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VIP

Bicyclic Peptides with Optimized Ring Size Inhibit Human Plasma Kallikrein and its Orthologues While Sparing Paralogous Proteases

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Peptide macrocycles can bind with high affinity and specificity to disease targets and hence are an attractive molecule class for the development of therapeutics. Several peptide macrocycles are already in clinical use, as for example the antibacterial peptide vancomycin, the immunosuppressant drug cyclosporine or the anticancer drug ocreotide.[1] The good binding properties result from the numerous molecular interactions formed at the relatively large contact interface between peptide and target, as well as from the limited conformational flexibility of the cyclic structures. Typically, peptide macrocycles bind to surfaces of several hundred square angstrom as, for example, the cyclic peptide CXCR4 antagonist CVX15 (400 Å^2),[2] a cyclic peptide with an RGD motif that binds to integrin $\alpha V\beta 3$ (355 Å^2),[3] or the cyclic peptide inhibitor upain-1 that binds to urokinase-type plasminogen activator (603 Å^2).[4]

We recently isolated potent bicyclic peptide inhibitors of different serine proteases from phage display libraries that proved to be highly specific.[5] One of the proteases was human plasma kallikrein (hPK), a plasma hydrolase that cleaves kininogen to produce bradykinin and activates coagulation factors XII and VII as well as plasminogen.[6] Excess plasma kallikrein activity plays a role in different diseases, including hereditary angioedema (HAE), a life-threatening disease characterized by local swelling in subcutaneous tissue. A protein-based inhibitor, ecallantide, was approved in 2009 for its treatment,[7,8] and this injectable drug is a significant advance in HAE therapy. PK inhibitors based on small peptides or peptidomimetics would potentially allow alternative application routes and are hence of interest. The most potent bicyclic PK inhibitor we isolated, PK15,[5] blocks human PK (hPK) with a K_i value of 2.9 ± 0.9 nM and at the same time does not inhibit the homologous human serine protease that shares the highest sequence identity with hPK, factor XIa (69% sequence identity) or any of the other human serine proteases tested.[5] High binding specificity is particularly important with this target because more than 70 trypsin-like serine proteases exist in humans, and many of these enzymes have vital physiological functions, for example, the coagulation factor thrombin or the fibrinolytic enzyme plasmin.[9] However, as a consequence of the high specificity, PK15 does not efficiently inhibit orthologous proteases, such as mouse PK (mPK; 81% sequence identity)[5] or rat PK (rPK; 81% sequence identity), preventing testing of its therapeutic effects in small laboratory animals.[10]

In this work, we aimed at generating bicyclic peptides inhibiting both human and murine PK but not any paralogous proteases. We speculated that inhibitors with the desired specificity profile could be obtained by generating binders to a surface region that is identical in PK orthologues but different in paralogues. To estimate whether this strategy is feasible, we compared the protein surfaces around the substrate binding site in hPK, rPK and human fXIa (hfXIa). As mentioned above, rPK and hfXIa share similar sequence identities with hPK (81% and 69%, respectively). Within a radius of 4 Å around the S1 subsite of the proteases, the 14 surface amino acids (mostly aligning the S1 pocket) are identical in hPK, hfXIa and rPK. Inhibitors binding exclusively to this region are expected to block all three proteases with a similar potency and to inhibit also many paralogous proteases (Figure 1; Table S1 in the Supporting Information). Further away from the S1 site, hfXIa differs more from hPK than rPK does—in the area between 4 and 8 Å around the S1 site, hPK and hfXIa differ in two amino acids (hPK→hfXIa: E146L, R222Q), while hPK and rPK are identical. In the area between 8 and 12 Å, hPK and hfXIa differ in seven amino acids (hPK→hfXIa: D60Y, S97A, G99S, F143Y, K147R, Y175H, Q225R), while hPK and rPK differ in only three amino acids (hPK→rPK: L41M, F143Y, K147R). In addition, the few amino acid mutations from hPK to rPK are conservative ones. We concluded that these different levels of conservation found for the two proteases around the active site should allow the development of bicyclic peptide with the desired specificity.

To generate inhibitors that bind to the relatively small surface area that is identical in hPK and rPK, we produced combinatorial libraries of bicyclic peptides with only three amino acids per loop and hence are significantly smaller than PK15, which has two six-amino acids loops (Figure 2). Genetically encoded combinatorial libraries of bicyclic peptides were generated by displaying linear peptides of the format AC-(Xaa)$_3$-C-(Xaa)$_3$-CA (library 3×3 A; Figure 2a) on phage and subsequent chemical cyclization of the three cysteine side chains with tris(-bromomethly)benzene (TBMB) (Figure 2b). In a second 3×3 library, the flanking exocyclic amino acids were randomized (library 3×3 B) (Figure 2a). Each library containing between 10^8 and 10^{10} random peptides was separately subjected to two to three rounds of iterative selection and amplification using im-

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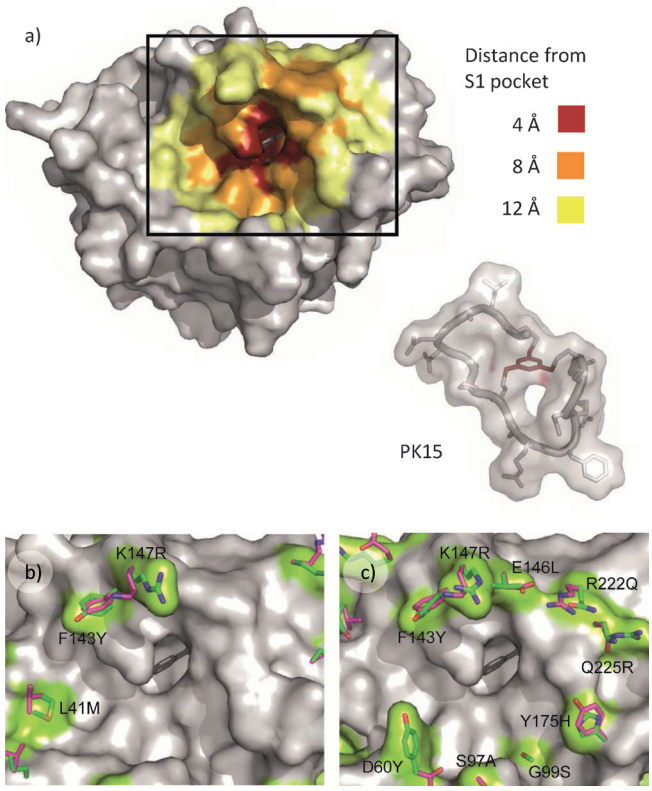

Figure 1. Comparison of the surface amino acids around the active site in hPK, rPK and hfXIa. a) Structure of hPK (PDB: 2ANW[14]) with surface representation. Atoms of the amino acids exposed to the surface and closer than 4, 8 and 12 Å to benzamidine (in grey) bound to the S1 pocket are colored as indicated. The NMR structural model of PK15 is also shown for size comparison. b) Surface of rPK with amino acids that differ to those in hPK highlighted in green. The rPK structure was generated by homology modeling based on the hPK structure (PDB: 2ANW[14]). The region around the active site shown is indicated by the frame in panel a. The side chains of amino acids that differ in rPK and hPK are shown as green (rPK) and pink (hPK) sticks. c) Surface of hfXIa with amino acids that differ to those in hPK highlighted in green. The hfXIa structure was generated by homology modeling based on the hPK structure (PDB: 2ANW[14]). The region around the active site shown is indicated by the frame in panel a. The side chains of amino acids that differ in hfXIa and hPK are shown as green (hfXIa) and pink (hPK) sticks.

mobilized hPK. Sequencing of isolated peptides revealed strong consensus sequences in both peptide loops (Figure 2c,d). Most peptides isolated had the consensus sequence F-Xaa-Xaa in the first loop and RV-Xaa in the second loop. Several of the peptides were chemically synthesized with a free N terminus and an amidated C terminus (indicated with an asterisk in Figure 2c,d) and were found to inhibit hPK with inhibitory constants (K_i) in the low nanomolar range (Table 1).

Next we assessed whether rPK was inhibited by these peptides. The protease was transiently expressed in mammalian cells as inactive precursor having an N-terminal proTEV substrate sequence, purified and activated. We also produced hPK in mammalian cells to compare the recombinant protein with the commercially available protein isolated from blood plasma. While in all recombinant proteins only a fraction of the proteases could be activated, the comparison of active recombinant hPK and plasma-derived hPK showed comparable catalytic properties ($hPK_{recombinant}$: $K_M = 120 \pm 28$ μM; hPK_{plasma}: $K_M = 165 \pm 26$ μM) and gave the same inhibitory constants in inhibition assays with PK15 ($hPK_{recombinant}$: $K_i = 2.9 \pm 1.2$ nM; hPK_{plasma}: $K_i = 2.9 \pm 0.9$ nM). Previously described peptide inhibitor PK15 derived from the 6×6 library inhibited recombinant rPK very poorly ($K_i = 2.1 \pm 0.9$ μM; Table 1). In contrast, the smaller bicyclic peptides derived from the 3×3 library potently inhibited rPK with K_i values in the low nanomolar range (Table 1). To assess the specificity of the 3×3 bicyclic peptides toward paralogous proteases, we measured the inhibition of several human trypsin-like class S1A serine proteases including hfXIa, which shares the highest sequence identity with hPK (69%) and the structurally less conserved but vital human serine proteases thrombin (36% sequence identity with hPK), plasmin (34% sequence identity with hPK), and factor XIIa (35% sequence identity with hPK). The bicyclic peptides inhibited hfXIa at nanomolar concentrations but not any of the other paralogues. This finding suggests that the smaller bicyclic peptides bind to the S1 substrate pocket and the rim of the pocket, regions that are identical in hPK, rPK and hfXIa.

To generate inhibitors with a slightly larger footprint that would not inhibit hfXIa, we generated combinatorial bicyclic peptide libraries with loops of five amino acids (AC-$(Xaa)_5$-C-$(Xaa)_5$-CA; library 5×5 A; Figure 2a,b). Affinity selection with this library yielded peptides having the sequence Xaa-WPAR in the first loop and LHQDL in the second loop or similar sequences (Figure 2e). This consensus sequence was not related to the sequence of the 3×3 bicyclic peptides, suggesting a different mode of interaction with hPK. Several of these bicyclic peptides inhibited hPK with K_i values in the low nanomolar or even sub-nanomolar range, and all of these hPK inhibitors also blocked rPK potently (Table 1). Specificity profiling with paralogous proteases revealed that the 5×5 bicyclic peptides were significantly more specific than the smaller 3×3 peptides. In particular, 5×5 bicyclic peptides only inhibited hfXIa at high micromolar concentrations or not at all at the highest concentration tested (Table 1). By inhibiting orthologous but not paralogous proteases, the 5×5 bicyclic peptides are suited for the testing of bicyclic peptide PK inhibitors in rat models.

In summary, by comparing structural models of target—and related nontarget—proteases to identify conserved regions in the vicinity of the active site and by modulating the loop size of our libraries of peptide macrocycles accordingly, we succeeded in identifying potent inhibitors of human, rat and monkey plasma kallikrein that do not also inhibit related human serum proteases. Our strategy should facilitate the evaluation of this type of peptide macrocycles in animal models, and furthermore, avoid unwanted off-target activities that would be detrimental to their clinical development.

Experimental Section

Cloning of phage libraries and phage panning: The bicyclic peptide phage libraries were cloned, and phage panning was performed according to the same principles as previously described.[5] Libraries based on filamentous phage were created using degenerate DNA primers containing NNK codons, where N is any of the four

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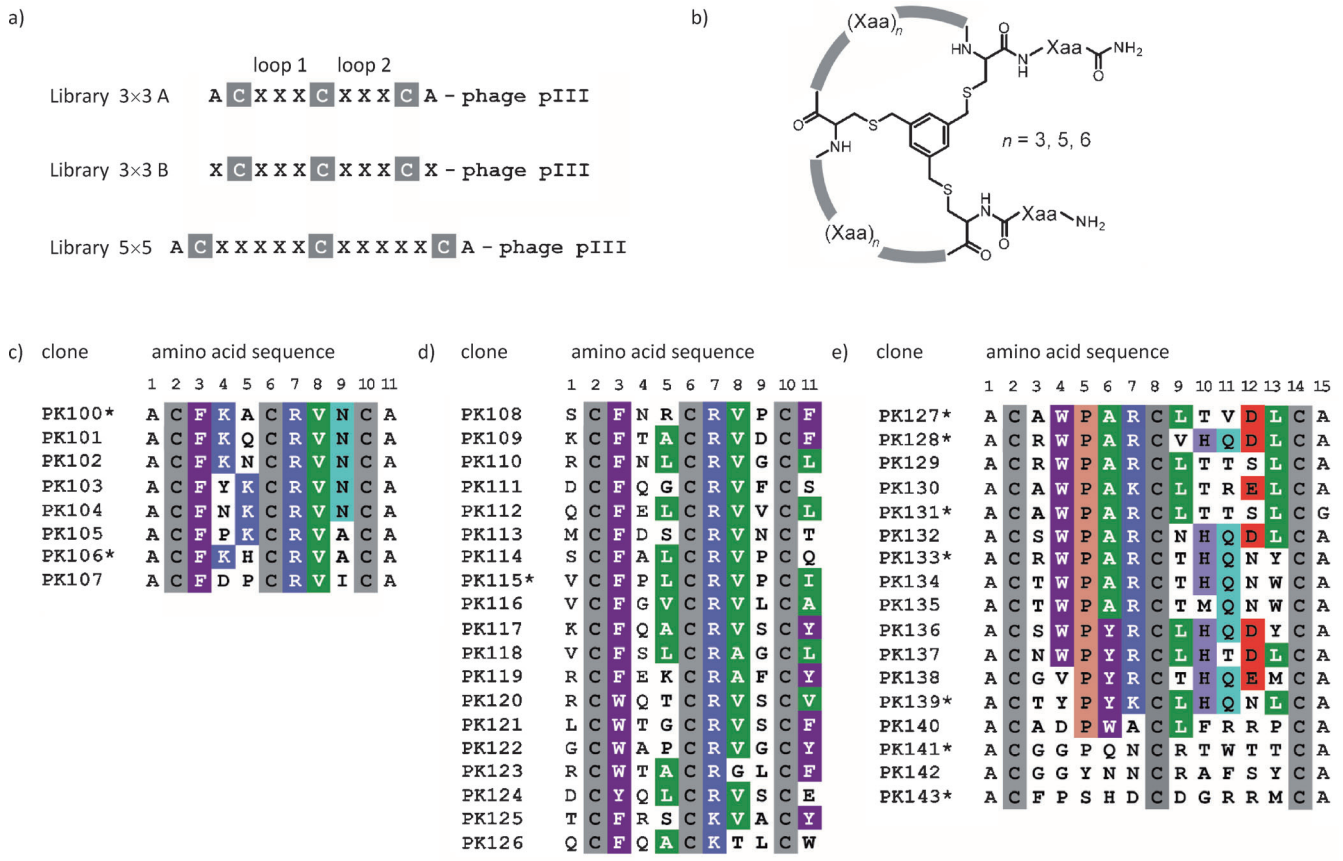

Figure 2. Phage selection of bicyclic peptides. a) Bicyclic peptide phage libraries. Random amino acids are indicated as "X", alanine as "A" and the constant three cysteine residues as "C". b) Format of chemically synthesized bicyclic peptide structures having loops of 3, 5 or 6 amino acids. The structures are generated by linking linear peptides via three cysteine side chains to tris(bromomethly)benzene (TBMB). Amino acids that vary in the bicyclic peptides are indicated with 'Xaa'. Sequences of bicyclic peptides isolated from c) library 3×3 A, d) library 3×3 B, and e) library 5×5. Similarities in amino acids are highlighted in color using the Rasmol color code. Bicyclic peptides that were characterized in inhibition assays are indicated with an asterisk.

Table 1. Target specificity of bicyclic peptides with different loop lengths indicated by the K_i values of these peptides against hPK and different paralogous and orthologous proteases.[a]

Peptide	$n \times n$[b]	K_i[c] [nM]						
		Orthologous proteases			Paralogous proteases			
		hPK	monkeyPK	rPK	hfXIa	hThromb	hPlasm	hfXIIa
PK15	6×6	2.9±0.9	2.9±0.6	2089±860	>50000	>50000	>50000	>50000
PK100	3×3	5.2±1.8	2.8±1.7	27.2±14.0	40.4±11.9	>50000	>50000	>50000
PK106	3×3	5.2±1.9	4.4±1.1	14.0±8.2	39.7±11.9	>50000	>50000	>50000
PK115	3×3	37.1±8.1	12.2±7.4	59.7±29.4	526±46	>50000	>50000	>50000
PK127	5×5	0.4±0.1	1.1±0.5	24.0±4.0	2502±231	>50000	>50000	>50000
PK128	5×5	0.3±0.0	0.4±0.1	11.0±2.0	19739±1912	>50000	>50000	>50000
PK131	5×5	0.9±0.1	2.5±0.8	38±7.9	12907±1490	>50000	>50000	>50000
PK133	5×5	2.9±1.2	2.0±1.1	6.6±2.3	>50000	>50000	>50000	>50000
PK139	5×5	12.2±4.8	9.4±1.5	37.5±14.2	>50000	>50000	24137±1796	>50000
PK141	5×5	4.3±0.2	2.0±0.6	16.6±0.9	>50000	>50000	>50000	>50000
PK143	5×5	5.3±2.8	6.0±2.8	27.7±11.8	>50000	>50000	>50000	>50000

[a] A K_i value of >50000 nM means that no inhibition was observed or that 50% inhibition was not reached at a bicyclic peptide concentration of 100 μM. [b] Number of amino acids in loops. [c] Human plasma kallikrein (hPK); Monkey plasma kallikrein (monkeyPK); Rat plasma kallikrein (rPK); Human factor XIa (hfXIa); Human thrombin (hThromb); Human plasmin (hPlasm); Human factor XIIa (hfXIIa).

nucleotides and K is guanine or thymine. Phages were produced and purified by polyethylene glycol (PEG) precipitation using standard procedures. Cysteine residues were reduced with tris(2-carboxyethyl)phosphine (TCEP) prior to chemical reaction with tris(bromomethyl)benzene (TBMB). Activated human plasma kallikrein (hPK) purified from human plasma (Innovative Research, Novi, USA) was biotinylated and immobilized on streptavidin or avidin magnetic beads. Binders were eluted at low pH.

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Bicyclic peptide synthesis: Peptides were synthesized in house by standard solid-phase peptide synthesis using Fmoc-protected amino acids. As a solid support, Rink amide AM resin was used to obtain peptides with a free N terminus and an amidated C terminus. Peptides were eluted under reducing conditions and partially purified by precipitation. Crude peptide (0.5 mM) was reacted with TBMB (1 mM) in 80% aqueous buffer (20 mM NH_4HCO_3, 5 mM EDTA, pH 8.0) and 20% CH_3CN for 1 h at 30 °C. The product was purified by reversed-phase chromatography on a C_{18} column (Waters, XBridge BEH300 Prep 5 μM), and H_2O/0.1% trifluoroacetic acid (TFA) and CH_3CN/0.1% TFA were used as solvents. The pure bicyclic peptide was lyophilized and dissolved in H_2O. The mass was confirmed by electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI)–time of flight (TOF) mass spectrometry.

Cloning and expression of human, monkey and rat PK: The catalytic domain of human, monkey (*Macaca mulatta*) and rat PK was expressed in mammalian cells as an inactive precursor having a short peptide connected to the N terminus of the catalytic domain via a proTEV cleavage site. The expression vector was cloned, and the protein expressed, activated and purified as follows. Synthetic genes coding for a PK signal sequence, a polyhistidine tag, a proTEV cleavage site, mature catalytic domain of PK, and a stop codon were purchased from Geneart (Regensburg, Germany). The sequences are given in the Supporting Information. Plasmid DNA containing the synthetic genes for human, monkey and rat PK was prepared, and the gene was transferred into the pEXPR-IBA42 mammalian expression vector (IBA-BioTAGnology, Göttingen, Germany) using the restriction enzyme pair XhoI and HindIII. The ligated plasmids were transformed into XL-1 blue electrocompetent cells (Stratagene, Santa Clara, USA) and plated onto 2YT agar plates containing ampicillin (10 $\mu g\,mL^{-1}$). DNA from the three expression vectors (termed monkeyPK, rPK and hPK) was produced, and the correct sequences were confirmed by DNA sequencing (Macrogen, Seoul, South Korea). The three orthologous plasma kallikreins were expressed in mammalian cells as described in the Supporting Information. The polyhistidine-tagged protein was purified by nickel affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) resin, washing buffer (500 mM NaCl, 25 mM Na_2HPO_4, pH 7.4) and elution buffer (500 mM NaCl, 25 mM Na_2HPO_4, pH 7.4, 500 mM imidazole). The protein was further purified by gel filtration on a Superdex 75 column (GE healthcare, Uppsala, Sweden) using assay buffer (10 mM TrisCl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$). The protease was partially activated with proTEV (Promega, Madison, USA). Alternatively, the protease was partially (around 0.1–1%) activated via autocatalytic means by incubation at 4 °C for 24 h.

Protease inhibition assays: Residual activities were measured in buffer (150 μL) containing 10 mM TrisCl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% *w/v* bovine serum albumin (BSA), 0.01% *v/v* Triton-X100, and 5% *v/v* DMSO. Final concentrations of serine proteases were as follows: commercially available hPK (Innovative Research) 0.25 nM; recombinant hPK 125 nM; recombinant monkeyPK 20 nM; recombinant rPK 10 nM; commercially available hfXIa (Innovative Research) 6 nM; hThromb (Innovative Research) 10 nM; hPlasm (Molecular Innovations) 2.5 nM; hfXIIa (Innovative Research) 200 nM. Commercially available proteases were purified from human plasma; recombinant proteases were produced in house. Four-fold dilutions of inhibitors were prepared ranging from 100 μM to 0.4 nM for all proteases. For determination of the IC_{50} values, the following 7-amino-4-methylcoumarin (AMC)-derived fluorogenic substrates (Bachem) were used at final concentrations of 50 μM: Z-FR-AMC (for human, monkey and rat PK), Boc-FSR-AMC (for human factor XIa), Z-GGR-AMC (for human thrombin and human factor XIIa) and H-D-VLK-AMC (for human plasmin). Fluorescence intensity was measured with a Spectramax Gemini fluorescence plate reader (excitation at 355 nm, emission at 460 nm; Molecular Devices). The inhibitory constant (K_i) was calculated according to the Cheng–Prusoff equation: $K_i = IC_{50}/(1+([S]_0/K_M))$,[11] where IC_{50} is the functional strength of the inhibitor, $[S]_0$ is the total substrate concentration, and K_M is the Michaelis–Menten constant. The reactions were performed at 25 °C. IC_{50} values were determined using Excel software.

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[1] E. M. Driggers, S. P. Hale, J. Lee, N. K. Terrett, *Nat. Rev. Drug Discovery* **2008**, *7*, 608–624.
[2] B. Wu, E. Y. Chien, C. D. Mol, G. Fenalti, W. Liu, V. Katritch, R. Abagyan, A. Brooun, P. Wells, F. C. Bi, D. J. Hamel, P. Kuhn, T. M. Handel, V. Cherezov, R. C. Stevens, *Science* **2010**, *330*, 1066–1071.
[3] J. P. Xiong, T. Stehle, R. Zhang, A. Joachimiak, M. Frech, S. L. Goodman, M. A. Arnaout, *Science* **2002**, *296*, 151–155.
[4] G. Zhao, C. Yuan, T. Wind, Z. Huang, P. A. Andreasen, M. Huang, *J. Struct. Biol.* **2007**, *160*, 1–10.
[5] C. Heinis, T. Rutherford, S. Freund, G. Winter, *Nat. Chem. Biol.* **2009**, *5*, 502–507.
[6] A. P. Kaplan, B. Ghebrehiwet, *Mol. Immunol.* **2010**, *47*, 2161–2169.
[7] A. Banerji, *Allergy Asthma Proc.* **2010**, *31*, 398–406.
[8] M. M. Frank, *J. Allergy Clin. Immunol.* **2008**, *121*, S398–401 (for quiz, see page S419 of the same issue).
[9] G. M. Yousef, M. B. Elliott, A. D. Kopolovic, E. Serry, E. P. Diamandis, *Biochim. Biophys. Acta Proteins Proteomics* **2004**, *1698*, 77–86.
[10] E. D. Han, R. C. MacFarlane, A. N. Mulligan, J. Scafidi, A. E. Davis III, *J. Clin. Invest.* **2002**, *109*, 1057–1063.
[11] Y.-C. Cheng, W. H. Prusoff, *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.
[12] G. Backliwal, M. Hildinger, V. Hasija, F. M. Wurm, *Biotechnol. Bioeng.* **2008**, *99*, 721–727.
[13] L. Baldi, N. Muller, S. Picasso, R. Jacquet, P. Girard, H. P. Thanh, E. Derow, F. M. Wurm, *Biotechnol. Prog.* **2005**, *21*, 148–153.
[14] J. Tang, C. L. Yu, S. R. Williams, E. Springman, D. Jeffery, P. A. Sprengeler, A. Estevez, J. Sampang, W. Shrader, J. Spencer, W. Young, M. McGrath, B. A. Katz, *J. Chem. Biol.* **2005**, *280*, 41077–41089.

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