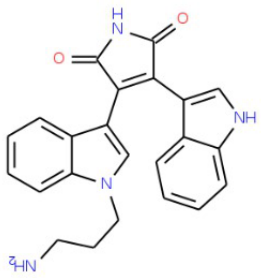
This note describes the application of the technology in identifying/de-convoluting true positive targets of a test-molecule BIS-III.

Background and Overall Goal

Bisindolylmaleimide-III (Bis-III) a known inhibitor of GSK3 protein and it induces apoptosis in the cancerous cell-lines. At 1µM, Bis-III inhibits 93% of PKCα kinase activity and also inhibits many other protein kinases including, S6K1, MAPKAP-K1, RSK2 and MSK1 with similar potency. Additionally, it inhibits PDK1, an important kinase in the insulin signaling pathway. In following experiments Shantani's technology was utilized to identify primary and secondary targets of BIS-III.



Step-1 & 2) Derivatization and Immobilization of Bis-III on Agarose Beads

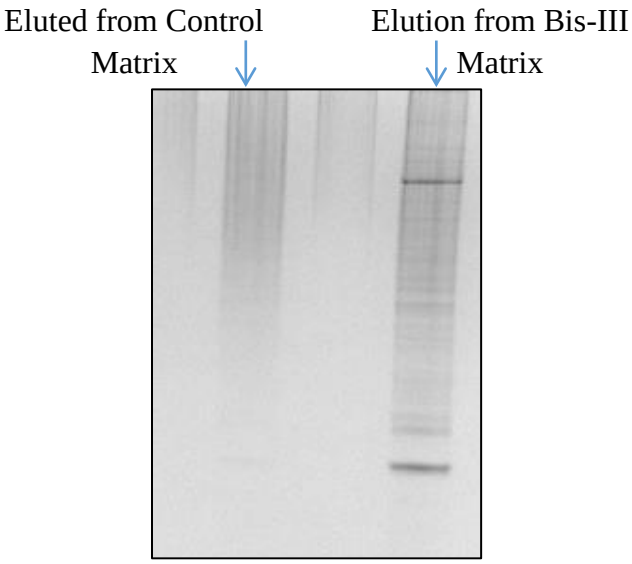
Bis-III molecule already has a primary amine at it non-active end and hence molecule derivatization was not needed. 25 µl of Bis-III Affinity matrix was prepared by immobilizing Bis-III on epoxy activiated agarose beads. For controls similar amount of beads was prepared by blocking the epoxy active side by amine based buffer. Prepared affinity matrix was extensive washed and then equilibrated in high salt buffer (1 M NaCl, 50 mM HEPES (pH 7.3), 0.1% Triton, 1 mM EDTA).

Step-3) Affinity Chromatography and Isolation of Target Proteins

25 µl each of Bis-III and control matrix was incubated with 2 mg/ml HeLa Cell Lysate for 3 hours at 4° centigrade. After incubation beads were centrifuged for 60 seconds at 3000 RPM using benchtop mini-centrifuge. Unbound protein fraction, the supernatant, was discarded. Beads were washed 2X with 100 µl of high salt buffer and 2X with 100 µl of low salt buffer (150 mM NaCl, 50 mM HEPES (pH 7.3), 0.1% Triton, 1 mM EDTA) by adding the buffer to beads and gently inverting the tubes upside down for 2 minutes followed by 60 seconds of centrifugation at 3000 RPM and remove of supernatant washes. Proteins bound to beads were then eluted by Elution buffer (1 mM Bis-III in 50 mM HEPES (pH 7.3)). Eluted proteins were precipitated using chloroform-methanol based methods.

Step-4) Protein Analysis and Identification

$1/10^{th}$ of precipitated protein was separated over 4-12 Bis-Tris SDS-PAGE gels and stained with silver-stain to establish if the Bis-III Matrix could enrich some differential proteins. Further, $1/10^{th}$ of precipitated proteins was used for western blotting to confirm the capture of primary target.



Typical Profile of Proteins separated using SDS-PAGE. Proteins were stained with silver-stain for visualization.
It was established that Bis-III Matrix enriched some proteins over control matrix.

GSK3-β Western-Blot

Western-Blot Analysis probing primary target protein GSK3-β established the specific capture of target protein using Bis-III specific matrix.

Rest of the 9/10 of the proteins was separated over a SDS-PAGE and stained with coomassie-blue stain. All the gel-bands from control and Bis-III elution lane were subjected to in-gel trypsin digestion protocol and proteins were identified using mass-spectrometry based workflow.

Step-5) Deconvolution of Targets and Conclusion

Specific targets of Bis-III were deconvoluted by comparing the protein identified in multiple Bis-III and control experiments (see TBB-Technical note). Out of 16 deconvoluted specific proteins following 4 proteins are known primary and secondary targets of Bis-III.

Uniprot ID	Protein Name	Number of Unique Peptides
P16083	NQO2	2
P55263	Adenosine Kinase	4
P49840	GSK3	2
O00764	Pyridoxal Kinase	3

This result confirms the capabilities of TBB in identifying targets of bioactive compounds.
