The potential therapeutic value of many proteins — including enzymes, receptors, cytokines, blood factors and peptides — can be realized by fusing them to the Fc region of human immunoglobulin G. Of the 46 monoclonal antibody (MAb) and MAb-derivative products approved by the FDA to date as human therapeutics, 10 are Fc-fusion proteins (Table 2). Among approved products, several structural variations are represented (Figure 4).

In BPI’s October 2014 issue, Part 1 of this review examined the structure and manufacturing of Fc-fusion proteins. It referred to 45 MAb and MAb-derivative products approved by the FDA. One additional Fc-fusion protein (Eloctate) was approved while this manuscript was in review. This month, we conclude with a detailed examination of such products that have received market authorization over the past 16 years. Tables, figures, and references are numbered continuously from Part 1.

**Fc-Fusion Proteins As Drugs**

Currently nine Fc-fusion proteins are FDA approved and marketed in the United States as biopharmaceutical products (Table 2). Other compounds are in late-stage clinical development (Table 3). For several clinical indications (e.g., autoimmune conditions such as rheumatoid arthritis), Fc-fusion proteins directly compete with MAbs that are designed to bind to similar molecular targets. Nine of the 10 approved Fc-fusion proteins are homodimers. They can be categorized into four groups based on ligand specificity (binding to one or multiple epitopes on a ligand molecule) and valency (stoichiometry of binding to ligand molecules):

- bivalent with single-ligand specificity
- monovalent with multiligand specificity
- multivalent with single-ligand specificity
- monovalent with single-ligand specificity.

**Group 1 — Single-Ligand Specificity in Bivalent Fusion Constructs:**

Alefacept, etanercept, abatacept, and belatacept are examples of homodimeric molecules comprising single-ligand binding domains derived from receptor ECDs. The fusion molecules directly compete with MAbs that are designed to bind to similar molecular targets. Nine of the 10 approved Fc-fusion proteins are homodimers. They can be categorized into four groups based on ligand specificity (binding to one or multiple epitopes on a ligand molecule) and valency (stoichiometry of binding to ligand molecules):

- bivalent with single-ligand specificity
- monovalent with multiligand specificity
- multivalent with single-ligand specificity
- monovalent with single-ligand specificity.

Figure 4: Structural variety of FDA-approved Fc-fusion proteins is illustrated. Typical receptor ECD-containing Fc fusions exhibit single-ligand specificity in bivalent fusion constructs such as (i) alefacept, and (ii) abatacept and belatacept (black dots in denote Cys–Ser mutations in the hinge); “cytokine traps” (iii) such as rilonacept, aflibercept, and ziv-aflibercept; and “peptibodies” (iv) such as the aglycosylated romiplostim with single-ligand specificity in multivalent fusion constructs (in which peptide mimetics represented by large red ovals are attached in tandem and separated by flexible spacers at the C-terminal end of IgG Fc); etanercept (i) and clotting factor Fc (v) FIX Fc and FVIII Fc, with single-ligand specificity in a monovalent fusion construct. Shown in yellow are domains of truncated FIX. Horizontal lines indicate interchain disulfide bonds. Glycosylation sites are indicated in the ligand-binding domains (small red ovals) and Fc domain (small gray ovals). This figure is reproduced and adapted with permission from (3).
CD45RO+ memory effector cells that express a high level of cell-surface CD2. By binding to CD2 on those T cells, alefacept effectively prevents APC cross-talk with T cells and therefore inhibits T-cell activation (23). Alefacept has a circulating half-life of 12 days in humans (24).

Abatacept and belatacept (Bristol-Myers Squibb’s Ocrenza and Nulojix products, respectively) consist of the ECD of human cytotoxic T lymphocyte-associated molecule-4 (CTLA-4) fused to the Fc domain. They were designed as receptor antagonists to block the interactions between CD80 or CD86 on APCs and CD28 on T cells, which provide the secondary costimulatory signal needed for T-cell activation. Abatacept incorporates the disulfide-linked homodimeric structure of CTLA-4, enabling the fusion protein to bind to CD86 with a stoichiometry of 1:2 (25).

That drug was developed by Bristol-Myers-Squibb and approved for the treatment of rheumatoid arthritis (26), with several trials ongoing for other indications. The company later developed a more potent, engineered version of CTLA-4 Fc (belatacept) for renal and liver transplant rejection (27). The new molecule contains two amino-acid substitutions (L104E and A29Y) in its CTLA-4 region, giving it higher affinity for both CD86 and CD80, and it is 10-fold more potent as measured by in vitro T-cell activation assays. Belatacept was approved in 2011.

Group 2 — Traps (Multiligand Specificity in Monovalent Fusion Constructs): Rilonacept, aflibercept, and ziv–aflibercept are examples of cytokine traps: homodimeric molecules made up of multiligand-binding domains derived from different receptor chains. Their ligand-binding domains combine functionally to form a single binding site, thus “trapping” a single ligand molecule between the two chains. The drugs are thus functionally monovalent and act as receptor antagonists.

Rilonacept (Sanofi Aventis and Regeneron’s Arcalyst product) is an example of a cytokine trap. Developed by Regeneron, it was approved in 2008 to treat cryopin-associated periodic syndromes (CAPS), a rare autoinflammatory disease. Using domains from different interleukin 1 (IL1) binding proteins, Regeneron fused the C-terminus of the IL1 receptor accessory protein (IL1RAcP) ligand-binding region to the N-terminus of the IL1R1 ECD, then fused this hybrid IL1 binding domain to human IgG1 Fc. Rilonacept is an IL1 antagonist with high affinity (Kd = 1.5 pM) and potency (IC50 = 6.5 pM). Its 8.6-day half-life enables weekly administration. By contrast, anakinra (Amgen’s Kineret product) is a recombinant non-glycosylated version of IL1RA produced in Escherichia coli that must be administered daily because of its rapid clearance in vivo (28).

Aflibercept (Sanofi Aventis and Regeneron’s Eylea product) is an antiangiogenic fusion protein designed to bind vascular endothelial growth factor A (VEGF-A), VEGF-B, and PIGF, all of which are implicated in tumor angiogenesis. The design of this molecule illustrates the use of protein engineering to combine domains of different receptors to overcome issues that were initially encountered with one or the other (29). Originally the VEGF trap molecule was created by fusing the first three domains of the VEGF receptor 1 to human IgG1 Fc. The protein was very potent when measured by in vitro tumor growth inhibition assays, but it exhibited a poor pharmacokinetic profile and nonspecific interactions that made it unsuitable as a clinical candidate. The developers deleted the entire high pI domain 1, thought to be responsible for nonspecific binding, and replaced the high pI domain 1, with one characterized by a lower pI and better binding (domain 3 from VEGF receptor 2), resulting in a final construct that actually improved the binding affinity in addition to lowering the pI (29).

Aflibercept thus has much improved pharmacokinetic properties and better antitumor activities, making it better than the sum of its parts. Developed by Regeneron, it was approved in 2011 (Eylea, formulated for intravitreal injection) to treat wet age-related macular degeneration. In 2012 the Zaltrap version (ziv–aflibercept, formulated for intravenous infusion) was approved for metastatic colorectal cancer. Net Eylea sales in the United States for 2012, its first full year on the market, were $800 million (3).

Group 3 — Peptibodies (Single-Ligand Specificity in Multivalent Fusion Constructs): Romiplostim (Amgen’s NPlate product) is a peptibody: a homodimeric peptide molecule that is specific for a single ligand. First identified using phage display technology, this 14–amino-acid peptide binds to thrombopoietin receptor (also known as c–Mpl on CD1110) (30), mimicking the activating effect of thrombopoietin (TPO). For the fusion

**Abbreviations Used Herein**

- ADCC: antibody-dependent, cell-mediated cytotoxicity
- Ang: angiopoietin
- APC: antigen presenting cell
- BAFF: B-cell activating factor
- CDC: complement-dependent cytotoxicity
- CHO: Chinese hamster ovary cells
- ECD: extracellular domain
- Fab: antigen binding, comprising VL, VH, CL, and CH1 domains
- Fc: effector, comprising hinge, CH2, and CH3 domains
- FcRn: neonatal Fc receptor
- GLP-1: glucagon-like peptide-1
- HEK-293: human embryonic kidney 293 cells
- ICD: intracellular domain
- IL-1: interleukin-1
- Kd: equilibrium dissociation constant
- LFA-3: lymphocyte function-associated antigen 3
- MAb: monoclonal antibody
- PEG: polyethylene glycol
- PIGF: placental growth factor
- RA: rheumatoid arthritis
- SLE: systemic lupus erythematosus
- TM: transmembrane domain
- TNF: tumor necrosis factor
- TPO: thrombopoietin
- VEGF: vascular endothelial growth factor
- VLDL: very low-density lipoprotein
Table 2: FDA-approved therapeutic Fc-fusion proteins (* indicates a product withdrawn from the market in 2011)

<table>
<thead>
<tr>
<th>Product</th>
<th>Molecular Construct (Expression System)</th>
<th>Ligand and Clinical Effect</th>
<th>Company</th>
<th>FDA Approval</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eloctate</td>
<td>FVIII-IgG1 Fc (HEK293)</td>
<td>Blood clotting factor (replacement therapy for hemophilia A)</td>
<td>Biogen Idec</td>
<td>2014</td>
<td>37</td>
</tr>
<tr>
<td>Alprolix</td>
<td>FIX-IgG1 Fc (HEK-293)</td>
<td>Blood clotting factor (replacement therapy for hemophilia B)</td>
<td>Biogen Idec</td>
<td>2014</td>
<td>38</td>
</tr>
<tr>
<td>Amevive (alefacet)</td>
<td>LFA3-IgG1 Fc (CHO)</td>
<td>Binds CD2 (inhibits T-cell proliferation in psoriasis and transplant rejection)</td>
<td>Astellas and Biogen Idec</td>
<td>2003*</td>
<td>23</td>
</tr>
<tr>
<td>Enbrel (etanercept)</td>
<td>TNFR2-IgG1 Fc (CHO)</td>
<td>Binds to soluble and membrane TNF (treatment of RA and plaque psoriasis)</td>
<td>Amgen and Immunex</td>
<td>1998</td>
<td>26</td>
</tr>
<tr>
<td>Orencia (abatacept)</td>
<td>CTLA4-IgG1 Fc (CHO)</td>
<td>Binds CD80 and CD86 (inhibits T-cell costimulation in RA)</td>
<td>Bristol-Myers Squibb</td>
<td>2005</td>
<td>30</td>
</tr>
<tr>
<td>Nulojix (belatacept)</td>
<td>CTLA4-IgG1 Fc (CHO)</td>
<td>Binds CD80 and CD86 (inhibits T-cell costimulation in transplant rejection)</td>
<td>Bristol-Myers Squibb</td>
<td>2011</td>
<td>31</td>
</tr>
<tr>
<td>Eylea (afibercept)</td>
<td>VEGFR1-VEGFR2-IgG1 Fc (CHO)</td>
<td>Binds VEGF-A, VEGF-B, and PIGF (treatment of wet age-related macular degeneration)</td>
<td>Regeneron and Sanofi Aventis</td>
<td>2011</td>
<td>33</td>
</tr>
<tr>
<td>Zaltrap (ziv-afiberecept)</td>
<td>VEGFR1-VEGFR2-IgG1 Fc (CHO)</td>
<td>Binds VEGF-A, VEGF-B, and PIGF (treatment of colorectal cancer)</td>
<td>Regeneron and Sanofi Aventis</td>
<td>2012</td>
<td>33</td>
</tr>
<tr>
<td>Arcalyst (riloncept)</td>
<td>IL-1R-IgG1 Fc (CHO)</td>
<td>Binds and neutralizes IL-1 in cryopyrin-associated periodic syndrome</td>
<td>Regeneron and Sanofi Aventis</td>
<td>2008</td>
<td>32</td>
</tr>
<tr>
<td>NPlate (romiplostim) Thrombopoietin-binding peptide–IgG1 Fc (E. coli)</td>
<td>Acts as an agonist on thrombopoietin receptor to stimulate production of platelets in refractory immune thrombocytopenia</td>
<td>Amgen</td>
<td>2008</td>
<td>35</td>
<td></td>
</tr>
</tbody>
</table>

Construct, multiple copies of the ligand-binding peptide are fused in series to each C terminus of a human IgG1 Fc dimer. The first c-Mpl-binding peptide is separated from Fc by a five–amino-acid flexible glycine linker. It is followed by another, longer glycine linker before the second copy of the c-Mpl-binding peptide, making it functionally bivalent.

Binding of romiplostim to c-Mpl activates several signaling pathways, including tyrosine phosphorylation of c-Mpl and of proteins in the original transduction pathways JAK, STAT, and MAPK. By design, the active peptide shares no amino acid sequence homology to endogenous TPO, eliminating all risk of eliciting an immune response that cross-neutralizes endogenous TPO. Glycosylation is not required for activity of the peptide-binding moiety, nor is it required for the Fc to bind to FcRn. So romiplostim can be produced by Escherichia coli.

GLYCOSYLATION is not required for activity of the peptide-binding moiety, nor is it required for the Fc to bind to FcRn. So romiplostim can be produced by Escherichia coli.

is composed of the 75-kDa soluble ECD of the TNFα-receptor II (TNFR II) fused to the Fc domain of human IgG1. Developed by Immunex and approved by the FDA in 1998, the drug was acquired by Amgen in 2002. It was the first successful commercial example of a soluble receptor–Fc-fusion protein used as a therapeutic. The drug neutralizes both membrane-bound and soluble forms of TNFα, thus reducing the inflammatory downstream effects of TNFα. Dimeric etanercept binds to trimeric TNFα with a stoichiometry of 1:1 (32). The TNFRII receptor ECD contains both N- and O-linked oligosaccharides.

Etanercept was initially approved for treatment of rheumatoid arthritis (RA); in addition, it has been approved for juvenile idiopathic arthritis, psoriatic arthritis, ankylosing spondylitis, and plaque psoriasis. The drug has had a significant impact in the treatment and management of RA (along with TNFα antagonist MAbs), and it is ranked among the most commercially successful biologics, with 2012 worldwide sales of nearly US$8.4 billion (33).

Subtle binding properties can make a dominating contribution to a molecule’s ultimate activity in a clinical setting, and Fc-fusion proteins and MAbs can differ in this regard. For example, both etanercept and infliximab neutralize soluble TNFα, but only infliximab can induce a clinical response in Crohn’s patients, although both drugs significantly improve RA (34). Direct comparison of the two drugs has shown that only infliximab can bind activated lymphocytes, induce apoptosis, and activate caspase 3 (35). These differences may result from differences in affinity (35) or stoichiometry (25) of the drugs for transmembrane TNFα, which is expressed by activated lymphocytes (35). Scallon et al. suggest that infliximab binds to TNFα with a stoichiometry of 3:1 and that the difference in stoichiometry may be an important factor in the clinical differences seen between the two drugs (32). Hence, designing optimal binding properties of a receptor for its intended
ligand is essential for such a drug to achieve a desired clinical activity.

The clotting factor Fc-fusion protein FIX Fc (Biogen Idec’s Alprolix product) is an enzyme–Fc fusion that was approved in 2014. It is an excellent example of the Fc-fusion protein paradigm used to convert an existing therapy — in this case, a recombinant enzyme limited by its short in vivo half-life — into a second-generation therapy with significantly improved clinical value. FIX plays an essential role in the coagulation cascade. Recombinant FIX was originally licensed in 1997 for treatment of hemophilia B. Because of its relatively short half-life (14–34 hours), frequent repeated intravenous infusions are required for prophylaxis (36).

Developed by Biogen Idec to require less frequent dosing due to an extended half-life, FIX Fc is likely to increase patient compliance and improve clinical outcomes (37). Several characteristics distinguish it from typical Fc-fusion constructs. Only one chain of the Fc dimer is fused to a monomeric FIX construct. They introduced a truncated enzyme. So Biogen Idec developed a recombinant B-domain–deleted factor VIII Fc and found in early work that the homodimer was not well secreted. Generation of the FVIII Fc monomer therefore was enabling to achieve a desired clinical activity.

FIX Fc is an example of the Fc-fusion paradigm being used to convert an existing therapy — an enzyme limited by its short in vivo half life — into a second-generation therapy with greatly IMPROVED clinical value.

### Table 3: Therapeutic Fc-fusion proteins in clinical testing

<table>
<thead>
<tr>
<th>Product</th>
<th>Molecular Construct (Expression System)</th>
<th>Ligand (Clinical Effect)</th>
<th>Company</th>
<th>Clinical Phase</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>trebananib (AMG386)</td>
<td>TIE2 mimetic peptide–IgG1Fc peptibody (E. coli)</td>
<td>Targets and binds to Ang1 and Ang2, prevents interaction with TIE2 receptor (antiangiogenic)</td>
<td>Amgen</td>
<td>Phase 3</td>
<td>62</td>
</tr>
<tr>
<td>blisibimod (A-623, AMG623)</td>
<td>BAFF–IgG 1 Fc (CHO)</td>
<td>Binds to BAFF and inhibits receptor interaction, decreasing B-cell survival (may be effective in SLE and RA)</td>
<td>Anthera and Amgen</td>
<td>Phase 3</td>
<td>63</td>
</tr>
<tr>
<td>dulaglutide (LY2189265)</td>
<td>GLP1 peptide analog–IgG Fc (CHO)</td>
<td>Mimics effects of GLP1 on insulin resistance and VLDL production</td>
<td>Eli Lilly and Company</td>
<td>Phase 3</td>
<td>64</td>
</tr>
<tr>
<td>CNTO 528</td>
<td>erythropoietin-mimetic peptide–IgG1Fc peptibody (CHO)</td>
<td>Binds and activates erythropoietin receptor, stimulating erythrocyte production</td>
<td>Centocor (J&amp;J)</td>
<td>Phase 1</td>
<td>65</td>
</tr>
<tr>
<td>CNTO 530</td>
<td>erythropoietin-mimetic peptide–IgG4Fc peptibody (CHO)</td>
<td>Binds and activates erythropoietin receptor, stimulating erythrocyte production</td>
<td>Edison (J&amp;J)</td>
<td>Phase 1</td>
<td>66</td>
</tr>
<tr>
<td>APG 101 (Apoecept)</td>
<td>CD95–IgG1Fc (CHO)</td>
<td>Blocks CD95 ligand (reducing cancer cell migration in malignant glioma)</td>
<td>Apogenix</td>
<td>Phase 2</td>
<td>67</td>
</tr>
</tbody>
</table>

### Fc-Fusion Proteins in Clinical Development

Fc-fusion proteins in clinical development include enzymes, receptor ECDs, and peptides with antagonist or agonist activities.

Also known as AMG386, the peptibody trebananib is a TIE2 mimetic peptide fused to the C-terminus of IgG Fc. It is produced as a homodimer in E. coli. The drug blocks binding of angiopoietin 1 and 2 to the receptor kinase TIE2, inhibiting angiogenesis in ovarian cancer (41). Developed by Amgen, it is currently in phase 3 clinical testing.

A variation on C-terminal fusion peptibodies, blisibimod (A623) consists of four peptides that bind to B-cell activating factor (BAFF) fused to the N-terminus of a human IgG1 Fc. BAFF is a member of the TNF family and is critical to development, maintenance, and survival of B cells. Blisibimod binds to BAFF and inhibits receptor interaction, decreasing B-cell survival (42). Anthera Pharmaceuticals is currently testing the efficacy of this product in a clinical phase 3 trial for treatment of systemic lupus erythematosus.

Dulaglutide is a glucagon-like peptide (GLP)-1 receptor agonist in which a modified GLP-1 peptide is fused to the N-terminus of a modified IgG4 Fc fragment. Developers at Eli Lilly and Company chose to use Fc-fusion IgG4 to minimize effector function (CDC and ADCC) of the fusion construct. They introduced
several mutations on the peptide to improve stability and solubility while decreasing immunogenicity. And they found that a flexible linker (GGGGS) between the peptide and the Fc domain improved the product’s in vitro activity (43). Phase 3 clinical testing has been completed for Type 2 diabetes treatment, and a marketing application is pending with the FDA.

Another peptibody platform is Centocor’s Mimetibody technology, in which a bioactive peptide sequence is genetically linked to the N-terminus of an Fc domain. The first example of this approach, CNTO 528 combines a 20–amino-acid erythropoietin mimetic peptide 1 (EMP1) — identified using a combinatorial phage library — with the Fc domain of human IgG1. EMP1 binds to and activates the erythropoietin receptor (44). That construct was replaced by CNTO 530, a second-generation Fc-fusion protein with improved biological and biophysical properties (45). But production challenges have been noted with this class of Fc-fusion molecules (46).

APG 101 is a CD95–IgG1 Fc receptor ECD fusion protein under development by Apogenix. It is a homodimer produced in CHO cells. APG101 blocks the CD95 ligand (CD95L, FasL, Apo-1L) from binding to CD95, reducing cancer-cell migration in malignant glioma and preventing early cell death in myelodysplastic syndrome (47). The product is currently in phase 2 clinical testing.

**NOVEL FC-BASED SCAFFOLDS**

The bifunctionality of Fc-fusion proteins can be exploited further. Gillies et al. engineered IL-2–IL-12 and IL-4–GM-CSF bispecific fusions in which the cytokines were positioned in tandem at the C-terminus of Fc (48). In such homodimeric constructs, both cytokine combinations were shown to have synergistic effects as antitumor agents. Moreover, increasing affinity of a binding protein for its target can be achieved using multivalent constructs. Development of heterodimeric Fc platforms based on strand-exchange engineered domains (SEED) — CH3 heterodimers composed of alternating segments of human IgA and IgG CH3 sequences — may allow for the existing homodimeric Fc-fusion platform to extend to including multiple specificities (49).

Multiple avidity has been engineered into Fc fusions to generate both tandem Fc-repeated homodimers (50) and hexameric constructs (51), thus mimicking the structure and therapeutic potential of IgM. A perceived drawback of using polymeric MAb’s, particularly in oncology, is that their large size limits tissue penetration. Although it is true that polymers naturally demonstrate slower penetration times, even intact IgM can reach implanted tumors and metastases in patients after intravenous or intraperitoneal administration (52). Slower penetration and accumulation could be an advantage in directing effector function against tumor cells (53).

Approaches that increase valency may be particularly relevant to the growing number of therapeutic antibody fragments entering the clinic: e.g., dromedary VHH minibodies (54), single-chain Fv-based antihuman-immune-deficiency (HIV) proteins (55), or nonantibody-based protein scaffolds that are characterized by smaller size and cysteine-free sequence (56). IgA and IgM also could serve as alternatives to the classic IgG backbone for therapeutic Fc-fusion proteins (57, 58).

**FC-FUSION PROTEINS AS BIOSIMILARS**

Fc-fusion proteins are just now entering clinical testing, and several clinical trials for biosimilar development are ongoing. Despite the number of MAbs and are manufactured using processes based on the MAb production platform. Fc-fusion proteins are typically homodimers and can be functionally monovalent, bivalent, or multivalent. Newer scaffolds include heterodimers capable of binding to multiple ligands and multimers with increased valency. Of the 46 MAb–based products currently approved by the FDA in the United States, 10 are Fc-fusion proteins. With more in clinical testing and a growing pipeline in preclinical development, the future of this drug class as a path to enable use of biologically active peptides in medicine is bright.

**ACKNOWLEDGMENTS**

The authors acknowledge Laura Shih for her assistance with graphics and have no conflicts of interest in publishing this article herein.
REFERENCES (REPEATED FROM PART 1)


REFERENCES (NEW FOR PART 2)


