Characterization of tryptophan-containing dipeptides for anti-angiogenic effects

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Abstract
Aims: In the pathogenesis of several diseases, neo-angiogenesis is increased (e.g. tumour growth). The peptide L-glutamyl-L-tryptophan (EW/IM862) has been claimed to exhibit inhibitory effects on tumour growth in vivo. However, the potential role of natural peptides with respect to anti-angiogenic properties is unsettled. The current study explores anti-angiogenic effects of the dipeptides WL, EW, IW and WE.

Methods and Results: Using a bottom-up strategy, we first evaluated the effects of the peptides on VEGFR-2 signalling and quantified their effects in different angiogenesis assays. WL consistently had the strongest effects on phosphorylation of VEGFR-2 and downstream signalling. Therefore, this peptide was chosen in comparison with EW to further assess anti-angiogenic properties. However, sprout formation in three-dimensional (3D) fibrin gel bead assay was significantly inhibited by EW only. Furthermore, vessel sprouting in the mouse aortic ring assay was decreased by the presence of WL and EW compared to control. Results from a chorioallantoic membrane assay showed that under vascular endothelial growth factor (VEGF) stimulation WL and EW decreased the number of blood vessels versus control. These results were in line with those obtained in a matrigel plug assay. The VEGF-induced increase in the haemoglobin content was nearly abolished when treatment was combined with either WL or EW application. In the murine model of oxygen-induced retinopathy, WL exhibited a small albeit significant anti-angiogenic effect.

Conclusion: Comprehensive screening of WL suggests an anti-angiogenic effect, demonstrated in in vitro, ex vivo and in vivo models. Thus, WL is a dipeptide with potential anti-angiogenic effects and is worthy for further exploration.

Keywords
angiogenesis, anti-angiogenic peptides, EW, tryptophan-containing peptides, WL
INTRODUCTION

Formation of blood vessels from pre-existing ones (i.e., angiogenesis) is a well-established influencing factor for many physiological as well as pathological processes. In adults and under normal conditions, it is a tightly regulated process underpinned by the balance between pro- and anti-angiogenic factors. In a limited number of physiological conditions, the balance can deviate toward enhanced angiogenesis, for example, during wound healing, proliferation of the uterine mucous membrane during the menstrual cycle, and placenta formation during pregnancy. On the contrary, a plethora of pathological conditions is associated with a shift of vessel remodelling towards the pro-angiogenic side, a condition denoted as angiogenic switch. The angiogenic switch is an indispensable step in the pathogenesis of many diseases, for example, psoriasis, rheumatoid arthritis, atherosclerosis, diabetic retinopathy, retinopathy of prematurity (ROP), age-related macular degeneration (AMD), tumour growth and metastasis.

One of the major regulators of angiogenesis as well as vasculogenesis is the vascular endothelial growth factor (VEGF) family, which comprises seven members (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and PIGF). Under pathological conditions, VEGFs promote an abnormal blood vessel phenotype and act as survival factors for endothelial cells in pathological blood vessel development. However, under physiological conditions the vasculature is largely independent of VEGFs for survival and stability. Thus, blocking the action of VEGFs under pathological conditions would restore the balance between the pro- and anti-angiogenic factors back to normal. In fact, VEGF-A neutralizing monoclonal antibodies, which block VEGF-A from binding to its receptors, have shown an impressive inhibitory effect on tumour growth and are used in the treatment of AMD. However, it should be noted that hypertension, bleeding and asymptomatic proteinuria are common side effects for such treatments. Additionally, because of the potential risks to the foetus, an anti-VEGF treatment is not recommended during pregnancy. Another effective treatment option for the prevention of pathological vessel growth are VEGF-receptor tyrosine kinase inhibitors, for example, SU5416. These inhibitors interfere with the VEGF signal transduction pathway. Again, side effects, for example, headache and thromboembolic occurrences, limit its usage. Thus, searching for anti-angiogenic therapy with fewer risks and minimal or no side effects remains a tempting target. Recently, some food ingredients, especially polyphenols, for example, resveratrol, tocotrienol, catechins and curcumin have been shown to exhibit anti-angiogenic effects. Their anti-angiogenic potency was proved in in vitro and in vivo assays, in which the production of VEGF or activation of the VEGF-receptor (VEGFR) have been suppressed. However, natural compounds may also exert side effects. For example, Brakenhielm et al (2001) showed that oral administration of resveratrol inhibited the growth of murine fibrosarcoma in mice; however, there was also a significant delay in wound healing.

Aside from the above-mentioned natural compounds, specific peptides have been claimed to mediate anti-angiogenic properties. The peptide L-glutamyl-L-tryptophan (EW/IM862) initially isolated from thymus has been shown to inhibit angiogenesis in preclinical studies using the chorioalantoic membrane assay. Although the exact mechanism of action remains unclear, this peptide exhibited immunomodulatory and inhibitory effects on tumour growth in vivo. It has been assumed that it inhibits the production of VEGF and the activation of natural killer cell function. Deplanque et al (2004) showed in their studies a significant drop in the VEGF concentration in plasma of patients with metastatic renal cell carcinoma, even though the clinical benefit was small. However, the evaluation of natural peptides with respect to anti-angiogenic properties is far from complete. Because such peptides might in principle serve as anti-angiogenic compounds and after topical application their degradation by endogenous peptidases would limit side effects, the current study was undertaken to more comprehensively address potential anti-angiogenic effects of tryptophan-containing dipeptides. Because there is no experimental gold standard to predict the anti-angiogenic potential of a compound, we established a line of assays implementing in vitro, ex vivo and in vivo models to test the peptides for anti-angiogenic properties. Using this approach, we started out by studying the natural peptides WL, EW, IW and WE for potential inhibition of VEGF and related signalling pathways. Following this molecular screening, we selected the most promising dipeptides and explored their effects in different angiogenic assays, to balance the problem of inter-assay variability.

RESULTS

2.1 Screening of tryptophan containing peptides for effects on VEGF signalling

To identify small tryptophan-containing peptides with potential anti-angiogenic effects, we used a bottom-up strategy by first evaluating the effects on VEGFR-2 signalling, namely phosphorylation of the VEGFR-2 and downstream signalling pathways Akt and ERK1/2 by using Western blot analysis. The degree of phosphorylation was assessed in the absence and presence of the dipeptides applied in two different concentrations in response to VEGF and without VEGF stimulation.

Without pre-incubation of VEGF, phosphorylation of the VEGFR-2 was not observed in the control group or in the peptide-treated groups (Figure 1). However, an inhibitory
influence of the peptides on the phosphorylation of ERK1/2 and Akt was detected. While WE had no effect, WL consistently decreased the phosphorylation of ERK1/2 and Akt in both concentrations (100 µmol/L and 1 mmol/L). IW and EW showed some inhibitory effects on phosphorylation at the highest peptide concentration tested (1 mmol/L).

A clear VEGFR-2, ERK1/2 and Akt phosphorylation was observed after incubation with VEGF compared to their corresponding control groups. The phosphorylation response was significantly reduced by high concentrations of IW and WL (1 mmol/L). WL also showed an inhibitory effect on ERK1/2 phosphorylation at a concentration of 0.1 mmol/L (Figure 1). Representative blots are shown in the online data supplement.

Since WL exhibited the most complete pattern of inhibition with respect to phosphorylation of VEGFR-2, ERK1/2 and Akt, this peptide was investigated further in various in vitro and in vivo studies. Besides WL, we also decided to evaluate the potential of EW because this peptide has been described to be effective in angiogenesis assays.18-23

2.2 Inhibitory effect of tryptophan-containing peptides on in vitro endothelial cell sprouting

Assessment of the effects of WL and EW on angiogenesis was first done using the 3D fibrin gel bead model.25,26 This assay measures the ability of HUVECs coated on the surface of cytodex microcarrier beads to form endothelial cell sprouts in a fibrin gel in response to stimulation by feeder cells (here SUM 149 cells), which synthesize and secrete growth factors.

![Figure 1](image1.png)

**FIGURE 1** Changes in phosphorylation of VEGFR-2, ERK1/2 and Akt after incubation with peptides in presence of VEGF (A and B) or absence of VEGF (C and D) in HUVECs determined with Western blot. Two peptide concentrations, 100 µmol/L (equivalent to 33.3 µg/mL for EW and WE; 31.7 µg/mL for WL and IW) A and C, and 1 mmol/L (equivalent to 333 µg/mL for EW and WE; 317 µg/mL for WL and IW) B and D, were used. Data are shown related to each respective control. Results are given as mean values ± SD, *P < .05 vs control, n = 5-7

![Figure 2](image2.png)

**FIGURE 2** Number of sprouts counted per bead in the 3D angiogenesis assay for PBS (control), endostatin 50 ng/mL (equivalent to 2.5 nmol/L; positive control), WL and EW (100 µmol/L; equivalent to 31.7 µg/mL for WL and 33.3 µg/mL for EW) treated groups. Data are from 48 h of incubation in fibrin gel with SUM 149 cells on top as feeding cells. Results are given as absolute mean values ± SD, *P < .05 vs control, n = 5-12
Results in Figure 2 showed a significant reduction in the number of sprouts formed in the presence of the peptide EW (0.29 ± 0.08 sprout/bead) compared to the control group (0.6 ± 0.1 sprout/bead). This effect was similar to the effect encountered with the positive control endostatin (0.36 ± 0.1 sprout/bead), a well-known endogenous angiogenesis inhibitor. On the contrary, WL did not show a significant decrease of sprouts (0.5 ± 0.1 sprout/bead) in this assay. Incubation with the single amino acids W or E did not affect the sprouting compared to control (data not shown).

2.3 | Characterization of WL and EW on endothelial cell differentiation and tube formation

The capability of endothelial cells to form tubes is fundamental in angiogenesis. Therefore, the effects of WL and EW were tested using a further in vitro angiogenesis assay, namely the matrigel tube formation assay. Tubule length and number of loops formed were calculated (Figure 3; representative pictures Figure S2). In this assay both peptides showed a similar inhibitory action. The response was quantitatively similar to the effect of endostatin. However, no effect was noted during co-incubation with the single amino acids W, E, I and L (data not shown).

2.4 | Inhibitory effect of the peptides on VEGF induced sprouting in the mouse aortic ring assay

In addition to the in vitro assays, the 3D fibrin gel bead assay and the matrigel tube formation assay, an ex vivo tissue sprouting 3D model was adopted to assess the effect of the dipeptides on angiogenesis. As shown in Figure 4, there was little angiogenic activity in the control group in absence of VEGF as indicated by the low number of sprouts compared to stimulation with VEGF. Baseline sprouting was not influenced by the single incubation with 1 mmol/L of EW or WL. However, mouse aortic rings were very sensitive to VEGF stimulation. A concentration of 30 ng/mL induced a substantial sprouting response (data not shown) and 15 ng/mL VEGF induced a threefold increase in sprout numbers (45 ± 7 sprouts/ring) compared to control (13 ± 3 sprouts/ring; Figure 4; representative pictures Figure S3). The latter concentration was used in all subsequent experiments. Compared to the VEGF-treated group, the simultaneous incubation of VEGF and WL showed a concentration-dependent reduction in the number of sprouts (Figure 4A) with the highest effect seen at 1 mmol/L (29 ± 5 sprouts/ring). In contrast, EW showed a significant anti-sprouting effect only at the highest concentration of 1 mmol/L (16 ± 4 sprouts/ring) (Figure 4B). Endostatin used as a positive control exhibited a clear anti-sprouting effect (17 ± 3 sprouts/ring) compared to the VEGF only treated group. No effect of the single amino acids W, E, I and L on sprouting in the aortic ring assay was seen (data not shown).

2.5 | Angiogenesis in CAM assay after incubation with WL and EW

In vivo models for angiogenesis represent a strategic extension for the previous in vitro experiments. As a first in vivo model, the CAM assay was employed. As seen in Figure 5, experiments in the CAM model revealed a more than doubled increase in blood vessel formation in the VEGF
(10 ng/mL) treated group as compared to the control group (PBS). Additionally, a potent anti-angiogenic effect for the dipeptides WL and EW was observed. Compared to stimulation with VEGF alone (228 ± 28%), WL and EW decreased the number of blood vessels to 74 ± 15% and 57 ± 26% respectively. The anti-angiogenic response of the peptides was similar to that of Lucentis®, which also showed a significant reduction in the number of blood vessels (102 ± 46%) compared to the VEGF-treated group. CAMs co-treated with peptides or Lucentis® and VEGF were not significantly different from the control group, which was treated only with PBS.
2.6 Effect of peptides on angiogenesis in matrigel plug assay

As a further in vivo model, the matrigel plug assay was used. After incubation for 5 days, the extent of the blood vessels formed was indirectly analysed by quantifying the haemoglobin content (Figure 6). The results from the matrigel plug assay gave further evidence for an anti-angiogenic potency of WL and EW. In the VEGF-treated group a significant increase to 166 ± 32% in the haemoglobin content compared to the control group was observed. This effect was nearly abolished in all groups in which VEGF treatment was combined with either 1 mM WL (107 ± 17%), 1 mmol/L EW (98 ± 22%), or 10 mg/mL Lucentis® (88 ± 12%) application.

2.7 Murine model of OIR

As a third in vivo assay, we studied the changes in pathological retinal angiogenesis using the mouse model of OIR in the presence of the peptide WL. Furthermore, the effect of the amino acid W was investigated to exclude any effect of the single amino acid after peptide degradation. In this assay, one specifically treated eye was compared to the contralateral eye, which served as a control (PBS) in each mouse. The results are shown in Figure 7. The mean number of pathological nuclei moderately decreased after WL injection to 26 ± 2 compared to control (35 ± 3). No changes between PBS and W injection were determined (38 ± 5 vs 38 ± 6), which again indicated that the single amino acid alone did not have any anti-angiogenic effect. As expected, the strongest effect was seen with Eylea® (2.4 ± 1.7 vs 27 ± 5), which was used as positive control.

3 DISCUSSION

The aim of our study was to identify natural dipeptides with a relevant anti-angiogenic effect. Because of the promising results of other studies with the peptide EW, we concentrated on tryptophan-containing dipeptides encrypted in the sequence of whey proteins, which are cost-efficient to obtain and which have the advantage of being used as a possible functional food in prevention. To support an anti-angiogenic effect of tested substances we combined the evidence obtained in several different assays. With this strategy we intend to balance the problem that different angiogenesis assays may result in highly variable predictions with respect to anti- or pro-angiogenic effects of a tested compound.

Initially we screened for anti-angiogenic effects of tryptophan-containing dipeptides by determining the effects on the VEGF pathway. VEGF exerts its stimulatory effect on endothelial proliferation and survival through binding to the extracellular domain of the VEGF receptor (VEGFR-2). This binding further leads to ERK1/2 activation, which influences cellular proliferation, and additionally to activation of Akt, which is associated with improved cell survival. Therefore, checking the potential molecular interactions of the dipeptides with the VEGF receptor and/or downstream signalling molecules Akt and ERK1/2 appeared to be a promising strategy. Using the tryptophan-containing dipeptides WL, EW, IW and WE, we found that the strongest effect on the signalling molecules was produced by WL. Using 1 mmol/L, WL exerts, beside IW, a significant inhibitory effect on VEGF induced receptor phosphorylation. As a possible explanation it could be considered, that this effect may be because of binding of the peptides to the VEGF receptor and thus preventing VEGF from fitting to its receptor, or binding to VEGF itself, which in turn might interfere with its binding to the receptor and prevent its full activation. Likewise, inhibition of VEGF induced ERK1/2 and Akt phosphorylation. This may be explained as a consequence of VEGFR-2 inhibition. EW did not directly influence the phosphorylation of VEGFR-2, thus the anti-angiogenic effect seemed to be VEGF-independent in this experimental model.

Based on these results we decided to investigate the effect of WL with in vitro and in vivo angiogenesis assays because WL showed the most potent impact on the VEGF receptor and downstream molecules. Even though EW had a poor effect in the VEGF studies, it was used as a positive control because of its anti-angiogenic effects described in several assays, for example, in the CAM angiogenesis model and in the Lewis lung tumour model. Moreover, this peptide was reported to be effective in phase
I and phase II studies against AIDS-Kaposi’s sarcoma. However, it should also be mentioned that in a phase III trial this effect could not be confirmed. In the study by Deplanque et al, the effect of EW after nasal application over 8 weeks in patients with metastatic renal cell carcinoma was investigated. It was shown that the plasma VEGF concentration had significantly decreased after 4 weeks treatment. However, a clinical benefit for EW as a single treatment agent could in this study not be demonstrated. Although the mechanism behind is not yet understood, the observed reduction of VEGF concentration makes EW attractive in a combination therapy with other chemotherapy or biological agents. Ferrario et al were able to demonstrate an enhancing effect in a combination therapy of photodynamic therapy (PDT) and parallel administration of EW (IM862) in mouse mammary carcinoma. The cure rate was increased by PDT and the additional administration of EW as compared to PDT alone. Whether similar effects also apply to WL has not yet been tested.

Further experiments were done in vitro using the 3D fibrin gel bead assay, which models the first step in angiogenesis and further the matrigel tube formation assay, which recapitulates endothelial cell differentiation and tube formation. EW showed in both assays an anti-angiogenic effect similar to the control substance endostatin, which is an endogenous angiogenesis inhibitor. In contrast, WL did not show a significant reduction of the number of sprouts compared to the control group using the 3D fibrin gel bead assay. However, cell differentiation and tube formation in the matrigel assay were significantly affected in the WL-group similar to the effect of EW. Explanations for these contrary results could be because of a possible degradation of WL by proteases, which may have been secreted by SUM 149 cells. For the HUVEC model, a good stability of WL against hydrolysis by endogenous peptidases has previously been reported by us.

To simulate a more complex 3D environment, the ex vivo mouse aortic ring sprouting assay was used. This robust assay bridges the gap between in vitro and in vivo models. The anti-angiogenic activity of both peptides observed in vitro was supported by the results from this mouse aortic ring sprouting assay. Based on the results of these in vitro and ex vivo experiments, the potential anti-angiogenic effects of WL and EW were also explored under in vivo conditions, because only in vivo assays may appropriately approach the complex processes during angiogenesis. Thus, three different in vivo angiogenesis assays were selected: CAM assay, matrigel plug assay and a murine model of OIR. All of them have strengths and weaknesses. The CAM assay is technically relatively simple and inexpensive. However, this assay is very sensitive to the oxygen tension, and it does not study mammalian tissue. Furthermore, the assay may rather reflect embryonic angiogenesis, and drugs that require metabolic activation cannot be assessed. The matrigel plug assay is also a frequently used method, although it is more time-consuming and more expensive. One advantage is that it does not require any surgical procedure because matrigel including the compound to be tested is injected subcutaneously into mice. A disadvantage of the matrigel assay is that the subcutaneous tissue is artificial and may have little relevance for pathological angiogenesis that occurs during tumour growth. The OIR model is a widely used and established way of studying vascular regression, vessel regrowth, and neovascular tuft formation reflecting processes seen in ROP and diabetic retinopathy. This model is regarded as appropriate for research on non-surgical treatments for ROP in particular, but also for angiogenesis in general. Therefore, we also used this in vivo assay to explore the anti-angiogenic effect of WL.

The results in all in vivo assays generally support the notion that WL may exert an anti-angiogenic effect as already suggested by the in vitro and ex vivo experiments. Results of the CAM assay indicate that both peptides, WL and EW, significantly decreased the number of blood vessels compared to control. The effects were similar to those of the well-known anti-VEGF drug Lucentis® (Ranibizumab, anti-VEGF antibody fragment), which is a standard treatment in AMD. Also, the results obtained in the matrigel plug assay lend support to an anti-angiogenic effect of WL and EW. Again, effects were similar to those observed using Lucentis®. Interestingly, both peptides were almost identically effective in these experiments. This could indicate that both peptides act through a similar mechanism. Here, as a potential interaction partner laminin should be considered, since the structure-analogous sequence MWWW has been identified in a functional domain of the laminin receptor, which is a key player in angiogenesis. The OIR reflects pathological angiogenesis similar to that which occurs in AMD. This disease is known to be associated with an increased supply of VEGF. This may well explain the efficacy of VEGF inhibitors, for example, Lucentis® or Eylea® (Afibercept) in this disease. Both compounds act as antibody fragments, which bind directly to VEGF and thus prevent its binding to the receptor. Eylea®, used as a control substance in our OIR experiments, greatly reduced the mean number of epiretinal vascular nuclei compared to control. A more attenuated, but still significant decrease was seen after injection of WL. This difference may be explained by a much higher binding affinity to VEGF in the case of Eylea® as compared to WL. Also, the larger molecular size may have contributed to the steric hindrance of receptor interaction in the case of Eylea®. Another aspect refers to the stability of compounds in the vitreous fluid. While we have shown previously that WL is relatively stable in human plasma, its stability in the vitreous fluid is also unknown.
is unknown. Thus, a possible degradation of WL may have contributed to the rather small effect in vivo.

### 3.1 Conclusion and further perspectives

Our comprehensive screening studies provide evidence for an anti-angiogenic effect of the dipeptide WL. This effect was explored in vitro, ex vivo and in vivo. It may be that the limited stability of the peptide, which is also found in the sequence of natural food protein, may decrease its potential anti-angiogenic use. However, this fact could also be seen as an advantage. Because WL is rapidly metabolized into two essential amino acids, this may largely eliminate side effects. While the bioavailability of WL is poor after oral application, other strategies of use may be considered. These embrace the topical application to the skin, encapsulated applications into the gastro-intestinal tract or local injections, for example, into the eye. Further, it seems worthwhile to consider chemical extensions of WL to improve stability or increase the anti-angiogenic potency. The formulation of WL as hydrogels or encapsulation in liposomes or nanoparticles may also be considered to achieve high local concentrations. Since so far, the mechanisms behind the anti-angiogenic effect of the peptides has not been clarified yet, this should be further pursued. It should be investigated whether the VEGF-reducing effect of EW is also detectable for WL and if other mechanisms such as anion-quadrupole interactions may be involved as they can strongly influence protein-protein and protein-membrane interactions. In conclusion, several lines of experimental evidence support the view that WL is a dipeptide with anti-angiogenic properties and is worthy of further exploration.

### 4 MATERIALS AND METHODS

#### 4.1 Materials

Chemicals used included L-tryptophyl-L-leucine, L-glutamyl-L-tryptophan, L-isoleucyl-L-tryptophan, and L-tryptophyl-L-glutamic-acid (all: purity >95%, Bachem, Bubendorf, Switzerland), endostatin (human recombinant, purity >98%, Sigma, Munich, Germany) and VEGF (PeproTech, Hamburg, Germany).

#### 4.2 Human umbilical vein endothelial cells (HUVECs) isolation and culturing HUVECs

Endothelial cells were freshly isolated from human umbilical cords collected from the obstetrics and gynaecology department, University Hospital Dresden, after obtaining consent from the donor, as described previously. Permission for use of umbilical cords for isolation of HUVECs had been given by the Ethikkommission of the Medical Faculty Carl Gustav Carus of TU Dresden.

#### 4.3 Cell culture treatments and Western blot

After incubation of HUVECs with WL, EW, IW and WE, VEGF was added in the desired concentration for 5 minutes, then the effect on VEGFR-2, Akt and ERK1/2-phosphorylation was analysed via Western blot. For details please see online data supplement.

#### 4.4 In vitro 3D-angiogenesis assay

The fibrin solution, containing fibrinogen, aprotinin, WL, EW, the amino acids tryptophan (W) and glutamic acid (E), and endostatin (everything dissolved in EGM-2) was mixed with dextran-coated Cytodex 3 microcarrier beads (Amersham Pharmacia Biotech; Uppsala, Sweden) coated with HUVECs and incubated for 2 hours until the gel had polymerized. Then, 200 μL of the SUM 149 cell (breast cancer cells) suspension (25 000 cells/mL), were added in each well on top of the gel. Then cells were incubated in a humidified incubator with 5% CO₂ for 48 hours. Details are described in the online supplement.

Bright field images from random beads (100 beads for each condition) were obtained using 10X objective in Zeiss Observer Z1 microscope. The numbers of sprouts were counted, and the length of the sprouts was measured using Axiovision software.

#### 4.5 In vitro matrigel tube formation assay

After matrigel is polymerized in each well of Ibidi μ-Slide Angiogenesis®, 50 μL of the cell suspension (HUVECs with peptides or endostatin in the desired concentrations) were added on top and incubated in a humidified incubator for 6 hours. Then cells were fixed with 4% formalin before they were analysed via bright field mosaic images. Please see details in the online data supplement.

#### 4.6 Mouse aortic ring sprouting assay

Experiments were conducted on male adult C57BL/6J mice, purchased from Charles River Laboratories, Sulzfeld, Germany as previously described with some
modified. Experiments were approved by the Landesdirektion Sachsen, Germany. Details are described in the online data supplement. In brief, the aorta was dissected, cut into small rings and incubated for 24 hours in serum-free Opti-MEM medium inside a humidified incubator at 37°C and 5% CO2. Aortic rings were embedded in collagen and treated with peptides or single amino acids without serum in PBS) were added to 50 µL of Drabkin’s Reagent. The absorbance was measured using a plate reader at wavelength 540 nm.

4.7 | In vivo chicken chorioallantoic membrane assay (CAM)

The chick chorioallantoic membrane model was used as described before. Different treatments (VEGF 10 ng/mL, Lucentis® 10 mg/mL, and peptides each 1 mmol/L dissolved in PBS) were introduced through a 1 cm² window over a sterile cover slip that was placed directly over the blood vessels. The window was closed with an adhesive strip and incubated at 37°C with 55% relative humidity for 5 days. Thereafter, the adhesive strip was removed and the piece of the membrane with the overlying cover slip was cut and put in a sterile petri dish, then washed carefully with PBS.

Images were taken with a Canon EOS 700D camera fixed with an adaptor over a dissecting Zeiss microscope. Blood vessels in the membrane area underlying the cover slip were counted manually using Image J to display the pictures.

4.8 | In vivo mouse matrigel plug assay

BALB/c mice aged 5-6 weeks, were purchased from Harlan Laboratories (Indianapolis, IN, USA). Experiments were performed in compliance with Public Health Service Policy on Humane Care and Use of Laboratory Animals and approved by the Albany VA Medical Center (Albany, NY, USA) Institutional Animal Care and Use Committee (IACUC). In these animal studies, conducted in the Albany VA Animal Facility, mice were maintained under specific pathogen-free conditions, with controlled conditions of temperature (20-24°C) and humidity (60%-70%) and a 12 hours light/dark cycle with ad libitum access to water and food. Mice were shaved on the back and injected with 300 µL of matrigel alone, or matrigel plus VEGF (10 ng/mL), or matrigel plus VEGF (10 ng/mL) and Lucentis (10 mg/mL), or matrigel plus VEGF (10 ng/mL) and peptides (each 1 mmol/L) subcutaneously in the midventral abdominal region, then left to solidify. After 5 days of incubation, mice were sacrificed by neck breaking, matrigel plugs were removed, and 300 µL of distilled water were added to each sample, which were then homogenized. All samples were centrifuged at 1600×g for 5 minutes and the supernatant was collected. Fifty microliters of the collected sample supernatant or distilled water (blank) were added to 50 µL of Drabkin’s Reagent. The absorbance was measured using a plate reader at wavelength 540 nm.

4.9 | In vivo mouse murine model of oxygen-induced retinopathy (OIR)

Studies of the model of oxygen-induced retinopathy were done according to a previously described protocol. Animal experiments were approved by the Landesdirektion Sachsen, Germany. Briefly, 7-day-old (P7) mice were exposed to 75% oxygen for 5 days together with their nursing mothers. The hyperoxia results in vessel regression in the developing retinas. At P12, the mice were returned to room air (21% O2). The resulting retinal ischemia leads to a hypoxic response in the retina, triggering pathologic neovascularization. At P17, mice were sacrificed, eyes were processed, and the quantification of epiretinal neovascular nuclei was performed as previously described. The angiogenic effect of the peptides was tested by intravitreal injections of 1 µL (1 mmol/L) of either WL or W into the right eye or as control PBS into the left eye of the same animal with a 33-gauge needle (Hamilton Bonaduz AG, Bonaduz, Switzerland). Under a surgical microscope in anaesthetized mice on postnatal day 14 of the OIR model. Intravitreal injections with Aflibercept (Eylea®, Bayer, Germany) with 25 µg were used as positive control.

4.10 | Statistical analysis

If not mentioned, data are given as mean ± SD. The number of experiments is indicated in the figure legends. The comparison of the data was performed by a one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test. The statistical significance of the data of the OIR model was determined using two-tailed unpaired student t-test. A P-value < .05 was considered to indicate a significant difference.

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CONFLICT OF INTEREST
The authors report no conflict of interest.

DATA AVAILABILITY STATEMENT
Data available on reasonable request from the authors.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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