VIP Protein Engineering Very Important Paper

DOI: 10.1002/ange.201410049

Rational Design of a Humanized Glucagon-Like Peptide-1 Receptor Agonist Antibody

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Abstract: *Bovine antibody BLV1H12 possesses a unique "stalk–knob" architecture in its ultralong heavy chain CDR3, allowing substitutions of the "knob" domain with protein agonists to generate functional antibody chimeras. We have generated a humanized glucagon-like peptide-1 (GLP-1) receptor agonist antibody by first introducing a coiled-coil "stalk" into CDR3H of the antibody herceptin. Exendin-4 (Ex-4), a GLP-1 receptor agonist, was then fused to the engineered stalk with flexible linkers, and a Factor Xa cleavage site was inserted immediately in front of Ex-4 to allow release of the N-terminus of the fused peptide. The resulting clipped herceptin–Ex-4 fusion protein is more potent in vitro in activating GLP-1 receptors than the Ex-4 peptide. The clipped herceptin–Ex-4 has an extended plasma half-life of approximately four days and sustained control of blood glucose levels for more than a week in mice. This work provides a novel approach to the development of human or humanized agonist antibodies as therapeutics.*

Agonist antibodies that bind and activate cell-surface receptors are being developed as powerful research and therapeutic tools. The selection of agonist antibodies from combinatorial antibody libraries has proven challenging, and relatively few examples exist to date.[1] The chemical conjugation of peptide agonists to antibody scaffolds requires highly selective chemistry and results in more complex and costly manufacturing processes.[2] We recently developed an alternative strategy based on the X-ray crystal structure of the bovine antibody BLV1H12 with an ultralong heavy chain CDR3 (CDR3H) region (Figure 1 A).[3] This unusual CDR3H region folds into a novel structural motif characterized by a solvent-exposed, antiparallel β-strand "stalk", terminating in a disulfide-crosslinked "knob" domain. We have used this unique "stalk–knob" structure to generate functional bovine antibody–CDR3H fusion proteins with the bovine granulocyte colony-stimulating factor (bGCSF), human erythropoietin (hEPO), and modified CXCR4-binding peptides.[4] The antiparallel β-strand motif allows correct folding of the fused peptide/protein and the immunoglobulin scaffold while preserving their respective pharmacological activities. More recently, we have successfully used a heterodimeric, antiparallel coiled-coil motif in place of the β-strand stalk to fuse bGCSF into the CDR3H region of antibody BLV1H12.[5] The resulting fusion protein has excellent thermodynamic stability and an in vitro biological activity similar to that of bGCSF.

In contrast to this family of bovine antibodies, most human antibodies have CDR loops of 8–16 residues, although a number with longer, protruding CDR loops have been found in some neutralizing antibodies to viruses.[6] However, the evolutionally conserved β-sheet frameworks of the bovine and human antibody variable regions suggested to us that the unique stalk–knob architecture of the bovine antibody could be transferred to human antibodies to generate human or humanized agonist antibodies. To test this hypothesis, we chose to initially examine herceptin as a candidate human antibody scaffold and exendin-4 (Ex-4) as a model peptide agonist. Herceptin, a humanized monoclonal antibody, has been used to treat HER2-positive breast cancer, and has not shown significant immunogenicity in clinical use.[7] The X-ray crystal structure of the herceptin Fab–HER2 complex suggests that like the bovine antibody BLV1H12, CDR3H of herceptin can be fused through a rigid stalk to incorporate a separately folded peptide agonist without significantly affecting the folding or activity of the antibody or the payload (Figure 1 A). Ex-4, a glucagon-like peptide-1 (GLP-1) receptor agonist, has potent glucose-lowering and insulin-sensitizing activities,[8] and exenatide, a synthetic version of Ex-4, is used to treat type 2 diabetes. However, in clinical practice, twice daily injections of Ex-4 are required because of its short plasma half-life (2–3 h).[9] To overcome this drawback, a number of long-acting versions of the GLP-1 receptor agonist have been developed including liraglutide (acylated GLP-1), dulaglutide (Fc–GLP-1 fusion; Fc = antibody fragment, crystallizable region), albiglutide (albumin–GLP-1 fusion), exenatide (extended release), and a fusion protein of an albumin-binding domain antibody to Ex-4.[10] We sought to determine whether fusion of Ex-4 into CDR3H of herceptin would result in a GLP-1 receptor agonist antibody with an extended half-life, as a result of an increase in size and the ability to bind FcRn. Herein, we show that using an engineered coiled-coil stalk, the Ex-4 peptide can be genetically fused into CDR3H of herceptin directly adjacent to a Factor Xa cleavage site for release of the N-terminus of the fused Ex-4 peptide,[11] to generate an agonist antibody specifically targeting the GLP1 receptor (GLP-1R; Figure 1 B and C). The resulting clipped herceptin–Ex-4 fusion protein has potent in vivo biological activity and a long serum half-life in mice. This work demonstrates the successful extension of

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201410049.

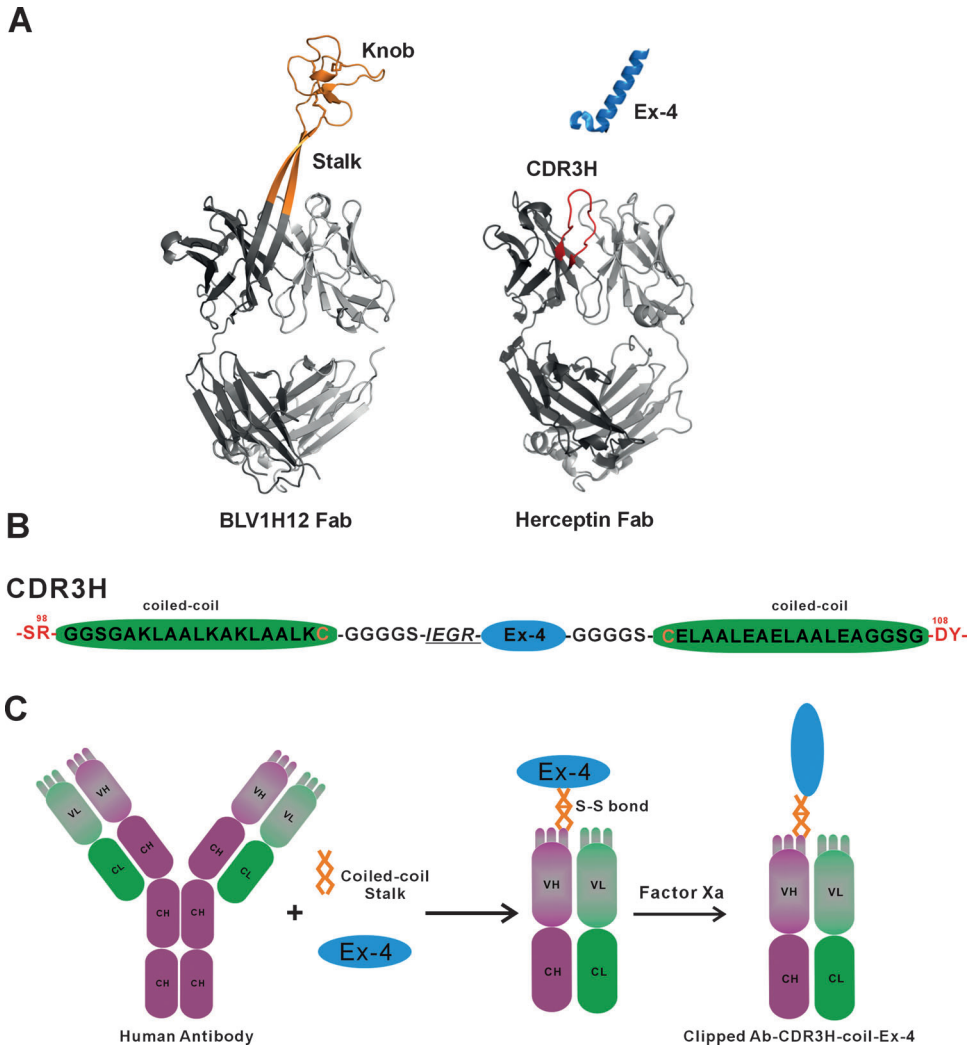

Figure 1. Design of a humanized GLP-1 agonist antibody. A) X-ray crystal structures of the bovine antibody BLV1H12 Fab fragment (PDB ID: 4K3D), the humanized antibody herceptin Fab fragment (PDB ID: 1N8Z), and Ex-4 (PDB ID: 3C5T). B) Map of the key elements of the herceptin–Ex-4 fusion. *IEGR*: factor Xa cleavage site, Cys in orange: engineered disulfide bond at the top of the coiled-coil stalk, numbered residues in red: parental CDR3H region of herceptin. C) Generation of the clipped herceptin–CDR3H–Ex-4 fusion protein using a coiled-coil stalk. The fusion protein was produced as a full-length IgG, and each IgG molecule contains two fused Ex-4 peptides.

this CDR3H fusion strategy not only to non-bovine antibodies, but also to agonist peptides and should facilitate the generation of therapeutic agonist antibodies.

Comparative analysis of the X-ray crystal structures of herceptin and bovine antibody BLV1H12 reveals high structural similarity in the canonical β-sheets of the variable domain (root mean square deviation (RMSD) <2.8 Å). The 13-residue (Ser97–Tyr109) CDR3H loop of herceptin has a β-strand conformation at its bottom (Ser97, Arg98, Asp108, and Tyr109) and forms extensive interactions with adjacent CDR loops from both the heavy and light chains (Figure 1A). Therefore, we reasoned that the β-strand stalk of bovine antibody BLV1H12 or a designed coiled-coil stalk might also allow fusion of a functionally active Ex-4 peptide into the structurally similar CDR3H of herceptin.[5] To test this notion, a gene fragment encoding Ex-4 with flexible GGGGS linkers at each end was fused with the coiled-coil stalk-forming sequences to connect the N- and C-termini of Ex-4 with the CDR3H region of herceptin.[5] However, fusion of Ex-4 into CDR3H may lead to a significant loss in activity, as an α-helical conformation and interactions between the N-terminal residues of Ex-4 and GLP-1R are critical for receptor activation.[12] To overcome this complication, a genetically encoded Factor Xa site was inserted immediately in front of the Ex-4 peptide (Figure 1B). In addition, a disulfide bond was introduced at the top of the coiled-coil stalk to further stabilize the clipped herceptin–CDR3H–Ex-4 fusion protein by covalently linking the two split heavy-chain fragments (Figure 1C). This entire cassette was then grafted into CDR3H of herceptin in place of the Trp99–Met107 loop to generate the herceptin–CDR3H–coil–Ex-4 fusion protein (Figure 1B). This fusion protein contains the IgG1 heavy-chain constant region with seven mutations (E233P, L234V, L235A, ΔG236, A327G, A330S, and P331S) to reduce complement-dependent and antibody-dependent cell-mediated cytotoxicities.[13] The non-clipped and clipped herceptin–Ex-4 fusion proteins were designated as herceptin–CDR3H–coil–Ex-4 and herceptin–CDR3H–coil–Ex-4 RN, respectively.

The herceptin–CDR3H–coil–Ex-4 fusion protein was expressed in freestyle HEK 293 cells by transient transfection. Secreted fusion protein was purified using protein G chromatography. The purified herceptin–Ex-4 fusion protein was then processed by Factor Xa protease to liberate the N-terminus of the Ex-4 peptide, followed by re-purification using protein G chromatography to remove the protease. The purified non-clipped and clipped herceptin–Ex-4 fusion proteins were analyzed by SDS-PAGE gels (Supporting Information, Figure S1). Protease cleavage at the N-terminus of the fused Ex-4 peptide results in two heavy-chain fragments that migrate at 13 and 43 kDa. Mass spectral analysis following treatment with peptide-*N*-glycosidase and DTT (DTT = dithiothreitol) revealed two heavy chain fragments of 13307 and 43458 Da (Figure S2 and S3), consistent with two

fused Ex-4 peptides per IgG molecule. The final yields are approximately 10 mg L^{-1} for the non-clipped herceptin–CDR3H–coil–Ex-4 fusion protein and 7 mg L^{-1} for the clipped herceptin–CDR3H–coil–Ex-4 RN fusion protein. Both the non-clipped and clipped herceptin–Ex-4 fusion proteins have solubilities of over 10 mg mL^{-1} in PBS (pH 7.4). The yields, solubilities, and stabilities suggest that the fusion proteins fold correctly, and recombinant expression of the resulting agonist antibodies is not significantly complex than the production of conventional antibodies.

Next, the activities of the herceptin–Ex-4 fusion proteins were examined using HEK 293 cells overexpressing GLP-1R and carrying a cAMP response element (CRE) luciferase (Luc) reporter. The Ex-4 peptide and the herceptin–Ex-4 and herceptin–Ex-4 RN fusion proteins all activate GLP-1R in a dose-dependent manner (Figure 2 A). The EC_{50} value of the non-clipped herceptin–CDR3H–coil–Ex-4 is 3.8 ± 0.2 nM. Following the release of the N-terminus of fused Ex-4 by Factor Xa, the potency of the herceptin–Ex-4 fusion protein increases by a factor of 380 ($EC_{50} = 0.01 \pm 0.001$ nM), which is three times larger than that for the Ex-4 peptide ($EC_{50} = 0.03 \pm 0.004$ nM). These results indicate that grafting of Ex-4 into CDR3H of herceptin results in an antibody chimera that potently activates GLP-1R. The comparable potency of the herceptin–Ex-4 RN fusion protein may result in part from the enhanced binding avidity that is due to the bivalent nature of the antibody–CDR fusion protein. The significant increase in potency following cleavage by Factor Xa is consistent with the critical role of the N-terminal residues of Ex-4 in activating GLP-1R. To examine the specificity of the herceptin–CDR3H–coil–Ex-4 RN fusion protein, HEK 293 cells overexpressing a glucagon receptor (GCGR) and a CRE-Luc reporter were treated with various concentrations of glucagon, Ex-4, herceptin, herceptin–CDR3H–coil–Ex-4, and the herceptin–CDR3H–coil–Ex-4 RN fusion protein. The herceptin–Ex-4 fusion proteins show no detectable activation up to 100 nM (Figure S4), indicating that like Ex-4, the non-clipped and clipped herceptin–Ex-4 fusion proteins specifically target GLP-1R. These results, together with the excellent physicochemical properties, indicate that substitution of peptide agonists for CDR3H of herceptin using a rigid, protruding stalk provides an efficient and straightforward approach to the generation of potent agonist antibodies.

Furthermore, the binding affinity of the herceptin–Ex-4 fusion proteins to the HER2 receptor was analyzed by ELISA using a recombinant human HER2-Fc chimera (Figure 2 B). Herceptin tightly binds the HER2 receptor with an EC_{50} value of 145 pM, whereas both the non-clipped and clipped herceptin–Ex-4 fusion proteins show no detectable binding to the HER2 receptor up to 20 nM, indicating that fusion of Ex-4 into CDR3H abrogates binding of herceptin to its cognate antigen. This is consistent with previous findings that CDR3H of herceptin plays a key role in binding to the HER2 receptor,[11d] and supports the use of the antibody herceptin as a general carrier for the generation of GPCR agonist antibodies with extended CDR3H regions.

To determine whether fusion of the Ex-4 peptide into the CDR3H region of herceptin increases its plasma half-life, we carried out a pharmacokinetic study of Ex-4 and the herceptin–CDR3H–Ex-4 RN fusion protein in mice. On the basis of in vitro activity assays of isolated mouse serum, the estimated half-lives by intravenous administration are 1.5 hours for Ex-4 and 2.4 days for herceptin–CDR3H–Ex-4 RN (Figure S5), assuming a one-compartment model with first-order elimination. Additionally, the herceptin–CDR3H–Ex-4 RN fusion protein subcutaneously injected into mice exhibited a plasma half-life of 3.9 days (Figure S6). Thus, fusion of Ex-4 into the CDR3H region of herceptin, with its N-terminus released by Factor Xa, significantly extends its plasma half-life. Notably, herceptin–CDR3H–Ex-4 RN has a longer plasma half-life in rodents compared to the acylated GLP-1 ($t_{1/2}$: 4 h in rats), Fc-GLP-1 fusion ($t_{1/2}$: 1.5 day in rat), albumin-binding domain antibody–GLP-1 fusion ($t_{1/2}$: < 20 h in rats), and PEGylated GLP-1 ($t_{1/2}$: 12 h in rat).[14]

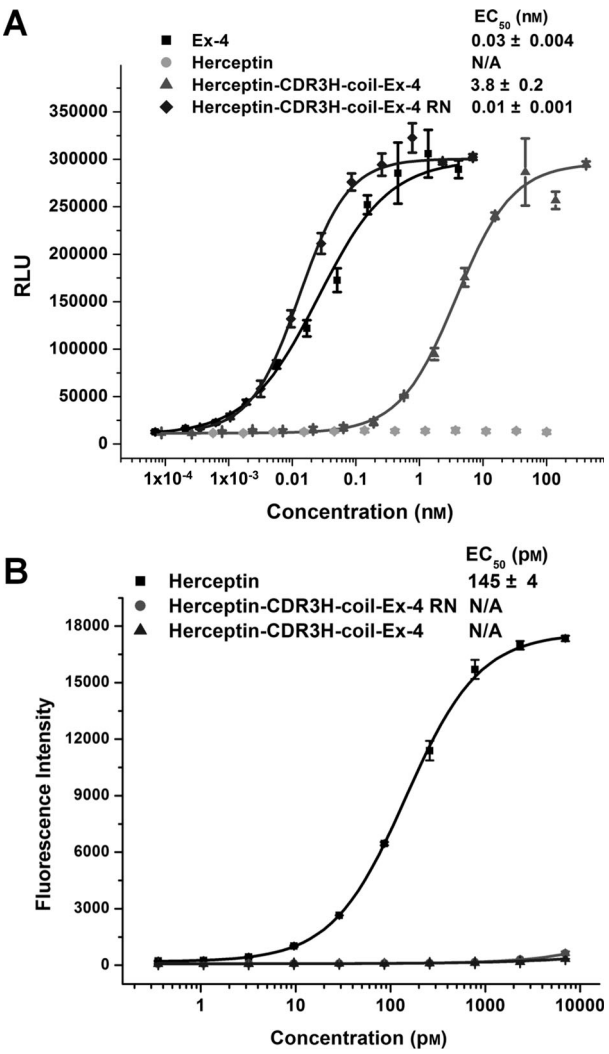

Figure 2. In vitro biological activities of herceptin–CDR3H–Ex-4 fusion proteins and their binding affinities to the HER2 receptor. A) Herceptin–CDR3H–Ex-4 fusion proteins activate GLP-1R in a dose-dependent manner using HEK 293 cells overexpressing GLP-1R and a CRE-Luc reporter. RLU = relative light units. B) ELISA-based analyses of binding to the HER2 receptor by herceptin, herceptin–CDR3H–coil–Ex-4, and herceptin–CDR3H–coil–Ex-4 RN fusion proteins using an anti-human kappa light chain antibody HRP conjugate.

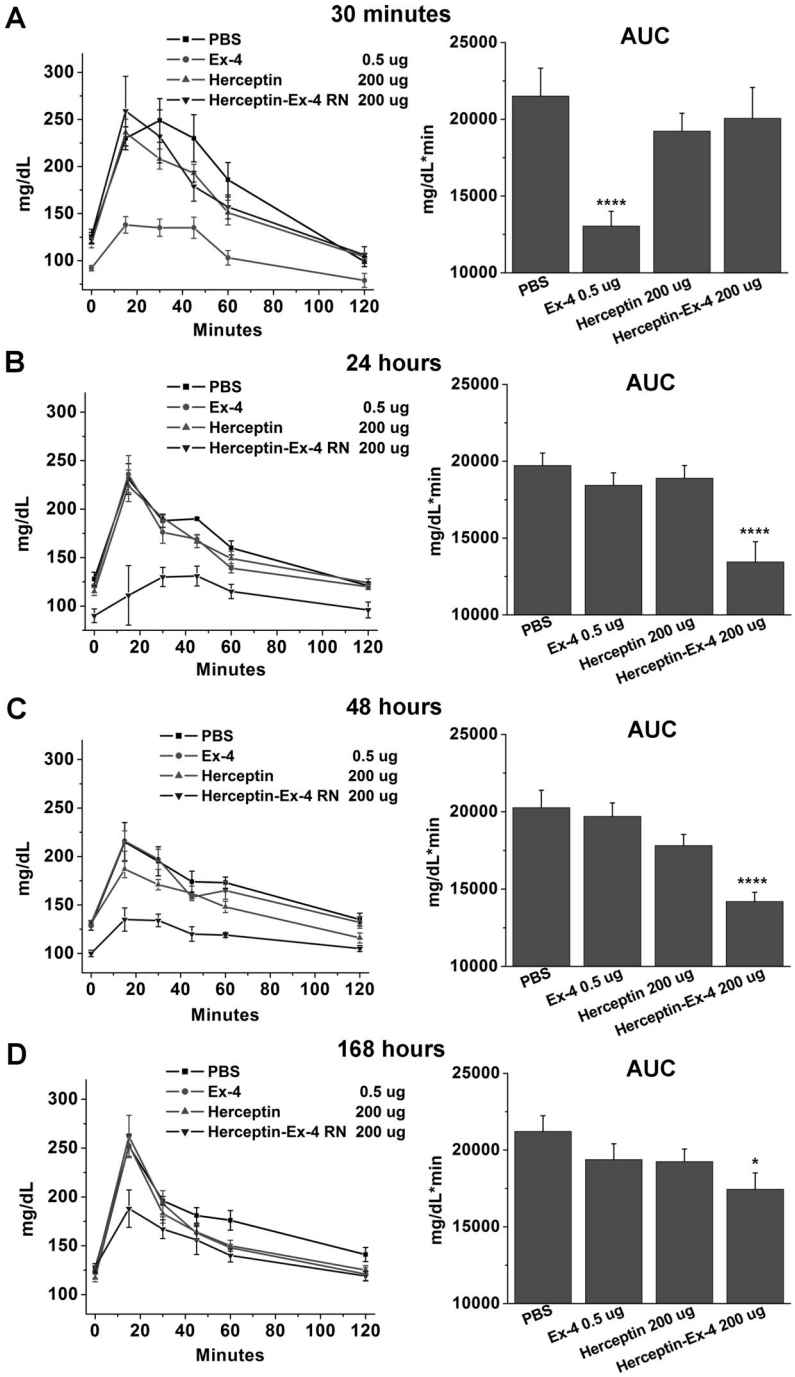

Figure 3. Pharmacodynamics of the herceptin–CDR3H–coil–Ex-4 RN fusion protein in mice. Single doses of vehicle (PBS, pH 7.4), Ex-4 peptide (20 μg kg^{-1}), herceptin (8 mg kg^{-1}), and the herceptin–Ex-4 RN fusion protein (8 mg kg^{-1}) were subcutaneously administered into CD1 mice ($N=5$). OGTTs (3 g kg^{-1} D-glucose; p.o.) were performed at 30 min (A) and 24 (B), 48 (C), and 168 h (D) after single-dose treatment. **** $p<0.0001$, * $p<0.05$ (one-way ANOVA test).

We next examined the time-dependent blood-glucose-lowering activity of the herceptin–CDR3H–Ex-4 RN fusion protein in mice using an oral glucose tolerance test (OGTT) at multiple time points after single-dose treatment. Mice treated with a single dose of the herceptin–CDR3H–Ex-4 RN fusion protein (8 mg (50 nmol)/kg; s.c. = subcutaneous injection) displayed sustained control of blood glucose levels for more than seven days, whereas mice treated with the Ex-4 peptide (20 μg (5 nmol)/kg; s.c. injection) or Herceptin (8 mg (50 nmol)/kg; s.c. injection) had significantly elevated blood glucose levels (Figure 3 and S7) after 24 hours. In addition, mice treated with various single doses of the herceptin–CDR3H–Ex-4 RN fusion protein (2, 4, and 6 mg kg^{-1}; s.c. injection) showed extended control of blood glucose levels in a dose-dependent manner (Figure S8). These in vivo activity data are consistent with the plasma half-lives of the fusion protein and Ex4.

Thus, the herceptin–CDR3H–Ex-4 RN fusion protein shows comparable biological activity to the Ex-4 peptide both in vitro and in vivo, but has a significantly extended half-life in rodents. This half-life will likely translate into greater than 1X/week dosing in humans. Moreover, the resulting humanized agonist antibodies are likely to have significantly lower immunogenicity in comparison with the bovine antibody derived fusions. One potential issue, however, arises from the possible immunogenicity of the coiled-coil linker sequences and the splice junctions. Although computational analysis of the linker sequences for T-cell epitopes predicts a low probability of immunogenicity,[15] further in vivo studies are ongoing to fully address this question. In conclusion, we have developed a novel approach to the generation of humanized agonist antibodies with an extended CDR3H, which show excellent biological activity and enhanced pharmacological properties. We are currently applying a similar strategy to GLP-1R/glucagon receptor co-agonists and GLP-2R agonists.

Received: October 13, 2014
Published online: December 29, 2014

Keywords: antibodies · peptide · pharmacology · protein engineering · receptors

[1] a) H. Zhang, K. Yea, J. Xie, D. Ruiz, I. A. Wilson, R. A. Lerner, *Chem. Biol.* **2013**, *20*, 734–741; b) H. Zhang, I. A. Wilson, R. A. Lerner, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 15728–15733; c) A. L. Wu, G. Kolumam, S. Stawicki, Y. Chen, J. Li, J. Zavala-Solorio, K. Phamluong, B. Feng, L. Li, S. Marsters, L. Kates, N. van Bruggen, M. Leabman, A. Wong, D. West, H. Stern, E. Luis, H. S. Kim, D. Yansura, A. S. Peterson, E. Filvaroff, Y. Wu, J. Sonoda, *Sci. Transl. Med.* **2011**, *3*, 113ra126.

[2] a) V. R. Doppalapudi, J. Huang, D. Liu, P. Jin, B. Liu, L. Li, J. Desharnais, C. Hagen, N. J. Levin, M. J. Shields, M. Parish, R. E. Murphy, J. Del Rosario, B. D. Oates, J. Y. Lai, M. J. Matin, Z. Ainekulu, A. Bhat, C. W. Bradshaw, G. Woodnutt, R. A. Lerner, R. W. Lappe, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 22611–

22616; b) J. Huang, T. Ishino, G. Chen, P. Rolzin, T. F. Osothprarop, K. Retting, L. Li, P. Jin, M. J. Matin, B. Huyghe, S. Talukdar, C. W. Bradshaw, M. Palanki, B. N. Violand, G. Woodnutt, R. W. Lappe, K. Ogilvie, N. Levin, *J. Pharmacol. Exp. Ther.* **2013**, *346*, 270 – 280.

[3] F. Wang, D. C. Ekiert, I. Ahmad, W. Li, Y. Zhang, O. Bazirgan, A. Torkamani, T. Raudsepp, W. Mwangi, M. F. Criscitiello, I. A. Wilson, P. G. Schultz, V. V. Smider, *Cell* **2013**, *153*, 1379 – 1393.

[4] a) Y. Zhang, D. Wang, L. de Lichtervelde, S. B. Sun, V. V. Smider, P. G. Schultz, F. Wang, *Angew. Chem. Int. Ed.* **2013**, *52*, 8295 – 8298; *Angew. Chem.* **2013**, *125*, 8453 – 8456; b) Y. Zhang, D. Wang, G. Welzel, Y. Wang, P. G. Schultz, F. Wang, *ACS Chem. Biol.* **2013**, *8*, 2117 – 2121; c) T. Liu, Y. Liu, Y. Wang, M. Hull, P. G. Schultz, F. Wang, *J. Am. Chem. Soc.* **2014**, *136*, 10557 – 10560.

[5] Y. Zhang, D. Goswami, D. Wang, T. S. Wang, S. Sen, T. J. Magliery, P. R. Griffin, F. Wang, P. G. Schultz, *Angew. Chem. Int. Ed.* **2014**, *53*, 132 – 135; *Angew. Chem.* **2014**, *126*, 136 – 139.

[6] a) E. O. Saphire, P. W. Parren, R. Pantophlet, M. B. Zwick, G. M. Morris, P. M. Rudd, R. A. Dwek, R. L. Stanfield, D. R. Burton, I. A. Wilson, *Science* **2001**, *293*, 1155 – 1159; b) R. Pejchal, L. M. Walker, R. L. Stanfield, S. K. Phogat, W. C. Koff, P. Poignard, D. R. Burton, I. A. Wilson, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 11483 – 11488.

[7] a) J. Baselga, D. Tripathy, J. Mendelsohn, S. Baughman, C. C. Benz, L. Dantis, N. T. Sklarin, A. D. Seidman, C. A. Hudis, J. Moore, P. P. Rosen, T. Twaddell, I. C. Henderson, L. Norton, *J. Clin. Oncol.* **1996**, *14*, 737 – 744; b) M. A. Bookman, K. M. Darcy, D. Clarke-Pearson, R. A. Boothby, I. R. Horowitz, *J. Clin. Oncol.* **2003**, *21*, 283 – 290.

[8] a) J. Eng, W. A. Kleinman, L. Singh, G. Singh, J. P. Raufman, *J. Biol. Chem.* **1992**, *267*, 7402 – 7405; b) A. A. Young, B. R. Gedulin, S. Bhavsar, N. Bodkin, C. Jodka, B. Hansen, M. Denaro, *Diabetes* **1999**, *48*, 1026 – 1034.

[9] D. J. Drucker, M. A. Nauck, *Lancet* **2006**, *368*, 1696 – 1705.

[10] a) L. B. Knudsen, P. F. Nielsen, P. O. Huusfeldt, N. L. Johansen, K. Madsen, F. Z. Pedersen, H. Thogersen, M. Wilken, H. Agerso, *J. Med. Chem.* **2000**, *43*, 1664 – 1669; b) M. B. DeYoung, L. MacConell, V. Sarin, M. Trautmann, P. Herbert, *Diabetes Technol. Ther.* **2011**, *13*, 1145 – 1154; c) J. B. Buse, M. Nauck, T. Forst, W. H. Sheu, S. K. Shenouda, C. R. Heilmann, B. J. Hoogwerf, A. Gao, M. K. Boardman, M. Fineman, L. Porter, G. Schernthaner, *Lancet* **2013**, *381*, 117 – 124; d) R. E. Pratley, M. A. Nauck, A. H. Barnett, M. N. Feinglos, F. Ovalle, I. Harman-Boehm, J. Ye, R. Scott, S. Johnson, M. Stewart, J. Rosenstock, *The Lancet. Diabetes & Endocrinology* **2014**, *2*, 289 – 297; Endocrinology **2014**, 2, 289 – 297; e) K. M. Dungan, S. T. Povedano, T. Forst, J. G. Gonzalez, C. Atisso, W. Sealls, J. L. Fahrbach, *Lancet* **2014**, *0*, 0; f) R. L. O'Connor-Semmes, J. Lin, R. J. Hodge, S. Andrews, J. Chism, A. Choudhury, D. J. Nunez, *Clin. Pharmacol. Ther.* **2014**, *0*, 0.

[11] a) J. Baselga, D. Tripathy, J. Mendelsohn, S. Baughman, C. C. Benz, L. Dantis, N. T. Sklarin, A. D. Seidman, C. A. Hudis, J. Moore, P. P. Rosen, T. Twaddell, I. C. Henderson, L. Norton, *Semin. Oncol.* **1999**, *26*, 78 – 83; b) M. M. Goldenberg, *Clin. Ther.* **1999**, *21*, 309 – 318; c) S. Shak, *Semin. Oncol.* **1999**, *26*, 71 – 77; d) H. S. Cho, K. Mason, K. X. Ramyar, A. M. Stanley, S. B. Gabelli, D. W. Denney, Jr., D. J. Leahy, *Nature* **2003**, *421*, 756 – 760.

[12] S. Runge, H. Thogersen, K. Madsen, J. Lau, R. Rudolph, *J. Biol. Chem.* **2008**, *283*, 11340 – 11347.

[13] a) K. L. Armour, M. R. Clark, A. G. Hadley, L. M. Williamson, *Eur. J. Immunol.* **1999**, *29*, 2613 – 2624; b) R. L. Shields, A. K. Namenuk, K. Hong, Y. G. Meng, J. Rae, J. Briggs, D. Xie, J. Lai, A. Stadlen, B. Li, J. A. Fox, L. G. Presta, *J. Biol. Chem.* **2001**, *276*, 6591 – 6604.

[14] a) F. Selis, R. Schrepfer, R. Sanna, S. Scaramuzza, G. Tonon, S. Dedoni, P. Onali, G. Orsini, S. Genovese, *Results Pharma Sci.* **2012**, *2*, 58 – 65; b) W. Glaesner, A. M. Vick, R. Millican, B. Ellis, S. H. Tschang, Y. Tian, K. Bokvist, M. Brenner, A. Koester, N. Porksen, G. Etgen, T. Bumol, *Diabetes/Metab. Res. Rev.* **2010**, *26*, 287 – 296; c) B. Rolin, M. O. Larsen, C. F. Gotfredsen, C. F. Deacon, R. D. Carr, M. Wilken, L. B. Knudsen, *Am. J. Physiol. Endocrinology Metabolism* **2002**, *283*, E745 – 752; d) W. Bao, L. J. Holt, R. D. Prince, G. X. Jones, K. Aravindhan, M. Szapacs, A. M. Barbour, L. J. Jolivette, J. J. Lepore, R. N. Willette, E. DeAngelis, B. M. Jucker, *Cardiovasc. Diabetol.* **2013**, *12*, 148.

[15] a) C. Lundegaard, O. Lund, M. Nielsen, *Bioinformatics* **2008**, *24*, 1397 – 1398; b) J. J. Calis, M. Maybeno, J. A. Greenbaum, D. Weiskopf, A. D. De Silva, A. Sette, C. Kesmir, B. Peters, *PLoS Comput. Biol.* **2013**, *9*, e1003266.

