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# **Sorafenib Prevents Human Retinal Pigment Epithelium Cells from Light-Induced Overexpression of VEGF, PDGF, and PIGF**

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## **Abstract**

**Background:** Cumulative light exposure is significantly associated with progression of age-related macular degeneration (AMD). Inhibition of vascular endothelial growth factor (VEGF) is the main target of current antiangiogenic treatment strategies in AMD. However, other growth factors, such as platelet-derived growth factor (PDGF) and placenta growth factor (PIGF), have a substantial impact on development of AMD. Previous reports indicate that sorafenib, an oral multikinase inhibitor, might have beneficial effects on exudative AMD. This study investigates the effects of sorafenib on light-induced overexpression of growth factors in human retinal pigment epithelial (RPE) cells.

**Methods:** Primary human RPE cells were exposed to white light and incubated with sorafenib. Viability, expression, and secretion of VEGF-A, PDGF-BB, and PIGF and their mRNA were determined by reverse transcription-polymerase chain reactions, immunohistochemistry, and enzyme-linked immunosorbent assays.

**Results:** Light exposure decreased cell viability and increased expression and secretion of VEGF-A, PDGF-BB, and PIGF. These light-induced effects were significantly reduced when cells were treated with sorafenib at a dose of 1 µg/mL.

**Conclusion:** The results show that sorafenib has promising properties as a potential antiangiogenic treatment for AMD.

## **Introduction**

Exudative age-related macular degeneration (AMD) is a leading cause of visual deterioration and legal blindness in people over age 60 in developed countries <sup>1</sup>. Early stages of this disease are characterized by the formation of drusen and atrophic changes in the retinal pigment epithelium (RPE) <sup>1</sup>. With progression of the disease, 10% of patients develop "wet" forms of AMD due to choroidal neovascularization (CNV) that often leads to a rapid loss of vision <sup>1</sup>. AMD is a multifactorial disease with several risk factors for development and progression, including ethnicity, gender, smoking, hypertension, genetics, diet, and sunlight exposure <sup>1-4</sup>.

CNV in the macular region often results in significant loss of central vision and high-resolution visual acuity and is the fundamental pathophysiologic process underlying this disease <sup>5 6</sup>. Without treatment, AMD can lead to irreversible loss of the ability to read, recognize faces, and drive <sup>5 6</sup>.

Up-regulation of vascular endothelial growth factor (VEGF) is a major critical event in the development of CNV <sup>7</sup>. Therefore, inhibition of VEGF is the main target of the currently used antiangiogenic agents in the treatment of AMD <sup>8</sup>. The mechanism of action of these VEGF inhibitors (e.g., bevacizumab and ranibizumab) is blocking VEGF in the extracellular space and thereby preventing access to its receptor <sup>9</sup>. Studies have demonstrated that treatment of AMD with VEGF inhibitors is safe and effective <sup>10-12</sup>. However, major prospective randomized trials revealed that despite an improvement in visual acuity, the neovascular tissue typically does not regress with VEGF inhibition alone and without continuous

treatment often re-progression occurs <sup>10 11</sup>. The reason for this lack of sustained effect might be due to redundant pathways. Previous studies indicated that several angiogenic growth factors, including platelet-derived growth factor (PDGF) and placenta growth factor (PIGF), are overexpressed in the vitreous and retinas in proliferative retinal disease and play a crucial role in the development and progression of AMD <sup>13-16</sup>. The reason for this could be that other molecular pathways remain active and binding VEGF in the extracellular space alone might have only limited effect on neovascular tissue. In addition, inhibition of a single molecule could induce a compensatory increase in stimulatory increase of alternative pathways.

Sorafenib (Nexavar, BAY 43-9006) is an oral multikinase inhibitor, approved for the treatment of several forms of cancer <sup>17</sup>. Previous case reports indicate that sorafenib, alone or in combination with other angiostatic drugs, might have a beneficial effect on exudative AMD <sup>18 19</sup>.

This study was designed to investigate the effect of sorafenib on light-induced growth factor expression in the RPE, which plays a key role in the development and progression of AMD <sup>1</sup>. Therefore, we exposed primary human RPE cells to wide-band white light (spectral range 400-700 nm). Cells were treated with sorafenib and the drug's effects on viability of the cells after light exposure were investigated. Expression of VEGF-A, PDGF-BB, and PIGF was determined.

## **Methods**

## ***Materials***

Sorafenib (Nexavar, BAY 43-9006 [N-(3-trifluoromethyl-4-chlorophenyl)-N-(4-(2-methylcarbamoyl pyridin-4-yl)oxyphenyl)urea]) was dissolved in 100% dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO) and diluted with DMSO and the cell culture medium to the desired concentration with a final DMSO concentration of 0.1% for in vitro studies. DMSO was added to cultures at 0.1% (v/v) as a solvent control.

## ***Ethics***

The methods of securing human tissue were humane, included proper consent and approval, complied with the Declaration of Helsinki, and were approved by the local ethics committee.

## ***Human RPE Cell Culture***

Primary RPE cells from four human donors (48, 52, 56, and 74 years old, obtained 3-10 h postmortem) without any history of eye disease were obtained from the Eye Bank of Ludwig Maximilian University and were prepared as previously described<sup>20</sup>. Dulbecco modified Eagle medium (DMEM; Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS; Biochrom, Berlin, Germany) was used as the cell culture medium. An increased accumulation of lipofuscin is a common characteristic of ageing RPE cells in vivo and lipofuscin is known to act as a photosensitizer. It has been implicated to play a certain role in the development and progression of AMD<sup>21-23</sup>. To investigate the effects of

white light on RPEs growth factor expression, we used primary RPE cells of passages 2 and 3 for our experiments. All investigated RPE cell cultures presented intracellular pigmented granules in phase-contrast microscopy. These granules contain, besides melanin and other pigments, a certain amount of lipofuscin. This characteristic disappears when RPE cells are cultured for long-term periods <sup>24-26</sup>.

### ***Cell Culture Treatment***

For all cell culture experiments, RPE cells were seeded in 35-mm tissue culture dishes and cultured on confluence in darkness. RPE cells were kept for 24 h in serum-free conditions. After the cells were washed with phosphate-buffered saline (PBS), they were illuminated for 60 min. Directly after illumination, PBS was replaced with serum-free cell culture medium and sorafenib was added with a final concentration of 1 µg/mL.

### ***Illumination of Cells***

A spot-light source (LC-8; Hamamatsu Photonics, Japan) from a mercury-xenon lamp equipped with an optic fiber as the light guide (spectral range: 400-700 nm) was used for illumination. The cell culture medium was replaced with PBS just before illumination. The plastic cover of the illuminated cell culture well was removed, and the cells were illuminated from above. They were illuminated (300 mW/cm<sup>2</sup>) for 60 min. The illumination power and spectral range were measured with a spectrometer (C10083MD; Hamamatsu Photonics). Directly after illumination, PBS was

replaced by serum-free cell culture medium, sorafenib was added with a final concentration of 1  $\mu\text{g}/\text{mL}$ , and the cells were kept in darkness for another 24 h. Then, the methylthiotetrazole (MTT) assay, enzyme-linked immunosorbent assay (ELISA) for detection of growth factor concentrations in cell culture supernants, epifluorescence microscopy, phase-contrast microscopy, and RNA isolation were performed.

### ***MTT Assay***

The tetrazolium dye-reduction assay (MTT; 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was used to determine the cell survival rate. The MTT test, which is well-established for the assessment of cell viability, was performed as described by Mosmann,<sup>27</sup> with some modifications. The medium was removed; the cells were washed with PBS; and 1000  $\mu\text{L}$  of MTT solution (1.5  $\mu\text{L}$  MTT stock, 2  $\text{mg}/\text{mL}$  in PBS, plus 28.5  $\mu\text{L}$  MEM) was added to each well. RPE cells were incubated at 37°C for 1 h. The formazan crystals that formed were dissolved by the addition of 1000  $\mu\text{L}$  DMSO per well. Absorption was measured by a scanning multiwell spectrophotometer at 550 nm (Molecular Probes). The results were expressed as the mean percentage of proliferation in the control.

To investigate the effects of different sorafenib concentrations on RPE cell viability, cells were brought to confluence, kept under serum-free conditions for 24 h, and then were treated with sorafenib concentrations of 0.5, 1, 1.25, 2.5, 5, 7.5, 10, 12.5, 15, 25, and 50  $\mu\text{g}/\text{mL}$  for another 24

h. Then the MTT assay was performed. To investigate the effects of sorafenib on RPE cell viability after light exposure, cells were illuminated and treated as described above. At 24 h after illumination the MTT assay was performed.

The experiments were performed in triplicate and repeated three times. The control cells were RPE cells of the same passage; they were kept in darkness, without exposure to any radiation.

#### ***Detection of VEGF-A, PDGF-BB, and PIGF Secretion by RPE Cells***

RPE cell cultures were grown to confluence and treated as described. Levels of VEGF-A, PDGF-BB, and PIGF in the culture supernatants were determined by ELISA. The supernatants were collected after 24 h, and the levels of VEGF-A, PDGF-BB, and PIGF were quantified using a VEGF-A, PDGF-BB, or PIGF Quantikine ELISA Assay Kit (R & D Systems) according to the manufacturer's instructions.

#### ***RNA Isolation and Real-Time PCR***

Total RNA was isolated from 10-cm Petri dishes by the guanidium thiocyanate-phenol-chloroform extraction method (Stratagene, Heidelberg, Germany). The structural integrity of the RNA samples was confirmed by electrophoresis in 1% Tris-acetate-EDTA agarose gels. The yield and purity were determined photometrically (BioPhotometer; Eppendorf, Hamburg, Germany). Real-time polymerase chain reaction (PCR) enables quantitative detection of small amounts of mRNA. After the

usual isolation of mRNA, this mRNA was transferred to complementary DNA (cDNA) through reverse transcriptase (RT). This cDNA was then used for the specific PCR. Quantification of VEGF-A, PDGF-BB, and PIGF mRNA was performed with specific primers with a LightCycler instrument (LightCycler System; Roche Diagnostics, Mannheim, Germany). Primers and probes were detected with ProbeFinder 2.04. All primers and probes were designed to cross intron/exon boundaries to avoid amplification of genomic DNA. All PCR products were sequenced to ensure product validity. Each 14- $\mu$ L reaction volume contained 1 $\times$  FastStart DNA Master Hybridization Probes Mix (Roche Diagnostics), 4 mM MgCl<sub>2</sub>, 0.5 mM of each primer, 0.2 mM *Taq*Man probe, and 2  $\mu$ L cDNA.

The amplification signals were detected in real time, which allowed accurate quantification of the amounts of the initial RNA template, because the system can select signals easily during the exponential amplification phase of PCR. The cDNA of RPE cells, either after incubation with sorafenib or untreated and then exposed to light, was amplified with specific primers for 40 cycles. Two oligonucleotides with different labeled fluorophores were hybridized to the amplified fragment during the annealing phase. When the two probes came in close proximity, fluorescence resonance energy transfer (FRET) developed between the two fluorophores. The emitted fluorescence was then measured by the LightCycler instrument. Hybridization probes were displaced during the extension step. Depending on the initial concentration of target genes, the signal intensity increased in different cycles, and these cycles were used

as the crossing point. The standard curve was made with three different probes of untreated RPE cells. For normalization of differences in the amount of total RNA added to each reaction, 18S rRNA was simultaneously processed in the same sample as an internal control. The level of VEGF-A, PDGF-BB, and PIGF mRNA was determined as the relative ratio (RR), which was calculated by dividing the level of VEGF-A, PDGF-BB, and PIGF mRNA by the level of the 18S rRNA housekeeping gene in the same samples. The ratios are expressed as decimals. All experiments were performed at least in triplicate and repeated three times. Table 1 lists the primers used for RT-PCR.

### ***Immunohistochemistry of RPE Cell Cultures***

Cultured RPE cells were grown on microscope slides and treated as described above. After incubation, cells were fixed with 4% phosphonoformic acid (PFA) for 15 min and subsequently washed twice with PBS containing 0.1% Triton X-100. Primary incubation of all samples was performed with a mouse anti-human VEGF-A antibody (Santa Cruz Biotechnology, Santa Cruz, CA), a mouse anti-human PDGF-B antibody (Santa Cruz Biotechnology), and a goat anti-human PIGF antibody (Santa Cruz Biotechnology), diluted 1:200 in PBS for 4 h at room temperature. After washing three times, the culture dishes and sections were further processed with a Texas red-conjugated rabbit anti-mouse secondary antibody or a fluorescein-conjugated rabbit anti-goat secondary antibody (diluted 1:500 in PBS; Santa Cruz Biotechnology) for 1 h at room temperature, rinsed in PBS, and mounted with Kaiser's glycerine jelly

(Merck, Darmstadt, Germany). Slides were investigated with a Zeiss Axio Imager fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany). All experiments were performed at least nine times with RPE cells from three different cell cultures.

### ***Statistical Evaluation***

All data were analyzed with SPSS 13.0 for Windows (SPSS, Chicago, IL, USA). For all statistical tests,  $p < 0.05$  was considered significant. Results of the MTT assay are presented as mean (SD) units of absorbance. Ten individual samples per group were measured in triplicate. The Mann-Whitney test was used. Results from VEGF-A, PDGF-BB, and PIGF ELISA are presented as mean (SD) ratios of each tested probe, which were normalized to the control. Results of the RT-PCR are presented as mean (SD) ratios of the investigated mRNA and 18S rRNA. Again, Mann-Whitney testing was applied; all experiments were performed at least in triplicate and repeated three times.

## **Results**

### ***Viability of Cells***

#### *Testing concentrations of sorafenib in primary RPE cells*

No gross abnormalities, such as abnormal shape and appearance, cellular lysis, cellular death, or other abnormalities, were detected with phase-contrast microscopy up to a concentration of 12.5  $\mu\text{g}/\text{mL}$  sorafenib in primary RPE cells after 24 h treatment. The number of cells counted in

phase-contrast microscopy correlated well with the quantitative results of the MTT test.

#### *MTT assay*

Sorafenib showed no significant toxic effects in the investigated RPE cell cultures (24-h exposure) at concentrations between 0.5 and 12.5 µg/mL. No significant decrease (Wilcoxon with a correction for multiple testing) in cellular viability was detected with RPE cells compared with the control. Concentrations of sorafenib between 15 and 25 µg/mL showed an increasing reduction of cell viability, but still >60% of the cells revealed activity. At a sorafenib concentration of 50 µg/mL a more rapid, dose-dependent and significant reduction of viability was seen (Fig. 1).

#### *Testing the effects of sorafenib on viability of RPE cells after light exposure*

When cells were illuminated with unfiltered white light (300 mW/cm<sup>2</sup>) for 60 min, a significant reduction of RPE cell viability was detected (Fig. 2). In contrast, cells treated with sorafenib, 1 µg/mL, immediately after illumination showed a significantly smaller less decrease in viability compared to cells that were not treated with sorafenib (Fig. 2).

#### ***Expression of VEGF-A, PDGF-B, and PlGF mRNA***

VEGF-A, PDGF-BB, and PlGF mRNA expression was detected in every sample. All detected mRNA levels of VEGF-A, PDGF-BB, and PlGF were normalized to those of 18S rRNA; the values are expressed as the RR of VEGF-A/18S, PDGF-BB/18S, or PlGF/18S. Illumination with white light for

60 min led to a significant increase in VEGF-A, PDGF-B, and PIGF mRNA expression in RPE cells (Fig. 3). Treatment of RPE cells with sorafenib, 1 µg/mL, directly after light exposure significantly reduced this light-induced increase in VEGF-A, PDGF-BB, and PIGF mRNA after 24 h (Fig. 3).

### ***Immunohistochemical Detection of VEGF-A, PDGF-B, and PIGF Expression in RPE Cells***

For verification that the light-induced increase of VEGF-A, PDGF-B, and PIGF mRNA transcription translates into increased protein synthesis, immunohistochemistry was performed 24 h after illumination.

In both untreated and non-illuminated control cells and cells that were treated with sorafenib, 1 µg/mL, and had no illumination, only a faint staining for VEGF-A, PDGF-B, and PIGF was observed (Fig. 4). After 60 min of light exposure, the expression of VEGF-A, PDGF-B, and PIGF was markedly increased.

In contrast, for RPE cells that were illuminated for 60 min and had sorafenib at a dose of 1 µg/mL added directly after illumination, staining showed only a weak expression of VEGF-A, PDGF-B, and PIGF, which was comparable to those cells that served as non-illuminated controls (Fig. 4).

### ***Detection of VEGF-A, PDGF-B, and PIGF Secretion of Human RPE Cells***

To investigate the effect of sorafenib on VEGF-A, PDGF-BB, and PIGF secretion after light exposure, a quantitative detection of VEGF-A, PDGF-

BB, and PIGF in cell culture supernants was conducted using the ELISA method 24 h after illumination. In our experimental set-up, light exposure led to a significant increase of VEGF-A, PDGF-BB, and PIGF in cultured RPE cells after 60 min of light exposure. In contrast, when cells were treated with sorafenib, 1 µg/mL, directly after light exposure, sorafenib treatment decreased the amount of VEGF-A, PDGF-BB, and PIGF significantly compared to those cells that were not treated with sorafenib after illumination (Fig. 5).

## **Discussion**

RPE forms the outer blood–retina barrier to facilitate selective transport between the choroidal blood vessels and the outer retina. As a phagocytic system it is essential for the renewal of photoreceptors and plays a major role in rod and cone photoreceptor integrity<sup>1</sup>. AMD is a disease of the elderly population<sup>1</sup>. Loss and degeneration of RPE cells, particularly in the macular center, is pathognomonic for this disease<sup>1 21</sup>. With advancing age, RPE cells undergo an increase in pleomorphism and accumulate metabolic debris from remnants of incomplete degradation of phagocytized rod and cone membranes<sup>1 21</sup>. The incomplete degradation of phagocytized photoreceptor membranes results in a continuous increase in intracellular lipofuscin in the RPE cells over time. This lipofuscin accumulation seems to be associated with deterioration in cellular function potentially resulting in a higher sensitivity to radiation damage<sup>22 28-30</sup>. Furthermore, the formation of drusen in Bruch’s membrane might

compromise RPE cell function by impeding fluid transport and inducing inflammatory responses, which have been implicated in the development of AMD and CNV <sup>31-33</sup>. Once damaged, RPE cells secrete inadequate amounts of growth factors, such as VEGF, PDGF, PlGF, and others <sup>13-16</sup>. An imbalance between these growth factors in the eye potentially leads to aberrant angiogenesis resulting in the formation of dysfunctional blood vessels and CNV <sup>34</sup>.

Cumulative light exposure is thought to induce proangiogenic factors in RPE cells and to promote the development of exudative AMD <sup>16 35</sup>. Previous studies have demonstrated that light exposure can lead to an increased production of VEGF-A in RPE cells <sup>20 36 37</sup>. The reason for this could be that continuous light exposure induces sublethal cellular stress, which results in an up-regulation of proangiogenic VEGF-A secretion <sup>36</sup>.

In accordance with these findings, our results clearly indicate that light exposure not only leads to an up-regulation of VEGF-A but also to an increased expression and secretion of PDGF-BB and PlGF in primary human RPE cells.

VEGF-A, a member of the VEGF family, is a dimeric 36–46-kDa glycosylated protein with a predominant role in the development of pathologic angiogenesis in AMD <sup>1 7</sup>. A secondary important role of VEGF-A is the induction of increased vascular permeability <sup>1 6 7 15 16</sup>. In RPE cells out of the macular region of patients with AMD VEGF-A is significantly increased, and vitreous VEGF-A levels are significantly higher in patients with AMD and CNV, compared to healthy control subjects <sup>38 39</sup>. Therefore,

VEGF-A has become the main target of the currently used antiangiogenic agents in the treatment of AMD<sup>8</sup>. However, endothelial cells and pericytes that form the structure of neovascular tissue seem not to respond to VEGF-A inhibition alone and currently used anti-VEGF agents seem to have little effect on existing capillaries<sup>15 16</sup>. In addition, not all attributes of CNV are related to vessels, but many of the structural abnormalities associated with CNV are related to fibroblasts, damage of the RPE, and the remodeling of the nascent extracellular matrix<sup>15 16</sup>.

PlGF, a member of the VEGF family, induces increased vascular permeability, proliferation, chemotaxis, and angiogenesis and may act synergistically with VEGF-A in the development of CNV<sup>15 16</sup>. Both VEGF-A and PlGF have a recruiting effect on pericytes during angiogenesis, either in the maturation process of endothelial tubes, but as well in the early phase of angiogenesis, where both growth factors act as an integral part of sprouting capillaries<sup>16 40 41</sup>.

PDGF-BB, another growth factor expressed by the RPE, acts as a survival factor for retinal pericytes. PDGF-BB is the key regulator of the pericyte endothelial bond and known to recruit pericytes in the development of CNV<sup>15 41-43</sup>. Pericytes are an important component of the choroidal neovascular complex, providing stabilization of vessels and are thought to limit the penetration of anti-VEGF agents<sup>15 41-43</sup>. In addition, PDGF is thought to have an important role in fibroplasia as a wound healing response in CNV<sup>15 43 44</sup>.

Recent studies demonstrated that combined inhibition of VEGF and PDGF enforces vessel regression by interfering with pericyte-mediated endothelial cell survival and produced inhibition and regression of corneal neovascularization and CNV compared to anti-VEGF treatment alone<sup>42 43</sup>.

Therefore, a combined approach of targeting several key molecules or pathways in a complex disease state as AMD might lead to significant improvements in outcome and seems to be worthwhile<sup>45</sup>.

The multikinase inhibitor sorafenib was initially identified as an RAF kinase inhibitor, but it also inhibits several receptor tyrosine kinases involved in angiogenesis. Sorafenib's antiproliferative and antiangiogenic efficacy seems to be derived from its blocking capability of the RAF/mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK (RAF/MEK/ERK) cascade and its effect on receptor tyrosine kinases, including VEGF receptor 2 (VEGFR2), VEGFR3, PDGF receptor, FLT3, Ret, and c-Kit<sup>17 46-49</sup>. Furthermore, sorafenib interacts with hypoxia-inducible factors 1 and 2 and seems to have an influence on expression of growth factors, such as VEGF and PDGF<sup>17 46-48</sup>.

In this study, sorafenib at a concentration of 1 µg/mL led to a significant reduction of the light-induced increase in expression and secretion of VEGF, PlGF, and PDGF in primary human RPE cells. Moreover, this attenuation of light-induced growth factor overexpression was accompanied by a significant improvement of light-induced decrease in RPE cell viability. One potential reason for this might be that sorafenib does not only interact with growth factor regulation, but also interferes

with certain proteins within the intrinsic apoptotic cascade. The exact mechanisms are not completely understood, however, it has been demonstrated that sorafenib inhibits for example the phosphorylation of the initiation factor eIF4E which is known to regulate the translation of a large number of mRNAs, including member so the Bcl-2 family <sup>17 48</sup>. Further research is warranted.

Sorafenib appears to be comparable to systemic anti-VEGF medications in its safety profile <sup>11 50</sup>. The most common side effects after oral administration in patients treated for renal cell carcinoma (RCC) were dermatologic symptoms such as rash or hand-foot skin reactions, diarrhea, and low-grade hypertension. Severe adverse events have been described but are rare <sup>50 51</sup>. In addition, in our experimental set-up, sorafenib did not have a significant influence on RPE cell viability after a 24-h exposure up to a concentration of 12.5 µg/mL. This is more than 10-fold higher than the sorafenib concentration of 1 µg/mL effective in our experiments.

The standard dosage of sorafenib as oral treatment for RCC is 800mg per day. From several clinical trials we know that after a daily dose of 800mg sorafenib, plasma levels are within the range of 10µg/mL, after 200mg per day around 4.5µg/mL can be reached <sup>52-54</sup>. To date, there is no data regarding the penetration of sorafenib into ocular tissue, neither after systemic, nor after topic application, available and this issue needs further investigation. However, the sorafenib concentration of 1 µg/mL effective in our experiments, might be achievable even with a low dose treatment.

Our data provide the first evidence that sorafenib effectively interacts in human RPE cells with three important targets of AMD treatment: VEGF, PlGF, and PDGF. Due to its activity against multiple targets and pathways in angiogenesis, sorafenib seems to be promising as a potential treatment for AMD<sup>55</sup>. Another substantial difference between sorafenib to the currently used extracellular VEGF inhibitors might be that the substance potentially acts upstream and downstream in the angiogenic cascade and therefore potentially prevents transcription and protein production of growth factors in the tissue from where they are derived<sup>17 55</sup>.

Recent reports have demonstrated beneficial effects of sorafenib in AMD treatment. Kernt et al reported about a patient with occult CNV who experienced visual improvement and resolution of macular edema under oral sorafenib treatment due to advanced RCC, where intravitreal bevacizumab treatment failed<sup>19</sup>. Other reports describe improvement in visual acuity under low-dose sorafenib treatment alone or in combination with intravitreal ranibizumab<sup>18</sup>.

In this in vitro study sorafenib had a significant impact on growth-factor expression in the human RPE. The RPE is only one component interacting in retinal neovascular disease and not all aspects of mechanisms of action of sorafenib regarding growth factor expression and signaling are completely understood. Its additional effects on receptor tyrosine kinases and other cell types involved in the development of AMD need further investigation. However, the data presented in this in vitro study indicate that sorafenib provides properties to reduce light-induced growth factor

overexpression in human RPE and, therefore, might prevent development and progression of CNV in vivo. Further experimental and clinical studies will have to substantiate our in vitro findings. Nevertheless, sorafenib seems to be a promising adjunctive treatment for patients with AMD.

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## Figure Legends

**Fig. 1.** Viability of primary human RPE cells after treatment with the various concentrations of sorafenib, measured by a colorimetric test (MTT). Untreated RPE cells of the same passage served as controls. The results shown are the mean percentage of control cell survival from three experiments, each performed in triplicate with error bars indicating SEM. Sorafenib concentrations of up to 12.5 mg/mL showed no significant reduction in viability (Wilcoxon with a correction for multiple testing) compared to controls.

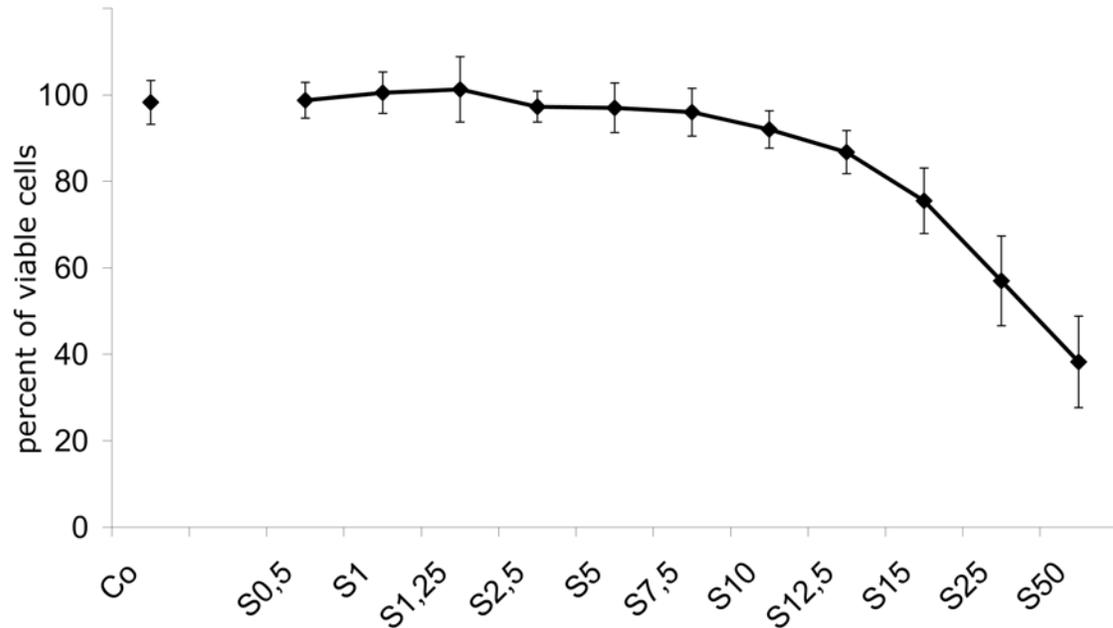
**Fig. 2.** Viability of primary human RPE cells after illumination with plain white light (black column) and after incubation with sorafenib at a concentration of 1  $\mu$ g/mL (light gray column), as measured by a colorimetric test (MTT). The decrease in cellular viability was significantly reduced when cells were additionally incubated with sorafenib after 60 min of illumination. Error bars: SD.

**Fig. 3.** VEGF-A, PDGF-BB, and PIGF mRNA expression of primary RPE cells after illumination with plain white light (black columns) and after incubation with sorafenib at 1  $\mu$ g/mL (light gray columns), as investigated by quantitative RT-PCR. X-axis: relative ratio (RR) of VEGF-A, PDGF-BB, or PIGF mRNA normalized to 18s rRNA, expressed in decimal format. Y-axis: time of illumination.

**Fig. 4.** Immunohistochemical staining of VEGF-A, PDGF-B, and PIGF expression in RPE cells. Both untreated and non-illuminated control cells

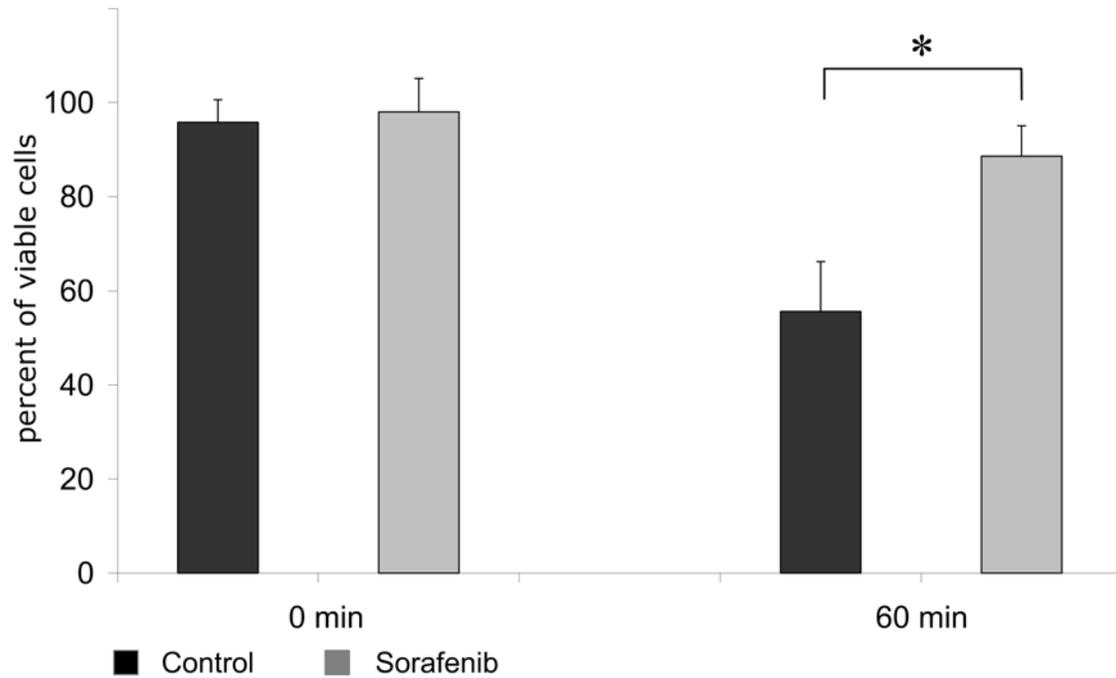
and cells that were treated with sorafenib, 1  $\mu\text{g}/\text{mL}$ , and had no illumination presented only a faint staining for VEGF-A, PDGF-B, and PIGF. After 60 min of light exposure, the expression of VEGF-A, PDGF-B, and PIGF was markedly increased. In contrast, RPE cells that were illuminated and had sorafenib added directly after illumination showed only a weak staining of VEGF-A, PDGF-B, and PIGF, which was comparable to those cells that served as non-illuminated controls.

**Fig. 5.** Inhibitory effect of sorafenib on light-induced secretion of VEGF-A, PDGF-BB, and PIGF, as investigated by ELISA: untreated RPE cells after illumination with plain white light (black columns) and after additional treatment with sorafenib (light gray columns). Each value was normalized to a standard curve of VEGF-A, PDGF-BB, or PIGF and expressed as concentration in nanograms per milliliter. Data values are means  $\pm$  SD.



**Fig. 1:**

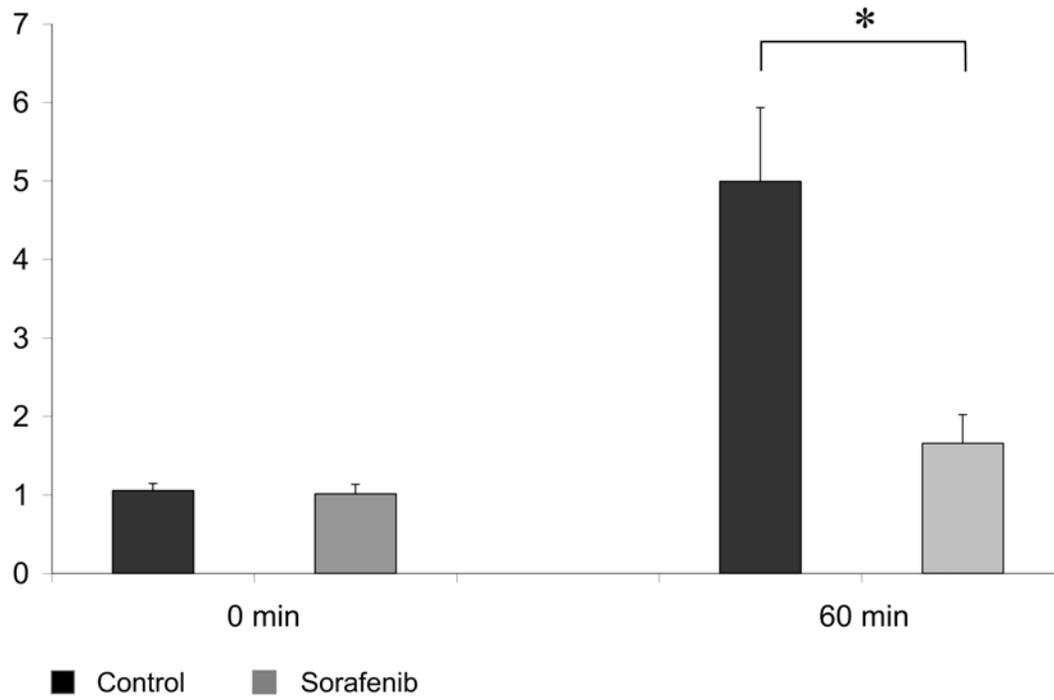
Viability of primary human RPE cells after treatment with the various concentrations of sorafenib, measured by a colorimetric test (MTT). Untreated RPE cells of the same passage served as controls. The results shown are the mean percentage of control cell survival from three experiments, each performed in triplicate with error bars indicating SEM. Sorafenib concentrations of up to 12.5 mg/mL showed no significant reduction in viability (Wilcoxon with a correction for multiple testing) compared to controls.



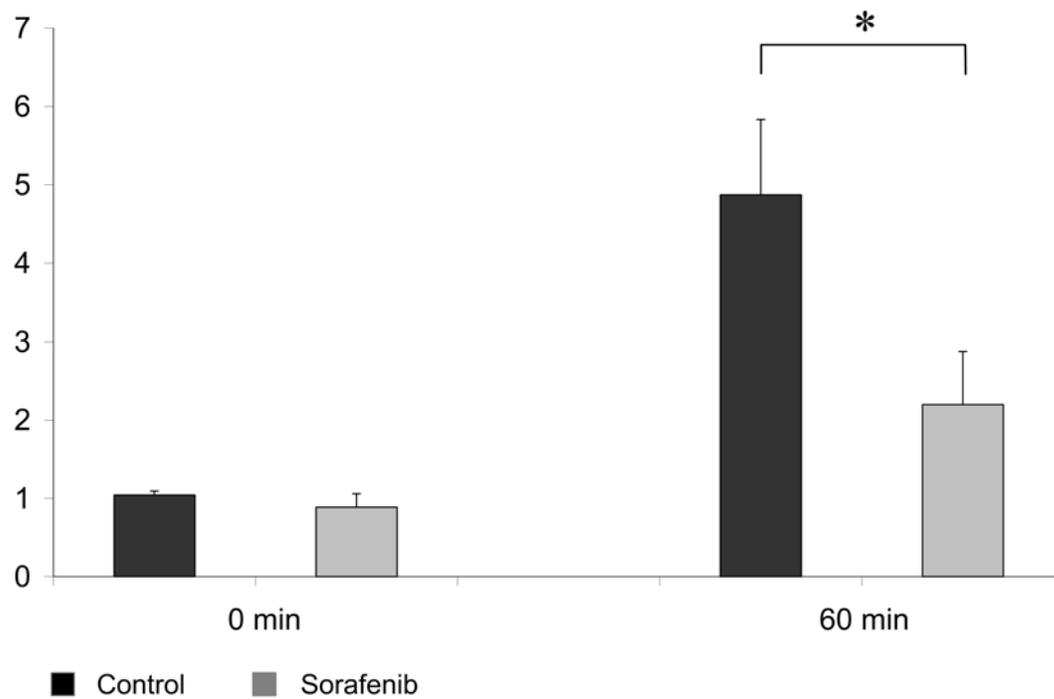
**Fig. 2.:**

Viability of primary human RPE cells after illumination with plain white light (black column) and after incubation with sorafenib at a concentration of 1  $\mu\text{g}/\text{mL}$  (light gray column), as measured by a colorimetric test (MTT). The decrease in cellular viability was significantly reduced when cells were additionally incubated with sorafenib after 60 min of illumination. Error bars: SD.

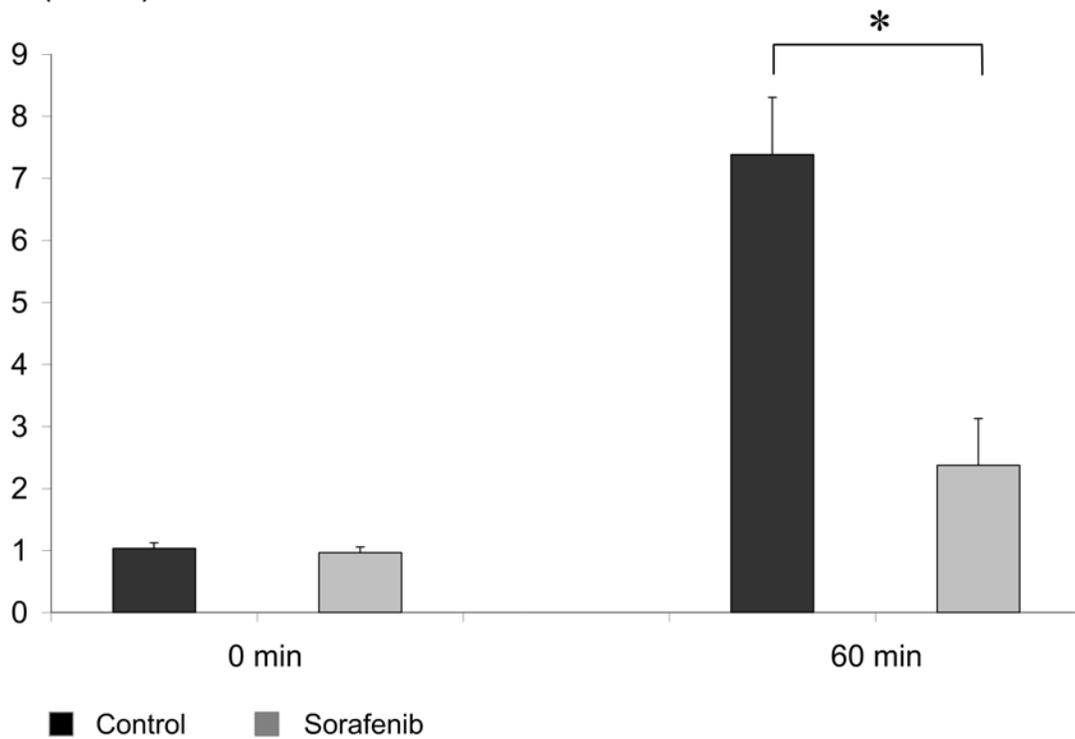
### VEGF-A-RNA (RT-PCR)



### PDGF-RNA (RT-PCR)

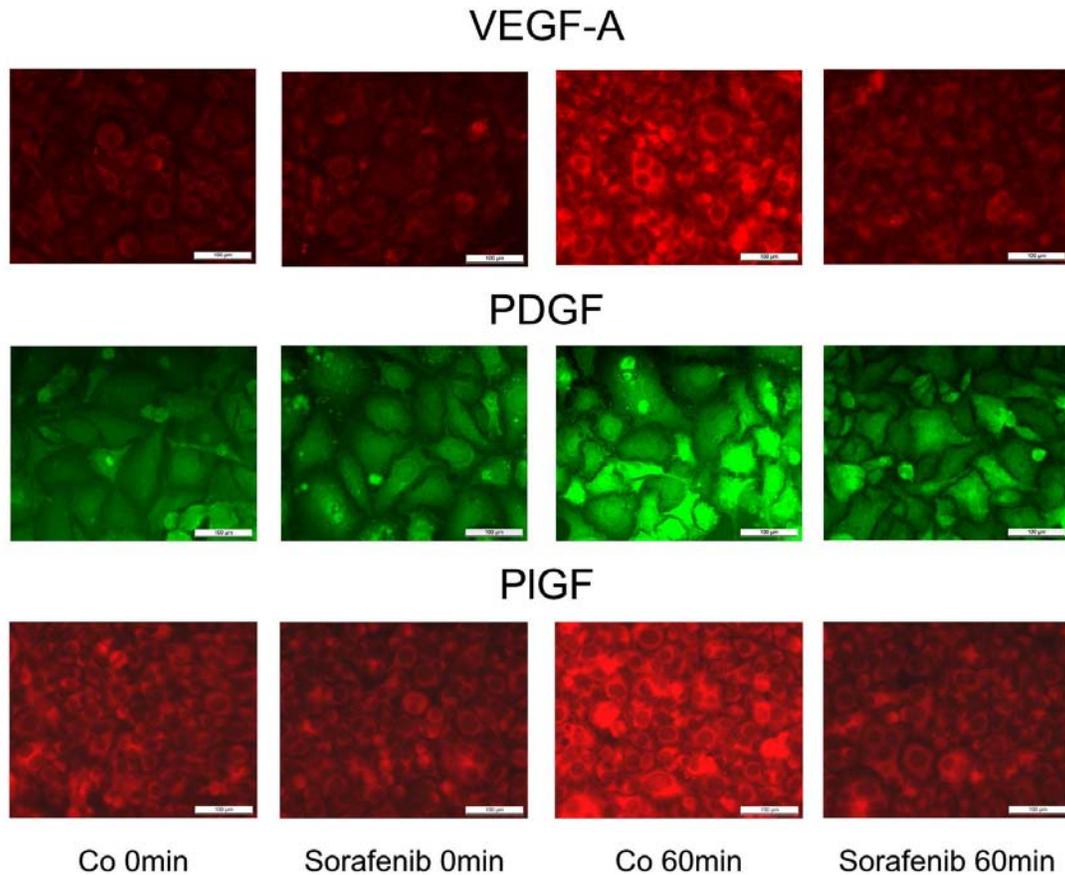


PIGF-RNA  
(RT-PCR)



**Fig. 3:**

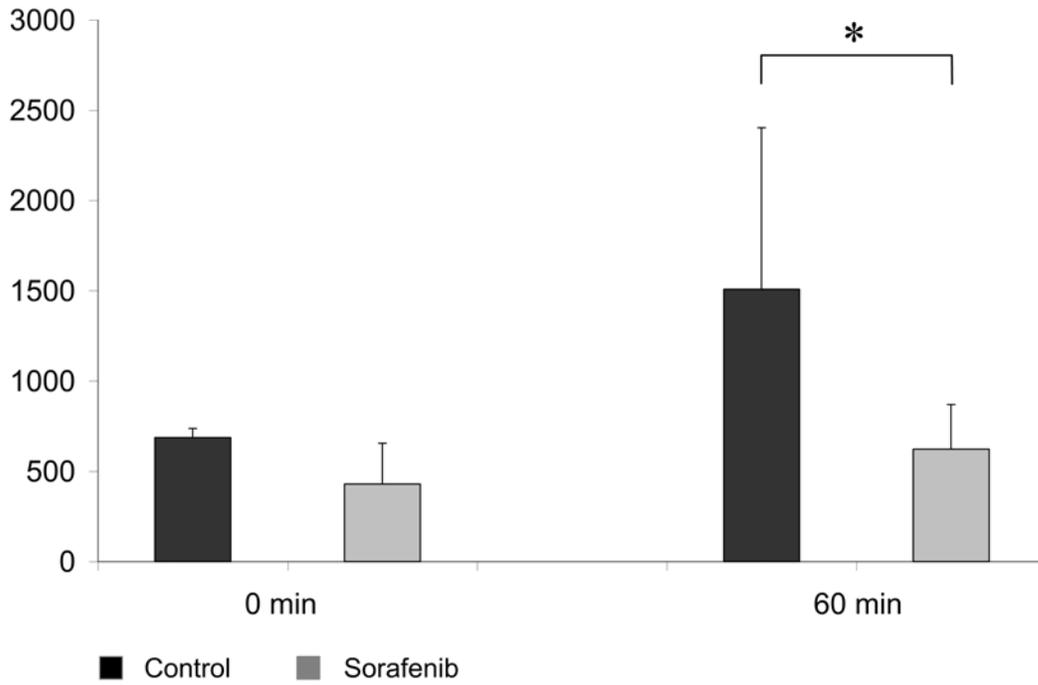
VEGF-A, PDGF-BB, and PIGF mRNA expression of primary RPE cells after illumination with plain white light (black columns) and after incubation with sorafenib at 1  $\mu\text{g}/\text{mL}$  (light gray columns), as investigated by quantitative RT-PCR. X-axis: relative ratio (RR) of VEGF-A, PDGF-BB, or PIGF mRNA normalized to 18s rRNA, expressed in decimal format. Y-axis: time of illumination.



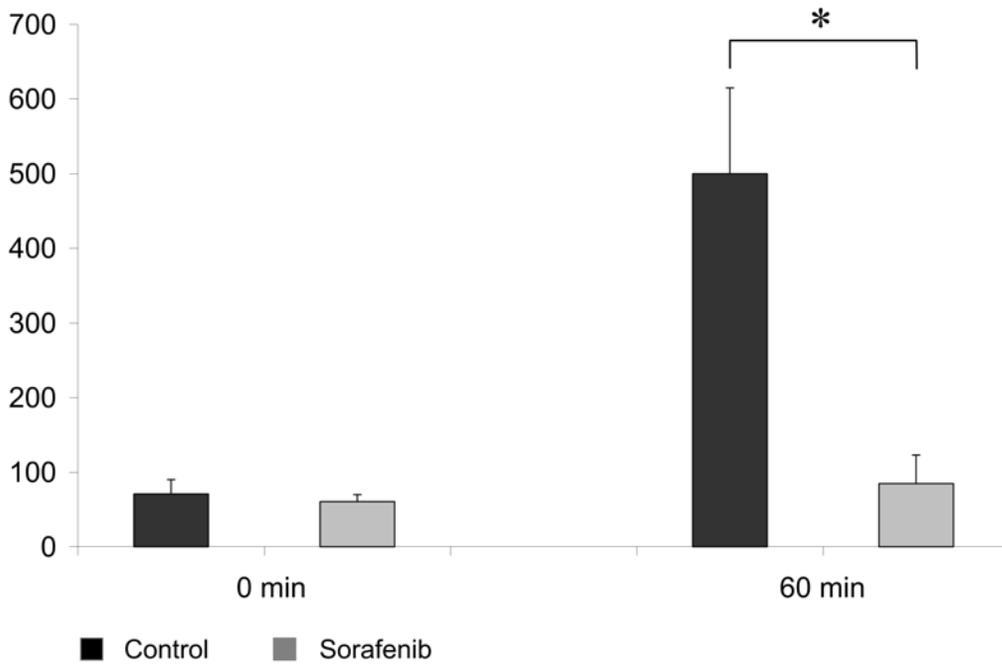
**Fig. 4:**

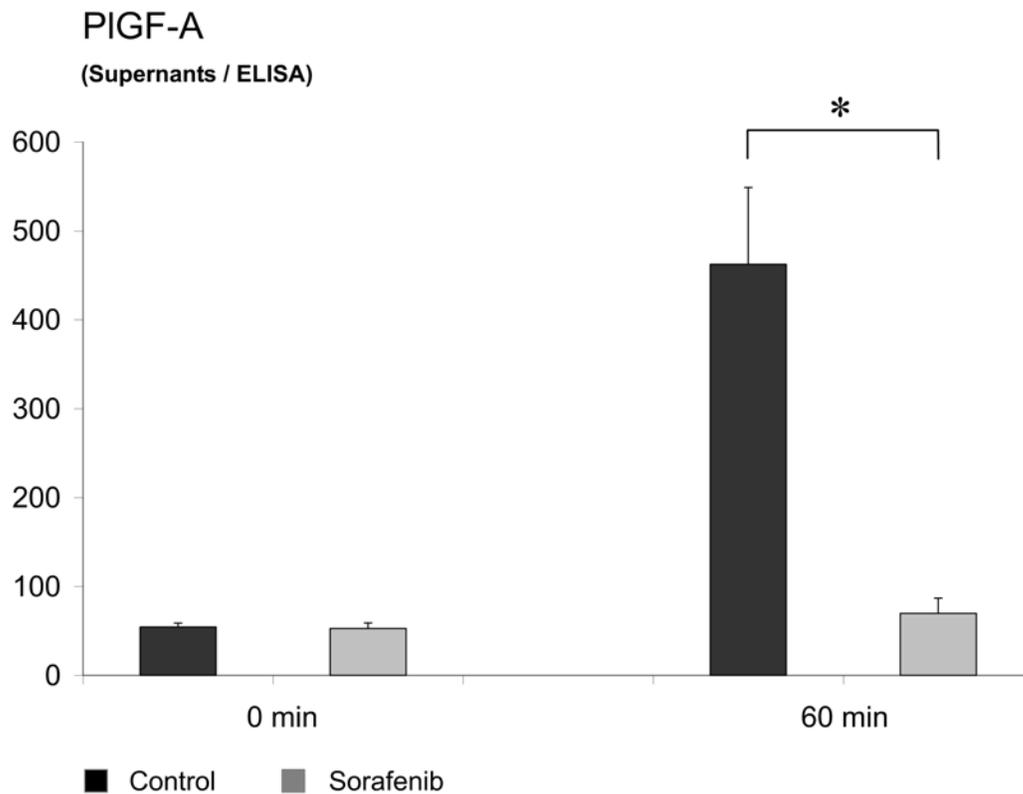
Immunohistochemical staining of VEGF-A, PDGF-B, and PIGF expression in RPE cells. Both untreated and non-illuminated control cells and cells that were treated with sorafenib, 1  $\mu\text{g}/\text{mL}$ , and had no illumination presented only a faint staining for VEGF-A, PDGF-B, and PIGF. After 60 min of light exposure, the expression of VEGF-A, PDGF-B, and PIGF was markedly increased. In contrast, RPE cells that were illuminated and had sorafenib added directly after illumination showed only a weak staining of VEGF-A, PDGF-B, and PIGF, which was comparable to those cells that served as non-illuminated controls.

### VEGF-A (Supernants / ELISA)



### PDGF-BB (Supernants / ELISA)





**Fig. 5:**

Inhibitory effect of sorafenib on light-induced secretion of VEGF-A, PDGF-BB, and PIGF, as investigated by an ELISA method: untreated RPE cells after illumination with plain white light (black columns) and after additional treatment with sorafenib (light gray columns). Each value was normalized to a standard curve of VEGF-A, PDGF-BB, or PIGF and expressed as concentration in nanograms per milliliter. Data values are means  $\pm$  SD.

**Table 1:**

Primers used for RT-PCR

Target	Length	Position	AT (°C)	% GC	Sequence
<b>VEGF-A</b>	18	1540-1557	60	56	tgcccgctgctgtctaat
	18	1592-1609	60	61	tctccgctctgagcaagg
<b>PIGF</b>	18	335-352	60	61	ggctgtcccttgctcc
	18	395-412	59	61	cagacaaggcccactgct
<b>PDGF-BB</b>	18	1108-1125	60	56	tgatctccaacgcctgct
	20	1156-1175	59	50	tcatgtcaggccaactcg