High-Throughput Interrogation of Ligand Binding Mode Using a Fluorescence-Based Assay**

Paweł Śledź, Steffen Lang, Christopher J. Stubbs, and Chris Abell*

Structure-based ligand discovery has benefitted from extensive developments in recent years.[1–4] In particular, the application of high-throughput crystallography has vastly accelerated these efforts.[5] This approach is based on the rapid generation of protein–ligand complexes through the soaking of protein crystals with ligands and subsequent automated X-ray data collection and structure determination. However, suitable soakable crystals are not available for many proteins, especially for those complexes in which protein–protein interactions or structural changes play a role. Consequently, there is a need for more high-throughput and crystallographically independent methods for assessing the binding mode of molecules to select candidates for further analysis.

This has become particularly important to us while developing more potent ligands against the polo-box domain (PBD) of polo-like kinase 1 (Plk1). The PBD domain is responsible for the proper cellular localization of Plk1 through an array of phosphorylation-dependent protein–protein interactions mediated by the phosphopepitide-binding groove.[6,7] Disrupting these interactions has shown potential as a strategy for anticancer therapy.[8] The discovery of an auxiliary flexible pocket on the PBD surface, which is involved in binding a peptide derived from polo-box interacting protein 1 (PBIP1), has been recently reported.[9,10] This flexible hydrophobic pocket recognizes its ligand through hydrophobic interactions of four aromatic residues (Y417, Y421, Y481, and F482) with F71PBIP1 and an additional hydrogen bond between the hydroxy group of Y417 and the backbone nitrogen of D72PBIP1 (Figure 1a).[9]

Our objective was to prepare more potent ligands of the PBD by improving binding to this auxiliary pocket by optimizing the hydrophobic interactions whilst retaining the key hydrogen bond. To efficiently distinguish specific recognition by the pocket from nonspecific hydrophobic interactions, we adapted a fluorescence-based thermal shift (FTS) assay.[11] In this assay the ability of a molecule to stabilize the protein during its thermal unfolding is quantified by its thermal shift (ΔTm): the difference in the protein unfolding temperature in the presence and absence of a ligand. FTS has been successfully applied in fragment and high-throughput screening campaigns,[12,13] however, it generally provides very limited information on binding and it is not always appropriate for weakly binding ligands.

We designed a procedure through which information on the binding mode can be obtained using FTS. When a modified protein that cannot fully engage a ligand in the selected binding mode is used, ligands adopting such a binding mode are expected to stabilize the mutant protein against thermal unfolding to a lesser extent than the wild-type protein; ligands not engaging in the selected binding mode are expected to bind similarly to both mutant and wild-type protein (Figure 1b). The difference in thermal shift of a ligand for the wild-type and mutant protein (ΔΔTm) is therefore a measure of how beneficial the pocket binding is for a particular ligand. Such an experimental setup eliminates errors arising from the concentration dependence of the assay,[14] as a single stock solution of the ligand can be used. Also noteworthy is that since in this particular case we were working with elaborated ligands with significant ΔTm values, the sensitivity of the assay (ΔTm > 1.0 K) was not a problem and identification of ligands with a significant ΔΔTm value (higher than 2.0 K) was straightforward.

For the proof-of-principle study, we first obtained three different mutants of the PBD to assess their ability to distinguish binding in the hydrophobic pocket (Figure 1a). The Y417A mutation was expected to prevent the formation
of the key hydrogen bond with the ligand. A double mutant with an additional Y421A mutation was also prepared to further reduce the possible scope of hydrophobic interactions.\textsuperscript{[9]} To assess the specificity of these effects, we prepared a Y481K mutant, which changed the distal end of the pocket, where interaction with the ligand is limited. These mutants were subsequently tested against two PBIP1-derived ligands—FDPLHspTa (1a), a peptide that binds in the pocket, and ADPPLHspTa (1b), which does not. Both the Y417A and Y417A/Y421A mutants exhibited detectable and significant $\Delta T_m$ values for 1a (3.7 and 3.2 K, respectively), while $\Delta T_m$ values for 1b were within the experimental error (1.2 and 0.1 K). This was further confirmed by the study with the Y481K mutant, which gave negligible $\Delta T_m$ values for 1a (−0.8 K) and 1b (−1.0 K).

We used the double-mutant Y417A/Y421A to further probe the molecular recognition of the pocket (Table 1). A series of analogues of PBIP1-derived peptide 1a with phenylalanine replaced by other acetylated hydrophobic and aromatic amino acids (2a–f) were prepared and tested in the FTS binding mode assay. Interestingly, the pocket turned out to be very specific for hydrophobic aromatic residues. Tryptophan-bearing 2e exhibited a significant $\Delta T_m$ (3.3 K), indicating hydrophobic pocket binding, in contrast to histidine-bearing 2f ( $\Delta T_m = 0.5$ K). None of tested aliphatic residues (2a–d) showed $\Delta T_m$ values higher than 1.0 K.

To further study the molecular recognition of the pocket, we expanded the series of PBIP1-derived peptides to include non-amino acid N-terminal residues, focusing on, but not limited to, hydrophobic aromatic ring systems (Table 1). As expected, neither of the tested aliphatic ring compounds 3a,b showed binding in the pocket. Only two out of the four simple phenylalanine analogues 3c–f gave $\Delta T_m$ values exceeding 2.0 K. Peptide 3d with a phenethyl substituent showed slightly better stabilization than the original PBIP1 peptide 1a. Two simple analogues of 3d, however, showed no indication of hydrophobic pocket binding. Ligand 3e, lacking one methylene group, was too short to comfortably insert the phenyl ring to the bottom of the pocket, while the $p$-methoxy substituent in analogue 3e appeared to cause a steric clash at the bottom of the pocket. Interestingly, 3,4-dichloro-substituted 3f was found to bind well in the hydrophobic pocket. The two ortho-chloro substituents in the ring mimic the presence of an additional benzene ring because of their size and hydrophobicity. Three peptides incorporating heterocyclic single-ring systems 3g–j were also prepared and tested in the assay. Interestingly, the pocket showed significantly higher selectivity for thiophene-derived 3h over pyridine-based 3g or furan-based 3j, even though for 3h the observed $\Delta T_m$ value of 2.5 K was not as high as observed for 1a. In addition, the two double-ring aromatic systems 3j,k were tested, both giving high $\Delta T_m$ values indicative of hydrophobic pocket binding. The structures of these compounds suggest that they are able to insert their benzene rings into the pocket, reinforcing the suggestion of its selectivity for aromatic moieties.

Analysis of peptide binding to the protein by isothermal titration calorimetry (ITC; Table 1 and Table S1 in the Supporting Information) revealed a clear correlation between

| R | WT $T_m$ (K) | DM $T_m$ (K) | $\Delta T_m$ (K) | $K_0$ (µM) |
|---|---|---|---|---|
| 1a | 12.9 | 9.7 | 3.2 | 160 |
| 1b | 9.8 | 9.7 | 0.1 | 960 |
| 2a | 10.3 | 10.0 | 0.3 | 1100 |
| 2b | 10.9 | 10.7 | 0.2 | 1200 |
| 2c | 11.5 | 10.5 | 1.0 | 1100 |
| 2d | 11.0 | 10.5 | 0.5 | 950 |
| 2e | 13.6 | 10.3 | 3.3 | 160 |
| 2f | 9.3 | 8.8 | 0.5 | 1200 |
| 3a | 12.5 | 11.5 | 1.0 | 240 |
| 3b | 11.6 | 11.3 | 0.3 | 310 |
| 3c | 12.2 | 11.0 | 1.2 | 190 |
| 3d | 15.4 | 11.4 | 4.0 | 66 |
| 3e | 11.6 | 11.0 | 0.6 | 280 |
| 3f | 15.8 | 11.2 | 4.8 | 21 |
| 3g | 9.9 | 9.7 | 0.2 | 790 |
| 3h | 12.7 | 10.2 | 2.5 | 140 |
| 3i | 9.9 | 8.8 | 1.1 | 1000 |
| 3j | 16.0 | 11.9 | 4.1 | 20 |
| 3k | 16.0 | 11.2 | 4.8 | 40 |

Table 1: The results of the assay for the series of modified peptides (R–DPPLHspTa–NH₂). $\Delta T_m$ values are shown for the wild-type (WT) and double-mutant Y417A/Y421A (DM) protein; the difference between them ($\Delta \Delta T_m$) and the $K_0$ value against the wild-type protein is also shown. The color code corresponds to that used in Figure 2 a,b.
the $\Delta T_m$ for wild-type protein and binding affinity ($pK_D$) (Figure 2a). The peptides are clustered into three groups (designated weak, medium, and tight binders), with the initial pocket-binding peptide 1a being in the middle group. The correlation between $\Delta\Delta T_m$ and $K_D$ furnishes additional insight into the binding mode (Figure 2b). Peptides identified in the assay as pocket binders have in general shown higher affinity than those not utilizing the pocket, and all the most potent ligands (3d, 3f, 3j, and 3k, colored purple) also bind to the pocket exhibiting the highest $\Delta T_m > 4.0$ K. None of the peptides designated as weak binders (in blue) showed pocket binding. The seven compounds clustered together as medium binders in the center of the plot of $pK_D$ against $\Delta T_m$ (Figure 2a) are resolved in the plot of $pK_D$ against $\Delta\Delta T_m$ (Figure 2b). Peptides 3a-e (in red) gain additional binding affinity apparently from pocket-independent hydrophobic interactions, in contrast to similarly potent 1a, 2b, and 3h (in green), shown to be pocket binders. These two different effects responsible for the increased affinity of our ligands can be readily distinguished by $\Delta\Delta T_m$ values, allowing for informed binder optimization.

To confirm the binding modes, we co-crystallized two representative examples with the PBD, the leucine-based peptide 2a ($\Delta\Delta T_m = 0.3$ K) and the most potent ligand 3j ($\Delta\Delta T_m = 4.1$ K). As predicted, peptide 2a turned away from the pocket, corroborating the result of the assay (Figure 2c). In contrast, 3j bound in a way similar to 1a, retaining the key hydrogen bond and the benzene ring of thiophene was inserted into the pocket, mimicking the phenylalanine of 1a (Figure 2d).

Our FTS-based assay allowed us to rapidly interrogate the binding mode of a series of peptides binding to the PBD of Plk1. The approach is high throughput, as all the FTS experiments described in Table 1 could be performed in duplicate on a single 96-well plate in less than two hours. The results of this assay allowed us to readily observe both the correlation between binding mode and ligand affinity as well as the selectivity of the pocket for particular aromatic moieties. They validate $\Delta\Delta T_m$ as a valuable parameter in the structure-based ligand-discovery process, allowing for much deeper insight into structure–activity binding-mode relationships than a purely activity-oriented screen.\textsuperscript{[13]} As our experimental setup is generalizable, we believe that its adaptation for other targets may be useful, especially for challenging systems like protein interfaces.

Received: April 5, 2012
Published online: June 22, 2012

Keywords: binding mode · drug discovery · ligand design · protein structures · structure–activity relationships

[1] V. L. Nienaber, P. L. Richardson, V. Klighofer, J. J. Bouska, V. L. Giranda, J. Greer, Nat. Biotechnol. 2000, 18, 1105 – 1108.
[2] V. M. Sánchez-Pedregal, M. Reece, J. Meiler, M. J. J. Blommers, C. Griesinger, T. Carlonagno, Angew. Chem. 2005, 117, 4244 – 4247; Angew. Chem. Int. Ed. 2005, 44, 4172 – 4175.
[3] P. Sledz, H. L. Silvestre, A. W. Hung, A. Ciulli, T. L. Blundell, C. Abell, J. Am. Chem. Soc. 2010, 132, 4544 – 4545.
[4] C. W. Murray, T. L. Blundell, Curr. Opin. Struct. Biol. 2010, 20, 497 – 507.
[5] T. L. Blundell, H. Jhoti, C. Abell, Nat. Rev. Drug Discovery 2002, 1, 45 – 54.
[6] K.-Y. Cheng, E. D. Lowe, J. Sinclair, E. A. Nigg, L. N. Johnson, EMBO J. 2003, 22, 5757 – 5768.
[7] S.-M. Yun, T. Moulac, D. Lim, J. K. Bang, J.-E. Park, S. R. Shenoy, F. Liu, Y. H. Kang, C. Liao, N.-K. Song, et al., Nat. Struct. Mol. Biol. 2009, 16, 876 – U104.
[8] K. Strebhardt, A. Ullrich, Nat. Rev. Cancer 2006, 6, 321 – 330.
[9] P. Śledź, C. J. Stubbs, S. Lang, Y.-Q. Yang, G. J. McKenzie, A. R. Venkitaraman, M. Hyvönen, C. Abell, Angew. Chem. 2011, 123, 4089 – 4092; Angew. Chem. Int. Ed. 2011, 50, 4003 – 4006.

[10] F. Liu, J.-E. Park, W.-J. Qian, D. Lim, M. Gräber, T. Berg, M. B. Yaffe, K. S. Lee, T. R. Burke, Nat. Chem. Biol. 2011, 7, 595 – 601.

[11] M.-C. Lo, A. Aulabaugh, G. Jin, R. Cowling, J. Bard, M. Malamas, G. Ellestad, Anal. Biochem. 2004, 332, 153 – 159.

[12] D. J. Parks, L. V. Lafrance, R. R. Calvo, K. L. Milkiewicz, V. Gupta, J. Lattanze, K. Ramachandren, T. E. Carver, E. C. Petrella, M. D. Cummings, et al., Bioorg. Med. Chem. Lett. 2005, 15, 765 – 770.

[13] A. W. Hung, H. L. Silvestre, S. Wen, A. Ciulli, T. L. Blundell, C. Abell, Angew. Chem. 2009, 121, 8604 – 8608; Angew. Chem. Int. Ed. 2009, 48, 8452 – 8456.

[14] P. Cimmperman, L. Baranauskiene, S. Jachimovicciute, J. Jachno, J. Torresan, V. Michailoviene, J. Matuliene, J. Sereikaite, V. Bumelis, D. Matulis, Biophys. J. 2008, 95, 3222 – 3231.

[15] F. Liu, J.-E. Park, W.-J. Qian, D. Lim, A. Scharow, T. Berg, M. B. Yaffe, K. S. Lee, T. R. Burke, ACS Chem. Biol. 2012, 7, 805 – 810.