Double-CLIPS technology for the mimicry of structurally complex antibody binding sites on proteins
Smeenk, L.E.J.

Citation for published version (APA):
Smeenk, L. E. J. (2013). Double-CLIPS technology for the mimicry of structurally complex antibody binding sites on proteins
MIMICRY OF A DISCONTINUOUS BINDING SITE ON HUMAN VASCULAR ENDOTHELIAL GROWTH FACTOR (hVEGF)

This chapter has been patented in: L. E. J. Smeenk, J. H. Van Maarseveen, N. S. Dailly, P. Timmerman, WO2012057624, 2012
6.1 GENERAL INTRODUCTION

With the promising results obtained for mimicry of discontinuous epitopes on hFSH in chapter 4 and hCG in chapter 5, the possibilities to use double-CLIPS technology to mimic even more challenging discontinuous binding sites, like the one of Bevacizumab (Avastin) on human Vascular Endothelial Growth Factor (hVEGF), is highly interesting. The similarity between hVEGF on one hand and hFSH and hCG on the other is found most evidently in the cys-knot moiety and the equal structure of a discontinuous hVEGF epitope that also consists of two loops, that in this thesis are likewise to hFSH and hCG referred to as β3 and β1-loop (Figure 6.1 A and B).

![Figure 6.1: Three representations of the discontinuous β1/β3 epitope of the hVEGF protein.](image)

Differences, however, are found in the 3D-structure of the proteins. In contrast to the heterodimeric topology of the glycoprotein hormones hFSH and hCG, hVEGF forms an antiparallel homodimer (Figure 6.1 C), covalently linked by two disulfide bridges (between C51 and C60). And unlike hFSH and hCG, a disulfide link between the neighboring loops of hVEGF is not present in the native structure of this protein. Smaller double-CLIPS mimics may therefore require the introduction of a non-native disulfide bond to physically hold the loops in the mimics in close proximity to each other. Such a constraint should be introduced at the most optimal amino acid positions, with ideally the smallest possible disturbance of the overall 3D-structure of the mimics. Further details on the design of similar VEGF-mimics containing an artificially introduced disulfide bond will be discussed in this chapter.
6.2 HUMAN VASCULAR ENDOTHELIAL GROWTH FACTOR (hVEGF)

6.2.1 human Vascular Endothelial Growth Factor
The platelet-derived growth factor (PDGF) family comprises another subfamily of the cysteine-knot growth factor family. PDGF’s are glycoproteins that regulate cell growth and division. In particular, they play a significant role in blood vessel formation, either spontaneous (i.e. vasculogenesis) or from pre-existing vessels (i.e. angiogenesis). The glycoprotein hVEGF is an interesting member of this family from a therapeutic point of view. It is normally expressed when the initiation of new blood vessel growth is required, for example during embryonic development, after muscle injury following exercise, or to bypass blocked blood vessels. The importance of hVEGF in normal blood vessel development is emphasized by the fact that deletion of a single hVEGF allele is already lethal to an embryo in development. Because uncontrolled angiogenesis is a characteristic of several forms of cancer, the hVEGF protein is a very interesting target for anticancer therapies. Several methods have been developed in treating cancer patients by blocking VEGF production or binding with its receptor. For example, in 1993, anti-peptide antibodies were shown to suppress tumor growth in vivo by blocking hVEGF and later, a DNA vaccine against the receptor hVEGF-R-2 was applied for active immunization to block angiogenesis, published by the groups of Niethammer and Li in 2002 and the group of Feng in 2004. Recently, toxic drugs such as tyrosine kinase receptor inhibitors were used in clinical trials, together with soluble receptors, VEGF-derived immunogens or mAbs against VEGF or its receptors. One such an antibody (Bevacizumab), reported by Ferrara et al., became the first commercially available angiogenesis inhibitor. It was approved for the treatment of breast, colorectal, renal cell and non-small-cell lung cancer in combination with cytotoxic chemotherapy, increasing the overall survival of cancer patients. Due to its dual effect of decreasing the formation of functional micro vascular networks of tumors (i.e. tumor starving) and blocking the maturation-inhibiting activity of hVEGF, a decrease in regulatory T-cells was observed, thus exerting an effect on the immune system. However, the expensive and cumbersome therapy of mAbs, together with the sometimes revealed side effects of their treatment, encourage the investigation of alternative strategies to block VEGF overproduction, like active immunization by anti-VEGF immunogens, for which preliminary results will be discussed in this chapter.

6.2.2 Anti-hVEGF Monoclonal Antibodies (mAbs) 293 and Bevacizumab (Avastin)
Two monoclonal antibodies 293 and Bevacizumab (hereafter named ‘Avastin’, being the trade name by Genentech/Roche) were selected for binding studies in order to monitor the success in the manufacturing of double-CLIPS mimics of hVEGF. The epitope of mAb 293 on hVEGF has never been clarified in detail, but is known to bind for a large part to the longer β3-loop of the protein. This in contrast to Avastin, in which case binding to hVEGF was clarified by X-ray. This data shows that Avastin evidently binds to a discontinuous epitope consisting of at least the β1/β3-loops on hVEGF and therefore is mainly used in this study to elucidate the resemblance of our hVEGF-mimics with the native hVEGF protein.
6.2.3 Structural Design of Double-CLIPS hVEGF-Mimics

The X-ray structure of hVEGF in complex with bevacizumab is shown in Figure 6.2 A-C, providing detailed information about the binding site of this humanized antibody on hVEGF. Direct interaction between the β3-loop and Avastin is clearly illustrated by a space-filling model that shows how the top of the β3-loop is completely engulfed in the binding “pocket” of the antibody (Figure 6.2 B). In addition, several hydrogen bonds are observed between the β3-loop residues G89-M94 and the Fab fragment (Figure 6.2 C). Investigation of the drop in binding affinities in alanine mutation studies of hVEGF in complex with Avastin by Muller et al.30 provided strong evidence that the β1-loop not only contributes to Avastin-binding in a direct way, but also in an indirect manner by stabilizing the strand-turn-strand structure of the β3-loop. This could be deduced from the numerous hydrogen bonds and short-range electrostatic interactions that exist between the β1-loop and the β3-loop, concluded from decreased binding affinities of mutants of hVEGF with A-substitutions: replacing the β3-loop positions M81, R82, I83, G88, Gln89, and Q92 for alanine residues showed a 20-100 fold decreases in affinity relative to the wild type and a much smaller but significant (5–8 fold) decreases in binding was found for mutations at the β1-loop positions Y45 and K48. This confirmed the assumption that an additional positive contribution of the β1-loop within double-CLIPS mimics is very likely to be observed in binding with Avastin.

![Figure 6.2](image_url)

**Figure 6.2:** X-ray structure of one monomer of hVEGF in complex to its antibody mAb Avastin (purple). (A) Structure indicating the β1 (green), β2 (yellow), β3 (blue), the N- and C-terminus (in red) of hVEGF. (B) Space-filling model. (C) Representation of the hydrogen bonds between residues of hVEGF and mAb Avastin.

Mimicry of the Avastin epitope on hVEGF using double-CLIPS compounds thus can become highly challenging and probably relies mainly on interactions between the residues within these loops in order to (spontaneously) form an optimal mimic. Design of the first hVEGF-mimic VEGF I (Figure 6.4) was mostly determined from X-ray data: The positions where the amino acids were replaced by cysteine within the scaffolded β1- and β3-loops were carefully chosen to involve residues pointing towards each other in the natural structure, being the red-colored amino acids M78 and M94 on the β3-loop and V33 and V52 on the β1-loop, shown in Figure 6.3. Note that in contrast to all previous mimics described for
hFSH and hCG, the mimic VEGF I presents a much longer β1-loop (20-mer) as compared to the β3-loop (17-mer) in order to retain the best scaffold position as close as possible to each other (see also Figure 6.1 A-C). Moreover, the absence of a natural covalent constraint on top of the β1/β3-loops makes the synthesis of mimics even more challenging. To ensure a positioning of both loops in close proximity, a non-natural disulfide link was introduced in some of the mimics by replacement of two amino acids on both the β1 and β3-loop for cysteine residues. As illustrated in orange in Figure 6.3, the glutamine residue 44 of the β1-loop and the lysine residue 84 of the β3-loop represent two amino acids that are located relatively close to each other and are pointing in the same direction. In addition, they are not highly essential for Avastin binding compared to a few other residues discussed above and may therefore be replaced by cysteine residues in order to introduce a disulfide bond between these residues without suppressing the binding towards the antibody too much. This resulted in the synthesis of VEGF II, as shown in Figure 6.4.

![Figure 6.3](image1.png)

**Figure 6.3:** X-ray structure of the β1/β3-entity of hVEGF, illustrating cysteine scaffold positions (red) and alternative cysteine positions for disulfide introduction (orange).

![Figure 6.4](image2.png)

**Figure 6.4:** Schematic representation of two candidate hVEGF epitope mimics VEGF II and VEGF III and their reference compounds VEGF-β3 4g, VEGF-β3 8g, VEGF 8f-8g, and VEGF I.
Another alternative to improve epitope mimicry for hVEGF was envisioned by applying longer peptide sequences, like in VEGF 8f-8g. These can have both a positive effect on the organization of the loops (i.e. by presenting more residues and thus more non-covalent connection points to induce the native structure of the protein) or a negative effect on the correct orientation of the loops (i.e. becoming too flexible when the number of amino acids is increased). Therefore, introduction of an extra constraint at the bottom of the loops was also investigated by increasing the lengths of the β1/β3-loops sufficiently far as to include the upper native disulfide bond of the original cys-knot fold (C_{57} - C_{102}) at the very bottom of this double-CLIPS mimic VEGF III (Figure 6.4).
6.3 ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA)

6.3.1 Evaluation of Discontinuous Binding Site Mimics for mAb 293

In order to investigate the additional benefits of the second β1-loop when attached to the β3-loop of hVEGF for binding to mAb 293 in ELISA, we first studied the double-CLIPS construct VEGF I and its single β3-loop variant VEGF-β3 4g. The improved titer of VEGF I compared to the single β3-loop was not extreme (Figure 6.5), though similar effects were earlier seen in the cases of mAb 5828/6602 on hFSH and mAb 3468 on hCG for the double-CLIPS mimics lacking a disulfide bond. In those cases, binding was enhanced mostly when the natural disulfide bond was added between the loops to enhance their topologies. Since no natural disulfide bond between the β1 and β3-loops is present in native hVEGF, this bond was initially not introduced in VEGF I. Now, based on the earlier results, an unnatural disulfide bond was added in the hVEGF mimic VEGF II. However, as can be seen from Figure 6.6, the addition of this constraint did not enhance the recognition of VEGF II by mAb 293 to any extent (titer of 6 μg/ml for VEGF II versus 4 μg/ml for VEGF I).

![Figure 6.5: Binding ELISA data for mAb 293 of double-CLIPS mimic VEGF I and the β3-loop VEGF-β3 4g.](image)

![Figure 6.6: Binding ELISA data for mAb 293. Left: binding of the double-CLIPS mimics VEGF I and VEGF II. Right: binding of a single loop (VEGF-β3 8g), a double-CLIPS (VEGF 8f-8g) and a disulfide-constrained double-CLIPS mimic (VEGF III).](image)

In an effort to further enhance the binding, we investigated several mimics with elongated β1- and β3-loops (e.g. VEGF-β3 8g, VEGF 8f-8g and VEGF III). The synthesis of the 32-mer β1-loop and 35-mer β3-loop turned out to be much more efficient as compared to the equally-sized loops of hFSH and hCG, which allowed us to examine whether the upper disulfide bond of the cys-knot motif could further assist in organizing the loops in the mimics (i.e. VEGF III). Much to our surprise, it was found that no significant beneficial binding was observed for mAb 293 in binding to the double-CLIPS VEGF-mimics 8f-8g and VEGF III (titer > 10.000) as compared to the shorter mimic VEGF I (titer 4283 ng/ml).
Even more remarkably, the control experiment presenting the corresponding single β3-loop (VEGF-β3 8g) revealed a higher titer (425 ng/ml) for mAb 293 than for any of the double-CLIPS mimics tested so far (Figure 6.6). Clearly, the epitope of mAb 293 is located mostly on the β3-loop, because this single entity binds the antibody much better than in the presence of a second loop. In addition to this, it was observed that the attachment of a second flexible β1-loop even disturbed the topology of the β3-loop, resulting in decreased recognition towards mAb 293 when double-CLIPS mimics are presented on the surface.

6.3.2 Evaluation of Discontinuous Binding Site Mimics for mAb Avastin

In the case of Avastin, the single β3-loop VEGF-β3 4g was already recognized better that the double-CLIPS mimic VEGF I, implicating that for this antibody the additionally presented β1-loop in VEGF I only influenced the binding negatively instead of improving the recognition (Figure 6.7).

Figure 6.7: Binding ELISA data for mAb Avastin of double-CLIPS mimic VEGF I and the β3-loop VEGF-β3 4g.

In contrast to mAb 293, the addition of a non-native disulfide bond between the β1- and β3-loop in VEGF II now gave a strongly improved recognition of Avastin (Figure 6.8), showing a titer of 833 ng/ml, only a factor of 10 less then native hVEGF (83 ng/ml).

Figure 6.8: Binding ELISA data for mAb Avastin. Left: binding of the double-CLIPS mimics VEGF I and VEGF II. Right: binding of a single loop (VEGF-β3 8g), a double-CLIPS (VEGF 8f-8g) and a disulfide-constrained double-CLIPS mimic (VEGF III).

These results encouraged us to further enhance binding of VEGF mimics with elongated β1- and β3-loops as presented in VEGF 8f-8g and VEGF III. Unfortunately, the potentially too unstructured mimics VEGF 8f-8g and VEGF III were not recognized any better than
VEGF I, indicating that increasing the peptide lengths for recognition to Avastin itself was not sufficient to mimic the epitope better (Figure 6.8).

From these results, it was concluded that the presented mimics of the epitope of Avastin on hVEGF may require a more advanced strategy, or combinations of the ones presented until now, in order to provide stronger binding titers. Notably, in case of binding to Avastin, VEGF II showed more promising results than the single β3-loop, illustrating that possible improvement of hVEGF mimics may be found when the results of VEGF II and III are combined, by additionally constraining the loops of VEGF III with a disulfide bond as presented in VEGF II. It seems that the difficulties observed for designing successful hVEGF mimics basically have a structural origin, because the use of elongated peptide loops without an additional constraint (i.e. VEGF 8f-8g) did not significantly enhance antibody binding, while at the same time the use of shortened double-CLIPS fragments (i.e. VEGF I) showed only decreased binding as compared to the single β3-loop variants. Therefore, increased recognition is to be expected when using the elongated that have additional constraint built into the loops. This could, for example, be achieved when the additional non-native disulfide bond is introduced in the longer VEGF III mimic in a similar way as it was introduced in VEGF II. It is expected that the designed mimic VEGF IV pre-organizes the individual loops towards the native structure, while the disulfide bond located just above the CLIPS-scaffolds “binds its legs together”, thus creating a sufficiently well-organized mimic that should bind much better to Avastin. As an alternative, other positions at the loops can be used to connect the CLIPS-scaffolds, like in VEGF-mimic V, that is also expected to result in enhanced Avastin-binding.

In addition to this, the epitope of Avastin may not be purely based on the β1/β3-entity alone, since the N-terminus of the second monomeric fragment in hVEGF seems also to be involved in the interaction with Avastin, as shown in Figure 6.2. In the future, it would be highly interesting to synthesize the proposed alternative mimics and test them for binding with Avastin, or to investigate the effects of introducing a third entity to both loops, as will be discussed in the Appendix to this thesis.
6.4 SUMMARY

Mimicry of the highly challenging discontinuous β1/β3-loop epitope on hVEGF was the general topic of this chapter. The monoclonal antibodies 293 and Avastin were included in these studies to compare the recognition of the potential hVEGF mimics VEGF I-III and the single β3-loop controls. The epitope on hVEGF differs mostly from those on hFSH and hCG by the absence of a native disulfide bond covalently connecting the loops that forces the loops into an aligned orientation. The design of mimic VEGF I was purely based on X-ray data in combination with hFSH- and hCG-data for the best scaffold positions, but did not show appropriate antibody binding. Recognition of mAb 293 was optimal for VEGF-β3 loop 8g (35-mer; titer 425 ng/ml), while addition of the corresponding β1-loop, as in double-CLIPS mimics VEGF 8f-8g and I-III, only reduced binding towards this antibody (Table 6.1). In sharp contrast to this, binding of Avastin to the β3-loop mimic 8g was almost 10-fold improved upon linking an additional β1-loop as in VEGF-mimic II (titer for 8g and II was 5810 and 833 ng/ml, respectively). The increase was only observed when the top of the loops were constrained with a non-native disulfide bond (as in VEGF II) while increasing just the loop lengths (as in VEGF 8f-8g) or introduction of a natural disulfide bond at the bottom of the elongated loops (as in VEGF III) only reduced binding.

<table>
<thead>
<tr>
<th>Compound</th>
<th>mAb 293 titer (ng/ml)</th>
<th>mAb Avastin titer (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hVEGF</td>
<td>24.26</td>
<td>82.82</td>
</tr>
<tr>
<td>VEGF-β3 4g</td>
<td>&gt;10000</td>
<td>4844</td>
</tr>
<tr>
<td>VEGF-β3 8g</td>
<td>425.4</td>
<td>5810</td>
</tr>
<tr>
<td>VEGF 8f-8g</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>VEGF I</td>
<td>4283</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>VEGF II</td>
<td>6347</td>
<td>833.2</td>
</tr>
<tr>
<td>VEGF III</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
</tr>
</tbody>
</table>

As can be seen from Table 6.1, the titers of VEGF II for Avastin are approaching that hVEGF and only differ by a factor of ~10. This strongly encourages investigation the combination of multiple disulfide constraints to further support the mimics to adopt a native-like VEGF structure, and in this way to design fully optimized hVEGF-mimics with recognition for Avastin approaching that of native hVEGF.
6.5 EXPERIMENTAL SECTION

**Binding Studies in ELISA**
Polystyrene 96-well plates (Greiner, Germany) were treated with 100 µl/well of 0.2% glutaric dialdehyde in phosphate-buffer (0.1 M, pH 5) for 4 h at room temperature while shaking, followed by washing (3x10 min) with phosphate-buffer (0.1 M, pH 8). Then, the wells were coated with 100 µl/well of a 1 µg/ml solution of hVEGF or a 10 µg/ml solution of hVEGF-mimic in phosphate-buffer (0.1 M, pH 8) overnight at 37 °C. After washing with 1% Tween-80 (3x) and stabilizing in horse serum (4% in PBS/1% Tween-80/3% NaCl) for 30 min, the plates were incubated with antibody (293 or Avastin) at various dilutions, starting with a standard antibody dilution of 3 µg/ml in the first well and threefold dilution steps in subsequent wells. Incubation was performed for 1 h at 37 °C, followed by washing with 1% Tween-80 (3x). Then, the plates were incubated with 100 µl/well of peroxidase-labeled rabbit-anti-mouse serum (1/1000 dilution in 4% horse serum, see above) for 1 h at 25 °C, followed by washing with 1% Tween-80 (4x). Finally, the plates were incubated with a 0.5 µg/ml solution of ABTS (2,2’-azinid(e)ethylbenzthiazolesulfonate) containing 0.006% H_2O_2 in citric acid/phosphate-buffer (0.1M each, pH 4). OD_{405nm}-values (AU) were measured after 45 min standing at room temperature in the dark.
6.6 REFERENCES

