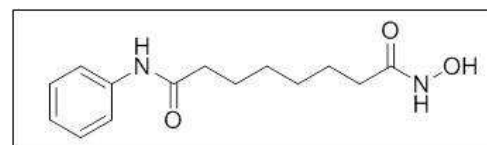


Isolation of drug target protein Histone Deacetylase Inhibitor (Vorinostat)

Summary

Histone deacetylase (HDAC) is an enzyme that performs deacetylation of the histone which is the main configuration factors in chromatin structure, and vorinostat (SAHA) is known as one of the HDAC inhibitors. We show the result of experiment here to purify the targets of vorinostat in certain condition. We immobilized vorinostat binding proteins.



Vorinostat

As the result, several binding proteins were separated from HeLa cell extracts. Four types of HDAC were identified by western blotting, and ECHS1 (Enoyl CoA hydratase, short chain 1) was identified by mass spectrometry in this experimental condition. Type and amount of the purified proteins may be changed dependent on the amount of compounds immobilized on FG beads. Therefore, it may be necessary to examine several conditions.

Experimental Information

Vorinostat does not have available functional group. So, we synthesized an amino-derivative of vorinostat. When we synthesize a derivative, we introduce a new functional group and position it in a way that does not alter the activity of the compound.

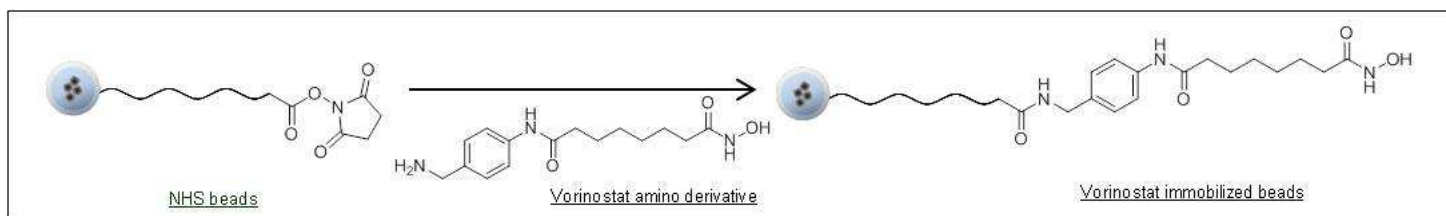
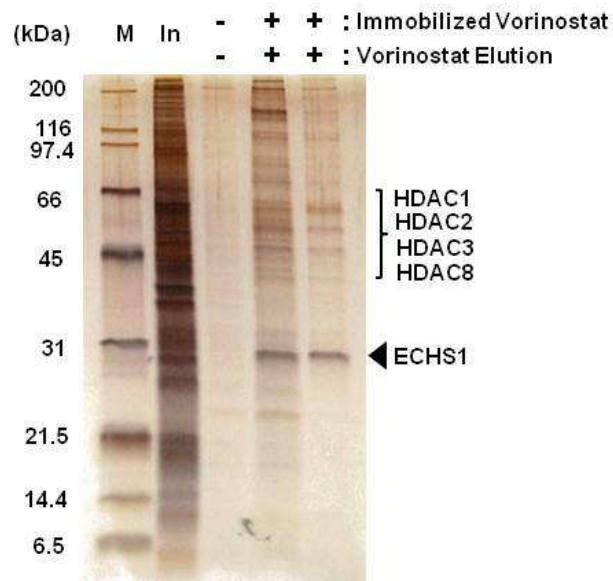


Fig. Immobilization of Vorinostat amino derivative on NHS beads

Result

Several binding proteins were separated using FG beads. Several proteins were eluted with free vorinostat, and it was suggested that these proteins specifically interact with vorinostat. Four type of HDAC were confirmed by western blotting, and ECHS1 was identified by mass spectrometry.



Materials and Method

• Materials

1. Vorinostat amino derivative
2. NHS beads
3. HeLa cell extracts (cytosolic fraction) ... 3mg/ml
4. Binding & Washing Buffer (20mM HEPES-NaOH (pH7.9), 100mM KCl, 1mM MgCl₂, 0.2mM CaCl₂, 0.2mM EDTA, 10%(v/v) glycerol, 0.1% NP-40, 1mM DTT, 0.2mM PMSF)
5. Elution Buffer (0.0625M Tris-HCl (pH6.8), 0.005% BPB, 2% SDS, 10% glycerol, 5% 2-mercapto ethanol)
6. Vorinostat
7. Rabbit polyclonal anti-HDAC1 (Santa Cruz)
8. Rabbit polyclonal anti-HDAC2 (Santa Cruz)
9. Rabbit polyclonal anti-HDAC3 (Santa Cruz)
10. Rabbit polyclonal anti-HDAC8 (Santa Cruz)
11. Anti-Rabbit IgG, HRP-Linked Whole Ab Sheep (GE)
12. Starting Block™ (TBS) Blocking Buffer (Thermo)
13. TBS-T buffer (137mM NaCl, 2.68mM KCl, 25mM Tris-HCl, pH7.4, 0.1w/v% Tween-20)

• Methods 1 (Immobilization)

1. Apply
0.5mg NHS beads and 0.3mM vorinostat amino derivative in 100ul DMF
0.5mg NHS beads and NO vorinostat amino derivative in 100ul DMF (Negative Control)
2. Reaction
 - 1) Immobilization 70 min at RT
 - 2) Masking
Inactivate unreacted NHS according to recommended conditions.

• Methods 2 (Affinity purification)

1. Wash
Wash beads with washing buffer 3 times at 4°C (on ice).
2. Add sample solution
Add 1000ul HeLa cell extracts to beads.
3. Reaction
Incubate for 240min at 4°C.
4. Wash
Remove sample solution. Wash beads with washing buffer 3 times at 4°C (on ice).
5. Elution
Vorinostat elution: —
Add 40ul elution buffer and resuspend beads. Boil for 5min at 98°C and remove beads.
Vorinostat elution: +
Add 30ul of 25mM vorinostat in washing buffer and resuspend beads. Place the tube on ice for an hour to allow bound proteins to elute by tapping at intervals. After spin down, separate magnetically. Transfer the supernatant to a new clean tube. Add 10ul of 4x elution buffer to the tube. Boil for 5min at 98°C.
6. Analyze the samples by SDS-PAGE and silver staining

• Methods 3 (Western blotting)

1. Perform SDS-PAGE and transfer the protein to a PVDF membrane.
2. Block the membrane with the blocking buffer for 15min at room temperature.
3. Dilute the anti-HDAC antibody with the blocking buffer to 1/200.
4. Incubate for 60min at room temperature.
5. Dilute the secondary antibody with the TBS-T buffer to 1/1000.
6. Incubate for 60min at room temperature.
7. Wash the membrane with TBS-T buffer three times.
8. Detect with a chemiluminescence substrate.

• Methods 4 (MS)

1. Excising the bands from the gel.
2. Wash the band pieces, then dry the band pieces.
3. Add 500ul/band of 10mM DTT/25mM NH₄HCO₃.
4. Incubate for 60min at 56°C.
5. Add 500ul/band of 55mM iodeacetamide/25mM NH₄HCO₃.
6. Incubate for 60min at room temperature.
7. Wash the band pieces with 200ul of 25mM NH₄HCO₃ for 10min.
8. Wash the band pieces with 500ul of 25mM CH₃CN/NH₄HCO₃ (50:50 v/v) for 10min.
9. Dry the band pieces.
10. Add 10ul/band of 10ug/ml trypsin in 25mM NH₄HCO₃.
11. Incubate overnight at 37°C.
12. Add 50ul/band of 25mM CH₃CN/trifluoroacetic acid/ultrapure water (50:5:45 v/v).
13. Incubate for 30min at room temperature.
14. Transfer supernatant to a new clean tube.
15. Dry the peptides in the tube.
16. Injection on the MS.

• FG beads information

Product name	NHS beads
Product number	TAS8848N1141
Storage temperature	-20°C
Storage buffer	Isopropyl alcohol
Size of beads	190nm ± 20nm
Functional groups	200 - 300 nmol/mg