



RESEARCH ARTICLE

Efficacy of aflibercept (EYLEA®) on inhibition of human VEGF *in vitro*

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ARTICLE INFO

Article history:

Received 12 January 2017

Received in revised form 9 February 2017

Accepted 14 February 2017

Keywords:

EYLEA®

HUVEC

Aflibercept

VEGF

VEGF-trap

ABSTRACT

Introduction: Pathological formation of blood vessels plays a key role in the growth and metastasis of tumors and also in several serious ophthalmological diseases such as wet age-related macular degeneration (AMD) or diabetic retinopathy. In AMD treatment, aflibercept (tradename EYLEA®) is used to deactivate the underlying pathological neovascularisation. Aflibercept is a recombinant fusion protein which binds to vascular endothelial growth factor (VEGF) receptors, thereby inhibiting VEGF pathway activation. VEGF is one of the most important angiogenesis factors.

Objective: This analysis investigates lasting efficacy of aflibercept *in vitro* for later application as therapeutic agent against macular degeneration (AMD).

Material and methods: VEGF-ELISA assays were performed to investigate binding affinities at different aflibercept concentrations. The impact of VEGF on the proliferation of human umbilical vein endothelial cells (HUVEC) was investigated using proliferation assays. Moreover, time-dependent kinetic studies were performed to analyze different aflibercept storage durations with regard to its inhibitory capabilities on human VEGF.

Results and conclusion: Our results reveal that aflibercept significantly lowers the amount of unbound VEGF as well as the proliferation rate of HUVEC. Moreover, in contrast to specifications given by the manufacturer, aflibercept retains its full inhibitory effect up to at least 120 h after transference from the original vial into the injection syringe.

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1. Introduction

In the course of embryonic development and during wound healing, vascular endothelial growth factor (VEGF) is one of the most important factors for *de novo*-formation of new blood vessels (Bao et al., 2009). Besides endothelial cells, it is also produced by a range of other cell types such as fibroblasts (Nissen et al., 1998), neutrophils (Gaudry et al., 1997) and macrophages (Berse et al., 1992). As a platelet derived growth factor (PDGF) homologue, VEGF is involved in a variety of processes relating to wound healing. As previously illustrated for pathophysiological processes during tumor expansion, cancer cells are also challenged by hypoxia, which causes the overexpression of HIF1 α and, in the course, the release of VEGF. Apart from the prominent example of cancer pathophysiology, abnormal angiogenesis also occurs in several

ophthalmological diseases, such as diabetic macular edema (DME) or age-related macular degeneration (AMD). AMD is characterized by damage to the retinal epithelium due to chronic inflammation. This process can, for instance, be enhanced by a genetic defect involving ocular collagen or elastic fibers (Marneros et al., 2007). In advanced AMD, referred to as wet or exudative AMD, choroidal neovascularisation (CNV) occurs along with a higher expression of VEGF. At this stage there is a risk that newly formed choroidal microvessels penetrate Bruch's membrane resulting in subretinal fluid accumulation, lipid deposition, hemorrhage, retinal pigment epithelium detachment and fibrosis. Severe visual loss or sudden and irreversible blindness can be the consequence (Jager et al., 2008).

One promising approach in cancer research is to prevent the tumor from growing and expanding by reducing its oxygen and nutrient supply. Beginning at a certain size, neoplastic tissue relies on the development of new blood vessels for its nutritional supply. Consequently, one therapeutic aim has been the development of new antiangiogenic agents involving the VEGF pathway as a potential target. Aflibercept binds to VEGF at a much higher affinity

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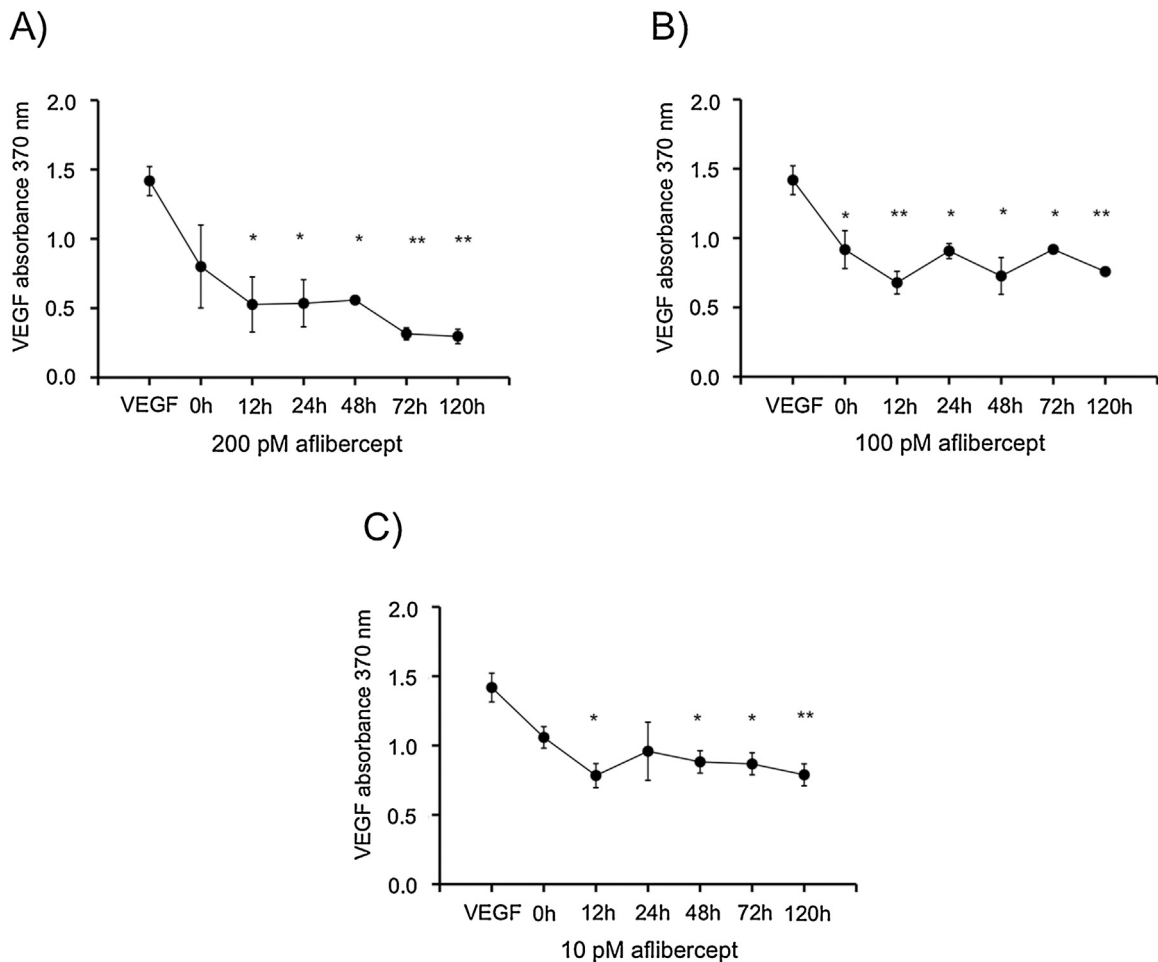


Fig. 1. Proliferation and inhibitory properties of aflibercept.

BrdU-graph of HUVEC cells with aflibercept concentrations of 200 pM (A), 100 pM (B) and 10 pM (C) plotting the VEGF absorbance at 370 nm against the storage times of aflibercept. *p < 0.05; **p < 0.01 using the t-test for two samples. The diagram represents the mean of four samples at the different storage times and the corresponding SEM. VEGF = as control.

than natural VEGF receptors (VEGF-trap) (Balaratnasingam et al., 2015). Working as a competitive antagonist, aflibercept prevents dimerization and autophosphorylation of VEGF receptors (VEGFR), thereby inhibiting the signaling cascade that normally leads to the transcription of proangiogenic factors.

Not only cancer therapy can benefit from a VEGF-trap. As already mentioned above, several ophthalmological diseases such as diabetic retinopathy or wet AMD are characterized by neovascularisation (Adamis et al., 1994). Thus, aflibercept attracted notice in treatment of these ophthalmological diseases, and, after its licensing in November 2012, has become the mainstay in the treatment of wet AMD. Furthermore, aflibercept has exhibited a favorable safety profile in clinical trials, as serious ocular adverse events have been only infrequently observed (McKibbin et al., 2015). Besides its application in the treatment of wet AMD, aflibercept therapy is also used in macular edema secondary to retinal vein occlusions, impaired vision caused by diabetic macular edema or by myopic choroidal neovascularization. For these purposes, aflibercept is injected intravitreally by an ophthalmologist, where it is expected to decrease *de novo* formation of blood vessels. Phase II studies analyzing the influence of aflibercept on wet AMD have shown improvements in visual acuity (Press Release of Regeneron, 2007). Nevertheless, VEGF inhibitors, such as aflibercept, are neither able to permanently prevent pathological neovascularization nor to cure wet AMD. Rather, they help to minimize the structural damage of the

macula by limiting the degenerative phase of the disease (Jager et al., 2008).

The aim of the present study has been to analyze the efficacy of various aflibercept concentrations to inhibit VEGF. Furthermore, we performed time dependent kinetics to determine the storage stability of aflibercept after opening the manufacturer's original vial.

2. Material and methods

2.1. Cell lines

For *in vitro* investigations, an immortalized human umbilical vein endothelial cell line (HUVEC-Tert) from the Department of Surgery, Division of Molecular and Experimental Surgery, Friedrich-Alexander-University Erlangen-Nürnberg (E. Naschberger) was used. HUVEC cells are a human vascular endothelial cell model which is frequently used to study VEGF dependent angiogenesis and tumorigenesis *in vitro*.

2.2. Cultivation of HUVEC

Cells were cultured under standard conditions (37 °C, 21% O₂, 5% CO₂) as described before (Schoppmann et al., 2004). In brief, HUVEC were cultivated in serum-containing medium (500 ml medium 200 (ThermoFischer Scientific, Germany)) containing 10 ml low serum

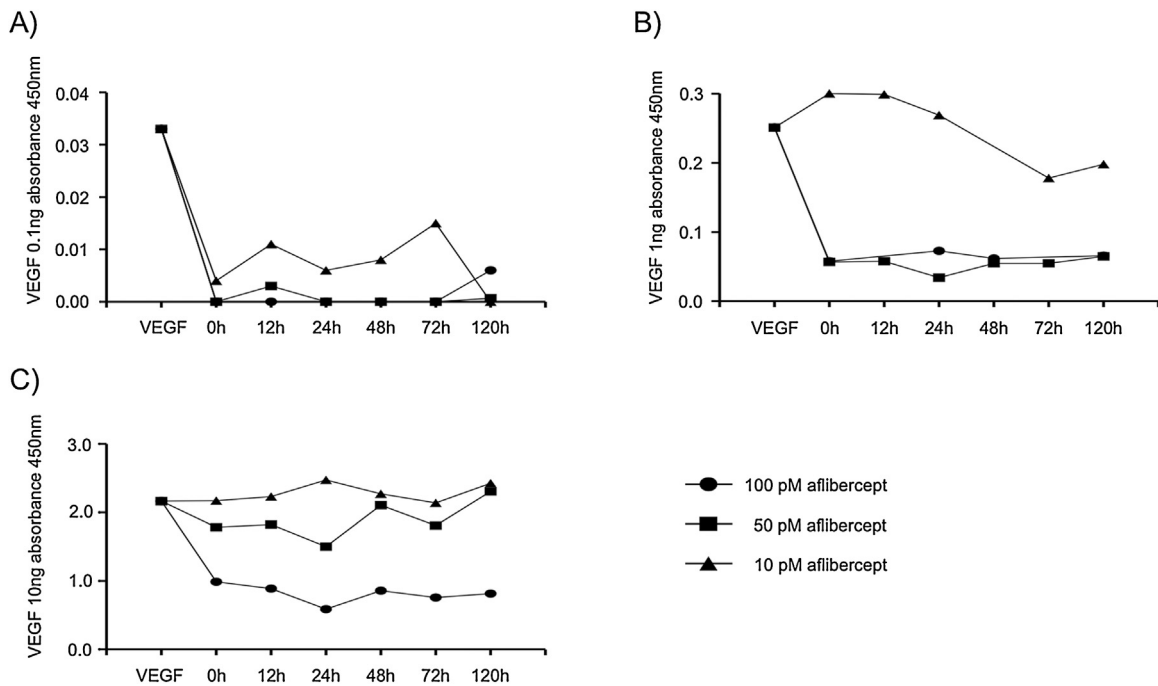


Fig. 2. ELISA—binding affinity of aflibercept.

Graphs of the three aflibercept concentrations plotting the VEGF absorbance at 450 nm against the respective storage time of aflibercept. (A) with 0.1 ng/ml VEGF, (B) with 1 ng/ml VEGF and (C) with 10 ng/ml VEGF. VEGF= as control.

growth supplement (LSGS, ThermoFischer Scientific, Germany). At confluence, which was determined by light microscopy, cells were passaged or frozen for storage in liquid nitrogen. For this purpose, cells had to be washed with 1–2 ml of sterile 1x PBS before splitting. Cells were subsequently detached with 6 ml trypsin-EDTA (1 ml trypsin-EDTA per 25 square-millimeter of the tissue culture flask) for 5 min in an incubator. After cellular detachment, trypsin digestion was stopped by using at least the double amount of FCS containing medium.

2.3. Pharmaceuticals

EYLEA® (BAYER AG, Leverkusen, Germany) containing the active substance aflibercept (also known as VEGF-trap) was used for subsequent experiments. To apply the drug intravitreally, aflibercept normally is delivered in a special injection vial with an injection needle. For the experiment, six original vials containing aflibercept were opened before use at different time points (0 h, 12 h, 24 h, 48 h, 72 h, 120 h) and stored at 4 °C. The drug was drawn up from the vial in a sterile injection syringe using the injection needle. To avoid crystallization and contamination, the needle was removed and the syringe containing the drug was stored at 4 °C up to 120 h. Briefly, before starting the experiment, the aflibercept solution was transferred into sterile reaction tubes, which were kept on ice.

2.4. Cell proliferation assay

In order to determine cell proliferation of stimulated cells, BrdU labelling (5-bromo-2'-deoxyuridine) was directly applied to cultured cells as described previously by [Schroeder et al. \(2016\)](#). HUVECs were incubated with a solution containing 1 ng/ml recombinant human VEGF (rhVEGF₁₆₅) (293-VE; R&D Systems, USA) as well as aflibercept at concentrations of 10 pM, 100 pM or 200 pM. Furthermore, the six different storage durations were examined performing this procedure. Cell proliferation was determined by

means of enzyme linked immunosorbent assay (ELISA) with a CLARIOstar ELISA reader (BMG Labtech, Germany).

2.5. Human VEGF-ELISA

To detect unbound VEGF, a human VEGF Quantikine ELISA Kit (DVE00; R&D Systems, USA) was used according to specifications given by the manufacturer. Determination of time-dependent VEGF kinetics by ELISA were performed using three different aflibercept concentrations: 10 pM, 50 pM and 100 pM. Furthermore, each of the three aflibercept concentrations was treated with one of the three varying rhVEGF₁₆₅ (293-VE; R&D Systems, USA) concentrations: 0.1 ng/ml, 1 ng/ml and 10 ng/ml. This strategy was used to determine whether the potential of aflibercept was sufficient to inhibit binding of even high rhVEGF₁₆₅ concentrations. The amount of unbound rhVEGF₁₆₅ was determined by ELISA using the ELISA reader (CLARIOstar, BMG Labtech).

2.6. Statistics

Results were evaluated using computer software GraphPad Prism V5 (GraphPad, La Jolla, CA, USA). This was done by calculating mean and standard error (SEM) for values. Kruskal–Wallis and t-test for two samples (each storage time of aflibercept was compared to the negative control) served as statistical tools for determination of p value.

3. Results

3.1. Proliferation (BrdU) assay

The time kinetic proliferation assay (0, 12, 48, 72 and 120 h) of stimulated HUVEC cells shows that the proliferation rate is significantly decreased after using aflibercept concentrations of 10 pM, 100 pM and 200 pM compared to the mean absorptions of rhVEGF₁₆₅ without aflibercept ([Fig. 1A–C](#)).

Fig. 1C demonstrates that even low concentrations of aflibercept are able to decrease the proliferation of HUVECs *in vitro* at all investigated time points (12, 48, 72 and 120 h).

3.2. Human VEGF ELISA—binding assay

Fig. 2A displays the chart for the lowest rhuVEGF₁₆₅ concentration ($c = 0.1$ ng/ml). The results ($c = 0.1$ ng/ml) show that samples with 100 pM of the VEGF-trap aflibercept still show obviously smaller amounts of unbound VEGF than pure-rhuVEGF₁₆₅ samples.

Results for rhuVEGF₁₆₅ (1.0 ng/ml) show that samples with 100 pM and 50 pM of VEGF-trap reveal obviously smaller amounts of unbound rhuVEGF₁₆₅ than pure-rhuVEGF₁₆₅ samples (cf. Fig. 2B). In contrast, 10 pM aflibercept increases the VEGF absorbance compared to the negative control even after 24 h. Only absorbance values at 72 and 120 h aflibercept-storage-time were slightly lower.

Regarding the chart of the highest rhuVEGF₁₆₅ concentration (10 ng/ml) in Fig. 2C absorption of aflibercept samples differed less from the negative control than it was recognized for the preceding example. Nevertheless, still a difference was apparent, especially in view of the 100 pM aflibercept concentration. However, as it turned out for the two analyzed rhuVEGF₁₆₅ concentrations, no change of the inhibitory effect of aflibercept could be observed after different storage times up to 120 h.

In summary, absorbance values of the samples, which were treated with aflibercept concentrations of 100 pM and 50 pM, were consistently lower compared to the negative control. This tendency could be noticed for each of the three rhuVEGF₁₆₅ concentrations. For 10 pM of aflibercept lower absorptions were only attained by employing a very low VEGF₁₆₅ concentration.

4. Discussion

The recombinant fusion protein aflibercept is used for the treatment of distinct ophthalmological diseases such as wet AMD. As Holash et al. demonstrated using a VEGF-trap-binding assay that aflibercept functions as a potent VEGF blocker (Holash et al., 2002). Based on these findings we analyzed the inhibitory effect of aflibercept up to a storage period of 120 h after opening the manufacturer's original vial and transferring the drug to a sterile injection syringe. The performed human VEGF-ELISA revealed that aflibercept significantly lowers the amount of unbound VEGF for rhuVEGF₁₆₅ concentrations of 0.1 ng/ml, 1 ng/ml and 10 ng/ml using a 100 pM dose of aflibercept. Using a minimal rhuVEGF₁₆₅ concentration of 0.1 ng/ml, an aflibercept concentration of 10 pM was sufficient to functionally inhibit VEGF. For rhuVEGF₁₆₅ concentrations of 1 ng/ml and 10 ng/ml, results of absorbance measurements demonstrate that VEGF was completely captured by the decoy inhibitor. For a rhuVEGF₁₆₅ concentration of 1.0 ng/ml above a dose of 50 pM aflibercept an obvious inhibitory effect of the trap was still apparent. Even 10 ng/ml of rhuVEGF₁₆₅ could apparently be inhibited by an aflibercept concentration of 100 pM. In addition, and most importantly, none of the results revealed a loss of the inhibitory activity of aflibercept, as the manufacturer's specifications claim. An equally strong decrease of the rhuVEGF₁₆₅ amount caused by aflibercept could be demonstrated, regardless of whether the drug was used immediately or was first stored for up to 120 h outside the manufacturer's original vial.

BrdU assay confirmed these findings. The measured absorbance reflects the amount of proliferating cells; i.e. low absorbance values reflect a strong inhibition of proliferation. With regard to HUVEC, a concentration of 200 pM aflibercept resulted in a significantly reduced proliferation rate of the cells. Hence, it can be concluded that this dose of VEGF-trap inhibits binding of VEGF to its natural receptors. For lower concentrations of aflibercept (100 pM

and 10 pM) the inhibitory effect on VEGF could be shown as well, even though not in a comparative intensity. As already revealed by ELISA, here again no differences concerning the inhibitory potential of aflibercept comparing the storage times were detected. Even 200 pM of aflibercept was able to significantly decrease the proliferation rate of HUVEC for all investigated storage times of the drug up to 120 h. Very low concentrations of 100 pM and 10 pM aflibercept still reveal sufficient capacity to inhibit VEGF even after 120 h of storage time, although the proliferation rate of the cells was not decreased as strongly as using 200 pM aflibercept.

In conclusion, our results reveal that aflibercept is a very potent VEGF inhibitor, efficient at concentration magnitudes lower than those published by the manufacturer. Moreover, and, this is the most important finding with regard to the practical and economical application of the pharmaceutical, aflibercept remains its inhibitory activity even after a storage period of 120 h. This is in strong contrast to the manufacturer's specifications that claim only a maximum of 6 h of stability of aflibercept after transferring it to the injection syringe.

Author contributions

M.S., W.L. and L.B. contributed to the conception and design of the study. M.S., K.H. performed the experiments. E.N. and W.L. provided HUVEC and regarding buffers. M.S., K.H., W.L., H. S., F.P. and L.B. contributed to the analysis and interpretation of data. All approved the submitted manuscript.

Acknowledgments

We thank Anke Fischer-Gößwein, Maïke Hemmerlein and Jessica Braun for their excellent laboratory support. This work was supported by the Ernst und Berta Grimmke Stiftung (Program Grant 1/15).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aanat.2017.02.005>.

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