JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
pubs.acs.org/JACS

# Rational Design of Humanized Dual-Agonist Antibodies

Yong Zhang,[#] Yan Liu,[#] Ying Wang, Peter G. Schultz,* and Feng Wang*

California Institute for Biomedical Research (Calibr), 11119 North Torrey Pines Road, La Jolla, California 92037, United States

Supporting Information

**ABSTRACT:** The ultralong heavy chain complementarity determining region 3 (CDR3H) of bovine antibody BLV1H12 folds into a novel "stalk-knob" structural motif and has been exploited to generate novel agonist antibodies through replacement of the "knob" domain with cytokines and growth factors. By translating this unique "stalk-knob" architecture to the humanized antibody trastuzumab (referred to hereafter by its trade name, Herceptin, Genentech USA), we have developed a versatile approach to the generation of human antibody agonists. Human erythropoietin (hEPO) or granulocyte colony-stimulating factor (hGCSF) was independently fused into CDR3H, CDR2H, or CDR3L of Herceptin using an engineered "stalk" motif. The fusion proteins express in mammalian cells in good yields and have similar *in vitro* biological activities compared to hEPO and hGCSF. On the basis of these results we then generated a bi-functional Herceptin-CDR fusion protein in which both hEPO and hGCSF were grafted into the heavy- and light-chain CDR3 loops, respectively. This bi-functional antibody fusion exhibited potent EPO and GCSF agonist activities. This work demonstrates the versatility of the CDR-fusion strategy for generating functional human antibody chimeras and provides a novel approach to the development of multi-functional antibody-based therapeutics.

A recently solved X-ray crystal structure of the bovine antibody BLV1H12 revealed a novel structural motif in which an ultralong heavy chain complementarity determining region 3 (CDR3H) folds into a solvent exposed, antiparallel $\beta$-strand "stalk" that terminates with a "knob" domain stabilized by three disulfide bonds. This unusual CDR3H domain has been exploited to replace the "knob" region of BLV1H12 with cytokines, growth factors, and G protein-coupled receptor (GPCR) ligands to generate novel antibody agonists.[1−3] Moreover, we have shown that this $\beta$-strand "stalk" can be replaced with a heterodimeric coiled-coil motif while preserving the folding and function of the fused "knob" domain.[4]

Unlike this family of bovine antibodies with an ultralong CDR3H, human antibodies typically consist of CDR loops of 8−16 residues. Given the evolutionally conserved $\beta$-sheet frameworks of the bovine and human antibody variable regions, the question arises whether this "stalk-knob" motif can be transferred to the CDR loops of human antibody scaffolds. Here we show that protein agonists can be genetically fused into distinct CDRs of the humanized anti-HER2 receptor monoclonal antibody trastuzumab (referred to hereafter by its trade name, Herceptin, Genentech USA)[5−8] to generate humanized mono-functional and bi-functional antibody chimeras targeting cytokine receptors. The resulting Herceptin-CDR fusion proteins have excellent physicochemical properties and comparable *in vitro* biological activities relative to native protein agonists. This work illustrates the versatility of scaffold-based engineering and provides a new approach for generation of multi-functional antibody chimeras.

Human EPO is a 34 kDa glycoprotein produced in the kidney that stimulates erythroid progenitor differentiation and maturation.[9,10] By fusing hEPO into the ultralong CDR3H of bovine antibody BLV1H12, we generated an antibody-hEPO chimera with excellent potency and prolonged serum half-life in mice.[1] However, the bovine-derived antibody scaffold would likely be immunogenic if used therapeutically.[11−13] Comparative analysis of the X-ray crystal structures of the humanized antibody Herceptin and bovine antibody BLV1H12 indicates high structural similarity in the $\beta$-sheet frameworks of the variable domain (RMSD <2.8 Å), suggesting that the CDR regions of Herceptin can be fused through rigid "stalks" to a separately folded protein agonist without significantly affecting folding of the antibody or fusion partner. The 13-residue CDR3H loop of Herceptin has a $\beta$-strand conformation at its bottom and forms extensive interactions with adjacent CDR loops from both the heavy and light chains (Figure 1A). Therefore, we reasoned that the $\beta$-strand "stalk" of bovine antibody BLV1H12 or the designed coiled-coil "stalk" might also allow fusion of hEPO into CDR3H of Herceptin without interfering with the immunoglobulin fold.[4] To test this notion, a gene fragment encoding hEPO with flexible GGGGS linkers at each end was fused with the $\beta$-strand "stalk"-forming sequences (ascending strand: $H_2N$-ETKKYQS-COOH; descending strand: $H_2N$-SYTYNYE-COOH) from bovine antibody BLV1H12. This entire cassette was then grafted into the CDR3H of Herceptin with the original Trp99−Met107 loop removed to generate the Herceptin-CDR3H-beta-hEPO fusion protein (Figure 1B,C). Similarly, Herceptin-CDR3H-hEPO with a coiled-coil "stalk" (Herceptin-CDR3H-coil-hEPO) was created by using the coiled-coil sequences ($H_2N$-GGSGAKLAALKAKLAALK-COOH and $H_2N$-ELAALEAELAALEAGGSG-COOH) from a previously engineered bovine antibody[4] to connect the N- and C-termini of hEPO with the CDR3H region of Herceptin. To minimize complement-dependent and antibody-dependent cell-mediated cytotoxicities, the heavy chain constant regions of the Herceptin-CDR fusion proteins were replaced with the human IgG4 heavy chain constant region containing the three mutations S228P, F234A, and L235A.[14]

**Received:** October 13, 2014
**Published:** December 10, 2014





DOI: 10.1021/ja510519u
*J. Am. Chem. Soc.* 2015, 137, 38−41

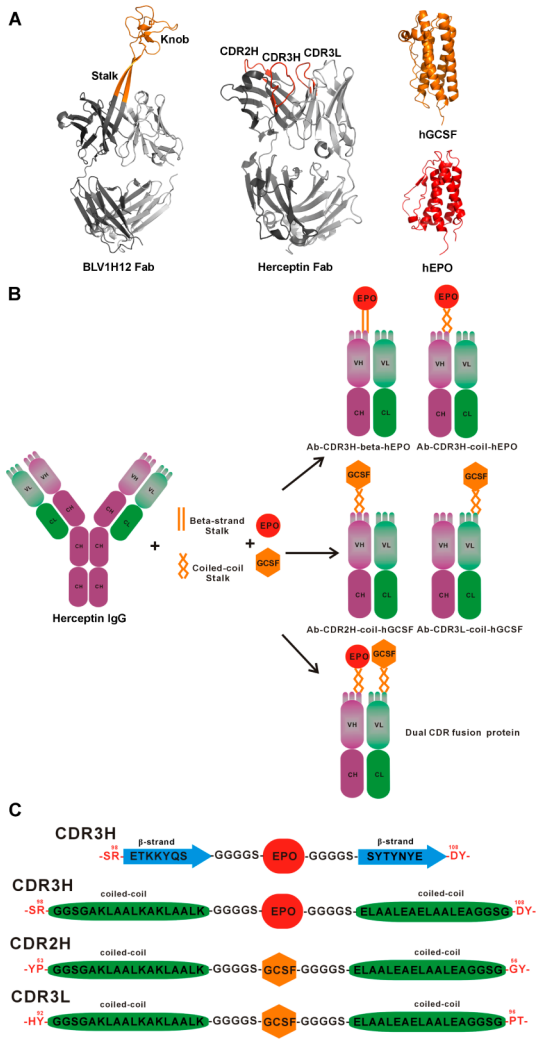


**Figure 1.** Design of humanized mono- and dual-agonist antibodies. (A) X-ray crystal structures of bovine antibody BLV1H12 Fab fragment (PDB ID: 4K3D), humanized antibody Herceptin Fab fragment (PDB ID: 1N8Z), human GCSF (PDB ID: 1CD9), and human EPO (PDB ID: 1EER). (B) Scheme for generation of monofunctional and bifunctional human antibody-CDR fusion proteins using $\beta$-strand and coiled-coil "stalks". All of the antibody-CDR fusion proteins were produced as full-length IgGs and each IgG molecule contains two fused peptides or proteins. Only the Fab domains of the antibody fusions are depicted. (C) Maps of the key elements of the Herceptin-CDR fusion proteins. Residues from the parental Herceptin CDR regions are numbered and highlighted in red.

Both Herceptin-CDR3H-hEPO fusion proteins were expressed in freestyle HEK 293 cells by transient transfection. Secreted fusion proteins were purified using protein G chromatography and analyzed by SDS-PAGE (Supporting Information (SI), Figure S1). Under non-reducing conditions, both Herceptin-CDR3H-beta-hEPO and Herceptin-CDR3H-coil-hEPO migrate as a single band higher than 200 kDa due to glycosylation. In the presence of 50 mM dithiothreitol (DTT), the light chains of the Herceptin-hEPO fusion proteins migrate at 25 kDa and the heavy chains of the Herceptin-hEPO fusion proteins migrate at 80 kDa. After treatment with peptide-*N*-glycosidase and DTT, mass spectral analyses indicate mass increases of 20 493 and 22 005 Da for the heavy chains of the Herceptin-CDR3H-beta-hEPO and Herceptin-CDR3H-coil-hEPO, respectively, relative to that of Herceptin (SI, Figures S2 and S3). This corresponds to two fused hEPO proteins per IgG molecule. The final yields of the Herceptin-CDR3H-hEPO fusion proteins are similar, ~17 mg/L, and both proteins can be concentrated to over 10 mg/mL in PBS (pH 7.4) without aggregation and show no loss of stability and activity after long-term storage at −20 °C. The yields and solubilities of the Herceptin-based hEPO fusion proteins are comparable to those of the bovine antibody-CDR3H-hEPO fusion protein, suggesting that the $\beta$-strand and coiled-coil "stalks" are both effective in promoting correct folding of fusion proteins to a non-bovine antibody scaffold.

Next the biological activities of both Herceptin-CDR3H-hEPO fusion proteins were determined using human TF-1 cells that are growth-dependent on hEPO.[15] Prior to treatment, cells were washed three times to remove residual granulocyte macrophage colony-stimulating factor (GM-CSF) from the growth media, followed by treatment with various concentrations of Herceptin, hEPO, and Herceptin-CDR3H-hEPO fusion proteins for 72 h. Cell viability in each well was quantified using an AlamarBlue assay. hEPO and both Herceptin-CDR3H-hEPO fusion proteins stimulate TF-1 cell proliferation in a dose-dependent manner (Figure 2A), whereas Herceptin itself has no proliferative activity, indicating that the observed activities of the Herceptin-CDR3H-hEPO fusion proteins result from the fused hEPO. $EC_{50}$ values are 0.1 ± 0.02 nM for hEPO, 0.1 ± 0.01 nM for Herceptin-CDR3H-beta-hEPO, and 0.1 ± 0.01 nM for

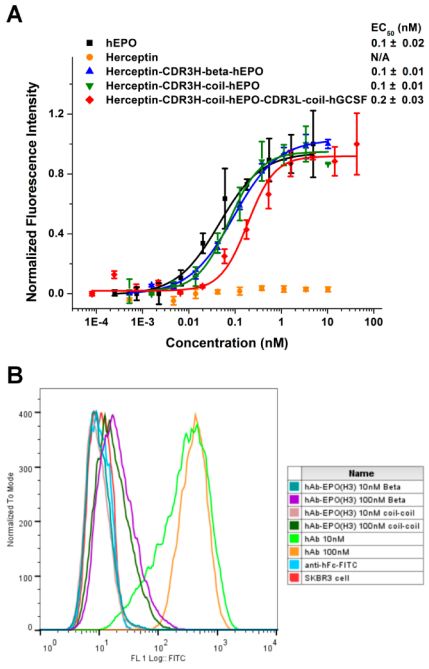


**Figure 2.** *In vitro* biological activities of Herceptin-CDR3H-hEPO fusion proteins and their binding affinities to HER2 receptor. (A) Herceptin-CDR3H-hEPO fusion proteins stimulate proliferation of human TF-1 cells in a dose-dependent manner. Cells cultured in RPMI-1640 medium with 10% FBS were treated with various concentrations of hEPO, Herceptin, and Herceptin-CDR3H-hEPO fusion proteins. Cell viability was quantified using an Alamar Blue (Life Technologies, CA) assay. (B) Flow cytometric analyses of binding to HER2-overexpressing SKBR3 cells by Herceptin (hAb), hAb-CDR3H-beta-hEPO and hAb-CDR3H-coil-hEPO fusion proteins. Cells cultured in DMEM medium with 10% FBS were incubated with 10 or 100 nM of hAb and hAb-CDR3H-hEPO fusion proteins, followed by staining with FITC anti-human IgG Fc for flow cytometric analysis.


DOI: 10.1021/ja510519u
*J. Am. Chem. Soc.* 2015, 137, 38−41

Herceptin-CDR3H-coil-hEPO, indicating that the fusion proteins have similar activity to hEPO in proliferating TF-1 cells. The proliferative activities of both Herceptin-CDR3H-hEPO fusion proteins are comparable to that bovine antibody-CDR3H-hEPO fusion protein ($EC_{50}$ = 0.03 ± 0.01 nM).[1] These results indicate that these two forms of "stalks" are equally effective in productively fusing hEPO into the CDR3H of Herceptin.

We next carried out flow cytometric analysis using HER2-overexpressing SKBR3 cells to examine if the Herceptin-CDR3H-hEPO fusion proteins bind HER2 receptor. Herceptin tightly binds SKBR3 cells (saturated binding at 10 nM), whereas the Herceptin-CDR3H-hEPO fusion proteins have no detectable binding to the cells at 100 nM (the highest concentration used in the experiment) (Figure 2B), indicating that fusion of hEPO into CDR3H abrogates binding of Herceptin to its cognate antigen.

The frameworks supporting the different CDR loops in the hypervariable region of most human antibody scaffolds are structurally similar, suggesting that it should also be possible to fuse proteins into different human CDRs and maintain their correct folding. To explore this possibility, as well as the generality of this approach, hGCSF was inserted into CDR2H or CDR3L of Herceptin using the coiled-coil "stalk". Human GCSF is a 20 kDa cytokine that is used in patients receiving chemotherapy to accelerate recovery from neutropenia.[16,17] The X-ray crystal structure of Herceptin shows that the CDR2H loop exhibits a similar conformation to that of CDR3H (Figure 1A). The 8-residue CDR2H loop connects anti-parallel $\beta$-strands and forms extensive interactions with the nearby CDR1H loop, making it also potentially suitable for the engraftment of proteins. To test this notion, a Herceptin-CDR2H-coil-hGCSF fusion protein was created by replacing Thr54-Asn55 in CDR2H of Herceptin with hGCSF using the coiled-coil "stalk". Multiple hydrogen bonds formed by the rest of the residues in CDR2H were retained to stabilize the grafted "stalk" in the resulting Herceptin-CDR2H fusion protein. In addition to CDR2H, CDR3L may serve as a suitable site for the fusion of proteins, since CDR3L and CDR3H are structurally similar and many residues in CDR3L form intra- and inter-loop interactions that can potentially stabilize a CDR3L-fusion protein. Thus, we also replaced Thr93-Pro95 in CDR3L of Herceptin by hGCSF using a coiled-coil "stalk" to generate a Herceptin-CDR3L-coil-hGCSF fusion protein. Both fusion proteins contain the human IgG1 heavy chain constant region with seven mutations (E233P, L234V, L235A, ΔG236, A327G, A330S, and P331S) for reduced complement-dependent and antibody-dependent cell-mediated cytotoxicities.[18,19]

The Herceptin-hGCSF fusion proteins were expressed and purified using the same method described above. The purified proteins were analyzed by SDS-PAGE (SI, Figure S4). Mass spectral analyses indicated mass increases of 23 179 Da for the heavy chain of the Herceptin-CDR2H-coil-hGCSF and 22 724 Da for the light chain of the Herceptin-CDR3L-coil-hGCSF relative to those of Herceptin (SI, Figures S5 and S6). The final yields are ~7 mg/L for the Herceptin-CDR2H-coil-hGCSF and 11 mg/L for the Herceptin-CDR3L-coil-hGCSF. Both proteins can be concentrated to over 10 mg/mL in PBS (pH 7.4) without aggregation. The yields and solubilities of the Herceptin-hGCSF fusion proteins are comparable to those of the Herceptin-hEPO fusion proteins.

Next the proliferative activities of the Herceptin-hGCSF fusion proteins were determined using mouse NFS-60 cells which are responsive to GCSF stimulation.[20,21] Both Herceptin-

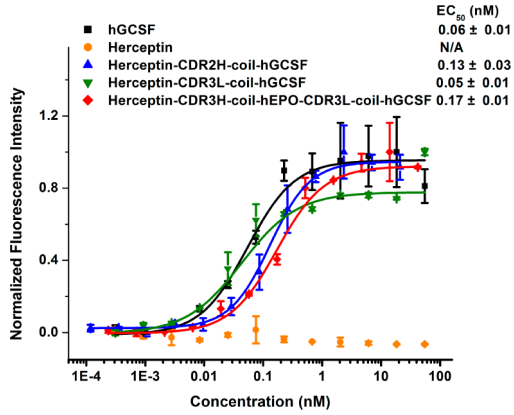


**Figure 3.** *In vitro* biological activities of the Herceptin-CDR-hGCSF fusion proteins. Herceptin-CDR-hGCSF fusion proteins stimulate proliferation of mouse NFS-60 cells in a dose-dependent manner. Cells cultured in RPMI-1640 medium with 10% FBS and 0.05 mM 2-mercapoethanol were treated with various concentrations of hGCSF, Herceptin, and Herceptin-CDR-hGCSF fusion proteins. Cell viability was quantified using an AlamarBlue (Life Technologies, CA) assay.

hGCSF fusion proteins induce proliferation of NFS-60 cells in a dose-dependent fashion (Figure 3), whereas Herceptin itself has no proliferative activity. The potencies of the Herceptin-hGCSF fusion proteins ($EC_{50}$ = 0.13 ± 0.03 nM for the Herceptin-CDR2H-coil-hGCSF; $EC_{50}$ = 0.05 ± 0.01 nM for the Herceptin-CDR3L-coil-hGCSF) are comparable to that of hGCSF ($EC_{50}$ = 0.06 ± 0.01 nM). Thus, fusion of hGCSF into either CDR2H or CDR3L results in similar proliferative activity, indicating that in addition to CDR3H, CDR2H and CDR3L can serve as sites for creation of functional antibody-CDR fusion proteins. To examine the binding affinities of the Herceptin-hGCSF fusion proteins to HER2 receptor, flow cytometric analysis was again carried out using the HER2-overexpressing SKBR3 cells. Similar to Herceptin-CDR3H-hEPO, fusion of hGCSF into CDR3L of Herceptin eliminates its binding to HER2 receptor (SI, Figure S7). In contrast, Herceptin-CDR2H-hGCSF shows significant binding to HER2 receptor at 100 nM, indicating that modification of CDR2H has a lesser effect on binding of Herceptin to HER2 receptor than insertion into CDR3H or CDR3L. This is consistent with a previous finding that CDR2H of Herceptin is less involved in binding to HER2 receptor than CDR3H and the light chain CDRs.[8] Therefore, CDR3H and CDR3L of Herceptin are more suitable sites for generation of mono-functional antibody-CDR fusion proteins.

The above results suggest that bi-functional and multi-functional antibody fusion proteins can be generated by incorporating two or more distinct proteins into different CDR loops of a single antibody. To test this hypothesis, hEPO and hGCSF with coiled-coil "stalks" were fused into CDR3H and CDR3L of Herceptin, respectively, to generate a bi-functional antibody-CDR fusion protein targeting both the EPO and GCSF receptors. On the basis of the X-ray crystal structure of Herceptin, it was expected that the fused cytokines would be relatively well separated and not sterically interfere with each other in binding to their cognate receptors. In addition, recent studies have revealed that hEPO in combination with hGCSF synergistically ameliorate anemia in patients with myelodysplastic syndrome and promote tissue plasticity and functional recovery in patients with ischemia.[22−24] Similar to the mono-functional Herceptin-CDR fusion proteins, the resulting Herceptin-CDR3H-coil-hEPO-CDR3L-coil-hGCSF fusion pro-


DOI: 10.1021/ja510519u
J. Am. Chem. Soc. 2015, 137, 38−41

tein stably expresses in mammalian cells and the final yield of the bi-functional Herceptin-CDR3H-coil-hEPO-CDR3L-coil-hGCSF fusion protein is ~26 mg/L (SI, Figures S8 and S9). The purified protein can be concentrated to over 10 mg/mL in PBS (pH 7.4) without aggregation The increased masses for the antibody chimera match those of the fused hEPO and hGCSF proteins in the heavy and light chains.

The biological activities of the Herceptin-CDR3H-coil-hEPO-CDR3L-coil-hGCSF fusion protein were examined using human TF-1 and mouse NFS-60 cells. Compared with the proliferative activities of hEPO and hGCSF proteins and their corresponding Herceptin-CDR fusion proteins, the bi-functional Herceptin-hEPO-hGCSF fusion protein shows slightly lower EPO ($EC_{50}$ = 0.2 ± 0.03 nM) and GCSF ($EC_{50}$ = 0.17 ± 0.01 nM) activities (Figures 2 and 3). These results indicate that simultaneous engraftment of hEPO and hGCSF with coiled-coil "stalks" into CDR3H and CDR3L regions of Herceptin does not significantly affect the activities of the individual proteins, and supports the notion that the CDR loops can be utilized to generate multi-functional antibody chimeras. Flow cytometric analysis again revealed that the bi-functional Herceptin-CDR3H-coil-hEPO-CDR3L-coil-hGCSF fusion protein has no detectable binding affinity to HER2 receptor at 100 nM (SI, Figure S7). The slightly reduced hEPO and hGCSF activities of the bi-functional fusion protein may be caused by unfavorable interactions between the bound receptors and the noncognate cytokine and/or other CDR loops, which can likely be minimized by grafting into other CDR loops or tuning the lengths of the respective "stalks". Due to the fixed stoichiometry of the fused agonists, it may be necessary in some cases to tune the potencies of the individual agonist activities by engineering other CDR loops and/or modifying the structure of the fused polypeptide. It is likely that the bivalent nature of antibody-CDR fusions also affects potency, although such bivalent interactions could differentially affect the individual fusion proteins in a multi-functional antibody. Finally, it is likely that other human antibodies, such as Synagis, a humanized monoclonal antibody against the F protein of respiratory syncytial virus (RSV),[25] can provide alternative scaffolds for production of therapeutic antibody-CDR fusion proteins.

Although it has recently been shown that smaller peptides like T-cell and B-cell epitopes can be grafted into different CDRs of IgGs to elicit an immune response,[26] the antibody-CDR fusion strategy using a rigid "stalk" provides a general strategy for the fusion of peptides and larger, functional proteins that correctly fold and retain potent biological activities. Moreover, this approach allows the generation of both mono-functional and multi-functional human antibody-protein fusions. Ongoing studies include analysis of the *in vivo* pharmacology of bi-functional fusion proteins, generation of other bi-functional antibodies, such as leptin/GLP-1 and GLP-1/glucagon dual agonist antibodies, as well as exploring the possibility of using similar approaches to combine the antigen binding affinities of two or more distinct antibodies. Finally, we are carrying out SAR studies to determine the role of the rigid "stalk" motifs in promoting and stabilizing the folding of the antibody-CDR fusion proteins.

## ■ ASSOCIATED CONTENT

### Supporting Information

Experimental methods and results. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION


### Corresponding Authors

schultz@scripps.edu
fwang@calibr.org

### Author Contributions

#Y.Z. and Y.L. contributed equally to this work.


### Notes

The authors declare no competing financial interest.

## ■ REFERENCES


(1) Zhang, Y.; Wang, D.; Welzel, G.; Wang, Y.; Schultz, P. G.; Wang, F. *ACS Chem. Biol.* **2013**, *8*, 2117.
(2) Zhang, Y.; Wang, D.; de Lichtervelde, L.; Sun, S. B.; Smider, V. V.; Schultz, P. G.; Wang, F. *Angew. Chem., Int. Ed.* **2013**, *52*, 8295.
(3) Liu, T.; Liu, Y.; Wang, Y.; Hull, M.; Schultz, P. G.; Wang, F. *J. Am. Chem. Soc.* **2014**, *136*, 10557.
(4) Zhang, Y.; Goswami, D.; Wang, D.; Wang, T. S.; Sen, S.; Magliery, T. J.; Griffin, P. R.; Wang, F.; Schultz, P. G. *Angew. Chem., Int. Ed.* **2014**, *53*, 132.
(5) Baselga, J.; Tripathy, D.; Mendelsohn, J.; Baughman, S.; Benz, C. C.; Dantis, L.; Sklarin, N. T.; Seidman, A. D.; Hudis, C. A.; Moore, J.; Rosen, P. P.; Twaddell, T.; Henderson, I. C.; Norton, L. *Semin. Oncol.* **1999**, *26*, 78.
(6) Goldenberg, M. M. *Clin. Ther.* **1999**, *21*, 309.
(7) Shak, S. *Semin. Oncol.* **1999**, *26*, 71.
(8) Cho, H. S.; Mason, K.; Ramyar, K. X.; Stanley, A. M.; Gabelli, S. B.; Denney, D. W., Jr.; Leahy, D. J. *Nature* **2003**, *421*, 756.
(9) Fisher, J. W. *Exp. Biol. Med. (Maywood)* **2003**, *228*, 1.
(10) Syed, R. S.; Reid, S. W.; Li, C.; Cheetham, J. C.; Aoki, K. H.; Liu, B.; Zhan, H.; Osslund, T. D.; Chirino, A. J.; Zhang, J.; Finer-Moore, J.; Elliott, S.; Sitney, K.; Katz, B. A.; Matthews, D. J.; Wendoloski, J. J.; Egrie, J.; Stroud, R. M. *Nature* **1998**, *395*, 511.
(11) Kato, M.; Miura, K.; Kamiyama, H.; Okazaki, A.; Kumaki, K.; Kato, Y.; Sugiyama, Y. *Drug Metab. Dispos.* **1998**, *26*, 126.
(12) Leroy-Viard, K.; Rouyer-Fessard, P.; Beuzard, Y. *Blood* **1991**, *78*, 1596.
(13) Reichel, C.; Gmeiner, G. *Handb. Exp. Pharmacol.* **2010**, 251.
(14) Glaesner, W.; Vick, A. M.; Millican, R.; Ellis, B.; Tschang, S. H.; Tian, Y.; Bokvist, K.; Brenner, M.; Koester, A.; Porksen, N.; Etgen, G.; Bumol, T. *Diabetes Metab. Res. Rev.* **2010**, *26*, 287.
(15) Hoang, T.; Paradis, E.; Brady, G.; Billia, F.; Nakahara, K.; Iscove, N. N.; Kirsch, I. R. *Blood* **1996**, *87*, 102.
(16) Cooper, K. L.; Madan, J.; Whyte, S.; Stevenson, M. D.; Akehurst, R. L. *BMC Cancer* **2011**, *11*, 404.
(17) Aritomi, M.; Kunishima, N.; Okamoto, T.; Kuroki, R.; Ota, Y.; Morikawa, K. *Nature* **1999**, *401*, 713.
(18) Armour, K. L.; Clark, M. R.; Hadley, A. G.; Williamson, L. M. *Eur. J. Immunol.* **1999**, *29*, 2613.
(19) Shields, R. L.; Namenuk, A. K.; Hong, K.; Meng, Y. G.; Rae, J.; Briggs, J.; Xie, D.; Lai, J.; Stadlen, A.; Li, B.; Fox, J. A.; Presta, L. G. *J. Biol. Chem.* **2001**, *276*, 6591.
(20) Bai, Y.; Ann, D. K.; Shen, W. C. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 7292.
(21) Shirafuji, N.; Asano, S.; Matsuda, S.; Watari, K.; Takaku, F.; Nagata, S. *Exp. Hematol.* **1989**, *17*, 116.
(22) Chen, F.; Liu, Q.; Zhang, Z. D.; Zhu, X. H. *Microcirculation* **2013**, *20*, 416.
(23) Jadersten, M.; Malcovati, L.; Dybedal, I.; Della Porta, M. G.; Invernizzi, R.; Montgomery, S. M.; Pascutto, C.; Porwit, A.; Cazzola, M.; Hellstrom-Lindberg, E. *J. Clin. Oncol.* **2008**, *26*, 3607.
(24) Yu, J. H.; Seo, J. H.; Lee, J. E.; Heo, J. H.; Cho, S. R. *Neurosci. Bull.* **2014**, *30*, 107.
(25) Subramanian, K. N.; Weisman, L. E.; Rhodes, T.; Ariagno, R.; Sanchez, P. J.; Steichen, J.; Givner, L. B.; Jennings, T. L.; Top, F. H., Jr.; Carlin, D.; Connor, E. *Pediatr. Infect. Dis. J.* **1998**, *17*, 110.
(26) Brumeanu, T. D.; Bot, A.; Bona, C. A.; Dehazya, P.; Wolf, I.; Zaghouani, H. *Immunotechnology* **1996**, *2*, 85.



DOI: 10.1021/ja510519u
*J. Am. Chem. Soc.* 2015, 137, 38−41