<table>
<thead>
<tr>
<th>Title</th>
<th>An evaluation tool for FKBP12-dependent and -independent mTOR inhibitors using a combination of FKBP-mTOR fusion protein, DSC and NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Sekiguchi, Mitsuhiro; Kobashigawa, Yoshihiro; Kawasaki, Masashi; Yokochi, Masashi; Kiso, Tetsuo; Suzumura, Kenichi; Mori, Keitaro; Teramura, Toshio; Inagaki, Fuyuhiko</td>
</tr>
<tr>
<td>Citation</td>
<td>Protein Engineering, Design and Selection, 24(11): 811-817</td>
</tr>
<tr>
<td>Issue Date</td>
<td>2011-11</td>
</tr>
<tr>
<td>Doc URL</td>
<td><a href="http://hdl.handle.net/2115/47434">http://hdl.handle.net/2115/47434</a></td>
</tr>
<tr>
<td>Type</td>
<td>article</td>
</tr>
<tr>
<td>File Information</td>
<td>PEDS24-11_811-817.pdf</td>
</tr>
</tbody>
</table>

Hokkaido University Collection of Scholarly and Academic Papers: HUSCAP
An evaluation tool for FKBP12-dependent and -independent mTOR inhibitors using a combination of FKBP-mTOR fusion protein, DSC and NMR

Mitsuhiro Sekiguchi¹, Yoshihiro Kobashigawa², Masashi Kawasaki¹, Masashi Yokochi², Tetsuo Kisó¹, Ken-ichi Suzumura¹, Keitaro Mori¹, Toshio Teramura¹ and Fuyuhiko Inagaki²,4

¹Analysis and Pharmacokinetics Research Labs, Department of Drug Discovery, Astellas Pharma Inc., Tsukuba 350-8585, Japan, ²Department of Structural Biology, Faculty of Advanced Life Science, Hokkaido University, Sapporo, Hokkaido 001-0021, Japan and ³Pharmacology Research Labs, Department of Drug Discovery, Astellas Pharma Inc., Tsukuba 305-8585, Japan

4To whom correspondence should be addressed.
E-mail: finagaki@pharm.hokudai.ac.jp

Received June 23, 2011; revised July 30, 2011; accepted August 5, 2011

Edited by Haruki Nakamura

Mammalian target of rapamycin (mTOR), a large multi-domain protein kinase, regulates cell growth and metabolism in response to environmental signals. The FKBP rapamycin-binding (FRB) domain of mTOR is a validated therapeutic target for the development of immunosuppressant and anticancer drugs but is labile and insoluble. Here we designed a fusion protein between FKBP12 and the FRB domain of mTOR. The fusion protein was successfully expressed in Escherichia coli as a soluble form, and was purified by a simple two-step chromatographic procedure. The fusion protein exhibited increased solubility and stability compared with the isolated FRB domain, and facilitated the analysis of rapamycin and FK506 binding using differential scanning calorimetry (DSC) and solution nuclear magnetic resonance (NMR). DSC enabled the rapid observation of protein–drug interactions at the domain level, while NMR gave insights into the protein–drug interactions at the residue level. The use of the FKBP12–FRB fusion protein combined with DSC and NMR provides a useful tool for the efficient screening of FKBP12-dependent as well as -independent inhibitors of the mTOR FRB domain.

Keywords: DSC/FRB domain/NMR/fusion protein

Introduction

Microcalorimetry and nuclear magnetic resonance (NMR) are useful tools for the assessment of protein–ligand interactions (Bewlley et al., 2004; Crump et al., 2004). Isothermal titration calorimetry is often used for the direct measurement of thermodynamic parameters of protein–ligand interactions, while differential scanning calorimetry (DSC) provides information on protein unfolding. Ligand association is generally accompanied by an increase in the transition temperature (T_M) of unfolding so that DSC could provide a convenient tool for detecting protein–ligand interactions (Waldron and Murphy, 2003). NMR, on the other hand, provides information on protein–ligand interactions at the residue level. A combination of DSC and NMR enables the rapid and detailed assessment of protein–ligand interactions and can help in the search for and design of drug candidates.

FK506 (Kino et al., 1987a,b) and rapamycin (Vezina et al., 1975; Sehgal et al., 1987) associate with the same cellular target, the 12-kDa FK506-binding protein (FKBP12), a peptidylprolyl cis/trans isomerase (PPlase). FK506 (Fig. 1a) was first isolated from Streptomyces tsukubaensis in Tsukuba, and rapamycin (Fig. 1b) from Streptomyces hygroscopicus in Easter Island. Both of these reagents have been used clinically as immunosuppressants in transplant patients. However, their pharmacological mechanisms after association with FKBP12 show significant differences. The FK506–FKBP12 complex acts as an inhibitor of calcineurin, an intracellular Ca^{2+}-dependent phosphatase (Liu et al., 1991; Schreiber, 1992). In contrast, the rapamycin–FKBP12 complex associates with the FKBP rapamycin binding (FRB) of mammalian target of rapamycin (mTOR) to inhibit the biological activity of mTOR (Huang et al., 2003).

mTOR is a large protein kinase (2549 residues) that contains a number of distinct functional domains from the N- to C-terminus, including 12 HEAT (1–1480) repeats, an FAT domain (1513–1910), an FRB domain (2181–2484), a catalytic domain (2181–2484) and an FATC domain (2517–2549), mTOR was found in two functionally and structurally distinct multiprotein complexes, mTORC1 and mTORC2. The rapamycin-sensitive complex, mTORC1, transmits nutrient availability signals to control numerous cellular functions (Heitman et al., 1991; Schmelzle and Hall, 2000; Wullschleger et al., 2006), whereas the rapamycin-insensitive complex, mTORC2, regulates actin cytoskeleton assembly and activates a protein kinase, AKT (Jacinto et al., 2003; Sarbassov et al., 2004, 2005). Thus, mTOR plays a key role in the regulation of cell growth and survival, and is a critical target for cancer therapy (Chan, 2004; Rosner et al., 2008).
Recently, the solution structure of the FRB domain in the ligand-free state was reported (Dames, 2008), and its interactions with new potential inhibitors have been characterized by solution NMR (Leone et al., 2006; Veverka et al., 2008). In these reports, the FRB domain was expressed as an inclusion body in Escherichia coli, solubilized using a chaotropic reagent, refolded to the native state and then further purified by chromatography. However, the solubility and stability of the FRB domain were quite low at ambient temperature. Hence, a highly soluble and stable FRB construct is required to screen inhibitors of the mTOR FRB domain.

Here we prepared a fusion protein between FKBP12 and mTOR FRB (Fig. 2a). The fusion protein was successfully expressed as a soluble form in E. coli and could be purified by simple two-step chromatography. We characterized the interactions of the fusion protein with FK506 and rapamycin, and demonstrated that DSC enables the rapid observation of the protein–drug interactions at the domain level, while NMR gives insights on the protein–drug interactions at the residue level. The use of the fusion protein of FKBP12 and the mTOR FRB domain combined with DSC and NMR methods provides a useful tool for the efficient screening of FKBP12-dependent as well as -independent inhibitors of the mTOR FRB domain.

Materials and method

Construction of the expression plasmid

The FKBP12 expression vector was constructed as follows. The coding sequence of human FKBP12 was amplified using the nucleotides FKBP12-f (5'-CCTCTAGACATATGAGTGCAGGTGGAAACC-3') and FKBP12-r (5'-AGACTCAGAGATTATCATTCCAGTTTTAGAAGCTCC-3'), digested using NdeI and XhoI, and cloned into pGBHPS (Kobashigawa et al., 2009) using the same sites.

The expression plasmid for the mTOR FRB domain was constructed as follows. The fragment encoding the human mTOR FRB (2015–2114) domain was amplified using the oligonucleotides FRB-f (5'-CCTCTAGACATATGGAGCTGATCCGAGTGGCC-3') and FRB-r (5'-AGACTCGAGATTACTGCTTTGAGATTCGTCGGAAC-3'), digested using NdeI and XhoI, and cloned into pGBHPS (Kobashigawa et al., 2009) using the same sites.

The expression plasmid for FKBP12–FRB was constructed as follows. The fragment encoding the human mTOR FRB (2015–2114) domain was amplified using the oligonucleotides FRB-f (5'-CCTCTAGACATATGGAGCTGATCCGAGTGGCC-3') and FRB-r (5'-AGACTCGAGATTACTGCTTTGAGATTCGTCGGAAC-3'), digested using NdeI and XhoI, and cloned into pGBHPS (Kobashigawa et al., 2009) using the same sites.

The expression plasmid for FKBP12–FRB was constructed as follows. The fragment encoding the human mTOR FRB (2015–2114) domain was amplified using the oligonucleotides FRB-f (5'-CCTCTAGACATATGGAGCTGATCCGAGTGGCC-3') and FRB-r (5'-AGACTCGAGATTACTGCTTTGAGATTCGTCGGAAC-3'), digested using NdeI and XhoI, and cloned into pGBHPS (Kobashigawa et al., 2009) using the same sites.

The expression plasmid for FKBP12–FRB was constructed as follows. The fragment encoding the human mTOR FRB (2015–2114) domain was amplified using the oligonucleotides FRB-f (5'-CCTCTAGACATATGGAGCTGATCCGAGTGGCC-3') and FRB-r (5'-AGACTCGAGATTACTGCTTTGAGATTCGTCGGAAC-3'), digested using NdeI and XhoI, and cloned into pGBHPS (Kobashigawa et al., 2009) using the same sites.

Fig. 1. Chemical structures of FK506 (a) and Rapamycin (b).

Fig. 2. (a) Schematic representation of the construction of the FKBP12-mTOR FRB fusion protein. (b) SDS-PAGE analysis for purification of the FKBP12–FRB fusion protein. Lane 1: after Ni-NTA purification, lane 2: after HRV 3C protease digestion lane 3: after purification by gel filtration chromatography.
purification, the ligation product was amplified by PCR using the oligonucleotides FKBP12-f and FRB-r2, digested using NdeI and XhoI and cloned into pGBKPS (Kobashigawa et al., 2009) using the same sites.

The FKBP38 expression vector was constructed as follows. The fragment encoding the human FKBP38 PPI domain (92–210) domain was amplified using the oligonucleotides FKBP38-f (5’-CCCTAGACATATGGAGTGCTGGACATTC-3’) and FKBP38-r (5’-AGACTGAGATTACTCAGGCTCAAGGCCGTCC-3’), digested using NdeI and BamHI. The linker was constructed as GSGSSGSGSSGSG, as same as FKBP12–FRB. The three PCR products were then linked using ligase high V.2 (TOYOBO). After purification, the ligation product was amplified by PCR using the oligonucleotides FKBP38-f and FRB-r2, digested using NdeI and XhoI and cloned into pGBKPS (Kobashigawa et al., 2009) using the same sites.

The expression plasmid for FKBP38–FRB was constructed as follows. The fragment encoding the human mTOR FRB domain (2015–2114) domain amplified as previously mentioned. The fragment encoding human FKBP38 (92–210) was amplified using the oligonucleotides FKBP38-f and FKBP38-r2 (5’-GGACGCGGCCTGACCTGGAGGATCCTCT-3’), and digested using NdeI and BamHI. The linker was constructed as GSGSSGSGSSSGGT, as same as FKBP12–FRB. The three PCR products were then linked using ligase high V.2 (TOYOBO). After purification, the ligation product was amplified by PCR using the oligonucleotides FKBP38-f and FRB-r2, digested using NdeI and XhoI and cloned into pGBKPS (Kobashigawa et al., 2009) using the same sites.

Protein expression and purification
FKBP12, FKBP38 PPI domain and the FKBP12–FRB, FKBP38–FRB fusion protein were expressed in E.coli at 25°C as GB1-fusion proteins. The GB1, hexahistidine tags and the HRV3C protease cleavage site was fused to the N-terminus of FKBP12. For DSC measurements, the proteins were expressed in E.coli in 2×YT medium. For NMR, the proteins were isotopically 13C- and 15N-labeled by growing E.coli Rosetta2 (DE3) in M9 minimal medium containing 15NH4Cl, 13C-glucose and Celtone-CN (Spectral Stable Isotopes) as the sole nitrogen and carbon sources. Protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM at 16°C. The cells were then cultured overnight at 16°C. The GB1- and hexahistidine-tagged FKBP12–FRB and FKBP38–FRB fusion protein were purified using Ni-NTA resin (Quiagen), and the GB1 and hexahistidine tags were removed by HRV3C protease. The samples were further purified using a Superdex 75 gel filtration column (GE Healthcare). The total yields of FKBP12, FKBP38 PPI domain and the FKBP12–FRB, FKBP38–FRB fusion protein were 40, 25, 24 and 17 mg/l, respectively.

DSC measurements
Calorimetric measurements were carried out with a VP-DSC microcalorimeter (MicroCal) at a scanning rate of 1°C/min from 293 to 353 K. All scans were obtained at a protein concentration of 0.1 mM for both FKBP12 and FKBP38 PPI domain, and the FKBP12–FRB and FKBP38–FRB fusion protein. Ligand concentrations were 1 mM for both rapamycin and FK506. All scans were acquired in 20 mM Tris-HCl (pH 8.0) and 150 mM NaCl. All the DSC data were analyzed using Origin 7.0 software (MicroCal).

NMR spectroscopy
FKBP12 and FKBP38 PPI domain, and the FKBP12–FRB and FKBP38–FRB fusion proteins were dissolved in 20 mM NaPi buffer (pH 7.2) or 20 mM Tris-HCl and 150 mM NaCl (pH 8). Rapamycin or FK506 (100 mM in DMSO-d6) were added to 250 μl of protein-containing sample. All NMR spectra were obtained on Varian Unity INOVA 600 or 800 spectrometers at a temperature of 25°C. The data were processed using VnmrJ 2.2D and Olivia (http://fermi.pharm.hokudai.ac.jp/). Backbone resonance assignments were obtained using a standard set of triple-resonance experiments.

Results and discussion
Design, expression and purification of the fusion protein between FKBP and FRB
The isolated FRB domain was neither soluble nor stable enough for NMR analysis at 25°C. The NMR sample was precipitated, and the signal intensity was reduced within several hours (data not shown). A highly soluble and stable protein tag (e.g. GB1, thioredoxin and MBP) is generally attached to the target protein to improve solubility and stability. Here, instead of attaching a general solubilizing tag, we used a highly stable and soluble protein, FKBP12, as a fusion tag of the FRB domain (Fig. 2a). This also facilitates the screening of FKBP12-dependent mTOR FRB inhibitors.

According to the crystal structure of the FKBP12–rapamycin–FRB ternary complex (Choi et al., 1996; Liang et al., 1999), the C-terminus of FKBP12 is located near the N-terminus of the FRB domain, indicating that the termini could be connected by a covalent linker. The N-terminus of the FRB domain (E2014 to W2023) is flexible in the crystal structure. Considering that the distance from FKBP12 E107 to FRB H2024 is 28 Å, a minimum of seven additional amino acid residues would be required for spanning this distance. Thus, the FKBP12–FRB fusion protein with a linker of 13 amino acid residues in length was designed.

The FKBP12–FRB fusion protein was expressed in E.coli with the GB1 and hexahistidine tags attached to the N-terminus (Kobashigawa et al., 2009). The supernatant was
purified using Ni-NTA resin (Quiagen), and the tags were removed by HRV3C protease digestion. The fusion protein was further purified by gel filtration using Superdex 75 (GE Healthcare) to a single band in sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis (Fig. 2b). The total yield of the FKBP12–FRB fusion protein was 34 mg/l, which was sufficient for the subsequent biophysical analyses. In addition, the fusion protein was stable and soluble enough for solution NMR measurements >1 week at 25°C.

Fig. 4. (a) Overlay of the $^1$H–$^{15}$N HSQC spectra of the FKBP12–FRB fusion protein in the absence (black) and presence (red) of FK506. (b) Chemical shift differences between the FKBP12–FRB fusion protein in the presence and absence of FK506. (c) Overlay of the $^1$H–$^{15}$N HSQC spectra of the FKBP12–FRB fusion protein in the absence (black) and presence (red) of rapamycin. (d) Chemical shift differences between the FKBP12–FRB fusion protein in the absence and presence of rapamycin. (e) Overlay of the $^1$H–$^{15}$N HSQC spectra of the FKBP38–FRB fusion protein in the absence (black) and presence (red) of rapamycin. (f) Chemical shift differences between the FKBP38–FRB fusion protein in the absence and presence of rapamycin.
Fig. 5. (a) Overlay of the $^1$H–$^{15}$N HSQC spectra of isolated FKBP12 (black) and the FKBP12–FRB fusion protein (red). (b) Chemical shift differences of FKBP12 residues between isolated FKBP12 and the FKBP12–FRB fusion protein. (c) Overlay of the $^1$H–$^{15}$N HSQC spectra of isolated FKBP12 (black) and the FKBP12–FRB fusion protein (red) in the presence of rapamycin. (d) Chemical shift difference of the FKBP12 residues between the isolated FKBP12 and FKBP12–FRB fusion protein in the presence of rapamycin. (e) The FKBP12 residues shifted upon fusion with FRB domain in the presence of rapamycin were mapped on the crystal structure (1NSG) of the FKBP12–FRB–rapamycin ternary complex. Red represents chemical shift differences $>$0.3 p.p.m., orange of 0.2 and yellow of 0.1 p.p.m. Black represents Pro or missing residues. FKBP12 was shown in ribbon, FRB in surface and rapamycin in stick. FRB residues in contact with FKBP12 in the crystal structure of the FKBP12–FRB–rapamycin ternary complex (Liang et al., 1999) were colored cyan. (f) Open-book representation of (e).
Differential scanning calorimetry

We carried out DSC measurements for FKBP12 and the FKBP12–FRB fusion protein in the absence and presence of two inhibitors, FK506 and rapamycin. The DSC curve of FKBP12 in the ligand-free state exhibited a single peak (black) with a transition midpoint ($T_M$) of 65.8 ($\pm$0.1)$^\circ$C. On addition of FK506 (blue) or rapamycin (red), the $T_M$ was shifted to 77.6 ($\pm$0.4)$^\circ$C or 76.5 ($\pm$0.4)$^\circ$C, respectively, demonstrating the thermodynamic stabilization of the FKBP12 via the drug association (Fig. 3a). DSC analysis of the FKBP12–FRB fusion protein exhibited two separated peaks ($T_M$ of 43.8 ($\pm$0.4) and 49.6 ($\pm$0.1)$^\circ$C) in the ligand-free state (black) (Fig. 3b). This revealed that the two protein moieties, FKBP12 and FRB, were independent or only weakly interacting in the ligand-free state. The FKBP12 moiety of the FKBP12–FRB fusion protein was less stable than the isolated FKBP12 domain in the DSC analysis. Destabilization effect by fusion with labile protein was reported for other protein systems (Blondel et al., 1996; Novokhatny and Ingham, 1997; Auton et al., 2010), and was assumed to be general.

On addition of FK506, one of the peaks was shifted to a higher temperature ($T_M$ from 49.6 ($\pm$0.1) to 70.5$^\circ$C ($\pm$0.2), while the other did not change (blue) ($T_M$ from 43.8 ($\pm$0.4) to 43.9 ($\pm$0.2)$^\circ$C) (Fig. 3b). Since FK506 is known to bind to FKBP12, but not to FRB, the shifted DSC peak could be assigned to the FKBP12 moiety. Finally, the DSC curve of the FKBP12–FRB fusion protein exhibited a single peak (red) with a $T_M$ of 74.9 ($\pm$0.3)$^\circ$C (Fig. 3b) in the presence of rapamycin. This indicated that a ternary complex was formed among the FKBP12 moiety, rapamycin and the FRB moiety, which is consistent with a previous report (Laura et al., 2005). Thus, DSC measurements definitely identify the ligand association in the fusion protein.

NMR spectroscopy

Next, we used an NMR spectroscopy to monitor the association of rapamycin or FK506 to the FKBP12 protein or FKBP12–FRB fusion protein at the residue level. In FKBP12, 97% of the resonance assignments of the main chain atoms were established without or with rapamycin. In the fusion protein, resonance assignments for 79% of the main-chain atoms were obtained for the ligand-free state, 79% for the FK506-bound state and 80% for the rapamycin-bound state, respectively. Figure 4a shows an overlay of the $^1$H–$^{15}$N HSQC spectra of the FKBP12–FRB fusion protein without ligand binding and with FK506. Figure 4b shows the amide group chemical shift difference in the FKBP12–FRB fusion protein between the ligand free and FK506-bound forms. Marked chemical shift changes were observed in the FKBP12 moiety, but only negligible chemical shift changes were observed in the FRB moiety. Residues with large chemical shift changes were mostly located in the previously reported FK506 binding surface (Kissinger et al., 1995).

However, the addition of rapamycin to the FKBP12–FRB fusion protein caused marked chemical shift changes among the residues of both the FKBP12 and FRB moieties (Fig. 4c and d), particularly from R18 to S39 and F46 to M66 in the FKBP12 moiety, and I2021 to E2052 and D2102 to V2106 in the FRB moiety. These residues were shown to be located in the interface in the ternary complex (Choi et al., 1996; Liang et al., 1999). Thus, NMR could clearly distinguish two different drug-binding modes. Next, we searched for drug candidates that associate with the FRB moiety but not with the FKBP12 moiety. We replaced the FKBP12 moiety with the PPI domain of FKBP38, which does not associate with FK506 or rapamycin. The FKBP38 PPI domain–FRB fusion protein (denoted as FKBP38–FRB) was also soluble and stable, and to this fusion protein we added rapamycin and recorded the NMR spectra. The residues with large chemical
shift changes on addition of rapamycin were assigned to the FRB moiety but not to the FKBP38 moiety (Fig. 4e and f). Thus, the FKBP38–FRB fusion protein is useful for the screening of drugs that bind only to the FRB moiety.

**NMR analysis and comparison of the FKBP12–FRB and FKBP38–FRB fusion proteins with the isolated FKBP1s**

Figure 5a shows an overlay of the $^1$H–$^{15}$N HSQC spectra of FKBP12 and the FKBP12–FRB fusion protein. Chemical shift difference was small except for T21 and the C-terminal residues. C-terminus and T21 exhibited chemical shift difference of $\approx$0.2 p.p.m. (Fig. 5b). Since FRB was fused to the C-terminus of FKBP12 via the 13 residue linker, the chemical shift change of the C-terminal residue was caused by fusion artifact. In the 3D structure of FKBP12, T21 was located close to the C-terminus so that, it is assumed that the chemical shift difference of T21 is also caused by fusion artifact. Thus, we concluded that the FKBP12 and FRB moieties in the fusion protein are independent with each other. Figure 5c shows the overlay of the HSQC spectra of FKBP12 and the FKBP12–FRB fusion protein complexed with rapamycin. Large chemical shift difference was observed around R42 to E61 and A81 to T96 (Fig. 5d). They were mapped on the crystal structure of the FKBP12–rapamycin ternary complex (Choi et al., 1996; Liang et al., 1999). F46 to M49 of the helix region, L50 to I56 and A81 to T96 loop regions were located in the interface between FKBP12 and FRB (Fig. 5e and f). Previous NMR analysis revealed that affinity of FRB for rapamycin was $\approx$2000-fold enhanced by association of the drug with FKBP12 (Lau et al., 2005) by protein–protein interaction. Our data support their observation. Moreover, extensive protein–protein interaction in the FKBP12–FRB-rapamycin ternary complex was also supported by our DSC analysis that only single peak was observed for the FKBP12–FRB fusion protein in the presence of rapamycin.

Next, we compared $^1$H–$^{15}$N HSQC spectra of the isolated FKBP38 PPI domain and FKBP38–FRB fusion protein in the absence of rapamycin (Fig. 6a and b). NMR spectra of FKBP38 moiety was superimposed well with the isolated FKBP38 PPI domain. This indicates that the inter-protein interaction was negligible in the fusion protein. This is similar to the generally used solubility enhancement tag (denoted as SET), such as GB1 (Zhou et al., 2001; Zhou and Wagner, 2010), while it remains poorly understood how the SET enhances the solubility of fused proteins. This was also unclear from our experiment using FKBP12–FRB and FKBP38–FRB fusion proteins, and further analysis will be required to understand the stability and solubility enhancement mechanism by protein fusion.

**Conclusion**

In the present paper, we showed that the stability and solubility of the mTOR FRB domain could be improved by the attachment of FKBP12 or FKBP38. This enabled the application of this fusion protein to biophysical analysis. We demonstrated that DSC detected ligand association at the domain level, and NMR provided information on protein–ligand interactions at the residue level. The present method will be useful not only in the search for FKBP12-dependent or -independent inhibitors of the mTOR FRB domain but also as a general tool to assess protein–ligand interactions.

**Funding**

This work was supported by the Targeted Proteins Research Program, the Funding Program for World-Leading Innovative R&D on Science and Technology, and a Grant-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Science and Culture, Japan. Funding to pay the Open Access publication charges for this article was provided by Mitsubishi Seikiguchi of Astellas Pharmaceutical company.

**References**