

ORIGINAL PAPER



***In vitro* and *in ovo* experimental study of two anti-VEGF agents used in ophthalmology**

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Abstract

Anti-vascular endothelial growth factor (anti-VEGF) monoclonal antibodies can inhibit neovascularization and also to block the growth of several tumor cell lines. Treatment with anti-VEGF drugs like Bevacizumab (Avastin[®]) and Aflibercept has proven optimistic results in various malignant diseases. The present study was aimed to investigate Bevacizumab and Aflibercept *in vitro* effects on two human melanoma cell lines (A375 and SK-Mel-28), as well as on a healthy cell line (HaCaT human keratinocytes), followed by characterization of the *in ovo* effects on the chorioallantoic membrane (CAM). Our data indicated that Bevacizumab and Aflibercept decreased human melanoma cells viability in a dose-dependent way, a more significant effect was obtained for Aflibercept. Regarding the safety profile of the active compounds tested, they showed a low-moderate irritation score. In the case of the tested samples, the vascular capillaries were not majorly affected. In both cases, the only notable change was the appearance of a slight vascular coagulation. The viability of the embryos after application was good, they survived more than 24 hours after testing the compounds on the CAM.

Keywords: Bevacizumab, Aflibercept, melanoma, chorioallantoic membrane.

Introduction

Neovascularization represents the ability of tumoral cells to develop aberrant blood vessel, to assure proliferation and growth, pathway mediated by pro-angiogenic factors [1]. On the other hand, progression of new blood supply is mandatory in wound recovery. The vascular endothelial growth factor (VEGF) mechanism of action is one of the most important angiogenic route, the development of antiangiogenic therapies can lead to clinical improvement in neoplasm treatments and pathologies associated with neovascular complications [2].

Anti-VEGF monoclonal antibodies can inhibit neovascularization and also to block the growth of several tumor cell lines [3]. Treatment with anti-VEGF drugs like Bevacizumab (Avastin[®]) and Aflibercept has proven optimistic results [4] in various malignant diseases [5]. Bevacizumab, one of the humanized monoclonal antibodies used in therapy, has, through its biological mechanism and antagonizing effect on VEGF function in endothelial cells, an antiangiogenic effect. Based on angiogenesis inhibitory effect, Bevacizumab was originally used to treat metastatic colorectal cancer. In fact, Bevacizumab has

proven to be effective not only in treating cancers of all stages, but also in various chronic conditions, such as neovascular complications of the eye, including wet age-related macular degeneration and proliferative diabetic retinopathy [6].

Aflibercept, a VEGF-targeted agent and anti-placental growth factor (anti-PIGF) agent, can suppress angiogenesis which is associated with the reduction of tumor vasculature and size. Regarding the biological mechanism of action of Aflibercept, it has an antagonistic effect on both VEGFs and PIGFs, thus blocking the binding to VEGF receptors and exerting an anti-angiogenic effect [7]. Compared to Bevacizumab, which has a selective VEGF-A mechanism of action, Aflibercept has a more complex mechanism of action, with affinity for both VEGF-A and VEGF-B [8]. Clinical studies have shown good results after treatment with Aflibercept alone or in combination with other anti-neoplastic drugs [5].

Aim

The aim of the present study was to evaluate Bevacizumab and Aflibercept *in vitro* effects on two human melanoma

cell lines (A375 and SK-Mel-28), as well as on a healthy cell line (HaCaT human keratinocytes), followed by characterization of the *in ovo* effects on the chorioallantoic membrane (CAM).

Materials and Methods

Cell culture

The human melanoma cell lines [A375 (code CRL-1619) and SK-Mel-28 (code HTB-72)] were purchased from the *American Type Culture Collection* (ATCC, USA). The HaCaT cells (human keratinocytes) were provided by the University of Debrecen (Hungary). As a culture medium for A375 and HaCaT cell lines, it was used high Glucose Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) and for SK-Mel-28 cell line was used Eagle's Minimum Essential Medium (EMEM; Sigma-Aldrich). A 1% antibiotic mixture (Penicillin/Streptomycin – Pen/Strep, 10 000 IU/mL; Sigma-Aldrich) and 10% Fetal Bovine Serum (FBS; Gibco, Thermo Fisher Scientific) were added to all media used. The cells were maintained in standard conditions – 37°C and humidified atmosphere containing 5% carbon dioxide (CO₂).

MTT assay

Bevacizumab and Aflibercept were evaluated for their effect on A375 and SK-Mel-28 melanoma cell lines and HaCaT human keratinocytes viability using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The procedures were carried out in the same manner as indicated earlier [9]. 1×10⁴ cells/well were sown in 96-well culture plates and allowed to attach for 24 hours. Afterwards, the cells were stimulated with various concentrations of 1, 5, 10, 25, 50, 100, 150 and 300 µg/mL for 72 hours. The control group consisted of cells that had been treated with DMEM. After 72 hours, the cells were treated with 10 µL of 5 mg/mL MTT solution made using an MTT kit (Sigma-Aldrich) and incubated for another three hours. The resultant formazan crystals were shattered in 100 µL of the lysis solution provided with the MTT kit. At 570 nm, the absorbance was determined using a microplate reader (xMark™ microplate spectrophotometer, BioRad).

CAM assay

A biological model represented by the CAM of the hen's egg was used to determine the irritant potential of Bevacizumab and Aflibercept. For this purpose, chicken eggs from a local farmer were used. To perform the experiment, eggs were prepared respecting the following steps: (i) eggs were washed and disinfected with 70% alcohol (*v/v*) after which they were dated and placed in the incubator, (ii) on the 3rd day of the experiment, a small perforation was performed on the eggs, through which a volume between 5 mL and 8 mL depending on the size of the egg, so that the CAM detaches from the inner shell of the egg, and (iii) on the 4th day, a hole was cut in the eggshell for easy visualization of the CAM and vascular plexus. Afterward, the eggs were taped and placed in the incubator until the day the experiment began.

Hen's egg test (HET)–CAM assay

For this experiment, the highest concentrations tested in the cell viability test (1000 µg/mL) of the two active

substances were used. In parallel, a negative control represented by distilled water and a positive control represented by 1% Sodium Dodecyl Sulfate (SDS) were tested. Samples and controls were applied in a volume of 600 µL on the CAM surface. This volume ensures the complete coverage of the blood vessels and thus, obtaining a uniform effect at the level of the CAM. The following vascular changes were followed for five minutes: hemorrhage (H), lysis (L) and vascular coagulation (C). These vascular effects were monitored using a stereomicroscope (Discovery 8 stereomicroscope, Zeiss, Göttingen, Germany). Images of the CAM were also taken at T0 (before applying the sample) and T5 (five minutes after applying the sample) using AxioCam 105 color microscope camera (Zeiss). At the end of the experiment, the photographs were processed using the ImageJ v 1.50e program (US National Institutes of Health, Bethesda, MD, USA). To quantify the irritant effect, the calculation formula for determining the irritation score (IS) was applied [10, 11]:

$$IS = 5 \times \frac{301 - H}{300} + 7 \times \frac{301 - L}{300} + 9 \times \frac{301 - C}{300}$$

Depending on the IS value, substances can be classified as shown in Table 1.

Table 1 – Classification of substances according to irritation score (IS) [12]

| | |
|-------------------|-----------|
| Non-irritating | IS: 0–0.9 |
| Irritating | IS: 1–8.9 |
| Severe irritating | IS: 9–21 |

Statistical analysis

The results were expressed as mean ± standard deviation (SD). The statistical analysis was performed using Graph Pad Prism 5. One-way analysis of variance (ANOVA) followed by Dunnett's *post-hoc* test (**p*<0.05; ***p*<0.01; ****p*<0.001) was used for comparison among groups.

Results

MTT assay

The effect of Bevacizumab was assessed on A375 and SK-Mel-28 human melanoma cell lines and on HaCaT human keratinocytes after a stimulation period of 72 hours and compared to the unstimulated cells (the control group). As for Bevacizumab, it causes a decrease in the number of viable tumor cells. The strongest effect was seen at the highest dose tested (1000 µg/mL) – cell viability was 84.6% compared to control (Figure 1).

It can be observed that the effect of Bevacizumab on SK-Mel-28 melanoma cell line affects tumor cells viability but without statistical difference. A dose-dependent decrease can be observed (Figure 2).

After a stimulation period of 72 hours with Bevacizumab on HaCaT keratinocytes, a mild decrease in cell viability was observed (Figure 3).

The same concentrations were used to evaluate the effect of Aflibercept on the two melanoma cell lines and on the keratinocytes. The compound produced a significant dose-dependent decrease of tumor cells viability. Significant differences were observed starting with the dose 250 µg/mL. The effect obtained following stimulation with Aflibercept is stronger than the one obtained for Bevacizumab (Figure 4).

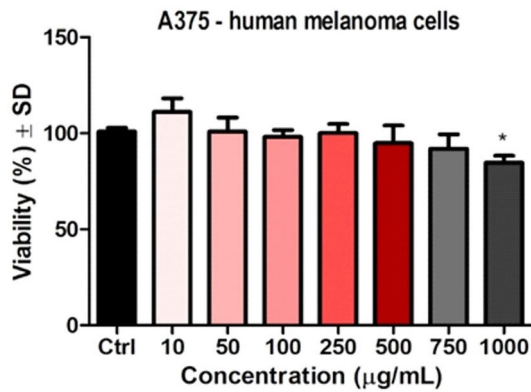


Figure 1 – Viability recorded for A375 human melanoma cell line after 72 hours treatment with Bevacizumab (10, 50, 100, 250, 500, 750 and 1000 µg/mL). The results are highlighted as a percentage and represent the comparison between the tested cells and the control group represented by the unstimulated cells. One-way ANOVA and Dunnett’s post-hoc tests were used for statistical analysis (* $p < 0.05$). ANOVA: Analysis of variance; SD: Standard deviation.

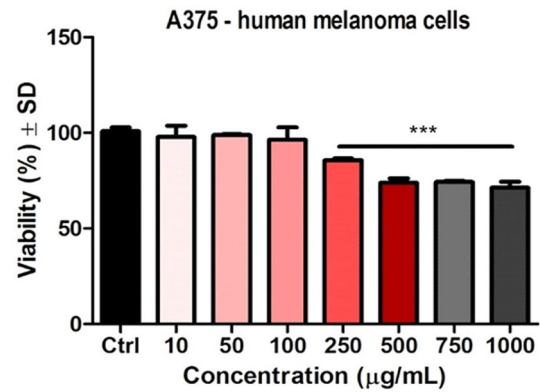


Figure 4 – Viability recorded for A375 human melanoma cell line after 72 hours treatment with Aflibercept (10, 50, 100, 250, 500, 750 and 1000 µg/mL). The results are highlighted as a percentage and represent the comparison between the tested cells and the control group represented by the unstimulated cells. One-way ANOVA and Dunnett’s post-hoc tests were used for statistical analysis (** $p < 0.01$).

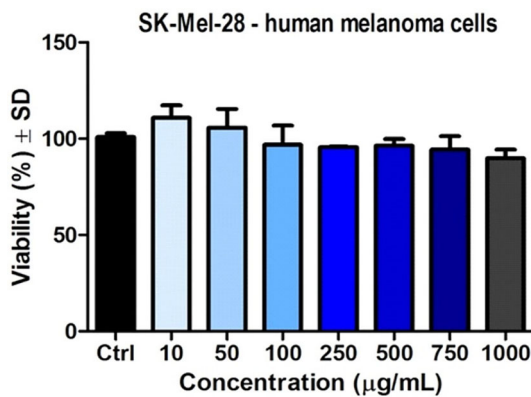


Figure 2 – Viability recorded for SK-Mel-28 human melanoma cell line after 72 hours treatment with Bevacizumab (10, 50, 100, 250, 500, 750 and 1000 µg/mL). The results are highlighted as a percentage and represent the comparison between the tested cells and the control group represented by the unstimulated cells. One-way ANOVA and Dunnett’s post-hoc tests were used for statistical analysis.

Aflibercept was also investigated on SK-Mel-28 cell line. At 72 hours post-stimulation, the compound produced a significant decrease in melanoma cells viability (Figure 5).

After a stimulation period of 72 hours of Aflibercept on HaCaT keratinocytes, a mild decrease of cell viability was noticed. HaCaT cells viability was higher after treatment with Aflibercept compared to the effect obtained after stimulation with Bevacizumab (Figure 6).

HET-CAM assay

The HET-CAM method was applied to verify the vascular irritant potential of Bevacizumab and Aflibercept. This method was selected because it has the advantage of being easy to achieve, providing an image of the toxic potential of the substance by calculating the IS. For a comprehensive and accurate assessment of the irritant potential, the active substances were tested in parallel with the negative control (distilled water) and the positive control (1% SDS). The values of the IS obtained both for the two active substances and for the positive and negative control are presented in Table 2.

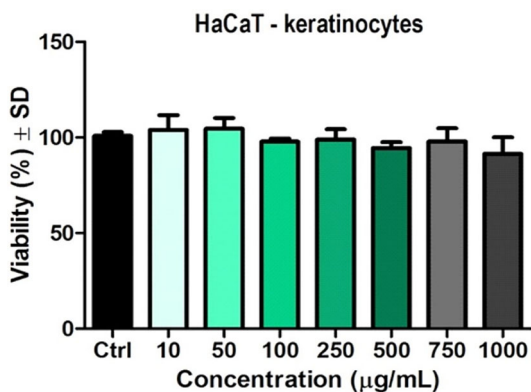


Figure 3 – Viability recorded for HaCaT human keratinocytes cell line after 72 hours treatment with Bevacizumab (10, 50, 100, 250, 500, 750 and 1000 µg/mL). The results are highlighted as a percentage and represent the comparison between the tested cells and the control group represented by the unstimulated cells. One-way ANOVA and Dunnett’s post-hoc tests were used for statistical analysis.

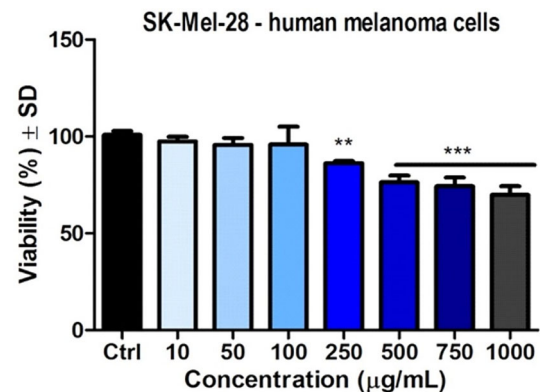


Figure 5 – Viability recorded for SK-Mel-28 human melanoma cell line after 72 hours treatment with Aflibercept (10, 50, 100, 250, 500, 750 and 1000 µg/mL). The results are highlighted as a percentage and represent the comparison between the tested cells and the control group represented by the unstimulated cells. One-way ANOVA and Dunnett’s post-hoc tests were used for statistical analysis (** $p < 0.01$; *** $p < 0.001$).

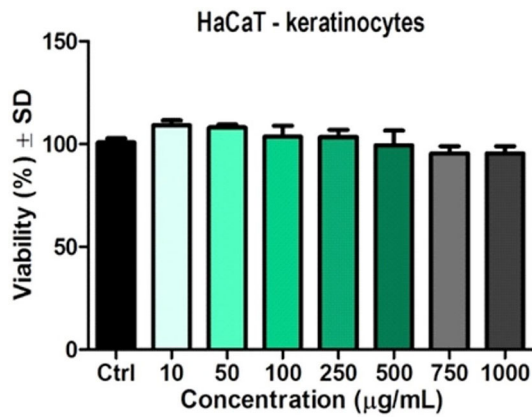


Figure 6 – Viability recorded for HaCaT human keratinocytes cell line after 72 hours treatment with Aflibercept (10, 50, 100, 250, 500, 750 and 1000 µg/mL). The results are highlighted as a percentage and represent the comparison between the tested cells and the control group represented by the unstimulated cells. One-way ANOVA and Dunnett's post-hoc tests were used for statistical analysis.

The highest value of the IS, as expected, was obtained in the case of the positive control. In this case, the value of the IS was 19.57. At the opposite pole was the negative control, distilled water, where the IS was 0.15. Regarding the safety profile of the active compounds tested, they showed a low-moderate IS. In the case of Bevacizumab, the IS was 1.64, and in the case of Aflibercept, the calculated

IS was 2.13. These values are in the range 1–8.9, which means that these substances have a low irritant potential. In Figure 7, the changes produced at the level of the vascular plexus can be observed after the application of the negative and positive controls and the samples. In the case of distilled water, there were no changes in the vascular capillaries to suggest an irritating effect. On the other hand, in the case of SDS, at the capillary level there were signs of major irritation, such as vascular lysis, coagulation and hemorrhage. These changes were recorded in the first seconds after the administration of the sample, which underlines the strong irritating effect of SDS. In the case of the tested samples, the vascular capillaries were not majorly affected. In both cases, the only notable change was the appearance of a slight vascular coagulation. In addition, the viability of the embryos after application was good, they survived more than 24 hours after testing the compounds on the CAM (Figure 7).

Table 2 – Irritation score (IS) values for positive control (1% SDS), negative control (distilled water), Bevacizumab and Aflibercept

| | 1% SDS | H ₂ O | Bevacizumab | Aflibercept |
|--------|--------|------------------|-------------|-------------|
| IS | 19.57 | 0.15 | 1.64 | 2.13 |
| tH [s] | 17 | 300 | 300 | 300 |
| tL [s] | 20 | 299 | 289 | 285 |
| tC [s] | 25 | 298 | 256 | 243 |

SDS: Sodium dodecyl sulfate; tC: Time of coagulation; tH: Time of hemorrhage; tL: Time of lysis.

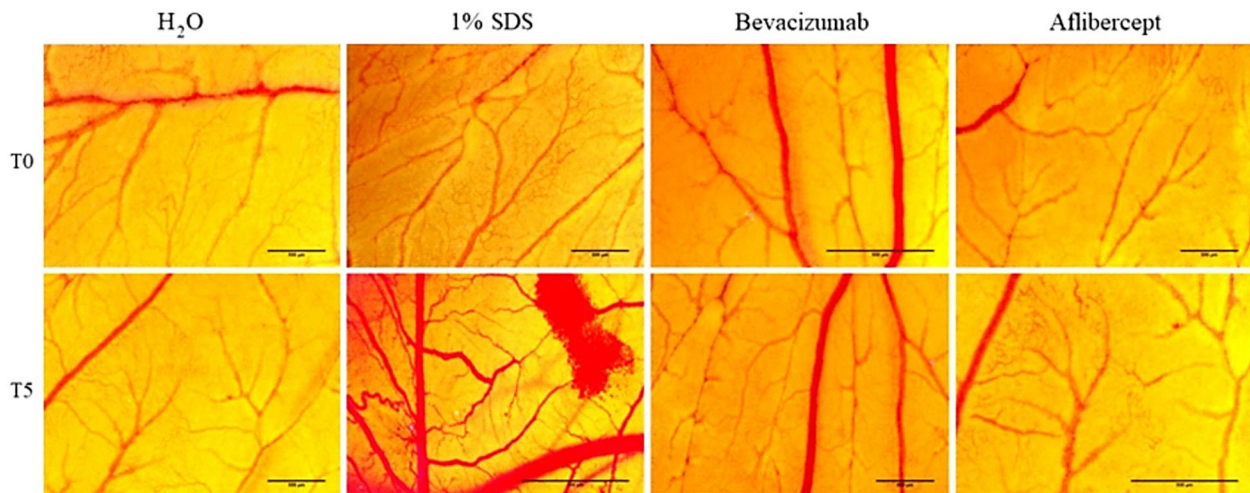


Figure 7 – Stereomicroscope images of the CAMs inoculated with H₂O (used as negative control) and 1% SDS (used as positive control) and test samples (Bevacizumab and Aflibercept). CAMs: Chorioallantoic membranes; H₂O: Distilled water; SDS: Sodium Dodecyl Sulfate.

Discussions

Bevacizumab and Aflibercept are humanized monoclonal immunoglobulin G1 antibodies currently studied in various types of cancers acting by inhibiting the angiogenesis process [13].

Combined therapy can lead to the use of lower doses of the compounds involved and thus there are fewer side effects. Bevacizumab is considered to have a modest activity as a single compound and that association with different agents can be beneficial in different types of cancers [14]. The compound is used with Carboplatin and Paclitaxel in non-small cell lung cancer, with Interferon-alpha for

metastatic renal carcinoma and with 5-Fluorouracil for metastatic colon cancer [13]. Bevacizumab was previously administered with Carboplatin and Paclitaxel in a double-blind, randomized study where 214 patients with melanoma were involved [15]. The authors of the *Bevacizumab Advanced Melanoma* (BEAM) study concluded that there was no significant enhancement of the progression-free survival rate after the addition of Bevacizumab to therapy [15]. Other authors consider that the study brought beneficial evidence to the scientific community [16]. The side effects that appeared after treatment with Bevacizumab were neutropenia, peripheral neuropathy, hypertension, and arterial thromboembolic events [13].

In a phase 2 study, Aflibercept given in monotherapy improved with 50% the progression-free survival rate for four months at least for patients with melanoma. A side effect of the treatment with Aflibercept was severe hypertension [13].

In a study that included patients with inoperable stage III or IV cutaneous or uveal melanoma treatment with Aflibercept at a concentration of 4 mg/kg i.v. administered every two weeks improved the progression-free survival [17]. Several side effects were observed including hypertension, proteinuria, renal failure, hyponatremia, and gastrointestinal bleeding [17].

Our data indicate that the HaCaT cells were less affected by the treatment with Aflibercept compared with the effect produced by Bevacizumab. Parisi *et al.* had similar results when they evaluated the effect on healthy cells, on primary human retinal pigment epithelium cells and on scleral fibroblasts [18]. Their data indicated that Bevacizumab decreased primary human retinal pigment epithelium cells and scleral fibroblasts proliferation more than Aflibercept [18].

Evaluation of the irritant potential of an ophthalmic substance is an important step to take before administering it to human subjects. The HET-CAM method is an easy method, used as an alternative to the Draize rabbit eye test, to check for eye irritation [19]. With the implementation of the HET-CAM method, following the experiments performed, a good correlation was observed between the results obtained by this method and those obtained *in vivo* by the Draize eye irritation test method. Thus, this method is suitable for evaluating the eye irritant potential of various ophthalmic substances and solutions [20].

Following the testing performed in this article, it was found that both Bevacizumab and Aflibercept in a concentration of 1000 µg/mL have a low IS (1.64 and 2.13, respectively), ranging from 1–8.9, which suggests that these substances are safe for ophthalmic application.

Bevacizumab is a monoclonal antibody that has found utility in ophthalmic practice in a variety of conditions, such as diabetic retinopathy, neovascular glaucoma or neovascular disorders of the anterior segment [21]. In a similar study by Pandit *et al.* [22], a formulation of Chitosan (CS)-coated Poly(lactic acid-co-glycolic acid) (PLGA) nanoparticles of Bevacizumab was tested by the HET-CAM assay. Following this study, it was found that this new formulation of Bevacizumab is not irritating to the CAM, being safe for ophthalmic application, the ISs obtained being very similar to those obtained in the present study.

Aflibercept is also a monoclonal antibody with a mechanism of action like Bevacizumab, but with a higher affinity for VEGF. Intravitreal administration of this substance is currently used in medical practice for a variety of ophthalmic pathologies such as macular edema, central retinal vein occlusion or myopic choroidal neovascularization [23]. Regarding its toxic and irritating effects, various *in vitro* studies have been performed, but, to our knowledge, its effect has not been tested *in ovo*, at the level of the CAM. Preliminary *in vitro* studies have shown that the concentration of 1000 µg/mL of Aflibercept does not show cytotoxic effects in the cell lines of primary cultures of human trabecular meshwork cells, human scleral fibroblasts, and a retinal pigment epithelial cell line [24]. *In vivo*

studies in rabbit eyes have suggested that short-term administration of Aflibercept does not cause eye irritation [25]. On the other hand, a 6-month clinical trial found that intravitreal administration of Aflibercept may cause corneal endothelial alterations, but these potential side effects have not been studied in detail [26].

☞ Conclusions

Bevacizumab and Aflibercept decreased human melanoma cells viability in a dose dependent manner, a more significant effect was obtained for Aflibercept. Regarding the safety profile of the active compounds tested, they showed a low-moderate IS. In the case of the tested samples, the vascular capillaries were not majorly affected. In both cases, the only notable change was the appearance of a slight vascular coagulation. The viability of the embryos after application was good, they survived more than 24 hours after testing the compounds on the CAM. Compounds that target the VEGF are of interest in the therapy of metastatic melanoma. Although there are multiple studies that present the positive effect of Bevacizumab and Aflibercept, including clinical trial, several side effects were also reported. Additional studies, including *in vitro* and *in vivo* analysis are needed in order to have an evidence of the mechanism of action involved and of the effects of the combined therapy in patients with melanoma.

Conflict of interests

The authors declare that they have no conflict of interests.

Authors' contribution

Mădălina-Casiana Palfi and Ovidiu Muşat equally contributed to the manuscript.

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