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Preparation and characterization of vorinostat-coated beads for profiling of novel target proteins

Congcong Lu[a], Kai Zhang[a,b,*], Yi Zhang[c], Minjia Tan[c], Yijun Li[a], Xiwen He[a], Yukui Zhang[a,d]

[a] State Key Laboratory of Medicinal Chemical Biology & Department of Chemistry, Nankai University, Tianjin 300071, PR China
[b] Department of Biochemistry and Molecular Biology & Tianjin Key Laboratory of Medical Epigenetics, Basic Medical School, Tianjin Medical University, Tianjin 300070, PR China
[c] The Chemical Proteomics Center and State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, PR China
[d] National Chromatographic Research and Analysis Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, PR China

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ABSTRACT

Inhibitors of histone deacetylases (HDACs) have been considered to be new anticancer agents. As a key inhibitor of HDAC, vorinostat can cause growth arrest and death of a broad of transformed cells and interact with a variety of substrates. A comprehensive analysis of proteins interacting with HDAC inhibitors is of great importance in understanding molecular mechanisms of the drugs. Here, we reported the preparation and characterization of vorinostat-coated beads for profiling of novel target proteins of vorinostat (a key HDAC inhibitor). The enriched proteins were further analyzed by HPLC–MS/MS. Besides the known substrates, there were also several novel enriched protein candidates, one of which was a metalloenzyme α-enolase (ENO-1). According to our best knowledge, it is the first time that ENO-1 has been detected as a potential target of vorinostat through chemoproteomics approach. Further competition analysis indicated that ENO-1 may be co-enriched as a substrate complex. Our results demonstrated that the chemical probe combined with proteomics approach may be developed as a potential tool to identify target proteins of drugs.

1. Introduction

Protein lysine acetylation, including histone acetylation and non-histone protein acetylation, plays a major role in regulating chromatin structure, transcriptional activity and metabolic pathways, thus contributing to diverse cellular process like transcription, cell cycle regulation, apoptosis and senescence [1–4]. This dynamic and reversible post-translational modification (PTM) is regulated by histone deacetylases (HDACs) and histone acetyl transferases (HATs) [5,6]. Three major classes of mammalian HDACs have been extensively described, of which classes I and II are zinc-dependent metallohydrolases and class III are NAD^+-dependent deacetylases [4,5]. Recent advances have shown that the development of cancer is intimately associated with HDAC expression [7–9]. Treatment of tumor cells with HDAC inhibitors (HDACIs) results in growth arrest, differentiation and/or apoptosis of many cancer cells [7,10]. HDACIs have emerged as exciting anticancer agents and several classes of HDACIs have been found to have potent and specific anticancer activities [10–12].

To understand the anticancer activity mechanisms of HDACIs, it is necessary to elucidate the target proteins of the drugs [13]. Small molecular probe-based proteomics approach has become a potential tool for profiling targets of the drugs [14]. A series of novel target proteins of HDACI have been revealed by combining affinity capture and mass spectrometry analysis [14,15]. These studies further indicate that the HDACIs mode of action could be considerably broader and more complicated than original understanding of altering epigenetic changes [16,17].

Suberoylanilide hydroxamic acid (SAHA, also named Vorinostat or Zolinza), is a key second-generation hydroxamate HDACI of classes I and II for the treatment of refractory cutaneous T-cell lymphoma [13,18]. Vorinostat can cause growth arrest and death of a broad of transformed cells and have little or no toxic effects on normal cell [13]. Recent evidence indicates that, vorinostat may interact with a variety of substrates including chromatin proteins,

* Corresponding author at: Department of Biochemistry and Molecular Biology, Tianjin Medical University, Tianjin 300070, PR China. Tel.: +86 22 23494962.
E-mail address: zhangkai@nankai.edu.cn (K. Zhang).

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transcription factors, metabolic enzymes, and cell structure proteins [19].

In this study, we aimed to profile potential targets and substrate complexes of vorinostat in a whole-cell lysate combining vorinostat-coated beads and HPLC–MS/MS analysis. A vorinostat-based small molecule probe was designed and synthesized, then immobilized onto the surface of ECH sepharose beads. We investigated the effect of different conditions on proteomics profiling and further identified 58 protein candidates using the probe in the optimized condition. Bioinformatic analysis indicated that most identified proteins were involved in metabolic process, protein biosynthesis, cell cycle and differentiation. Besides known complexes and substrates, there were also several novel enriched proteins in this analysis, one of which was a metalloenzyme α-enolase (ENO-1). As a novel, potential target of vorinostat, ENO-1 was detected and confirmed in the study. Further western blot competition analysis indicated that ENO-1 may be co-enriched as a substrate complex rather than direct interaction with vorinostat. This study showed that the probe-based approach holds a great potential for identification of novel candidates interacting with HDACIs.

2. Experimental

2.1. Reagents and materials

Suberic acid monomethyl ester was purchased from Suzhou BEC Biological Technology Co., Ltd (Suzhou, China). Hydroxybenzotriazole (HOBt), dicyclocarbodiimide (DCC) and *p*-phenylenediamine were obtained from Sinopharm Chemical Reagent Beijing Co., Ltd. (Beijing, China). ECH Sepharose 4B was purchased from GE Healthcare Life Sciences (Pittsburgh, Pennsylvania, USA). *N*-(3-Dimethylaminopropy)-*N*′-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were both purchased from J&K (Beijing, China). Petroleum ether, ethyl acetate, acetic acid, methanol and ethanol were purchased from Concord Technology Co., Ltd. (Tianjin, China). The other chemicals unless otherwise mentioned were all purchased from Sangon Biotech (Shanghai, China). All chemicals were used as received without further purification. The water in this work was distilled water unless otherwise mentioned.

2.2. Synthesis of vorinostat-based small molecule probe (SAHA-probe)

As shown in Fig. 1, the SAHA-probe was synthesized in two-step procedure with mild conditions according to Gediya's protocol with minor modifications [20]. In brief, suberic acid monomethyl ester was condensed with *p*-phenylenediamine in a typical coupling reaction for amide bond formation using HOBt and DCC in DMF. After stirred at room temperature (RT) for 4 h, the mixture was added in cold stirring water. The precipitate was filtered and the crude product Compound A was placed on a short pad of silicagel and eluted with petroleum ether and ethyl acetate (1:4). Hydroxylamine was prepared by mixing hydroxylamine hydrochloride with potassium hydroxide in methanol. After stirred at RT for 2 h, the product was obtained by adding water and neutralizing with acetic acid. The precipitate was filtered off and the resulting product SAHA-probe was dried using an infrared heating lamp.

2.3. Cell culture and preparation of cell lysate

Hela cells were cultured with modified RPMI-1640 medium (Thermo Fisher Scientific, Beijing, China) supplemented with 10% fetal calf serum (FBS, Invitrogen, Carlsbad, California, USA) at 37 °C with 5% CO_2 in atmosphere. After being washed twice with ice-cold phosphate-buffered saline (PBS), cells were harvested and lysed by the use of Cell Lysis Kit (Sangon Biotech, Shanghai, China, containing both lysis buffer and protease inhibitors). The cell lysate was incubated on ice for 30 min and centrifuged at 4 °C at 12,000 × *g* for 10 min. The supernatant was taken as extracted proteins and stored at −20 °C until further use. The protein concentration was approximately 400 μg/mL determined by Bradford assay (Sangon Biotech, Shanghai, China).

2.4. Proteomics-based vorinostat targets identification

Vorinosta-coated beads (SAHA-CB) were prepared by immobilizing SAHA-probes on ECH Sepharose 4B using a typical coupling reaction for amide bond formation using EDC and NHS. Before use, ECH Sepharose beads were washed each two times with distilled water (adjusted to pH 4.5 with HCl), followed by NaCl (0.5 M). Then, beads were resuspended in reaction buffer (0.1 M PBS, pH 6.0) and EDC and NHS were added as free flowing powder. After 1-h end-over-end rotation at RT, SAHA-probes (dissolved in DMSO) were added into the mixture. Keep rotating the mixture overnight at RT. The product was washed thoroughly with alternating pH three cycles to remove the extra small molecules. Each cycle consists of a wash with 0.1 M Tris–HCl buffer (pH 8.0, containing 0.5 M NaCl), a wash with 0.1 M acetic acid/sodium acetate (pH 4.0, containing 0.5 M NaCl) followed by DMSO. The SAHA-CB were stored in isopropanol at −20 °C and washed thrice by distilled water before use. As a control experiment, DMSO without SAHA-probe was employed to perform control-Beads (Ctrl-Beads) for verifying the effect of vorinostat in chemoproteomics profiling.

About 30 μL drained SAHA-CB were incubated with 150 μL cell lysate on an end-over-end shaker at 4 °C overnight. After removing the supernatant by centrifugation, SAHA-CB were washed extensively twice with lysis buffer and ice-cold PBS, followed by HPLC water to remove proteins non-specifically enriched through protein–protein interactions. Then, targets were denatured in 50 μL SDS-PAGE sample buffer by boiling for 10 min, analyzed by 10% SDS-PAGE and detected by silver staining (Pierce Silver Stain Kit, Thermo Scientific, Rockford, Illinois, USA) according to the standard laboratory procedure. Besides harsh protein-denaturing conditions, different eluting buffers were also tried, including 1% SDS, 0.5 M and 2.5 M NaCl, 0.2 M HAc and 1.0 M EDTA, with end-over-end rotation for 15 min at RT. After SAHA-CB firstly eluted with above elution agents, target proteins eluted with water and heat denaturation by boiling for 10 min were further studied.

2.5. Western blot analysis of SAHA-CB binding to ENO-1

Competition analysis using free vorinostat as a competitor binding ENO-1 was manufactured. SAHA-CB was incubated with cell lysate or purified ENO-1 (Prospec-TechnoGene Ltd., Ness-Ziona, Israel) overnight at 4 °C, which had been preincubated with different concentration vorinostat (0.0 μM, 0.5 μM, 5.0 μM and 50 μM for cell lysate, 0.0 μM, 0.1 μM and 50 μM for purified ENO-1, respectively) for 2 h on an end-over-end shaker at 4 °C.

Proteins were separated by SDS-PAGE and electrotansferd to 0.45 μm nitrocellulose (Millipore Corporation, Billerica, Massachusetts, USA). The membrane were incubated in blocking buffer (TBST with 5% nonfat dry milk) for 1 h at RT and then incubated in anti-ENO-1 antibody (1:2000) (Cell Signalling Technology, Inc., Boston, Massachusetts, USA) with gentle agitation overnight at 4 °C after being washed three times with TBST. After rinsed thrice with TBST, the membrane was incubated with HRP-conjugated anti-rabbit (1:20,000) (Santa Cruz Biotechnology, Inc., Shanghai, China) for 1 h at RT. Finally, immunoreactive bands were revealed using