Measuring the Conformational Effects of RORγ Residues Upon Small Molecule Ligand Binding

Daniel Benjamin[1,2], Faraz Choudhury[1,2]
Immuto Scientific Inc. Madison WI
University of Wisconsin- Madison, Madison WI

Summary

- This project was in collaboration with Professor Patrick Griffin of the Department of Molecular Medicine, Scripps Research Institute.
- RORγ is a nuclear receptor found in hematopoietic stem cells and regulates Programmed Cell Death 1 (PD1) expression. Modulating RORγ activity is attractive for developing therapeutics for autoimmune disorders and cancers.
- Inverse agonists SR2211 and SR19355 are endogenous ligands which disrupt cell-based activity of RORγ.
- Here we use PLIMB to measure conformational differences of RORγ's tryptophan 317 (W317) residue upon binding to SR2211 and SR19355.
- This technical brief outlines the process of analyzing PLIMB data on a single amino acid level.

Experimental Conditions

Samples of RORγ alone, RORγ treated with SR2211, and RORγ treated with SR19355 were prepared in PBS buffer and exposed in triplicates to PLIMB for 2 seconds. Following PLIMB exposure, samples were precipitated, denatured, digested with trypsin, and prepared for mass spectrometry analysis. The samples were analyzed in a data-dependent fashion with an Orbitrap Fusion Lumos mass spectrometer.

Data analysis was performed using Protein Metrics software. The '.raw' data files were submitted to Byos® (Protein Metrics) for a database search using the sequence of RORγ, and automated generation of extracted ion chromatograms ('XIC') of the precursor ions. The peptides are identified using MS and MS/MS criteria, and the proportion of oxidized species is calculated based on relative areas of modified and unmodified peptides. The sequence coverage of RORγ was 94% as shown in **Figure 1**.

The percent modification of the RORγ peptides was compared in samples containing RORγ alone to samples treated with SR2211 and SR19355. This analysis will be considered a peptide-level analysis, as it does not consider the modification on each individual residue of a specific peptide. As we will discuss in the results and discussion, a residue-level analysis of the PLIMB data was later performed.

Results and Discussion

Mass Spectrometry Coverage

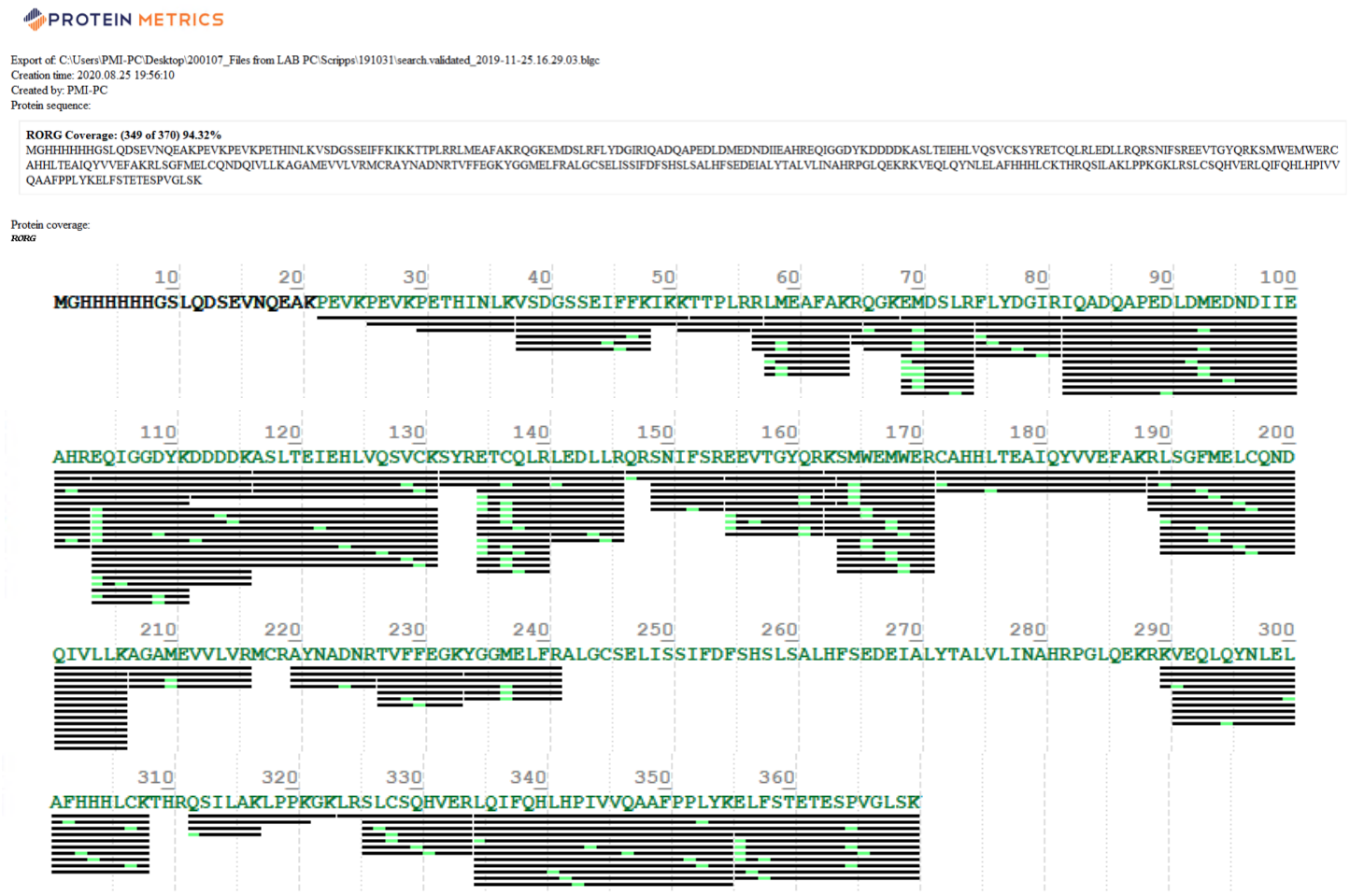


Figure 1: Coverage map of the tryptic digest of RORγ shows peptides that were detected by mass spec, as shown in the black bars, and specific residues that showed oxidative labelling, as shown in the green bars.

Residue Level Footprinting Analysis

After analyzing data on the peptide level, residue level analysis was performed. This was performed as follows:

- Find most abundant modifications for a particular peptide (**Figure 2**).
- Verify the modifications by their Delta Mod Score and MS^2 plots (**Figure 3**).
- Find XIC peaks which consistently correspond to particular verified modification (**Figure 3**).
- Set the XIC intervals for each modified peptide over the peak which corresponds to that particular modification (**Figure 4**).
- Protein Metrics Biologic software will then calculate the percent modification of a particular modified peptide based on the manually set XIC intervals. The resulting data can be used to infer differences in solvent accessibility of a particular residue of a protein in different conditions (**Figure 4**).

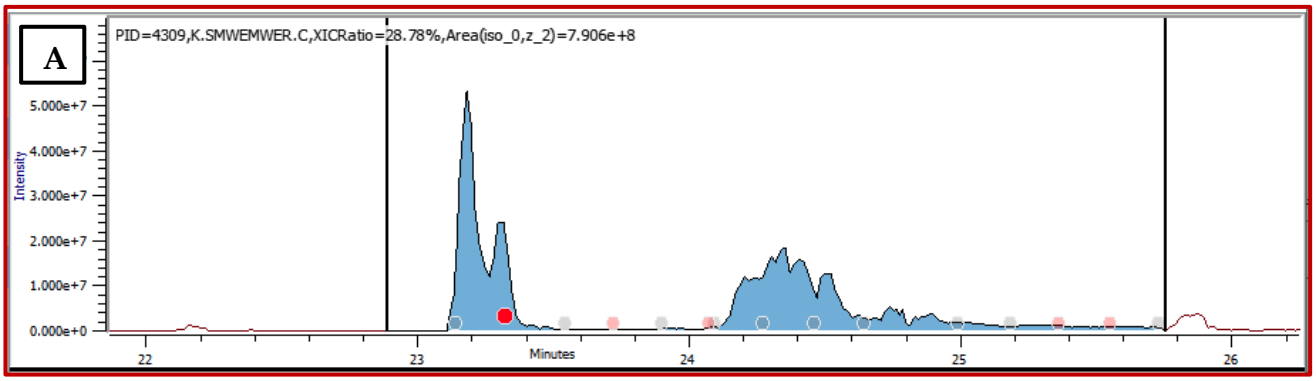
Peptides

wemwer.

Row#	MS Alias name	z	Sequence	Mod. Summary	Score	Delta Score	Delta Mod. Score	XIC area summed	XIC AUC
1	191031_RORBG_...	2	K.SMWEMWER.C		14.11 - 530.88	14.1 - 530.9	14.1 - 530.9	1.5e+10	1.048e+9 - 2.613e+9
2	191031_RORBG_...	2	K.SmWEMWER.C	M2(Dioxidation/31.9898)	51.79 - 292.28	51.8 - 292.3	0.0 - 45.7	8.52e+8	8.523e+7 - 1.579e+8
3	191031_RORBG_...	2	K.SmWEMWER.C	M2(Oxidation/15.9949)	159.82 - 518.70	159.8 - 518.7	0.3 - 129.6	6.87e+9	5.444e+8 - 1.000e+9
4	191031_RORBG_...	2	K.SMWEmWER.C	M5(Dioxidation/31.9898)	58.49 - 314.44	58.5 - 314.4	0.0 - 34.8	1.12e+9	3.606e+7 - 1.666e+8
5	191031_RORBG_...	2	K.SMWEmWER.C	M5(Oxidation/15.9949)	131.00 - 479.52	131.0 - 479.5	0.3 - 98.1	6.85e+9	5.211e+8 - 1.079e+9
6	191031_RORBG_...	2	K.SMwEMWER.C	W3(Dioxidation/31.9898)	59.69 - 437.69	59.7 - 437.7	0.1 - 100.3	1.42e+9	1.070e+8 - 2.341e+8
7	191031_RORBG_...	2	K.SMwEMWER.C	W3(Oxidation/15.9949)	100.13 - 433.17	100.1 - 433.2	0.7 - 24.2	5.22e+9	2.685e+8 - 1.000e+9
8	191031_RORBG_...	2	K.SMWEMwER.C	W6(Dioxidation/31.9898)	59.40 - 397.47	59.4 - 397.5	0.4 - 47.6	1.03e+8	6.942e+6 - 1.726e+7
9	191031_RORBG_...	2	K.SMWEMwER.C	W6(Oxidation/15.9949)	202.76 - 315.43	202.8 - 315.4	19.4 - 34.3	2.31e+7	1.608e+6 - 4.722e+6
10	191031_RORBG_...	3	K.SMWEMWER.C		97.02	97.0	97.0	2.51e+6	2.51e+6

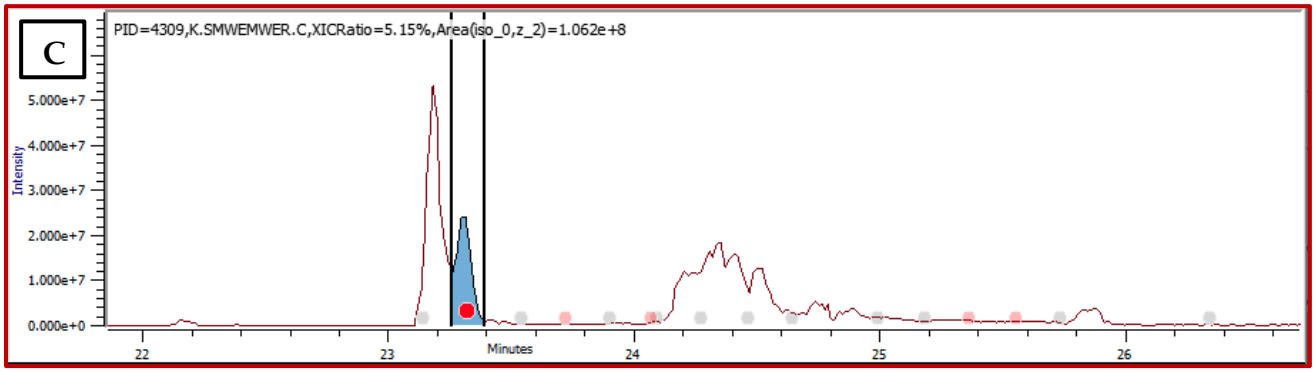
Figure 2: Identified modifications of peptide 'SMWEMWER' in are listed in Protein Metrics Byologic software after performing a search. Here, the list of peptides was reduced to oxidation and dioxidation, occurring on M2, W3, M5, and W6, as these are the most abundant modifications on this particular peptide.

Figure 3: Protein Metrics Byologic will automatically generate a list of modified peptides which it identifies during a search. Localizing a specific modification to a particular residue is done by analyzing the MS^2 data (**Figure 3A**). A list of MS^2 hits that were identified as a modified residue will be generated (**Figure 3B**). Each hit will be assigned a "Delta Mod Score" which is a measure of the confidence in the assigned localization of the modification to a particular residue (shown as a red letter). Selecting a MS^2 hit shows where on the extracted ion chromatogram (XIC) plot the MS^2 data was collected. **Figure 3C** shows two XIC plots: the top corresponds to the modified peptide with the dark red dot showing the location of the selected MS^2 hit, and the bottom corresponds to the wild type, or unmodified, peptide. Selecting the MS^2 hits with the highest Delta Mod Score will allow the user to locate a particular peak on the XIC plot which corresponds to a localized modification.



B

Sequence (unformatted)	Mod. Names	Protein name	Mod. AAs	Var. Pos. Protein	MS Id / MS Alias name	1 / 191031_RORBG_alone_1 (%)	2 / 191031_RORBG_alone_2 (%)	3 / 191031_RORBG_alone_3 (%)
SMWEMWER	Dioxidation/31.9898	RORG	M	165		3.49	2.78	2.52
				168		4.09	3.31	2.52
			W	166		4.06	3.31	3.74
				169		0.301	0.299	0.206
	Oxidation/15.9949	RORG	M	165		19.9	18.6	16
				168		19.9	17.9	17.2
			W	166		8.37	9.61	16
				169		0.0905	0.0363	0.0754



D

Sequence (unformatted)	Mod. Names	Protein name	Mod. AAs	Var. Pos. Protein	MS Id / MS Alias name	1 / 191031_RORBG_alone_1 (%)	2 / 191031_RORBG_alone_2 (%)	3 / 191031_RORBG_alone_3 (%)
SMWEMWER	Dioxidation/31.9898	RORG	M	165		3.49	3.28	2.52
				168		4.09	3.92	2.52
			W	166		4.06	3.92	3.74
				169		0.301	0.353	0.206
	Oxidation/15.9949	RORG	M	165		19.9	22	16
				168		19.9	2.84	17.2
			W	166		8.37	11.4	16
				169		0.0905	0.043	0.0754

Figure 4: The XIC intervals for a particular localized modification will likely cover a large area before manual validation, as seen in **Figure 4A**. This is because the intervals will be automatically set to include many false-positive hits (pink dots). The calculated percent modification for a particular modified residue shown in this example can be seen circled in **Figure 4B**. Once a particular peak on the XIC plot has been identified corresponding to the given modification, the XIC intervals can be manually set around that peak (**Figure 4C**). This will then update the calculated percent modification based on the area between the manually set XIC intervals (**Figure 4D**).

Ligand-Induced Conformational Effects

- Residue level analysis was performed to identify conformational changes to specific residues of RORγ when treated with SR2211 and SR19355.
- PLIMB data suggests that Tryptophan 317 (W317) was shown to increase in solvent accessibility when treated with SR211 and to decrease when treated with SR19355 (see **Figure 5**).
- These results corroborate data generated by x-ray crystallography (see **Figure 6**).

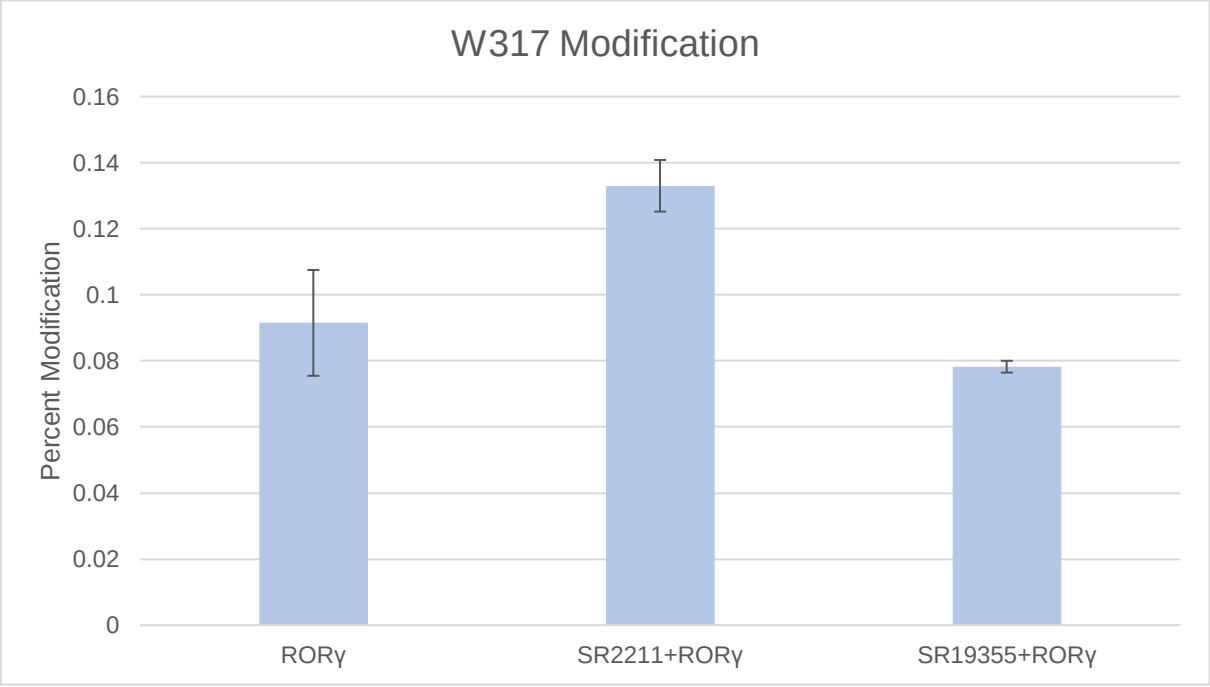


Figure 5: Here, residue specific PLIMB data is shown. The results suggest that SR2211 increases the solvent accessibility of RORγ's W317, while SR19355 decreases solvent accessibility of W317.

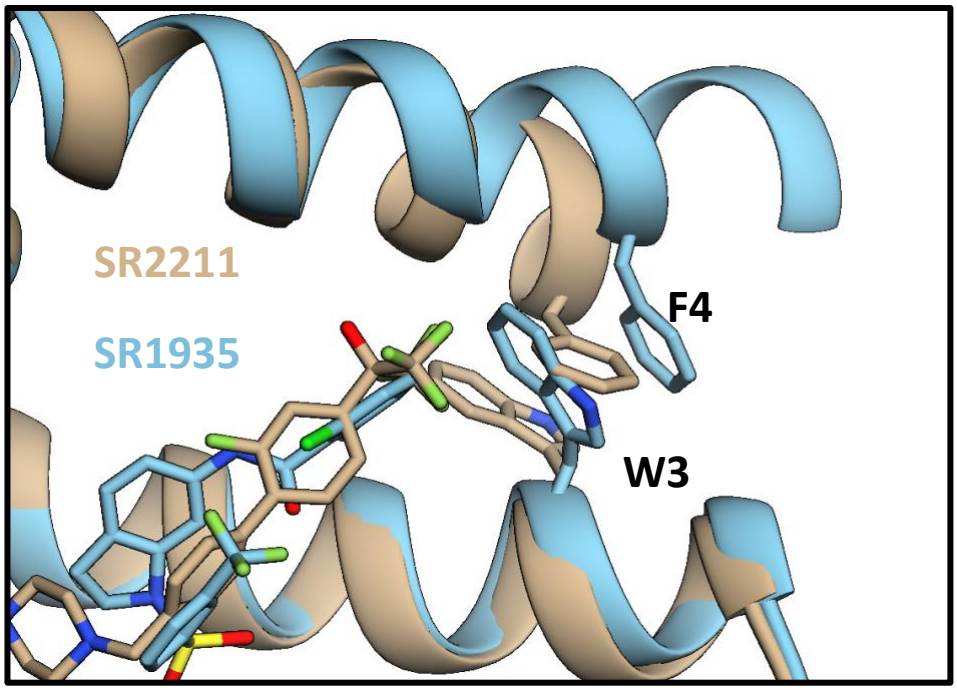


Figure 6: RORγ inverse agonists have multiple modes of action where the rotomeric state of W317 repositions helix 11-12: SR19355 stabilizes *gauche* rotomer of W317, and SR2211 clashes with and displaces W317. (Source: Strutzenberg *et al.*)

Conclusions

- PLIMB data analyzed by Protein Metrics software can generate structural information on a single amino acid-level resolution
- Minute changes in solvent accessibility induced by small molecule binders can be measured with PLIMB.
- The effects of SR2211 and SR19355 on RORγ's W317 solvent accessibility generated with PLIMB corroborate data generated by x-ray crystallography.

Immuto Scientific Inc.
Madison, WI
immutoscientific.com
info@immutoscientific.com