Discovery of the improved antagonistic prolactin variants by library screening

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Prolactin (PRL), a potent growth stimulator of the mammary epithelium, has been suggested to be a factor contributing to the development and progression of breast and prostate cancer. Several PRL receptor (PRLR) antagonists have been identified in the past decades, but their in vivo growth inhibitory potency was restricted by low receptor affinity, rendering them pharmaceutically unattractive for clinical treatment. Thus, higher receptor affinity is essential for the development of improved PRLR antagonistic variants with improved in vivo potency. In this study, we generated Site 1 focused protein libraries of human G129R-PRL mutants and screened for those with increased affinity to the human PRLR. By combining the mutations with enhanced affinities for PRLR, we identified a novel G129R-PRL variant with mutations at Site 1 that render nearly 50-fold increase in the antagonistic potency in vitro.

Keywords: Ba/F3 assay/library screening/prolactin receptor antagonist/scintillation proximity assay/surface plasmon resonance

Introduction

Breast cancer is the most common form of cancer in women and the second leading cause of cancer-related death in western countries. Prolactin (PRL), a potent growth factor for the mammary epithelium, has been suggested as an important factor in the development and progression of breast cancer (Clevenger et al., 1995; Vonderhaar, 1999; Goffin et al., 2005). Low levels of PRL receptor (PRLR) expressed in breast cancer cell lines have been found to mediate PRL-induced expansion of cancer cell lines and synergize with estrogen in a growth-promoting effect (Rasmussen et al., 2010). Increased plasma PRL concentration was observed in breast cancer patients, which was found to correlate with poor prognosis and the occurrence of metastatic breast cancer (Tworoger and Hankinson, 2008). Previous attempts to reduce the circulating levels of PRL with dopamine agonists failed in clinical trials with breast cancer patients (Bonneterre et al., 1988). The extrapituitary production of PRL may explain the disappointing outcome of these trials. Hence, PRLR antagonists capable of targeting the PRLR hereby abrogating the pro-tumorigenic effect of PRL could present an attractive alternative approach for developing a novel therapy for treatment of human breast and prostate cancer.

PRL, similar to growth hormone (GH), has two distinct binding sites, binding Site 1 (BS1) and 2 (BS2), through which it forms a 1:2 triplex with two copies of its receptor. PRLR belongs to the cytokine hematopoietic receptor superfamily. These receptors are composed of three domains including an extracellular ligand-binding domain (ECD), a hydrophobic transmembrane domain and an intracellular domain containing a proline-rich motif. Formation of the ternary complex between PRL and two PRLRs initiates downstream activation via the associated Janus kinases.

PRLR antagonists have been developed based on the same principles as those used for GH, namely introduction of mutations at BS2 (Chen et al., 1991). The PRL mutants are expected to occupy the receptor by forming inactive 1:1 complexes by high-affinity binding via BS1 but fail to interact constructively with the second PRLR through BS2, which is indispensable for the activation and signaling of the receptor. Therefore, impaired binding imposed by the BS2 mutation (G129R) constitutes the mechanistic basis for the antagonistic properties. Comparison of the 1:2 PRLR complex structure with those of free PRL and the 1:1 PRL: PRLR complex indicates that the structure of PRLR undergoes significant changes when binding to the first, but not the second receptor. This further implies that the second PRLR moiety adapts to the 1:1 complex rather than the opposite (Brouin et al., 2010). Another study (Qazi et al., 2006) also suggested the presence of pre-existing homodimers of the receptor in the absence of PRL. Therefore, receptor dimerization is a necessary, but not sufficient, event for receptor activation and notably, not strictly ligand dependent. Several PRL variants have been reported as PRLR antagonists (Goffin et al., 2005): G129R-PRL, S179D-PRL, Δ1–14-G129R-PRL and Δ41–52-PRL (DePalatis et al., 2009). Δ1–9-G129R-PRL was shown to be devoid of residual agonism in every cell or animal model in which it has been tested to date. G129V-PRL was found to be an antagonist by stabilizing the hormone and constraining its intrinsic flexibility (Jomain...
et al., 2007). The most well-characterized PRLR antagonist is G129R-PRL. The G129R substitution results in a PRL molecule, which retains the ability to bind to the PRLR, though its apparent affinity is reduced by a factor of 10 as shown in binding competition experiments (Goffin et al., 1996). It has been shown in vivo with T-47D cells that G129R-PRL in combination with tamoxifen exhibits at least an additive effect on the inhibition of proliferation (Chen et al., 1999). It has also been shown that G129R-PRL can inhibit tumor growth in vivo (Chen et al., 2002). However, a literature (Goffin et al., 2005) states that high doses of PRLR antagonists are necessary to obtain effects in vivo. The antagonistic properties of PRL variants based on the mechanism of impaired BS2 binding can be tuned by changing the relative BS1 and BS2 affinities. Thus, our goal to improve the antagonistic properties of G129R-PRL variants was to maturate its BS1 affinity.

In this study, libraries were generated based on G129R-PRL sequence with mutagenesis in a few key regions defined on the basis of published mutagenesis and structural data, including hydrogen exchange-MS, nuclear magnetic resonance and X-ray data of the complex of PRLR and an antagonistic PRL variant, Δ1–11 (Q12S, G129R)-PRL (Svensson et al., 2008). The libraries were then screened for PRLR binding using a scintillation proximity assay (SPA) and surface plasmon resonance (SPR). Series of single mutation sites, which enhanced the BS1 affinity, were identified. By combining the single mutations, one antagonist with significantly improved affinity was discovered. It exhibited nearly 50-fold increased in vitro potency compared with G129R-PRL in cell-based assays, and notably, devoid of residual agonism. The potent antagonists are potential drug candidates to be used as inhibitors of the growth-promoting actions of PRL.

Materials and methods

Construction, expression and purification of PRLR and PRLR-BIRA

PRLR and PRL variants used throughout this work are based on human sequences. The ECD of PRLR is defined as residue 1–210 of PRLR. Unless specified, PRLR used in the following experiments refers to the ECD of PRLR. The coding sequence of PRLR was amplified from a cDNA library (human cDNA library universal quick-clone cDNA II, Clontech 637260) and cloned into a pET32a (+) vector. Primers 5′-GGGAGATACATATGTCTCAGTTACCTCCTGG AAAACC-3′ and 5′-GGGCTCGAGCTACTAATCATTCATG GTGAAAGTCATAG-3′ were used to introduce the NdeI and XhoI restriction sites to the PRLR insert. A biotinylation tag (BIRA) with an amino acid sequence GLNDIFEAQKIEWHR was added at the C-terminus to allow chemical modification, and it was confirmed to have no effect on the binding of PRL to its receptor (data not shown). The size of the library was estimated by transforming 1 μl of the PCR product to the competent cells, the libraries were analyzed by sequencing 100 clones to assess the mutation rate and the distribution of the mutated residues.

SPA assay

One hundred nanograms of the plasmid harboring the PRLR library was electrooporated into 50 μl of E. coli Origami (DE3) (Novagen) cells and were then plated on 20 × 20 cm² plates containing Luria–Bertani medium supplemented with ampicillin. After incubation at 37°C overnight, single colonies were automatically picked using the QPix2 colony picker and transferred to 96-deep-well plates to prepare seed cultures. The seeds were diluted 12.5 folds into new 96-well plates with the Biomek FX liquid handler (Beckman), and then harvested after induction overnight with 20 μM IPTG at 25°C.

The SPA assay was performed in 96-well plates using a liquid handler. Cells were lysed by incubating with CellLytic Express lysis buffer (Sigma) at room temperature for 1 h followed by three times dilution with water. Lysate (15 μl) was added into 85 μl of assay buffer (50 mM Tris, pH 8.0, 0.05% Triton X-100, 0.2% bovine serum albumin) containing 0.3 mg of streptavidin SPA beads (RPNQ0066V, GE Healthcare), 0.1 μCi of tritium labeled wtPRL and 150 nM of PRLR-BIRA. The reaction mixture was incubated at room temperature for 3 h before the scintillation signal was
counted by a luminescence counter (MicroBeta TriLux, PerkinElmer). Two rounds of SPA screening were carried out in a high throughput procedure 1% clones were cherry-picked as positive candidates for both sequencing analysis and SPR analysis.

**Construction, expression and purification of the PRL analogs**

Combinational constructs including two or more of the S61A, D68N and Q73L mutations were generated according with the QuickChange® II XL site-directed mutagenesis kit (Stratagene) using pET32a-Ser-(G129R, K190R)-PRL as the template. Purification of PRL mutants was conducted using the published protocol (Zhu et al., 2000) with minor modifications. Briefly, cells from 5 ml of cell culture (OD<sub>600</sub>=2.5) were lysed with Celllytic Express lysis buffer (Sigma). The lysate was filtered and then incubated with 160 μl of pre-equilibrated PRLR-coupled beads for 15 min at room temperature. After washing with 10 bead volumes of PBS, the target protein was eluted with 80 μl of 50 mM citric acid (pH 2.9) and neutralized with 0.5 M Na<sub>2</sub>HPO<sub>4</sub>. The concentration of PRL mutants purified with this method was generally above 0.1 mg/ml, suitable for direct SPR analysis for the determination of the dissociate constant (K<sub>off</sub>). The above-described affinity purification was also applicable in larger scale.

**SPR assay**

Binding of the PRL mutants to PRLR ECD was investigated on a Biacore T100 instrument (Biacore). A total of 1500 resonance units of PRLR-BIRA were coupled to a CM5 Biacore chip at pH 4.0 and the binding curves were generated by flowing the PRL analogs diluted in HBS-EP buffer (BIACore) in five concentrations ranging from 3.13 to 50 nM across the chip. All response data were globally fit to a 1:1 interaction model to extract the rate constants at 25°C. The SPR affinity data and ratio were normalized by flowing the PRL analogs diluted in HBS-EP buffer (BIAcore) in five concentrations ranging from 3.13 to 50 nM across the chip. All response data were globally fit to a 1:1 interaction model to extract the rate constants at 25°C using Biacore T100 control software (version 1.1, Biacore). G129R-PRL was included in each SPR assay as an internal control. Both the SPR affinity data and ratio were normalized based on the internal control. For the purpose of SPR screening, only one concentration (6.25 nM) of the PRL analogs was run through the chip and only the K<sub>off</sub> values were evaluated.

**Ba/F3-PRLR proliferation assay**

The Ba/F3 cell line stably expressing PRLR was generated following the published protocol (Bernichtein et al., 2003). The parental Ba/F3 cells were cultured at 37°C in 5% CO<sub>2</sub> in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 50 U/ml of penicillin, 50 μg/ml streptomycin and 1 ng/ml IL-3 as a mitogenic stimulator. Log-phase cells were electroporated with a PRLR expression plasmid based on vector pEGFP-N1 (Clontech). The transfected cell population was selected by several passages in geneticin-containing medium and the subcloned cells were maintained with 1 ng/ml wtPRL instead of IL-3. Several Ba/F3-PRLR cell lines were successfully established and one of them was chosen for the followed proliferation assays for the analysis of the antagonistic and agonistic activities of the PRL analogs. Prior to conducting the proliferation assays, Ba/F3-PRLR cells were starved for 24 h in 10% FCS RPMI-1640 medium without PRL, and were subsequently distributed in 96-well plates at a density of 2 × 10<sup>4</sup> cells/well in a final volume of 100 μl.

For the analysis of agonistic activity, 50 ng wtPRL combined with 50 μl of PRL analogs in serial dilutions were added after the starvation period. Following 3 days of culturing, cell proliferation was determined by measuring OD<sub>490</sub> 2 h after adding 30 μl of CellTiter 96<sup>®</sup> AQueous One Solution Reagent (Promega) to each well. Data analysis was performed with the program Prism 4 (Graphpad) and all data are presented as mean ± SEM.

For the analysis of antagonistic activity, 50 μl of wtPRL or PRL analogs in serial dilutions were allocated to culture wells after the starvation period. To detect the residual proliferation-stimulating potency of the PRL analogs, 20 μl of a more sensitive reagent, alara blue (Invitrogen), was added to each well 3 days later. Fluorescence emission at 590 nm with an excitation wavelength of 550 nm was measured after 2.5–3 h of colorimetric reaction using an enzyme-linked immunosorbent assay plate reader (PHERA star BMG Labtech). All the proliferation experiments were performed at least twice in triplicates.

**Results**

**Construction of the BS1 mutant libraries**

In the LibMixNew library, we aimed to obtain mutations at both the N-terminus (residues 23–83) and the C-terminus (residues 173–199) using mega primers generated from both regions. After sequence analysis of randomly picked clones from the library, 86% was observed to have 1–3 mutations, but most mutations were limited to the 23–83 region. This might be due to a low chance to incorporate mutations into short PCR products using the error-prone PCR strategy. PRL BS1 mutations were initially constructed in the framework of G129R-PRL. After several rounds of initial expression, screening, selection and sequencing, the K190R mutation was found to appear very frequently among the hits. SPR analysis demonstrated that the K<sub>off</sub> of the (G129R, K190R)-PRL mutant was reduced to half of that of G129R-PRL. Therefore, in subsequent rounds of the mutatation procedure, (G129R, K190R)-PRL was used to replace G129R-PRL as the template to generate the libraries. Not all mutation sites that were repeatedly selected by SPA could increase the PRLR-binding affinity. Throughout the whole SPA screening process, at least four mutations, V24D, Y28N, M36L and F80L, were frequently found among the hits. For these four mutations, in contrast to K190R, no enhanced K<sub>off</sub> was observed as revealed by the SPR assay (data not shown).

**Identification of the single mutations with improved affinities**

Using published mutagenesis and structural data on the interaction between PRL and PRLR via BS1, the libraries aimed to cover all residues directly involved in BS1 interactions as well as residues in spatial vicinity of BS1 were designed. The SPA technique was applied to detect PRLR binding of the PRL BS1 mutants in cell lysates of single clones derived from the libraries. First, PRLR-BIRA was immobilized to streptavidin-SPA beads by biotin–streptavidin interaction. Then the cell lysates were allowed to compete with tritium-labeled wtPRL for binding to PRLR. Thus, lysates of clones exhibiting a low scintillation read out were interpreted as producers of G129R-PRL variants efficiently competing with
labeled wtPRL. Two rounds of SPA screening were carried out in a high-throughput procedure capable of screening 4800 PRL variants per day. After SPA screening, 1‰ clones were cherry picked as positive candidates, expressed and purified in small amounts, and evaluated by measuring the $K_{D}$ values and their agonistic and antagonistic activities were evaluated using a PRLR stably expressing Ba/F3 cell proliferation assay. (S61A, D68N, G129R, K190R)-PRL, (S61A, Q73L, G129R, K190R)-PRL, and (S61A, D68N, Q73L, G129R, K190R)-PRL were referred as NN100, NN099, NN094 and NN096, respectively.

**Generation of the improved PRLR antagonists by combining single mutations with the high-affinity**

In attempts to further improve the BS1-binding affinity, the mutations identified with increased BS1 affinity were combined in a two by two manner with site-directed mutagenesis. The combinational mutants were characterized by measuring the PRLR-binding affinity with SPR assay (Table II) and their agonistic and antagonistic activities were evaluated using a PRLR stably expressing Ba/F3 cell proliferation assay. (S61A, D68N, G129R, K190R)-PRL, (S61A, Q73L, G129R, K190R)-PRL, (D68N, Q73L, G129R, K190R)-PRL and (S61A, D68N, Q73L, G129R, K190R)-PRL and (S61A, D68N, Q73L, G129R, K190R)-PRL are referred as NN100, NN099, NN094 and NN096, respectively.

**Identification of the improved PRLR antagonists using the Ba/F3-PRLR cell-based assays**

Ba/F3 cell stably expressing PRLR has been used for the reference bioassay of PRL lactogenic activity for more than one decade (Sasaki et al., 1996). After being optimized and systematically evaluated (Bernichtein et al., 2003), this cell model has been demonstrated to be much more sensitive and robust than several other breast cancer cell line models. Ba/F3-PRLR also avoids the species-specific problem existing in the highly sensitive rat Nb2 cell model. Following a published transfection protocol (Bernichtein et al., 2003), Ba/F3 cells stably expressing PRLR were selected with geneticin resistance. Substitution of IL-3 by PRL ensured the selection of cells dependent on PRL in their growth. One of the established transfection protocol (Bernichtein et al., 2003), Ba/F3 cells stably expressing PRLR were selected for the antagonistic and agonistic assays in this study.

**Table I. PRLR binding properties of the best hits as measured by SPR assay**

<table>
<thead>
<tr>
<th>PRL mutation sites</th>
<th>$K_{m}^a$ (M$^{-1}$s$^{-1}$) $\times 10^{4}$</th>
<th>$K_{m}^b$ (s$^{-1}$) $\times 10^{-4}$</th>
<th>$K_{D}$ (nM)</th>
<th>Relative gain of affinity $a,b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>S61A, G129R</td>
<td>17.4</td>
<td>12.6</td>
<td>7.7</td>
<td>1.7</td>
</tr>
<tr>
<td>D68N, G129R</td>
<td>21.2</td>
<td>9.4</td>
<td>4.6</td>
<td>2.5</td>
</tr>
<tr>
<td>Q73L, G129R, K190R</td>
<td>22.1</td>
<td>5.1</td>
<td>2.4</td>
<td>4.7</td>
</tr>
<tr>
<td>G129R, K190R</td>
<td>24.1</td>
<td>10.7</td>
<td>4.6</td>
<td>2.4</td>
</tr>
<tr>
<td>G129R</td>
<td>19.4</td>
<td>21.1</td>
<td>11.3</td>
<td>1.0</td>
</tr>
</tbody>
</table>

$a$The values listed are the averages of at least two experiments, which varied ± 15%.

$b$Fold increases in affinity compared with G129R-PRL, calculated as the ratios of $K_{D}$ values.

**Table II. PRLR binding properties of the best combinational mutants as measured by SPR assay**

<table>
<thead>
<tr>
<th>Name</th>
<th>PRL mutation sites in addition to the G129R, K190R backbone</th>
<th>$K_{m}^a$ (M$^{-1}$s$^{-1}$) $\times 10^{4}$</th>
<th>$K_{m}^b$ (s$^{-1}$) $\times 10^{-4}$</th>
<th>$K_{D}$ (M) $\times 10^{-10}$</th>
<th>Relative gain of affinity $a,b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NN100</td>
<td>×</td>
<td>23.9</td>
<td>57.1</td>
<td>25.7</td>
<td>4.4</td>
</tr>
<tr>
<td>NN099</td>
<td>×</td>
<td>33.7</td>
<td>30</td>
<td>9.2</td>
<td>12.3</td>
</tr>
<tr>
<td>NN094</td>
<td>×</td>
<td>26.2</td>
<td>27.6</td>
<td>10.9</td>
<td>10.4</td>
</tr>
<tr>
<td>NN096</td>
<td>×</td>
<td>26.7</td>
<td>24.6</td>
<td>9.5</td>
<td>12</td>
</tr>
</tbody>
</table>

$a$The values listed are the averages of at least two experiments, which varied ± 20%.

$b$Fold increases in activities compared with G129R-PRL, calculated as the ratios of $K_{D}$ values.

Fig. 1. (A) Ba/F3-PRLR competition assay of PRL analogs selected for improved antagonistic binding to PRLR as measured by SPR. G129R-PRL was used as a standard. Affinities were reported as the $IC_{50}$ values and were results of at least two independent experiments performed in triplicate. Data analysis was performed in the program Prism 4 (Graphpad) and all data are presented as mean ± SEM. NN100: 7.81 $\times 10^{-9}$ M, NN099: 5.84 $\times 10^{-9}$ M, NN094: 3.26 $\times 10^{-9}$ M, NN096: 1.36 $\times 10^{-8}$ M, G129R: 1.13 $\times 10^{-7}$ M, which varied ± 20%. (B) Relative antagonistic activities were calculated by fold decreases in $IC_{50}$ compared with G129R-PRL.
exhibited 12.7-fold, 14.8-fold and 27.9-fold increased in vitro potency compared with G129R-PRL, respectively. The most potent combinational variant was NN096, which demonstrated 49.1-fold of higher potency.

In the cell agonistic proliferation assay, wtPRL reached the maximum stimulation activity at 1 nM with an EC$_{50}$ of $1.02 \times 10^{-10}$ M. G129R-PRL reached the highest stimulation activity at 110 nM with an EC$_{50}$ of $3.2 \times 10^{-9}$ M. The highest proliferation rate under G129R-PRL stimulation is only 12% compared with that of wtPRL. Compared with wtPRL and G129R-PRL, all four PRL combinational variants exhibited undetectable agonistic activities in the concentration range tested ($1.0 \times 10^{-14}$–$1.0 \times 10^{-8}$ M) (Fig. 2).

Discussion

In this study a series of BS1 affinity improving mutation sites (S61A, D68N, Q73L and K190R) in PRL were discovered by screening the PRL libraries based on the G129R-PRL sequence and by introducing additional mutations in and around the BS1 region. The individual mutations introduced in the framework of G129R-PRL only resulted in modest increases in the BS1-binding affinity (Table I). However, when mutated in combination, the affinity improvements approaching a factor of 12 could be obtained (Table II). Two methods, SPR and the Ba/F3-PRLR assay, were used to characterize the binding and functional properties of the PRL variants. SPR was applied to measure the kinetic parameters ($K_{on}$ and $K_{off}$) in the binding of PRL variants to PRLR, and the ratio of the kinetic rate constants provides a measure of the BS1 affinity ($K_f$) to the receptor for comparison. The Ba/F3-PRLR assay was employed to assess the interaction between the PRL variants with the PRLR on the cell surface in competition with wtPRL, by which antagonistic property of each variants expressed as IC$_{50}$ values was evaluated. Conducted in the absence of wtPRL, the Ba/F3-PRLR assay also provides a measure of the ability of the PRL variants to functionally activate the receptor (residual agonism).

Unsurprisingly, no simple correlation between BS1-binding affinity measured by SPR and the functional response showed in the Ba/F3-PRLR assay was observed, confirming that an improvement in the affinity does not necessarily translate into an improved function (Haugh, 2004).

A closer scrutiny of the affinity data on the combinational constructs (Table II) revealed that the effect of a given mutation is dependent on its framework. If the affinity data of NN099 were compared with those of (Q73L, G129R, K190R)-PRL, 2.6(12.3/4.7)-fold affinity improvement can be observed by introducing the S61A mutation in (Q73L, G129R, K190R)-PRL. A similar comparison of NN094 and (Q73L, G129R, K190R)-PRL showed that the D68N mutation resulted in an affinity gain corresponding to a factor of 2.2 (10.4/4.7) when introduced into (Q73L, G129R, K190R)-PRL. However, simultaneous mutation of both S61A and D68N in the (Q73L, G129R, K190R)-PRL framework to form NN096 resulted in an affinity gain of 2.6 (12.0/4.7), similar to the ratios obtaining by introducing the S61A and D68N mutations separately (2.6 and 2.2, respectively). No synergistic effect on introducing both the S61A and D68N mutations were observed.

By virtue of the published crystal structure of the complex between a PRL variant and the extracellular domain of the PRL receptor, the BS1 interactions have been described in atomic details (Svensson et al., 2008), and speculations about the structural basis for the effects of the S61A mutation were discussed. Using information from the crystal structure, we attempt to rationalize the affinity improving effects associated with D68N, Q73L and K190R mutations. D68 and Q73 are situated immediately before and after a short helical segment (K69-A72) present in the long loop connecting helix 1 and helix 2. From this short helix, several receptor contacts are made to the receptor. The effects of the D68N and Q73L mutations might be mediated by improved direct contacts, or the mutations may stabilize the segment in a conformation favorable for receptor interaction. K190 is in the complex structure not in close proximity to the residues in the receptor chain. Mutation of K190 to R does not immediately provide possibilities for creating additional receptor contacts, which would require significant conformation changes in the backbone of the hormone or/and its receptor.

Although the effects of the mutations are not readily explained on a structural basis, an interesting observation is made when mapping the S61A, D68N, Q73L and K190R mutation sites onto the three-dimensional structure of the PRL.
variant, Δ1–11 (Q12G, G129R)-PRL taken from the complex structure with PRLR (PDB entry 3D48) (Fig. 3). Notably, all the affinity-improving mutations are positioned at the outer rim of BS1 comprising H27, H30, N31, I51, T52, A54, I55, N56, P66, E67, D68, K69, E70, A72, Q73, H173, R176, R177, H180, K181, D183, N184, Y185, K187, L188, C191, R192, N197 and N198 as defined by Svensson (Svensson et al., 2008), whereas none is positioned centrally in the highly optimized binding interface. This implies that strategies for further optimizing high-affinity protein–protein interactions should have particular focus on residues not directly involved in binding, but spatially close to the wild-type interface.

The aim of the present work has been to generate improved, pure PRLR antagonists by increasing Site 1 affinity and/or decreasing Site 2 affinity to abolish the undesired residual agonistic activity of G129R-PRL. It was reported that deletion of nine amino acids in the N-terminus of G129R-PRL led to the best pure antagonist Δ1–9-G129R-PRL. The hypothesis was that the N-terminus was restricted to Site 2 binding and could be directly involved in the interaction with the second PRLR chain. This effect was emphasized especially in the G129R context (Jomain et al., 2007). In our study, all the BS1 improved mutants were screened in the context of G129R. The mutation site K190R, which is not directly involved in binding, might have effect on the BS2 interaction.

The strategies applied during the screening process favoured the identification of hits with reduced $K_{\text{off}}$ instead of an increased $K_{\text{on}}$. In the preliminary screening, the SPA method was applied. It has been shown that disparity between the $K_i$ value determined at different time points could identify compounds that dissociate slowly based on the SPA method (Heise et al., 2007). In the secondary screening process, the technical limitation of high through-put SPR screening determined that only $K_{\text{off}}$ can be accurately measured when only single protein concentration was applied. Putting together, the best hits were fished out based on their $K_{\text{off}}$ due to screening strategies. The $K$ values of screening outcome (Tables I and II) perfectly matched our assumption. Meanwhile, none of the best candidates showed agonistic activity. This observation is consistent with a study of mathematically modeling of human growth hormone (hGH) (Haugh, 2004). It was concluded in that paper that the affinity-matured hGH variants with greatly reduced off-rates were not expected to show enhanced bio-potency. On the other hand, a receptor agonist or antagonist with an enhanced on-rate was always predicted to show a proportional gain in potency.

In the previous study, it was shown that mutants Δ1–9-G129R-PRL and Δ1–14-G129R-PRL, similar to G129R-PRL, displayed 10-fold reduced affinity to the PRLR (Jomain et al., 2007). In our study, all the combinational variants showed 10-fold higher affinities to PRLR as compared with G129R-PRL. Notably, all combinational variants listed in Fig. 1 exhibited undetectable agonistic activities. These pure antagonists with higher affinity will be attractive as new therapeutic reagents in the future.

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