A quantitative analysis to unveil specific binding proteins for bioactive compounds

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The mechanisms of action of bioactive compounds discovered via ‘black box’ assays using phenotypic indicators generally remain unknown, with the major challenges being the identification of target proteins. In this study, we aimed to develop an efficient methodology to unveil target proteins that are rarely characterised. Proof-of-concept experiments were performed using N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), a well-known calmodulin (CaM) antagonist, as a bait compound. The results showed that in our methodology CaM was identified as a W-7-specific binding protein in the cytosolic fraction of a rat brain extract, whereas other proteins acquired in the same experiment were recognised as non-specific binding proteins. The binding affinity of CaM to W-7 is not very high (dissociation constant: 1.6 × 10⁻⁴ M), showing that the recognition specificity is applicable to compounds with very low binding affinities. Keywords: calmodulin/differential analysis/fluorescence polarization/protein identification/W-7

Introduction

The discovery of low-molecular-weight bioactive compounds offers significant potential for drug discovery and development. These compounds generally possess major advantages in their desirable properties, such as high absorbability, high permeability and long-term stability. In particular, cell membrane permeability is a unique and advantageous property because it enables the compound in question to reach a protein target inside a cell. Phenotypic assays that employ live cells are available to screen and identify active compounds by directly targeting intracellular proteins (Lipinski, 2000; Kerns and Di, 2003). Although many such compounds have distinct pharmacological effects, their mechanisms of action remain unclear. So-called bait compounds have provided a recent breakthrough for the clarification of target proteins (Oda et al., 2003; Guiffant et al., 2007; Cong et al., 2012), and hence a leap forward in understanding the mechanisms of action of bioactive compounds acquired through phenotypic assays. A well-known case is that of FKBP12, the target protein for the immunosuppressant molecule FK506 (tacrolimus), using affinity resin and another well-known case is that of Rho-associated protein kinase as a target protein for Y-27632 via photoaffinity labeling (Harding et al., 1989; Uehata et al., 1997). These identification, undoubtedly, have significantly contributed to the elucidation of the underlying biological mechanism of drug action and the development of molecules as drugs. However, on the other hand, only a few target proteins that are directly linked to therapeutic events have been successfully identified despite major challenges and advances in protein analytical techniques (Kosaka et al., 2005; Terstappen et al., 2007). Recently, a quantitative method for profiling protein–compound interaction by using stable isotope labeling with amino acids in cell culture (SILAC) was reported (Sharma et al., 2009). Although this method was instrumental for the quantitative identification of specific proteins in cell lysates that bind to bait compounds, the SILAC method is unlikely to be applied for the general tissues that are not cultured. Moreover, such conventional methods, which analyse proteins bound to a bait compound, introduce a number of potential biases owing to the complex elution process (washing out unbound proteins, dissociation of bound protein from bait using competitors or denaturants, etc.).

Here, we present a solution for characterizing authentic target proteins of a bioactive compound from cytosolic samples; we have developed a new concept aimed for general utility by combining different techniques. In this method, affinity-based scoring of bound proteins is performed by quantitative differential analysis of the unbound fractions (i.e. the amount of protein lost in the fraction, which does not bind to a bait compound). Following the differential analysis, the identified candidate proteins are verified by estimating their binding capacity and specificity for the bait compound by a fluorescence polarisation (FP) assay, which employs a fluorescently labeled bait compound. Calmodulin (CaM) and N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), an established CaM antagonist, were employed for the proof-of-concept experiments (Hidaka et al., 1981; Nishikawa and Hidaka, 1982). W-7 can easily be immobilized on a resin for the affinity analysis, and can also undergo an additional reaction with a fluorophore for the FP analysis via an intramolecular primary amine that is not essential for CaM binding (Tanaka et al., 1982; Osawa et al., 1998). All methods were systematically developed and validated on an experiment-to-experiment basis and well designed for versatility. The results obtained in a series of analyses are presented below and are discussed in light of the validity and utility of this methodology.
Materials and methods

Reagents

W-7 hydrochloride was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). CyDye and Cy5 N-hydroxysuccinimide (NHS) ester for the FP assay were purchased from GE Healthcare (Little Chalfont, UK). Lyophilized CaM was purchased from Biomol International (Farmingdale, NY, USA). Mouse monoclonal anti-CaM antibody was obtained from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-tubulin were obtained from Sigma-Aldrich (St Louis, MO, USA). All other reagents were of analytical grade.

Preparation of rat brain cytosolic sample

Whole rat brain tissue was suspended in 8 ml of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.4) containing 150 mM NaCl, 6% glycerol, 1 mM dithiothreitol and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The suspension was homogenised on ice in a Potter-type Teflon® homogeniser. The homogenate was centrifuged twice at 10 000 g for 10 min to separate the insoluble components from the soluble components. Supernatants corresponded to the cytosolic fraction and were stored at −80°C until use. The protein concentration was determined to be 3.6 mg protein/ml by Coomassie brilliant blue staining in the Bradford assay.

Immobilisation of W-7 on an affinity resin under anhydrous conditions

N-hydroxysuccinimide-activated sepharose-4FF resin (1 ml, GE Healthcare) was washed twice with fresh isopropanol alcohol (IPA) (10 ml, IPA). Equivalent volumes of IPA and W-7 (30 mM in dimethyl sulfoxide (DMSO)) were then sequentially added to the resin. The immobilisation reaction was initiated by the addition of triethylamine (1 : 200 v/v). The homogenate was centrifuged twice at 10 000 g for 10 min to separate the insoluble components from the soluble components. Supernatants corresponded to the cytosolic fraction and were stored at −80°C until use. The protein concentration was determined to be 3.6 mg protein/ml by Coomassie brilliant blue staining in the Bradford assay.

Optimisation of affinity resin volume

Aliquots of W-7 (0.6–80 µl each) and EtN resins (80 µl) were suspended in 1 ml of a rat brain cytosolic sample overnight at 4°C with gentle shaking. The sample contained 360 µg protein and corresponded to the original rat brain cytosolic preparation diluted 1 : 10 in Tris-based buffer. The supernatants were then separated from the resins as unbound fractions by centrifugation at 860 g for 2 min at 4°C using an EX-126 model centrifuge (Tomy Seiko, Tokyo, Japan) and a 0.45-µm membrane filter unit (Millipore, Billerica, MA, USA). Residual proteins on the resins or the filters were washed out with Tris-based buffer (1 ml) and combined with the unbound fractions. This recovery process was performed twice. Next, the resins were suspended in 1 ml of 6 M guanidine containing 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate and agitated for 15 min at room temperature. The supernatants were separated as bound fractions by centrifugation at 860 g for 2 min. This process was performed twice.

Finally, the unbound and bound fractions were concentrated using ultracentrifugation and a 10 000 MW cut-off filter (Millipore) followed by buffer exchange to Tris-based buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis. In this process, the bound fractions were adjusted to a final volume of 0.2 ml (5 × sample) for SDS-PAGE analysis and 1 ml (1 × sample) for immunoblot analysis, whereas the unbound fractions were prepared in a final volume of 1 ml (1 × sample) for both analyses. An aliquot of each fraction (10 µl) was electrophoresed in an SDS-PAGE gel, followed by transfer to a polyvinylidene fluoride membrane for immunoblotting. CaM was detected using mouse monoclonal anti-CaM antibody (1 : 1000 dilution) and horseradish peroxidase-conjugated anti-mouse IgG (1 : 1000 dilution) and an enhanced chemiluminescence Plus detection kit (GE Healthcare). ECL was measured with a LAS-3000 imaging system (Fujifilm, Tokyo, Japan).

Two-dimensional electrophoresis analysis

Unbound fractions from EtN and W-7 resins were obtained with 5-µl aliquots of W-7 and EtN resins as described above, but without the recovery process for retrieval of residual proteins; accordingly, the concentration step using ultrafiltration was eliminated. An aliquot of the unbound fraction obtained from the W-7 resin (50 µl) was labeled with cyanine 3 (400 µM, 2 µl, Cy3) for 1 h on ice following removal of impurities using a two-dimensional (2D) Clean-Up Kit (GE Healthcare), whereas a corresponding aliquot obtained from the EtN resin (50 µl) was labeled in the same manner with cyanine 5 (400 µM, 2 µl, Cy5). The labeled samples were mixed and applied to a pH 3–11 non-linear, 13-cmimmobilised pH gradient strip (GE Healthcare) for isoelectric focusing, which was initiated at 500 V. The voltage was gradually increased to 3500 V within 1.5 h and then kept constant for an additional 8 h using a Pro3900 power supply (Anatech, Tokyo, Japan). Separation in the second dimension was performed on a 7.5–18% linear gradient polyacrylamide gel after the immobilized pH gradient strip was reduced and alkylated. The gel was visualized using a LAS-3000 imaging system, and the appearance of protein spots in the combined unbound fractions was analyzed with DeCyder 2D Software (GE Healthcare).

Protein identification

Coomassie-stained spots were selected and in-gel digested overnight at 37°C with sequencing grade trypsin (Roche Diagnostics, Mannheim, Germany) following reduction and alkylation of the cysteine residues. Peptide fragments were then extracted with 50% acetonitrile containing 0.1% trifluoroacetic acid. The peptide fragments were analysed using an linear trap quadrupole linear ion trap mass spectrometer.
(Thermo Fisher Scientific, Waltham, MA, USA). HPLC was performed using an Atlantis-C18 column (0.075 × 50 mm, Waters). Mobile phases A and B consisted of 0.1% formic acid and 0.1% formic acid in 80% acetonitrile, respectively. The samples were eluted with a step gradient (10–40% B over 30 min, 40–100% B over 0.1 min, 100% B over 4.9 min, 100–10% B over 0.1 min and 10% B over 9.9 min) at a flow rate of 200 nl/min. Peptide matching and protein searches were performed using SEQUEST software (Gatlin et al., 2000).

**FP assay**

Cy5-labeled W-7 (Cy5-W-7) was obtained as described in our previous manuscript (Arai et al., 2010). For the saturation binding assay, serially diluted CaM, GAPDH and β-tubulin solutions (5 µl of each) were prepared in Tris-based buffer with 0.01% Triton X-100 (assay buffer) to give final concentrations of 0.15–150, 0.3–300 and 0.15–150 µM, respectively. Next, 5 µl of a 1 µM Cy5-W-7 solution (diluted 100-fold in assay buffer from a 100 µM stock in DMSO) was added to each protein solution. Following a 5-min equilibration at room temperature, FP was measured using a ViewLux Microplate Imager (PerkinElmer, Waltham, MA, USA).

Competition binding assays were performed in the presence of unlabeled W-7. W-7 was serially diluted in DMSO to give final concentrations ranging from 2 mM to 0.2 µM. Aliquots of the W-7 solutions were diluted 1:50 with assay buffer, and the diluted solutions (5 µl of each) were then transferred to a 384-well microplate. The premixed CaM/Cy5-W-7, GAPDH/Cy5-W-7 and tubulin/Cy5-W-7 solutions were prepared at final concentrations of 10 µM for CaM, 5 µM for GAPDH, 50 µM for β-tubulin and 1 µM for Cy5-W-7 to give FP values of ~300 mP. Five-microliter aliquots of the mixtures were then added to the unlabeled W-7 solutions. FP was measured after equilibration for 5 min. The degree of polarization was defined as follows:

\[
P = \frac{(I_|| - I_\perp)}{(I_|| + I_\perp)} \times 1000
\]

where \(I_||\) and \(I_\perp\) represent the fluorescence intensities of parallel and perpendicularly polarised emission, respectively. These values were corrected by subtraction of the background fluorescence signal, which was derived from all assay components other than the fluorescent probe. All experiments were performed in triplicate.

**Results and discussion**

**Immobilization of the bait compound**

The goals of this study were to develop and demonstrate the utility of a methodology for the efficient and reliable identification of the target protein for a bioactive compound that has an underlying mechanism of action. This work focused on CaM and its antagonist, W-7 (Fig. 1), as a suitable model system. W-7 is readily immobilized on an affinity resin via an intramolecular primary amine that is not essential for CaM binding, and the binding affinity of W-7 to CaM is also relatively lower (dissociation constant: \(1.6 \times 10^{-5}\) M) than that of other bioactive compounds. Thus, the success of our model-validation approach can broaden the applicability of our methodology. W-7 was first immobilized on the sepharose 4FF affinity resin through the addition of W-7 (30 µmol) to the resin (1 ml). According to the manufacturer’s instructions, the density of NHS groups on the resin surface is 16–23 µmol/ml. Therefore, the molar amount of W-7 added to the resin slightly surpassed that of the NHS groups. Furthermore, the difference in the concentration of W-7 in the supernatant before and after the coupling reaction was 20.7 µmol, based on measurement by HPLC. This difference (consumption) suggests that almost all of the NHS groups on the resin were substituted by W-7.

**Optimization of affinity resin volume**

Affinity resins are typically used for protein purification, and excess quantities of resin are generally applied in experiments for the identification of target molecules in the same way as they would be employed for protein purification. Hence, there has been no significant discussion regarding the volume of resin that is appropriate for target protein studies. Thus, it was important to conduct such an investigation to optimize the affinity resin volume. The details of the experiments for optimizing resin volume, including a rough dynamic interaction analysis of CaM with W-7 immobilised on resin, are shown in Supplementary Fig. S1. The results showed that W-7 immobilised on the resin retained its conformational space and behaved similarly as free compounds. Therefore, it should be possible to roughly optimise the resin volume necessary for general models by using inhibitory (or enhancing) activity as an indicator in the phenotypic assay. Considering this, we employed a resin volume of 5 µl (corresponding to ~0.1 µmol W-7), in which ~80% of CaM in the cytosolic sample moved into the bound fraction.

**Affinity-based differential analysis using 2D difference gel electrophoresis**

Bound fractions are often used in affinity resin-based searches for target proteins. It is possible to identify proteins that bind directly to a bait compound that is immobilized on the resin by analyzing the bound fraction. However, a complex process (washing out unbound proteins, dissociation of bound protein from bait and removal of competitors or denaturants used for dissociation) is needed to first acquire
this fraction, thereby introducing a number of potential biases. On the other hand, the presence of bound proteins can be inferred by monitoring protein decreases in the unbound fraction after application of the sample to the resin. As the unbound fractions are acquired simply under physiological conditions, artificial biases can be prevented, and the results are likely to be highly quantitative and reproducible. Thus, the affinity-based differential analysis was developed in this work by taking advantage of the unbound fraction.

The affinity-based differential analysis presented here allows the quantification of the bait-binding proteins as priority targets; i.e. proteins in unbound fractions are quantitatively analyzed and scored by evaluating their loss from this fraction as their affinities for the bait. Protein concentrations in unbound fractions acquired from the EtN resin and the W-7 resin (5 μl of each) were calculated to be 2.8 and 2.0 mg protein/ml, respectively. Without equalizing the protein concentrations in the two fractions, the proteins were labeled with Cy5 and Cy3, respectively, mixed together, and subsequently subjected to 2D electrophoresis (Fig. 2 and Supplementary Fig. S2). The fluorescence intensity (FI) of individual proteins spots were quantified, and the affinity score as an indicator of binding affinity to the W-7 bait was calculated using the following equation:

\[
\text{Affinity score} = \frac{1}{\text{CF}} \times \frac{\text{FI of Cy3}}{\text{FI of Cy5}}
\]

where CF is the correction factor that was determined for taking into account the difference of labeling efficiency by using an internal standard protein added in equal quantities to both the samples prior to fluorescent labeling. From this equation, proteins with scores approaching 1 were shown to have a relatively strong affinity for W-7. For the 537 spots detected via 2D-DIGE analysis, 19 spots (including isoforms and post-translationally modified forms of the same protein) were found to have an affinity score of >0.5. These spots were excised, and the products were analyzed by in-gel digestion followed by liquid chromatography-mass spectrometry (LC/MS). The identified proteins are shown in Table I. Two spots (8, 12) on the acidic side and low-molecular-weight portion (left-hand side, bottom portion) of the gel were identified as CaM. These had affinity scores of 0.83 and 0.72, respectively, and these scores agreed with the ratio of CaM detected in the bound and unbound fractions using immunoblotting (see Supplementary data). The differential scores between the two spots (believed to be isoforms of CaM) suggested a slight difference in affinity for W-7. The protein showing the highest affinity score was N-ethylmaleimide-sensitive factor (spots 3–6). The scores for the four spots detected for this protein (including post-translational modifications) were all 1.0; i.e. these proteins were not detected in the unbound fraction obtained from the W-7 resin. GAPDH exhibited the second-highest score, with two spots for GAPDH (1, 2) displaying affinity scores of 1.00. Affinity scores for other spots of GAPDH (7, 9–11, 13) varied from 0.84–0.70. Parathymosin (14–16), elongation factor 1-alpha 1 (17), elongation factor 1-beta 1 (18) and pyridoxal kinase (19) were also identified as proteins with possible high affinity for W-7. The largest spot on the acidic (left-hand side) side of the gel was β-tubulin (20), for which the affinity score was 0.46.

The high reproducibility of the results was confirmed by repeatedly obtaining unbound fractions from the EtN and W-7 resins and subjecting them to 2D-DIGE analysis.

**Fig. 2.** 2D-DIGE analysis of the combined unbound fractions from EtN and W-7 resins. Unbound proteins obtained from EtN resin (a) and W-7 resin (b) were subjected to 2D electrophoresis. Proteins present at reduced amounts in the unbound fraction from the W-7 resin compared with the EtN resin were identified, indicating a higher affinity for W-7. Spots with high-affinity scores were identified by in-gel digestion followed by LC/MS. Spots 1, 2, 7, 9–11 and 13 were identified as GAPDH (including several modified forms); spots 3–6 were identified as N-ethylmaleimide-sensitive factor; and spots 8 and 12 were identified as CaM. Additional spots were 14–16, parathymosin; 17, elongation factor 1-alpha 1; 18, elongation factor 1-beta 1; 19, pyridoxal kinase; and 20, β-tubulin.
Specific binding analysis using the FP assay

Rather than their initial identification, the major hindrance in the search for target proteins is their validation as authentic binding proteins. The affinity-based differential analysis described above contributes to the prioritization of potential binding proteins by using the affinity index. However, it is still difficult to eliminate all proteins that can bind to the bait compound non-specifically or indirectly. If such proteins remain, validation experiments such as protein X-ray crystallography, signal pathway analysis or gene knockout may not be practicable because these procedures are both labor- and cost-intensive. Here, a useful method is proposed to validate target proteins by performing a specific binding analysis using the FP assay, which is based on the physical property of small molecules to rotate more rapidly than large molecules in solution. Therefore, a small fluorescent molecule produces a high FP when it binds to a large molecule (Freyssinet et al., 1978). The unique features of FP allow the development of high-performance assays in a microplate format. On the other hand, fluorescent labeling complicates the use of low-molecular-weight compounds in FP assays, because the labeling can influence the binding of these molecules to target proteins. Nonetheless, as already mentioned above, W-7 can be covalently bound to a fluorophore by an additional reaction via an intra-molecular primary amine, resulting in the easy preparation of biologically active, fluorescently labeled W-7. This approach should be widely applicable to similar studies for target protein identification of a compound.

In this investigation, the FP assay employed Cy5-W-7 (Fig. 1, previously prepared by us) as the bait (Arai et al., 2010). The specific binding of GAPDH, β-tubulin and CaM (commercially available proteins with high-affinity scores) to W-7 was then evaluated. First, to confirm the W-7-binding properties of these proteins, a saturation study was performed with a fixed probe concentration. As shown in Fig. 3a, each protein showed a concentration-dependent increase in the FP value upon association with Cy5-W-7. Because this increase reflected an increase in the amount of the protein-Cy5-W-7 complex, the direct binding properties of these proteins to fluorescently labeled W-7 were verified. The EC50 values and Hill coefficients for GAPDH and CaM binding to W-7 were calculated by curve fitting (Table II). As suggested by the affinity scores (Table I), GAPDH and CaM had almost identical EC50 values. The dose-response curve with β-tubulin was not a simple sigmoid, suggesting that β-tubulin binds to W-7 in a heterogeneous manner. Since a maximum FP value was not obtained, it was not possible to calculate an EC50 value or a Hill coefficient for this protein. Beta-tubulin in solution at room temperature spontaneously forms tubule structures (Hamel and Lin, 1981), and this process may have increased the mass per molecule in an initial dose- and incubation time-dependent manner, resulting in an unobservable saturation of the FP value.

Table I. Proteins with high-affinity scores for binding to W-7 as determined by the affinity-based differential analysis

<table>
<thead>
<tr>
<th>No.</th>
<th>Identified protein</th>
<th>Affinity score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glycereraldehyde-3-phosphate dehydrogenase</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>Glycereraldehyde-3-phosphate dehydrogenase</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>Ethylmalic enzyme-sensitive factor</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>Ethylmalic enzyme-sensitive factor</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>Ethylmalic enzyme-sensitive factor</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>Ethylmalic enzyme-sensitive factor</td>
<td>1.0</td>
</tr>
<tr>
<td>7</td>
<td>Glycereraldehyde-3-phosphate dehydrogenase</td>
<td>0.84</td>
</tr>
<tr>
<td>8</td>
<td>Calmodulin</td>
<td>0.83</td>
</tr>
<tr>
<td>9</td>
<td>Glycereraldehyde-3-phosphate dehydrogenase</td>
<td>0.83</td>
</tr>
<tr>
<td>10</td>
<td>Glycereraldehyde-3-phosphate dehydrogenase</td>
<td>0.82</td>
</tr>
<tr>
<td>11</td>
<td>Glycereraldehyde-3-phosphate dehydrogenase</td>
<td>0.76</td>
</tr>
<tr>
<td>12</td>
<td>Calmodulin</td>
<td>0.72</td>
</tr>
<tr>
<td>13</td>
<td>Glycereraldehyde-3-phosphate dehydrogenase</td>
<td>0.70</td>
</tr>
<tr>
<td>14</td>
<td>Parathymin</td>
<td>0.59</td>
</tr>
<tr>
<td>15</td>
<td>Parathymin</td>
<td>0.58</td>
</tr>
<tr>
<td>16</td>
<td>Parathymin</td>
<td>0.58</td>
</tr>
<tr>
<td>17</td>
<td>Elongation factor 1-alpha 1</td>
<td>0.56</td>
</tr>
<tr>
<td>18</td>
<td>Elongation factor 1-beta 1</td>
<td>0.55</td>
</tr>
<tr>
<td>19</td>
<td>Pyridoxal kinase</td>
<td>0.50</td>
</tr>
<tr>
<td>20</td>
<td>β-Tubulin</td>
<td>0.46</td>
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</table>
Next, to confirm that GAPDH, β-tubulin and CaM bind to W-7 via a specific binding mode, a competition study was performed using unlabeled W-7. If a protein specifically recognizes W-7, the Cy5-labeled W-7 that initially forms the complex with the protein is substituted by the addition of unlabeled W-7, causing fluorescence depolarization. The results of the competition study are shown in Fig. 3b. As expected, a decrease in the FP value for CaM was observed depending on the concentration of unlabeled W-7. In contrast, the FP value for GAPDH or β-tubulin was not even slightly reduced, despite the addition of large quantities of unlabeled W-7. These results revealed that although GAPDH and β-tubulin both bind to W-7, the interactions are not specific, and these proteins are therefore not targets for W-7. Because GAPDH exhibited favorable profiles in the affinity score and the saturation study in the FP assay, we expected that this protein would be a new target for W-7. However, the results of the competition study did not support this view. On the other hand, this case exemplifies the utility of performing specific binding analysis to validate/invalidate potential protein targets.

Target-based drug discovery has become a major pharmacological approach since the emergence of -omics technologies (Knowles and Gromo, 2003; Lindsay, 2003). However, this approach is highly information-dependent and has plateaued as the number of new proteins suitable for drug targeting has gradually decreased. Antibody drugs are playing an increasingly large role in drug discovery for the inhibition of protein–protein interactions for which the development of small compounds has proven difficult (Waldmann, 2003; Beck et al., 2010). Although the appeal for the development of low-molecular-weight drugs may be fading, they still retain noteworthy features, such as membrane permeability. Thus, phenotype-based drug discovery protocols that emphasize the value of small compounds will likely be reevaluated. The new methodology reported herein is anticipated to make an important contribution for elucidating the underlying biological mechanism of action of drug candidates at a molecular and cellular level toward discovery research in this area.

**Supplementary data**

Supplementary data are available at PEDS online.

**References**