Identification of small molecules that inhibit the histone chaperone Asf1 and its chromatin function

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The eukaryotic genomic is packed into chromatin, which is important for the genomic integrity and gene regulation. Chromatin structures are maintained through assembly and disassembly of nucleosomes catalyzed by histone chaperones. Asf1 (anti-silencing function 1) is a highly conserved histone chaperone that mediates histone transfer on/off DNA and promotes histone H3 lysine 56 acetylation at globular core domain of histone H3. To elucidate the role of Asf1 in the modulation of chromatin structure, we screened and identified small molecules that inhibit Asf1 and H3K56 acetylation without affecting other histone modifications. These pyrimidine-2,4,6-trione derivative molecules inhibited the nucleosome assembly mediated by Asf1 in vitro, and reduced the H3K56 acetylation in HeLa cells. Furthermore, production of HSV viral particles was reduced by these compounds. As Asf1 is implicated in genome integrity, cell proliferation, and cancer, current Asf1 inhibitor molecules may offer an opportunity for the therapeutic development for treatment of diseases. [BMB Reports 2015; 48(12): 685-690]

INTRODUCTION

In eukaryotic cells, genomic DNA forms a highly ordered structure called chromatin, which is composed of nucleosomes. The nucleosome core is composed of 147bp of DNA wrapped around the histone octamer containing two copies each of histones H2A, H2B, H3 and H4 (1). Chromatin structure and stability largely affect the gene expression and genome integrity.

Chromatin remodeling factors have an effect on the chromatin states, and controls the access of other factors to DNA to regulate its transcription, DNA replication, and DNA repair through covalent modification of histones or nucleosome assembly/disassembly in the choice of various histones (2).

When chromatin is not regulated correctly due to dysfunction of chromatin remodeling factors, many diseases occur, including several types of cancers. In recent years, there has been increasing interest in developing small molecules targeting the chromatin remodeling factors, especially histone modifying enzymes. Inhibitors of histone deacetylase complex (HDACi; SAHA, MS-275), histone demethylase (HDMi; GSK-J1), histone methyltransferase (HMTi; BIX-01294), and DNA methyltransferase (DNMTi; 5-Azacytidine, Hydralazine) have been shown to possess anticancer activity, and are further being investigated for clinical therapy for a range of diseases (3-5). However, far too little attention has been paid to histone chaperones as targets of epigenetic therapy.

Histone chaperones, in addition to simple mediation of nucleosome dynamics, modulate most aspects of chromatin functions, directly or indirectly, through assistance of histone modification (6). Asf1, originally identified in budding yeasts with its impact on heterochromatin silencing, is the most conserved histone H3/H4 chaperone protein from yeast to humans, and has been implicated in multiple functions in chromatin, including transcription, replication, and repair. Asf1 contributes to chromatin functions by delivering histone H3/H4 onto DNA by coupling with other chaperones, such as CAF1 for nucleosome assembly during DNA replication, or HIRA complex for nucleosomes required during replication-independent chromatin formation such as transcription and senescence (6, 7). Asf1 is also an essential cofactor for histone acetylation on some residues of histone H3/H4. The best-known Asf1-dependent acetylation target site is H3K56 in both budding yeast (8-10) and metazoans (11). Yeast Asf1 forms a complex with a fungal specific histone acetyl transferase (HAT), Rtt109, to promote H3K56 acetylation (12). Kcat of Rtt109 is increased by about 100-fold by Asf1. In metazoans, CBP/p300 is the corresponding HAT that acts with Asf1a isoform to mediate H3K56 acetylation (9). H3K56 acetylation in mammals is implicated in DNA replication, genome stability, stem cell pluripotency, and...
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RESULTS AND DISCUSSION

Screening of Asf1 inhibitor compounds
To identify small molecules that interfere with the chromatin function of Asf1, medicinal chemistry initially screened the chemical compound library from InterBioScreen for molecules that would have an inhibitory effect on Asf1-histone H3/H4 interaction, based on the crystal structure of Asf1/H3/H4 complex (17, 18). From a total of 260,000 compounds screened, 151 small molecules were identified as probable inhibitors. These candidates were evaluated individually by the binding assay (as described in Materials and Methods) to see whether they had an effect on the interaction between GST-Asf1a and H3 in vitro (Fig. 1A). Two compounds (compounds #1-20 and #1-71) had an inhibitory effect on Asf1/H3 binding (Fig. 1B). These compounds were pyrimidine-2,4,6-trione derivatives with a substitution group at R2 (#1-71) and an additional phenethyl group at R1 (#1-20). A series of PYT derivatives have previously been identified as matrix metalloproteinase (MMP) inhibitors, PPARγ agonists, or efficient drug candidates for a neurodegenerative disease such as Amyotrophic lateral sclerosis (ALS); however, they have never been studied as histone chaperone inhibitors (19-21). According to the distinct structural motif, 49 relevant derivatives were selected from the library and tested in the in vitro binding assay. This gave us further 6 additional hits, as shown in Fig. 1C. These compounds reduced the interaction between Asf1a and H3 in vitro in the range of 20-50 μM concentration (Fig. 2, left panel). There are two closely related isoforms of Asf1 in humans, termed Asf1a and Asf1b. They have a highly conserved N-terminal region (155 residues, 84% identical) that provides a binding platform for histone H3/H4, which is sufficient for most of Asf1’s functions (6, 22). Thus, it is likely that these compounds also have an ability to reduce the interaction between Asf1b and H3. As expected, these compounds affected

cancers (11-16). However, the mechanism by which Asf1 and H3K56 are functionally combined in these cellular processes is not clear, except that the loss of H3K56 acetylation due to H3 binding defective mutation of Asf1 (V94R) indicates that the H3 binding of Asf1 is critical for H3K56 acetylation (9). Chromatin needs to be highly dynamic to mediate appropriate regulation of gene expression and maintenance of genome integrity. This provoked considerable pharmaceutical interests for the development of small molecule inhibitors against various chromatin remodeling factors, mostly targeting covalent modification of histones or DNA. In this study, we sought to modulate chromatin by targeting the nucleosome assembly/disassembly pathway. For this purpose, we tried to find small molecules that inhibit Asf1’s histone chaperoning activity, and focused to determine whether they could affect chromatin functions contributed by Asf1.
the in vitro interaction between Asf1b and H3 (Fig. 2, right panel). Working concentrations of all compounds were in a similar range for both Asf1a and Asf1b, indicating that the small molecules might exert their inhibitory effects to the conserved structural features of the N-terminal domains involved in H3 binding.

Small molecule inhibitors reduced Asf1-mediated nucleosome assembly in vitro

Asf1 is a histone chaperone; this activity can be measured in vitro as it generates various supercoiled DNA isomers in the presence of topoisomerase I through the incorporation of histone subunits onto naked DNA that are easily resolved on agarose gels. Addition of Asf1 to a relaxed plasmid DNA induced the appearance of fast-migrating supercoiled forms through nucleosome formation (Fig. 3, lane 5). To investigate whether the compounds that compromise the histone interaction of Asf1 also affect the nucleosome assembly activity, we carried out the in vitro nucleosome assembly assay, with GST-Asf1a and core histones in the presence of compounds. Each compound efficiently reduced the formation of supercoiled isoforms, indicating that they were able to inhibit nucleosome assembly mediated by Asf1a in vitro (Fig. 3A, compare lane 5 and lanes 6-11). As nucleosome assembly is the fundamental activity of Asf1 as a central H3/H4 chaperone of both replication-dependent and replication-independent histone deposition pathways, our data provide the potential use of these compounds as useful molecules to understand Asf1’s chromatin function.

Asf1 inhibitory compounds reduced histone H3K56 acetylation in HeLa cells

We next examined the inhibitory role of compounds by testing whether they can affect the level of H3K56 acetylation in cells. H3K56 acetylation is in part mediated by Asf1, when in combination with Rtt109 in yeast and p300/CBP in mammals. HeLa cells were first treated with siRNAs that target Asf1a or Asf1b, either individually or simultaneously. H3K56 acetylation decreased immediately within 24 hr, specifically when Asf1a is depleted by siRNA, confirming previous reports that Asf1a, but not Asf1b, is required for acetylation of H3K56 in cells (11) (Fig. 4A, left panel). In addition, the reduction of K56 acetylation detected by Asf1a knock down at early time points was not observed after a 72 hr incubation, regardless of sustained depletion of Asf1a (Fig. 4A, right panel). Since K56 acetylation is coupled to many aspects of DNA function, its level is often dynamically and reversibly changed depending on the cell cycle stages, recovery time after DNA damage, and the relative contribution of different HATs such as p300/CBP and hGcn5, and HDACs such as SIRT1/2 and HDAC1/2 (11, 15, 23, 24). In this study, as Asf1-dependent H3K56 acetylation could be detected at early time points, we treated cells with Asf1 inhibitor compounds for 24 hr. As shown in Fig. 4B, all compounds resulted in a substantial decrease of H3K56 acetylation to various extents (Fig. 4B). Whereas, other histone modifications that occur through Asf1-independent manner, such as H3K56 monomethylation, H3 K4 acetylation, H3 K4 dimethylation, were not altered. The inhibitory effect of the small molecules differed, probably due to their stability and different cell permeability properties. However, consistent with the requirement of direct binding between Asf1 and H3 for K56 acetylation, small molecules with activity of interfering Asf1/H3 interaction were effective in blocking the acetylation of H3K56 in cells tested.

Asf1 inhibitor compounds reduced viral production

Asf1 is critical in maintenance of cellular DNA, as it controls the histone flow for de novo nucleosome assembly on newly synthesized DNA during DNA replication. In addition, its histone deposition activity is required for efficient viral DNA replication. Asf1 plays an important role together with the cellular transcription factor HCF-1, in the production of herpes simplex virus (HSV)-1 and varicella zoster virus (VZV) (25, 26). Incorporation of histone H3 onto HSV-1 DNA and viral replication, are thus decreased by Asf1 depletion (27). If Asf1 is indeed the authentic target of the identified compounds, they...
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Fig. 4. In vivo function of chromatin mediated by Asf1 is affected by Asf1 inhibitors. (A) Role of Asf1 isomers in H3K56 acetylation. HeLa cells were treated with siRNAs (200 pmol) targeting Asf1a and Asf1b, either separately or simultaneously, for 24 hr (left panel) and 72 hr (right panel). Depletion of Asf1 and the effect on H3K56 acetylation was determined by immunoblotting assay with whole cell extracts and specific antibodies recognizing Asf1a, Asf1b, and H3K56Ac. (B) Asf1 inhibitors specifically reduced the level of H3K56 acetylation. HeLa cells were treated with each of the Asf1a inhibitors (40 μM) for 24 hr and whole cell lysate was prepared for immunoblotting, and probed with specific antibodies recognizing different histone modification. H3 and actin served as loading controls. (C) HSV-1 yield was decreased by treatment of #2-03 and #2-32. Vero cells treated with DMSO (control) or 80 μM of each compound were infected with HSV-1 and viral yields in the culture supernatants were determined using plaque assay. Error bars represent the standard deviations of three independent experiments. Significance of difference was evaluated (*P < 0.05, **P < 0.005).

could possibly affect viral DNA synthesis also.

To investigate the effect of Asf1 inhibitor compounds on the viral DNA accumulation, we treated Vero host cells with DMSO control, or 80 μM of compounds, #2-03 and #2-32, for 8 hr. As shown in Fig. 4C, quantitative analysis of viral DNA at 20 hr post-infection showed significant reduction of viral DNAs from cells treated with #2-03 or #2-32, as compared to control, indicating that these compounds efficiently inhibit the activity of Asf1 required for viral DNA replication.

As a histone chaperone, Asf1 regulates most of the processes involving histones. First of all, it localizes at the active replication forks to mediate efficient chromatin duplication, where H3K56 acetylation is believed to be important for genome replication and integrity. Hence, rapidly proliferating cells such as cancer cells show increased H3K56 acetylation with increased levels of Asf1a/b, which makes Asf1 an attractive target for therapeutic intervention for cancer. Furthermore, H3K56 acetylation is a histone mark, more abundant in fungal species than in mammals, and proposed as a pharmacological target for the treatment of fungal infectious diseases caused by Candida albicans (28). In this regard, many efforts have been made to validate Asf1 as a biological target and to propose a new therapeutic approach. For example, a series of N-acyl hydrazones have recently been reported as potential Asf1 inhibitors with moderate interference of Asf1-H3/H4 binding (29).

Here, we showed that Asf1 and its chromatin function can be interfered by PYT derivatives. Some PYT derivatives with diverse substitutions have been described as inhibitors of matrix metalloproteinase, and have antitumoral, antiangiogenic, and antimetastatic activities (20, 21). In this study, we have shown that PYT scaffold derivatives have an anti-Asf1 activity. As elevated expression of Asf1 is associated with high mitotic index and poor tumor grade with clinical relevance of disease outcome, targeting Asf1 will be a therapeutic opportunity for use in the treatment of cancer (11, 30). Although Asf1 inhibition activity of the current PYTs identified is not as strong as required and relatively high concentrations have been used for this study, they have a potential to work best with further optimization of molecular structure or in combination with other chromatin inhibitors, to be more potent epigenetic modulators having therapeutic possibilities.
MATERIALS AND METHODS

Preparation of GST-Asf1a

*Escherichia coli* harboring pGEX-hAsf1a or pGEX-hAsf1b were induced by IPTG (Duchefa) and cells were harvested and resuspended in ice-cold PBS containing 1% Triton X-100, 0.5 M NaCl, and protease/phosphatase inhibitors. Cells were lysed by sonication and whole cell extract was incubated with glutathione-agarose beads (Sigma-Aldrich). GST-proteins were eluted with elution buffer [20 mM Tris-HCl (pH 7.5), 10% glycerol, 0.1% NP-40, 0.35% 2-mercaptoethanol, 20 mM reduced glutathione].

*In vitro* H3 binding assay

GST-Asf1a or b were incubated with 20 μl glutathione-agarose beads for 30 min at room temperature. Beads were washed 4 times with 1 ml of wash buffer, and then equilibrated with 1 ml histone binding buffer [30 mM HEPES-KOH (pH 7.5), 5% glycerol, 100 mM NaCl, 0.1% Triton X-100, 0.1 mM EDTA, and 0.1 mg/ml BSA]. Beads were incubated with 1.25 μg of calf-thymus histone H3 (Roche 11 034 758 001) or 3 μg of HeLa core histones (purified with Active Motif core histone purification kit; 40025), in the presence or absence of Asf1 inhibitor candidates (InterBioScreen Ltd, Russia), for 1 hr at room temperature, with rotation. Beads were washed 4 times with 1 ml of histone binding buffer. Histones co-precipitated with GST-Asf1a beads were analyzed by polyacrylamide gel electrophoresis (PAGE), followed by Coomassie staining or immunoblotting.

Virtual screening

The X-ray crystal structure of Asf1 (PDB entry: 2HUE) was selected as the receptor model in the virtual screening with docking simulations (17). The docking library for Asf1, comprising approximately 260,000 synthetic and natural compounds, was constructed from the latest version of the chemical database distributed by InterBioScreen Ltd. (http://www.ibscreen.com). We used the AutoDock program in the virtual screening because the excellence of its scoring function over those of the others had been demonstrated for various target proteins (31, 32). Finally, the compounds with the calculated binding free energy of lower than −25 kcal/mol were selected as virtual hits and tested for their inhibitory activity against Asf1.

*In vitro* nucleosome assembly assay

0.1 μg of relaxed pBR322 plasmid DNA (Topogen) and 5U of wheat germ Topoisomerase I (Promega) were incubated for 30 min at 37°C in Topo I buffer [50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 20% glycerol]. Meanwhile, 0.33 μg of HeLa core histones and 0.5 μg of purified GST or GST-hAsf1a were incubated for 30 min at 37°C in nucleosome assembly buffer [10 mM Tris-HCl (pH 7.5), 125 mM NaCl, 2 mM MgCl2, 0.5 mM DTT, 0.1 mg/ml BSA], with or without the small molecule inhibitors. The two reaction mixtures were combined and further incubated for 1 hr at 37°C. Reaction was stopped by addition of 60 μl of stop solution (20 mM EDTA, 1% SDS, 200 μg/ml pronase) followed by incubation at 37°C for 30 min. Plasmids were purified with phenol-chloroform-isooamylalcohol extraction and precipitated with ethanol. DNA was analyzed on 1.2% agarose gel (50 V, 12 hr, 4°C), and visualized by ethidium bromide staining.

siRNA treatment

For RNA interference assays, cells were transfected with a control siRNA (AccuTarget™ Negative control siRNA, Bioneer) or siRNAs against Asf1a and Asf1b (ST Pharm, Korea). Transfection of siRNAs was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The sequences of siRNAs are as following: AAGUGAAGAAUACGAUCAAGUTT (si-Asf1a) and AACAAGAGAUCAGACCCGUTT (si-Asf1b).

Western blot analysis

For western blotting, cells were lysed in NETN lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% NP-40, and inhibitors of proteases and phosphatases]. After 10 min incubation on ice, the cell lysate was recovered by centrifugation (13,000 rpm at 4°C). Proteins were resolved by SDS-PAGE and subjected to immunoblotting with appropriate antibodies. Protein bands were visualized by ECL detection reagent (Abfrnter, Korea). Following antibodies were used in this study: anti-Asf1a (Cell Signaling), anti-Asf1b (Cell Signaling), anti-actin (Millipore), anti-H3 (Abcam), anti-H3K56Ac (Active-motif), anti-H3K56Methyl (Active-motif), anti-H3K4Ac (Active-motif), anti-H3K4Methyl (Millipore).

Virus experiment

HSV-1 (strain KOS/k12) was provided by Patricia G. Spear (Feinberg School of Medicine of Northwestern University, Chicago, IL, USA) (33). African green monkey kidney cells (Vero cells) were maintained and infected with HSV-1 according to standard protocols. Briefly, cells were treated with 80 μM of compounds or DMSO vehicle for 8 hr before HSV-1 infection. Cells were infected with virus particles at a MOI (multiplicity of infection) of 0.2 in serum-free DMEM medium for 1h at 37°C in 5% CO2 chamber with rocking, after which serum-supplemented medium was added. Cells were further incubated for 20 hr for determination of viral yield in the culture supernatants, using the plaque assay. For the plaque assay, Vero cells in a 6-well plate (1 × 10⁶ cells/well) were infected with the serially diluted virus samples. After 1 hr incubation, the culture medium was removed and 2 ml of methylocellulose overlay was added to each well, and incubated for 3 days. The methylcellulose was then removed, and the cell monolayer was stained with 1% crystal violet in 50% ethanol for 20 min. The cells were then washed with tap water and the numbers of plaque were counted.

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