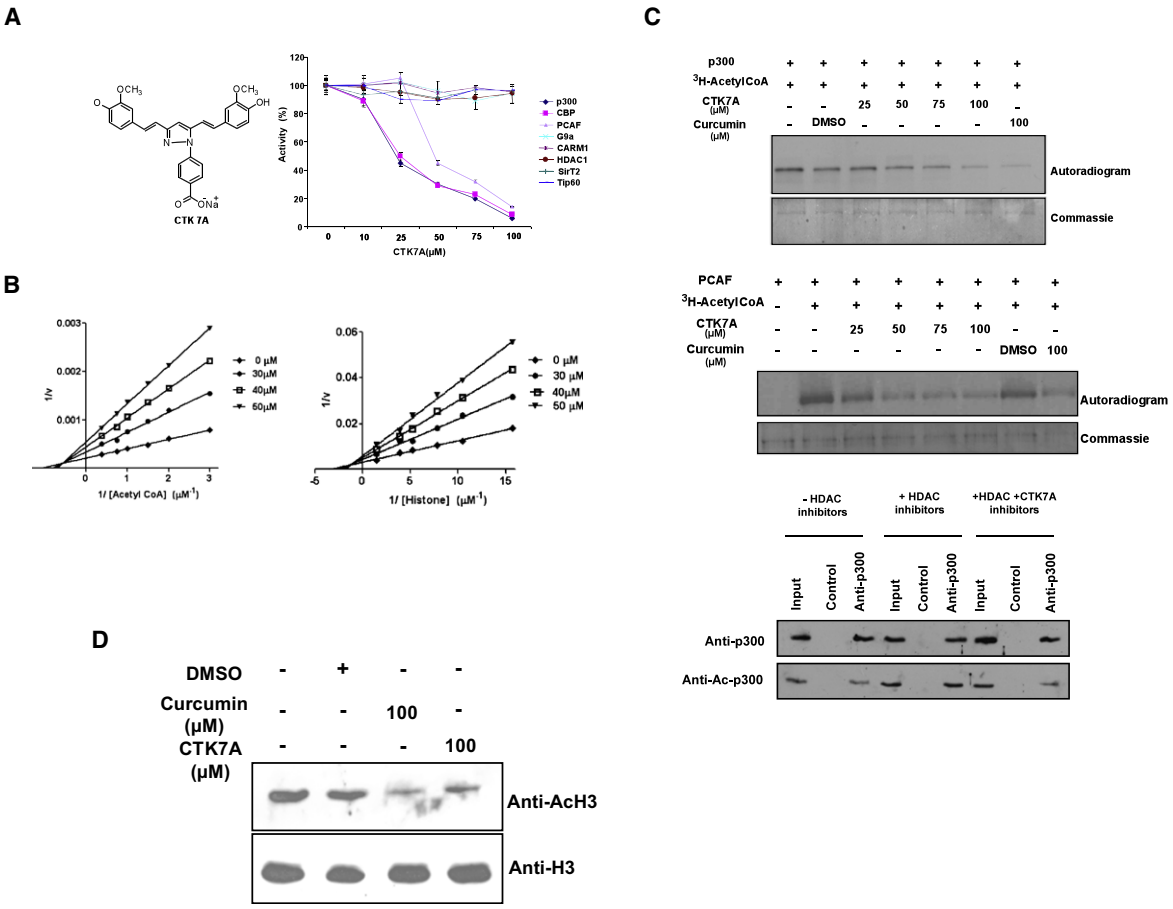

Figure 3. CTK7A Is a Water-Soluble HATi

(A) Structural formula of CTK7A. Sodium 4-(3,5-bis(4-hydroxy-3-methoxystyryl)-1*H*-pyrazol-1-yl)benzoate (left panel). Inhibition curves for various recombinant HATs, HMTs, and HDACs (right). Reactions were done in duplicate and error bars reflect the SD from at least two independent experiments.

(B) Lineweaver-Burk plots showing mixed inhibition of p300 by CTK7A. Each experiment was performed three times, and reproducibility was within 15%. Representative figure is shown.

(C) CTK7A inhibits p300fl (top) and PCAF autoacetylation (middle). Gel fluorography assay were performed using curcumin and DMSO as a positive and solvent control respectively. Reaction mixtures were resolved on 8% SDS-PAGE. CTK7A inhibits p300 autoacetylation in KB cells as shown by IP assay (bottom). Cell lysates were immunoprecipitated with anti-p300 antibody and control antibody (IgG). The immunoprecipitates were analyzed by western blotting with anti-p300 or anti-ac-p300 antibody (see Experimental Procedures for detail).

(D) CTK7A inhibits histone acetylation in KB cells. In vivo histones were isolated from CTK7A or curcumin-treated KB cells at the indicated concentration for 24 hr and were immunoblotted with anti-acetylated H3 and anti-H3 antibody.

enhanced the level of H3K14 acetylation in a concentration-dependent manner (Figure 2G), which was similar to the concomitant increase in NPM1 and GAPDH levels upon GSNO treatment as mentioned above (Figure 2B). Taken together, these data suggest that hyperacetylation of histones in oral cancer could be achieved by the overexpressed and autoacetylated p300 in a NO-dependent manner.

CTK7A Is a HAT Inhibitor

These results clearly demonstrate that hyperactivity of lysine acetyltransferase p300 could be one of the factors responsible for oral cancer manifestation. Therefore, the inhibitor of p300 HAT activity would be useful to verify the possible involvement of the acetyltransferase(s). We have previously shown that curcumin is specific inhibitor of p300/CBP (Balasubramanyam et al., 2004). Curcumin and several of its derivatives has been reported to have anticancer properties (Anand et al., 2008). However, among them, 4-{3,5-bis-[2-(4-hydroxy-3-methoxy-phenyl)-ethyl]-4,5-dihydro-pyrazol-1-yl}-benzoic acid (also known as HBC) has been shown to posses antiproliferative activities against several human cancer cells (Shim et al., 2002). HBC was further shown to inhibit the cell cycle progression of colon cancer cells via antagonizing of Ca^{2+}/calmodulin functions (Shim et al., 2004). Using curcumin as a synthon, we first made HBC and then in order to get a water-soluble derivative made a salt of that, namely, CTK7A (Figure 3A). CTK7A was found to inhibit HAT p300/CBP and PCAF but did not affect the activity of other histone modifying enzymes like G9a, CARM1, Tip60, HDAC1, and SIRT2, even at 100 μM (Figure 3A). Further kinetic analysis revealed that CTK7A-mediated p300 inhibition follows a mixed type of inhibition for both of the substrates, acetyl-CoA, and core histones (Figure 3B). Replot of the slopes and

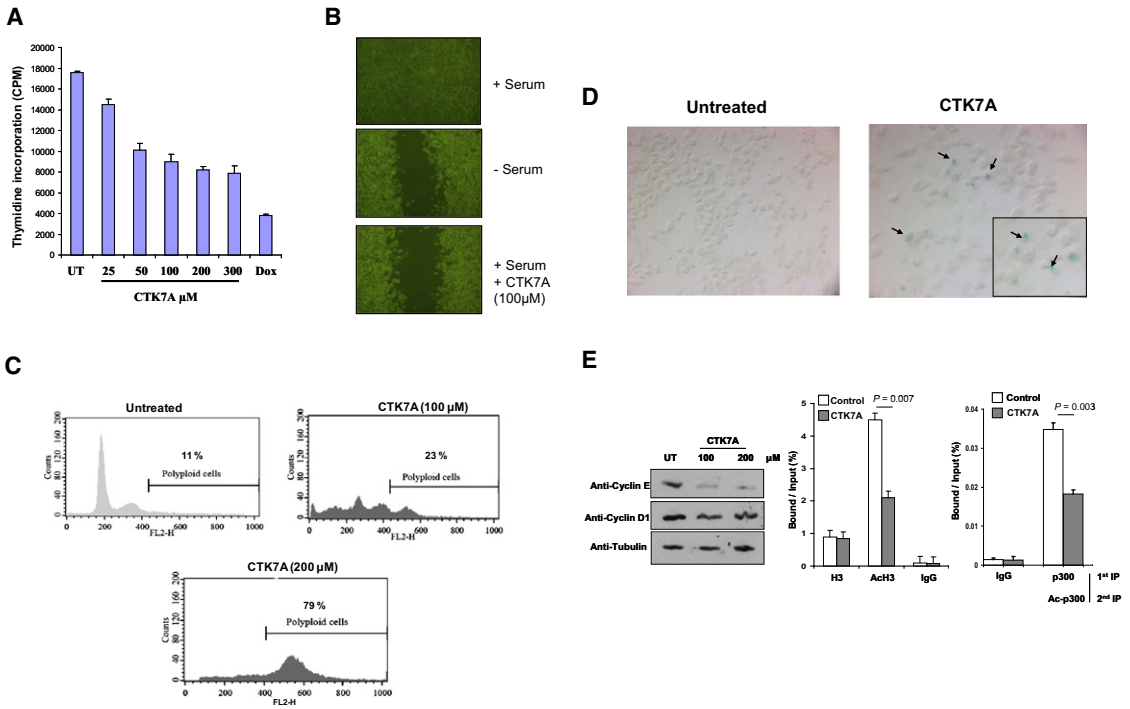

Figure 4. CTK7A Inhibits the Growth of KB Cells and Induces Senescence-like Growth Arrest

(A) CTK7A inhibits the growth of KB cells. Cell proliferation was examined by [^{3}H]-thymidine incorporation assay after treatment with different concentrations of CTK7A as indicated. Doxorubicin (100 μM) was used as a positive control. Each experiment was performed in duplicate and error bars reflect the standard deviation from three independent experiments.

(B) CTK7A inhibits wound healing. Cells with wounds of constant diameter were treated with CTK7A (100 μM) for 24 hr along with 10% serum. The wound photographs were taken under phase-contrast microscope. Serum positive (top) and negative (middle) cells act as positive and negative controls, respectively. Each experiment was performed three times. Representative figure is shown.

(C) CTK7A induces polyploidy in KB cells. KB cells were incubated with the indicated doses of CTK7A for 24 hr and were analyzed by flow cytometry. In CTK7A- (100 and 200 μM) treated cells, cell populations with DNA content >4N (polyploidy) were drastically increased compared with untreated KB cells (control). Experiment was performed three times. Representative figure (data) is shown.

(D) CTK7A induces senescence-associated β-gal expression (SA-β-gal) in KB cells. KB cells were incubated with 100 μM of CTK7A for 24 hr. Cells were stained for SA-β-gal. CTK7A treatment induces the SA-β-gal expression in treated cells (right) as compared with untreated, control (left). Experiment was performed two times. Representative figure is shown.

(E) Immunoblot indicating levels of cyclin E upon CTK7A treatment in KB cells (left). ChIP analysis at the promoter of cyclin E after CTK7A treatment (100 μM) for 24 hr in KB cells (middle). ChIP was performed using antiH3 and anti-acetylated H3 antibodies. IgG was used as a control. A reChIP assay was also done by carrying out first immunoprecipitation with anti-p300 and then anti-Ac-p300 antibody at the cyclin E promoter after CTK7A treatment (100 μM) for 24 hr in KB cells. Values are relative to immunoprecipitated input DNA. Results are the mean ± SD of three independent ChIP experiments, one-way ANOVA. See also Figure S7.

the intercepts were linear. Kis for the acetyl-CoA and core histone were 13.8 and 18.6 μM while Kii for the same were 110.6 and 67.8 μM, respectively. The presence of Kii > Kis further confirms the occurrence of mixed type of inhibition with the line intersecting above the abscissa. However, as expected, CTK7A efficiently inhibited the autoacetylation of p300 and PCAF (Figure 3C) in a concentration-dependent manner in vitro. Furthermore, CTK7A also inhibited the enhanced autoacetylation of p300, mediated by a cocktail of HDAC inhibitors in KB cells (Figure 3C). To elucidate the effect of CTK7A on histone acetylation in a cellular system, KB cells were treated with CTK7A. As expected, it potently inhibited the acetylation of histone H3 in KB cells (Figure 3D). These results suggest that the water-soluble HATi, CTK7A, inhibits histone acetylation in the cellular system at least partially through the inhibition of p300 autoacetylation.

CTK7A Inhibits Cell Proliferation and Induces Senescence-like Growth Arrest

Because p300/CBP is a master regulator and is involved in the regulation of cell cycle progression, proliferation, and differentiation (Goodman and Smolik, 2000), we next investigated the growth-inhibitory properties of CTK7A on KB cells. CTK7A caused a dose-dependent inhibition of KB cells proliferation, observed by thymidine-incorporation assay (Figure 4A). The antiproliferative activity of CTK7A was further assessed by a wound healing assay. We observed that CTK7A-treated cells showed a reduction in wound healing activity in the presence of serum (Figure 4B). Cells with or without serum were used as positive and negative controls, respectively (Figure 4B). These results suggest an antiproliferative role of CTK7A in KB cells. Based on the above results, the effect of CTK7A on cell cycle progression was also determined. KB cells, when treated with increasing

concentrations of CTK7A, showed a dramatic increase in the percentage of polyploid cells (>4N) in a concentration-dependent manner (Figure 4C).

Induction of polyploidy is often associated with senescence, which is known to be associated with antitumor processes (Ota et al., 2006). We found that CTK7A induced the expression of SA-β-gal, a marker for senescence (Figure 4D). This is consistent to the earlier report in which the role of p300 in regulating proliferation and senescence of human melanocytes was shown using the p300 acetyltransferase activity specific inhibitor, Lys-CoA (a substrate analog of acetyl-CoA) (Thompson et al., 2004; Bandyopadhyay et al., 2002). Lys-CoA has been shown to inhibit cell proliferation and induce senescence-like growth arrest as monitored by the expression of SA-β-gal (Bandyopadhyay et al., 2002).

Cyclin E is a critical regulator of senescence. Overexpression of cyclin E helps the cells to escape from BRG1- and RAS-induced senescence (Bandyopadhyay et al., 2002). Given that p300/CBP regulates the expression of many cell cycle genes (Goodman and Smolik et al., 2000), we determined the effect of CTK7A on the expression of cyclin E. We observed that CTK7A downregulated cyclin E expression in a dose-dependent manner in KB cells, whereas expression of cyclin D1 was marginally affected (Figure 4E). We have also tested the affect of other HATi, namely, RTK1 and curcumin on the cyclin E expression and found that they are able to inhibit the expression of the cyclin E (Figure S7A). Previous reports suggest that cyclin E gene expression in human tumor cells and in mouse embryonic fibroblasts is regulated by reversible acetylation of promoter-proximal histones (Bandyopadhyay et al., 2002; Sambucetti et al., 1999). To address the effect of CTK7A on acetylation status at the cyclin E promoter, chromatin immunoprecipitation (ChIP) assays were performed, which clearly demonstrated that CTK7A inhibited the acetylation of H3 at the cyclin E promoter (Figure 4E). A sequential ChIP (reChIP) assay was also performed with an anti-p300 antibody, followed by an anti-ac-p300 antibody, to estimate the acetylation status of p300 upon CTK7A treatment. reChIP assays showed the presence of p300 at the cyclin E promoter and that the p300 autoacetylation is inhibited upon CTK7A treatment (Figure 4E). Collectively, these results imply that cyclin E downregulation could be a direct effect of CTK7A-mediated inhibition of p300/CBP HAT activity. The FACS analysis shows an increase in the pre-G1 peak upon the HATi treatment which indicate the cellular apoptosis. In agreement with this observation, it was found that the treatment of CTK7A could lead to activation of caspase-3, a key effector molecule in the apoptosis pathway (Figure S7B). Furthermore, we found that the HATi-mediated caspase activation leads to PARP cleavage (Figure S7B). However, we do not observe induction of senescence and apoptosis in HeLa and HEK293T cells (Figure S7C and S7D). We also observed apoptosis with the p300 HATi RTK1 but not with the inactive derivative RTK2 (data not shown).

CTK7A Inhibits Tumor Growth

The in vitro- and cell line-based studies prompted us to investigate the effect of CTK7A on cell line-derived oral tumor growth in xenografted mice. We found that CTK7A is not toxic to the mice after intraperitoneal (i.p.) administration. There was no observed

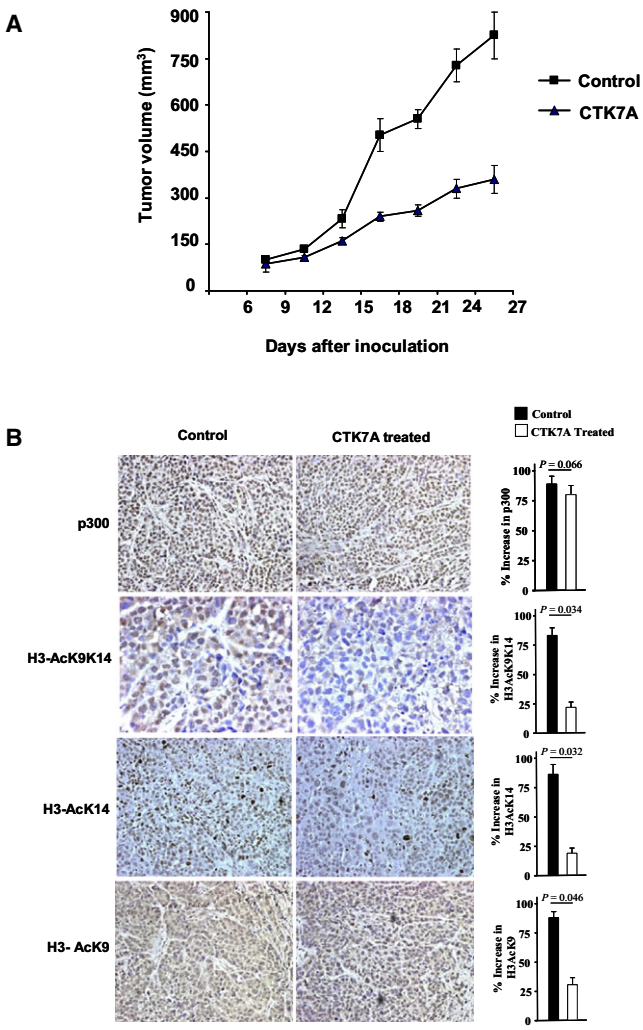

Figure 5. CTK7A Inhibits Xenograft Tumor Growth in Nude Mice

(A) Nude mice carrying the KB cell xenografts were treated with phosphate-buffered saline (control) or with 100 mg/kg body weight CTK7A intraperitoneally twice a day. Tumor volumes (mm^3) were determined as described in Experimental Procedures. One-way ANOVA revealed that tumor sizes were significantly different (p = 0.034, n = 16, mean ± SD).

(B) CTK7A inhibits histone acetylation in nude mice. KB cell tumors from control and CTK7A-treated mice were used for immunohistochemical detection with indicated antibodies as performed earlier. Representative images (40× magnification) are shown. Respective P values are shown, sample size; n = 16, mean ± SD, one-way ANOVA. See also Figure S8.

weight loss at doses up to 100 mg/kg body weight twice a day during 1 month of treatment (data not shown).The treatment of CTK7A has no apparent toxicity in the animals as revealed by the H&E staining (Figure S8A). In order to test the effect of CTK7A on tumor growth, we inoculated KB cells (2 × 10^6 cells) in nude mice in the right and left flanks and treated with 100 mg/kg body weight twice a day (see Experimental Procedures). CTK7A showed strong antitumor activity. KB cell tumors were about 50% smaller in mice treated with CTK7A than in control mice (Figure 5A). The differences in the tumor size between xenografts (control versus treated) were found to be statistically significant (p = 0.034). The levels of H3 acetylation

and that of other proteins (e.g., p300, GAPDH, NPM1) were determined using IHC analysis of xenografted tumors. CTK7A-treated mice tumors showed a marginal effect with respect to p300 expression (Figure 5B). We observed that CTK7A decreased the levels of H3K9, K14 acetylation (as probed by anti-H3AcK9AcK14, anti-H3AcK14, and anti-H3AcK9 antibodies) and ac-p300 (Figure S8B). CTK7A was found to inhibit H3K14 acetylation more potently than H3K9 acetylation (Figure 5B). These data are consistent with the antiproliferative effect of CTK7A on KB cells. Further, the levels of GAPDH, NPM1, and iNOS were also found to be downregulated in CTK7A-treated mice (Figure S8B), which could also be caused by the antiproliferative effect of CTK7A. Moreover, the level of COX2, which is induced in many cell types by mitogens, growth factors, cytokine, and tumor promoters and has been implicated in many cancers, including oral cancer (Fukumura et al., 2006), was also found to be suppressed in CTK7A-treated tumors (Figure S8B). We also observed downregulation of the proliferation marker, Ki67, in CTK7A-treated tumors (Figure S8B), which further support the antiproliferative nature of the HATi, CTK7A.

Taken together, all of these results suggest that the water-soluble HATi, CTK7A, by virtue of its HAT-inhibitory and antiproliferative activity, could lead to the inhibition of tumor growth in xenografted mice.

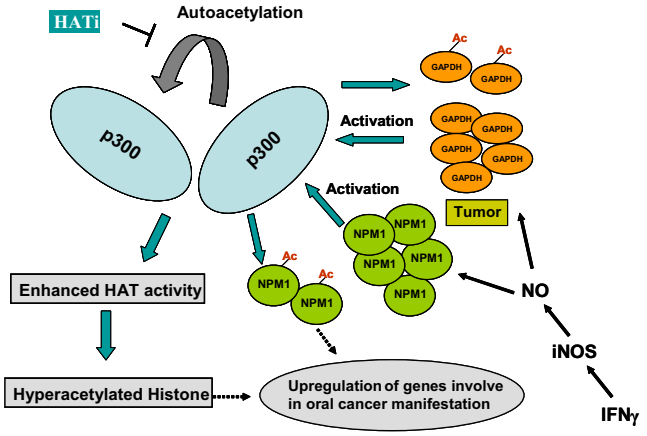

Figure 6. Proposed Model to Explain the Putative Epigenetic Signaling Pathway that Causes Hyperacetylation of Histone and Nonhistone Proteins

NO triggers the upregulation of NPM1 and GAPDH, which in turn enhance the autoacetylation of p300. Highly active p300 acetylates histones and histone chaperone NPM1, which presumably enhances gene expression for oral cancer manifestation.

DISCUSSION

Although reversible acetylation of histone and nonhistone proteins is one of the most well-studied epigenetic marks, the regulation of enzymatic machinery involved in these phenomena has gained attention only recently. Dysfunction of HDACs and the consequent hypoacetylation of histone and nonhistone proteins have been causally related to cancer manifestation in a few cases (Bolden et al., 2006). Reports regarding dysfunction of HATs and cancer are rather scarce. Here, we show that, in OSCC, histone H3 (predominantly at K14) is hyperacetylated in grade II oral tumors. Associated overexpression and hyperacetylation of p300 suggest that autoacetylation of p300 could be one of the key factors responsible for histone hyperacetylation. We found that the histone chaperone NPM1, which is also overexpressed in the oral tumors (Shandilya et al., 2009), induces the autoacetylation of p300, presumably through its protein chaperone activity (Szebeni and Olson, 1999). These results indicate a new function of the multifunctional nucleolar protein, NPM1. Furthermore, these studies also establish an epigenetic signal, IFN-γ-dependent NO synthesis, which could be acting as a basic stimuli for the NPM1- and GAPDH-mediated enhanced autoacetylation of p300 and thereby the downstream effects (Figure 6).

The correlation of histone modifications (especially acetylation) and cancer manifestation is not yet well established. However, in the case of prostate cancer (Seligson et al., 2005) and hepatocarcinomas (Bai et al., 2008) altered histone modifications were observed but the molecular mechanisms (and the enzymatic machineries) behind the altered modifications are not yet elucidated. Recently, increased acetylation of H3K56, a target of p300/CBP, was also observed in multiple cancers (Das et al., 2009). We have demonstrated that the hyperacetylation of histones, a histone chaperone (NPM1) and the metabolic protein, GAPDH, is caused by the hyperactive, autoacetylated p300. The unique positive loop involved in this process of hyperacetylation could be a common mechanism in certain types of cancer manifestation.

Oral cancer is an inflammation-related disease. Inflammation can lead to NO production that can impact the initiation and progression stages of cancer (Fukumura et al., 2006). Our findings also provide a link between NO production and overexpression of cell proliferative marker genes such as NPM1 and GAPDH. These observations are consistent with the report in which enhanced iNOS activity was detected in human tumor cell lines (Jenkins et al., 1994; Asano et al., 1994) and in patient tumor samples of various histogenetic origins (Fukumura et al., 2006). NOS activity has also been found to be associated with tumorigenesis, proliferation, and expression of important signaling components linked to cancer development (Fukumura et al., 2006). It is known that IFN-γ, a proinflammatory cytokine, activates iNOS to produce NO (Fukumura et al., 2006). We have found that IFN-γ treatment in an oral cancer cell line (KB cells) induced NO production, the inhibition of which (IFN-γ) repressed acetylation of NPM1 and GAPDH. Our results show that the IFN-γ treatment enhanced the nuclear translocation of GAPDH, which could be activating the p300 autoacetylation and thus its HAT activity. Although similar nuclear localization of GAPDH has been reported in other cases (Sen et al., 2008; Hara et al., 2005), the molecular mechanisms that induce nuclear translocation of GAPDH in KB cells remain elusive. Overexpressed NPM1 and GAPDH (nuclear) enhanced the autoacetylation of p300, which in turn induced histone hyperacetylation. The hyperacetylated histones and NPM1 may favor the expression of genes responsible for oral cancer progression (Figure 6). NPM1- and GAPDH-induced autoacetylation of p300 may also lead to the formation of a more dynamic transcriptional preinitiation complex (Black et al., 2006), facilitating transcription. The histone chaperone activity of NPM1 may also be used for transcriptional activation as reported earlier (Swaminathan et al., 2005). Our

results are also supported by the fact that p300-mediated acetylation of NPM1 augments its oncogenic potential and has been implicated in oral cancer manifestation (Shandilya et al., 2009). Cancer cells have a higher energy requirement and increased ribosome biosynthesis. Increased levels of GAPDH and NPM1 may help in metabolic requirements and in ribosome biogenesis, respectively, in oral cancer.

Here, we have shown that the p300/CBP HATi, CTK7A, could efficiently inhibit oral tumor growth in xenografted mice. CTK7A induced polyploidy and senescence-like growth arrest in the oral cancer cell line. Induction of senescence is known to contribute to the treatments of chemotherapy and ionization radiation (Dimri, 2005). Promotion of polyploidy followed by senescence-like growth arrest has also been documented for the p300/CBP-specific HATi, Lys-CoA (Bandyopadhyay et al., 2002). Mechanistically, inhibition of histone acetylation leads to the repression of cyclin E expression, which is an important regulator of senescence. We have also observed that CTK7A treatment leads to the repression of histone acetylation, NPM1 expression, iNOS production and a significant reduction in the tumor growth in nude mice. Induction of apoptosis may also be partially responsible for the observed antitumor activity of CTK7A.

Our current observations establish the causal relationship of histone hyperacetylation and oral cancer manifestation. Most significantly, this study elucidates the mechanisms of hyperacetylation and identifies a new candidate protein, NPM1, as a regulator of p300. With the recent discoveries of several new HAT inhibitors, these molecules are being recognized as potential antineoplastic therapeutics (Selvi and Kundu, 2009; Cole, 2008). In oral cancer, p300 acetyltransferase activity may be considered as an important target for therapy. However, the complete epigenetic language that derives OSCC remains elusive.

SIGNIFICANCE

Dysfunction of epigenetic regulation has been linked to many diseases. In the present study, we have found that histones are hyperacetylated in malignant oral tumors due to overexpression and hyperacetylation of histone acetyltransferase (HAT) p300. The autoacetylation of p300 is induced by NPM1 and GAPDH, which are overexpressed in the malignant oral tissue. Significantly, NPM1 is a factor discovered in this study as a positive regulator of p300 autoacetylation. Furthermore, our studies establish the interferon-γ (IFN-γ) dependent nitric oxide (NO) signal transduction pathway as an epigenetic signal for the hyperacetylation in oral cancer. The inhibition of p300 acetyltransferase activity by the newly synthesized HATi in the xenografted nude mice system leads to the arrest of tumor growth which could be a potential antineoplastic therapeutic strategies, particularly in OSCC.

EXPERIMENTAL PROCEDURES

Reagents

Chemicals used were from Sigma unless stated otherwise.

Cell Culture, Whole-Cell, and Tissue Lysate Preparation

KB (oral squamous cell carcinoma) and HeLa cells were maintained in Dullbecco's modified Eagle medium (DMEM) while KOSC-2 (oral squamous cell carcinoma) cells were maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS) at 37°C with a 5% CO_2 atmosphere in a humidified incubator. For IFN-γ and GSNO treatment, KB cells were incubated in the presence of IFN-γ 10 ng/ml for an additional 16 and 24 hr, respectively. Immunofluorescence staining of cells for confocal microscopy was carried out as described previously (Shandilya et al., 2009). CTK7A treatment was done for 24 hr followed by acid extraction of histone and immunoblotting as per standard protocol (Balasubramanyam et al., 2004). For whole-cell and tissue lysate preparation, RIPA buffer (50 mM Tris-HCl [pH 7.4], 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, EDTA 1 mM, phenylmethylsulfonyl fluoride [PMSF], 1 mM Na_3VO_4, 1 mM NaF, and protease inhibitor cocktail [Roche]) was used.

Transfection Assay

Transient transfections were carried out with 70%–80% confluent KB cells for 36 hr by using lipofectamine (Invitrogen) according to the manufacturer's protocol. The pCMV-Flag mammalian-NPM1 construct was described before (Shandilya et al., 2009).

Immunoprecipitation Assay

Whole-cell extract were prepared using RIPA buffer as mentioned above. The preblocked protein-G Sepharose-bound antibody (as indicated) (4 μg) was incubated with 500 μg whole-cell lysate overnight at 4°C. After extensive washes, bead-bound proteins were analyzed by western blotting with indicated antibodies. For HDAC inhibitors treatment, 5 mM sodium butyrate, 5 mM nicotiamide, and 100 ng/ml TSA were added 10 hr before cells were harvested (Thompson et al., 2004). IgG was used as a control.

Chromatin Immmunoprecipitation Assay

The ChIP and re-ChIPassays was performed as described earlier (Shandilya et al., 2009; Bandyopadhyay et al., 2002) using indicated antibodies. KB cells were treated with CTK7A and cells were grown for 24 hr. Real-time PCR analysis was performed using primers for the cyclin E promoter region: 5′-GGCGGGACGGGCTCTGGG-3′ and 5′-CCTCGGCATGATGGGGCTG-3′.

Chemical Synthesis

CTK7A was synthesized as described in Supplemental Experimental Procedures.

HAT, HDAC, and HMTase Assay

In vitro acetylation, deacetylation and methyltransferase assays were performed as previously reported (Mantelingu et al., 2007; Balasubramanyam et al., 2004). For SirT2 deacetylase assay, 50 ng of bacterially expressed, recombinant enzyme was added in the presence or absence of cofactor NAD^+.

Kinetic analysis of p300 HAT inhibition was performed as reported earlier (Balasubramanyam et al., 2004) in the presence of (0, 30, 40, and 50 μM) CTK7A. The obtained values were plotted as a lineweaver burk plot using Graph pad Prism software. For p300 autoacetylation assay, reactions of p300 full-length (80 ng) were carried out in HAT assay buffer at 30°C for 10 min with or without the protein (NPM1) followed by addition of 2 μl of 4.7 Ci/mmol ^{3}H-acetyl-CoA (NEN-PerkinElmer) and were further incubated for another 10 min in a 30 μl reaction. GAPDH was used as positive control (Sen et al., 2008). The radiolabeled acetylated p300 were processed by fluorography followed by autoradiography. p300 or PCAF autoacetylation inhibition assay were performed under similar reaction conditions as described above with minor changes. HATi were added in place of protein substrate and 1 μl of 4.7 Ci/mmol ^{3}H-acetyl-CoA was used.

Cell-Based Assays

Senescence-associated β-gal (SA-β-gal) activity analysis was performed as described earlier (Bandyopadhyay et al., 2002). Immunofluorescence assay was performed as previously described (Shandilya et al., 2009).

SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures,one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2010.06.014.

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