Reviving B-Factors: Retrospective Normalized B-Factor Analysis of c-ros Oncogene 1 Receptor Tyrosine Kinase and Anaplastic Lymphoma Kinase L1196M with Crizotinib and Lorlatinib

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S Supporting Information

ABSTRACT: Structure-based drug design (SBDD) is commonly leveraged in rational drug design. Usually, ligand and binding site atomic coordinates from crystallographic data are exploited to optimize potency and selectivity. In addition to traditional, static views of proteins and ligands, we propose using normalized B-factors to study protein dynamics as a part of the drug optimization process. A retrospective case study of crizotinib and lorlatinib bound to both c-ros oncogene 1 kinase (ROS1) and anaplastic lymphoma kinase (ALK) L1196M related normalized B-factors to differences in binding affinity. This analysis showed that ligand binding can have protein-stabilizing effects that start near the ligand but propagate through nearby residues and structural waters to more distal motifs. The potential opportunities for analyzing normalized B-factors in SBDD are also discussed.

KEYWORDS: Normalized B-factor, temperature factor, ALK, ROS1, L1196M, crizotinib, lorlatinib

Proteins are flexible and highly dynamic with motions ranging from rapid vibrations on the time scale of picoseconds and a magnitude of under 0.5 Å to conformational changes that take up to 10³ seconds and sweep out greater than 10 Å.¹ X-ray analysis of protein crystal structures with and without bound ligands has been a critical tool in structure-based drug design (SBDD). While there has been much work on understanding the conformation of the amino acid residues and secondary structure of proteins,^{1–4} less work has focused on the use of protein dynamics in SBDD.

B-factors, also known as temperature factors or atomic displacement parameters, are calculated parameters in X-ray crystal structures that reflect disorder of atoms from their ideal equilibrium positions due to both thermal motion and positional disorder. Crystallographic studies done at varying temperatures have shown that dynamics have a much larger effect on atomic B-factors in general than does the crystal lattice disorder.² B-factors provide insight into the dynamic motion of proteins in the crystalline state, which can be used as a model for motion in the solution state. In fact, typical protein crystals are composed of 40–60% solvent as measured by volume. B-factors provide insight into the dynamic motion of proteins in the crystalline state, which can be used as a model for motion in the solution state. Normalized B-factor measurements are utilized to compare motion across multiple structures. Normalized B-factor (B') is an expression of the B-factor (B) in units of standard deviation (σB) about the mean value (μB , eq 1). This value is calculated across all heavy atoms in a protein–ligand complex crystal structure.

$$B' = (B - \mu B) / \sigma B \quad (1)$$

Interestingly, while B-factors show a very large spread across high-resolution cocrystal structures, normalized B-factors show a more narrow distribution.⁴ Normalized B-factors have been used as a metric for analysis of protein structures to examine motion in various sections of the protein, such as secondary structures vs random coils, active site vs nonactive site, and buried vs solvent-front residues.^{1,4} More recently, normalized B-factor analysis has been used to link specific clinically relevant anaplastic lymphoma kinase point mutations to structural disorder that correlates with constitutive activity.⁵ While there are many published examples that make use of normalized B-factor analyses,^{6–10} to our knowledge there are no known examples of use cases associated with structure-based drug design. The use of normalized B-factor is compelling in the study of ligand–protein dynamics because it constitutes an experimental measure of dynamics that validates *in silico* calculations such as molecular dynamics simulations. It is also complementary to other experimental methods such as hydrogen–deuterium exchange mass spectrometry (HDX)¹¹ and nuclear magnetic resonance (NMR) experiments. While all three are highly valuable tools to investigate the motion of ligand–protein complexes, normalized B-factor analysis is uniquely capable of achieving atomic resolution.

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In most cases, the interaction of a reversibly bound ligand with a protein induces stabilization or “cooling” of the protein–ligand complex in rough correlation with ligand potency.¹² Ligands may impact the stabilization of proximal and distal residues. These differences in stabilization, as measured by normalized B-factor, can be exploited in drug design as part of the ligand optimization process, particularly through analysis of specific changes to binding site residues. As a proof of concept for using normalized B-factor analysis to explain differences in potency, we retrospectively examined binding of first and second generation anaplastic lymphoma kinase (ALK) and c-ros oncogene 1 kinase (ROS1) inhibitors crizotinib and lorlatinib (Figure 1). Clinically, ALK and ROS1 oncogenic translocations

Figure 1. Structures of crizotinib (1) and lorlatinib (2).

define a subset of non-small-cell lung cancers (NSCLC).^{13–16} In addition, both proteins show mutations in the kinase domain as an acquired resistance mechanism to ALK or ROS1 inhibitor therapy. In the case of ALK, gatekeeper mutations (ALK-L1196M) predominate.^{17,18}

Crizotinib was approved by the US Food and Drug Administration (FDA) for the treatment of ALK-positive lung cancer in 2011.^{19,20} While it was originally developed as an inhibitor of mesenchymal–epithelial transition factor (c-MET), it is also potent against oncogenic drivers ALK and ROS1. Crizotinib had a biochemical activity against ROS1 of 0.6 nM and a corresponding cellular IC₅₀ values of 51 nM and 3.9 nM (Table 1). Lorlatinib was developed as a next generation ALK/

Table 1. Crizotinib and Lorlatinib Potency against ROS1^{21,22}

compd	ROS1 K _i (nM)	HCC78 ^a Cell pROS1 IC ₅₀ (nM)	BaF3 ^b Cell pROS1 IC ₅₀ (nM)
crizotinib	0.6 ± 0.2	51 ± 23	3.9 ± 0.92
lorlatinib	<0.025	0.19 ± 0.14	0.23 ± 0.22

^aHCC78 cell line containing an endogenous SLC34 A2-ROS1 fusion.

^bBaF3 cell line with an engineered CD74-ROS1 fusion.

ROS1 inhibitor and is currently in Phase III clinical trials. Lorlatinib improved central nervous system (CNS) exposure and broad spectrum mutated ALK/ROS1 inhibition.^{21,22} Lorlatinib achieves biochemical ROS1 activity of <0.025 nM, which is >24-fold more potent than crizotinib. Furthermore, lorlatinib has cellular ROS1 IC₅₀ values of 0.19 and 0.23 nM in ROS1-fusion cellular assays, making it 17- to over 250-fold more potent than crizotinib.

Kinase domain constructs of ROS1 (I1934 through E2232, heterogeneously phosphorylated on Y2114 and Y2115 with predominant +3P state) were prepared. Notably, this construct was used for both crystallography and biochemical assays.^{21,22} The cocrystal structures of ROS1 with crizotinib and lorlatinib were solved, and their coordinates were previously described.^{22,23} These structures have a high degree of overlap

considering ligand and protein conformation. In order to complete a ligand binding analysis based on dynamics, we exploited normalized B-factors calculated from these cocrystal structures. While relatively homogeneous in global conformation, there are a few notable differences between ROS1 bound with crizotinib and lorlatinib (Figure 2a).¹ Most apparently, the glycine rich loop (P-loop) and activation loop (A-loop) are unresolved in the structure with crizotinib. This implies increased motion in these nonresolved regions for the structure of ROS1 with crizotinib. Examination of the cocrystal structures colored by normalized B-factor showed that there is cooling of the residues leading to and following the P-loop for ROS1 bound to lorlatinib (Figure 2b). Furthermore, the same effect is seen in the residues comprising the A-loop.

Figure 2. (a) Phospho-ROS1 stabilized with crizotinib (orange; PDB code 3ZBF) and lorlatinib (green; PDB code 4UUX). (b) Same as panel a, colored by normalized B-factor. (c) Per-residue (C α atom) $\Delta B'$ for ROS1 kinase domain stabilized with crizotinib or lorlatinib.

While coloring the cocrystal structures by normalized B-factor is useful in identifying these differences, a format more amenable to visualizing differences was achieved by extracting normalized B-factors for both complexes and calculating the per-residue $\Delta B'$ at each backbone C α atom (Figure 2c). The delta is calculated by subtracting the normalized B-factor for lorlatinib from the normalized B-factor of crizotinib. We have defined $\Delta B'$ greater than 0.64 as the threshold for significant changes in the structures with ROS1. This threshold was set at a constant (1.65) times the median absolute deviation (MAD) for $\Delta B'$ of the analyzed ROS1 structures, where the constant was chosen to be consistent with an upper 95% bound from a normal distribution. We chose to employ this cutoff to focus on the most statistically significant differences between structures. Therefore, variation within the red and blue line is considered insignificant. Apart from a small section of the α -I helix (residues 2225–2226) that is barely above this threshold, lorlatinib stabilizes ROS1 more than crizotinib. This region is far from the inhibitor binding site and at a solvent exposed region of the

protein that is unlikely to have a significant impact on inhibitor binding potency. Both structures show similar crystal packing, which leads us to believe that it is not influencing the conclusions drawn here.

The ALK gatekeeper mutation (L1196M) is often observed as an acquired resistance mechanism to crizotinib. Since this residue makes contact with the inhibitor and has been shown to activate the kinase,⁵ this piqued our interest in further examining normalized B-factor differences in ALK when bound with crizotinib and lorlatinib. Kinase domain constructs of ALK L1196M, D1093 through V1411, with a noncleavable N-terminal six histidine tag were purified for use in structural biology studies and in the biochemical assay. While a heterogeneously phosphorylated activated protein was used for the enzyme assay, nonphosphorylated enzyme was used for protein crystallization. In an ALK L1196M biochemical K_i assay, crizotinib and lorlatinib were 8.2 and 0.7 nM, respectively, which represents a 12-fold difference in activity (Table 2).¹⁹ In the

Table 2. Crizotinib and Lorlatinib Potency against ALK L1196M¹⁹

compd	ALK L1196M K_i (nM)	Cell p-EML4-ALK L1196M IC_{50} (nM)
crizotinib	8.2	843
lorlatinib	0.7	21

cellular assay, lorlatinib is approximately 40-fold more potent. Normalized B-factor analysis of ALK L1196M bound with crizotinib and lorlatinib still show similar trends to what was described above with ROS1.

For ALK L1196M, a $\Delta B'$ greater than 0.42 (derived from MAD) was defined as the threshold for significant changes between structures. Relative to ALK L1196M bound with crizotinib, ALK L1196M bound with lorlatinib showed cooling in the P-loop and A-loop via analysis of normalized B-factors (Figure 3). Similar to ROS1, a section of the P-loop in ALK

Figure 3. Graphical depiction of the difference in normalized B-factors for ALK L1196M stabilized with crizotinib (PDB code 2YFX) and lorlatinib (PDB code 4CLJ).

L1196M was completely unresolved when bound to crizotinib and fully resolved when bound with lorlatinib. The residues leading to and following the break showed preferential cooling in the presence of lorlatinib. Although ALK L1196M bound either crizotinib or lorlatinib had an unresolved section of the A-loop, the residues leading to and following the break showed preferential cooling with lorlatinib.

While the above-described differences in stabilization of ROS1 and ALK protein structures upon binding of crizotinib and lorlatinib are valuable, this general trend has been documented in the literature previously.¹² Whereas the

differential stabilization of proteins by inhibitors of different potencies has been noted, to our knowledge these observations have not yet been leveraged to provide insight for SBDD. We believe that differences in normalized B-factors can be readily exploited for use in SBDD through comparison of specific protein regions and residues for pairs of inhibitors.

Focusing in on the P-loop of ROS1, the residues starting at L1945 showed increased cooling in the presence of lorlatinib relative to crizotinib (Figure 4). This effect continued through

Figure 4. (a) C-terminal lobe of pROS1 stabilized with crizotinib (unresolved P-loop; PDB code 3ZBF) or lorlatinib (resolved P-loop; PDB code 4UXL) colored by the normalized B-factor. (b) C-terminal lobe per residue ($C\alpha$ atom) $\Delta B'$ in graphical format.

the P-loop peaking at L1951 with a large difference in $\Delta B'$ of almost 1.2 before a section of three amino acids (G1954, A1955, and F1956) that are unresolved in the structure with crizotinib, resulting in a gap in the plot. The unresolved P-loop and higher $\Delta B'$ of residues leading to the break strongly implied significant motion in this region of the protein. Following the P-loop, the $\beta 2$ and $\beta 3$ sequences also showed significant cooling in the presence of lorlatinib. This effect dissipates at L1982 as the α -C-helix begins.

For the discrepancies in stabilization of the P-loop, $\beta 2$, and $\beta 3$, structures can be traced back to specific ligand–residue interactions. L1951, which is on the P-loop proximal to the inhibitors, showed a large difference in normalized B-factor for binding with crizotinib versus lorlatinib. This difference is conserved across all atoms in the residue (Figure 5a).

When bound to crizotinib, L1951 sits slightly higher above the binding site than when bound with lorlatinib (Figure 5b). Lorlatinib contains an *N*-methyl amide that crizotinib lacks. This methyl group is directed up toward the P-loop and the carbonyl of L1951. It forms a close contact with the carbonyl oxygen (3.4 Å) that stabilizes the P-loop. Similarly, the carbonyl group of lorlatinib forms a stabilizing close contact with the side chain of V1959 (3.6 Å). The cooling effect is also conserved across all atoms in the residue, although the difference is less significant than what was observed at L1951 (Figure 5a). These specific ligand–protein interactions not only stabilize the P-loop but also may be a contributing factor in more distal stabilization of

Figure 5. (a) Per-atom normalized B-factor for pROS1 L1951, V1959, and V1979 stabilized with crizotinib (orange) and lorlatinib (green). (b) ATP binding site of pROS1 stabilized with crizotinib (heated, orange; PDB code 3ZBF) and lorlatinib (cooled, green; PDB code 4UXL). (c) P-loop, β_2 , and β_3 regions of pROS1 with lorlatinib (PDB code 4UXL).

the β_2 and β_3 region through a hydrogen bonding network as exhibited by V1979 (Figure 5c).

Shifting focus to ALK L1196M, the P-loop showed a similar trend to ROS1 when bound with crizotinib and lorlatinib. The sequence of L1118 through Y1131 is significantly cooled with lorlatinib relative to crizotinib (Figure 6). Two amino acids in this region, G1123 and V1124, are completely unresolved in ALK L1196M when bound with crizotinib, implying a higher degree of motion in these residues.

The difference in ALK L1196M P-loop stability can be linked to specific protein–ligand interactions. During the optimization process of lorlatinib for ALK and activating mutants of ALK, a *cis*-*N*-methyl amide was used as a macrocyclic linker that

Figure 6. Per-atom normalized B-factor for ALK L1196M stabilized with crizotinib (heated; PDB code 2YFX) and lorlatinib (cooled; PDB code 4CLJ). (a) Structure colored by normalized B-factor. (b) Graph of the difference in normalized B-factors.

provided productive interactions to the G-loop and stabilizing structural waters (Figure 7a).²¹ Lorlatinib forms a number of

Figure 7. (a) ATP binding site of ALK L1196M stabilized with crizotinib (heated, orange; PDB code 2YFX) and lorlatinib (cooled, green; PDB code 4CLJ). (b) Per-atom normalized B-factor for L1122 of ALK L1196M stabilized with crizotinib (orange) and lorlatinib (green).

significant interactions with both L1122 and G1123, which crizotinib lacks. The methyl group on the amide of lorlatinib makes productive stabilizing interactions with the carbonyl groups of both L1122 and G1123. Additionally, the carbonyl group of the lorlatinib amide forms a water-mediated interaction with the carbonyl group of G1123. These productive interactions show preferential cooling with lorlatinib relative to crizotinib in this region (Figure 7b) as well as throughout the P-loop (Figure 6).

The catalytic lysine (K1150) also showed differences in normalized B-factors between the two ligand bound structures of ALK L1196M. When cocrystallized with lorlatinib, there is a water-mediated interaction between the lysine and carbonyl group of the ligand (Figure 8a). This set of interactions through

Figure 8. (a) ATP binding site of ALK L1196M stabilized with crizotinib (heated, orange; PDB code 2YFX) and lorlatinib (cooled, green; PDB code 4CLJ). (b) Per-atom normalized B-factor for K1150 of ALK L1196M stabilized with crizotinib (orange) and lorlatinib (green).

the water network may aid in cooling of the lysine side chain relative to the structure with crizotinib, which lacks this carbonyl group and lysine interaction. While the overall magnitude of the difference is smaller than the other examples discussed herein, the differences increase along the side chain from the carbon atom closest to the backbone to the terminal amine (Figure 8b). Furthermore, the water molecules that are hypothesized to be a part of this network of stabilizing interactions are absent in the structure with crizotinib.

While the aforementioned examples are a retrospective observation, they illustrate how differences in a normalized B-factor could be used in structure-based drug design in a predictive way. Analyses using normalized B-factors allowed visualization of differences in amino acid conformation and dynamics impact potency. While computational chemistry could also be used to interrogate this difference, normalized B-factors

are experimentally derived and are already part of the PDB crystal structure data package.

In summary, lorlatinib significantly improved biochemical and cell-based inhibition of both ROS1 and ALK L1196M relative to crizotinib (Table 1). This enhancement in potency correlated well with crizotinib and lorlatinib trends in dynamics quantified by normalized B-factors for both ROS1 and ALK cocrystal structures. These differences in normalized B-factor derive from specific interactions in this retrospective analysis. These analyses show how a normalized B-factor can be used to support SBDD.

Normalized B-factor data and analysis adds value to the use of cocrystal structure data in structure-based drug design. It provides information on the dynamics of the protein–ligand complex, which can aid in singling out specific differences that are beneficial or detrimental to binding. We have found that normalized B-factor analysis is most productive when comparisons result from small structural changes and specific residue-based observations, as exemplified by the retrospective case study above. Changes in normalized B-factor can show strengthening or weakening of specific interactions of interest. Even small changes in ligand structure can change the binding affinity in a number of ways: lipophilicity-driven changes or changes caused by protein strain, ligand strain, desolvation penalty, etc.²⁴ Exploiting normalized B-factors, traditional structural analysis/SBDD and lipophilic efficiency together can help deconvolute these differences and have a significant impact on successful drug design. Normalized B-factor analysis is particularly useful when the absolute position of binding site residues is conserved between inhibitors of different potencies. In this case, using B' to aid in analyzing the strength of protein–ligand interactions has high potential to add additional insight that is not otherwise obvious. In addition to use in the analysis of protein–ligand complexes, normalized B-factors can be leveraged to improve modeling of prospective compounds.

■ ASSOCIATED CONTENT

Supporting Information

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Normalized B-factor computations and crystal structure summary (PDF)

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Notes

The authors declare the following competing financial interest(s): The authors are employees and shareholders of Pfizer.

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■ ABBREVIATIONS

ALK, anaplastic lymphoma kinase; ROS1, c-ros oncogene 1 receptor tyrosine kinase; SBDD, structure-based drug design; PDB, Protein Data Bank

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