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Antiviral activity of Taurisolo® during bovine alphaherpesvirus 1 infection

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ABSTRACT

Bovine alphaherpesvirus 1 (BoAHV-1), the pathogen causing Infectious Bovine Rhinotracheitis (IBR) and predisposing to polymicrobial infections in cattle, provokes farm economic losses and trading restrictions in the world. However, nontoxic antiviral agents for BoAHV-1 infection are still unavailable, but plant extracts, such as flavonoid derivatives possess activity against BoAHV-1. Taurisolo®, a nutraceutical produced by Aglianico grape pomace, has recently shown promising antiviral activity. Herein, the potential activity of Taurisolo® during BoAHV-1 infection in Madin Darby bovine kidney (MDBK) cells was tested. Taurisolo® enhanced cell viability and reduced morphological death signs in BoAHV-1-infected cells. Moreover, Taurisolo® influenced the expression of bICP0, the key regulatory protein of BoAHV-1, and it strongly diminished virus yield. These effects were associated with an up-regulation of aryl hydrocarbon receptor (AhR), a transcription factor involved in microbial metabolism and immune response.

In conclusion, our findings indicate that Taurisolo® may represent a potential antiviral agent against BoAHV-1 infection. Noteworthy, AhR could be involved in the observed effects and become a new target in antiviral therapy.

1. Introduction

Bovine alphaherpesvirus 1 (BoAHV-1), belonging to the *Alphaherpesvirinae* subfamily, causes an infection of the upper respiratory tract known as infectious bovine rhinotracheitis (IBR), conjunctivitis and genital disorders, which are often accompanied by lowered fertility, abortions, and reduced milk production. The virus, together with other pathogens, causes the bovine respiratory disease complex, leading to pneumonia and occasionally death in cattle (Muylkens et al., 2007; Jones 2019). BoAHV-1 establishes long-term latent infection in sensory neurons of the infected animals, and the reactivation from latency may prompt virus shedding and spread to susceptible animals (Muylkens et al., 2007; Jones 2019). Eradication programs are followed by several European countries, but IBR is still endemic in many regions, increasing the risk of virus transmission to free herds (Iscaro et al., 2021). IBR is currently listed in the Regulation 2016/429/EU and its Annexes (Animal Health Law), and outbreaks of IBR in IBR-free member states or in IBR-free zones of European countries should be subject to notification. Thus, IBR is responsible for large economic losses and trade restrictions (Iscaro et al., al.,2021). Vaccination is still the most efficient way to prevent the disease (Righi et al., 2023). To date, a potential antiviral effect against BoAHV-1 is due to conventional synthetic drugs (acyclovir, fenbendazole, famciclovir, ivermectin) administered alone or in combination with natural agents (Yuan et al., 2016; Chang and Zhu 2020; Yesilbag et al., 2021). Indeed, very few nontoxic medicinal compounds to fight BoAHV-1 infection are described, although plant extracts have been studied as potential new antiviral drugs. Polyphenols, for example, have been demonstrated to have noticeable anti-herpesvirus activity (Vilhelmova-Ilieva et al., 2014; Boubaker-Elandalousi et al., 2014; Annunziata et al., 2018a, 2018b). Furthermore, flavonoid derivatives have been shown to have antiviral activity against BoAHV-1 (Akula et al., 2002; Boubaker-Elandalousi et al., 2014; Zhu et al., 2018). Specifically, polyphenols and flavonoids derivatives have been shown to have antiviral activity in vitro in MDBK cells. For

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instance, genistein, a soy isoflavone, inhibits BoAHV-1 replication (Akula et al., 2002); *Thymus capitata*, reach in polyphenols and flavonoids, inhibits the viral replication by interfering with the early stages of viral adsorption and replication (Boubaker-Elandalousi et al., 2014); curcumin, a constituent of the spice turmeric inhibits BoAHV-1 entry into MDBK cells (Zhu et al., 2015); and kaempferol exhibits a robust antiviral activity against virus replication (Zhu et al., 2018).

Taurisolo®, a grape pomace polyphenolic extract obtained from the Aglianico cultivar grape, is a nutraceutical containing several substances such as catechin, resveratrol, procyanidins, epicatechin, flavonoids, and organic acids (gallic, syringic, caffeic, p-coumaric, ferulic) (Annunziata et al., 2019a). It has been shown that Taurisolo® reduces oxidative stress and oxidative damage in aged rats (Annunziata et al., 2020, 2021a,b; Badolati et al., 2020). Moreover, it decreases the levels of trimethylamine-N-oxide, a cardiovascular risk factor (Annunziata et al., 2019a,b) and preserves the vascular function against ox-inflamm-aging process and the consequent cardiovascular accidents (Martelli et al., 2021). In addition to antioxidant properties, it has recently demonstrated that Taurisolo® possesses antiviral activity against herpes simplex virus (HSV) type 1 and 2 (Zannella et al., 2023). Moreover, during a clinical trial, in patients affected by SARS-CoV-2 pneumonia, the administration of a Taurisolo® aerosol formulation reduced both the duration and the severity of symptoms (Sanduzzi Zamparelli et al., 2022).

As previously reported, BoAHV-1 is a useful model for anti-herpesvirus compounds testing (Akula et al., 2002; Boubaker--Elandalousi et al., 2014; Zhu et al., 2018; Fiorito et al., 2017a, 2022; Chang and Zhu 2020; Yesilbag et al., 2021). Thus, this study aimed to assess the potential antiviral activity of Taurisolo® against BoAHV-1.

2. Materials and methods

2.1. Production of Taurisolo®

Taurisolo® is a nutraceutical supplement containing a polyphenol extract from Vitis Vinifera cv 'Aglianico' grapes, collected in Montemarano (Avellino, Italy, Coordinates: 40°54058″ N 14°59054″ E) during the autumn harvest. The first production of the polyphenol extract was optimized at the NutraPharmaLabs of the Department of Pharmacy, University of Naples Federico II (Naples, Italy). Then, the MB-Med Company (Turin, Italy) performed large-scale production (Badolati et al., 2020; Annunziata et al., 2021a, b). Briefly, the production of polyphenol extract involved the extraction of grapes with water (50 °C). The extract was subsequently filtered and concentrated through a spray-drying method with maltodextrins (40–70%) to have a fine microencapsulated powder. The polyphenol profile of Taurisolo® was studied by High Performance Liquid Chromatography-diode array detector (HPLC-DAD, Jasco Inc., Easton, MD, USA) analysis. Taurisolo® consists of various molecules, such as organic acids (gallic, syringic, caffeic, p-coumaric, ferulic), catechin, procyanidins, quercetin, resveratrol and, rutin (Badolati et al., 2020; Annunziata et al., 2021a,b).

2.2. Cell cultures and virus infection

The bovine cell line Madin Darby Bovine Kidney (MDBK) (American Type Culture Collection, CCL22) was cultivated in Dulbecco's modified Eagle's minimal essential medium (DMEM) and incubated at 37 °C and 5% CO_2 (Longo et al., 2009; Fiorito et al., 2011). BoAHV-1 (Cooper strain, accession number: KU198480) was used. Both for virus stocks growth and virus titration MDBK cells were utilized (Fiorito et al., 2008a,b; 2020; 2021).

Taurisolo® was dissolved in DMEM to a final concentration of 0.1, 0.5, 1, 1.5 and 3 mg/mL. Monolayers of MDBK cells were infected or not with BoAHV-1, at a multiplicity of infection (MOI) of 0.1, 1, 5 or 10, in the presence or not of Taurisolo®, to obtain four groups: uninfected or infected cells, Taurisolo® treated infected and uninfected cells. After 1 hour of adsorption at 37 °C, cells were incubated and processed at 1, 3, 6, 12, 24, 48, 72 and 120 h post infection (p.i.). BoAHV-1 was in culture medium throughout the course of the experiment.

2.3. Cell viability

Trypan blue (TB) (Sigma-Aldrich) exclusion test was used to assess cell viability (Fiorito et al., 2008b). At 48 h post treatment, trypsin was added to the cells that were mixed with TB and counted by TC20 automated cell counter (Bio-Rad). Cell viability was obtained as the percentage of living cells over the total cell number. Results were reported as the mean ± S.D. of three independent experiments in duplicate.

2.4. Cell proliferation

3-(4,5-dimethyl-2-thiazolyl)−2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was performed to examine cell proliferation (Fiorito et al., 2008a; 2011; Santamaria et al., 2011). Several concentrations of Taurisolo® (0.1, 0.5, 1, 1.5 and 3 mg/mL) were evaluated after 48 h p.i. in a preliminary assay of cell viability and then we selected 0.5 mg/mL Taurisolo® for MTT experiments. Briefly, BoAHV-1 infected (MOI 0.1) cells, incubated or not with Taurisolo® (0.5 mg/mL) were tested by MTT assay after 48 h p.i.. Results were the mean ± S.D. of four independent experiments in duplicate.

2.5. Examination of cell morphology

Cell morphology was evaluated using Giemsa staining (Fiorito et al., 2020; 2022). Monolayers of MDBK were infected or not with BoAHV-1, at MOI of 5, in the presence or absence of Taurisolo® (0.5 mg/mL), and after 24 h of incubation, Giemsa staining and light microscopy (ZOE Cell Imager, Bio-Rad Laboratories) were performed. The criteria described to explore cell death features were used (Leite et al., 1999; Banfalvi et al. 2017).

2.6. Immunofluorescence staining

In order to study the influence of Taurisolo® on both bICP0 and AhR expression in BoAHV-1-infected cells, immunofluorescence staining was carried out at 24 h p.i. (Altamura et al., 2018; Fiorito et al., 2022). The antibodies, dissolved in 5% bovine serum albumin-TBST, were: anti-AhR (Sigma-Aldrich) (1:250), anti-bICP0 polyclonal rabbit (a.a. 663–676) serum (1:800), kindly supplied by Prof. M. Schwyzer and Prof. Cornel Fraefel (University of Zurich, Switzerland), Texas Red goat anti-rabbit (Thermo Fisher Scientific) (1:100). DAPI (1:1000) was used as nuclear counter-staining. Microscopy and photography were valued by ZOE Fluorescent Cell Imager (Bio-Rad Laboratories). Fluorescence signals from microscopy-generated images were quantified by ImageJ (National Institutes of Health, Bethesda, MD, USA) software.

2.7. BoAHV-1 infection

Cells were infected with BoAHV-1 at MOI 1, in the presence or not of Taurisolo®, and processed at 0, 1, 3, 6, 12, 24, 48, and 72 h p.i. by real-time PCR for BoAHV-1 quantification. Furthermore, viral cytopathic effects (CPE) were evaluated at light microscope until 120 h p.i. (De Martino et al. 2010; Fiorito et al., 2021).

2.8. Viral nucleic acids extraction

Nucleic acids extraction was carried out from 200 μL of cell

supernatant by using the King Fisher Flex System (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with the Mag Max Viral Pathogen kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA), according to the instructions of the manufacturer. Nucleic acids were dissolved in 80 µL of elution buffer. DMEM was utilized as a negative process control.

2.9. Real-Time PCR for quantification of BoAHV-1

BoAHV-1 was quantified in all the samples (cells infected with BoAHV-1 at MOI 1, in the presence or not of Taurisolo®, at 0, 1, 3, 6, 12, 24, 48, and 72 h p.i.) by quantitative real-time PCR, as described (Fiorito et al., 2022). Briefly, the detection was carried out on a Quant Studio 5 Real-Time PCR thermal cycler (Thermo Fisher Scientific, Waltham, Massachusetts, USA) in a total volume of 25 µL containing: 5 µL of nucleic acids extract, 12.5 µL of TaqMan Universal PCR Master Mix 2X (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 1 µL (4.5 µM) of primer forward gBF (5′- TGTGGAC-CTAAACCTCACGGT-3′), 1 µL (4.5 µM) of primer reverse gBR (5′-GTAGTCGAGCAGACCCGTGTC-3′) and 1 µL (3 µM) of probe gB-P (FAM-5′-AGGACCGCGAGTTCTTGCC GC-3′-TAMRA). The thermal profile consisted of initial denaturation for 15 min at 95 °C, 45 cycles of amplification for 15 s at 95 °C and, 45 s at 60 °C (OIE Manual of Terrestrial Animals Cap. 3.4.11. par B.1.3.1 2017). Then, quantification was performed by a standard curve, analysing serial dilutions of the quantified extracted virus (from 1×10^7 to 1×10^1 $TCID_{50}$/mL) and plotting the $TCID_{50}$/mL versus the threshold cycle (Ct) (Fiorito et al., 2022; Cerracchio et al., 2022a,b, 2023).

2.10. Statistical analysis

Data are presented as mean ± S.D. GraphPad InStat Version 3.00 for Windows 95 (GraphPad Software, San Diego, CA) was used to analyze one-way ANOVA with Tukey's post-test). $p < 0.05$ was judged statistically significant.

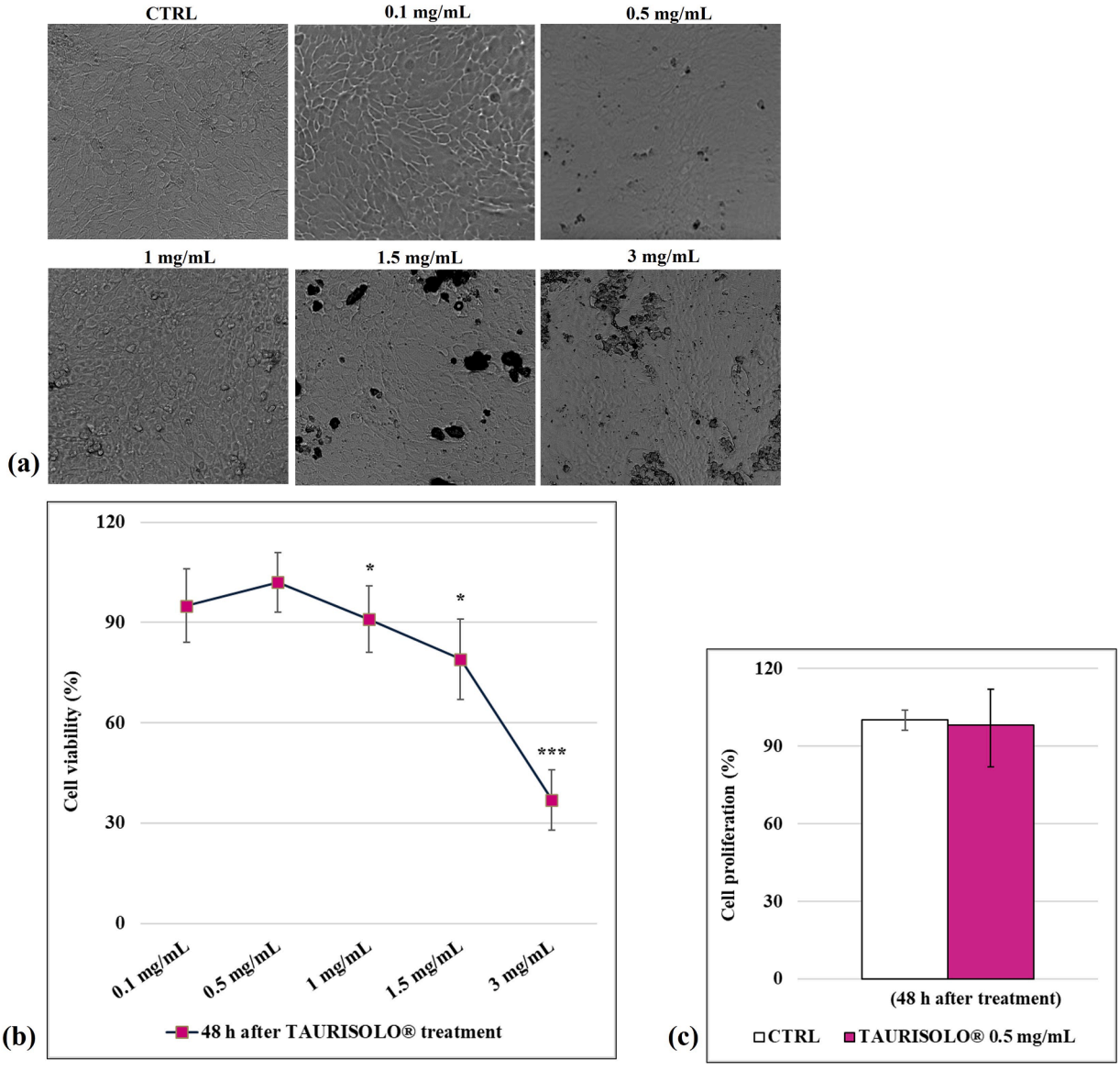


Fig. 1. Identification of Taurisolo® IC_{50} at different doses and doses-response curve in MDBK cells. (a) Microscope cells were stained with TB and scored by automated cell counter. (b) Dose-response curve of MDBK cells treated with Taurisolo® at different concentrations (0.1, 0.5, 1, 1.5, 3 mg/mL). At 48 h after treatment, cell viability was determined by TB staining while cells were attached to wells and counted under a light. (c) Dose-response curve of MDBK cells treated with Taurisolo® (0.5 mg/mL) for 48 h and assessed by MTT assay. Significant differences between control and Taurisolo®-treated cells are indicated by probability *p*. *** $p < 0.001$ and * $p < 0.05$. Scale bar 100 µm.

3. Results

3.1. Taurisolo® reduces MDBK cell death during BoAHV-1 infection

To examine the effect of Taurisolo® during BoAHV-1 infection, cell viability by TB was first assessed, then cell proliferation by MTT assay was analyzed. The assessment of IC_{50} values of Taurisolo® and the dose-response curve were performed by treating cells with different doses of Taurisolo®, as above described. Inhibition of cell growth was detected in MDBK cell treated with Taurisolo® IC_{50} values ranging between 1.5 and 3 mg/mL, at 48 h, while there were no significant alterations after treatment with lower concentrations of this nutraceutical (Fig. 1a,b). So, we choose to select the concentrations of 0.1 and 0.5 mg/mL to continue the experimental design. To confirm that the 0.5 mg/mL concentration is biocompatible and not cytotoxic, the mitochondrial redox activity of MDBK cells was analysed by MTT assay. After 48 h of exposure, Taurisolo® at 0.5 mg/mL did not produce significant ($p > 0.05$) time-dependent changes in the mitochondrial dehydrogenase's activity compared to control cells (Fig. 1c).

Finally, to evaluate the effect of Taurisolo® during BoAHV-1 infection, MDBK cells were infected with BoAHV-1 at MOI of 0.1 or 10 and treated or not with 0.1 mg/mL and 0.5 mg/mL of nutraceutical. After 48 h of treatment, cell viability and proliferation were evaluated. As shown in Figs, 2a,b, Taurisolo® was able to increase – in a significant way ($p < 0.01$) - the cell viability during BoAHV-1 infection already at 0.1 mg/mL, and its effect resulted stronger at the concentration of 0.5 mg/mL. At this last concentration, the nutraceutical also increased cell proliferation ($p < 0.05$) in MDBK cells (Fig. 2c).

Therefore, the concentration of Taurisolo® at 0.5 mg/mL was confirmed to be used throughout the study.

Altogether, at the end of BoAHV-1 infection, in the presence of a nontoxic dose of 0.5 mg/mL, Taurisolo® significantly reduced the cytotoxicity induced by BoAHV-1 infection of MDBK cells.

3.2. Taurisolo® reduces distinctive features of morphological cell death during BoAHV-1 infection in MDBK cells

To explore the effects of Taurisolo® in BoAHV-1-infected cells, we performed Giemsa staining, which allows to check the main differences between apoptosis and necrosis in cell morphology and evaluated it by light microscopy (Banfalvi et al., 2017). Taurisolo® exposed MDBK cells showed no cytomorphological changes when compared to controls (Fig. 3). Indeed, untreated infected cells, showed increased intercellular spaces and changes in cellular morphology such as chromatin condensation, fragmentation of nuclei, pyknosis and cell shrinkage, suggesting apoptosis activation) (Leite et al., 1999; Banfalvi et al., 2017) (Fig. 3, arrow). Furthermore, in infected cells, we observed typical signs of necrosis, due to nuclear and cytoplasmic swelling with chromatin appearing uniformly dense because of plasma membrane break (Fig. 3, arrowhead) (Leite et al., 1999; Banfalvi et al., 2017). On the other hand, only a few necrotic cell death features were detected in BoAHV-1 infected cells exposed to Taurisolo® (Fig. 3, arrowhead).

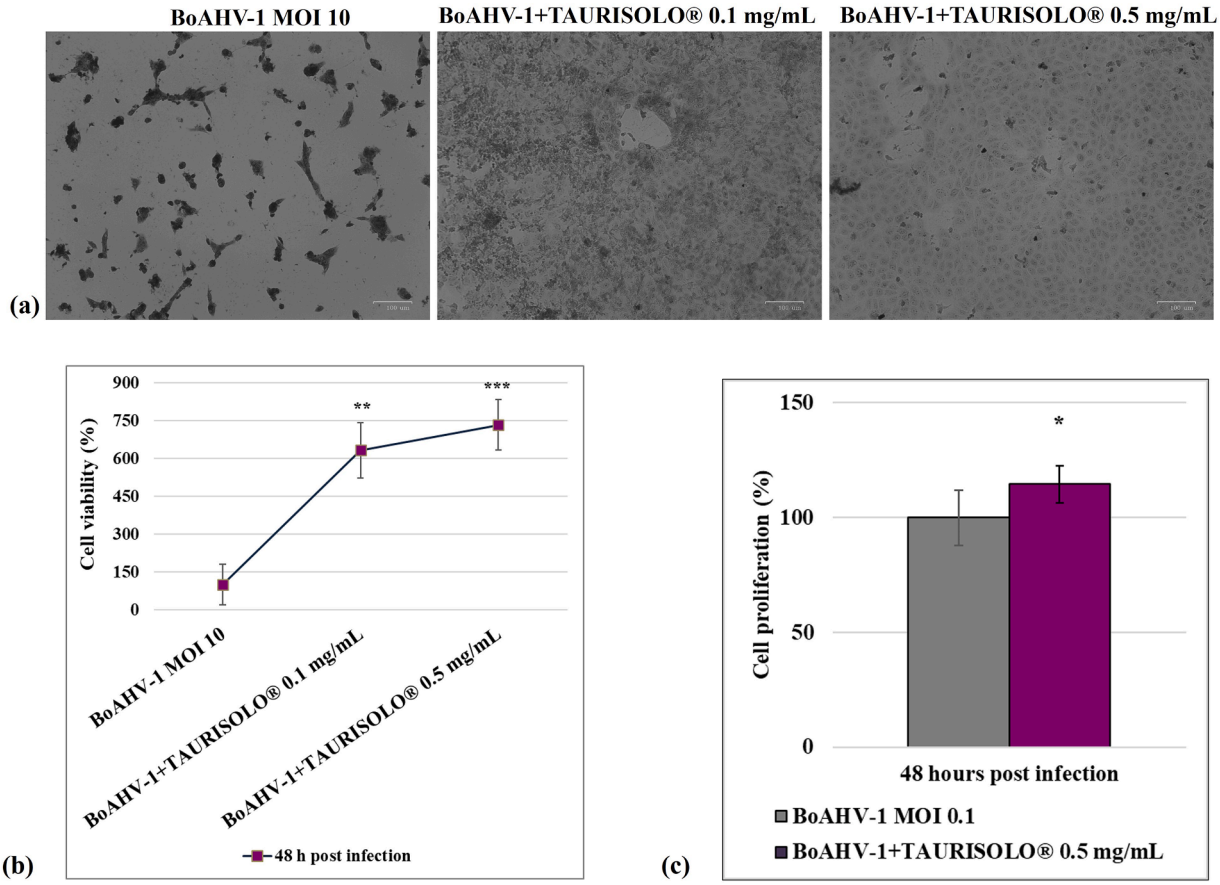


Fig. 2. Taurisolo® reduces cell death during BoAHV-1 infection. (a) Taurisolo® decreases cell death during BoAHV-1 infection in MDBK cells. Cells were infected with BoAHV-1, in the presence or absence of Taurisolo®. At 48 h p.i., cells were examined under a light microscope. In Taurisolo®-treated infected cells, only a few signs of cell death were found. Scale bar 100 μm. (b) Dose–response curve of MDBK cells infected with BoAHV-1 and treated with Taurisolo® at different concentrations (0.1, 0.5 mg/mL). At 48 h after treatment, cell viability was assayed by using TB staining while cells were attached to wells and counted under a light microscope. (c) Dose–response curve of MDBK cells infected with BoAHV-1, treated with Taurisolo® (0.5 mg/mL) for 48 h and analyzed by MTT assay. Significant differences between analysed groups are indicated by probability *p*. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

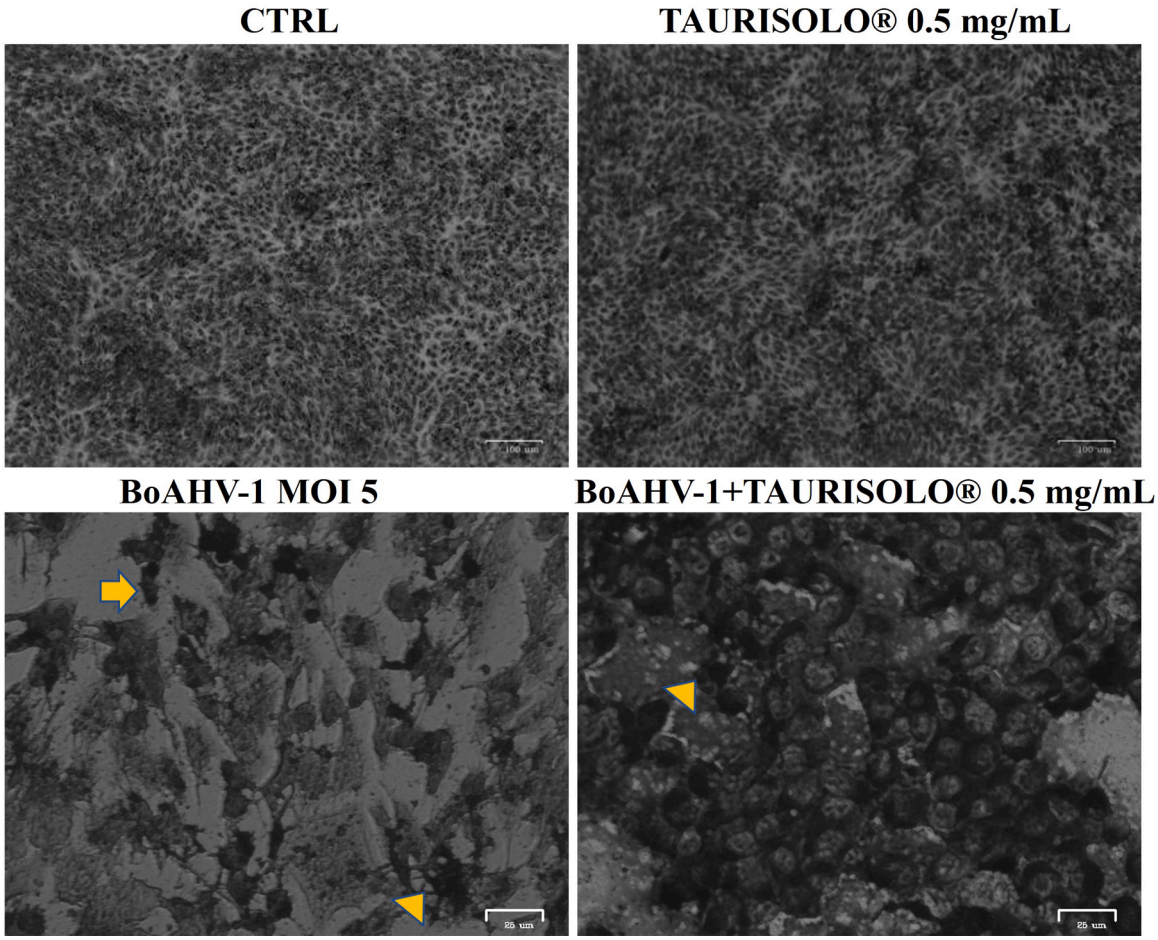


Fig. 3. Taurisolo® decreases morphological cell death features during BoAHV-1 infection. Taurisolo® reduces morphological cell death features during BoAHV-1 infection in MDBK cells. Cells were infected with BoAHV-1, in the presence or absence of Taurisolo®. At 24 h p.i., cells after Giemsa staining were examined under a light microscope. Photomicrographs showed no morphological alterations in Taurisolo® uninfected groups compared to control. By comparing BoAHV-1-infected cells to the control, some cells displayed apoptotic features, attributable to pyknotic nuclei and nuclear fragmentation (arrow), or necrosis marks, such as nuclear and cytoplasmic swelling (arrowhead). In Taurisolo®-treated infected cells, only a few signs of necrosis were found (arrowhead). Scale bar 100 and 25 μm.

Overall, these findings revealed that MDBK cells infected with BoAHV-1 and exposed to Taurisolo® were less subjected to apoptotic or necrotic cell death.

3.3. Taurisolo® reduces virus yield and downregulates the expression of bICP0 during BoAHV-1 infection in MDBK cells

To investigate Taurisolo® effects on virus production in MDBK BoAHV-1-infected cells, virus titer and viral CPE were analysed. Hence, MDBK cells were infected with BoAHV-1 at MOI of 1, in the presence or not of Taurisolo® at the nontoxic concentration of 0.5 mg/mL and processed. Following BoAHV-1 infection, a statistically significant ($p < 0.001$) decline in virus titer was found from 12 to 72 h post infection in cells treated with Taurisolo® (Fig. 4a).

Furthermore, at 24 h p.i., a marked CPE was detected in infected groups, caused by the development of syncytia and by destruction of cellular sheet, whereas these features were noticeably reduced in Taurisolo®-treated infected cells (Fig. 3). Interestingly, these characteristics were confirmed also in treated cells infected with BoAHV-1 at MOI 1 after 120 h p.i. (Fig. 4b). These results suggested that the nutraceutical significantly reduces virus yield and CPE during BoAHV-1 infection in MDBK cells.

In addition, to further investigate Taurisolo® influence in BoAHV-1 infection, we explored the expression of bICP0, the key protein concerning the transcription of BoAHV-1 (Wirth et al., 1992; Muylkens et al., 2007; Fiorito et al., 2013; Jones, 2019). After 24 h p.i., the expression of bICP0 in BoAHV-1 infected cells (MOI 10) was considerably downregulated in the presence of Taurisolo® (Fig. 5a). This result was confirmed by integrated measurement of density fluorescence (Fig. 5b).

These findings show that bICP0 viral protein, expressed in BoAHV-1-infected cells, was reduced in infected cells exposed to Taurisolo®.

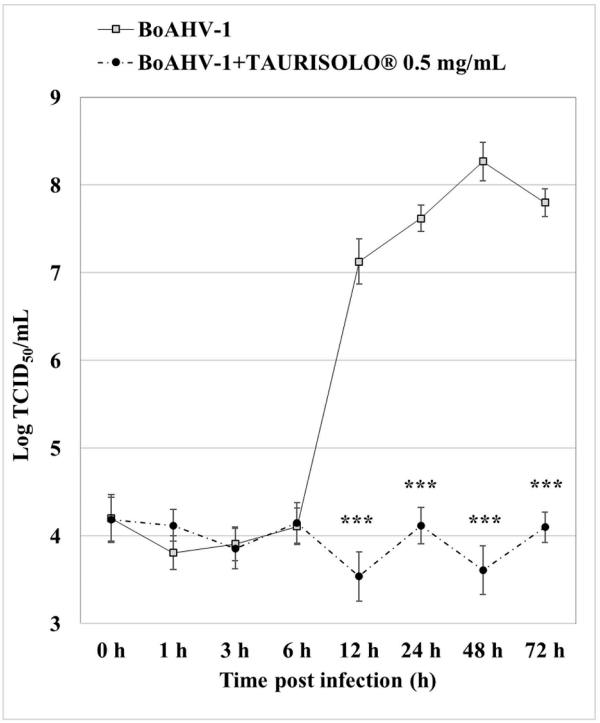
3.4. Taurisolo® upregulates the expression of AhR during BoAHV-1 infection in MDBK cells

To investigate the possible action of Taurisolo® in the regulation of AhR, we performed immunofluorescence analysis. Taurisolo® strongly stimulated AhR activation in MDBK cells until 120 h of treatment (Fig. 6a), and a remarkable stimulation of AhR in Taurisolo®-treated cells was detected during infection with BoAHV-1 after 24 h p.i. (Fig. 6b). These results were confirmed by integrated measurement of density fluorescence (Fig. 6c, d).

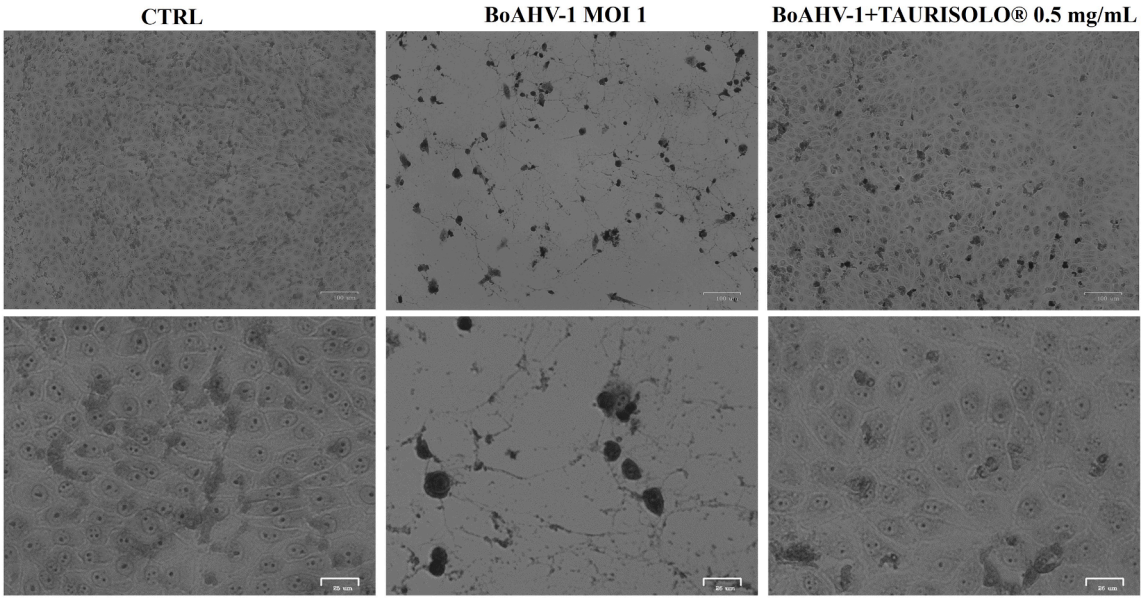
These results showed the Taurisolo® capability to stimulate AhR cellular receptor in both BoAHV-1 infected and uninfected MDBK cells.

4. Discussion

Pharmaceutical research is strongly involved in the development of novel therapeutic strategies against viral diseases in order to minimize



(a)



(b)

Fig. 4. Taurisolo® decreases virus yield during BoAHV-1 infection in MDBK cells. Cells were infected with BoAHV-1 in the presence or absence of Taurisolo®. (a) For viral growth curves, MDBK cells were infected with BoAHV-1 in the presence or absence of Taurisolo®. At indicated times, virus titer was evaluated by real-time PCR. Significant differences between BoAHV-1-infected cells and Taurisolo®-treated infected cells are indicated by probability *p.* *** $p < 0.001$. (b) For CPE evaluation, cells were infected with BoAHV-1 in the presence or absence of Taurisolo®. At 120 h p.i., CPE was observed. Scale bar 100 and 25 μm.

antiviral agents' toxicity and fight antibiotic-resistance phenomena, as well. In this context, plant extracts have been extensively explored as nontoxic natural remedies. To date, anti-herpesvirus natural compounds are scarce; for instance, anti-herpesvirus activity was detected in polyphenols (Vilhelmova-Ilieva et al., 2014; Boubaker-Elandalousi et al., 2014; Annunziata et al., 2018a), as well as in flavonoid derivatives (Akula et al., 2002; Boubaker-Elandalousi et al., 2014; Zhu et al., 2015, 2018). Taurisolo® is an extract which mainly contains polyphenols and flavonoids, and, in this study, we showed its capability to induce an excellent defense reaction against BoAHV-1 activity, in infected MDBK cells, by stimulating a significant improvement in cell viability and proliferation.

Generally, BoAHV-1 promotes cell death in a cell-type dependent manner (Geiser et al., 2008; Fiorito et al., 2020). Herein, during BoAHV-1 infection, morphological analysis of MDBK showed cell death hallmarks that were remarkably decreased by treatment with Taurisolo®. Interestingly, BoAHV-1 infected MDBK cells treated with the proteasome inhibitor MG-132 (Fiorito et al., 2017a,2021) and the fungal metabolite 3-O-methylfunicone (OMF) (Fiorito et al., 2022) showed comparable morphological features of cell defense. Furthermore, a significant decrease in BoAHV-1 replication was detected in Taurisolo® treated infected cells, which showed a relevant decrease both in CPE and

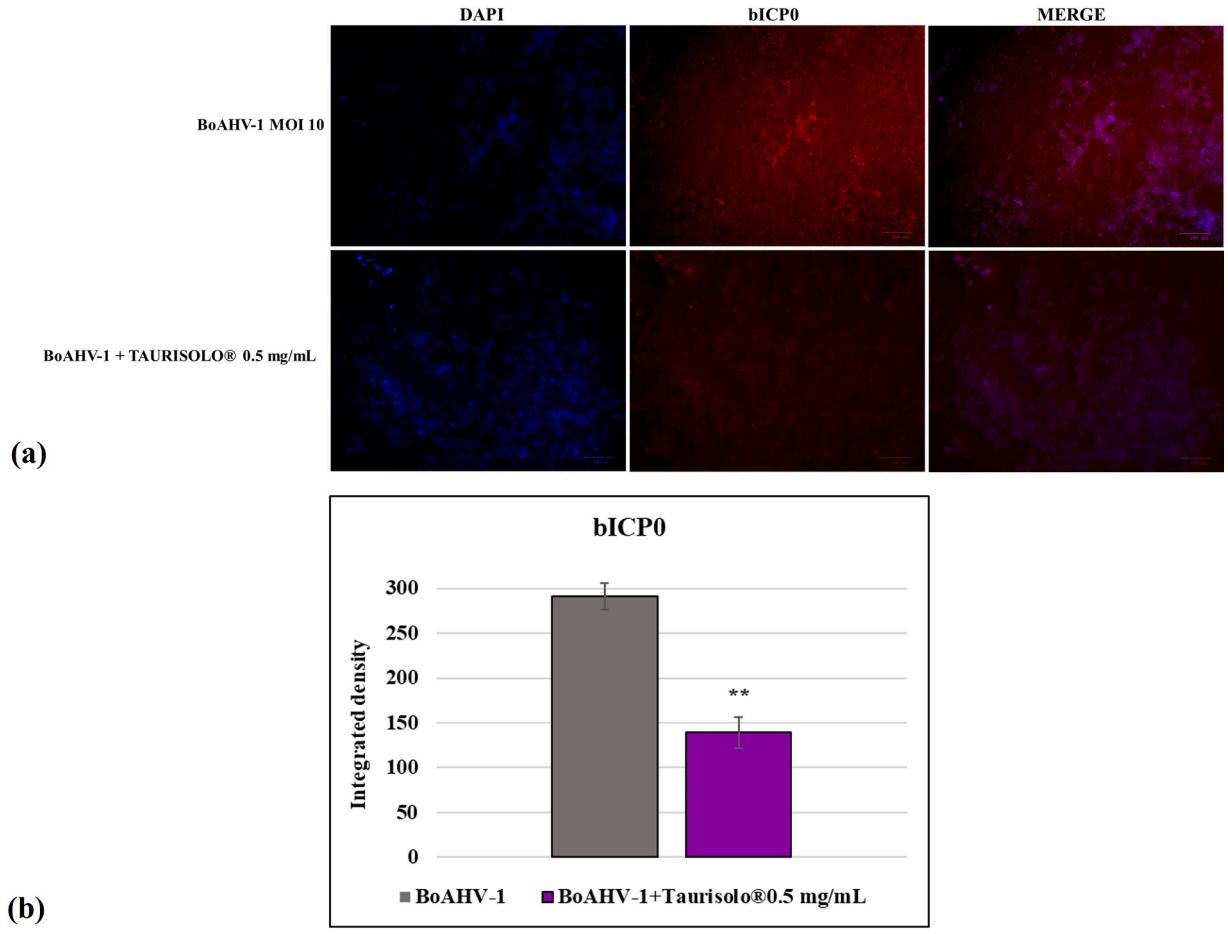


Fig. 5. Taurisolo® decreases the expression of bICP0 during BoAHV-1 infection in MDBK cells. Cells were infected with BoAHV-1, in the presence or absence of Taurisolo®, and after 24 h p.i, immunofluorescence staining for bICP0 (red fluorescence) was performed as described in the Method section. Nuclei were counterstained with DAPI. (a) During infection, bICP0 was localized both in nucleus and cytoplasm. In the presence of Taurisolo®, the expression of bICP0 was markedly reduced during BoAHV-1 infection. Scale bar 100 μm. (b) Bars correspond to the mean ratio produced from the integrated density (product of the area and mean intensity of fluorescence) calculated by ImageJ of the bICP0 expression. Error bars are standard deviation measurement. Significant differences between unexposed infected groups and Taurisolo®-treated infected cells are indicated by probability p. ** $p < 0.01$.

in virus yield. These findings suggest that polyphenols and flavonoids extract could act as anti-herpesvirus agents by influencing the early phases of infection (Chattopadhyay et al., 2009; Boubaker-Elandalousi et al., 2014). Indeed, Taurisolo® blocks HSV-1 and HSV-2 infection, in co-treatment as well as in pre-treatment, showing an inhibitory action in the early phases of both herpesviruses (Zannella et al., 2023). Indeed, gene expression of herpesviruses occurs in three phases named immediate-early, early, and late. The bovine homologue of HSV-1 ICP0, bICP0, regulates all three stages and promotes productive infection (Fraefel et al., 1994; Inman et al., 2001; Fiorito et al., 2010; Jones 2019). In our study, Taurisolo® remarkably reduced the expression of bICP0, in bovine cells and similar anti-BoAHV-1 activities were previously reported in the presence of chemical (MG-132) or natural (OMF) compounds (Fiorito et al., 2017a,2021,2022). Furthermore, Taurisolo® stimulated the activation of AhR in MDBK cells, also during BoAHV-1 infection. AhR can be activated by both endogenous and exogenous organic substrates, like tryptophan metabolites, bilirubin, biliverdin, environmental pollutants (dioxin), and microbial metabolites (Bock, 2021). After activation and translocation into the nucleus, AhR may stimulate target genes like AhR repressor, detoxifying monooxygenases (CYP1A1 and CYP1B1) and cytokines. Recent evidence suggests that diet products can activate and/or inhibit the AhR signaling pathway. Noteworthy, dietary flavonoids are the greatest class of natural AhR ligands, and some agonists/antagonists of AhR are used in clinical practice for cancer treatment (Yang et al., 2019). AhR is also implicated in the host response to viruses, such as coronaviruses (Tang et al., 2005; Grunewald et al., 2020; Giovannoni et al., 2021; Cerracchio et al., 2022a,b). Furthermore, targeting AhR may enhance the host response to herpesvirus infections (Chen et al., 2021; Torti et al., 2021). The first evidence of the involvement of AhR in inhibitory activity against BoAHV-1 due to OMF, a secondary metabolite produced by *Talaromyces pinophilus*, was recently observed in MDBK (Fiorito et al., 2022), a cell line which expresses the AhR (Fiorito et al., 2014, 2022).

Numerous AhR ligands with different properties have been identified: endobiotic, phytochemical, microbiota-generated ligands as well as xenobiotics/drugs (Bock 2020). Moreover, AhR signaling influences the immune response to different microorganisms (Torti et al., 2021). The replication of herpesviruses (cytomegalovirus, HSV-1, HSV-2, BoAHV-1) was stimulated by the activation of AhR. Dioxin (TCDD), a toxic environmental contaminant, through AhR, might provoke immune-suppression and enhances responsiveness to infectious agents (Fiorito et al. 2017b, c; Bock 2021). Veiga-Parga et al., showed that during HSV-1 infection in mice, dioxin-treated mice had the highest virus titers (2011). Particularly, if AhR was induced before infection, an increased number of mice died because of herpes encephalitis. Whereas, if AhR stimulation happened after HSV-1 infection, the disease features were ameliorated (Veiga-Parga et al., 2011). These findings also highlight the influence of timing in AhR activation in balancing pathology

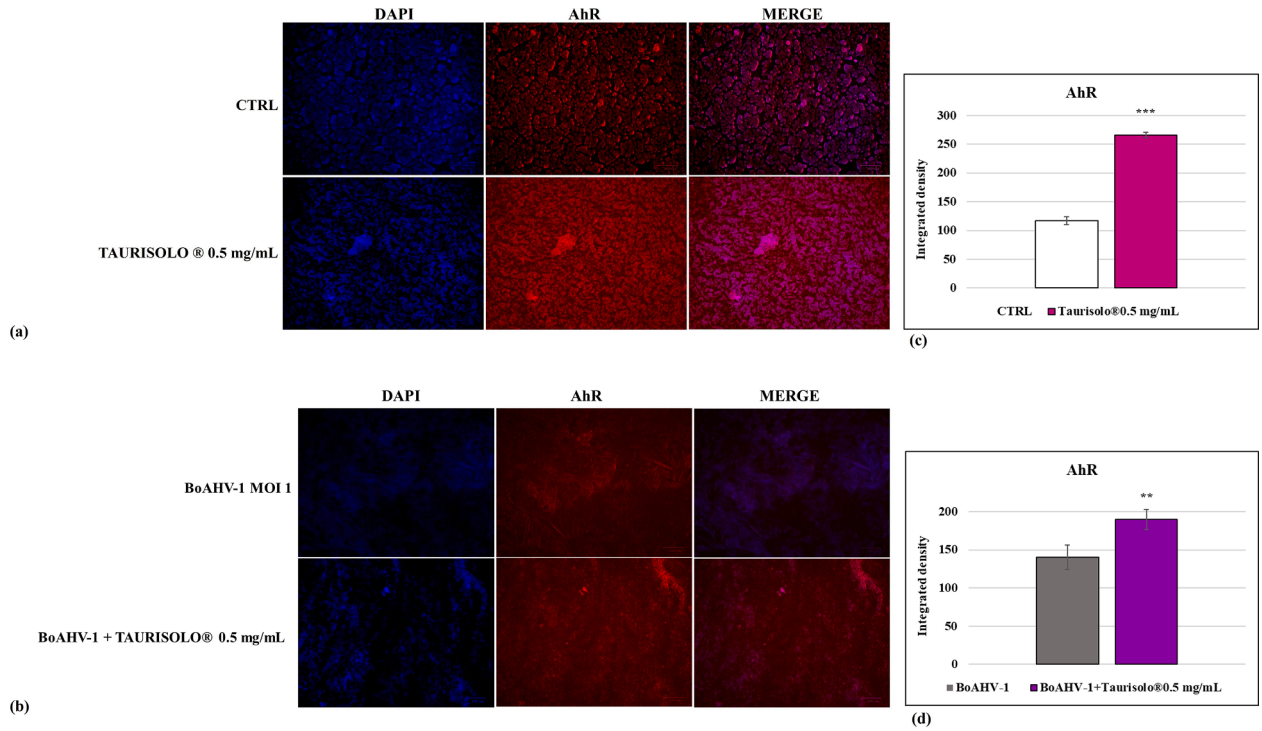


Fig. 6. Taurisolo® induces the expression of AhR during BoAHV-1 infection in MDBK cells. Representative microphotographs of uninfected (control) or infected cells (BoAHV-1), Taurisolo® -treated infected (BoAHV-1+Taurisolo®), and uninfected cells (Taurisolo®), stained with immunofluorescence for AhR (red fluorescence), as described in the Methods section. Nuclei were counterstained with DAPI. (a) AhR was expressed in MDBK cells and localized in both the nucleus and cytoplasm. The expression of AhR until 120 h after treatment was induced in the presence of Taurisolo®. (b) During infection, the expression of AhR was drastically reduced. In the presence of Taurisolo®, at 24 h p.i., the expression of AhR was remarkably enhanced during BoAHV-1 infection. Scale bar 100 μm. (c-d) Bars are the mean ratio produced from the integrated density (product of the area and mean intensity of fluorescence) calculated by ImageJ of the AhR expression. Error bars are standard deviation measurement. Significant differences between control cells and Taurisolo® exposed groups, as well as between unexposed infected groups and Taurisolo®-treated infected cells are indicated by probability p. *** $p < 0.001$ and ** $p < 0.05$.

and antiviral defensive immunity (Torti et al., 2021).

Taurisolo® is primarily composed of polyphenols and flavonoids, arylic nature molecules, which may interact with AhR. Flavonoids such as quercetin might be indirect AhR agonists (Bock 2020). The well-known polyphenol resveratrol has been recently recognized as a non-selective antagonist of AhR (Coelho et al., 2022). However, the hypothetical interaction between Taurisolo® and AhR in MDBK cells requests further investigations.

Overall, our findings revealed a promising antiviral activity of Taurisolo® during BoAHV-1 infection in MDBK cells, by affecting AhR. Hence, AhR may be a new target to develop potential antiviral treatments.

CRediT authorship contribution statement

CI, GCT, EN, RS and FF: conceived and designed research. **CC, MGA, MP, MGF, FPN and FS**: performed experiments. **CC, MGA, MP, MGF, LDM, FS, CI, GCT, EN, RS and FF**: contributed to the data interpretation. **CC, MGA, CI, MGF, RS, and FF**: wrote the manuscript and prepared the figures. All authors read and approved the manuscript.

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Data available with the paper

All data supporting the findings of this study are available within the paper.

Statements and declarations

This article does not contain any studies with human participants performed by any of the authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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