

Impact of Nintedanib and Anti-Angiogenic Agents on Uveal Melanoma Cell Behavior

Vera E. Pawlik, Svenja R. Sonntag, Salvatore Grisanti, Aysegül Tura, Vinodh Kakkassery, and Mahdy Ranjbar

Department of Ophthalmology, University of Lübeck, Lübeck, Germany

Correspondence: Vera E. Pawlik, Department of Ophthalmology, University of Lübeck, Lübeck 23562, Germany; vera.pawlik@uksh.de.

VK and MR authors contributed equally to this work and should be considered senior authors.

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PURPOSE. The purpose of this study was to investigate the direct impact of the combined angiokinase inhibitor nintedanib as well as the anti-angiogenic agents ranibizumab, bevacizumab, and aflibercept on the primary uveal melanoma (UM) cell line Mel270 and liver metastasis UM cell line OMM2.5.

METHODS. The metabolic activity, viability, and oxidative stress levels were analyzed by the Thiazolyl Blue Tetrazolium Bromide (MTT), LIVE/DEAD, and reactive oxygen species (ROS) assays. Expression of intracellular VEGF-A₁₆₅ and VEGF receptor-2 was detected by immunofluorescent staining. The secretion of VEGF-A₁₆₅ into the cell culture supernatants was evaluated by VEGF-A₁₆₅ ELISA.

RESULTS. Nintedanib, at a concentration of 1 µg/mL, resulted in a median reduction of metabolic activity (for Mel270 of approximately 38% and for OMM2.5 of 46% compared to the untreated control) without exerting toxicity in either cell line, whereas the other 3 substances did not result in any changes (which also means that none of the 4 substances led to an increased cell death). Moreover, nintedanib (1 µg/mL) induced oxidative stress in the Mel270 by approximately 1.2 to 1.5-fold compared to the untreated control, but not the OMM2.5 cells.

CONCLUSIONS. Nintedanib could suppress the growth of UM cells in a concentration-dependent manner. The metastatic UM cell line OMM2.5 was not sensitive to the pro-oxidant activity of nintedanib. This study was the first to investigate nintedanib in the context of UM. We propose further investigation of this substance to elucidate its effects on this tumor entity with the hope of identifying advantageous therapeutic options for future adjuvant tumor therapies.

Keywords: uveal melanoma (UM), nintedanib, in vitro, anti-VEGF, cell culture

Uveal melanoma (UM) is a rare entity of intraocular malignancy that develops from uveal melanocytes.¹ The incidence varies depending on the investigated population (between less than one to over 9 cases per million).^{2,3} UM is a disease of elderly adults which peaks between 70 and 80 years of age.⁴ There are several treatment options available, including radiotherapy,^{5–7} surgical resection,⁸ or the most radical approach of enucleation⁹ or exenteration of the orbit.¹⁰ These therapies target a local tumor control, which is often well achievable.¹¹ However, despite treatment, approximately half of the patients will develop metastases later in life,¹² with the liver as the main metastatic site.^{13,14} Unfortunately, treating metastatic UM remains a significant challenge due to the lack of effective standard therapies and genetic heterogeneity between the different tumors of the individual patients. Patients with UM are often enrolled in traditional chemotherapy or clinical trials for cutaneous melanoma despite giving the fact that both tumor entities differ immensely¹⁵ and therefore UM is known for not responding well to chemotherapy or immunotherapy like check point inhibitors, possibly, among others, due to its immunosuppressive microenvironment.¹⁶ Lately,

since 2022, the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) approved the immunotherapeutic substance Tebentafusp, however, which was restricted to patients with UM characterized by HLA-A*02:01 positivity. It is known to be the first immunotherapy that provides significant survival advantages.¹⁷

The concept of anti-angiogenesis is a method of tumor therapy that has been established since the 1970s, yet its complexity is not fully understood.^{18,19} Unfortunately, anti-angiogenic agents have shown limited success in previous clinical studies involving patients with UM. It is critical to consider the limitations of these previous clinical studies, including small cohorts, lack of randomization, non-placebo-controlled study design, and a mixing of patient populations between cutaneous melanoma and UM. However, some effects regarding the extension of stable disease were observed in certain cases.²⁰ Hence, there should be continued increase the focus on the cellular biological mechanisms of how such substances affect UM to better understand the reasons behind their efficacy or failure in lieu thereof. This approach should also involve the investigation of novel anti-angiogenic substances.

VEGF-A induced autocrine signaling was implicated in the malignant behavior of different tumor entities.^{21–31} Possible effects relate to stimulation of VEGF-A secretion,³¹ alteration of cell survival,^{24,26,28} tumor cell invasiveness,²⁸ or migration,³² or even resistance to chemotherapy.²⁷ However, previous studies have shown conflicting findings regarding the role of VEGF-A or its receptors in the growth of UM cells.^{33–35} For instance, it was shown that the presence of UM itself could stimulate VEGF and its receptors in otherwise unaffected ocular tissue in a paracrine manner.³⁶ In addition, VEGF-A secretion was stimulated by hypoxic conditions in the UM cell lines Mel270 and OMM2.5.³⁷ Although the anti-VEGF antibody bevacizumab was able to slow down UM growth in a mouse model after intraperitoneal injection,³⁸ another study demonstrated the paradoxical growth of UM in mice under intravitreal bevacizumab therapy.³⁹ Likewise, the proliferation of the human melanoma cell lines Mel285 and OMM2.3 was reduced through bevacizumab, whereas the expression of VEGF-A mRNA was stimulated by bevacizumab under hypoxic conditions.³⁹ Additionally, in clinical practice, several case reports have documented instances where UM tumor growth was observed in patients following intravitreal administration of bevacizumab.^{40,41} Conversely, intravitreal anti-VEGF therapy is routinely used for radiation-induced macular edema following UM irradiation.⁴² This practice may warrant critical reconsideration in the future, depending on the evolving understanding of the underlying pathomechanisms.³⁹ These incongruent and to some extent contradictory findings illustrate the complexity of the VEGF-related signaling pathways in UM and the urgent need for further investigation.

Most UM cell lines express VEGF-A₁₆₅ and other forms of pro-angiogenic factors, such as the basic fibroblast growth factor (FGF),^{37,43–46} as opposed to the normal melanocytes.⁴⁴ Additionally, VEGF receptors have been detected in the cytosol of UM cells, suggesting that autocrine and paracrine signaling may occur.^{37,46} Given that the development of UM metastasis can only occur hematogenously and UM is well vascularized,^{33,47} the objective of this study was to investigate the direct impact of the anti-VEGF agents ranibizumab,⁴⁸ bevacizumab,⁴⁹ and aflibercept,⁵⁰ as well as the triple angiokinase inhibitor nintedanib, which can block the receptors of VEGF, FGF, and platelet-derived growth factor,^{51–53} on the UM cells. The study design thus offers the opportunity to compare the cell behavior of primary tumor and metastasis. Additionally, it allows for a comparison among three ophthalmologically established substances and a new molecule that has not yet been explored in the field of ophthalmology, specifically in the context of UM. The rationale behind the use of nintedanib in this study was to investigate whether broad receptor inhibition could potentially have a stronger or different effect in the tumor cells compared to the other three tested substances, possibly through addressing overlapping signaling pathways.

METHODS

Cell Lines and Culturing

The UM cell lines Mel270 and OMM2.5 were obtained from Professor Martine J. Jager (Leiden, The Netherlands). Mel270 is a UM cell line originating from the primary tumor, whereas OMM2.5 is established from a liver metastasis of the same patient with UM (additional information about the reasons for choosing the two cell lines are provided

in the supplementary material).^{54,55} This provided us with the opportunity to comparatively assess the behaviors of the primary tumor cell line and cells from a metastasis, as both tumor cell lines have been derived from the same patient, but on the other hand could presumably differ in behavior from each other due to evolutionary changes in the cells during metastatic processes.^{56,57} Cells were maintained in the complete culture medium which consisted of RPMI 1640 with 2 mM L-Glutamine (BioWhittaker; Lonza, Verviers, Belgium), 10% fetal bovine serum (FBS; Biochrom GmbH, Berlin, Germany), and 1% penicillin/streptomycin (Biochrom GmbH, Berlin, Germany),⁵⁸ whereas the serum-starved culture medium contained only 1% FBS.⁵⁹ After cultivation at 37°C and 5% CO₂ until predominant confluence, the culture was passaged for the performance of different assays.

The test substances included the VEGF-A inhibitors ranibizumab^{48,60} (in concentrations of 62.5, 125, and 250 µg/mL; Novartis Pharma, Basel, Switzerland), bevacizumab^{49,61} (125, 250, and 500 µg/mL; Hoffmann-La Roche AG, Basel, Switzerland), and aflibercept⁵⁰ (250, 500, and 1000 µg/mL; Bayer Vital GmbH, Leverkusen, Germany), as well as the multi-angiokinase inhibitor nintedanib^{51–53} (0.001, 0.1, and 1 µg/mL; Selleck Chemicals LLC, Houston, TX, USA). The stock solution of nintedanib was prepared by dissolving in dimethylsulfoxide (DMSO; Sigma-Aldrich Chemie GmbH, Steinheim, Germany). As previously described,^{62,63} the final concentration of DMSO, which ranged between 0.0005% and 0.05% (v/v) for 0.01 to 1 µg/mL nintedanib, respectively, was not considered to be interfering with the following experiments. Cells that were incubated in complete or serum-starved medium without the addition of test substances served as controls. Please note that additional concentrations were tested for Thiazolyl Blue Tetrazolium Bromide (MTT) assay (see below).

Thiazolyl Blue Tetrazolium Bromide Assay

Cells were seeded into microtiter plates (Life Sciences, Corning, NY, USA) at a concentration of 5×10^3 cells/100 µL/well in full medium and allowed to attach overnight, followed by the incubation in fresh full medium with or without the test substances (at concentrations of 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, and 1 µg/mL for nintedanib and 1, 10, 50, 125, 250, 500, 750, and 1000 µg/mL for the other 3 substances) for 48 hours. The stock solution of MTT (5 mg/mL in PBS; Gibco by Lifetechnologies, Carlsbad, CA, USA) was added at a dilution of 1:5 (v/v) and the cells were incubated further for 3.5 hours.^{64,65} Measurement of absorbance (UV/visual absorption spectroscopy in optical density [OD]) was performed using a spectrophotometer (Molecular Devices GmbH, Biberach an der Riss, Germany) at 570 nm⁶⁴ after dissolving the MTT crystals with DMSO.⁶⁶ Negative controls were performed by measuring wells without cells but with the addition of MTT solution to prevent false positive results.

Reactive Oxygen Species Assay

Cells were seeded into black microtiter plates (Thermo Fisher Scientific, Roskilde, Denmark) in full medium at a density of 5×10^5 cells/100 µL/well and allowed to attach overnight, followed by a change to serum-starved medium for another 24 hours and then incubation with the test

substances in fresh serum-starved medium for a further 24 and 48 hours. Reactive oxygen species (ROS) detection was performed by a fluorometric assay, as described by the manufacturer (Lifetechnologies, Eugene, OR, USA), which uses a fluorometer (SpectraMax i3x; Molecular Devices GmbH, Biberach an der Riss, Germany) at the wavelengths of 487 nm and 532 nm (relative fluorescence units [RFU]).⁶⁷ Blanks were measured from the cells that were incubated with the same substance concentrations but without adding the assay reagent. The blanks were later subtracted from the corresponding values to exclude the possible autofluorescence. In addition, at the beginning of the experiments, negative controls were performed without cells but with cell medium and ROS solution.

Live/Dead Assay

Cells were seeded into 8-well chamber slides (Thermo Scientific, Rochester, NY, USA) at a concentration of 100,000 cells/400 μ L/well in full medium, allowed to attach overnight, followed by serum-starvation (1% FBS) for another 24 hours and incubated for 24 and 48 hours with the test substances that were diluted in fresh serum-starved medium. The fluorescent LIVE/DEAD Cell Imaging Assay (488/570 nm; Lifetechnologies, Eugene, OR, USA) was performed following the manufacturer's instructions.^{68–71} Cells were visualized under 100x magnification using a fluorescent microscope (Leica DMI 6000B, Wetzlar, Germany) with a monochrome digital camera (DFC350FXR2; Leica) and the filter sets Cy5/Y3 (exposure time 1030 ms, intensity 3, and gain 6.1) and Alexa488/L5 (exposure time 545 ms, intensity 3, and gain 6.1). Negative controls were performed by microscoping chamber slides without addition of fluorescent solution.

Three digital images per well were randomly taken (Leica Application Suite Advanced Fluorescence software 2.7.0.9329), blinded, and quantified using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).⁷² Cell survival was calculated by building a quotient out of the area of living cells and the total cell area for each image.

Immunofluorescent Staining

UM cells were seeded, cultured, and incubated similarly to the LIVE/DEAD assay. After 24 or 48 hours of incubation, the cells were fixed with paraformaldehyde (4% in PBS; MERCK KGaA, Darmstadt, Germany), permeabilized with Triton X-100 (0.1% in PBS; Serva Electrophoresis GmbH, Heidelberg, Germany), and blocked for at least 30 minutes in normal goat serum (2% in PBS; Sigma-Aldrich, Millipore, Temecula, CA, USA). The anti-human primary antibodies against human VEGF-A₁₆₅ (Human VEGFA PAb, polyclonal, IgG, rabbit; Thermo Scientific, Rockford, IL, USA) or VEGF receptor-2 (Pierce CD309 / VEGFR2 Antibody (4B4), monoclonal, IgG1, mouse; Thermo Scientific, Rockford, IL, USA) were diluted 1:300 in Tris-buffered saline with 0.1% bovine serum albumin. Cells were incubated with the primary antibodies overnight at 4°C, followed by the secondary antibodies goat anti-rabbit IgG (H+L) Alexa Fluor 488 (Millipore, Temecula, CA, USA; 1:400 in PBS) or goat anti-mouse IgG (H+L) DyLight 550 (Thermo Scientific, Rockford, IL, USA; 1:400 in PBS) for 1 hour at 37°C. Cell nuclei were counterstained with DAPI (0.001 μ g/mL in PBS, Invitrogen by Thermo Fisher Scientific, Paisley, UK). Negative controls

were performed by adding only the secondary antibody without the primary antibody.

Three images per well were randomly taken using the same microscope and camera as in the LIVE/DEAD assay under 400x magnification (DAPI/A4 with an exposure time of 100 ms, intensity 4, and gain 6.1; Alexa488/L5 with an exposure time of 2000 ms, intensity 5, and gain 6.1; Cy5/Y3 with an exposure time of 700 ms, intensity 4, and gain 6.1).

After blinding all images, semiquantitative measurement of positive stain was calculated using ImageJ. For each image, the positive fluorescent area for VEGF-A₁₆₅ or VEGFR2 was divided through positive DAPI area and multiplied with cell count of each image, so that a value of mean expression for VEGF-A₁₆₅ or VEGFR2 per cell was provided.

VEGF-A₁₆₅ Enzyme-Linked Immunosorbent Assay

The supernatants of cells were collected after the incubation with or without the test substances for 24 hours. VEGF-A₁₆₅ in the culture medium was detected by ELISA following the manufacturer's instructions and also negative controls were done according to these instructions (Thermo Scientific Pierce Biotechnology, Rockford, IL, USA).⁷³

Statistical Analysis

Data were analyzed using the software Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) as well as Graphpad Prism 7 and 8 (Graphpad Software, LLC, USA). Supplementary Table S1 gives an overview about the performed sample sizes, technical replicates, and repetition rates for each experiment. Values were normalized to the medians of the corresponding controls so that the results are shown as "percentage of control." Descriptive statistical analysis was done by calculating the median, interquartile range (IQR), minimum, and maximum for each group and presented as box-and-whisker plots. The comparison of two groups was performed by using the exact Mann Whitney *U* test. *P* values < 0.05 were considered as significant.

RESULTS

Optimal Dosage of Nintedanib, Ranibizumab, Bevacizumab, and Aflibercept on the Mel270 and OMM2.5 Cells Based on the Metabolic Activity

The extent of metabolic activity was evaluated by the MTT test after the incubation of the Mel270 and OMM2.5 cells for 48 hours with or without the test substances in complete medium with 10% serum. Nintedanib was administered at the concentration range of 0.005 to 1 μ g/mL whereas ranibizumab, bevacizumab, and aflibercept were provided at the doses between 1 and 1000 μ g/mL.

In the Mel270 cells, the highest tested concentration of nintedanib (1 μ g/mL) resulted in an approximately 38 or 45% decrease in the median metabolic activity compared to the untreated control or 0.5 μ g/mL Nintedanib, respectively (*P* < 0.001 and *P* = 0.002, respectively; Fig. 1A). Incubating the Mel270 cells with ranibizumab (Fig. 1B), bevacizumab (Fig. 1C), or aflibercept (Fig. 1D) resulted in no concentration-dependent tendencies or differences compared to the control group.

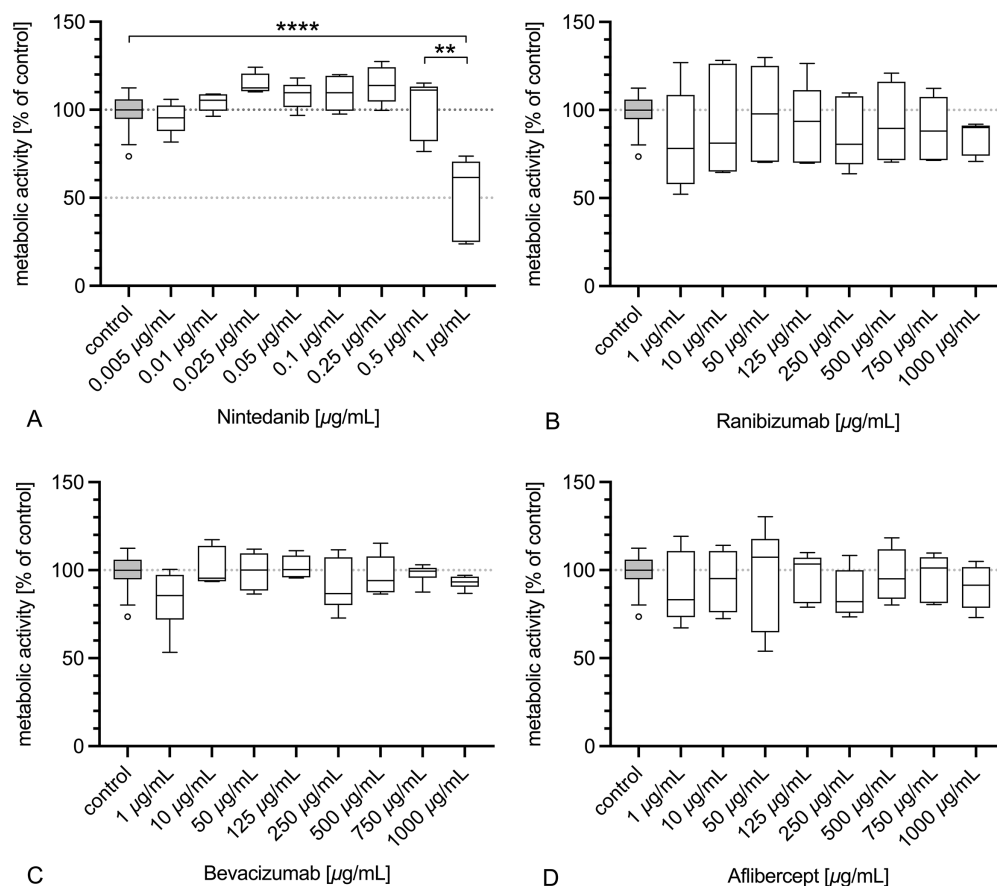


FIGURE 1. Metabolic activity of the cell line Mel270 as a percentage of untreated control as determined by the MTT assay after 48 hours of incubating the test substances. Cells were incubated in full medium with increasing concentrations of (A) nintedanib (0.005 to 1 µg/mL), (B) ranibizumab (1 to 1000 µg/mL), (C) bevacizumab (1 to 1000 µg/mL), and (D) aflibercept (1 to 1000 µg/mL; $n = 3$) for 48 hours. Absorption spectroscopy was taken in the unit (OD; optical density) at 570 nm. The results were normalized to the medians of the untreated controls of every repetition (n), respectively, and are therefore presented as the percentage of untreated control (%; lacking any unit). For the control group statistically simple outliers (degrees) were detected as shown in the figure, that represent values of more than 1.5-fold of the interquartile range in Tukey's box plots. None of the three different VEGF-A inhibitors had any impact on metabolic activity compared to the untreated control group, that also applies to the concentrations of nintedanib between 0.005 and 0.5 µg/mL. In contrast, the incubation with the highest concentration of nintedanib (1 µg/mL) decreased metabolic activity compared to the control ($P < 0.001$ ****) and in comparison to the lower tested concentrations of nintedanib ($P = 0.002$ **), as determined by the Mann Whitney U test.

The highest tested concentration of nintedanib (1 µg/mL) could also reduce the median metabolic activity of the OMM2.5 cells by approximately 46 or 52% compared to the control group and 0.5 µg/mL nintedanib, respectively ($P < 0.001$ and $P = 0.002$, respectively; Fig. 2A). For ranibizumab (Fig. 2B), bevacizumab (Fig. 2C), or aflibercept (Fig. 2D), there were neither concentration-dependent trends nor any indication of a difference compared to the control group.

Based on these findings, three concentrations of each substance were chosen for the subsequent experiments. The concentrations of ranibizumab, bevacizumab, and aflibercept were determined by considering their intraocular concentrations after a regular intravitreal injection, assuming a vitreous volume of 4 mL.^{74,75} Specifically, ranibizumab was tested at the concentrations of 62.5, 125, and 250 µg/mL, bevacizumab at 125, 250, and 500 µg/mL, and aflibercept at 250, 500, and 1000 µg/mL. Intravitreal clinical doses resemble each medium dosage, flanked by halved and doubled concentrations. As no information was available for nintedanib's intraocular use, its concentrations were solely based on the results of the MTT assay, and the 3 concentrations of 0.01, 0.1, and 1 µg/mL were selected.

This decision was also substantiated by the available pharmacological data for nintedanib on extraocular human cells.^{51,76}

Viability and Toxicity in Response to the Test Substances

In the Mel270 cells, the LIVE/DEAD assay revealed no evidence of changes in viability after the 24- or 48-hour incubation with any of the test substances compared to the control group, with the percentage of viable cells being in a narrow range around 100% of the control (Figs. 3A, 3B, Supplementary Fig. S1).

In the OMM2.5 cells, the median viability values after the nintedanib treatment for 24 hours were also similar to the median of the control group, except for a slight reduction to 99% for 1 µg/mL Nintedanib, indicating lower cell survival in response to the highest concentration of this inhibitor ($P = 0.003$; Fig. 3C, see Supplementary Fig. S1). However, this effect could only become apparent graphically through the scaling of the ordinate (see Fig. 3C).

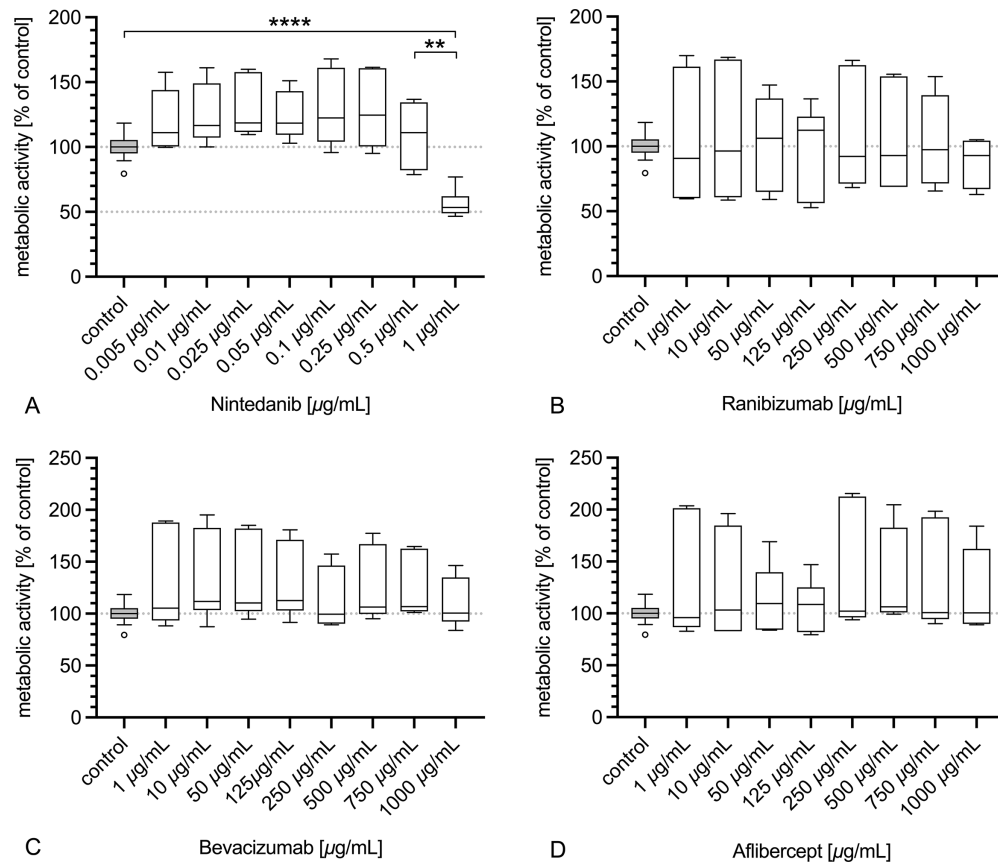


FIGURE 2. Metabolic activity of the cell line OMM2.5 as the percentage of untreated control as determined by the MTT assay after 48 hours of incubating the test substances. Cells were incubated in full medium with (A) nintedanib (0.005 to 1 µg/mL), (B) ranibizumab (1 to 1000 µg/mL), (C) bevacizumab (1 to 1000 µg/mL), and (D) aflibercept (1 to 1000 µg/mL) in increasing concentrations ($n = 3$) for 48 hours. Absorption spectroscopy was taken in the unit (OD; optical density) at 570 nm. The results were normalized to the medians of the untreated controls of every repetition (n), respectively, and are therefore presented as the percentage of untreated control (%; lacking any unit). For the control group, statistically simple outliers (degrees) were detected as shown in the figure, that represent values of more than 1.5-fold of the interquartile range in Tukey's box plots. Similar to the results for the cell line Mel270 (see Fig. 1), OMM2.5 also showed a lower metabolic activity in response to the highest concentration of nintedanib (1 µg/mL) compared to the untreated control group ($P < 0.001$ **** and $P = 0.002$ ** as determined by the Mann Whitney U test). Lower concentrations of nintedanib (0.005 to 0.05 µg/mL) as well as all concentration groups of the three substances ranibizumab, bevacizumab, and aflibercept (1 to 1000 µg/mL) did not result in any effects compared to the control. As opposed to the data of nintedanib, the remaining test substances did not indicate any effects on metabolic activity.

No difference in viability was detected after the 48-hour exposure of the OMM2.5 cells to nintedanib at any of the tested concentrations (Fig. 3D, Supplementary Fig. S1). Likewise, the incubation of the OMM2.5 cells with ranibizumab, bevacizumab, or aflibercept for 24 and 48 hours at the indicated concentrations did not influence the viability compared to the control (see Figs. 3C, 3D, Supplementary Fig. S1).

Oxidative Stress in Response to the Test Substances

Incubation of the Mel270 cells with 1 µg/mL of nintedanib for 24 hours resulted in an almost 1.5-fold increase in the accumulation of ROS compared to the control ($P < 0.001$) and a 1.2-fold elevation compared to the intermediate concentration of nintedanib at 0.1 µg/mL ($P = 0.002$; Fig. 4A). Similarly, the Mel270 cells that were incubated for 48 hours with 1 µg/mL nintedanib demonstrated 1.4-fold higher levels of oxidative stress compared to the control ($P = 0.01$; Fig. 4B). The prolonged exposure to the highest concentration of aflibercept (1000 µg/mL) for

48 hours could also intensify the oxidative stress by almost 1.3-fold compared to the control ($P = 0.001$; see Fig. 4B), whereas the shorter incubation with aflibercept for 24 hours failed to induce any effects (see Fig. 4A).

No notable differences in the ROS levels were detected in the Mel270 cells that were treated with ranibizumab or bevacizumab after 24 or 48 hours compared to the controls (see Figs. 4A, 4B). Likewise, the extent of oxidative stress was not altered in the OMM2.5 cells in response to any of the test substances (Figs. 4C, 4D).

Expression of VEGFA₁₆₅ and VEGFR2

In the Mel270 cells, the immunoreactivity for VEGFA₁₆₅ was not significantly altered in response to any of the test substances compared to control after 24 or 48 hours (Figs. 5A, 5B, see Supplementary Fig. S1). Likewise, the OMM2.5 cells that were exposed to the test substances for 24 hours did not exhibit any differences in the intracellular VEGFA₁₆₅ levels with regard to the control (Fig. 5C, see Supplementary Fig. S1). However, the incubation of OMM2.5 cells with the highest dose of aflibercept (1000 µg/mL) for

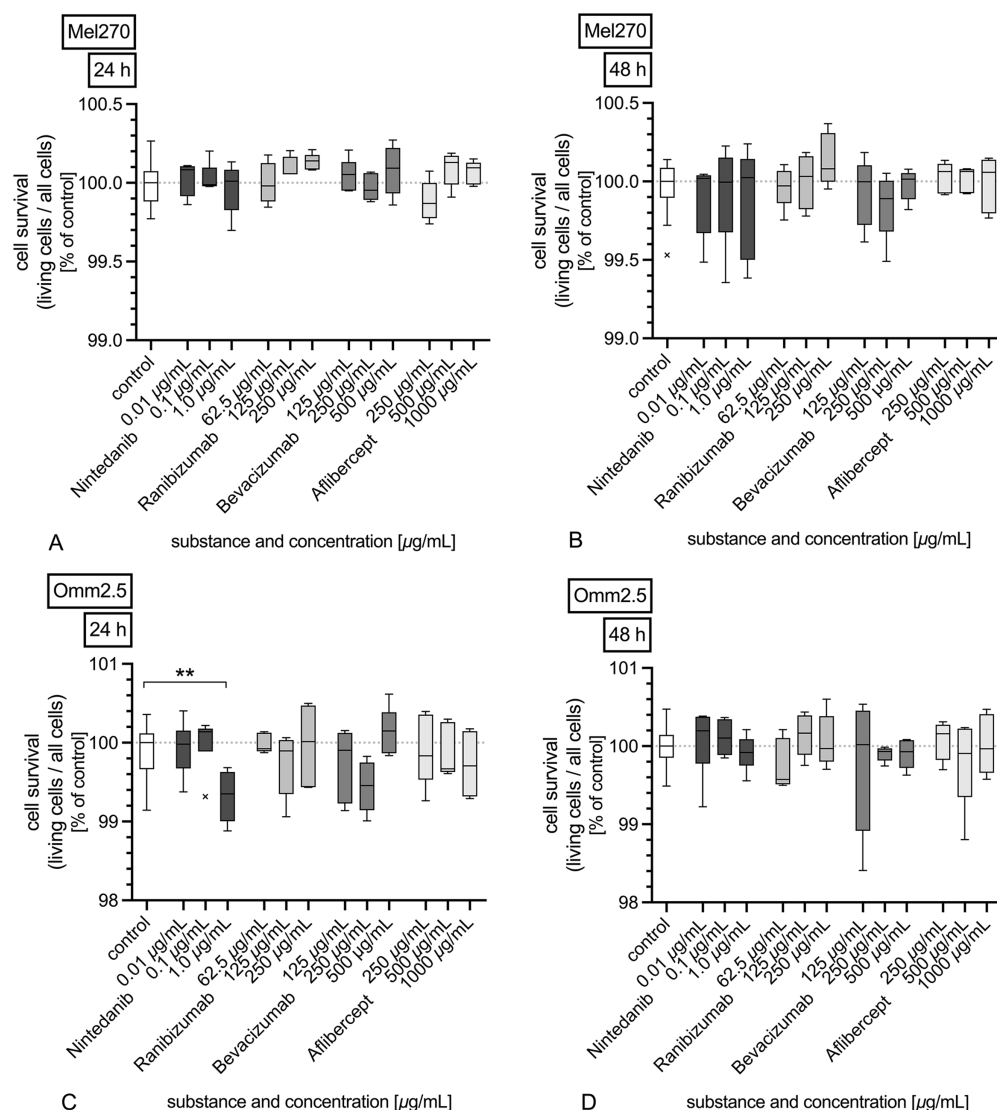


FIGURE 3. LIVE/DEAD assay demonstrating the cell survival as percentage of untreated control following incubation with nintedanib (0.01; 0.1 and 1 µg/mL), ranibizumab (62.5; 125 and 250 µg/mL), bevacizumab (125; 250 and 500 µg/mL), and aflibercept (250; 500 and 1000 µg/mL) at low, medium, and high concentrations ($n = 3$) for two different incubation durations (24 hours or 48 hours, respectively). The LIVE/DEAD assay aims to discriminate between living cells and dead cells by staining them in different fluorescent dyes (green or red, respectively). Cell survival was calculated by building a quotient out of the area of living cells (pixel) and the total cell area (pixel) for each image. Afterward, data were normalized to the medians of the untreated control of every repetition (n) and therefore data are presented as percentage of untreated control. For a few tested groups statistically extreme outliers ($^{\circ}$) were detected as shown in the figure, that represent values of more than three-fold of the interquartile range in Tukey's box plots. (A) After 24 hours of incubation, the cell line Mel270 did not exhibit any evidence for substance- or concentration-dependent changes in cell survival or toxic effects. (B) Similarly, after 48 hours of incubation, no evidence for changes in cell survival or toxic effects for the cell line Mel270 was shown. (C) In contrast, a 24-hour incubation with the high concentration of nintedanib (1 µg/mL) on the cell line OMM2.5 showed a slight decrease in cell survival in comparison with the untreated control ($P = 0.003$ **, Mann Whitney U test). However, the ordinate axis was expanded to accentuate this finding and all concentration groups had a cell survival of approximately 99%, ruling out any relevant toxic effects. (D) After the 48 hours of incubation of the OMM2.5 cells with different test substances, there was no evidence of substance- or concentration-dependent changes in cell survival or toxic effects.

48 hours resulted in a 1.4-fold increase in the VEGFA₁₆₅ expression compared to the control ($P = 0.004$; Fig. 5D), whereas aflibercept at lower doses or the remaining test substances failed to induce any effects on the intracellular VEGFA₁₆₅ in the OMM2.5 cells after 48 hours (see Fig. 5D, Supplementary Fig. S1).

No significant differences were observed in the expression of VEGFR2 in the Mel270 or OMM2.5 cells regardless of the test substance and incubation time (Fig. 6, see Supplementary Fig. S1).

Extracellular VEGFA₁₆₅ Levels in Response to the Test Substances After 24 Hours

By performing ELISA, we were not able to detect any VEGFA₁₆₅ in the supernatants of the Mel270 and OMM2.5 cells that were incubated with the anti-VEGFA agents ranibizumab, bevacizumab, and aflibercept ($n = 2$ experiments, data not shown). In both the Mel270 and OMM2.5 cells, the extracellular VEGFA₁₆₅ levels tended to be slightly reduced in response to the highest concentration of

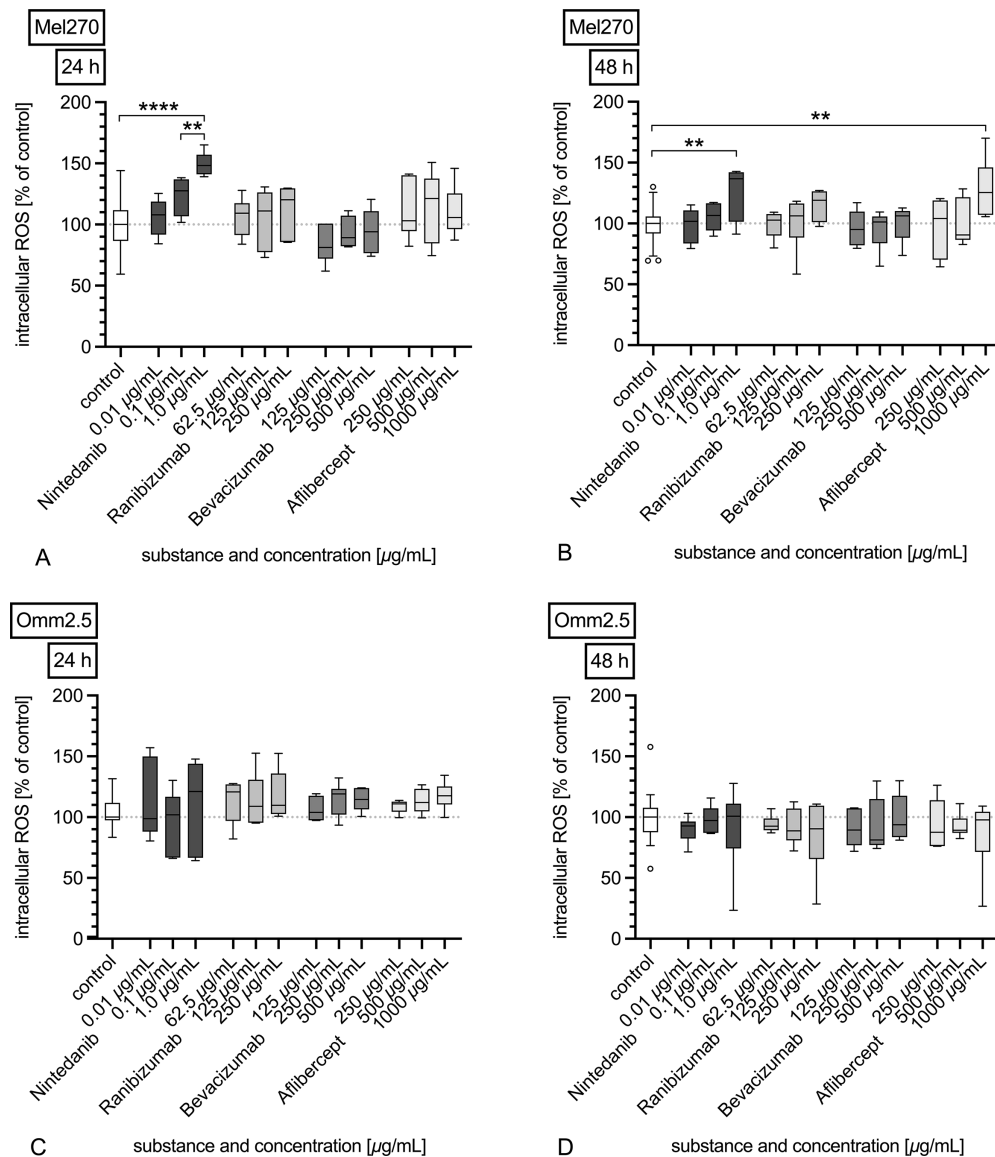


FIGURE 4. Intracellular reactive oxygen species (ROS) in the cell lines Mel270 and OMM2.5 (as percentage of the untreated control) after 24 or 48 hours of incubation with nintedanib (0.01; 0.1 and 1 $\mu\text{g/mL}$), ranibizumab (62.5; 125 and 250 $\mu\text{g/mL}$), bevacizumab (125; 250 and 500 $\mu\text{g/mL}$), or aflibercept (250; 500 and 1000 $\mu\text{g/mL}$) at low, medium, and high concentrations ($n = 3$). The amount of intracellular ROS is interpreted as intracellular oxidative stress level of the cells. Values were measured by fluorometry (relative fluorescence units [RFUs]) at the wavelengths of 487 nm and 532 nm. Data were normalized to the medians of the untreated control of every repetition (n) and therefore data are presented as percentage of untreated control. For the control groups statistically simple outliers (degrees) were detected as shown in the figure, that represent values of more than 1.5-fold of the interquartile range in Tukey's box plots. (A) Incubation of the Mel270 cells with nintedanib for 24 hours increased the intracellular ROS levels in a dose dependent manner, with the highest oxidative stress detected for 1 $\mu\text{g/mL}$ ($P < 0.001$ **** and $P = 0.002$ **). (B) The oxidative stress in response to 1 $\mu\text{g/mL}$ nintedanib was sustained after 48 hours in the Mel270 cells ($P = 0.01$ **). The 48-hour incubation of the Mel270 cells with aflibercept at the highest concentration (1000 $\mu\text{g/mL}$) also resulted in a higher ROS level than the untreated control ($P = 0.001$ **). For the cell line OMM2.5, no substance or concentration dependent changes in intracellular ROS could be detected neither for (C) 24 hours of incubation, nor for (D) 48 hours.

nintedanib compared to the intermediate and lower doses of this inhibitor, which did not reach significance (Figs. 7A, 7B).

DISCUSSION

Incubation of the Mel270 and OMM2.5 cells with the multi-angiokinase inhibitor nintedanib at the highest tested concentration (1 $\mu\text{g/mL}$) resulted in a reduction of metabolic activity without showing explicit toxic effects. In contrast to OMM2.5, the Mel270 cells furthermore presented an

elevated oxidative stress under treatment with 1 $\mu\text{g/mL}$ nintedanib. The VEGF-A inhibitors ranibizumab, bevacizumab, and aflibercept were able to fully block the secreted VEGF-A₁₆₅ in the medium supernatants (data not shown), whereas nintedanib failed to exert any effects on the extracellular VEGF-A₁₆₅. However, ranibizumab, bevacizumab and aflibercept remained mostly inefficient by comparison with controls regarding the metabolic activity and oxidative stress. We were not able to detect any alterations in the cellular expression of VEGF-A₁₆₅

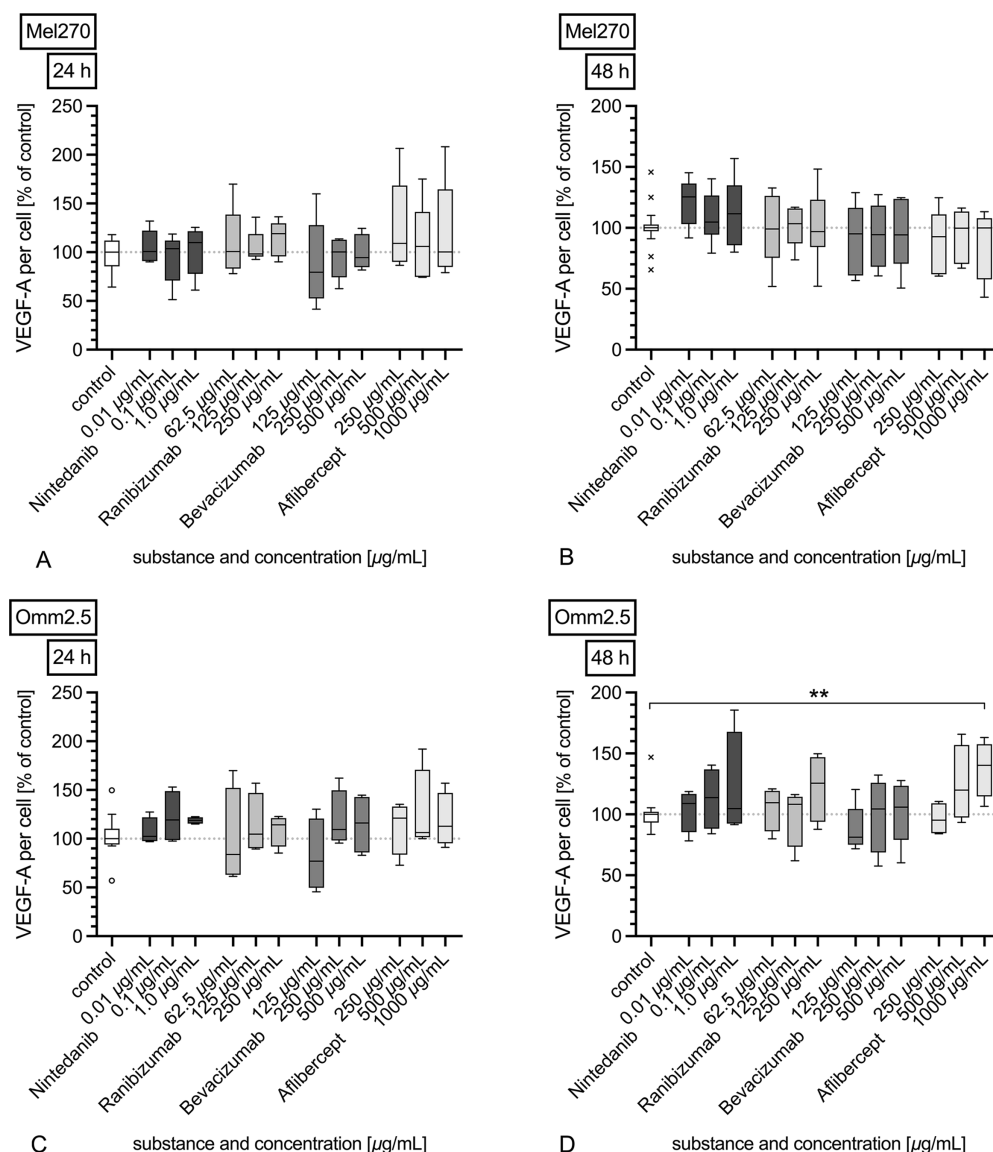


FIGURE 5. Immunofluorescent staining for intracellular VEGF-A₁₆₅ in the cell lines Mel270 and OMM2.5. The VEGF-A₁₆₅ levels were measured semiquantitatively as fluorescent stained area (VEGF-A₁₆₅) of each image per mean cell nucleus area (DAPI; in pixels) and expressed as the percentage of the untreated control after 24 (A and C) or 48 hours (B and D) of incubation with nintedanib (0.01; 0.1 and 1 µg/mL), ranibizumab (62.5; 125 and 250 µg/mL), bevacizumab (125; 250 and 500 µg/mL), or aflibercept (250; 500 and 1000 µg/mL) at low, medium, and high concentrations. The replicate numbers were $n = 5$ (A), $n = 6$ (B), $n = 4$ (C), and $n = 5$ (D), respectively. For the control groups statistically simple (degrees) and extreme outliers (*) were detected as shown in the figure, that represent values of more than 1.5- or 3-fold of the interquartile range in Tukey's box plots. Data were normalized to the medians of the untreated control of every repetition (n) and therefore data are presented as percentage of untreated control. Incubation of the OMM2.5 cells with 1000 µg/mL aflibercept for 48 hours resulted in a higher amount of VEGF-A₁₆₅ in comparison with the control group ($P = 0.004$ **). No changes in intracellular VEGF-A₁₆₅ levels were detected for the remaining treatment groups (A–D).

or VEGFR2 in response to any of the test substances either.

The MTT assay is a long established, cost effective, and rapidly applied method as part of the drug discovery processes.^{64–66,77} It helps identify potential candidates by evaluating their impact on cell viability and proliferation, especially in the field of sensitivity testing just as in this present study.^{78–83} In this study, the MTT assay^{64,66,77,79–85} was performed to screen different concentrations of the four substances. Nintedanib, at a concentration of 1 µg/mL, showed an anti-proliferative effect by reducing the metabolic activity of the 2 UM cell lines. Such an effect of this substance

has not been reported for UM yet, but similar effects of nintedanib on lung cancer cell lines have been described, in which the dimensions of metabolic reduction have been comparable with our results.⁸⁶

Our findings, that none of the 3 other substances (ranibizumab, bevacizumab, or aflibercept) induced any toxic effects on the two UM cell lines, may initially appear to be contradictory to our earlier study, which demonstrated a significant reduction in the metabolic activity of the Mel270 and OMM2.5 cells that were treated with ranibizumab for 1 day.⁸⁷ However, our former MTT analysis was conducted by using a culture medium with 5% serum, whereas, in our

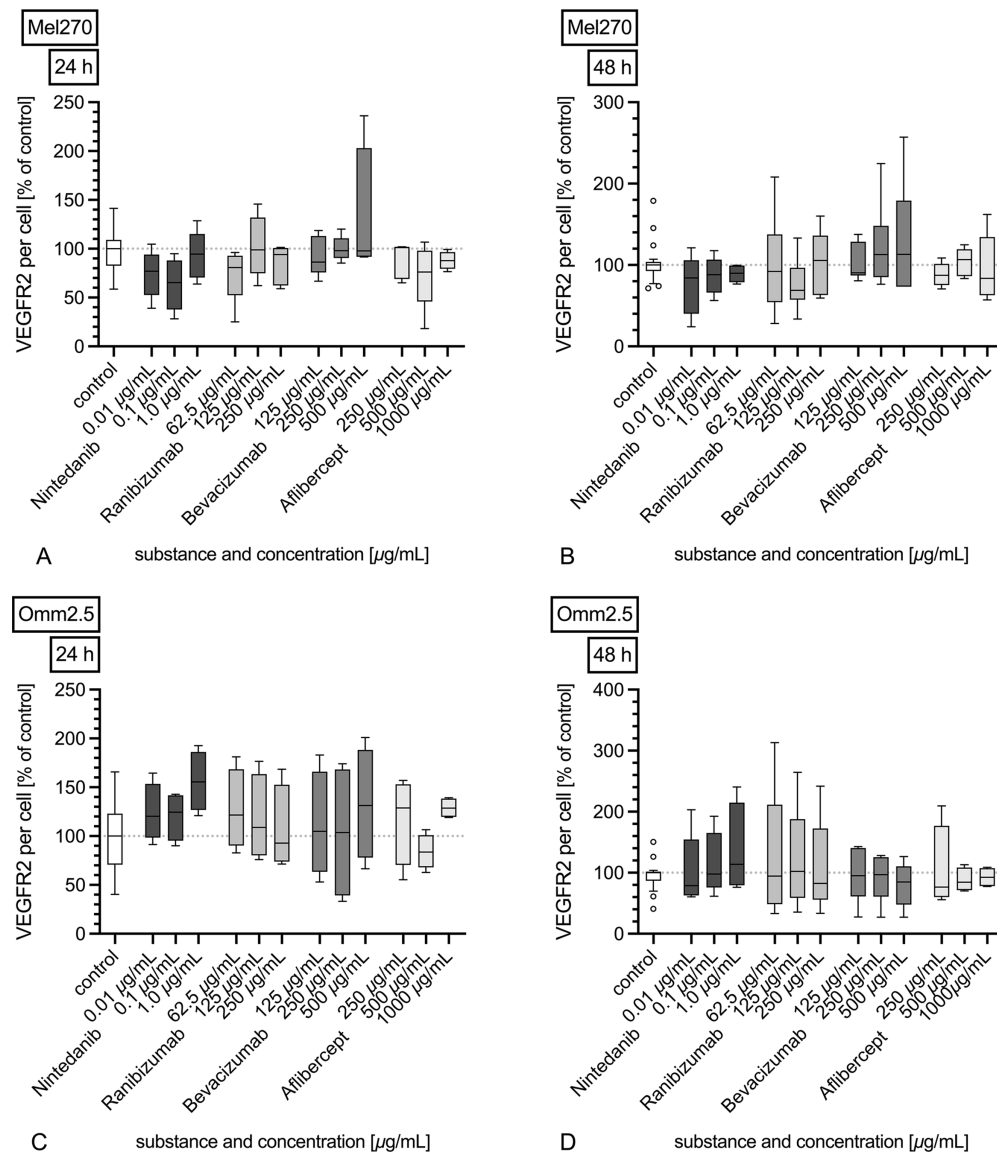


FIGURE 6. Expression of VEGF receptor-2 (VEGFR2) in the cell lines Mel270 and OMM2.5, as detected by immunofluorescent staining. The VEGFR2 levels were measured semiquantitatively as fluorescent stained area (VEGFR2) of each image per mean cell nucleus area (DAPI; in pixels) and expressed as the percentage of untreated control after 24 hours (A and C) or 48 hours (B and D) of incubation with nintedanib (0.01; 0.1 and 1 μg/mL), ranibizumab (62.5; 125 and 250 μg/mL), bevacizumab (125; 250 and 500 μg/mL), or aflibercept (250; 500 and 1000 μg/mL) at low, medium, and high concentrations with replicate numbers of $n = 6$ (A), $n = 7$ (B), $n = 4$ (C), and $n = 5$ (D). For the control groups statistically simple outliers (degrees) were detected as shown in the figure, that represent values of more than 1.5-fold of the interquartile range in Tukey's box plots. Data were normalized to the medians of the untreated control of every repetition (n) and therefore data is presented as percentage of untreated control. There was no evidence for substance- or concentration- dependent changes in the expression of VEGFR2 (A–D).

present work, the test substances were administered into the complete culture medium with 10% serum for the MTT assay. The higher serum levels in our current study might therefore have increased the abundance of VEGF-A₁₆₅ in the microenvironment, rendering the ranibizumab treatment inefficient for the suppression of metabolic activity. Our new results with ranibizumab, bevacizumab, and aflibercept are also in accordance with earlier findings that could not demonstrate any harm on nonmalignant ocular cells.^{88–90} On the other hand, in situ experiments with patients suffering from adenocarcinoma of the intestine showed an enhanced apoptosis of tumor cells under treatment with bevacizumab.⁹¹ Incongruent results were delivered by the research group

of Adamcic et al. that tested the proliferation of human melanoma cell lines in response to bevacizumab, where one cell line underwent no change, whereas the other cell line started to proliferate more.³⁰ The second cell line in that study, however, acquired an anti-proliferative phenotype in response to sunitinib, which is another inhibitor of multiple receptor tyrosine kinases that include the VEGFR2.³⁰ This shows how receptor inhibition versus substrate inhibition can have opposite effects, and how the actual results may depend on the cell culture entity. In connection with the previously mentioned “paradoxical” growth of UM in the eyes of patients following intravitreal bevacizumab administration, various explanatory approaches have also been

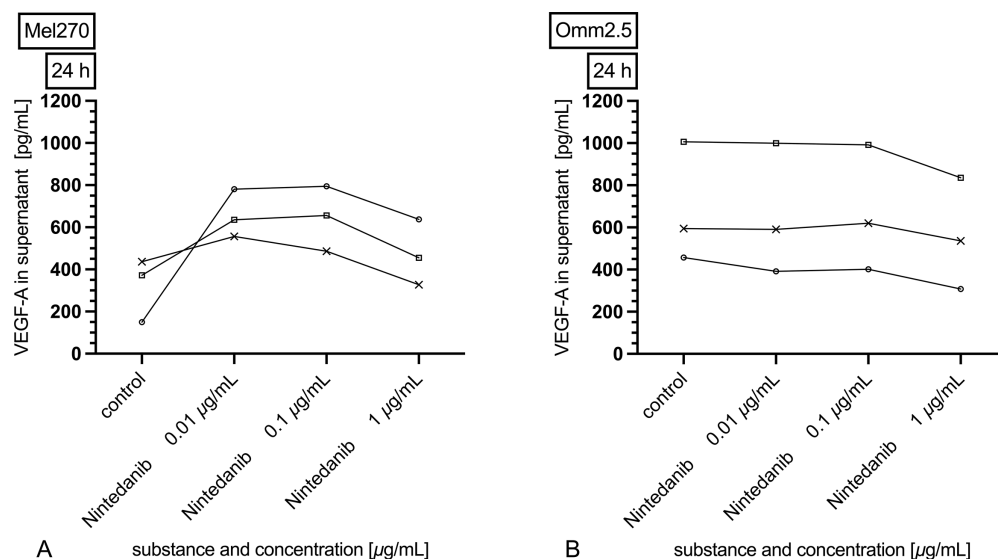


FIGURE 7. Extracellular VEGF-A₁₆₅ as determined by ELISA. The VEGF-A₁₆₅ concentrations (pg/mL) in the supernatants of the cell lines Mel270 (A) and OMM2.5 (B) are demonstrated for the untreated controls and cells that were treated with the indicated concentrations of nintedanib (0.01; 0.1 and 1 µg/mL) for 24 hours ($n = 3$ independent experiments that are individually shown [$\square \times \circ$]). The data for the three other substances ranibizumab, bevacizumab, and aflibercept are not shown due to the failure of ELISA to detect any VEGF-A₁₆₅ in the supernatants that had been incubated with these substances ($n = 2$), this could be a representation of competitive bindings sites of the ELISA test kit and the test substances themselves binding VEGF-A₁₆₅.

proposed.^{40,41} Different VEGF isoforms may contribute to both angiogenic and anti-angiogenic influences potentially shifting the tumor's milieu toward angiogenic and also proliferative properties by VEGF inhibition.⁹² A similar influence can occur through isoforms that either positively or negatively affect cell migratory or proliferative processes.⁹³ Additionally, activation of vasculogenic mimicry has been suggested as a resistance mechanism to VEGF-A inhibition,⁹⁴ as well as a potential rebound effect due to the cessation of such therapy.⁴⁰

Inducing oxidative stress or changing redox balancing is a generally used principle in cancer therapy.^{95–100} ROS are chemically reactive molecules that develop during cell metabolism. Although these molecules are often regarded as waste products that the cells have to get rid of to avoid DNA damage, on the other hand, ROS function as signaling molecules as well.^{101,102} These cell signaling pathways may have influence on cell survival, inflammation, and tumor progression.¹⁰³ Tumor cells often exhibit higher levels of ROS production compared to normal cells due to changes in redox balance.¹⁰³ Some anticancer drugs or radiation therapy use generation of ROS as part of their mechanisms of action to utilize the damaging effects of oxidative stress.^{95–98,100} In the literature, there are articles that attribute a protective effect to nintedanib against oxidative stress. For instance, Boxhammer et al., 2020, demonstrated in vitro that nintedanib at concentrations of 25 to 100 nM could protect against induction of ROS (via DCFDA assay) by Cyclosporin A in lung fibroblasts. However, the authors acknowledged that the underlying molecular mechanism remains unclear.¹⁰⁴ It is speculated that excessive ROS production may function as a signaling molecule for the release of growth factors. A number of growth factors, particularly PDGF, VEGF, and FGF (the receptors of which are inhibited by nintedanib), can themselves generate ROS, potentially creating a vicious cycle.^{105–108} On the other hand, VEGF/VEGFR signaling is involved in the modulation of

redox status of cells through multiple signaling pathways. It has been described that VEGF contributes to the reduction of the oxidative environment, thereby protecting cells from oxidative stress, in part by inducing antioxidant enzymes. Disruption of these protective systems through VEGF inhibition can lead to oxidative stress and cell damage.^{109–114} The primary tumor cell line Mel270 showed the elevation of ROS in the associated assay^{115–119} with the increasing concentrations of nintedanib, which can be interpreted as general oxidative stress¹¹⁹ induced by this substance. The pro-oxidant effect of nintedanib was shown after 24 hours as well as 48 hours, which corroborates this finding. Elevation of oxidative stress in Mel270 by nintedanib 1 µg/mL may be a sign for a potential anticancer drug effect. On the contrary, OMM2.5 did not show any changes in ROS levels in response to nintedanib. Thus, the metastatic cell line OMM2.5 could be less sensitive to this substance due to the possible adaptations that were acquired during the metastatic evolution. Such adaptation mechanisms for dealing with ROS and oxidative stress have indeed been reported for other cancer cells (like human breast cancer¹²⁰) by boosting the anti-oxidative capacity.¹⁰³ On the other hand, cell features could also be changed during cell lines' culture.⁵⁷

Due to the mode of operation of the three anti-VEGF substances ranibizumab, bevacizumab, and aflibercept – and the principle of the VEGF-A₁₆₅ ELISA, it could be shown that these substances were able to effectively block secreted VEGF-A₁₆₅ in the supernatants of both cell lines. However, it remains unclear in this experiment whether VEGF-A₁₆₅ secretion was influenced by these substances or whether the test substances interfered with the ELISA procedure by competing with the assay antibodies to bind VEGF.¹²¹ With ELISA, we observed a slight reduction in VEGF-A₁₆₅ secretion in supernatants of Mel270 and OMM2.5 cells that were incubated with nintedanib at 1 µg/mL. This conclusion should be regarded cautiously due to data spread. Semiquantitative

assessment of both intracellular VEGF-A₁₆₅ and VEGFR2 was not able to show any changes in response to any of the test substances. This may initially appear to contradict with our earlier findings, where we demonstrated a 25% to 45% reduction in intracellular VEGFA in the Mel270 and OMM2.5 cells that were exposed for 1 day to ranibizumab at the concentrations of 125 and 250 µg/mL.⁸⁷ However, in our former study, ranibizumab was introduced into a culture medium with 5% serum, whereas our current study was conducted by using a “lighter” medium with 1% serum for the treatment with the test substances. The depletion of some serum-derived factors in our current work may therefore have created a more stringent microenvironment that could have influenced the expression of VEGF-A, which remains to be further investigated. We therefore concluded that we could not provide evidence for a strong direct feedback loop between VEGF-A₁₆₅ or VEGFR2 secretion and VEGF-A₁₆₅ or VEGFR2 inhibition, although the previously discussed experiments were able to show auto- or paracrine influence on cell behavior.^{36,37,46,87}

Despite these achieved results, some aspects limit this work. For instance, the LIVE/DEAD assay or the immunostainings for VEGF-A₁₆₅ and VEGFR2 could only be analyzed in a semiquantitative manner, with the threshold for positivity of fluorescence being manually adjusted. However, taking three random pictures per well, blinding the images, and handling them in the same manner minimized intrapersonal deviation and made the analysis as objective as possible. The semiquantitative way of analysis has led to higher variability of results that potentially prohibited finding significant differences between the tested groups. We therefore suggest to explore not only possible alterations of VEGF-A₁₆₅ and VEGFR2 but also other relevant signaling pathways relevant for nintedanib like PDGF or FGF and its receptors through other experimental settings like the more quantitative approach of Western blot, flow cytometry¹²² or real time PCR.¹²³ For an even more precise analysis of the effects of the test substances on oxidative stress, it would have been advisable to include a baseline measurement of ROS in the experiments. This should be taken into consideration in the future. Our experiments were also restricted to only two UM cell lines, which limits possible conclusions for in vivo systems and even for cell culture experiments with other cell lines. Nevertheless, our work may offer interesting starting points for further research such as co-culturing or in vivo models. We would first suggest including additional UM cell lines. It may be beneficial to consider different characteristics of tumor aggressiveness in these cell lines, such as the very important monosomy 3 status^{124,125} or others like mitotic rate and the capacity for vasculogenic mimicry.¹²⁶ Additionally, we recommend broadening the study approaches by conducting co-culture experiments with other cell types, like endothelial cells, fibroblasts,^{127,128} or tumor lymphocytes.^{129–131} This would shift the focus to the possibilities of influencing the tumor microenvironment. Particularly for nintedanib, further exploration of possible antitumoral effects through modulation of the tumor microenvironment appears intriguing and should be investigated more closely for its potential in the context of UM. For instance, favorable effects of nintedanib's anti-fibrotic action have already been demonstrated in vitro for other melanoma cells as well as in a melanoma mouse model.^{132,133} Furthermore, inhibition of vasculogenic mimicry has been observed in cell culture experiments involving vemurafenib-resistant melanoma cells.¹³⁴ It may be of interest to investi-

gate whether the inhibition of TGF-β by nintedanib could lead to alterations in the preference of UM for the liver as its primary metastatic organ. This is prompted by findings indicating that TGF-β could enhance the adhesion of noninvasive UM cells to hepatic endothelium.¹³⁵ Certainly, such in vitro approaches cannot be directly extrapolated to in vivo models. However, if promising results continue to emerge, our next recommended step would involve evaluating the exploration of nintedanib in an animal model. Nintedanib has already been clinically approved for human use. Nevertheless, there is currently no available knowledge regarding the use of nintedanib in patients with UM. Whether the substance's mechanism of action could be effective and safe in human UM cannot be asserted at this time. The indispensable final step would involve investigating the effects of the substance in clinical research to gain insights.

Inhibiting tumor angiogenesis is an interesting approach for at least adjuvant tumor therapy,^{35,136} however, for UM, there has not yet been a breakthrough in this field of research.^{20,137} In clinical studies, angiogenesis inhibitors have only shown a tendency to stabilize the disease, but they have not provided a change in overall survival.^{138–143} Generally, the concept of anti-angiogenesis has its limitations also due to resistance mechanisms of tumors and the tumor microenvironment, which may bypass possible therapy effects.^{103,128,144–154} This study provides evidence that, unlike the VEGF-A₁₆₅ specific inhibition by ranibizumab, bevacizumab, and aflibercept, a more wide-ranging mechanism of action, provided by the multi-angiokinase inhibitor nintedanib, has an influence on UM cell behaviour, at least in our in vitro experiments. Discovering multiple different treatment options for UM is an important strategy in this field of research and novel anti-angiogenic drugs should among other mechanisms of action remain in the spotlight to gain knowledge. This could lead to different treatment options in consideration of personalized or precision medicine.¹⁵⁵ There could be patients whose tumors may possibly be more sensitive to influence of pro-angiogenic factors. Therefore, by understanding the molecular underpinnings of a patient's cancer, clinicians could select drugs like nintedanib as appropriate treatment options at least in combination with drugs of other target mechanisms. This could minimize the use of the one-size-fits-all approaches and reduce the risk of ineffective treatments.¹⁵⁵

We suggest further investigation of the mechanisms and effects of signaling pathways for nintedanib in UM. In addition, in vivo models should be established for this purpose in the future.

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