Characterizing Type III Kinase Inhibitor Using Laser-Free Flash Oxidation (Fox™) Hydroxyl Radical Protein Footprinting

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HRPF Introduction

The higher order structure (HOS) of a protein plays a critical role in a drug's stability, safety and biological function. Incorrect HOS or protein interactions are linked to adverse drug reactions which can result in further sickness or death. Advance techniques are required to robustly address the HOS of proteins. One such method is Hydroxyl Radical Protein Footprinting (HRPF). The Fox Protein Footprinting System is a novel HRPF method that uses a proprietary flash oxidation lamp to generate hydroxyl radicals (•OH) that irreversibly modify solvent exposed amino acid side chains. As solvent accessibility changes, the •OH modification concordantly changes.

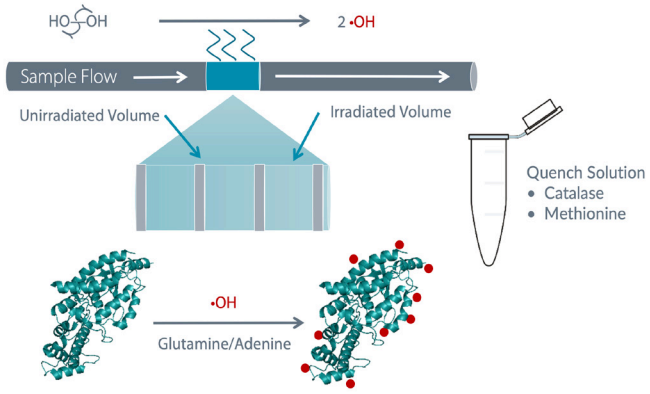

Figure 1: Schematic of an HRPF method, fast Photochemical Oxidation of Proteins (FPOP). With FPOP, protein is mixed with hydrogen peroxide and flown passed a pulsing light source which photolyzes the hydrogen peroxide into two •OH and modifies solvent exposed amino acids. Following labeling, the sample is deposited into a quench solution of catalase and methionine.

Abl Kinase Introduction

ABL1 is a protooncogene that encodes Abl tyrosine kinase and with ATP it is involved in cell division, adhesion, differentiation, and response to stress. The SH3 domain that negatively regulates Abl Kinase can be deleted resulting in an oncogene. ABL1 gene has also been found fused to translocation partner genes and is associated with gastrointestinal system cancer, colorectal and stomach cancer, and leukemia. Non-allosteric Abl kinase inhibitors have shown high levels of secondary resistance and severe off-target drug effects resulting in the need for allosteric inhibitors with high specificity for Abl kinase including, GNF-5 (Figure 2A). The allosteric inhibitor, GNF-5, binds specifically to Abl's myristate binding pocket inducing the active/open conformation of Abl (Figure 2B) to the inactive/closed conformation (Figure 2C).

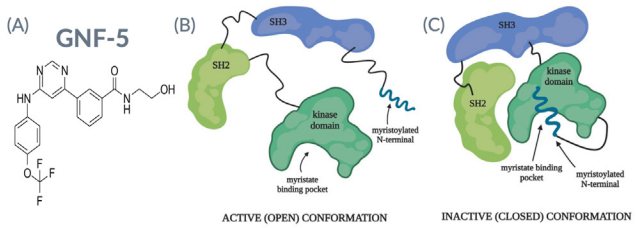

Figure 2: GNF-5 as an allosteric inhibitor for Abl Kinase. A) Chemical structure of GNF-5(PubChem CID: 44129660). B) Schematic of the active (open) and C) inactive (closed) conformation of Abl Kinase. (Adapted from Mol Cancer Ther. 2020 September ; 19(9): 1763–1769.)

Fox™ System Introduction

The Fox System is composed of a fluidics module, photolysis module, dosimeter module, and a product collector (Figure 3A). Protein in appropriate buffer and pre-mixed with H_2O_2 is injected on the sample loop connected to the fluidics module. Running buffer is used to push the protein sample passed the photolysis module which contains the fully enclosed high-pressure flash lamp (Figure 3B). The flash lamp has a pulse width <10 µs and reliably produces a broad-band spectral output from 200-300 nm wavelength along with some visible light. The broad spectrum of the lamp matches the UV absorbance spectrum of H_2O_2, resulting in effective production of •OH (Figure 3C). Immediately downstream of the photolysis module is the dosimeter module which monitors the effective •OH concentration by detecting the change in UV absorbance at 265 nm. Unoxidized tris does not absorb at 265 nm, but as the •OH concentration increases, the absorbance linearly increases (Figure 3D).

Fox Allosteric Inhibitor Characterization

Forty µM Abl Kinase was mixed with vehicle, ATP, or ATP+GNF-5 and aliquoted. Immediately before injection, a single aliquot was mixed with H_2O_2 and Tris buffer. The final labeling conditions were 10 mM Tris, 25 mM H_2O_2, 5 µM Abl ± 13.5 µM ATP ± 5.6 µM GNF-5. Samples were introduced to the Fox System using a 12 µL injection loop. Running buffer pushed the sample passed the Fox flash lamp, efficiently photolyzing H_2O_2 to form •OH. Downstream of the photolysis module, a dosimeter module accurately determines the effective •OH concentration by detecting the change in Tris absorbance at 265 nm. Finally, samples are collected in 20 µL of quench, 0.3 mg/mL catalase and 35 mM methionine, and underwent a tryptic digestion for bottom-up proteomics.

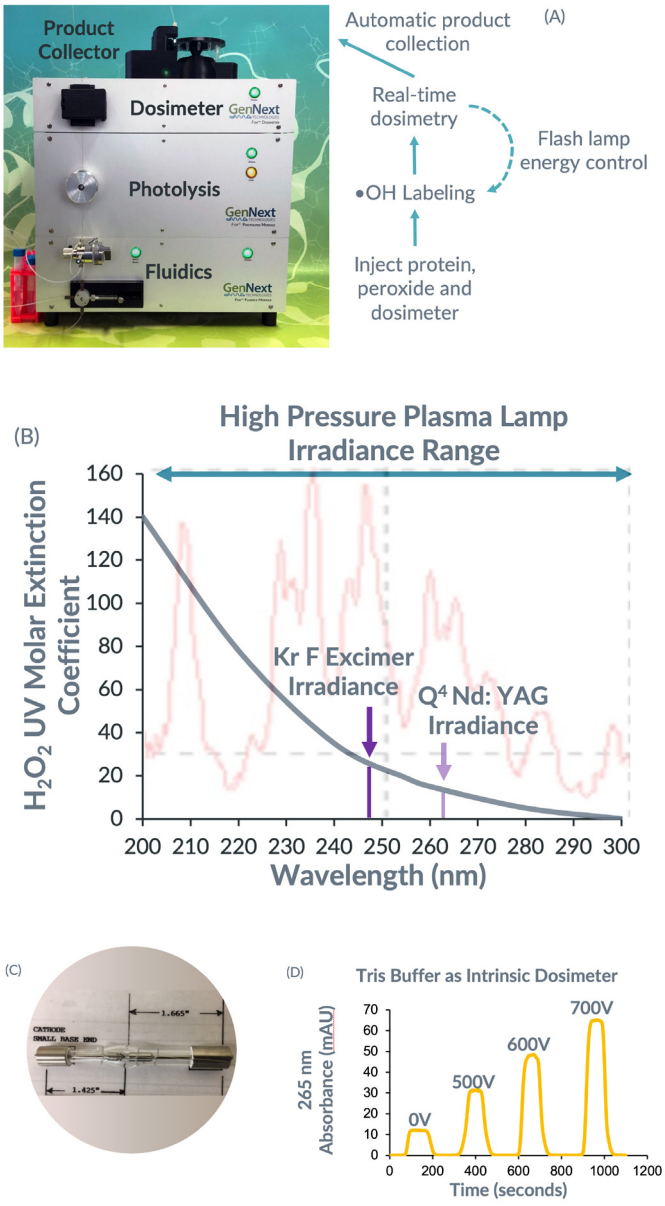

Figure 3: Fox System for laser free FPOP. A) Schematic of Fox system with the four modules (fluidics, photolysis, dosimeter, and product collector). B) High-pressure plasma lamp housed in the photolysis module. The plasma lamp withstands high peak energy, generates very small plasma arc for optimum focusing, and stable over many thousands of flashes. C) Emission spectrum of the Fox flash lamp with H2O2 UV molar extinction coefficient overlaid. The high-pressure nobel gas blend lamp emits broad spectrum UV irradiation from 200-300 nm wavelength to efficiently photolyze H_2O_2 to •OH.

Hydroxyl Radical (•OH) Dosimetry

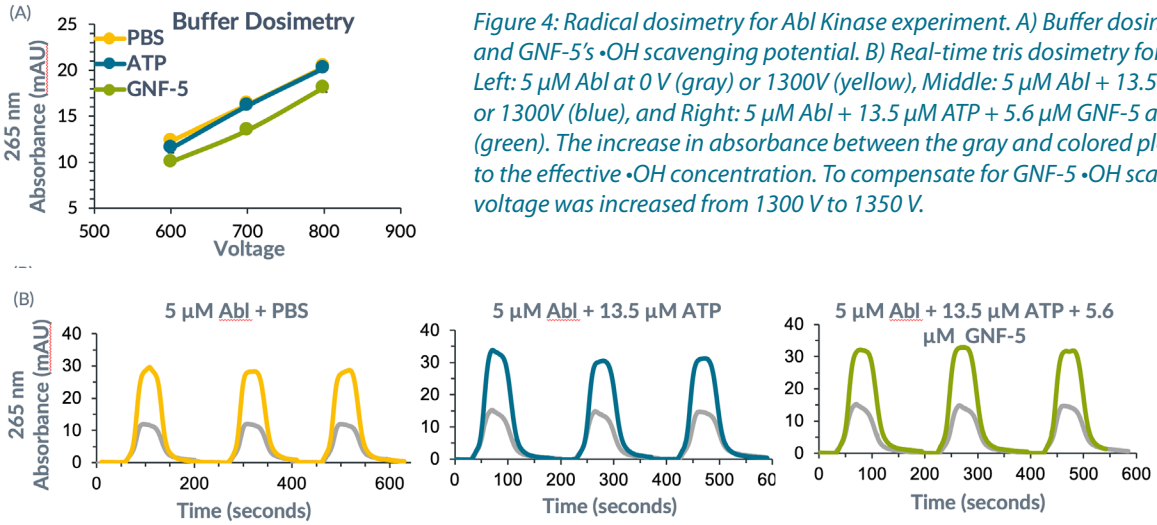

Figure 4: Radical dosimetry for Abl Kinase experiment. A) Buffer dosimetry to identify ATP and GNF-5's •OH scavenging potential. B) Real-time tris dosimetry for triplicate injections of Left: 5 µM Abl at 0 V (gray) or 1300V (yellow), Middle: 5 µM Abl + 13.5 µM ATP at 0 V (gray) or 1300V (blue), and Right: 5 µM Abl + 13.5 µM ATP + 5.6 µM GNF-5 at 0 V (gray) or 1350V (green). The increase in absorbance between the gray and colored plots is proportional to the effective •OH concentration. To compensate for GNF-5 •OH scavenging, the lamp voltage was increased from 1300 V to 1350 V.

Average Peptide Oxidation (APO)

Histograms

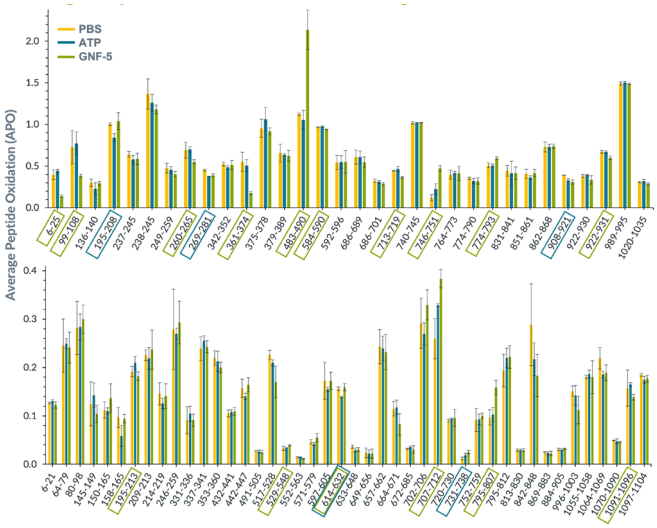

Figure 5: Average peptide oxidation (APO) of Abl Kinase alone (yellow) + ATP (blue) + Kinase Inhibitor GNF-5 (green). Error bars represent one standard deviation from an averaged triplicate measurement. Peptides with a significant change in oxidation after the addition of ATP or GNF-5 are outlined in a blue or green box, respectively, (p-value ≤ 0.05).

Volcano Plots

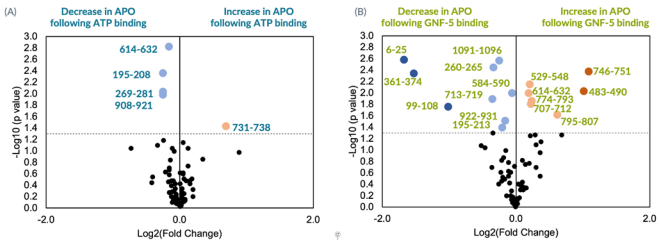

Figure 6: Average peptide oxidation (APO) volcano plots to identify peptides with a significant change in oxidation across conditions. A) Comparing APO in Abl Kinase alone vs with ATP and B) with ATP vs with ATP and GNF-5 . Peptides above the dotted line have a p-value less than 0.05. Peptides with a statistically significant change (above dotted line) and decrease in APO with a fold change less than two are light blue and more than two are dark blue. Increase in APO with a fold change less than two are light orange and more than two are red.

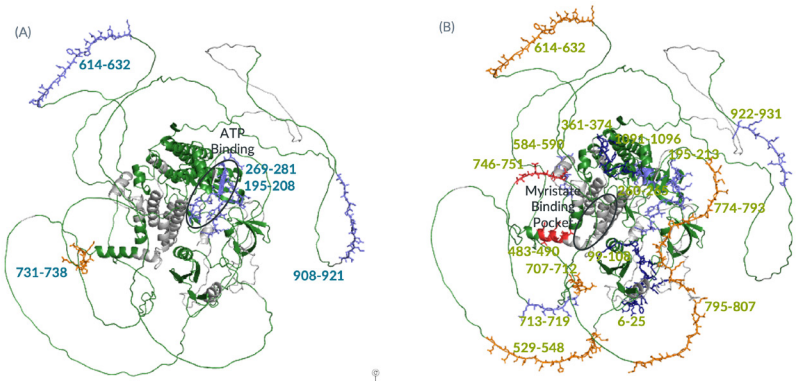

Figure 7: APO results mapped to AlphaFold Structure of Abl Kinase (AF-E9PT20-F1). A) Comparing APO between Abl Kinase alone and with ATP. B) Comparing APO between Abl Kinase with ATP and with GNF-5. Peptides with a significant decrease in oxidation less than two-fold are highlighted light blue and greater than two-fold are dark blue. Peptides with a significant increase in oxidation less than two-fold are highlighted orange and greater than two-fold are red.

Conclusions

- The Fox™ System identified both binding site and allosteric changes from binding for both a native ligand and an allosteric inhibitor for Abl Kinase.
- The Fox System is an easy and robust FPOP technique and replaces the difficult to maintain laser-based technique.
- Real-time •OH dosimetry improves reproducibility and quickly adjusts for varying background •OH scavenging.

GenNext has pioneered a superior, compact, cost-effective, and safe means of performing advanced HRPF analysis.

By replacing expensive, complicated, and hazardous lasers with our proprietary Flash Oxidation System, you can easily perform HRPF with a convenient benchtop instrument.

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