ORIGINAL ARTICLE

The effect of aflibercept and arsenic trioxide on the proliferation, migration and apoptosis of oral squamous cell carcinoma in vitro

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Abstract
Aflibercept and arsenic trioxide drugs apply a cytotoxic effect on some human cancer cell lines. However, no more study has followed the effects of both drugs, especially arsenic trioxide, on oral squamous cell carcinoma (OCC). We used three OCC lines as a model to show the effect of these drugs on the genetically complex disease and investigate its targeted therapy. In this study, three human OCC cell lines were used from different patients. We treated cell lines with both medications to detect the effect and relevant molecular basis. First, methyl thiazolyl tetrazolium (MTT) assay was performed to detect the cytotoxicity effect and cell growth. Second, flow cytometry, gene and protein expression were performed to evaluate the anti-angiogenic effect on OCC lines. Next apoptosis was analyzed by flow cytometry. Finally, clonogenesis capacity and cell migration were assessed by colony formation assay and wound healing, respectively. Aflibercept had no cytotoxic effect on the three OCC cell lines but decreased cell growth rate. Arsenic trioxide had a significant cytotoxic effect on three cell lines. Our results demonstrated that both drugs significantly decreased endoglin, VEGFA, and VEGFB expression. In addition, Migration and colony formation assays confirmed that these drugs have significant anti-proliferative and anti-migration effect on oral carcinoma cells. These results revealed that both medications might be a potential drug for the management of oral cancer patients.

Keywords Oral squamous cell carcinoma · OCC · Aflibercept · Arsenic trioxide

Introduction

Oral squamous cell carcinoma (OCC) accounts for over 90% of the oral malignancies [1, 2]. According to a Ferlay and et al. study in 2015, ∼300,000 new cases of oral malignancies were diagnosed worldwide and the mortality rate was about 145,000 [3]. Despite all the developments in treatment of OCC, the survival rate has remained low and satisfying improvement in the outcome of the patients has not been achieved [4]. Surgery, chemotherapy, and radiotherapy have been the first-line treatment for cancer for long [5]. Regardless of therapeutic advances, the treatment outcome remains disappointing, especially for cases diagnosed in advanced stages [6]. Moreover, the available treatment modalities for the primary and metastatic OCC are often limited, and are mainly comprised of palliative treatment. Thus, researchers are attempting to find new treatment strategies for OCC [7]. Another approach in cancer therapy is to use anti-angiogenic medications such as bevacizumab, aflibercept, sunitinib, pazopanib, ranibizumab, and arsenic trioxide [8]. Aflibercept has been effective on several tumors in vitro and ex vivo. It exerts its anti-angiogenic effects by regression of tumor vessels and vascular remodeling, and inhibition of neovascularization [9, 10].
Arsenic trioxide is a traditional Chinese medicine that has been used for many years. This medication is currently used clinically for treatment of acute promyelocytic leukemia by targeting PML/RARA; however, it has a wider range of activity [11]. A high concentration of arsenic trioxide can induce apoptosis in leukemic cells [12]. In this study we evaluated the response of three OCC cell lines, isolated from different patients, when treated with aflibercept and arsenic trioxide as anti-angiogenic and anti-proliferative drugs.

**Materials and methods**

**Cell lines and quality control**

Three human OCC cell lines (OCC-11, OCC-18, and OCC-20) were used in the study (Supplementary Table 1). The establishment and authenticated of all three cell lines were performed before freezing and banking in human and animal Iranian cell bank, Iranian biological Resource Center, Tehran, Iran. To detect bacterial, yeast, fungal contaminations, the supernatant of cells was cultured in tryptone soy broth and thioglycollate broth media for 14 days. In addition, mycoplasma contamination was evaluated using DNA staining with Hoechst, direct solid agar microbiological culture and mycoplasma PCR. Finally, for authentication of the cells STR assay was done [13]. The ethical committee of Tehran University approved this study (IR.TUMS.DENTISTRY.REC.1397.146) and supported by Tehran University of Medical Sciences (Grant #97-03-69-39,794).This study was approved by the ethics committee of Tehran University of Medical Sciences (IR.TUMS.DENTISTRY.REC.1397.146) and was financially supported by a grant from this university (Grant #97-03-69-39,794).

**Methyl thiazolyl tetrazolium (MTT) assay**

The MTT assay was performed to assess the cytotoxic effects of aflibercept and arsenic trioxide on cell proliferation [14]. The cell lines were seeded at a concentration of $5 \times 10^4$ cells/well for cytotoxicity testing, and $2 \times 10^3$ cells/well for the proliferation assay in a 96-well plate, and incubated at 37 °C overnight. The supernatant was replaced by a serum-free medium. The cells were then treated with the two drugs in the following groups:

- Control group: OCC cells $+$ culture medium.
- Aflibercept group: OCC cells $+$ culture medium $+$ 20, 100, 500 or 2500 µg/mL aflibercept.
- Arsenic trioxide group: OCC cells $+$ culture medium $+$ 5, 10, 15, 20, or 30 µM arsenic trioxide.

The results were read after 24, 48, and 72 h. The MTT solution (Sigma, St. Louis, USA) was added to each well at a final concentration of 250 µg/mL. The plates were incubated at 37 °C for 3 h and then, 150 µL of the MTT solvent was added to each well. The optical density of each well was measured by reading the absorbance of the wells at 570 nm wavelength (Van Meerloo et al. 2011).

The IC50 concentration of Arsenic trioxide and aflibercept were calculated as the drug concentration required to inhibit cell growth to 50% compared to untreated cells using Prism software (GraphPad Software, version 7.0).

**Effect of aflibercept and arsenic trioxide on expression of VEGF protein**

The western blot assay was performed to evaluate the effect of aflibercept and arsenic trioxide on the expression of VEGF by the three OCC cell lines. The cells were seeded into 6-well plates and incubated at 37 °C in complete culture medium overnight. Next, the cells were treated with aflibercept and arsenic trioxide at a final concentration of 100 µg/mL and 10 µM, respectively, for 48 h. Untreated cells served as the control group.

After 48 h, the cells were collected and centrifuged, and the expression of VEGF was assessed using VEGF Human Western Blot Kit (Abcam, Cambridge, MA, USA, Number code: ab46154) using 1:1000 concentration for primary antibody and 1:4000 for secondary antibody (Abcam, Cambridge, MA, USA, DB9572), according to the manufacturer’s instructions.

**Effect of aflibercept and arsenic trioxide on endoglin expression**

The flow cytometric assay was performed to assess the effect of aflibercept and arsenic trioxide on endoglin expression by the three OCC cell lines. The cells were cultured in complete culture medium at 37 °C overnight, and were then treated with aflibercept (100 µg/mL) and arsenic trioxide (10 µM). Untreated cells served as the control group. After 48 h, 2 µg/mL of endoglin conjugated primary antibody (Abcam, Cambridge, MA, USA, ab11415) was added, followed by incubation for a minimum of 30 min at 4 °C. The samples were analyzed with FlowJo software version 10.

**Apoptosis assay**

The three cell lines were treated with the two drugs to assess the apoptotic effect of the drugs. The assay was performed using the FITC Annexin V, Apoptosis Detection Kit I (BD Biosciences, San Jose, USA). The cells (100×10³ cells/well) were seeded in 6-well plates and treated with 100 µg/mL aflibercept and 10 µM arsenic trioxide, and incubated along with the non-treated control cells for 48 h. Annexin V and propidium iodide were added to the suspended cell tubes...
and incubated in the dark for 30 min at room temperature. Finally, the cells underwent flow cytometric analysis.

**Colony formation assay**

The colony formation assay was performed according to the protocol described by Franken [15]. Single cells were seeded in a 6-well plate (1 × 10² cells) and allowed to attach overnight and were then exposed to a final concentration of 100 µg/mL aflibercept and 10 µM arsenic trioxide for 48 h. Next, the medium was refreshed, and the cells were incubated at 37 °C. After 14 days, the cells were fixed and stained with 10% methylene blue in 70% ethanol. The number of colonies was counted and the fraction of survived cells was calculated as the ratio of the number of colonies in the treated sample to the number of colonies in the untreated sample. Five replicate wells were seeded for each drug.

**Scratch test**

The scratch test was performed to assess the effect of aflibercept and arsenic trioxide on cell migration [10]. The cell monolayer was scraped in a straight line to create a scratch with a p100 pipette tip. The cells were washed to remove the debris and smoothed the edge of the scratch and were then added with 5 mL of complete culture medium with 100 µg/mL aflibercept and 10 µM arsenic trioxide. Photographs were taken at 0, 24, and 48 h after incubation under an inverted microscope. The photographs were quantified using Image J software according to previously reported protocols [16]. Briefly, the captured images were changed to gray scale. The found edges were emphasized sharp changes in intensity with a white outline. These outlines blurred together when the image was blurred numerous times. The parts containing cells were white while the wound area stayed black. Then, the wound area selected manually threshold (Image → Adjust → Threshold). When the wound area was recognized, it was then calculated.

**Real-time PCR**

Total RNA was extracted using Trizol reagent (Sigma–Aldrich, Germany), according to the manufacturer’s instructions. Then, cDNA was synthesized and Real-time PCR was performed using SYBR Green on the StepOne™ Real-Time PCR System [17]. Following primers for genes were used: human ACTB (ß-actin; forward 5′-CTGGAA CGGTGAAAGTGACA-3′, reverse 5′-AAGGACTTCTCT GTACAATGTGA-3′), human VEGFA (forward 5′- ATCTACCCAATCGCTGTG -3′, reverse 5′- GAGGTCCAG TCCCAGATATCTG -3′), human VEGFB (forward 5′- AGCCCACTCGGATG-3′, reverse 5′- GTCATTCTCAC AGCAGT-3′), and human Endoglin (forward 5′-CTTCCT GGAGTTCCAACG-3′, reverse 5′-GTTGCCATTITGCTTG GA-3′). The relative mRNA expression levels were evaluated according to the ΔΔCt method. Relative quantification of gene expression was analyzed by calculating 2^−ΔΔCt of each cell line. Analysis was done using GraphPad Prism 7.0 and two-way ANOVA was used to calculate significant differences between different OCC cell lines.

**Statistical analysis**

Data from different experimentations expressed as mean ± standard deviation, and analyzed by one-way or two-way ANOVA using GraphPad Prism 7.0 software. p-value < 0.05 was considered statistically significant. For reproducibility, the MTT and colony formation assays were performed in five replicates, by three independent experiments. Other experiments were conducted in three independent biological replicates. The results of western blot test and the number of formed colonies were quantified by Image J and analyzed by GraphPad software.

**Results**

The result of contamination assays showed there is no contamination and STR assay detected no misidentified (Supplementary Figures S1 and S2).

**MTT assay**

To assess the possible cytotoxic effects of the two medications on cells, all three cell lines were exposed to 5–30 µM arsenic trioxide and 20–2500 µg aflibercept for 24, 48, and 72 h. The results revealed that aflibercept had no cytotoxic effect on the three OCC cell lines. In order to assess the effect of aflibercept on proliferation of OCC cells, we treated the cells with 20–2500 µg aflibercept, and the result was analyzed. A reduction in cell proliferation was observed in the aflibercept-treated group compared to non-treated cells. Aflibercept significantly decreased the proliferation of OCC cells; between OCC11 and OCC-18 [(24 h, p < 0.01 at 100 µg, and p < 0.0001 at 500 and 2500 µg) (48 h and 72 h, p < 0.0001 at 20, 100, 500, and 2500 µg)], between OCC-11 and OCC-20 [(24 h, p < 0.0001 at 500 and 2500 µg) (48 h p < 0.0001 at 20, 500, 2500 µg) (48 h p < 0.0001 at 100 µg, and 72 h at 500 µg) (72 h, p < 0.01 at 2500 µg)] and between OCC-18 and OCC-20 [(24 h, p < 0.0001 at 500 and 2500 µg, p < 0.01 at 100 µg) (48 h, p < 0.0001 at 20, 500, and 2500 µg) (72 h, p < 0.0001 at 20, and 100 µg)]. The results of the correlational analysis are shown in Fig. 1a–c. Arsenic trioxide showed significant cytotoxic effect (P < 0.05) (Fig. 1d–f). Arsenic trioxide induced significant cell death in OCC cells; between
OCC11 and OCC-18 [(24 h and 48 h, p < 0.0001 at 5 µM) (72 h, p < 0.0001 at 15 µM)] between OCC-11 and OCC-20 [(72 h, p < 0.0001 at 15 µM), and between OCC-18 and OCC-20 [(24 h, p < 0.001 at 5 and 15 µM, p < 0.05 at 30 µM) (48 h, p < 0.0001 at 5 µM) (48 h, p < 0.01 at 10, and 20 µM), (72 h, p < 0.05 at 20 µM)]. Occ-11 and OCC-18 [(24 h and 48 h, p < 0.0001 at 5 µM) (72 h, p < 0.0001 at 15 µM)] between OCC-11 and OCC-20 [(72 h, p < 0.0001 at 15 µM), and between OCC-18 and OCC-20 [(24 h, p < 0.001 at 5 and 15 µM, p < 0.05 at 30 µM) (48 h, p < 0.0001 at 5 µM) (48 h, p < 0.01 at 10, and 20 µM), (72 h, p < 0.05 at 20 µM)].

Half-maximal inhibitory concentration (IC50) values of treated OCC cells for aflibercept and arsenic trioxide were calculated and showed in Supplementary Table 2.

Selection of 10 µM arsenic trioxide was in accord with a previous study [18] and the selected concentration of aflibercept (100 µg) was based on the results of our previous study at 48 h [10].

Effect of aflibercept and arsenic trioxide on VEGF expression

The western blot assay was performed to assess the expression of VEGF at the protein level in treated and untreated OCC cell lines. Although the results of VEGF expression
showed no significantly differences between untreated cells, both drugs significantly decreased the expression of VEGF in all OCC cell lines in comparison with the control group (Fig. 2a–g). As shown in Fig. 2, the greatest reduction in VEGF expression was observed in OCC-20 cell line treated by arsenic trioxide in comparison with the control group (p < 0.0001) (Fig. 2f).

**Effect of aflibercept and arsenic trioxide on endoglin expression**

In order to study the expression of endoglin, three cell lines were treated with aflibercept (100 μg), and arsenic trioxide (10 μM), and underwent flow cytometry assay after 48 h. Aflibercept caused the greatest reduction in endoglin expression in all three cell lines compared with cells treated with arsenic trioxide and untreated cells. It is apparent from Fig. 3 that OCC-11 was more resistant to other groups when treated by aflibercept and arsenic trioxide (Fig. 3a–d).

**Effect of aflibercept and arsenic trioxide on cell apoptosis**

In order to assess whether aflibercept and arsenic trioxide induce cell death by apoptosis, OCC-11, OCC-18, and OCC-20 cell lines were treated with aflibercept (100 µg, 48 h) and arsenic trioxide (10 μM, 48 h) and then underwent flow cytometry. Analysis of the results indicated that both drugs significantly induced apoptosis compared to untreated groups (p < 0.0001). Beside, in OCC-11, the flowcytometric analysis showed that there are no significant differences of apoptotic cells between aflibercept and arsenic trioxide treated cells (Fig. 4b). However, a significant increase was noted in the percentage of apoptotic cells in OCC-18 and OCC-20 line (p < 0.01 and p < 0.001) treated by arsenic trioxide compared to aflibercept treated cells (Fig. 4c–d).

**Effect of aflibercept and arsenic trioxide on cell proliferation and colony formation**

The colony formation assay was performed to quantify the proliferation of treated cells. No colony formed after 14 days when the cells were treated with arsenic trioxide (Fig. 5a, c, e). Also, aflibercept significantly decreased the number of colonies in three primary cancer cell lines compared with the control group (p < 0.001).

**Effect of aflibercept and arsenic trioxide on cell migration**

The scratch test was performed in absence and presence of the drugs to assess the motility and migration of cells after exposure to aflibercept and arsenic trioxide. The results showed decreased cell migration following treatment with arsenic trioxide [OCC-11 (p < 0.001) and OCC-20, OCC-18 (p < 0.0001)] and aflibercept [OCC-11 and OCC-20 (p < 0.05)] at 24 h. After 48 h, only arsenic trioxide-treated OCC-11 cells showed a significant decrease (p < 0.01) and migration of cells decreased significantly in OCC-18 and OCC-20 (p < 0.0001). Also, the migration rate of aflibercept-treated cells decreased in OCC-18 and OCC-20 lines (p < 0.05) compared with the control group (Fig. 6a–f). Overall, our results indicated that both drugs reduced the migration and mobility of cancer cells.

**Effect of aflibercept and arsenic trioxide on VEGFA, VEGFB and endoglin genes expression**

The Real-Time PCR assay was performed to assess the expression of VEGFA, VEGFB and endoglin at the gene level in treated and untreated OCC cell lines. Treatment of OCC cells with aflibercept and arsenic trioxide induced a significant decrease of VEGFA, VEGFB, and endoglin gene expression compared to untreated cells in all groups (Fig. 7).

**Discussion**

A number of molecular factors are involved in tumorigenesis of OCC [5]. In the recent years, researchers have focused on genetic alterations in OCC to detect novel molecular targets which could be both effective and predictive for treatment response. Currently, some protocols have been established for sample collection, storage, detailed techniques, and proper analytical approaches in targeted therapy. Although Bonner et al. reported that cetuximab (Erbitux) is a medication for targeted therapy in OCC, this medication is used in combination with chemotherapy [19, 20]. It should be noted that in cancer, sometimes the formed vessels have pathological (abnormal) structures that resist the receipt of medications, including chemotherapeutic agents. Aflibercept can return the normal structure of such vessels to make them susceptible to various medications [21]. Aflibercept may be effective in combination with the conventional surgical procedures for treatment of oral cancer [10]. In addition, arsenic trioxide has anti-angiogenic effects, and can decrease the density of small vessels [5]. It can also prevent cell growth and differentiation because of its effects on various aspects of cellular activity [22]. It has been observed that arsenic trioxide induces apoptosis by rebooting the WNT signaling pathway in cancer cells, and prevents angiogenesis by inhibiting the vascular endothelial growth factor (VEGF) [5]. Recently, investigators examined the effects of arsenic trioxide on hematological malignancies and showed its optimal efficacy [23].
Fig. 2 Western blot assay to evaluate VEGF protein expression. Aflibercept (AF) and arsenic trioxide (AR) decreased VEGF expression by OCC cells at 48 h. a–b VEGF expression by OCC-11 cells treated with aflibercept and arsenic trioxide. c–d VEGF expression by OCC-18 cells treated with aflibercept and arsenic trioxide. e–f VEGF expression by OCC-20 cells treated with aflibercept and arsenic trioxide. There was a decreasing percentage of VEGF expression in all tested cell lines compared with the control group. g VEGF expression by untreated OCC cell lines. There was no significant difference of VEGF expression between untreated cell lines. Data are expressed as mean ± standard error of the mean.
We investigated different effects of aflibercept and arsenic trioxide on three primary OCC cell lines. Several reports have shown the anti-angiogenic effects of arsenic trioxide and aflibercept on different cancer types. Our results indicated that the tested drugs had different effects on each cell line isolated from different patients, which was in agreement with the personalized medicine concept. In reviewing the literature, impressive results have been reported for cancer treatment using personalized medicine [24–26]. However, only a limited number of targeted therapies have been implemented on OCC patients [27]. Our results indicated that treatment of OCC-18 and OCC-20 cell lines with arsenic trioxide significantly increased the number of apoptotic cells, unlike the aflibercept-treated cells. Interestingly, OCC-11 cells had less apoptotic response in comparison with the other two groups, suggesting that OCC cells isolated from different patients may show different responses to treatment with arsenic trioxide and aflibercept. In agreement with our results, Kumar et al. showed that arsenic trioxide significantly decreased the cell survival rate of OCC [28].
VEGF is the best known regulator of angiogenesis that controls proliferation, survival, and permeability of endothelial cells [29]. The previous studies reported that arsenic trioxide down regulates the expression of VEGFA and VEGFB genes in KG-1 cell line and U937 cell line [30]. As reported before, endoglin expressed in high level and play a significant role in the advanced level of such malignancies [31], we studied the anti-angiogenic activity of aflibercept and arsenic trioxide against three OCC cell lines, aiming to identify the most effective targeted drug in different patients. The flow cytometric analysis showed that endoglin expression significantly decreased in all treated groups. Surprisingly, OCC-11 was resistant to both aflibercept and arsenic trioxide. VEGF expression patterns confirmed the endoglin expression results, suggesting that aflibercept and arsenic trioxide induced anti-angiogenic effects on each cell line through a different mechanism. Also genes expression in mRNA level showed that both drugs down regulated the expression of VEGFA, VEGFB, and endoglin genes. This finding broadly supports other studies in this field. Giuliani et al. showed that aflibercept, with greater anti-angiogenic effects, might be effective in treatment of OCC in combination with the conventional surgical procedures [32].

Monitoring the patients taking aflibercept is highly recommended because of the common side effects of aflibercept including medication-related osteonecrosis of the jaw, ...
hypertension, and some clinical signs and symptoms of toxicity [32, 33]. On the other hand, there are limited studies that investigated the efficacy of aflibercept in comparison with other anti-angiogenic-targeted drugs in OCC patients.

The mechanism of action of arsenic trioxide as an anti-cancer drug involves apoptosis, induction of differentiation, inhibiting the mitochondrial permeability, and inducing the generation of reactive oxygen species [34, 35]. However, the anti-angiogenic effects of arsenic trioxide on cancers especially OCC have remained unclear.

Wang et al. claimed that arsenic trioxide in a combined treatment regimen can be a novel and effective therapeutic strategy for OCC [34]. Nakaoka et al. suggested a combination of arsenic trioxide and cisplatin as anti-apoptotic agents for OCC for the first time [36]. Additionally, it should be noted that arsenic trioxide is affordable and available.

It has been reported that anti-cancer drugs suppress the migration and colony formation of several cancer types [37]. For a clear understanding, we investigated the suppressive effects of aflibercept and arsenic trioxide on three OCC cell lines using the colony formation and migration assays. We found that these drugs inhibited cell migration and colony formation in OCC. In addition, we observed that cell migration significantly decreased in OCC-18 and OCC-20 in comparison with OCC-11. It is interesting to note that in all three cell lines, no colony formation observed when the cells were treated with arsenic trioxide for 14 days.

Fig. 5 Treatment with aflibercept and arsenic trioxide decreased colony formation in three OCC cell lines. For the colony formation assay, OCC-11 (a), OCC-18 (c), and OCC-20 (e) were treated with 100 µg/mL aflibercept and 10 µM arsenic trioxide. Fourteen days later, the cells were fixed and stained using the standard protocol and then photographs were quantified by ImageJ and analyzed by GraphPad. b, d, f Quantification of results in part a, c, e; b Colony number of OCC-11 cells treated with aflibercept (AF) and arsenic trioxide (AR). c Colony number of OCC-18 cells treated with aflibercept and arsenic trioxide. d Colony number of OCC-20 cells treated with aflibercept and arsenic trioxide. Data are expressed as mean ± standard error of the mean.
Fig. 6 For cell migration assay, first, OCC-11 (a), OCC-18 (c), and OCC-20 (e) cell lines were treated with 100 µg/mL aflibercept and 10 µM arsenic trioxide. Then, photographs were taken at 0, 24, and 48 h. Finally, photographs were quantified using ImageJ software and analyzed by GraphPad. (b, d, f) Quantification of images in part A. C, E (b) Relative migration in OCC-11 cells treated with aflibercept (AF) and arsenic trioxide (AR). (c) Relative migration in OCC-18 cells treated with aflibercept and arsenic trioxide. (d) Relative migration in OCC-20 cells treated with aflibercept and arsenic trioxide. Data are expressed as mean ± standard error of the mean.
Tandberg et al., in a recent study showed characterization of the mutational landscape of OCC which was demonstrated by single-site biopsy and could have implications for personalized medicine [38]. In future years, we can see the establishment of precision medicine modalities in an attempt improve the quality of life of patients with advanced head and neck SCC [7]. Different responses of OCC cells to drugs in our study showed heterogeneity of the responsible biomarkers in OCC patients. Accurate screening of specific biomarkers from a huge number of molecules in each patient is highly important to achieve personalized precision medicine in OCC [39].

On the other hand, OCC patients suffer from a lack of flexibility in their therapeutic strategy which leads to inadequate or excessive treatment [40]. Similarly, our results revealed that the drugs had different effects on different OCC cell lines. Clinically, different treatment responses in OCC patients show that even targeted therapy with major advances for treating the patients is beneficial only for a subset of patients not all of them [41].

**Conclusion**

Our study investigated the personalized medicine field for providing targeted therapies that it will definitely play a significant role against this deadly cancer. Considering the before-mentioned results and the advantages of arsenic trioxide and the optimal efficacy of this affordable drug for cell apoptosis, inhibition of colony formation, and reduction of endoglin and VEGF expression by OCC cells, we suggest future in vivo studies and clinical trials on this drug. Considering the heterogeneity of OCC, we should design ideal therapeutic methods such as targeted therapy and use optimal drugs for patients with genetically complex diseases.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11033-021-06341-w.

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**Author contributions** Study conception and design: SD, MA, MG, PF. Acquisition of data: MA, FP, SR, STS, MG. Analysis and interpretation of data: MA, MG, STS. Drafting of manuscript: SD, MA, PA, PF. Critical revision: PA, FP, SR, PF.

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**Data availability** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Declarations**

**Conflict of interest** The authors declare no conflict of interests.

**Consent to participate** Written informed consent was obtained from each subject or his or her legal representative before inclusion into the study.
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