MAPPING THE INTERACTIONS OF PKNB WITH SMALL MOLECULE INHIBITORS USING PLASMA INDUCED MODIFICATIONS OF BIOMOLECULES (PLIMB)

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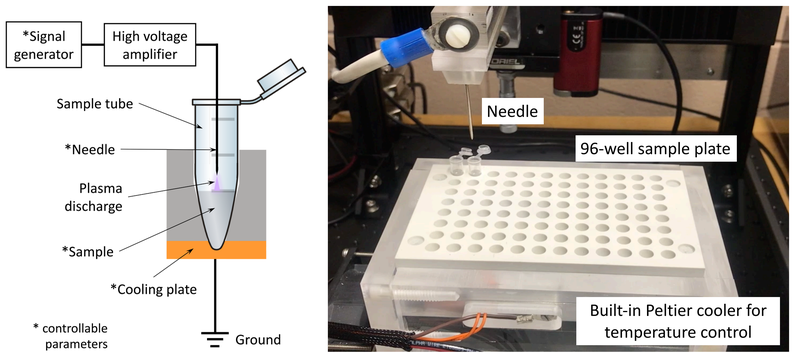
Introduction

Plasma Induced Modifications of Biomolecules (PLIMB) is a recently advanced technology that provides a uniquely powerful method for mapping protein-protein and protein-ligand interactions. PLIMB is a mass spectrometry (MS)-based protein footprinting technique that generates µs bursts of hydroxyl radicals from water, to measure changes in protein structure via altered solvent accessibility of amino acid side chains. Previously, PLIMB has been utilized to study protein-protein interactions and map the epitopes and paratopes of antibody:antigen pairs.

Here, we utilized PLIMB to characterize the interactions of PknB, a mycobacterial Ser/Thr kinase with two small molecule inhibitors, GSK690693 and GW779439X. PknB is essential for *M. tuberculosis* survival and is therefore an attractive drug target. Structure-based design of kinase inhibitors greatly benefits from understanding dynamic conformational changes in the target binding site. Furthermore, high resolution structural information of apo kinase structures is not always attainable, and rapid structural assessment of numerous small molecule-target interactions can assess structure-activity relationships.



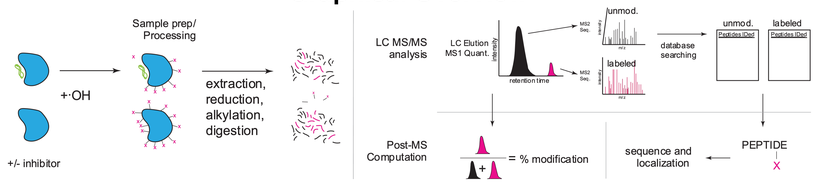
Schematic and photograph of the PLIMB setup

Key Benefits of PLIMB:

- Easy to use, automated, benchtop instrument
- High throughput (full analysis in under 48 hours) and high resolution (up to single amino acid level resolution)
- No need for protein crystallization or mutations
- In solution protein analysis in native state
- Sub-microsecond timescale hydroxyl generation
- Low sample consumption (<5 ug per sample)
- Temperature control (0°C to >80°C, ± 1°C)

Methods

Graphical Overview



Sample Preparation

Samples of PknB (1µM) and ligands (GSK690693 or GW779439X at 20 µM) or a DMSO only control for apo were mixed at a 1:1 molar ratio and incubated for 1 hour (Buffer = 10mM Tris pH 8.0, 150 mM NaCl, 1mM $MgCl_2$, 0.4% DMSO).

PLIMB Exposure

Samples of PknB alone and ligand bound PknB were prepared in triplicates and placed in 250 µL PCR tubes. The sample temperature was set to 2° C during the plasma exposure and were treated with PLIMB for 2.5 seconds. After the plasma exposure, 20 uL of 300 mM L-Met is added to each sample for a final of 50mM in order to quench the reaction.

Mass Spectrometry Preparation

Following PLIMB treatment, the samples were TCA precipitated and resolubilized into 8 M Urea in 50 mM ammonium bicarbonate (Am. Bi.). The samples were diluted to 4 M urea with 50 mM Am. Bi., reduced with 5 mM DTT, alkylated with 15 mM IAA, and then diluted to 1 M urea with 50 mM Am. Bi. The samples were digested overnight for 16 hours at 37° C with Trypsin and Lys-C concurrently at 1:20 protease:protein mass ratio. Following digestion, samples were acidified with neat formic acid to 1% final concentration. The samples were then desalted and concentrated using Omix tips (Agilent).

Mass Spectrometry and Data Analysis

Samples were analyzed on an Orbitrap Fusion LUMOS Tribid mass spectrometer. The mass spectrometry data was analyzed Protein Metrics software package. The raw data files were analyzed using Byos - Oxidative Footprinting workflow (Protein Metrics Inc.), which includes a database search followed by quantification of identified peptides. 90% sequence coverage of PknB was achieved. The % oxidation of the oxidized peptides from the samples with PknB alone were compared to those from the samples containing both PknB and small molecule inhibitors (GSK690693 and GW779439X).

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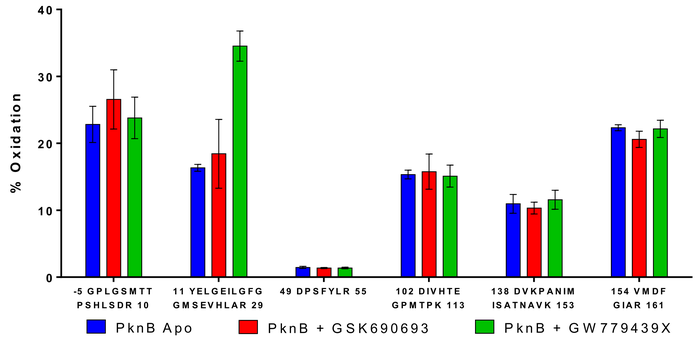
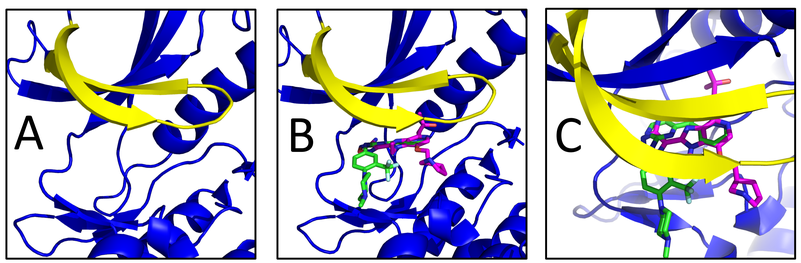
Results

The PLIMB analysis shows peptide 11-29 is more solvent exposed upon GW779439X binding.



Oxidation of PknB after 2.5 seconds PLIMB for PknB alone and PknB bound to small molecule inhibitors (GSK690693 and GW779439X).

This area corresponds to the PknB P-loop, a region frequently observed to undergo inhibitor-induced conformational changes in other kinases. These data suggest that GW779439X causes a conformational change in this region relative to apo or GSK690693-bound PknB. The crystal structure of PknB with GSK690693 shows some P-loop interactions, and computational predictions with GW779439X in PknB show less restriction in this region, suggesting different P-loop conformational positions are possible with this inhibitor. These data will be used to inform design of new inhibitors with optimal PknB binding.



PLIMB reveals changes in surface exposure of the P-loop of PknB. A) A model of PknB (blue) with ligand removed showing the P-loop (yellow) residues 11-29. B) The crystal structure (5U94) of GSK690693 (magenta sticks) in PknB shown relative to the P-loop with the docking-predicted pose of GW779439X overlaid (green sticks) from the side and C) from the top shows differences in active site occupancy by the inhibitor.