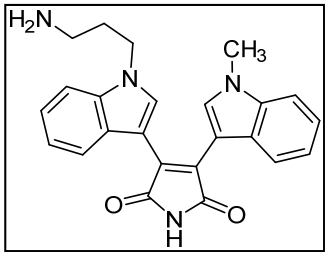
FG winds

Issue 3
September, 2014

Isolation of drug target protein (2)
Protein kinase inhibitors【Bisindolylmaleimide】

Summary

A protein kinase is a enzyme that modifies other proteins by adding phosphate groups to them (phosphorylation).
We show the result of experiment here to purify the targets of Bisindolylmaleimide Ⅷ (Bis Ⅷ) in certain condition. Bisindolylmaleimide compounds were originally reported as a potent inhibitor against Protein Kinase C. The inhibition of the other kinases such as GSK3α/β[1], CDK2[2] and Rsk[3] were also observed afterwards.
HeLa cell extracts we used was not treated by TPA or any other stimulator. And Bis Ⅷ was immobilized on FG beads (NHS beads) to perform affinity purification of bound proteins with Bis Ⅷ immobilized beads.
As the result, several binding proteins were separated and one of major proteins, bound to Bis Ⅷ immobilized beads was GSK3α/β, confirmed by western blotting and mass spectrometry in this experimental condition.
The major purified proteins may be changed dependent on the amount of compounds immobilized on FG beads.

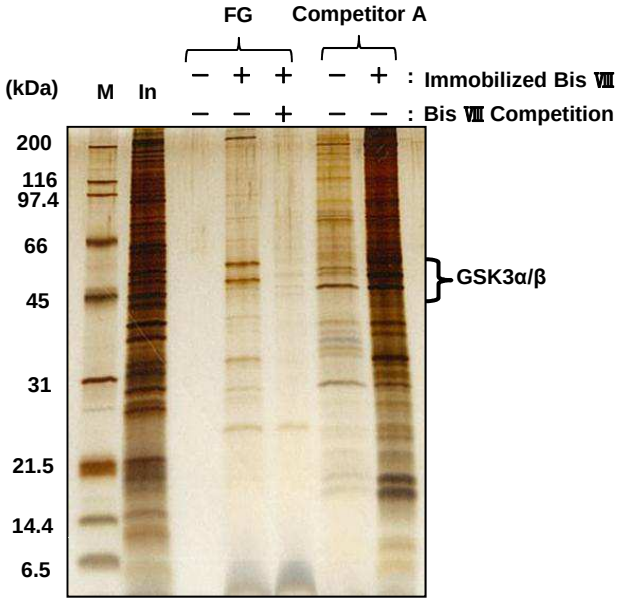


Bisindolylmaleimide Ⅷ

Result

1. Several binding proteins were separated using FG beads or Competitor A.
When free Bis Ⅷ was added to HeLa cell extracts before incubation with the beads, the yields of several proteins were reduced, which suggested that these proteins specifically interact with Bis Ⅷ.
Two major protein bands, which are supposed to be GSK3α/β[2] from their molecular weight, were selected for further identification experiments.
In addition, Their bands were confirmed as GSK3α/β by mass spectrometry.

2. We also compared FG beads with other commercially available magnetic beads.
We confirmed FG beads have extremely lower background.



1) Nihar R, Jena. Journal of Molecular Modeling 18 (2012) 631-644
2) Brehmer, D., Godl, K., Zech, B., Wissing, J., and Daub, H. Molecular & Cellular Proteomics 3.5 (2004) 490-500
3) Roberts, NA., Haworth, RS., Avkiran, M., J Pharmacol. 145(4) (2005) 477-489

Materials and method

■Materials

1. Bisindolylmaleimide Ⅷ
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. Mouse monoclonal anti-GSK3α/β (from Santa Cruz Biotechnology)
7. Anti-Mouse IgG, HRP-Linked Whole Ab Sheep (from GE Healthcare)
8. Starting Block™ (TBS) Blocking Buffer (from Thermo scientific)
9. TBS-T buffer (137mM NaCl, 2.68mM KCl, 25mM Tris-HCl, pH7.4, 0.1w/v% Tween-20)

■Methods 1 (Immobilization)

1. **Apply**
   FG beads
   0.5mg NHS beads and 0.1mM Bis Ⅷ in 100ul DMF
   0.5mg NHS beads and NO Bis Ⅷ in 100ul DMF (Negative Control)
   Competitor A
   0.5mg NHS beads and 1mM Bis Ⅷ in 100ul DMF
   0.5mg NHS beads and NO Bis Ⅷ in 100ul DMF (Negative Control)
2. **Reaction**
   1) Immobilization
   70min at r.t.
   2) Masking
   Inactivate unreacted NHS according to recommended conditions of each beads.

■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 (Bis Ⅷ Competition : －) or Bis Ⅷ added HeLa cell extracts (Bis Ⅷ Competition : ＋) to beads.
   (0.1mM Bis Ⅷ is added to HeLa cell extracts and preincubated for 120min before incubation with the 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**
   Add 40ul elution buffer and resuspend beads.
   Boil for 5min at 98ºC and remove beads.
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-GSK3α/β 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.

■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

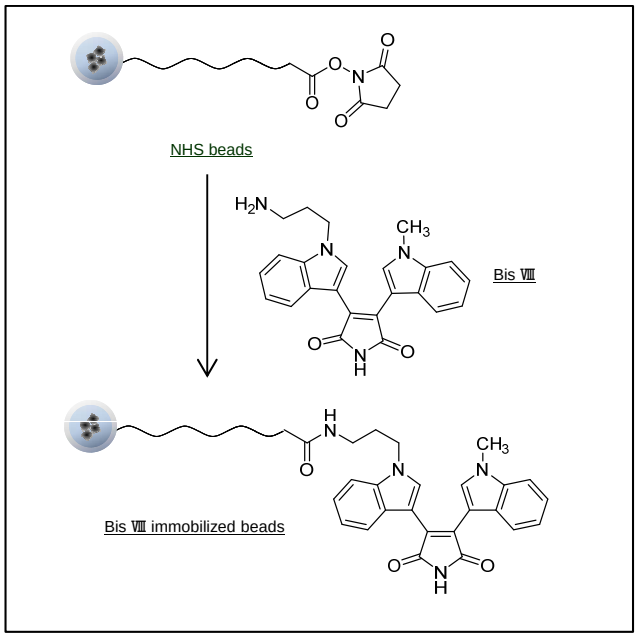


Fig.1 Immobilization of Bis Ⅷ on NHS beads

■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.