Local injection of receptor tyrosine kinase inhibitor MAE 87 reduces retinal neovascularization in mice

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Purpose: Retinal neovascularization occurs under the influence of angiogenic factors like vascular endothelial growth factor (VEGF). VEGF signaling is enhanced by insulin-like growth factor-1 (IGF-1). In vitro, the oxoindolinone MAE 87 inhibits angiogenic signal transduction by blocking tyrosine kinase receptors including VEGF receptor 2 (VEGFR-2), IGF-1R, fibroblast GF-1R and epidermal GFR. We investigated the effect of MAE 87 in vivo using the mouse model for oxygen induced retinopathy.

Methods: From postnatal day seven (P7) on, C57BL/6J mice were kept in a 75% oxygen environment for five days. On postnatal day 12 (P12) they received an intravitreal injection of MAE 87 in one eye and control substance in the fellow eye. The animals were sacrificed by intracardial perfusion with fluorescein-dextran solution on P17. Retinal whole mounts were prepared and ischemic retinopathy was evaluated in 26 animals using a standardized retinopathy score.

Results: After a single intravitreal injection of MAE 87 there were significantly less angioproliferative changes (blood vessel tufts, extra-retinal neovascularization, and blood vessel tortuosity) than in the fellow eye (p=0.007). The median retinopathy score (maximal 13) for the MAE 87 treated eyes was 6 (25th percentile: 5; 75th percentile: 7) and 8 for the control eyes (25th percentile: 5; 75th percentile: 10).

Conclusions: The tyrosine kinase inhibitor MAE 87 may be a promising substance for local treatment of retinal neovascularization. Due to its ability to inhibit not only the VEGF but also the IGF-1 cascade, MAE 87 may prove especially valuable for the treatment of diabetic retinopathy.

Retinal vasoproliferative disease is the most common cause of severe visual loss in people under the age of sixty in developed countries [1,2]. Patients at risk are mainly diabetics, prematurely born infants or patients with retinal vein occlusion. The only approved treatment consists in destroying parts of the peripheral retina by laser or cryocoagulation in order to diminish hypoxic tissue and thus reduce reactive neovascularization. Insight into the molecular mechanisms of neovascular eye disorders can provide new targets for novel nondestructive therapeutic agents.

Vascular endothelial growth factor (VEGF) is upregulated by hypoxia [3-7]. Increased intravitreal and intraretinal levels of VEGF are associated with retinal neovascularization not only in animal models [8-10] but also in patients with ischemic retinopathy [11-14]. VEGF overexpressed in transgenic mice induces retinal neovascularization [15]. These data suggest that VEGF signaling is a good target for pharmacological treatment of retinal neovascularization.

VEGF is not only a potent mitogenic factor for endothelial cells; it also induces vascular permeability and dilation [16]. Furthermore, it is the most important factor for tumor angiogenesis [17,18]. These biological activities are mediated by binding of VEGF to high-affinity transmembrane, autophosphorylating tyrosine kinase receptors. Three distinct VEGF receptors have been identified: VEGFR-1 (or Flt-1), VEGFR-2 (or murine Flk-1, human KDR) and VEGFR-3 (or Flt-4). VEGFR-1 and VEGFR-2 are predominantly expressed on vascular, VEGFR-3 on lymphatic endothelium [19]. Other well-characterized factors involved in angiogenesis are transforming growth factor, basic fibroblast growth factor (bFGF), growth hormone, epidermal growth factor (EGF) [20,21] and insulin-like growth factor (IGF) [22]. New insight into the role of IGF-1 during early development of the retinal vasculature and pathological retinal angiogenesis revealed this factor as an important partner of the VEGF-action by modulating intracellular VEGF-pathways and survival of endothelial cells [23].

The mouse model for oxygen induced retinopathy as introduced by Smith et al. [24] is widely used to study retinal neovascularization in vivo. Hyperoxia induces vessel regression via selective apoptosis of vascular endothelial cells [25,26]. When the animals are returned to room air, severe retinal hypoxia develops, VEGF is upregulated and retinal neovascularization appears [9,27]. In this model intravitreal injection of soluble VEGF receptor/IgG fusion proteins, VEGF anti-sense oligonucleotides or neutralizing VEGF antibodies each inhibited retinal neovascularization [28-31].

Indolinones such as MAE 87 have been shown to block directly a number of receptor tyrosine kinases, and thereby
inhibit biological processes such as angiogenesis [32,33]. Crystal structure analysis revealed that their oxoindole core serves as an anchor occupying the adenosine-binding pocket of the kinase [34]. In vitro MAE 87 was shown to inhibit autophosphorylation of tyrosine kinase receptors involved in retinal angiogenesis including VEGFR-2, IGF-1R, FGFR-1 and EGFR [35]. Here we show the antiproliferative effect of MAE 87 in vivo: a single intravitreal injection of MAE 87 reduced the angioproliferative retinopathy in the mouse model of oxygen induced retinopathy.

METHODS

Animals: Thirty-two C57BL/6J mice (5 litters), from Charles River Laboratories, Hamburg, Germany were used. All animal procedures adhered to the animal care guidelines by the Institute for Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals).

Mouse model of oxygen induced retinal neovascularization: The model we used (Smith et al. [24]) imitates retinopathy of prematurity. Mice are born at term with...
an incompletely developed retinal vascular system, a situation similar to that in prematurely born infants. In contrast to prematurely born infants however mice do not develop retinopathy. Retinopathy can nevertheless be induced by transient hyperoxia. On postnatal day seven (P7) the mice and their nursing mother were placed in an airtight incubator (own production) ventilated by a mixture of oxygen and air to a final oxygen fraction of 75 ± 2%. Oxygen levels were checked at least 3 times a day. On P12 the mice were returned to room air and intraocular injections were performed (see below). On P17 the animals were sacrificed by cardiac perfusion with a solution of 50 mg/ml fluorescein-labeled dextran in sodium chloride as described previously [36]. Both eyes were enucleated and fixed for 2 to 6 h in 4% buffered formaldehyde at room temperature. The anterior segment was cut off and the neurosensory retina carefully removed. The retina was cut radially and flat mounted in glycerin, photoreceptors facing downward. A cover slip was placed over the retina and sealed with nail polish. Retinal whole mounts were examined by fluorescence microscopy (BH2-RFC, Olympus, Hamburg, Germany). Figure 1 shows a fluorescein-dextran perfused normal retina from a 17 day old mouse kept at room air. The retinal vasculature is

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<td>central avascular area</td>
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<td>neovascularization</td>
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Retinal whole mounts were examined by fluorescence microscopy and ischemia induced retinopathy was quantified by evaluating four criteria. Points received for each criterion were summed to obtain the retinopathy score. A higher score (range: 0-13 points) indicates more severe retinopathy. This scoring system was adapted from Higgins et al. [37].
almost fully developed as a dense capillary network with two distinguishable layers.

Quantification of retinopathy: For quantification of the retinopathy we used a scoring system adapted from Higgins et al. [37]. To better meet the requirements of this study a few modifications were undertaken. The modified scoring system is shown in Table 1 and Figure 2. The slides were evaluated with the fluorescence microscope by two independent investigators in a blinded fashion. Retinopathy scoring criteria were central avascular area, circumscribed blood vessel tufts, presumed extra-retinal neovascularization (further described below), and tortuosity of vessels. For the purpose of scoring the central avascular area, the retina was divided into 3 concentric zones (Figure 2): the inner zone around the optic disc (A), the middle zone (B), and the outer zone (C). Vascular proliferations were quantified by counting blood vessel tufts and presumed extra-retinal neovascularization in each of 12 equally sized sections (“clock hours”) of the retina (Figure 2, numbers). The term “presumed” extra-retinal neovascularization is used for large clusters of neovascularization as in histological sections those usually grow into the vitreous. However, extra-retinal blood vessel growth can by principle only be presumed in flat mounted retinas with the vitreous removed. Tortuosity of the vessels was expressed as ratio of tortuous to straight major vessels leaving the optic disc. The retinopathy score was achieved by summing the points for each of the four criteria (Table 1): the higher the score, the worse the ischemic retinopathy (maximal score 13 points). For documentation, the retinal whole mounts were photographed using a digital camera (Hamamatsu C4742-95, Herrsching, Germany) connected to a fluorescence microscope (Zeiss Axiophot, Jena, Germany). Figure 3 illustrates a fluorescein-dextran perfused retina from a 17 day old mouse that had been exposed to hyperoxia from P7 to P12 and where no intravitreal injection had been performed. The retina shows the typical appearance of proliferative retinopathy. This retina has a retinopathy score (Table 1) of 12 based on the large central avascular area (2 points), blood vessel tufts (4 points), presumed extra-retinal neovascularization (3 points), and tortuosity of vessels (3 points).

Intraocular injections: The mice were fixed under a microsurgical microscope (Zeiss, Jena, Germany). Their mouth was firmly stuck in a tube for inhalation narcosis (2.5-3.5% of isoflurane in oxygen, Vapor 19.3, Dräger, Lübeck, Germany). Intravitreal injections were performed using glass pipettes with a diameter of approximately 150 µm at the tip made with a
standard pipette puller. The pipette was connected to a three directional tap with a 10 µl Hamilton syringe attached for injection. The lid fissure was opened and the eye proptosed by gentle pressure on the temporal upper eyelid. A drop of local anesthetic was administered. The eye was punctured at the upper nasal limbus and a volume of 2 µl of MAE 87 solution or control solution (see below), respectively, was injected in one eye each. Since reflux of a certain amount of intraocular fluid is unavoidable when removing the pipette from the injection site, the pipette was kept in place for 10 s to allow diffusion of the solution.

MAE 87: The chemical structure of the oxindolinone [34] is shown in Figure 4. MAE 87 was synthesized according to the procedure described by Kirkin et al. [34]. MAE 87 is presently not commercially available (to obtain MAE 87 contact Athanassios Giannis, University of Leipzig, Leipzig, Germany). The effect of MAE 87 on different protein kinases was tested by kinase assays as described previously [35]. For intraocular injections MAE 87 was dissolved in DMSO and PBS (DMSO/PBS=6/106). With an estimated globe volume of 14 µl, we injected 565 pmol of MAE 87 to reach a final concentration of calculated 40 µM in the vitreous, a concentration in which MAE 87 reduced in vitro kinase activity of FGFR-1, EGFR, IGF-1R, and VEGF-R2 by more than 50% in the kinase assay. The DMSO/PBS vehicle (see above) was injected in the fellow eye as control.

Statistics: The data were analyzed using the Wilcoxon signed rank test.

RESULTS
Animals: A total of 32 mice from 5 litters were used in these experiments. The results are based on the evaluation of 26 mice (52 retinas). Out of the 32 mice sacrificed for the experiment, 5 mice were lost for evaluation due to incomplete perfusion and 1 mouse due to damage of the retina during preparation of the retinal whole mount. In these experiments we did

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Figure 5. Effect of MAE 87 on oxygen induced retinopathy. Each dot represents both eyes of one or several (white numbers) mice (n=26). The x value of a dot indicates the retinopathy score of the control eye, its y value the score of the MAE 87 treated eye. Eyes treated with MAE 87 showed a significantly lower retinopathy score (Wilcoxon signed rank test, p=0.007).

Figure 6. Effect of MAE 87 on oxygen induced retinopathy in a 17 day old mouse. A: The retina of a MAE 87 treated eye (retinopathy score 6). B: The retina of the control eye (retinopathy score 12). The structure of the retinal vasculature is markedly better preserved in the MAE 87 treated eye (A). Magnification is 20x in both images. Scale bar represents 1000 µm.
not lose any adult mice or pups due to oxygen exposure or anesthesia.

**Oxygen induced retinal neovascularization:** The effectiveness of the receptor tyrosine kinase (RTK) inhibitor MAE 87 as an antiproliferative agent in vivo was studied in the murine model of oxygen induced proliferative retinopathy. The retinopathy was scored by evaluating the vascular pattern of retinal whole mounts under a fluorescence microscope after intracardial perfusion with fluorescein-dextran solution. Ischemic retinopathy was reliably induced in any of the mice (see individual scores in Figure 5). Yet retinopathy varied markedly between different mice (see score of control eyes on x-axis of Figure 5). This will be further discussed below.

**MAE 87:** MAE 87 was injected in the right eye and control solution in the left eye of each mouse. The retinopathy scores of the 52 evaluated retinas (26 treated, 26 control eyes) showed a highly significant (p=0.007, Wilcoxon signed rank test) reduction of ischemic retinopathy in the treated eyes compared to the individual control eyes. The retinopathy scores (right eye and left eye) of each mouse are shown in Figure 5. In this scatter plot each dot represents the retinopathy scores of both eyes of an animal (n=26). The x value of a dot indicates the retinopathy score of the control eye, its y value the score of the MAE 87 treated eye. Some dots are labeled with white digits that indicate the number of mice with identical pairs of retinopathy scores. Dots below the bisecting line represent cases where the treatment was effective: lower retinopathy scores (i.e., less retinopathy) in the MAE 87 treated eyes than in the control eyes. The median of the retinopathy score (maximal 13) was 6 (25th percentile 5, 75th percentile 7) for the MAE 87 treated eyes and 8 (25th percentile 5, 75th percentile 10) for the control eyes.

An example of retinal flat mounts of a MAE 87 treated eye and the control (fellow) eye of a hyperoxia exposed 17 day old mouse is given in Figure 6. The structure of the retinal vasculature of the MAE 87 treated eye (Figure 6A, retinopathy score 6) is markedly better preserved than in the control eye which shows a larger avascular area, more blood vessel tufts and presumed extra-retinal neovascularization as well as more pronounced blood vessel tortuosity (Figure 6B, retinopathy score 11).

**DISCUSSION**

**Oxygen induced retinal neovascularization:** Proliferative ischemic retinopathy was reliably induced in all mice as described by several other groups [9,24,27,28]. However, retinopathy can vary markedly between different mice even of the same litter. As illustrated in Figure 5, the retinopathy score of the control eyes (x-axis) ranges from 3 to 12. The high interindividual variance was also reported for a canine model of oxygen induced retinopathy [38] and emphasizes the necessity for intra-individual comparison (right eye versus left eye of each mouse).

**MAE 87:** A single intravitreal injection of the receptor tyrosine kinase (RTK) inhibitor MAE 87 led to a highly significant reduction of angioproliferative changes in the murine model of oxygen induced retinopathy. To our knowledge this is the first report on an intravitreal use of an RTK inhibitor aiming at the reduction of proliferative retinal disease. At the concentration used (40 µM) MAE 87 inhibits more than 50% of the activity of the receptor kinases of FGFR-1, EGF, IGF-1R and VEGFR-2 when using in vitro kinase assays as described previously [35]. All receptors are known contributors to angiogenesis [39-41], VEGFR-2 [19,42] and IGF-1R [23,43,44] being the best described for proliferative retinal disease.

IGF-1R promotes retinal neovascularization via supporting the VEGF-driven endothelial cell proliferation [45]. IGF-1 signaling plays an important role in proliferative diabetic retinopathy. Blocking both IGF-1 and VEGF signaling, MAE 87 might be considered a potentially useful therapeutic agent in this disease, especially during the initial phase of insulin therapy, when IGF-1 levels in serum and vitreous rise and diabetic retinopathy increases [45].

IGF-1 is also an important contributor to the physiological development of the retinal vasculature. However, when increased to a critical level, as in retinopathy of prematurity, IGF-1 triggers retinal neovascularization [23], a situation in which IGF-1 inhibitors like MAE 87 might prove effective. The major problem of antiangiogenic therapy in a developing organ will be to avoid inhibition of physiologic vessel growth. In mice the vessels of the peripheral retina develop at the end of the second week of life. Although we did not observe any major changes in our experiments, adverse effects on retinal vessel development were reported after systemic use of the VEGF-2 specific RTK-inhibitor PTK 787 in oxygen treated mice [27] and with intravitreal slow release pellets containing VEGF-2 antibodies in oxygen treated puppies [38].

It was shown previously that oral treatment with a tyrosine kinase inhibitor can reduce retinal neovascularization in the mouse model of oxygen induced retinopathy. Seo and colleagues [46] have demonstrated complete inhibition of retinal neovascularization after oral administration of the staurosorpine derivative CGP 41251, a partially selective kinase inhibitor that blocks phosphorylation by VEGFR-2 and platelet-derived growth factor receptor (PDGFR) as well as several isoforms of protein kinase C (PKC). In another study by the same group [27] the orally administered RTK inhibitor PTK 787 that blocks phosphorylation by VEGFR-2 and PDGFR, but not PKC, also led to complete inhibition of retinal neovascularization. Drugs that selectively block PDGFR kinase activity had no significant effect on retinal neovascularization. The authors concluded that blocking the VEGFR kinase was responsible for the antiproliferative effect.

Compared to systemic application, local administration of RTK inhibitors bears some advantages as well as possible drawbacks concerning safety and effectiveness. The lower risk of potential systemic side effects speaks in favor of local administration. Ozaki et al. [27] did not comment on systemic side effects of oral PTK 787 in mice. However, a clinical trial with PTK 787 in cases of advanced cancer revealed adverse drug effects like nausea, vomiting, fatigue, dizziness, ataxia, and raised blood pressure in 25 to 75% of the participants.
[47]. The risk of a hypertensive crisis was especially high in patients who already suffered from arterial hypertension.

The risk of intraocular injection and the short storage time of the drug in the eye are potential problems of intravitreal therapy. In our series we observed no intraocular injection. Before considering MAE 87 for the use in humans, additional aspects like longevity in the vitreous, duration of the therapeutic effect, and toxicity would have to be addressed in supplementary studies. If longevity proved to be short, frequent (and uncomfortable) injections would be required. Especially in diabetics, there may be an increased risk of vitreal hemorrhage with ocular injections. Yet, slow drug release devices such as intravitreal polymer pellets might facilitate long term local treatment.

MAE 87 is not highly specific to one particular RTK. However, the known receptors it can bind to are all proangiogenic. Therefore inhibition of neovascularization might be especially effective. Further experiments should reveal whether this theoretical advantage of MAE 87 holds true when comparing it to a more specific RTK inhibitor such as PTK 787. Yet, inhibiting several RTKs, MAE 87 might be prone to causing more adverse effects.

In summary, we showed that a single intravitreal application of the RTK inhibitor MAE 87 significantly reduces oxygen-induced retinal neovascularization in mice. MAE 87 interferes with different proangiogenic RTKs including VEGFR-2 and IGF-1R and seems therefore especially promising in the local treatment of diabetic retinopathy.

ACKNOWLEDGEMENTS

The authors thank Karl Boden for excellent experimental assistance and Flemming Staubach for critical discussion of the results.

REFERENCES


