Chapter 18

Measuring the Effect of Histone Deacetylase Inhibitors (HDACi) on the Secretion and Activity of Alpha-1 Antitrypsin

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Abstract

Alpha-1 antitrypsin deficiency (AATD) is a protein conformational disease with the most common cause being the Z-variant mutation in alpha-1 antitrypsin (Z-AAT). The misfolded conformation triggered by the Z-variant disrupts cellular proteostasis (protein folding) systems and fails to meet the endoplasmic reticulum (ER) export metrics, leading to decreased circulating AAT and deficient antiprotease activity in the plasma and lung. Here, we describe the methods for measuring the secretion and neutrophil elastase (NE) inhibition activity of AAT/Z-AAT, as well as the response to histone deacetylase inhibitor (HDACi), a major proteostasis modifier that impacts the secretion and function of AATD from the liver to plasma. These methods provide a platform for further therapeutic development of proteostasis regulators for AATD.

Key words Alpha-1 antitrypsin deficiency, Cellular proteostasis, Histone deacetylase inhibitor, Proteostasis regulators

1 Introduction

Alpha-1 antitrypsin deficiency (AATD) is a hereditary disorder caused by mutations in the Alpha-1 antitrypsin (AAT) gene [1]. AAT is the most abundant serine protease inhibitor (SERPIN) in the plasma that is synthesized in hepatocytes, and it plays a critical role in preventing the degradation of lung tissue by neutrophil elastase (NE). AAT is considered a metastable protein that is managed by a proteostasis system which encompasses protein synthesis, folding, degradation, and membrane trafficking systems [2–5]. This evolutionarily ancient system optimizes biological processes which facilitate the synthesis, processing, and maintenance of protein folds that elicit specific functions in response to different cellular environments [6]. However, AAT variants arising during the normal evolution of the genome may exceed proteostasis capacity and trigger disease phenotypes.

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The most common AAT variant that causes AATD is the Z-variant (E342K). This variant has a slow folding rate and an increased tendency to form polymers through a "loop-sheet" mechanism in the endoplasmic reticulum (ER) [7]. Z-AAT polymers result in proteotoxic stress in the ER, which triggers liver diseases such as neonatal hepatitis, cirrhosis, and hepatocellular carcinoma [8]. The misfolded Z-AAT conformation also decreases its trafficking efficacy along the secretory pathway, and therefore leads to a substantially reduced level of circulating AAT in the plasma that is required for antiprotease activity in the lung. Loss of functional AAT in the lung leads to emphysema and/or chronic obstructive pulmonary disease (COPD) [9]. Thus, AATD is considered to be a "protein conformational disease" [10, 11]. Understanding and managing the balance between evolutionarily diverse protein fold trajectories and the proteostasis system's ability to regulate the folding, trafficking, and function of proteins represents a largely untested mechanism for disease intervention in terms of the development of proteostasis targeted therapeutics [12].

The acetylation and deacetylation status of protein lysine residues, which is mediated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), has a major impact on protein folding properties and the proteostasis system [13]. It is well known that this modification of histone proteins controls the nucleosome structure and regulates gene expressions through epigenetic processes. Acetylation and deacetylation also impacts almost all proteostasis components and pathways, including: (a) the core chaperones that are responsible for assisting protein folding (e.g., Hsp90 [14], Hsp70 [15]; Hsp40 [16], Bip [17]; (b) the transcription factor HSF1, which is critical for stress response [18]; (c) Lys ubiquitination components (by direct competition), which are central in degradation processes [19]; and (d) cytoskeletal components that are responsible for membrane trafficking processes [20]. The key role of HATs and HDACs in proteostasis is further supported by the beneficial effects of HDAC inhibitors (HDACi) in many protein misfolding diseases, such as cystic fibrosis [21], Gaucher's disease [22], muscle atrophy [23], Niemann–Pick C [24], and neurodegenerative diseases [25].

We previously showed that HDACi suberoylanilide hydroxamic acid (SAHA) improved the secretion of functional of Z-AAT from less than 20% to approximately 50% in a Wild-Type (WT-AAT) cell line [26]. Here, we describe the detailed methods for measuring the HDACi effect on both the trafficking efficacy and NE inhibition activity of AAT and Z-AAT. AAT has three N-linked glycosylation sites. The processing states of oligosaccharides can demonstrate the trafficking efficacy of immature, core-glycosylated isoforms that reflect ER fractions as well as mature, complex-glycosylated isoforms that reflects Golgi fractions [27]. The mature isoform that is secreted from cells shows slower migration during sodium dodecyl

sulfate polyacrylamide gel electrophoresis (SDS-PAGE) than the immature isoform. In order to inhibit NE, AAT forms a covalent and irreversible complex with NE, which can be measured by SDS-PAGE as a readout for AAT function [28]. Therefore, this type of assay can serve as a platform to test proteostasis compounds that have therapeutic potential for AATD such as HDACi, which is described here.

2 Materials

2.1 Cellular Sample Materials

1. Cell lines: Human epithelial colorectal carcinoma cell line (HCT116) (*see* **Notes 1** and **2**).
2. Culture medium: Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS).
3. Suberoylanilide hydroxamic acid (SAHA) solution (Cayman Chemical, Ann Arbor, MI, USA): SAHA, 50 mM dimethyl sulfoxide (DMSO).
4. Cell culture equipment: Biosafety hood, 70% ethanol, incubator maintained at 37 °C and 5% CO_2, water bath, microscope, 12-well cell culture dish, pipettes, pipettor.
5. 1× PBS buffer: 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 15.2 mM $Na_2HPO_4/7H_2O$.
6. Cell lysis buffer: 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% (v/v) Triton X-100 containing protease inhibitor cocktail at 2 mg/mL.
7. Centrifuge capable of holding 1.5 mL tubes and reaching centrifugal force of 20,000 × *g* at 4 °C.

2.2 Immunoblotting AAT/Z-AAT

1. Bradford protein assay kit.
2. 8% and 10% SDS-PAGE gels.
3. 6× SDS-PAGE loading buffer: 30% glycerol, 120 mM Tris pH 7.0, 6% SDS, 0.6% Bromophenol Blue with 6% β-mercaptoethanol.
4. 1× running buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS, SeeBlue® prestained protein standard (Life Technologies, Grand Island, NY, USA).
5. SDS-PAGE materials: heating block, electrode/running tank.
6. Protein transfer materials: nitrocellulose membrane, wet-electroblotting systems (transfer tank, cassette, blot paper, glass tube, fiber pad, ice packs, glass dish, stir bar.
7. 1× transfer buffer: 25 mM Tris, 192 mM glycine.
8. Antibodies: AAT antibody (Immunology Consultants Laboratory, Inc. Portland, OR, USA), Hsp90 antibody (Enzo Life

Sciences, Plymouth Meeting, PA, USA.), peroxidase conjugated secondary antibody (mouse anti-goat IgG for AAT primary antibody, goat anti-rabbit IgG for Hsp90 antibody).

9. 1× TBST buffer: 25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4, 0.1% Tween 20.
10. Nonfat dry milk.
11. ECL Solution-1: 2.5 mM luminol, 0.45 mM p-coumaric acid, 0.1 M Tris pH 8.8.
12. ECL Solution-2: 0.02% hydrogen peroxide, 0.1 M Tris pH 8.8.
13. Film, developing cassette, dark room, developer.

2.3 Detecting the Complex of AAT/Z-AAT and NE

1. Human neutrophil elastase (Innovative research, MI, USA).
2. Phenylmethanesulfonylfluoride (PMSF) solution: PMSF, isopropanol.

3 Methods

3.1 Cell Culture Solution and AAT/Z-AAT Detection Solution

1. In order to prepare a stock solution for cell culture, dissolve SAHA in 50 mM DMSO of and store in aliquots at −20 °C.
2. In order to prepare a stock solution for AAT/Z-AAT detection, dissolve PMSF in isopropanol to a concentration of 20 mM (20×) and store it at −20 °C.

3.2 Cell Culture, Compound Treatment, and Sample Collection

1. Plate 10×10^4 WT-AAT and Z-AAT HCT116 cells in a 12-well tissue culture dish with DMEM containing 10% FBS and let them grow to confluent state (usually 2–3 days) (*see* **Note 3**).
2. When the cells are confluent, renew the culture medium (DMEM containing 10% FBS).
3. Add SAHA to a final concentration of 5 μM to the compound treated wells and an equal volume of DMSO for control wells (*see* **Note 4**).
4. Incubate the cells at 37 °C for 24 h.
5. At the end of the treatment, remove and discard the culture medium. Wash the cells twice with 1× PBS.
6. Add 350 μM of serum-free medium (only DMEM) to collect the secreted AAT/Z-AAT.
7. Incubate the cells at 37 °C for 2 h.
8. After 2 h, harvest the culture medium that contains the secreted AAT/Z-AAT (*see* **Note 5**).
9. Samples can be aliquoted and stored at −80 °C.

10. Put the culture dish on ice and wash the dish twice with cold 1× PBS.
11. Add 50 μL lysis buffer to each well.
12. Lyse the cells for 30 min on ice and rock the dish every 10 min.
13. After 30 min, scrape the wells and transfer the lysate to 1.5 mL centrifuge tubes.
14. Spin the lysate at 20,000 × *g* for 20 min at 4 °C.
15. Collect the supernatant of the lysate and transfer it to a new set of tubes (*see* **Note 6**).
16. Samples can be stored at −80 °C.

3.3 Measuring the Secretion of AAT/Z-AAT in Response to SAHA

1. Determine the protein concentration of the cell lysate supernatant by using the Bradford protein assay kit.
2. Prepare loading samples by adding SDS-PAGE loading buffer and heating the sample at 95 °C for 5 min (*see* **Note 7**).
3. Load 15 μg lysate supernatant samples to a 10% SDS-PAGE gel for probing the immature and mature AAT/Z-AAT.
4. Load 20 μL collected culture medium samples to another 10% SDS-PAGE gel for probing the secreted AAT/Z-AAT.
5. Load the prestained protein standard ladder on both gels.
6. Run the gel at 30 mA/gel and 200 V until the 50 kDa marker band nears the bottom (AAT is 52 kDa).
7. Stop the gel and perform a protein transfer to a nitrocellulose membrane at 500 mA of current and 100 V for 1.5 h.
8. Hsp90 is the loading control. The immature, mature, and secreted bands of AAT/Z-AAT (52 kDa of molecular weight) are found between the markers of 50 kDa and 64 kDa (See-Blue® prestained protein standard). When considering other proteins as loading controls, *see* **Notes 8** and **9**.
9. Cut the blot above 64 kDa in order to separate the intracellular AAT/Z-AAT from Hsp90.
10. Block the blots in 5% milk (diluted in 1× TBST) at room temperature for 1 h.
11. Dilute the AAT antibody and Hsp90 antibody in 1% milk with 1:2000 and 1:25,000 ratios, respectively.
12. Incubate the blots in the primary antibody at 4 °C on the rocker platform overnight.
13. On the next day, discard the primary antibody and wash the blots with 1× TBST buffer for 10 min three times.
14. Then incubate the blots in the peroxidase-conjugated secondary antibody (1:10,000 diluted in 1% milk) for 1 h at room temperature.

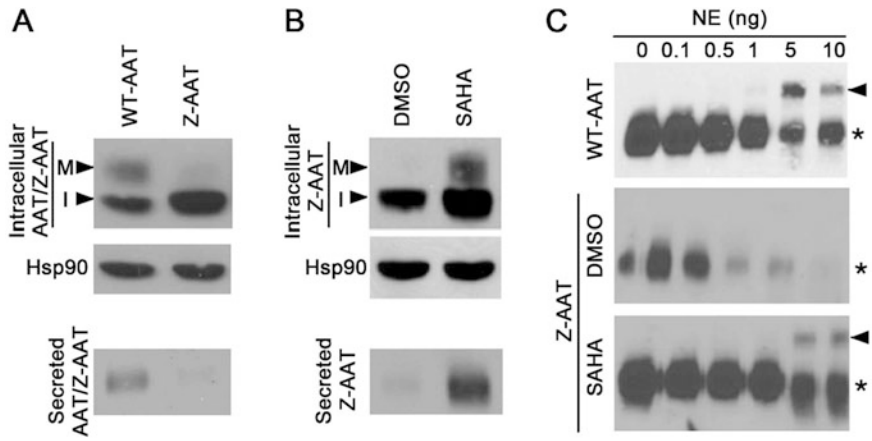

Fig. 1 Western blot shows the secretion and NE inhibition activity of Z-AAT with or without SAHA treatment. (**a**) Secretion of WT-AAT or Z-AAT. *M* mature fraction, *I* immature fraction. (**b**) Secretion of Z-AAT under DMSO or SAHA condition. (**c**) NE inhibition activity of WT-AAT and Z-AAT with or without SAHA treatment. The band indicated by *arrow* is the AAT-NE complex. The band labeled by *asterisk* is the unbounded AAT. This figure is derived from reference [26]

15. Discard the secondary antibody and wash the blots with 1× TBST buffer for 10 min three times.
16. After the wash, dab the blots on filter paper; place the blots on plastic wrap, add 2 mL of the ECL mixture (mix equal volumes of solution 1 and 2) to each blot, and incubate for 1 min.
17. Dab the excess liquid off and put blots in the developing cassette with the transparent sheet protector.
18. Perform the film exposure and developing in the dark room.

As shown in Fig.1a, the mature fraction (M) of WT-AAT migrates slower than the immature fraction (I). Compared to WT-AAT, the mature and secreted fraction of Z-AAT is significantly smaller, while the immature fraction is larger, indicating Z-AAT is largely retained in the ER. SAHA significantly increases both the mature and secreted fraction (Fig. 1b), indicating the correction effect of HDACi on the deficient secretion of Z-AAT.

3.4 Measuring the Antiprotease of AAT/Z-AAT in Response to SAHA

1. Prepare NE solution in 1× PBS buffer at 0.1, 0.5, 1, 2, 5, and 10 ng/μL.
2. Add 1 μL of each of the NE solutions to 20 μL of the culture medium that were collected in each experiment that contains secreted AAT or Z-AAT.
3. Tap the tubes several times to mix the solution, then briefly spin down the mixture.
4. Incubate the tubes at 37 °C for 30 min in order to perform the binding reaction.

5. After the reaction is complete, add SDS-PAGE loading buffer and heat the samples at 95 °C for 5 min (*see* **Note 10**).
6. Run the samples by using 8% SDS-PAGE and follow the same western blot procedure described above. Use the AAT antibody to detect the AAT-NE complex.

As shown in Fig.1c, the covalent complex between WT-AAT and NE is observed when 5 ng of NE was added in the solution (the band is indicated by the arrow). No complex is observed for Z-AAT. In contrast, after SAHA treatment, we observed the complex between Z-AAT and NE, which indicates that SAHA induced an increase in the antiprotease activity of secreted Z-AAT.

4 Notes

1. Although AAT is mainly synthesized and secreted from hepatocytes, the large amount of endogenous WT-AAT limits the use of the liver cell line to study the biology of the exogenously expressed Z-variant AAT. Therefore, we used a human epithelial colorectal carcinoma cell line (HCT116) with undetectable endogenous AAT to generate a stable cell line that expresses the exogenous WT-AAT or Z-AAT with FLAG and HA tags at the N-terminus [26].
2. All the materials listed need to be prepared in an aseptic environment or sterilized in order to avoid contamination.
3. For HCT116 cell maintenance, the medium (DMEM containing 10% FBS) needs to be renewed every 2 to 3 days, and a subcultivation ratio of 1:3 to 1:8 is recommended. The culture environment is 37 °C with 95% air and 5% carbon dioxide.
4. A dose-dependent increase effect of SAHA was observed in our previous study [26]. A dose of 0.5 μM of SAHA starts to show a corrective effect, and a dose of 5 μM of SAHA produces the maximal corrective effect. A dose higher than 5 μM is toxic to the cell.
5. After collecting the culture medium that contains secreted AAT/Z-AAT, it is better to add fresh medium back to the cells in order to prevent drying. This also provides more time to prepare the next step.
6. The pellet of the cell lysate can be kept for the analysis of aggregates of AAT/Z-AAT [29].
7. The loading volume of the collected culture medium can be normalized according to the protein concentration of the cell lysate.
8. Actin (43 kDa for either alpha or beta subunit) and tubulin (55 kDa for either alpha or beta subunit) are not recommended

for use as loading controls because they have molecular weights that are similar to AAT (52 kDa) and cannot be separated from AAT in SDS-PAGE.

9. GAPDH (36 kDa) can be used as a loading control for intracellular AAT. If GAPDH is used as a loading control, make sure that (a) GAPDH does not run out of the gel, and (b) cut the membrane below the 50 kDa marker band carefully to separate AAT and GAPDH. Ponceau staining of the membrane can provide a general view of how the samples run on the SDS-PAGE, which helps facilitate proper cutting.
10. It is recommended to add 1 mM of PMSF to stop the NE binding reaction before adding the SDS-PAGE loading buffer. After forming the covalent complex with NE, AAT undergoes large conformational changes and becomes disordered in some regions, which makes the complex very susceptible to being digested by protease, including NE. By adding PMSF immediately after the reaction, it is possible to detect the very weak Z-AAT and NE complex (data not shown).

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