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RETINAL DISORDERS

# Comparative antiproliferative and cytotoxic profile of bevacizumab (Avastin), pegaptanib (Macugen) and ranibizumab (Lucentis) on different ocular cells

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**Abstract**
*Aim* To compare the antiproliferative and cytotoxic properties of bevacizumab (Avastin), pegaptanib (Macugen) and ranibizumab (Lucentis) on human retinal pigment epithelium (ARPE19) cells, rat retinal ganglion cells (RGC5) and pig choroidal endothelial cells (CEC).
*Methods* Monolayer cultures of ARPE19, RGC5 and CEC were used. Bevacizumab (0.1–0.3 mg/ml), pegaptanib (0.025–0.08 mg/ml) or ranibizumab (0.04–0.125 mg/ml) diluted in culture medium were added to the cells. Expression of VEGF-receptors (VEGFR1 and VEGFR2) and von Willebrand factor (a marker for endothelial cells) were analysed by immunohistochemistry. CEC cells were stimulated with VEGF. Cellular proliferative activity was monitored by BrdU-incorporation into cellular DNA. For cytotoxicity assays cells were grown to confluence and then cultured in a serum-depleted medium to ensure a static milieu. MTT-test was performed after one day.
*Results* CEC and ARPE19 cells stained positively for VEGFR1 and VEGFR2. More than 95% of the CEC cells were positive for von Willebrand factor. Ranibizumab reduced CEC cell proliferation by 44.1%, bevacizumab by 38.2% and pegaptanib by 35.1% when the drugs were used at their established clinical doses. The differences, however, between the three drugs in respect to cell growth inhibition were not statistically significant. Only a mild antiproliferative effect of bevacizumab or pegaptanib on ARPE19 cells could be observed. Ranibizumab did not alter ARPE19 cell proliferation. No cytotoxicity on RGC5, CEC and ARPE19 cells could be seen.
*Conclusions* Bevacizumab, pegaptanib and ranibizumab significantly suppress choroidal endothelial cell proliferation. However, when used at the currently established doses none of the drugs was superior over the others in respect to endothelial cell growth inhibition. The biocompatibility of all three drugs — including the off-label bevacizumab — seems to be excellent when used at the currently recommended intravitreal dose.

**Keywords** Bevacizumab (Avastin) · Pegaptanib (Macugen) · Ranibizumab (Lucentis) · Ocular cells · Antiproliferative

This work is presented on behalf of the Tuebingen Bevacizumab Study Group.

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## Introduction

Randomized clinical trials have shown that the inhibition of vascular endothelial growth factor (VEGF) by either intravitreal pegaptanib (Macugen, Eyetech Pharmaceutical, New York, NY, USA) or ranibizumab (Lucentis, Genentech, San Francisco, CA, USA) results in a stabilisation or improvement of vision in patients with neovascular AMD [3, 7, 15, 18].

Moreover, VEGF has been identified as a major angiogenic stimulus in diabetic retinopathy and nevascularization due to retinal vascular occlusion [1, 5].

However, multiple promising case reports and case series suggested using off-label intravitreal bevacizumab (Avastin, Genentech, San Francisco, CA, USA) for various neovascular eye diseases [2, 17, 19]. Bevacizumab has been approved for the treatment of metastatic colorectal cancer as it has been shown to increase the survival time when it is added to chemotherapy with 5-flourouracil [9].

Although there is mounting evidence that intravitreal bevacizumab is safe [12–14, 20], its use remains controversial as no comparative — neither clinical nor experimental — data in respect to approved treatment modalities such as pegaptanib, ranibizumab or photodynamic therapy (PDT) exist.

This study compares the antiproliferative and cytotoxic properties of bevacizumab, pegaptanib and ranibizumab at the clinically used dose range on human retinal pigment epithelium cells (ARPE19), rat retinal ganglion cells (RGC5) and pig choroidal endothelial cells (CEC).

## Methods

### Cell culture

Pig choroidal endothelial (CEC) cells were isolated as described before [8]. The ARPE19 cell line was purchased from American Type Culture Collection (Manassas, VA, USA). The rat ganglion cell line (RGC5) was kindly provided by Professor Neeraj Agarwal (UNT Health Science Center, Fort Worth, TX, USA).

ARPE19 and RGC5 cells were maintained in Dulbecco's modified Eagle's medium. The CEC cells were maintained in EGM MV-Microvascular Endothelial Cell Medium (Cambrex Clonetics, Wokingham, UK).

Cell suspensions ($1–5 \times 10^3$ cells/ml) were seeded onto 96-well tissue culture plates. For proliferation assays Invitrogen-Gibco Advanced MEM medium containing 1% fetal bovine serum, 1% glucose, VEGF-A at 2 ng/ml, 50 U/ml penicillin G and 50 U/ml streptomycin was used. Bevacizumab, pegaptanib and ranibizumab were diluted with culture medium to obtain bevacizumab in concentrations of 0.1 mg/ml and 0.3 mg/ml, pegaptanib in concentrations of 0.025 mg/ml and 0.08 mg/ml and ranibizumab in concentrations of 0.04 mg/ml and 0.125 mg/ml.

### Immunohistochemistry

CEC and ARPE19 cells were stained for VEGF-Receptor 1 (Flt-1) and 2 (Flk-1) and CEC cells for von Willebrand factor as previously described [20]. The following primary antibodies were used:

1. Polyclonal goat antibody to VEGF-Receptor 1 (Flt-1 C-17: sc-316; Santa Cruz Biotechnology, Santa Cruz, CA, USA)
2. Monoclonal mouse antibody to VEGF-Receptor 2 (Flk-1 A-3: sc-6251; Santa Cruz Biotechnology, Santa Cruz, CA, USA)
3. Monoclonal mouse antibody to von Willebrand factor (M 0616; F8/86; Dako, Glostrup, Denmark)

### BrdU ELISA

Cellular proliferative activity was directly monitored by quantification of 5′-bromo-2′-deoxyuridine (BrdU)-incorporation into the genomic DNA during cell growth. DNA synthesis was assessed by a colorimetric cell proliferation ELISA assay (Calbiochem, LaJolla, CA, USA) according to the manufacturer's instructions. Absorbance was analysed with an ELISA reader (SLT Spectra 400 ATX, Salzburg, Austria) at dual wavelengths of 450–540 nm.

### MTT stationary toxicity assay

To exclude a possible cytotoxicity of bevacizumab and pegaptanib on CEC, RGC5 and ARPE19 cells cellular viability was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay. Bevacizumab, pegaptanib or ranibizumab in serum free medium was added to the cells. After 24 hours the cells were washed with PBS, and MTT at 0.5 mg/ml in serum free medium was added to the cells. After 2 hours of incubation formazan extraction was performed and the quantity was measured colorimetrically at 570 nm.

### Expression of results and statistics

Results were expressed as units of mean absorbance +/− SD for MTT and BrdU assays. All reported comparisons were statistically analysed with Bonferroni-adjusted unpaired t-tests using SPSS statistical software (version 13.0, SPSS Inc., Chicago, IL, USA).

## Results

### Immunohistochemistry results

A strong VEGFR1 expression in ARPE19 but only a weak one in CEC cells could be observed. VEGFR2, the main mediator for VEGF-induced mitogenesis and vascular permeability was strongly expressed in the cytoplasm of ARPE19 cells and CEC.

More than 95% of the CEC cells displayed immunoreactivity for the von Willebrand factor and thus were considered to be purely endothelial cells (Fig. 1).

### Antiproliferative activity of bevacizumab, pegaptanib and ranibizumab

To quantify the inhibition of cellular proliferation we used the BrdU to monitor cellular proliferation at the DNA level. A significant inhibitory effect on CEC cell growth could be observed for all three drugs ($p<0.05$).

A moderate growth inhibition could be observed when the medications were used at their usual clinical dose (ranibizumab reducing CEC cell proliferation by 44.1%, pegaptanib by 35.1% and bevacizumab by 38.2%). The differences, however, between the three drugs in respect to cell growth inhibition were not statistically significant (Fig. 2). All three drugs showed a dose dependant inhibition of endothelial cell growth. At the clinically relevant intravitreal doses — pegaptanib 0.08 mg/ml (equalling a total dose of 0.3 mg in 4 ml vitreal volume), bevacizumab 0.3 mg/ml (equalling 1.25 mg in the human vitreous), and ranibizumab 0.125 mg/ml (equalling a total dose of 0.5 mg in the vitreous) — the drugs seemed to be equivalent in respect to endothelial cell growth inhibition. Also, when higher concentrations of VEGF (25–50 ng/ml) were added to the CEC cells none of the drugs was significantly more effective in reducing cell proliferation than the other (data not shown).

Ranibizumab at the test doses caused no relevant inhibition of cell proliferation of ARPE19 cells (however, the proliferation was mildly inhibited by bevacizumab and pegaptanib) (Fig. 2). None of the drugs altered RGC5 cell proliferation (data not shown). Also, when VEGF in higher concentration was added to ARPE19 or RGC5 cells no relevant antiproliferative effect could be seen. In addition, both the ARPE19 and RGC5 cells only showed a mild increase of cell proliferation when VEGF was added to the medium (data not shown). Thus, the proliferation of ARPE19 and RGC5 cells seems not to be VEGF dependant.

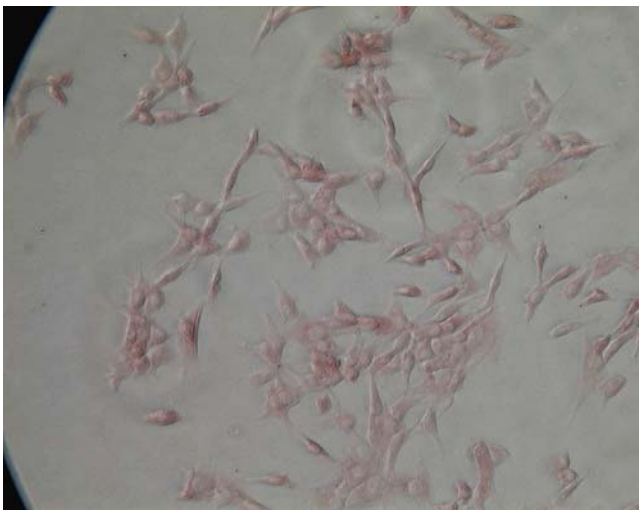

**Fig. 1** Immunohistochemistry for von Willebrand factor as an endothelial cell marker showing a strong expression in CEC cells

### Cytotoxicity assays

A stationary, confluent cell culture is better suited to detect a toxic drug effect than a proliferating culture and is more comparable to the natural situation within the eye (Fig. 3). No cytotoxicity was detectable for pegaptanib and bevacizumab at the respective concentrations that included the clinical dose for both drugs.

## Discussion

Ophthalmologists currently are confronted with the problem of choosing among several approved or off-label treatment options for their patients suffering from exudative AMD.

The safety and efficacy of ranibizumab in the treatment of neovascular AMD have been evaluated in two large phase III, multicenter, randomized, double-masked, controlled trials in different neovascular AMD patient populations. The trial results indicate that ranibizumab results not only in a slowing down of vision loss but also in a significant number of patients experiencing a clinically meaningful vision gain [3, 18]. In contrast, in clinical trials where patients received pegaptanib for neovascular AMD only stabilisation of visual acuity could be achieved in the majority of patients [7].

The low costs of off-label intravitreal bevacizumab led to its widespread use less than a year after its first successful application was published [17].

Unfortunately, to date no comparative clinical studies of these three drugs exist; but at least there is increasing evidence that the intravenous bevacizumab formulation is not harmful to the retina when it is injected into the vitreous [12, 13, 20].

In our retinal cell culture assays the biocompatibility of bevacizumab was as good as that of the approved drugs ranibizumab and pegaptanib.

The average inhibition of CEC cell growth was slightly stronger when ranibizumab was used. Nevertheless, this difference did not reach statistical significance. Moreover, such a trend in favour of bevacizumab — seen in cell culture — should not be over-interpreted as a better clinical efficacy.

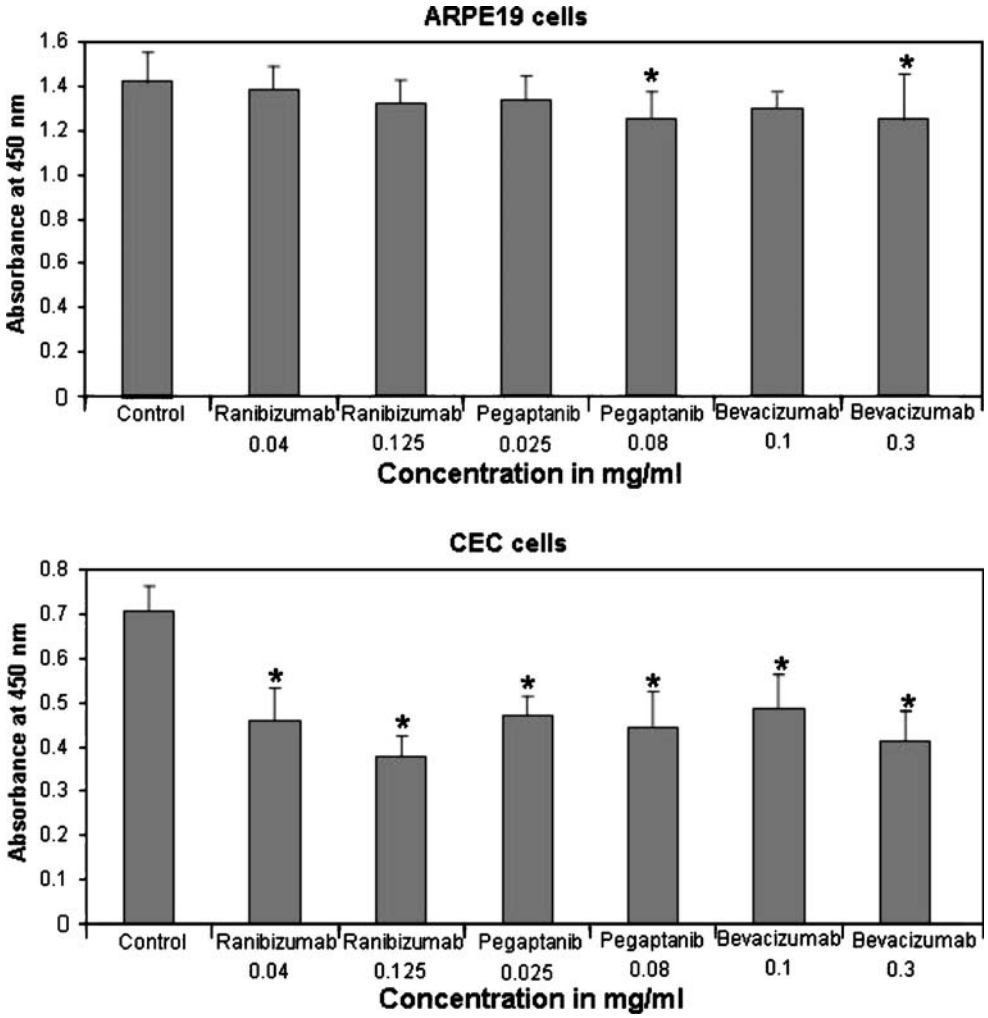


**Fig. 2** Proliferation assay using quantitative ELISA analysis of BrdU incorporation into ARPE19 (*top*) and CEC (*bottom*) cells during exposure to ranibizumab, pegaptanib and bevacizumab. The assay revealed a significant antiproliferative effect of each dose of bevacizumab, pegaptanib and ranibizumab on choroidal endothelial cells compared to control ($p<0.05$). However, pair-wise comparisons showed no differences between bevacizumab, pegaptanib and ranibizumab in respect to growth inhibition. The proliferation rate of RGC5 and ARPE19 cells was not affected by bevacizumab, pegaptanib nor by ranibizumab at the tested doses. *Indicates a statistically significant difference ($p<0.05$)

Our results suggest that the inhibition of CEC cell proliferation by bevacizumab is comparable to pegaptanib or ranibizumab and neither ranibizumab nor bevacizumab were superior to pegaptanib. However, the clinical efficacy involves multiple factors in addition to binding affinity and direct inhibition of cell proliferation, such as drug half life, biostability, retinal penetration, and non-antiangiogenic effects on vascular permeability and inflammation.

Our study did not compare the effect of ranibizumab, pegaptanib and bevacizumab on vascular permeability and the influence of the other factors mentioned above. Thus, no direct conclusion from our results can be drawn as to which of the three drugs may be more beneficial (or equally efficient) in patients suffering from neovascular AMD, diabetic macular edema or macular edema due to retinal vein occlusion.

The vitreal concentration of VEGF-A (a combination of all biologically active isoforms) in neovascular eye disease usually does not exceed 2 ng/ml [16, 21] (thus the VEGF concentration of 2 ng/ml we used in our endothelial cell experiments was sufficiently high). VEGF165 is the predominant isoform and seems to be crucial for the development of pathological ocular neovascularization [4, 10]. This may explain why pegaptanib that selectively inhibits VEGF165 was basically as effective in inhibiting CEC cell proliferation as the other drugs that block all VEGF isoforms.

It may be possible that human choroidal endothelial cells respond differently than porcine CEC cells. However, our CEC cells were strongly positive for human VEGFR2, the major mediator of VEGF induced mitogenesis, migration and endothelial cell growth [6]. Furthermore, human VEGF165 induces neovascularization in the myocardium of pigs [11]. Thus, it is reasonable to assume that human choroidal endothelial cells respond similarly to ranibizumab, pegaptanib and bevacizumab.

Moreover, bevacizumab, ranibizumab and pegaptanib seem to rather exclusively exhibit their biological effects on endothelial cells. Although the ARPE19 cell expressed

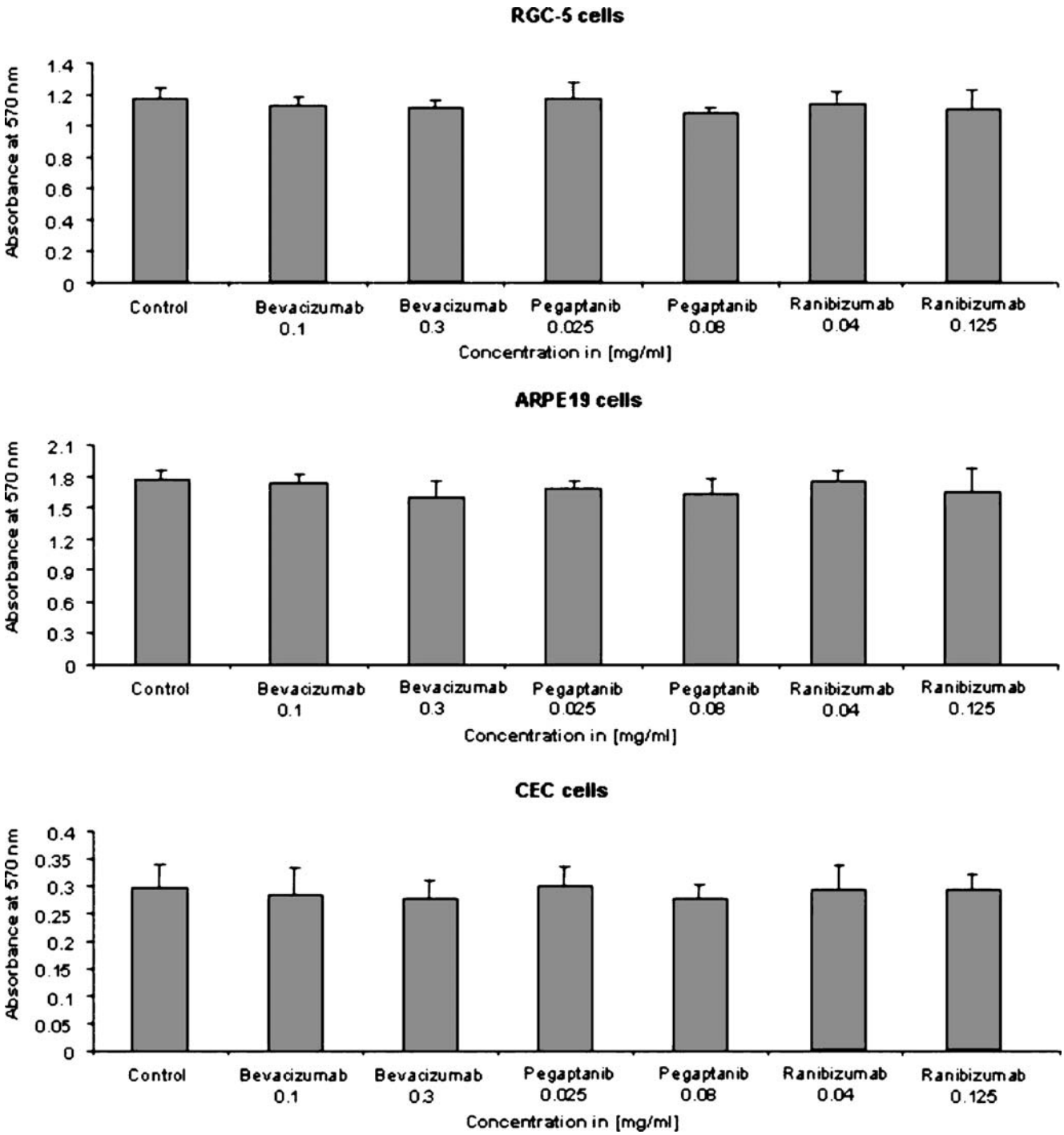


**Fig. 3** Toxicity determination in a stationary, confluent cell culture using MTT labelling. No significant cytotoxicity for bevacizumab, pegaptanib and ranibizumab could be seen in RGC5 (*top*), ARPE19 (*middle*) and CEC (*bottom*) cells

both VEGF-receptors no relevant reduction of RPE cell proliferation by ranibizumab, pegaptanib or bevacizumab could be observed. Thus, the VEGFR2 receptor on the ARPE19 cells unlike on the CEC cells may not mediate RPE cell growth. Nevertheless, it should be taken into account that the ARPE19 cell line is an immortalised cell line that may grow independently from VEGF inhibition or stimulation.

In conclusion, bevacizumab, pegaptanib and ranibizumab significantly suppress choroidal endothelial cell proliferation. The biocompatibility of all three drugs — including the off-label bevacizumab — seems to be excellent when used at the currently recommended intravitreal dose. However, when used at the established dose none of the drugs was superior over the others in respect to endothelial cell growth inhibition.

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