FG winds

Issue 4
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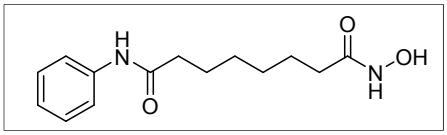
Isolation of drug target protein (3)
Histone Deacetylase Inhibitor【Vorinostat】

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 amino derivative on FG beads (NHS beads) to perform affinity purification of vorinostat binding proteins.
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.



Vorinostat

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.

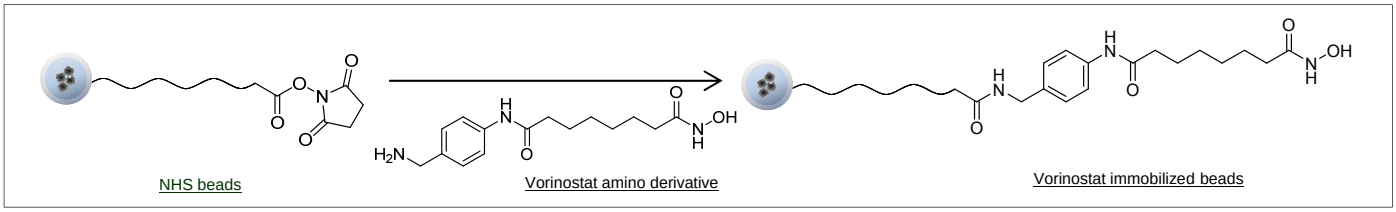
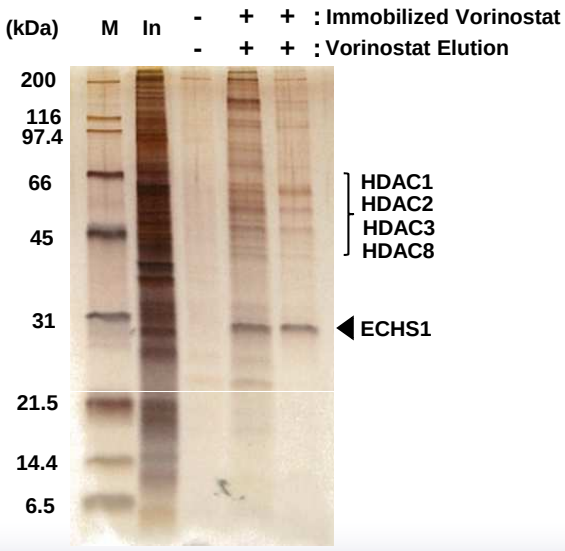


Fig.1 Immobilization of Vorinostat amino derivative on NHS beads

Result

1. 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 types 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 $MgCl_2$, 0.2mM $CaCl_2$, 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 (from Santa Cruz Biotechnology)
8. Rabbit polyclonal anti-HDAC2 (from Santa Cruz Biotechnology)
9. Rabbit polyclonal anti-HDAC3 (from Santa Cruz Biotechnology)
10. Rabbit polyclonal anti-HDAC8 (from Santa Cruz Biotechnology)
11. Anti-Rabbit IgG, HRP-Linked Whole Ab Sheep (from GE Healthcare)
12. Starting Block™ (TBS) Blocking Buffer (from Thermo scientific)
13. TBS-T buffer (137mM NaCl, 2.68mM KCl, 25mM Tris-HCl, pH7.4, 0.1w/v% Tween-20)

■FG beads information

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

■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

70min at r.t.

2) Masking

Inactivate unreacted NHS according to recommended conditions.

■Methods 2 (Affinity purification)

1. Wash

Wash beads with washing buffer 3 times at 4ºC (on ice).

2. Add sample solution

Add 1000ul HeLa cell extracts to beads.

3. Reaction

Incubate for 240min at 4ºC.

4. Wash

Remove sample solution.

Wash beads with washing buffer 3 times at 4ºC (on ice).

5. Elution

Vorinostat elution : -

Add 40ul elution buffer and resuspend beads.

Boil for 5min at 98ºC 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 4×elution buffer to the tube.

Boil for 5min at 98ºC.

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 NH_4HCO_3.
4. Incubate for 60min at 56℃.
5. Add 500ul/band of 55mM iodeacetamide/25mM NH_4HCO_3.
6. Incubate for 60min at room temperature.
7. Wash the band pieces with 200ul of 25mM NH_4HCO_3 for 10min.
8. Wash the band pieces with 500ul of 25mM CH_3CN/NH_4HCO_3 (50:50 v/v) for 10min.
9. Dry the band pieces.
10. Add 10ul/band of 10ug/ml trypsin in 25mM NH_4HCO_3.
11. Incubate overnight at 37℃.
12. Add 50ul/band of 25mM CH_3CN/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.