Complementary Backbone and Side Chain Analysis of Drug – Protein Interactions Combining Hydrogen/Deuterium Exchange and Protein Oxidative Mass Spectrometry

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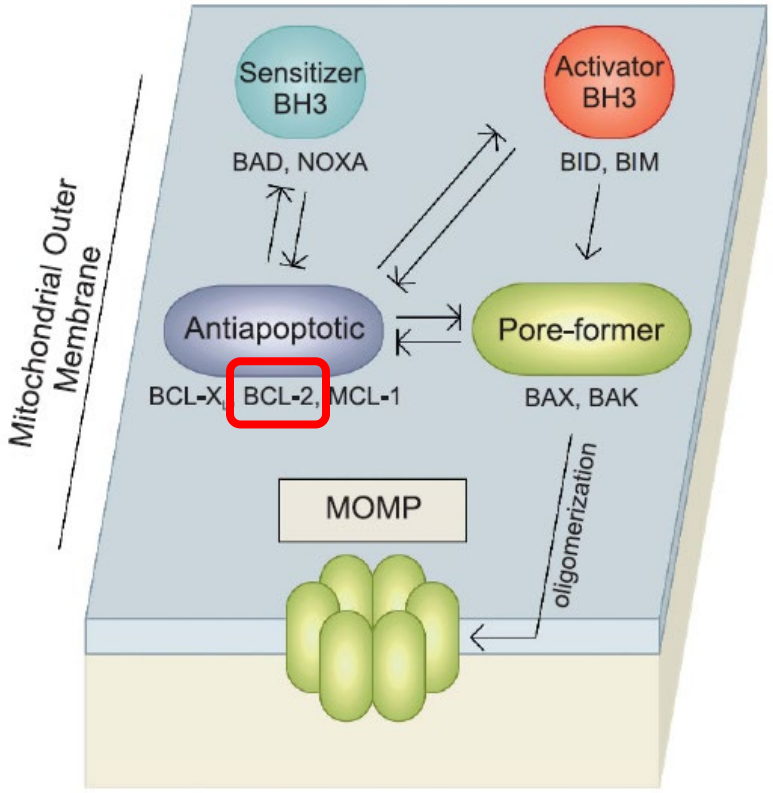
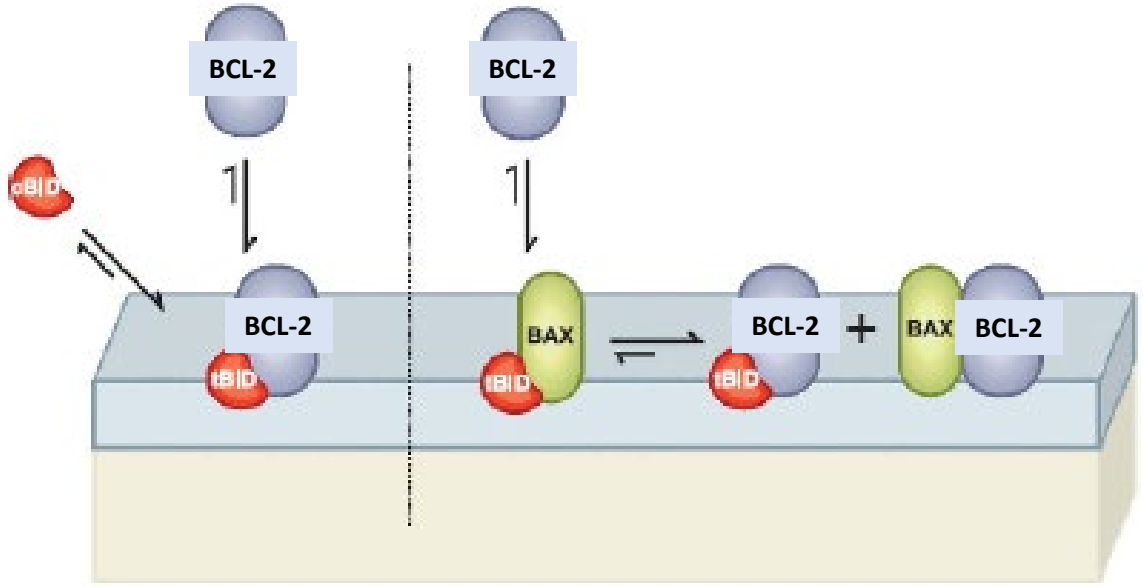
Project goals

- Evaluate if a Fenton-chemistry based Protein Oxidative Mass Spectrometry (OX-MS) approach with enhanced sequence resolution and experimental throughput can complement HDX-MS during drug discovery, development, and characterization
- Assess the ability of OX-MS to distinguish the binding profiles of two drugs bound to the anti-apoptotic protein BCL-2, Venetoclax (Abbvie and Genentech) and S55746 (Servier)
- Explore the structural insights provided by OX-MS analysis of drug-protein complexes

BCL-2 is an active target for the development of novel therapeutics

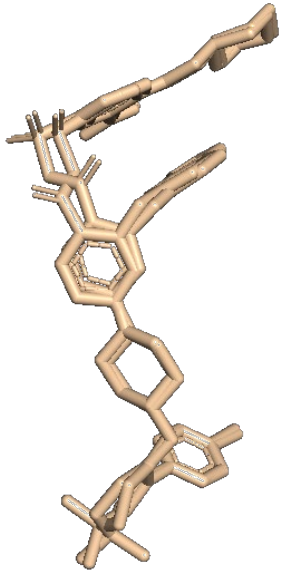


- BCL-2 is an anti-apoptotic protein
- BCL-2 is highly expressed in chronic lymphocytic leukemia
- BCL-2 enhances proliferation by inhibiting apoptosis

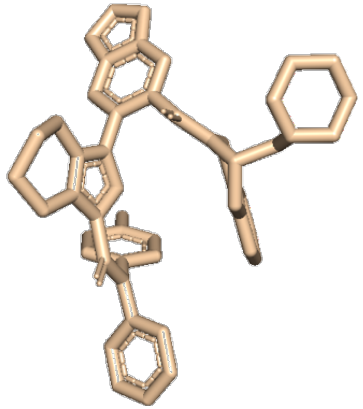


Kale et al. *Cell Death and Differentiation*, 2018

The small molecule therapeutics Venetoclax and S55746 target BCL-2

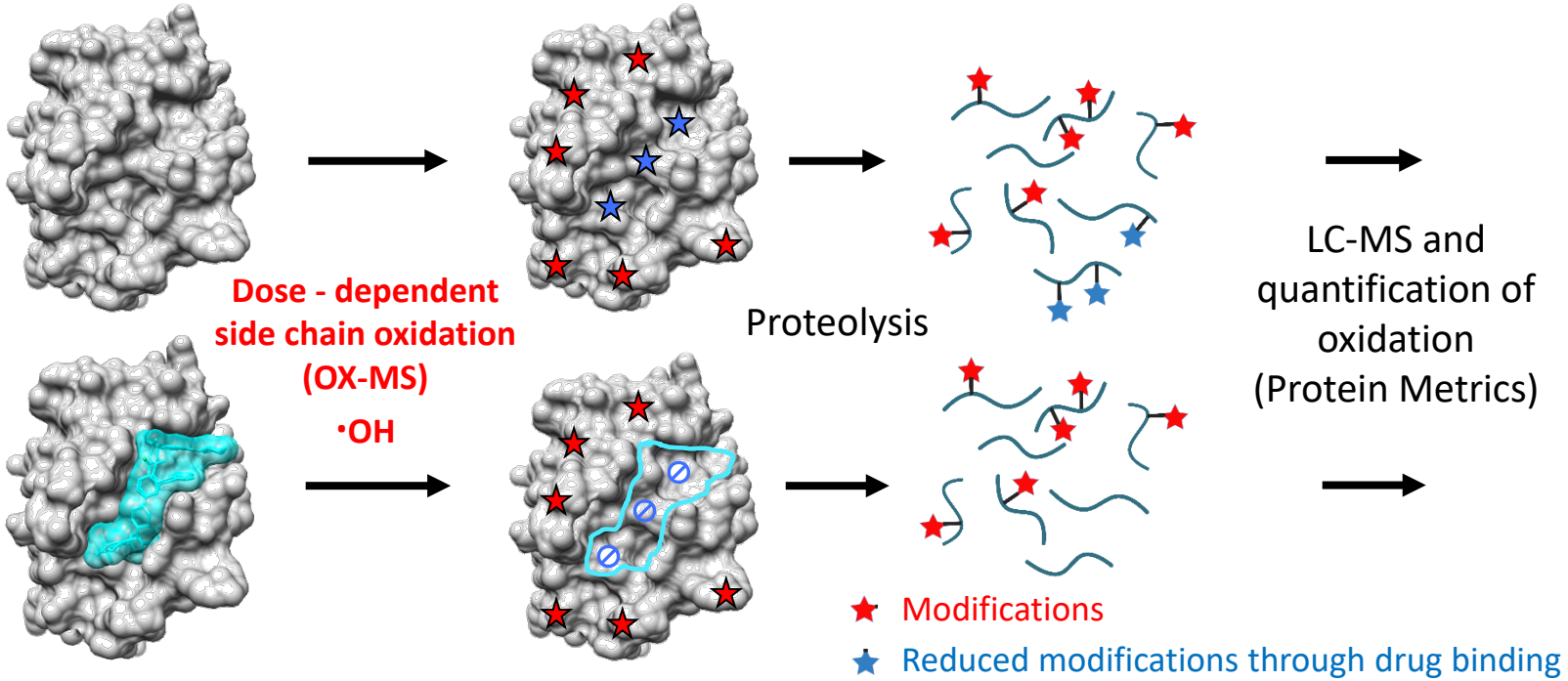


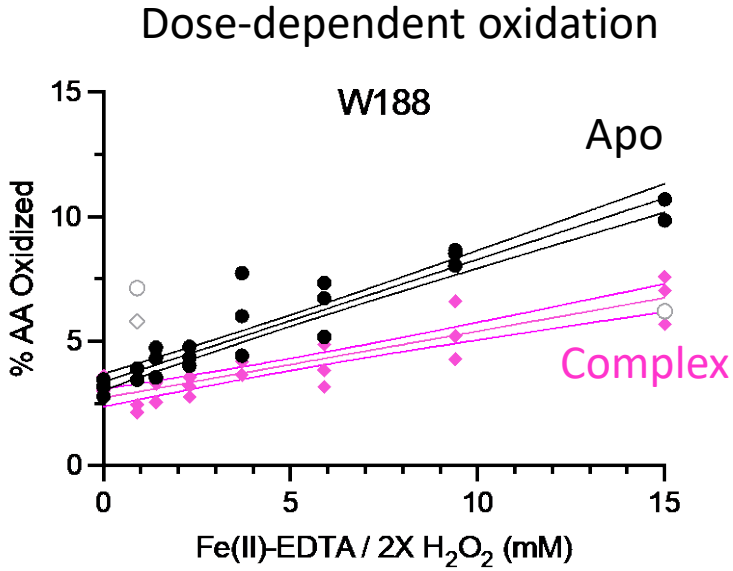
Venetoclax (ABT-199, GDC-0199-Abbvie and Genentech) is an FDA approved drug for the treatment of leukemias that binds to the BH3 domain of BCL-2, leading to the release of pro-apoptotic proteins and subsequent cell death (Birkinshaw et al. *Nat Commun*, 2019)



S55746 (Servier) is being investigated for its potential in treating hematological cancer that also binds to the BH3 domain of BCL-2 and has similar function of Venetoclax (Casara et al. *Oncotarget*, 2018)

Protein oxidative footprinting analyzed by mass spectrometry





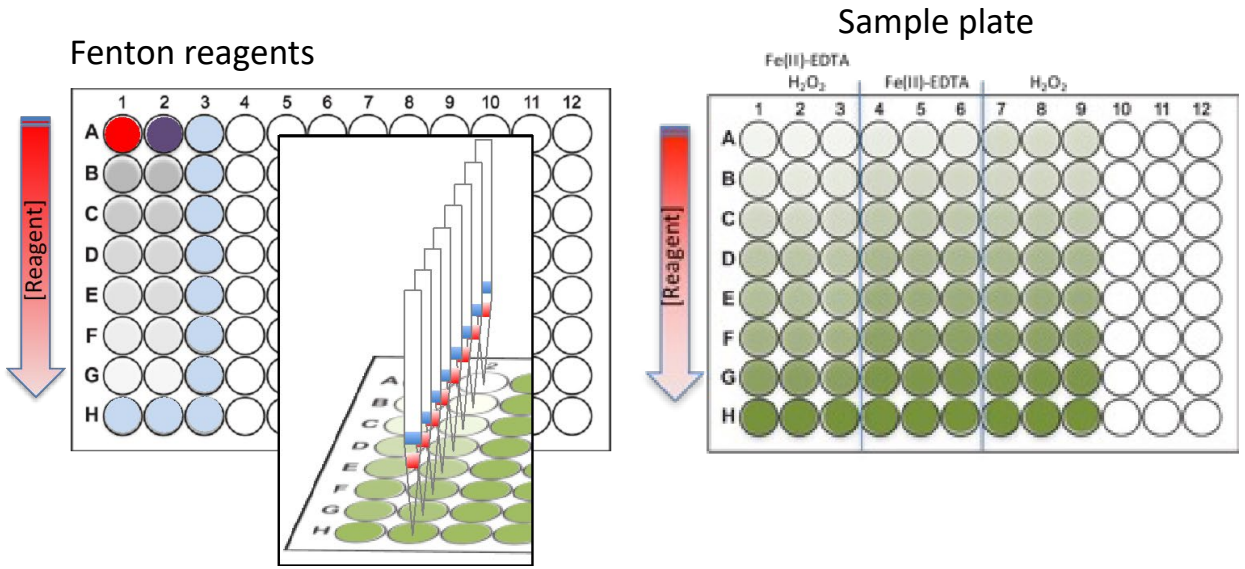
Methods of hydroxyl radical generation are being effectively used for protein oxidative footprinting

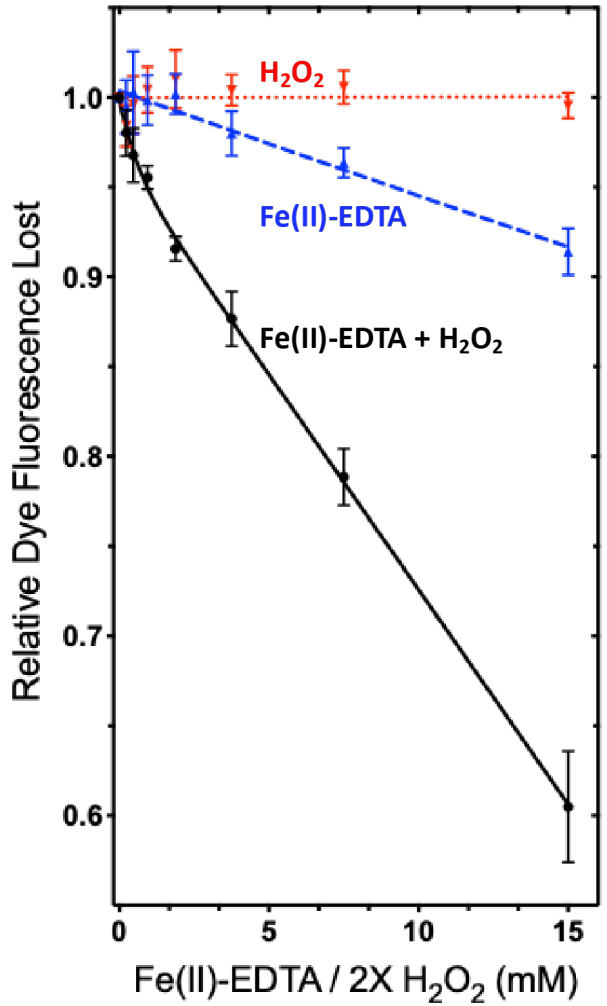
- UV laser photolysis of H_2O_2 (FPOP)
- X-ray (XFMS)
- Plasma discharge (PLIMB)
- UV flash lamp photolysis of H_2O_2 (FOX)
- **Fenton chemistry mediated by Fe(II)-EDTA (OX-MS)**

The OX-MS analysis presented in this talk quantitates changes in the extent of dose-dependent oxidation of single amino acid residues between free protein (black) and its drug complex (magenta)

A systematic and validated Fe(II)-EDTA method of hydroxyl radical generation for oxidative protein footprinting

Jessica R. Chapman, Max Paukner, Micheal Leser, Kai Wen Teng, Shohei Koide, Marlene Holder, Karim-Jean Armache, Chris Becker, Beatrix Ueberheide, and Michael Brenowitz – Submitted for publication



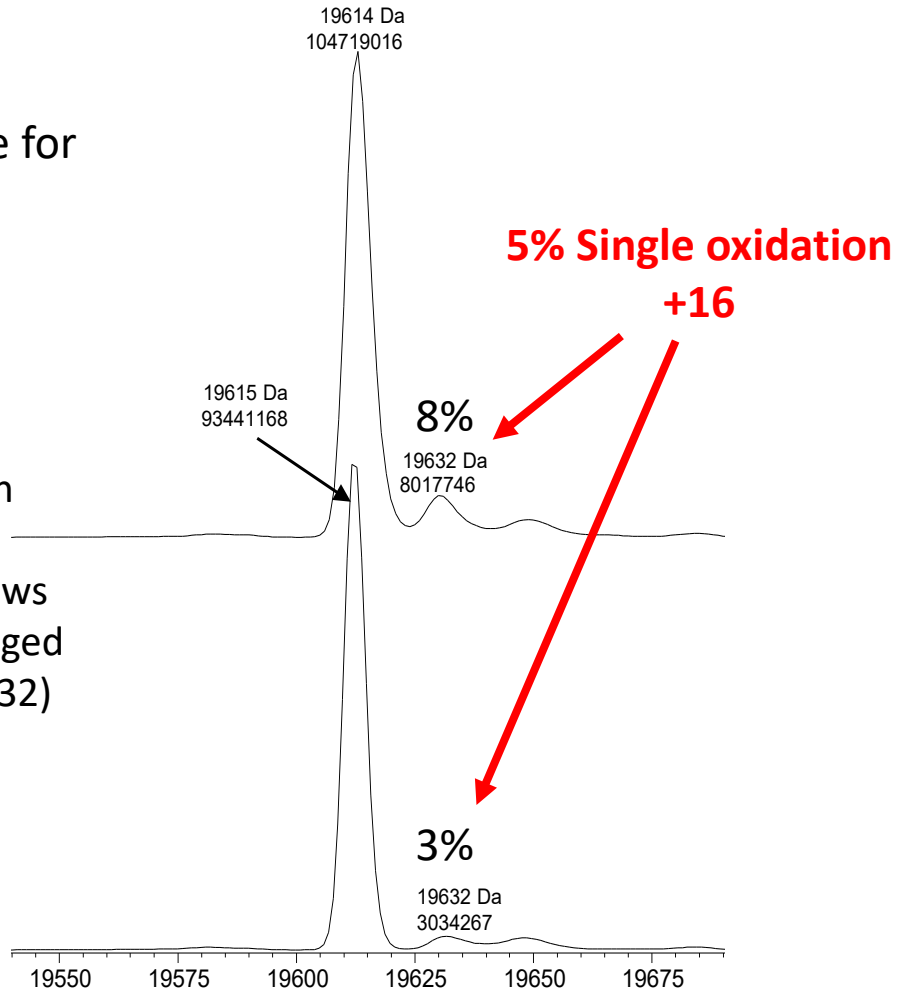


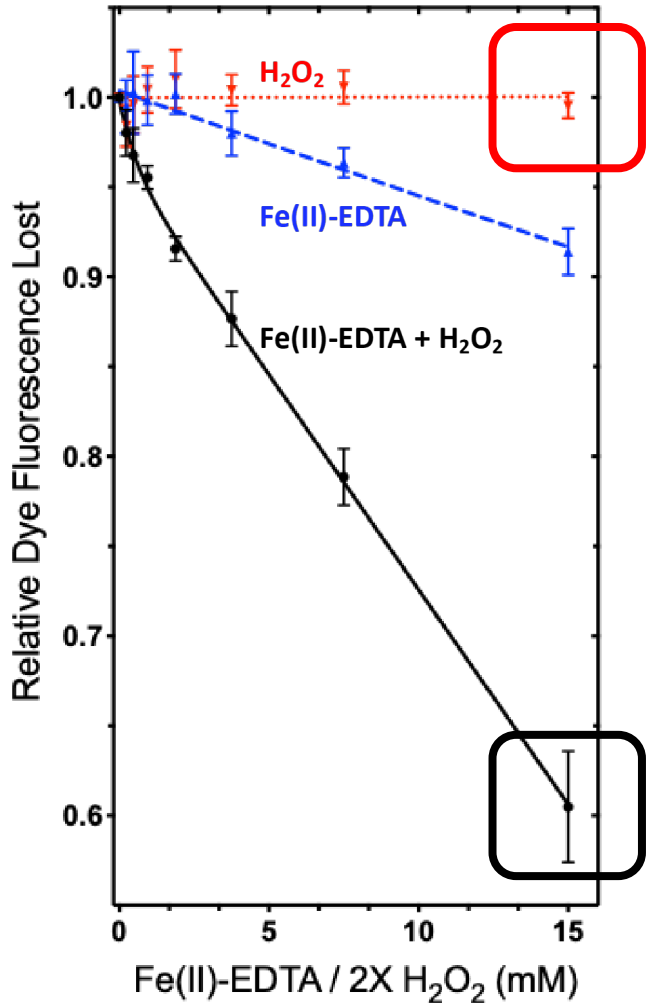
- Requires only inexpensive reagents and common laboratory equipment
- Provides robust and reproducible dose-dependent oxidation
- Multi-well plate format simplifies processing of protein samples for MS
- The detected side chain modifications are those previously reported using the other methods of hydroxyl radical generation

Protein oxidation is due to the Fe(II)-EDTA mediated Fenton chemistry. Oxidation by residual H_2O_2 is minimal

BCL-2 oxidized by 30 mM H_2O_2 alone for 2 min, the highest reagent concentration in the dose-response series was analyzed by intact MS

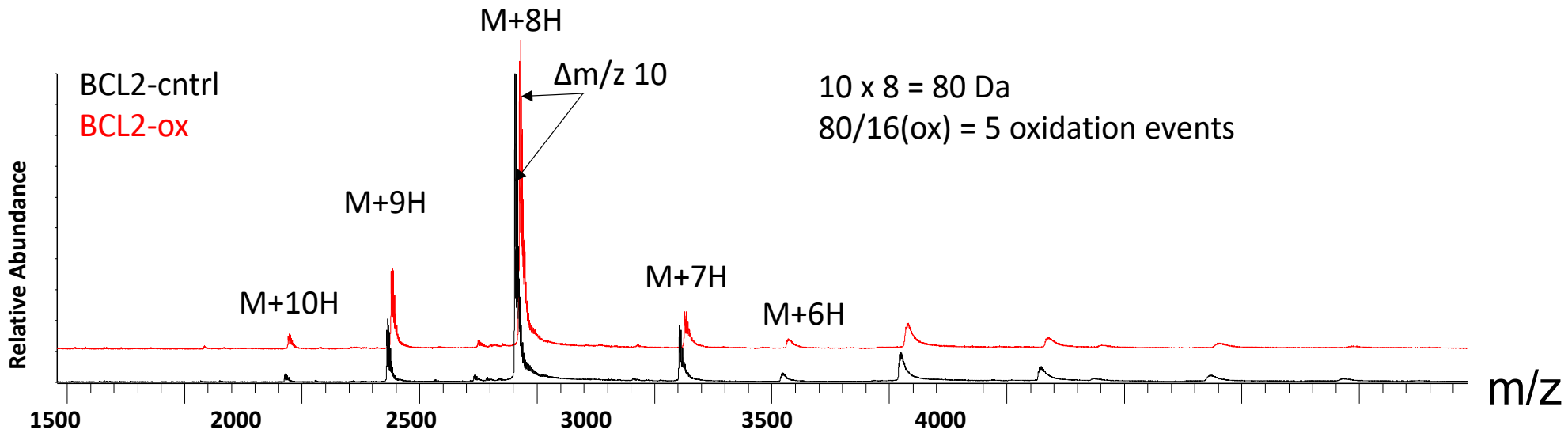
- The control BCL-2 sample shows 3% single oxidation (+16) that are either inherent or due to in-source oxidation (lower profile)
- The BCL-2 oxidized by H_2O_2 alone shows 8% single oxidation (+16) and unchanged trace amounts of double oxidation (+32) (upper profile)





Native-MS suggests that BCL-2 structure is unaffected by significant oxidative stress

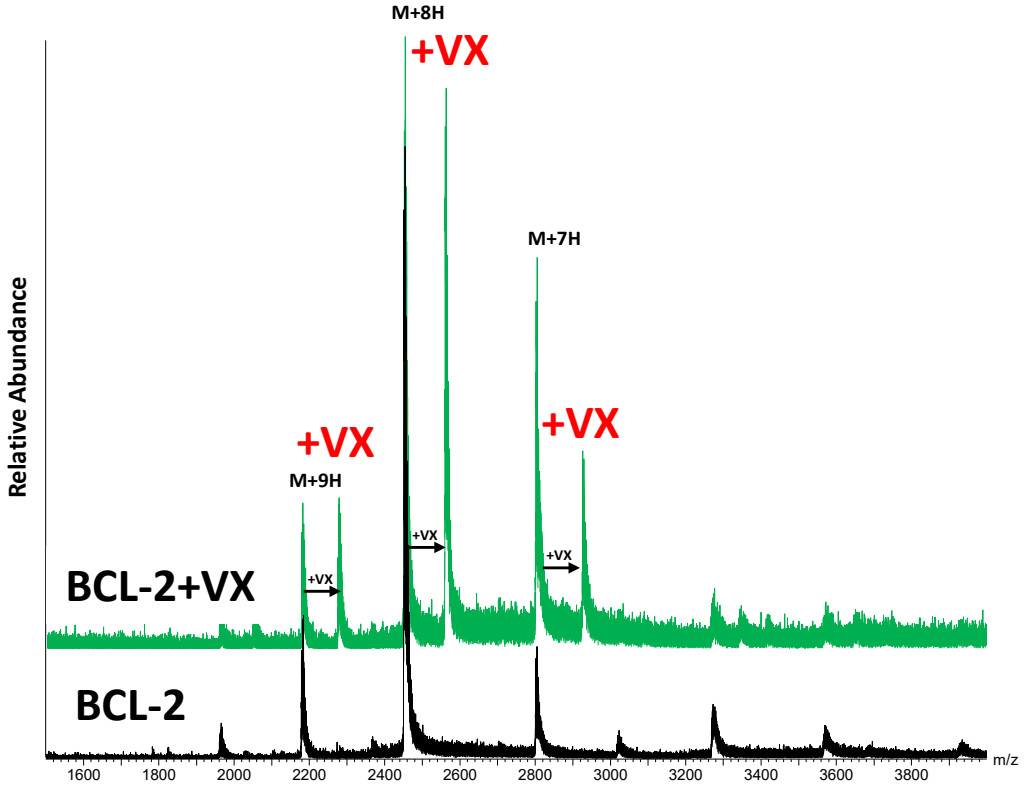
BCL-2 was stressed with 3% (900 mM) H_2O_2 for 2 minutes



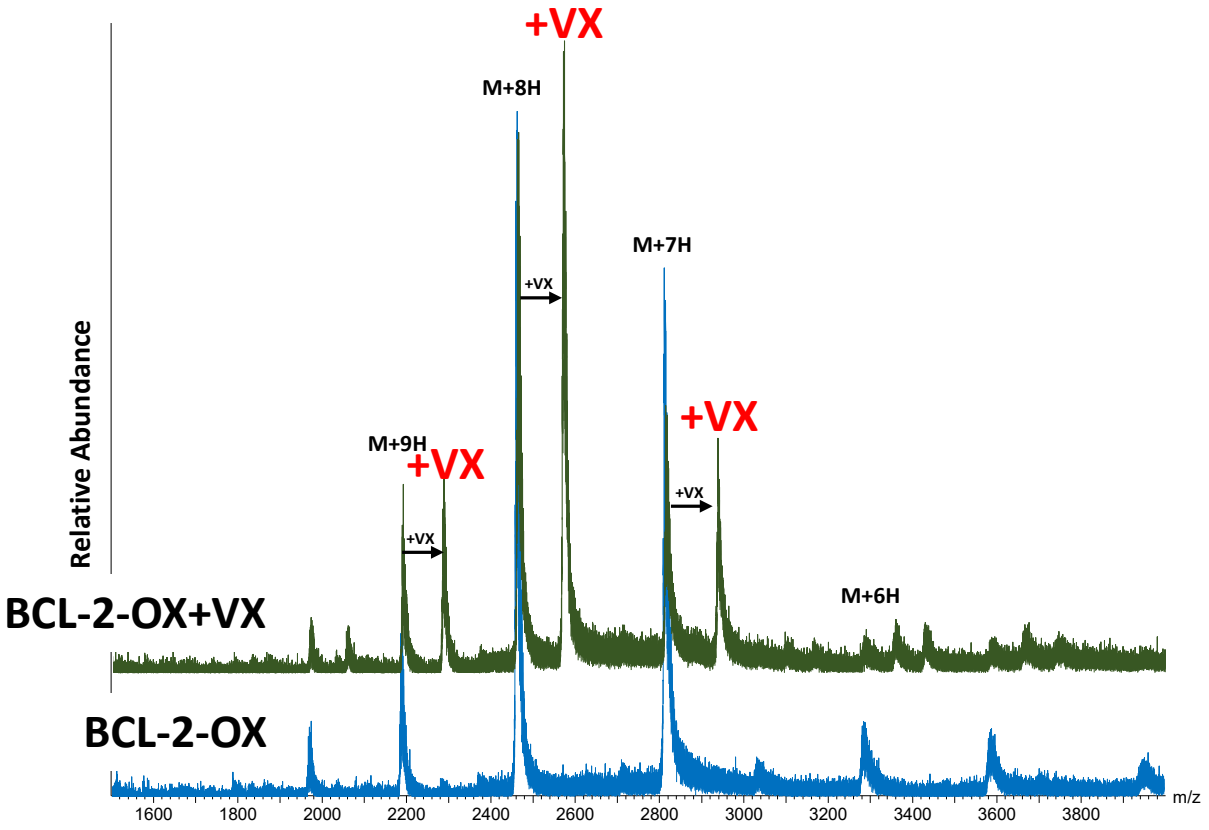
- The BCL-2 is significantly oxidized, with as many as 5 oxidation events per protein.
- The m/z distribution is unchanged
- No change in protein conformation

Native-MS suggests that BCL-2 structure is unaffected by significant oxidative stress

The oxidative stress does not affect Venetoclax binding to BCL-2

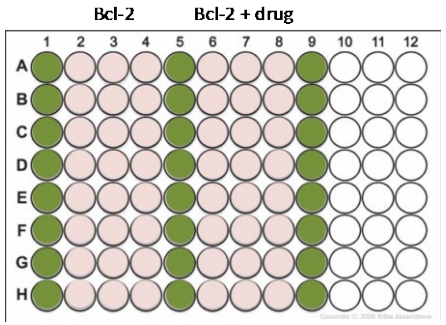


Binding of unoxidized BCL-2 with Venetoclax.



Binding of oxidized BCL-2 with Venetoclax.

Optimized LC-MS and oxidation analysis protocol



48 protein samples

Trypsin

Chymotrypsin

Chymotrypsin

Trypsin

Combined digests are prepped for injection

LC-MS - Orbitrap Fusion Lumos (Thermo Scientific)

- Longer LC gradient – Improved peptide separation
- Combined CID & HCD fragmentation MS2 and short dynamic exclusion time – More robust peptide detection

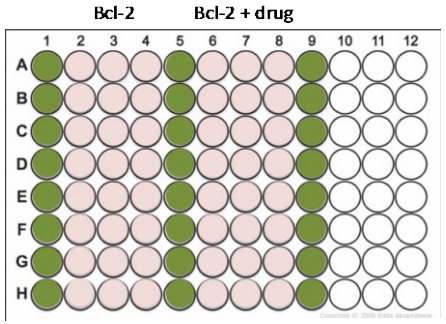
The multiple protease protocol yields improved coverage with smaller peptides that overlap in sequence

The amounts of unmodified and oxidized peptides are quantitated by the Protein Metrics software

Custom software plots the percent oxidized residue versus •OH dose (proportional to [Fe(II)-EDTA)

Linear regression and p-value statistical analysis by Prism™ software

Optimized LC-MS and oxidation analysis protocol -residue level analysis



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Trypsin

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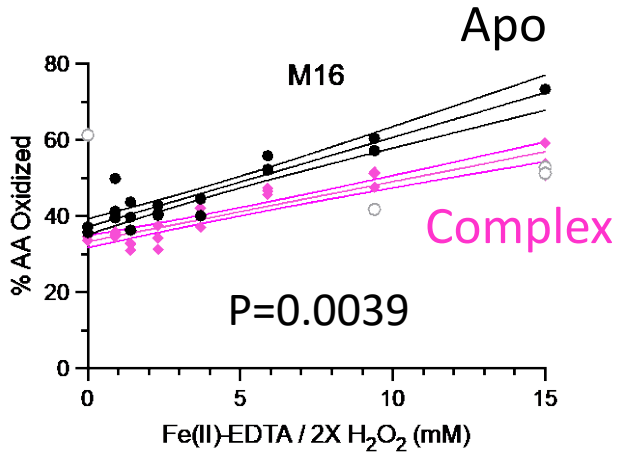
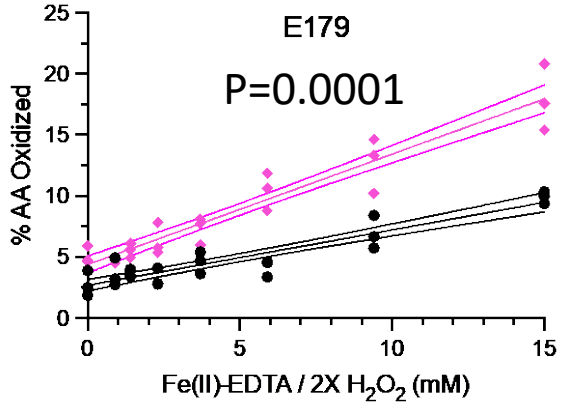
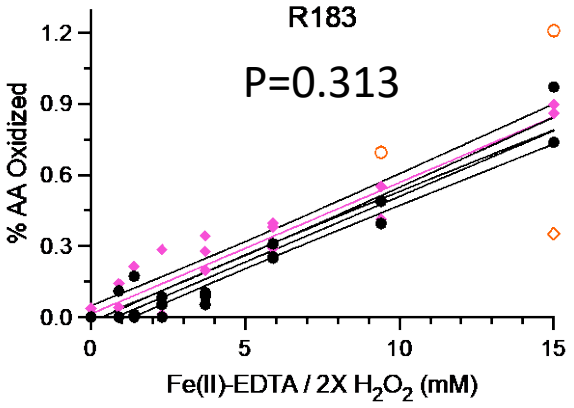
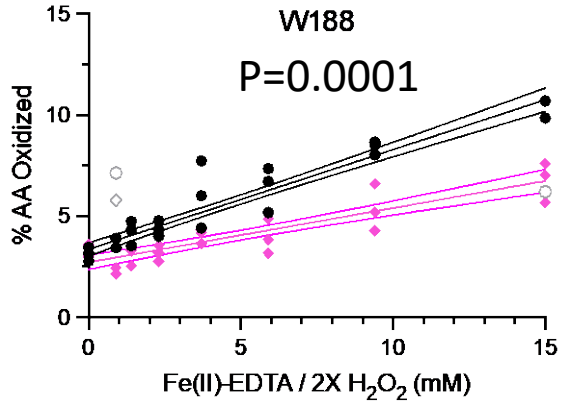
Combined digests are prepped for injection









Hydrogen deuterium exchange (HDX) and oxidation (OX) are complementary approaches to in-solution mapping of ligand binding to proteins

HDX-MS

- Principle: Exchange of **backbone hydrogen atoms** with deuterium in the protein.

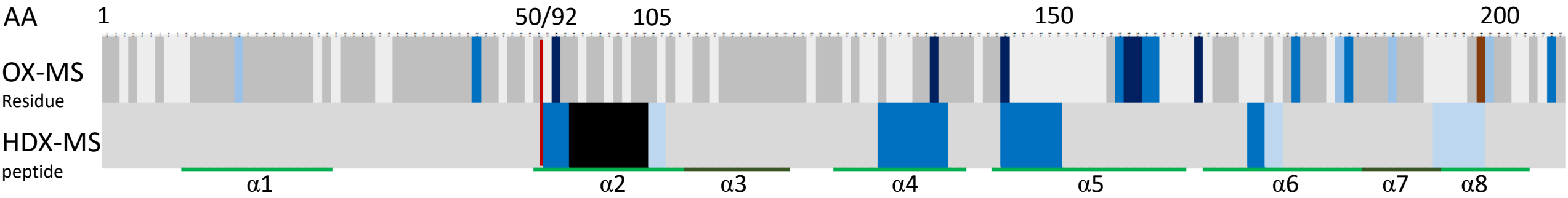
OX-MS

- Principle: Uses reactive oxygen species to selectively oxidize amino acid **side chains**.

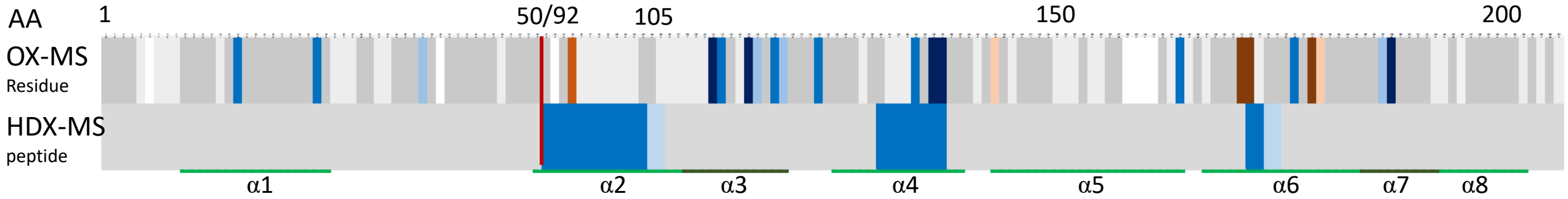
Both Information Provided: Insights into the **structural dynamics, folding, conformational changes of proteins, protein-protein, and protein-ligand interactions.**

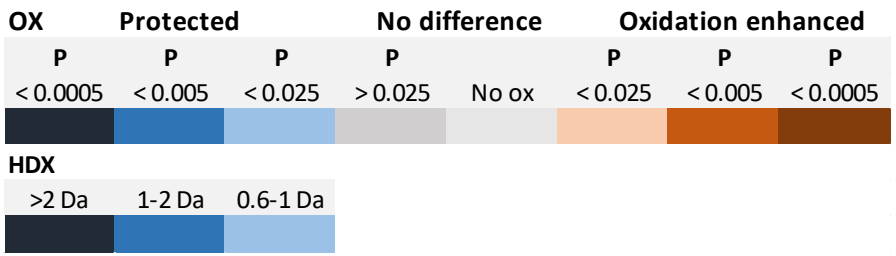
A heat map shows the residues whose oxidation (OX-MS) or isotope exchange (HDX-MS) changes upon drug binding

BCL-2 vs Venetoclax-BCL-2:



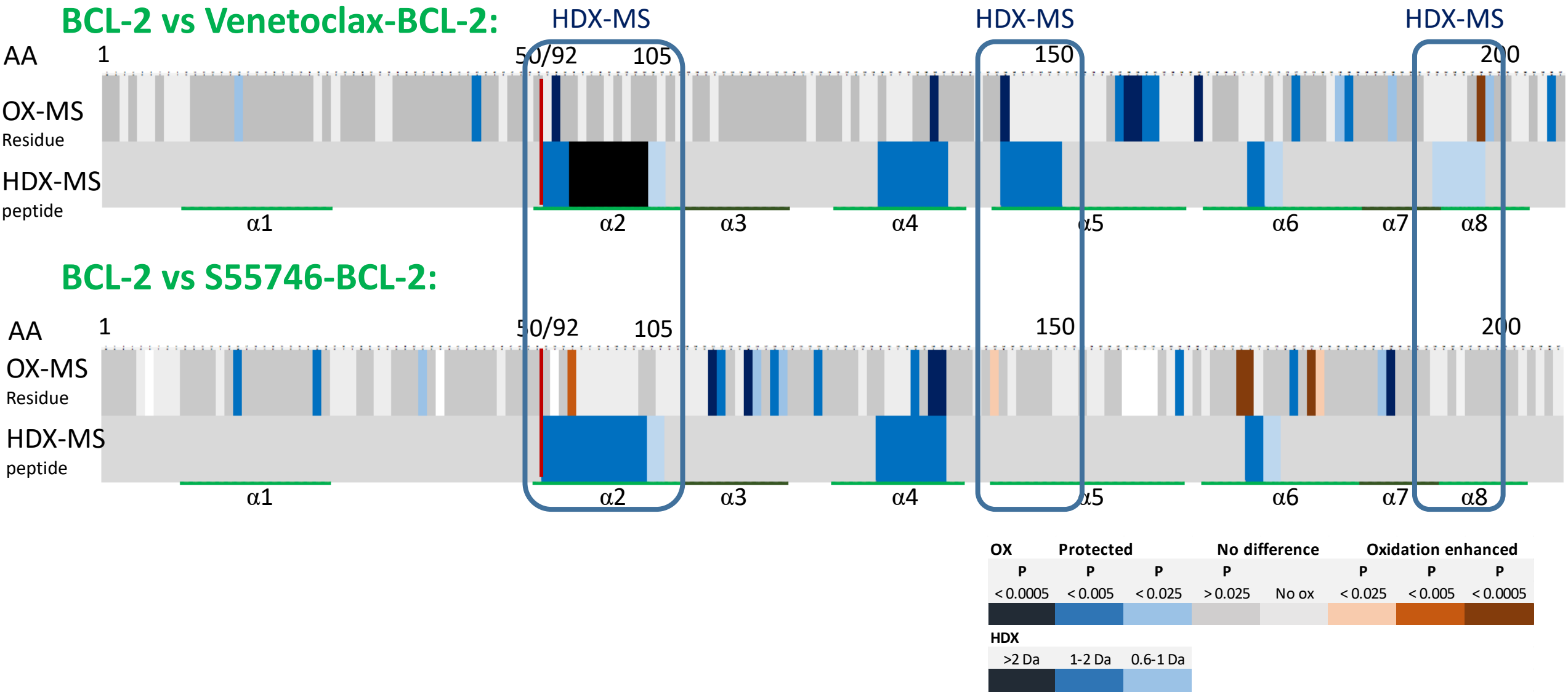
BCL-2 vs S55746-BCL-2:





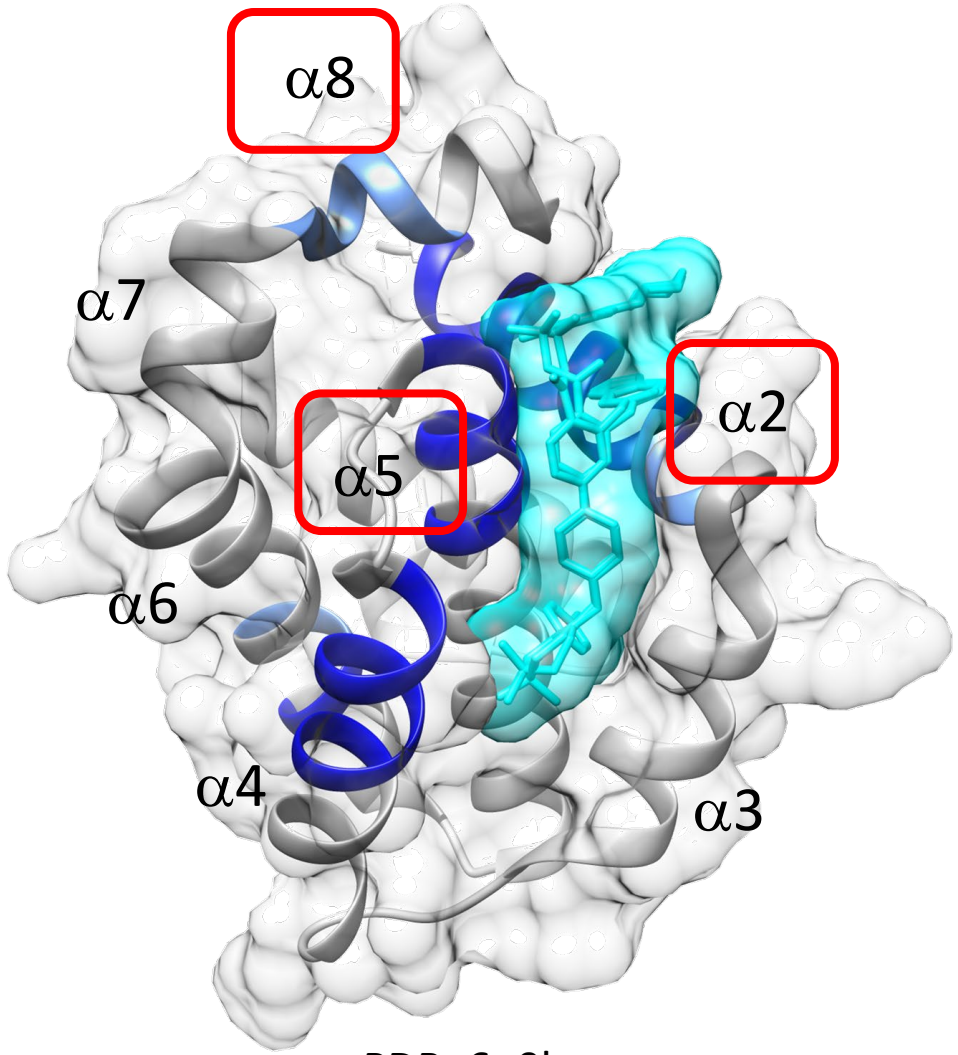
Nomenclature taken from:
Birkinshaw et al. *Nature Communication*, 2019

HDX-MS reveals distinct Venetoclax and S55746 BCL-2 exchange signatures



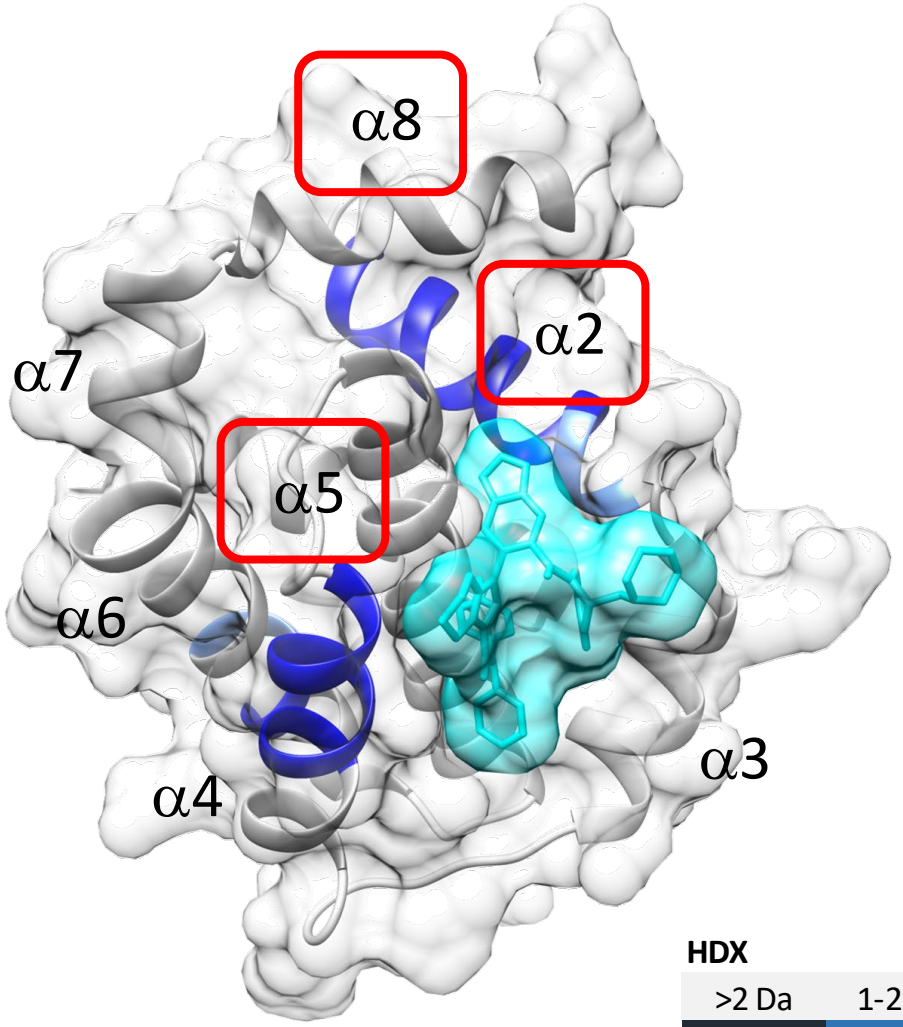
Mapping the HDX-MS results on co-crystal structures suggests that Venetoclax engages the backbone more directly than S55746

Venetoclax-BCL-2 complex



PDB: 6o0k

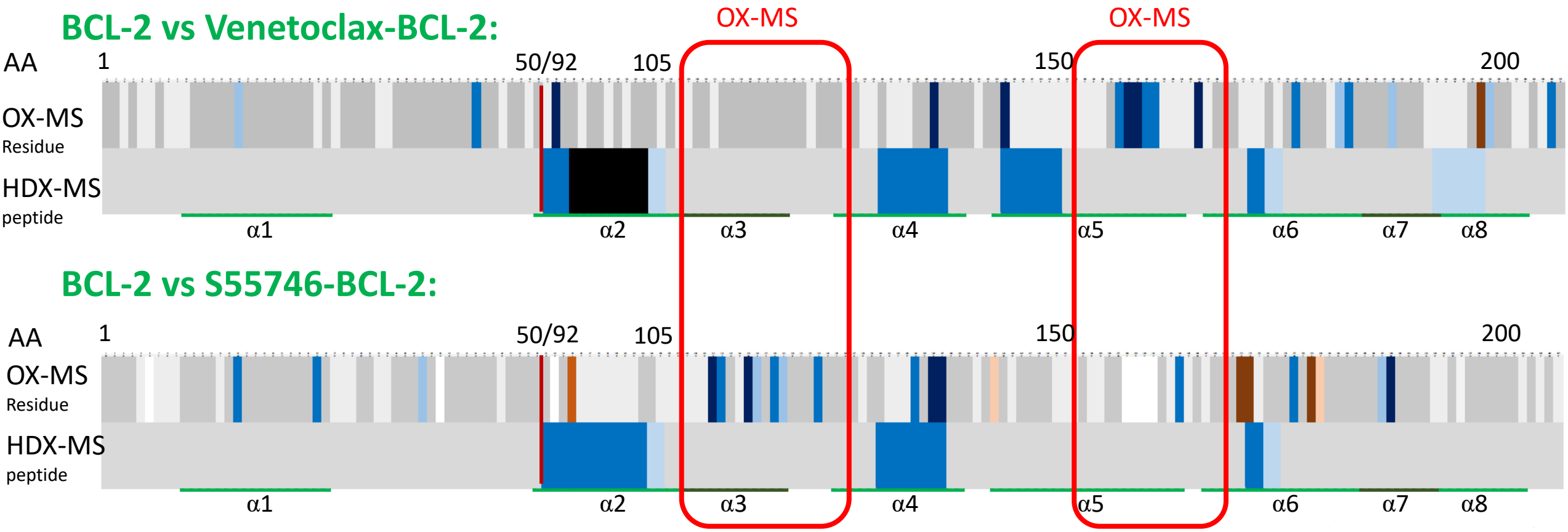
S55746-BCL-2 complex



HDX

>2 Da	1-2 Da	0.6-1 Da

OX-MS reveals distinct Venetoclax and S55746 BCL-2 binding signatures



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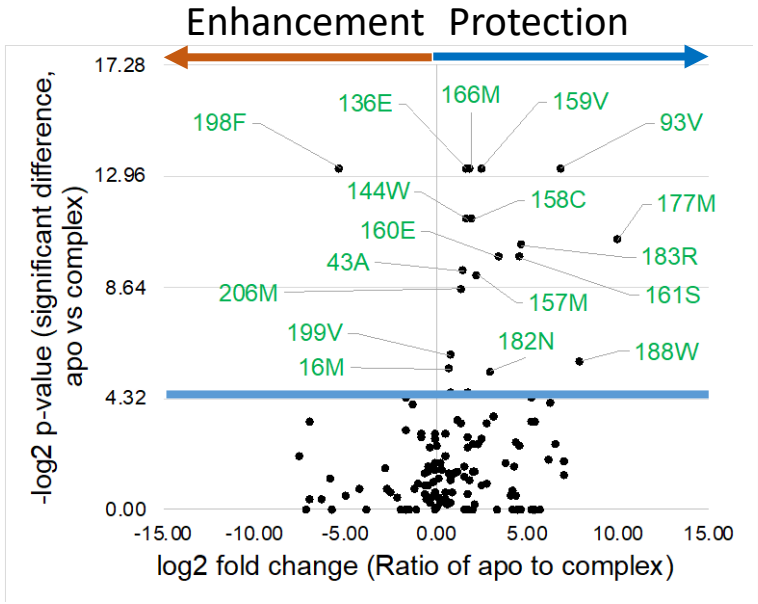
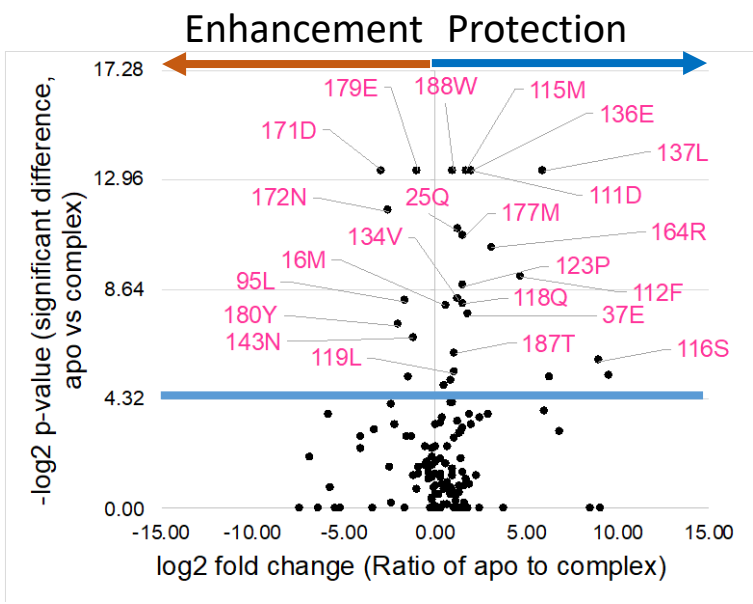
Birkinshaw et al. *Nature Communication*, 2019

OX-MS reveals distinct Venetoclax and S55746 BCL-2 binding signatures

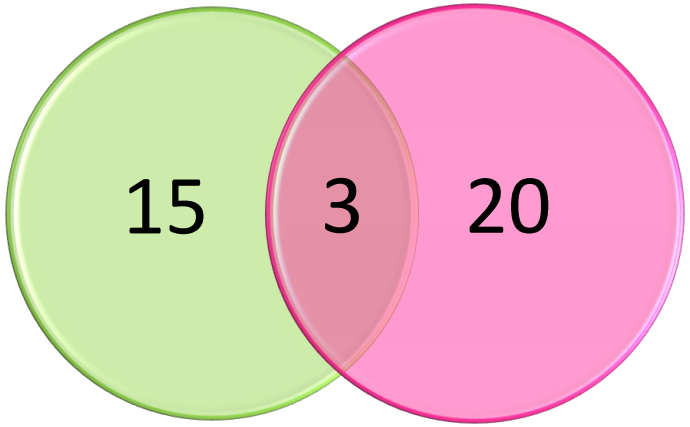
Venetoclax



S55746



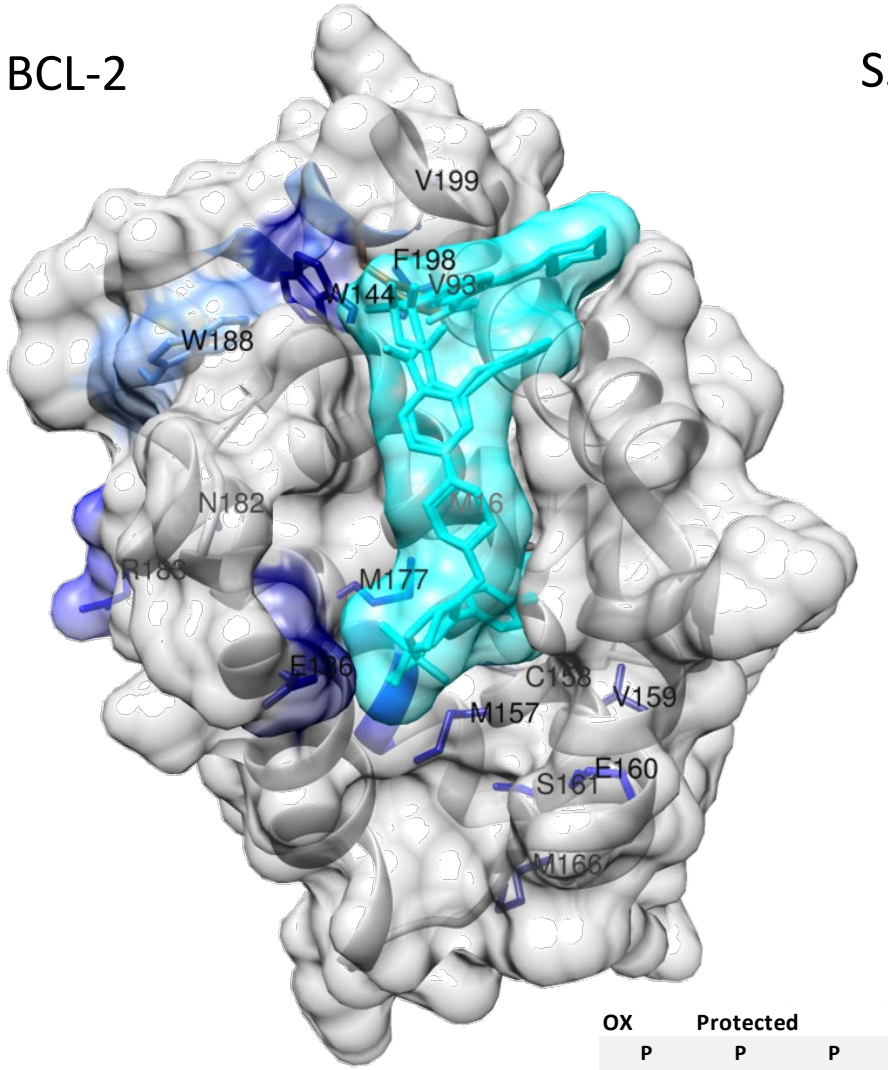
Fifteen residues are uniquely perturbed by Venetoclax



Twenty residues are uniquely perturbed by S55746

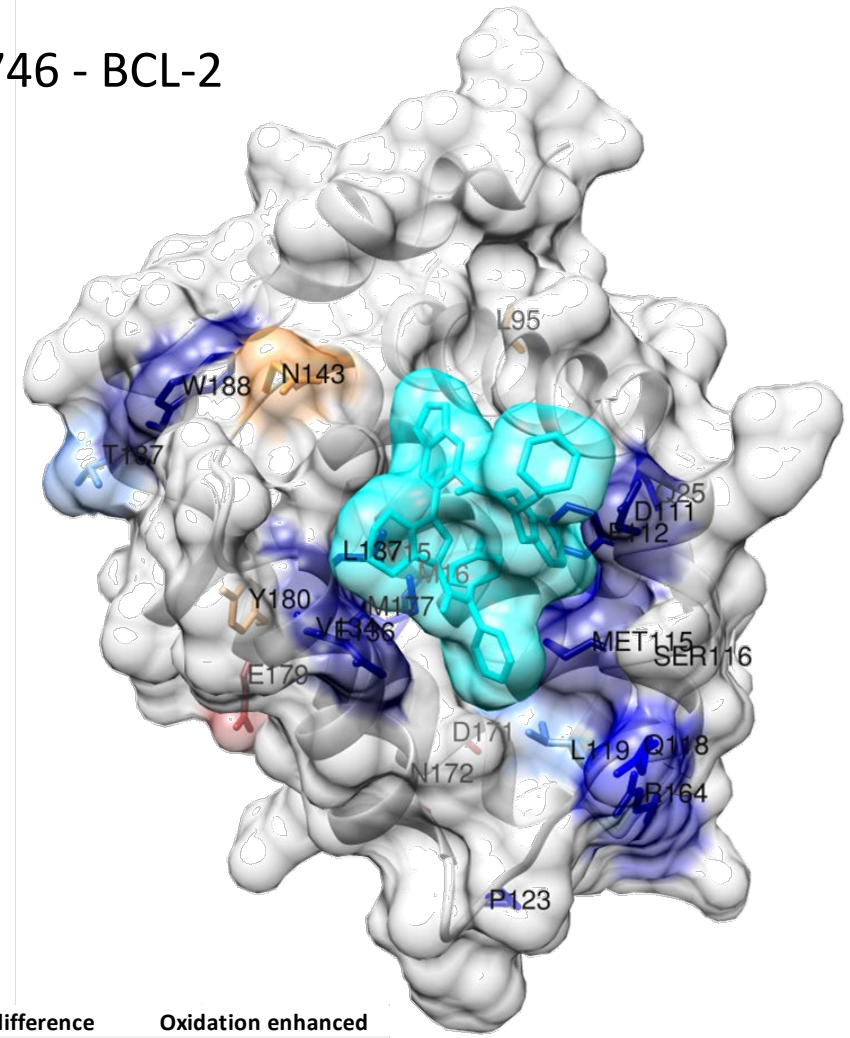
The OX-MS data is validated by comparison with solved structure of BCL-2 and its drug complexes

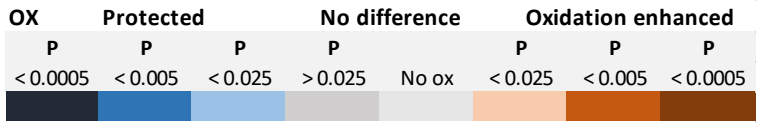
Venetoclax - BCL-2



PDB: 6o0k

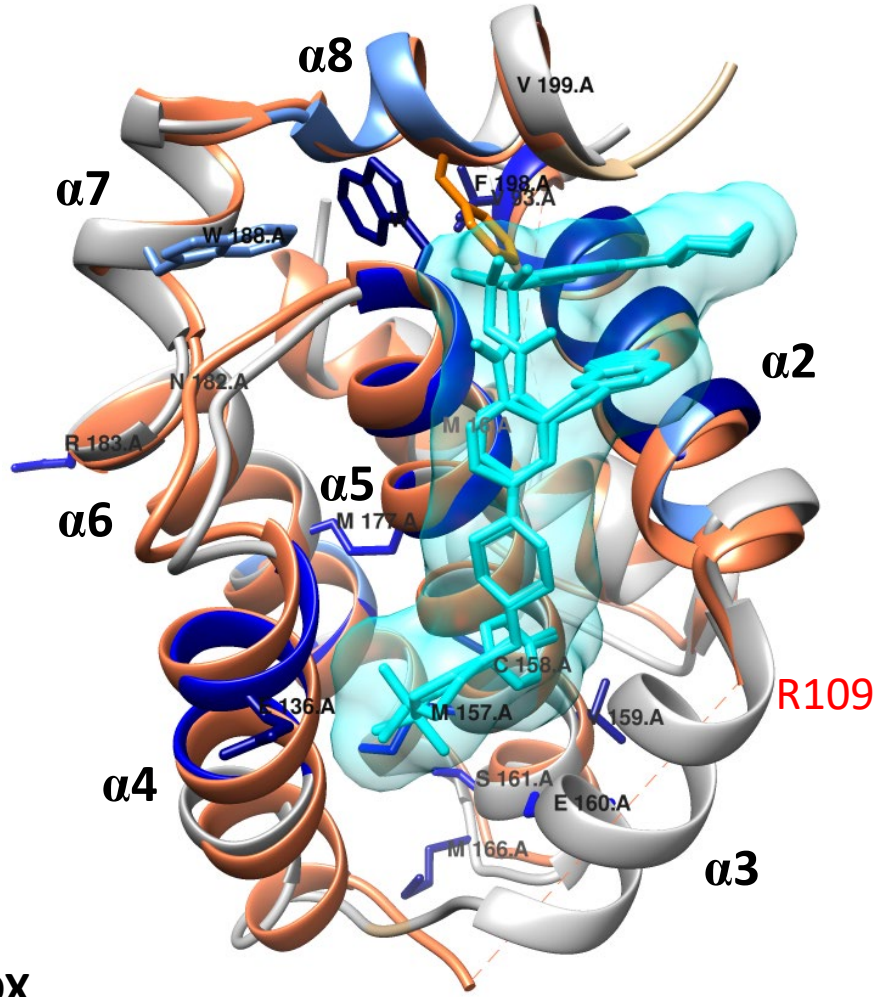
S55746 - BCL-2



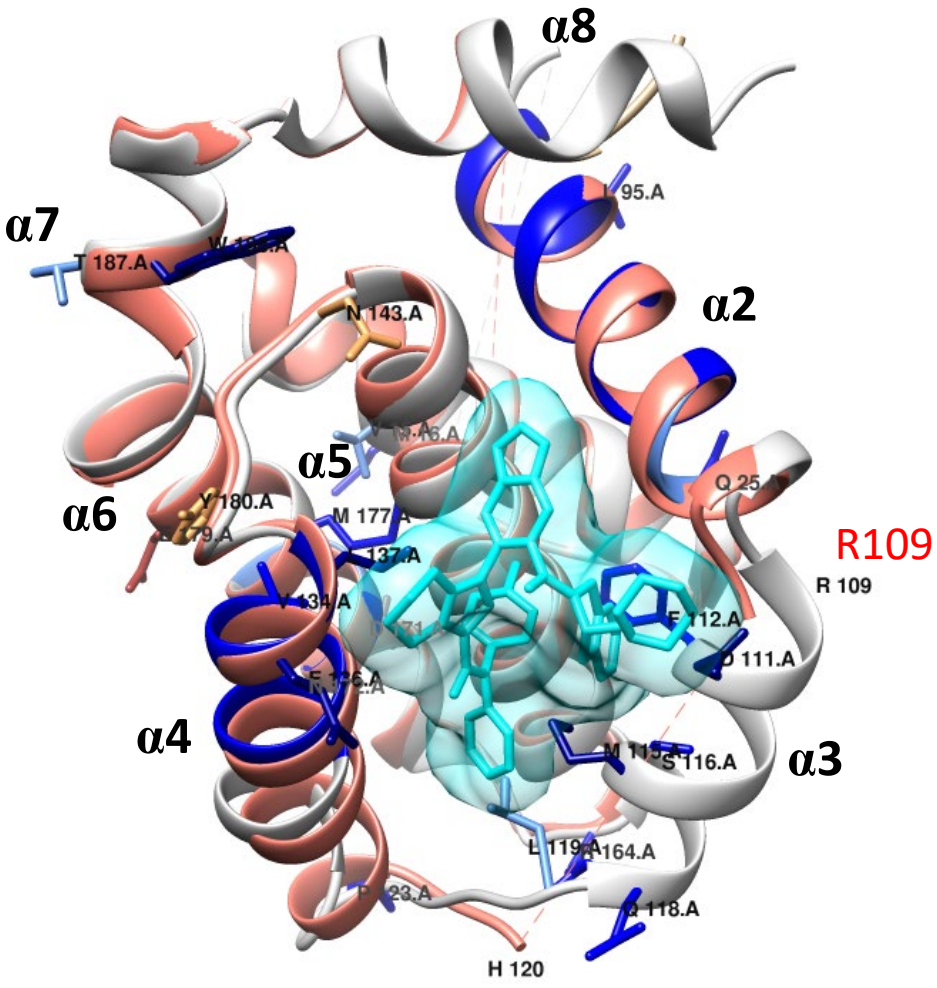


The backbone of the apo and drug bound BCL-2 structures closely aligned except for residues 109 – 120

Venetoclax-BCL-2 / **BCL-2**



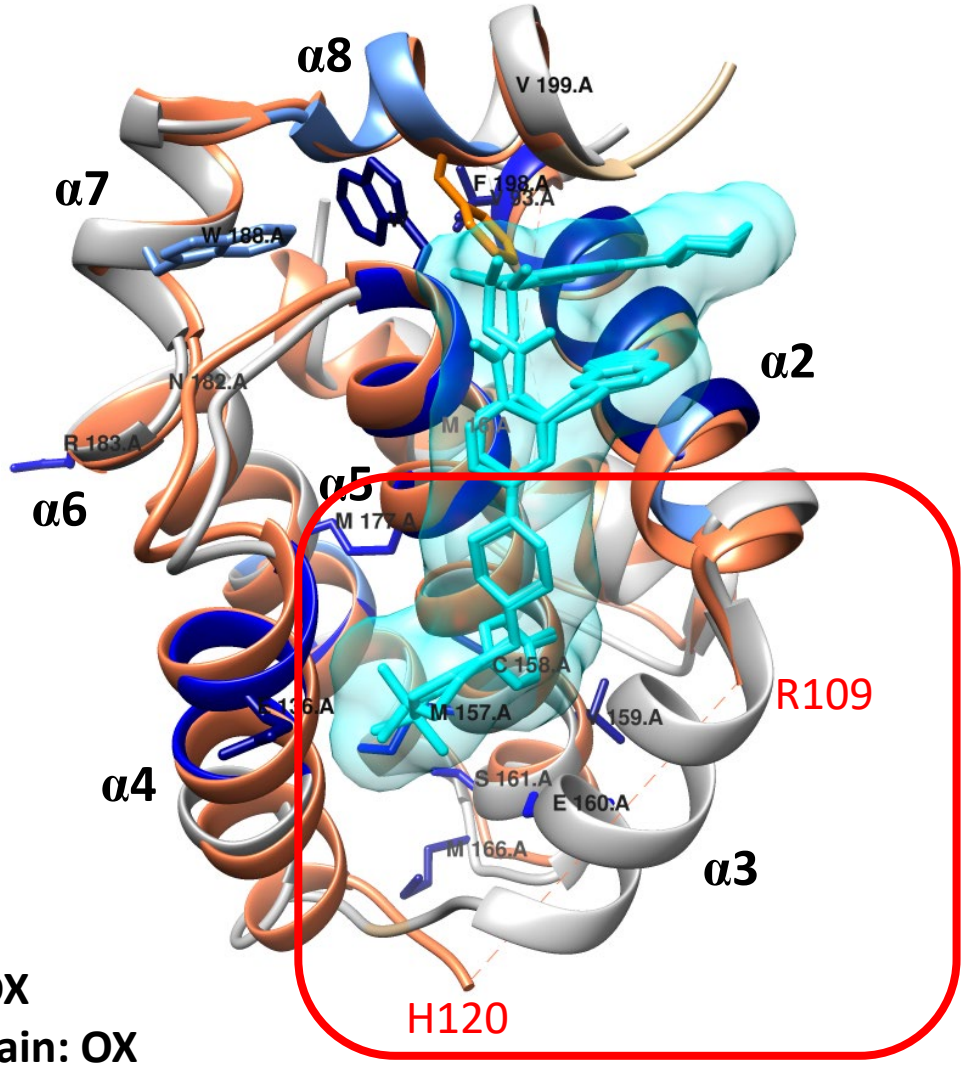
S55746-BCL-2 / **BCL-2**



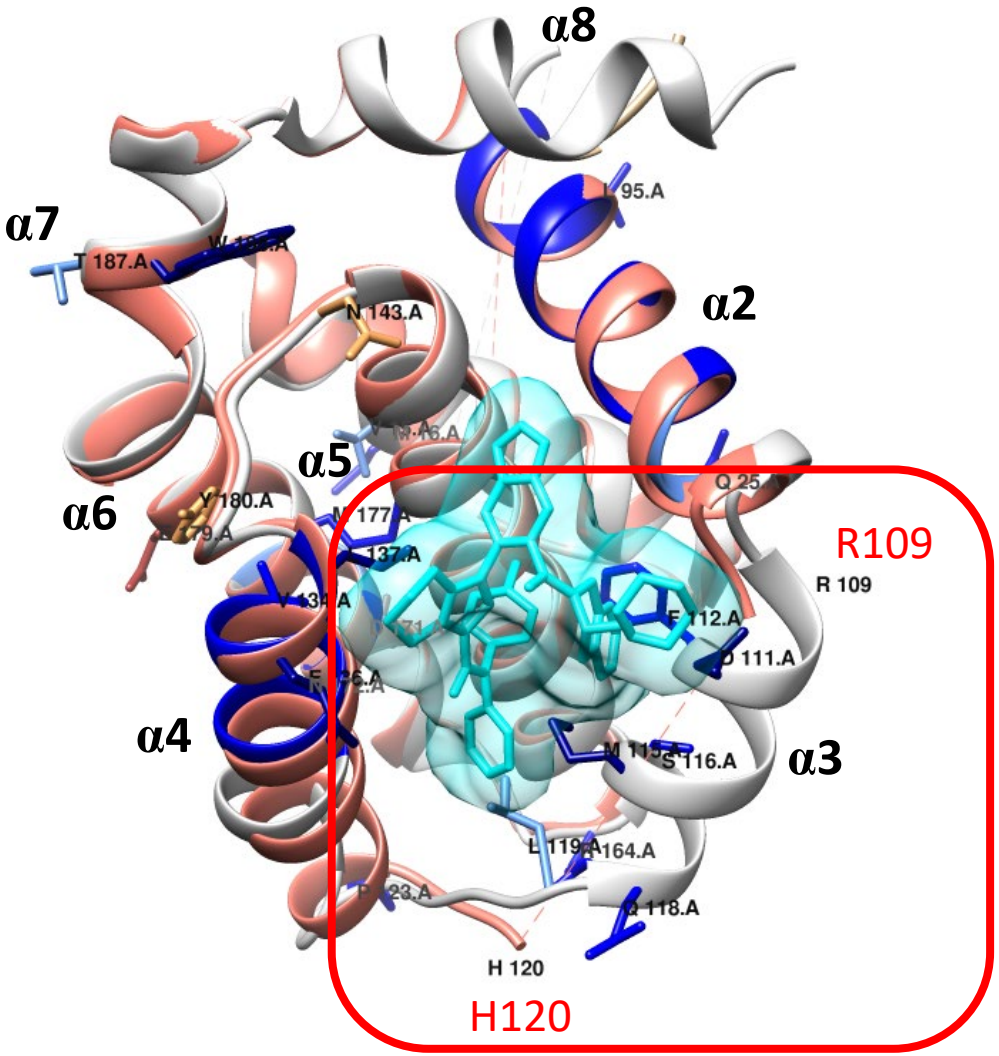
Blue ribbon: HDX
Colored Side chain: OX

Restructuring of residues 109 - 120 to helix α3 in both drug complexes protects clusters of residues from oxidation

Venetoclax-BCL-2 / BCL-2



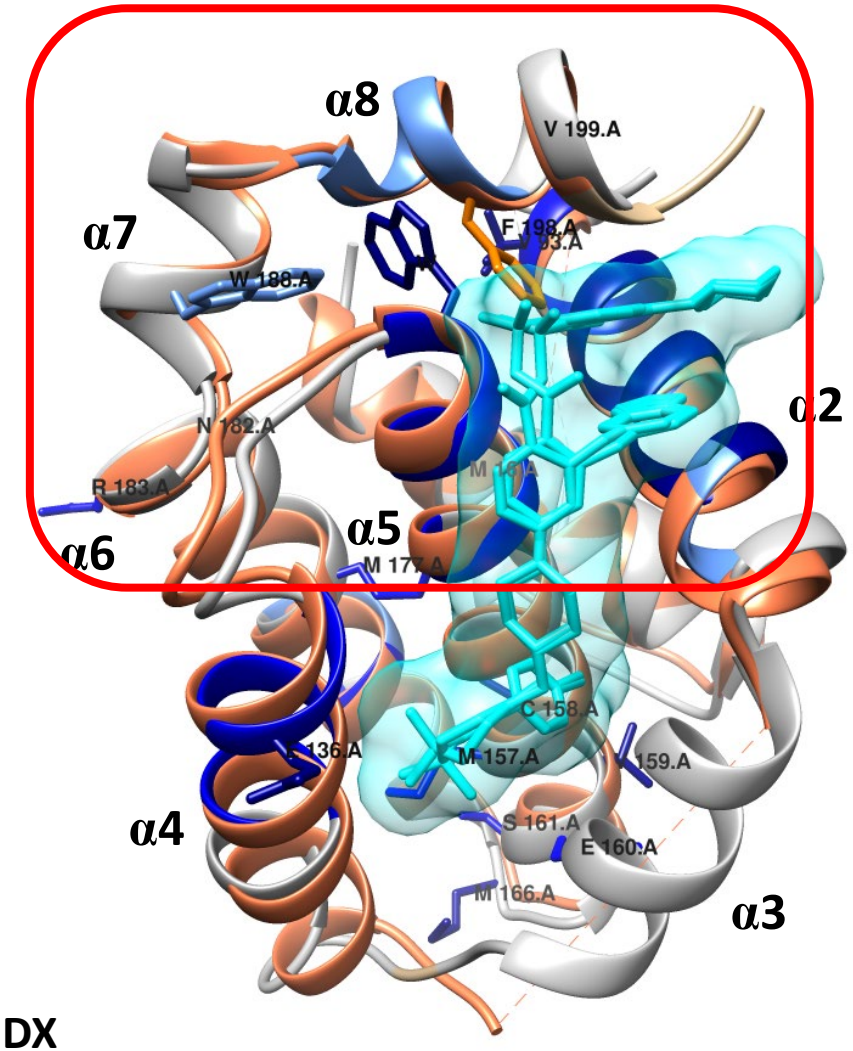
S55746-BCL-2 / BCL-2



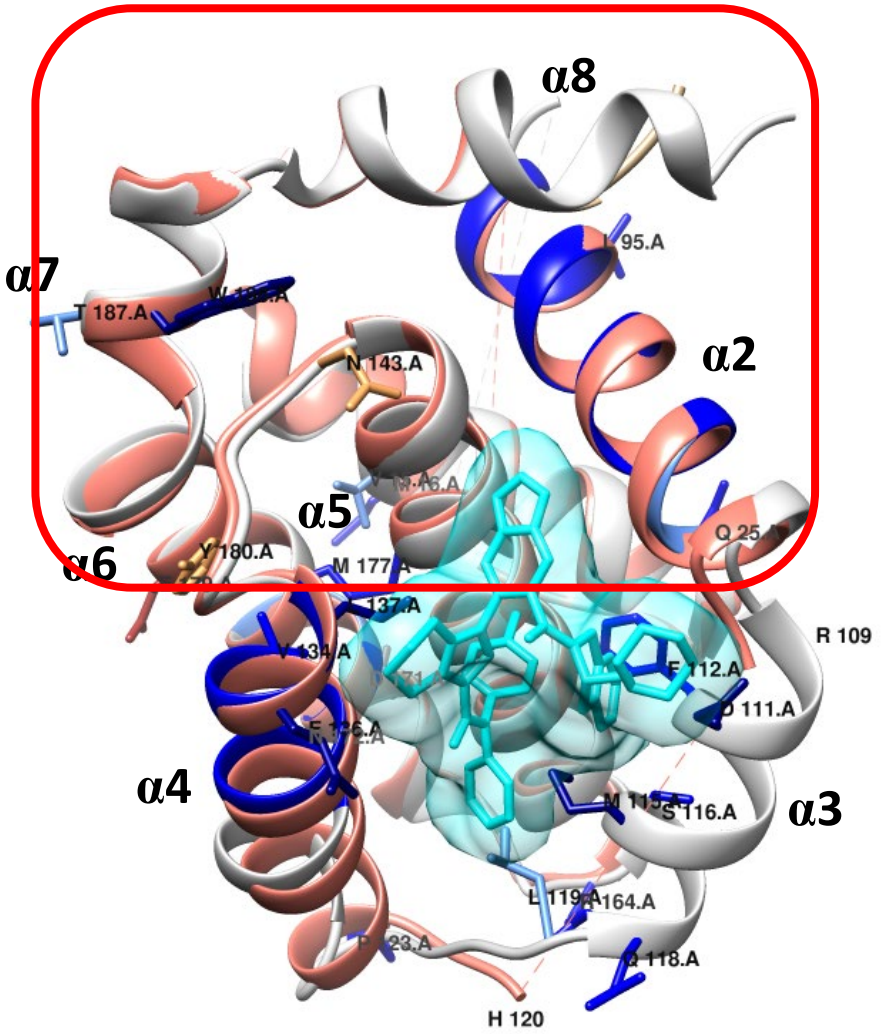
Blue ribbon: HDX
Colored Side chain: OX

Venetoclax causes strong protection of the residues in a pocket formed by helices α8, α7, α5, and α2

Venetoclax-BCL-2 / BCL-2



S55746-BCL-2 / BCL-2



Blue ribbon: HDX

Colored Side chain: OX

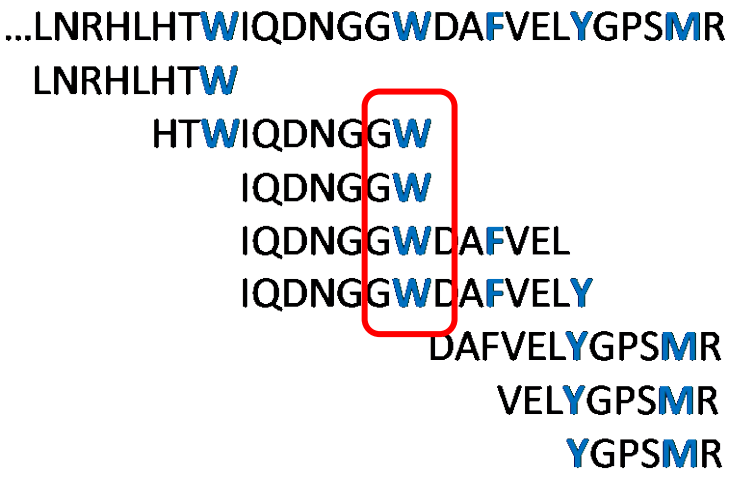
Summary and Conclusion

- OX-MS analysis clearly distinguishes the binding profiles of two drugs to BCL-2 demonstrating its ability to complement HDX-MS in screening candidate compounds during drug discovery and development
- OX-MS provides nuanced insight into the nature of small molecule complexes with proteins through sensitivity to structural changes resulting from both direct binding and allostery
- The precision and ease of implementation of Fenton chemistry mediated OX-MS has the potential to enhance pharmaceutical research and therapeutic development pipelines

Multi-enzyme digestion significantly improved sequence coverage, resolution and precision

- Get better protein sequence coverage 100%
- Maintain same instrument time
- The multitude of small overlapping peptides enables quantitation of individual residues

In addition to MS/MS

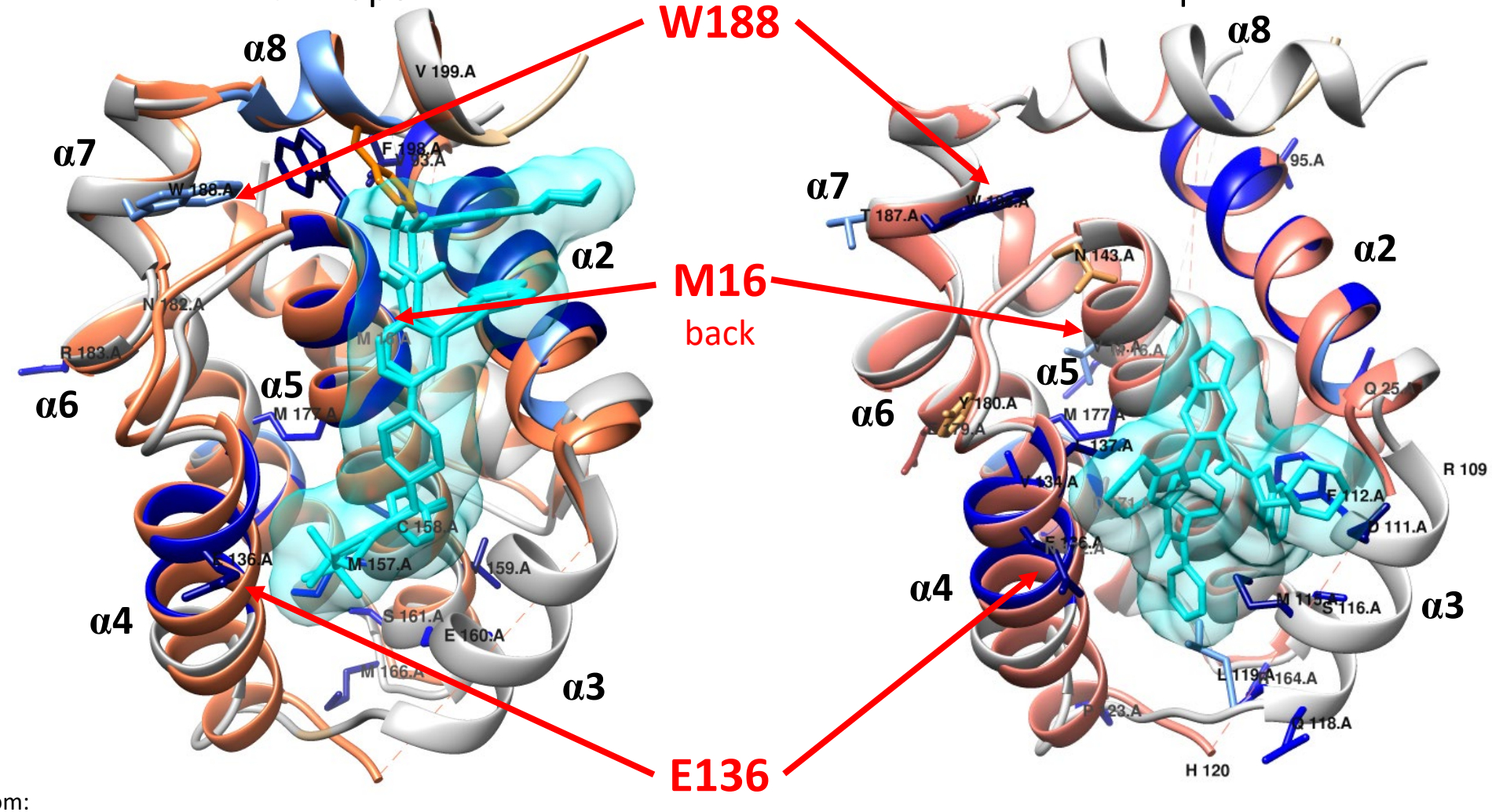


$$\text{Residue}_n\ (\%) = \frac{\sum \text{Peptides containing } \underline{\text{oxidized}}\ \text{residue}_n}{\sum \text{All peptides containing residue}_n}$$

OX of BCL-2 residues perturbed by both Venetoclax and S55746

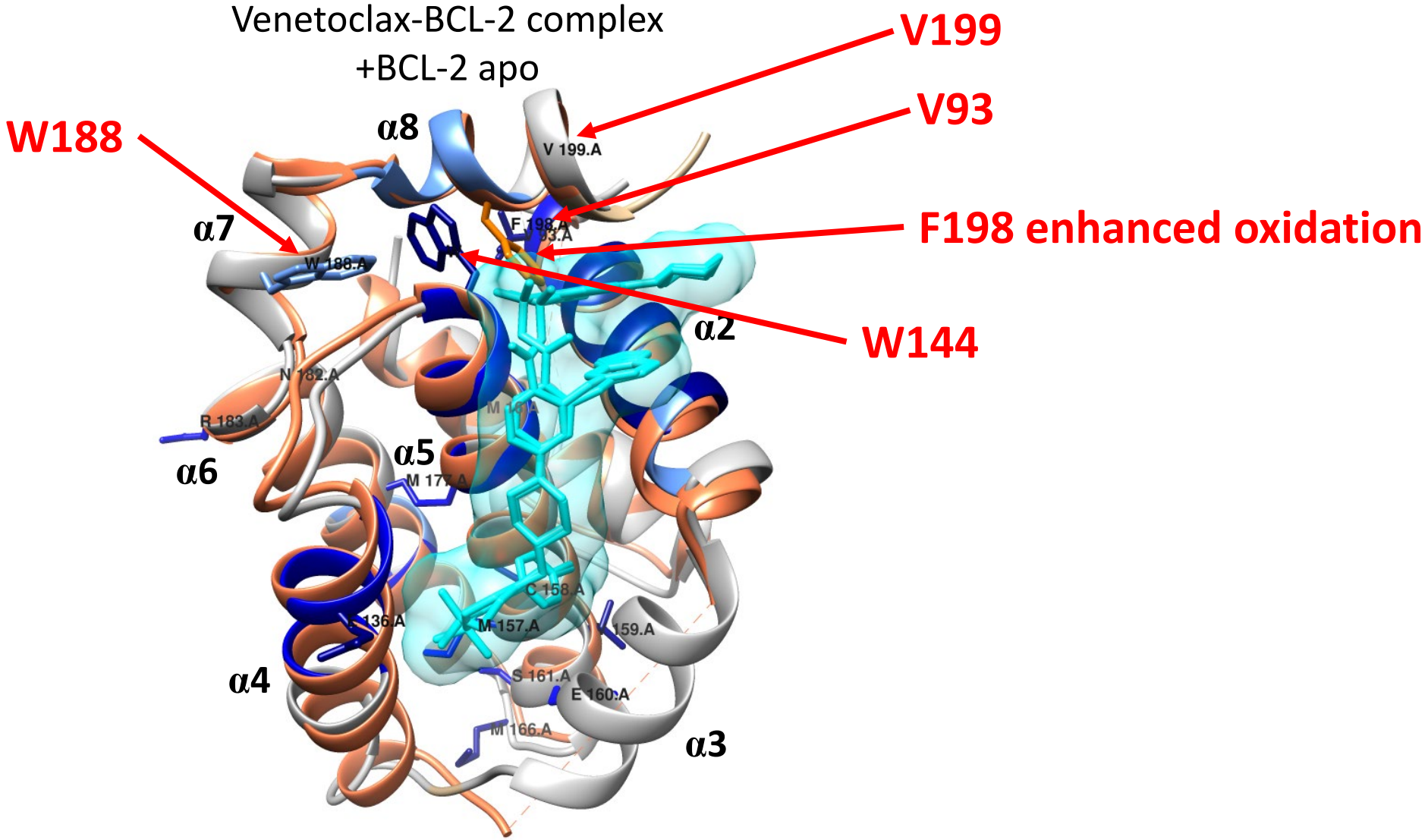
Venetoclax-BCL-2 complex
+BCL-2 apo

S55746-BCL-2 complex
+BCL-2 apo



Nomenclature taken from:
Birkinshaw et al. *Nature Communication*, 2019

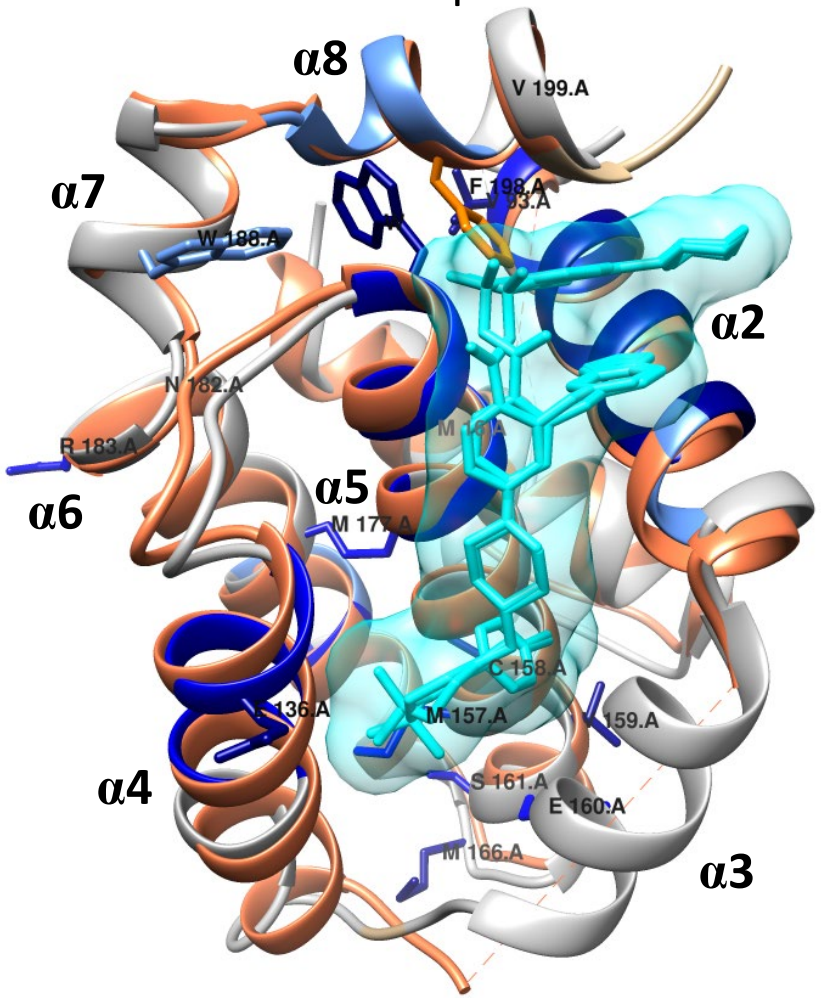
Venetoclax's impact on BCL-2 'top' structure



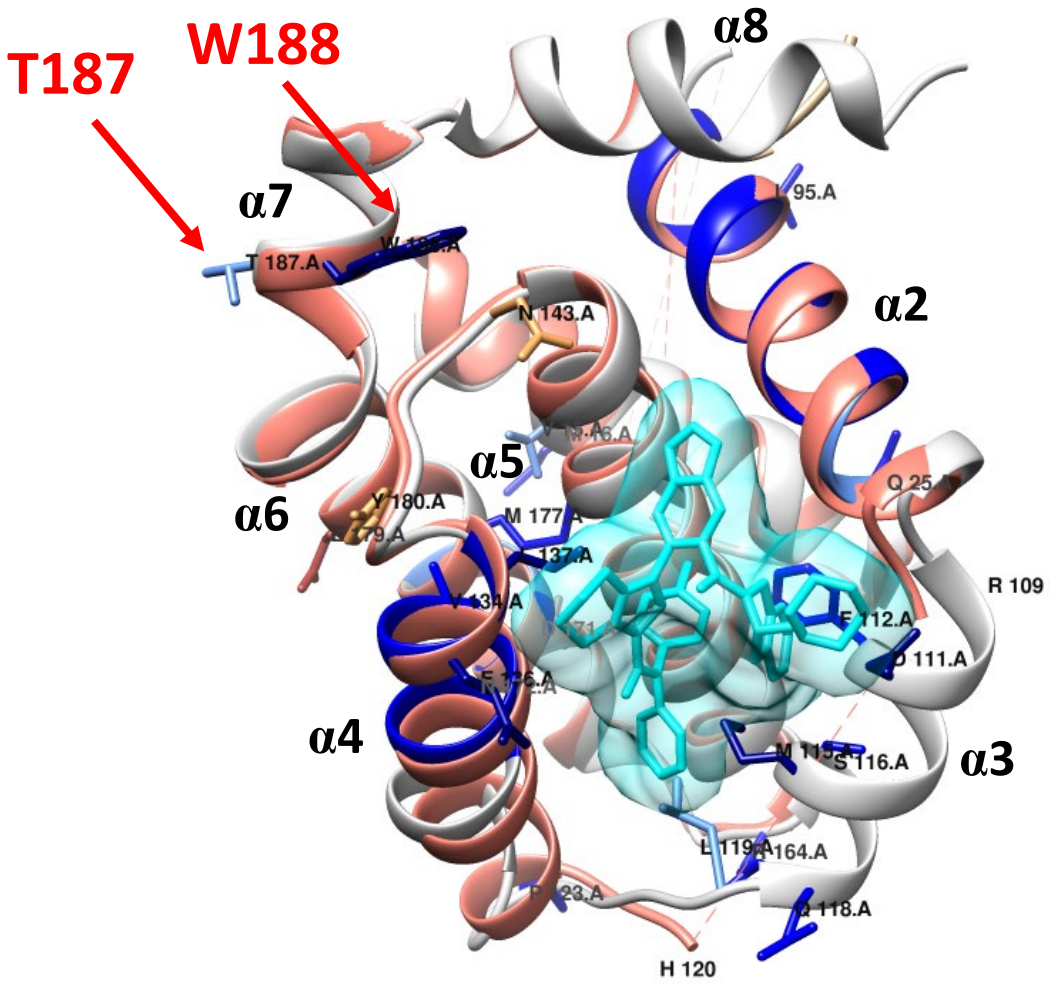
Nomenclature taken from:
Birkinshaw et al. *Nature Communication*, 2019

Venetoclax's greater impact on BCL-2 ‘top’ structure compared to S55746

Venetoclax-BCL-2 complex
+BCL-2 apo



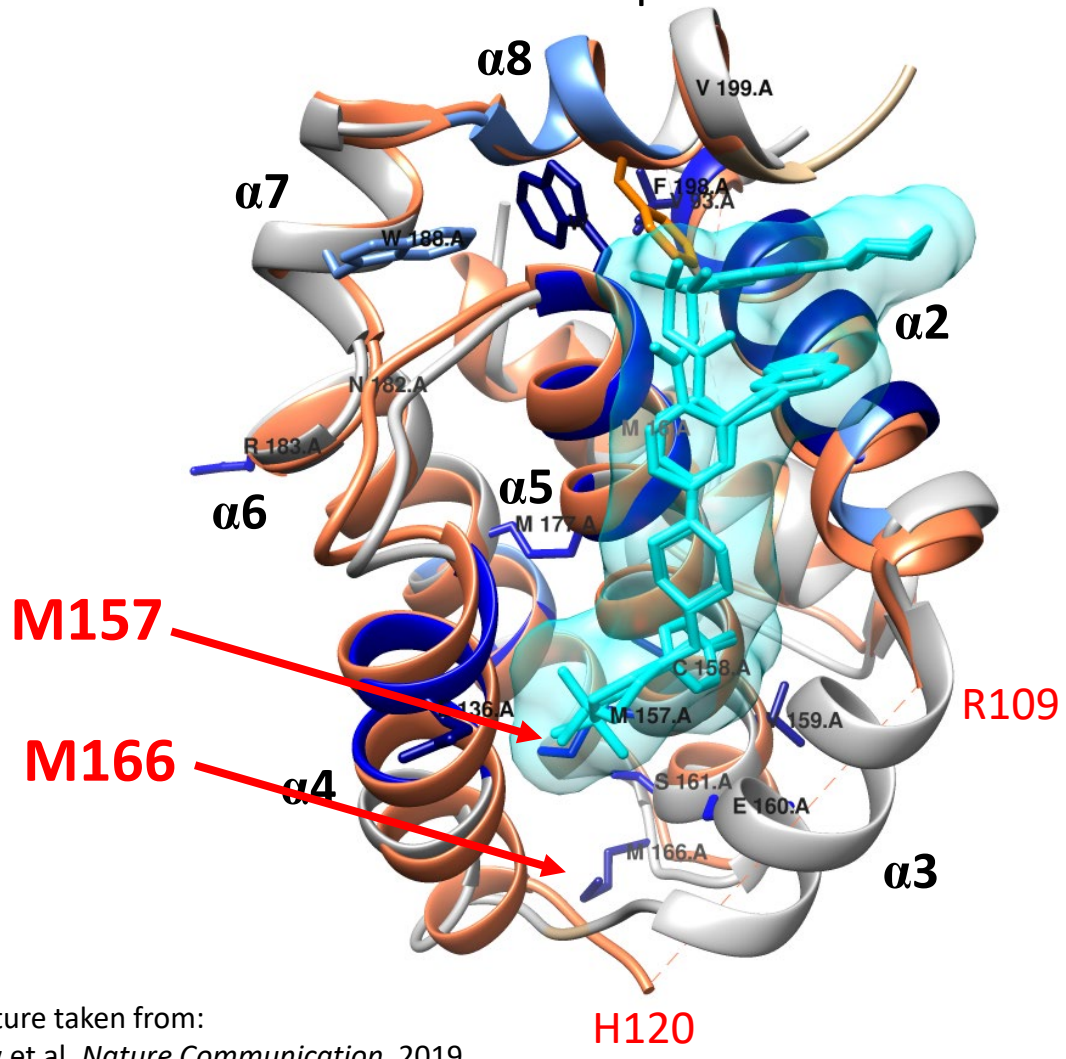
S55746-BCL-2 complex
+BCL-2 apo



Nomenclature taken from:
Birkinshaw et al. *Nature Communication*, 2019

Binding with drug induces BCL-2 ‘bottom’ structuring and oxidation protection in adjacent regions

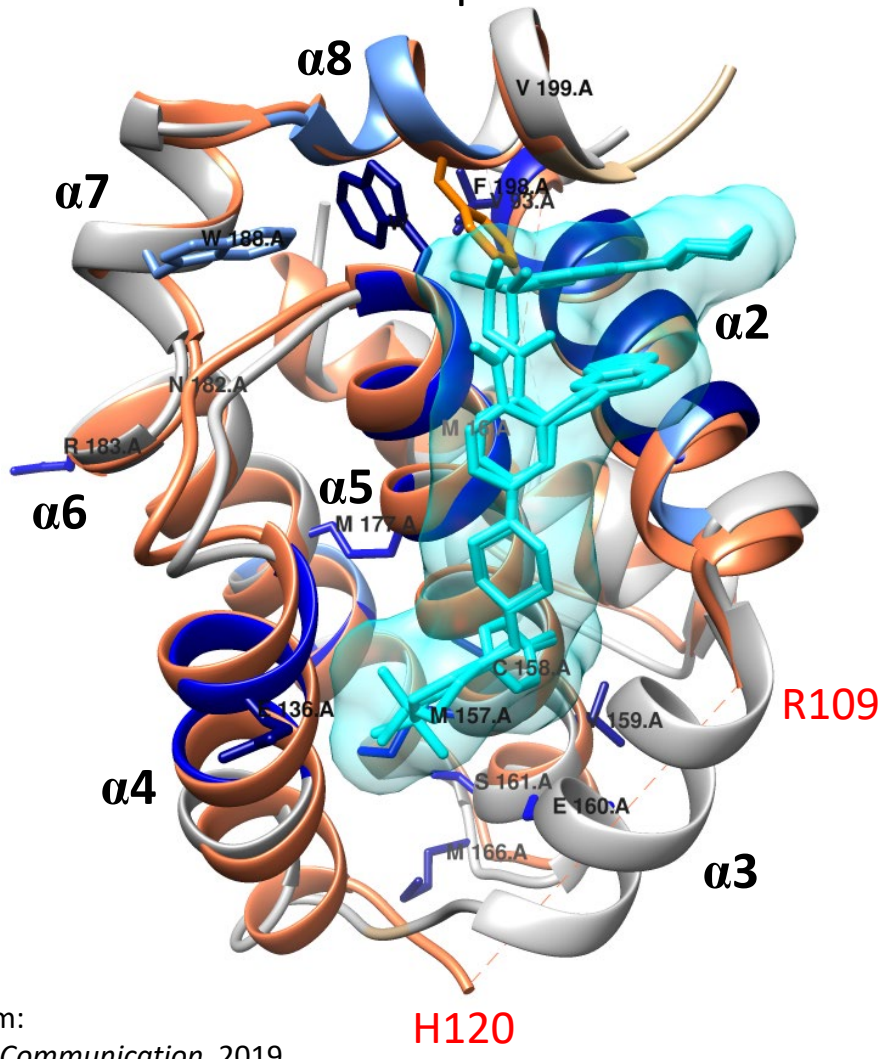
Venetoclax-BCL-2 complex
+BCL-2 apo



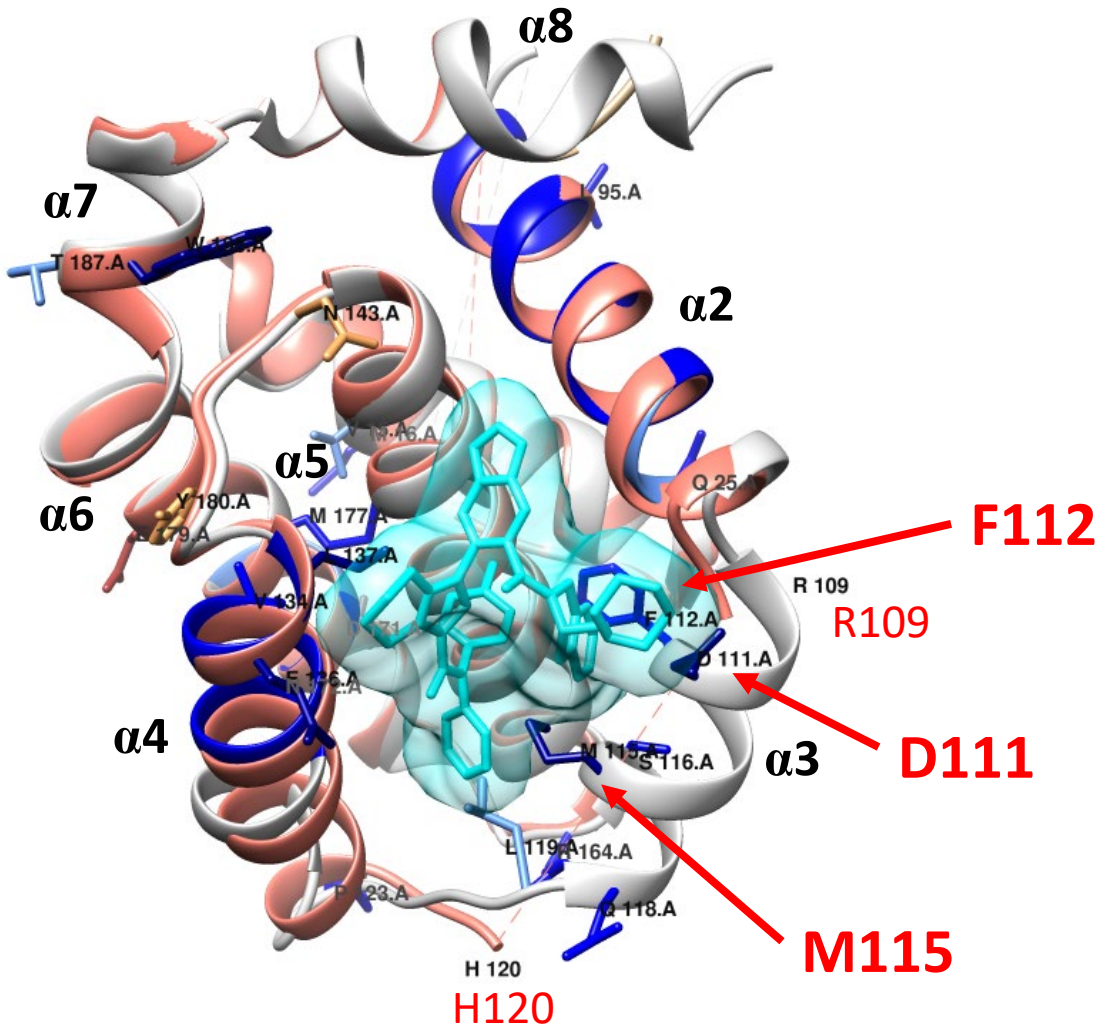
Nomenclature taken from:
Birkinshaw et al. *Nature Communication*, 2019

Binding with drug induces BCL-2 ‘bottom’ structuring and oxidation protection in adjacent regions

Venetoclax-BCL-2 complex
+BCL-2 apo



S55746-BCL-2 complex
+BCL-2 apo



Nomenclature taken from:
Birkinshaw et al. *Nature Communication*, 2019