Sorafenib prevents human retinal pigment epithelium cells from light-induced overexpression of VEGF, PDGF and PIGF

M Kernt, A S Neubauer, R G Liegl, C Hirneiss, C S Alge, A Wolf, M W Ulbig, A Kampik

ABSTRACT

Background Cumulative light exposure is significantly associated with progression of age-related macular degeneration (AMD). Inhibition of vascular endothelial growth factor (VEGF) is a major critical event in the development of CNV. Therefore, inhibition of VEGF is the main target of the currently used antiangiogenic agents in the treatment of AMD. The mechanism of action of these VEGF inhibitors (eg, bevacizumab and ranibizumab) is blocking VEGF in the extracellular space and thereby preventing access to its receptor. Studies have demonstrated that treatment of AMD with VEGF inhibitors is safe and effective. However, major prospective randomised 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 reprogression occurs. 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 retina in proliferative retinal disease and play a crucial role in the development and progression of AMD. 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. Previous case reports indicate that sorafenib, alone or in combination with other angiosstatic drugs, might have a beneficial effect on exudative AMD.

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. 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 was 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, USA).
**RNA isolation and real-time PCR**

Total RNA was isolated from 10 cm Petri dishes by the guanidinium 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 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 PlGF 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 μl reaction volume contained 1×FastStart DNA Master Hybridisation Probes Mix (Roche Diagnostics), 4 mM MgCl₂, 0.5 mM of each primer, 0.2 mM TaqMan probe and 2 μl 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 labelled fluorophores were hybridised to the amplified fragment during the annealing phase. When the two probes came into close proximity, fluorescence resonance energy transfer (FRET) developed between the two fluorophores. The emitted fluorescence was then measured by the LightCycler instrument. Hybridisation probes were displaced during the extension step. Depending on

**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. 20 Dulbecco modified Eagle medium (DMEM; Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS; Biochrom) 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 photosensitiser. It has been implicated to play a certain role in increased accumulation of lipofuscin is a common characteristic of ageing RPE cells in vivo, and lipofuscin is known to act as a photosensitiser. It has been implicated to play a certain role in increased accumulation of lipofuscin. This characteristic disappears when RPE cells are cultured for long-term periods. 24 26

**DNA isolation and real-time PCR**

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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 normalisation 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 PlGF mRNA was determined as the relative ratio (RR), which was calculated by dividing the level of VEGF-A, PDGF-BB and PlGF 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.

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<td>59</td>
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AT, adenine-thymidine; GC, guanine-cytosine.

Table 1 Primers used for RT-PCR

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 α 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 in 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 in viability was seen (figure 1).

MTT assay

When cells were illuminated with unfiltered white light (300 mW/cm²) for 60 min, a significant reduction in RPE cell viability was detected (figure 2). In contrast, cells treated with sorafenib, 1 μg/ml, immediately after illumination showed a significantly smaller less decrease in viability compared with cells that were not treated with sorafenib (figure 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 normalised 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 decrease in RPE cell viability (Wilcoxon with α correction for multiple testing). Untreated retinal pigment epithelium cells 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 α correction for multiple testing) compared with controls.

Figure 1 Viability of primary human retinal pigment epithelium cells after treatment with the various concentrations of sorafenib, measured by a colorimetric test (methylthiotetrazole). Untreated retinal pigment epithelium 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.
A decrease in cellular viability was significantly reduced when cells were additionally incubated with sorafenib after 60 min of illumination. Error bars: SD.

**Figure 2** Viability of primary human retinal pigment epithelium cells after illumination with white light (black column) and after incubation with sorafenib at a concentration of 1 μg/ml (light grey column), as measured by a colorimetric test (methylthiotetrazole). The decrease in cellular viability was significantly reduced when cells were additionally incubated with sorafenib after 60 min of illumination. Error bars: SD.

**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.1 AMD is a disease of older people population.2 Loss and degeneration of RPE cells, particularly in the macular centre, is pathognomonic for this disease.3, 21 With advancing age, RPE cells undergo an increase in pleomorphism and accumulate metabolic debris from remnants of incomplete degradation of phagocytosed rod and cone membranes.1, 22 The incomplete degradation of phagocytosed 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.22, 28 Furthermore, the formation of drusen in Bruch 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.31–33 Once damaged, RPE cells secrete inadequate amounts of growth factors, such as VEGF, PDGF, PlGF and others.34 An imbalance between these growth factors in the eye potentially leads to aberrant angiogenesis resulting in the formation of dysfunctional blood vessels and CNV.34

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

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

**Figure 3** (A) Vascular endothelial growth factor (VEGF)-A, (B) platelet-derived growth factor (PDGF)-BB and (C) placenta growth factor (PlGF) mRNA expression of primary retinal pigment epithelium cells after illumination with white light (black columns) and after incubation with sorafenib at 1 μg/ml (light grey columns), as investigated by quantitative RT-PCR. x-Axis, relative ratio (RR) of VEGF-A, PDGF-BB or PlGF mRNA normalised to 18s rRNA, expressed in decimal format; y-axis, time of illumination.
VEGF-A, a member of the VEGF family, is a dimeric 36–46 kDa glycosylated protein with a predominant role in the development of pathological angiogenesis in AMD. A secondary important role of VEGF-A is the induction of increased vascular permeability. 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 with healthy control subjects. Therefore, VEGF-A has become the main target of the currently used antiangiogenic agents in the treatment of AMD. 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. 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 remodelling of the nascent extracellular matrix.

PIGF, 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. Both VEGF-A and PIGF have a recruiting effect on pericytes during angiogenesis in the maturation process of endothelial tubes, but also in the early phase of angiogenesis, where both growth factors act as an integral part of sprouting capillaries.

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. Pericytes are an important component of the choroidal neovascular complex, providing stabilisation of vessels, and are thought to limit the penetration of anti-VEGF agents. In addition, PDGF is thought to have an important role in fibroplasia as a wound-healing response in CNV.

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 neovascularisation and CNV compared with anti-VEGF treatment alone.

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 worth while.

The multikinase inhibitor sorafenib was initially identified as an RAF kinase inhibitor, but it also inhibits several receptor

Figure 4  Immunohistochemical staining of vascular endothelial growth factor (VEGF)-A, platelet-derived growth factor (PDGF)-B, and placenta growth factor (PIGF) expression in retinal pigment epithelium cells. Both untreated and non-illuminated control cells and cells that were treated with sorafenib, 1 µg/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, retinal pigment epithelium 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 with those cells that served as non-illuminated controls.

Figure 5  Inhibitory effect of sorafenib on light-induced secretion of (A) Vascular endothelial growth factor (VEGF)-A, (B) platelet-derived growth factor (PDGF)-BB and (C) placenta growth factor (PIGF), as investigated by ELISA: untreated retinal pigment epithelium cells after illumination with plain white light (black columns) and after additional treatment with sorafenib (light grey columns). Each value was normalised to a standard curve of VEGF-A, PDGF-BB or PIGF and expressed as concentration in nanograms per millilitre. Data values are means ± SD.
tyrosine kinases involved in angiogenesis. Sorafenib’s anti-proliferative 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. 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.

In this study, sorafenib at a concentration of 1 μg/ml led to a significant reduction in the light-induced increase in expression and secretion of VEGF, PIGF, 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 not only interacts 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 for example that sorafenib inhibits the phosphorylation of the initiation factor eIF4E, which is known to regulate the translation of a large number of mRNAs, including members so the Bcl-2 family.

Further research is warranted.

Sorafenib appears to be comparable with systemic anti-VEGF medications in its safety profile. The most common side effects after oral administration in patients treated for renal cell carcinoma (RCC) were dermatological symptoms such as rash or effects after oral administration in patients treated for renal cell carcinoma (RCC) were dermatological symptoms such as rash or...

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 800 mg per day. From several clinical trials, we know that after a daily dose of 800 mg of sorafenib, plasma levels are within the range of 10 μg/ml, after 200 mg per day around 4.5 μg/ml can be reached. To date, there are no data regarding the penetration of sorafenib into ocular tissue after systemic or after topical 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.

These data provide the first evidence that sorafenib effectively interacts in human RPE cells with three important targets of AMD treatment: VEGF, PIGF and PDGF. Due to its activity against multiple targets and pathways in angiogenesis, sorafenib seems to be promising as a potential treatment for AMD. 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.

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

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 signalling 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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Competing interests None.

Patient consent Obtained.

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