



Research article

Fungal metabolite 6-pentyl- α -pyrone reduces canine coronavirus infection

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ABSTRACT

Canine coronavirus (CCoV) can produce a self-limited enteric disease in dogs but, because of notable biological plasticity of coronaviruses (CoVs), numerous mutations as well as recombination events happen leading to the emergence of variants often more dangerous for both animals and humans.

Indeed, the emergence of new canine-feline recombinant alphacoronaviruses, recently isolated from humans, highlight the cross-species transmission potential of CoVs.

Consequently, new effective antiviral agents are required to treat CoV infections. Among the candidates for the development of drugs against CoVs infection, fungal secondary metabolites (SMs) represent an important source to investigate. Herein, antiviral ability of 6-pentyl- α -pyrone (6 PP), a SM obtained by *Trichoderma atroviride*, was assessed against CCoV. During *in vitro* infection, nontoxic concentration of 6 PP significantly increased cell viability, reduced morphological signs of cell death, and inhibited viral replication of CCoV. In addition, we found a noticeable lessening in the expression of aryl hydrocarbon receptor (AhR), a strategic modulator of CoVs infection.

Overall, due to the variety of their chemical and biological properties, fungal SMs can decrease the replication of CoVs, thus identifying a suitable *in vitro* model to screen for potential drugs against CoVs, using a reference strain of CCoV (S/378), non-pathogenic for humans.

1. Introduction

Some of the coronaviruses (CoVs), positive-stranded RNA viruses, are responsible for different human and animal diseases with limited or not available treatment options. A canine coronavirus, like CCoV-II, an alphacoronavirus of the *Coronaviridae* family, not only provoke enteric symptoms as well as self-limited infections in dogs [1], but also lethal infection mainly in pups, due to extremely virulent CCoV-II strains [2–4]. Indeed, new combination of genetic material as well as mutation can occur developing new strains of

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CCoV, which may cause processes of spillover [1,5]. Recently, the isolation of new recombinant alphacoronaviruses (genotype II) was reported. Specifically, CCoV-HuPn-2018 and HuCCoV_Z19Haiti, were identified in children hospitalized with pneumonia in Malaysia, and in a health professional exhibiting symptoms of illness after visiting Haiti, respectively [5–7]. The genetic comparison between the two recombinant strains showed a very high (99.4%) similarity [5,7]. In addition, recombinant alphacoronaviruses emerge in different geographic locations independently, because of they have also been detected from humans in Thailand and in USA [5,8,9]. Therefore, due to emergence of new coronaviruses, often dangerous, discovering novel drugs with antiviral activity is strongly required.

The potential of natural products in drug discovery should be investigated [10]. Indeed, several compounds from various biological sources (i.e., medicinal plants, bacteria, fungi) are being studied with the aim to find and develop new nontoxic medicinal compounds. Fungi produce numerous bioactive secondary metabolites (SMs), some of which have been already used not only as antibiotics, but also as fungicides, regulators of plant growth and hormones. In addition, some fungal molecules revealed antiviral properties [11–13]. As an example, studies about 3-O-methylfunicone (OMF), which is a benzo- γ -pyrone obtained by *Talaromyces pinophilus*, revealed that this compound is able to induce a decline in the infectivity of CCoV [14], hepatitis C virus [15], and bovine herpesvirus 1 [16]. Recently, other funicone-like substances, vermistatin (VER) as well as penisimplicissin (PS) have been evaluated for their *in vitro* inhibitory activity towards CCoV infection [17]. The *Trichoderma* fungal metabolite 6-pentyl- α -pyrone (6 PP) shows a significant role against plant pathogens [18,19], and in anti-biofilm-producing bacteria activity [20,21], but the antiviral activity of 6 PP has never been explored up to now. Hence, herein the potential efficacy of 6 PP anti-CCoV was tested using A72, which are canine fibrosarcoma cells, and Crandell-Rees Feline Kidney Cell (CRFK), a cell line resulting from the feline kidney, both are suitable for studying CCoV [14, 22–24].

2. Materials and methods

2.1. Production and isolation of 6 PP

6-Pentyl- α -pyrone was isolated from *Trichoderma atroviride* strain P1 according to the method previously described [18]. Briefly, five 10 mm $\varnothing$ plugs, obtained from actively growing P1 cultures, were injected into 5 L conical flasks which contained 2.5 L of potato dextrose broth (PDB, HI-MEDIA, Pvt. Ltd., Mumbai, India). Two static cultures were then incubated, at 25 °C, for 30 days, and subsequently filtered under vacuum by a filter paper (Miracloth).

Culture filtrates (5L) were subjected to extraction using ethyl acetate (EtOAc, VWR International, LLC, Milan, Italy). The organic phase was first dried with sodium sulfate anhydrous (Na₂SO₄, VWR International), then evaporated under vacuum at 37 °C. Purification of 6 PP was achieved by flash column chromatography (with stationary phase containing silica gel, 100 g) using an elution gradient based on petroleum ether (Carlo Erba, Milan, Italy) and EtOAc (0–100% EtOAc). Characterization of purified 6 PP was achieved by gas chromatography-mass spectrometry (GC-MS) analysis according to the method previously reported [25].

2.2. Cell cultures and virus infection

The cultures of A72 and CRFK cells were carried out using Dulbecco's modified Eagle's minimal essential medium (DMEM), containing 1% penicillin/streptomycin, 10% fetal bovine serum (FBS), and incubated at 37 °C and 5% CO₂ [26,27]. The following Alphacoronavirus reference strain CCoV-IIa S378 (GenBank accession number KC175341) was utilized in the study. Virus stocks growth was obtained in A72 cells. A72 and CRFK were used for virus titration [28,29].

6 PP was solubilized in the vehicle dimethyl-sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MI, USA) at 0.1 g/mL concentration (stock solution). To obtain different concentrations, the stock solution was then diluted in DMEM. DMSO in DMEM (0.1% v/v) was vehicle control.

In order to obtain four groups: CCoV uninfected or infected cells, 6 PP treated infected and uninfected cells, A72 and CRFK cells, in monolayers, were infected or not with CCoV, at different multiplicity of infection (MOI), and were treated or not with 6 PP at different concentrations (0.001, 0.01, 0.1, 1 and 10 μ g/mL). One hour post adsorption at 37 °C, both cell lines were incubated, then processed at various times of infection.

2.3. Cell viability

A72 and CRFK cells, exposed to 6 PP, infected with CCoV, at MOI of 5 for A72, and at MOI of 1 for CRFK.

Cell viability, at 48 h post treatment, was assessed by Trypan Blue (TB) (Sigma-Aldrich) exclusion test, and it was obtained as a percentage of viable cells vs. control [30,31]. Three independent tests were assessed in duplicate, and results are exhibited as the mean $\pm$ standard deviation (S.D.). The calculation of the IC₅₀ in A72 and CRFK cells was assessed by IC₅₀ Calculator|AAT Bioquest (<https://www.aatbio.com/tools/ic50-calculator>).

2.4. Cell proliferation

In order to check cell proliferation, MTT assay was carried out [32–35]. In a first test of cell viability, five concentrations of 6 PP (0.001, 0.01, 0.1, 1 and 10 μ g/mL) were checked at 48 h p. i., and then we have chosen 0.1 μ g/mL 6 PP for MTT test. In brief, both A72 and CRFK cells were infected with CCoV (MOI 5 and MOI 1, respectively), in the presence or not of 6 PP (0.1 μ g/mL) were tested by MTT test at 48 h p. i.

2.5. Observation of cell morphology

We have performed Giemsa staining and acridine orange staining to study cell morphology [14,32] at 24 h and 48 h of infection on A72 and CRFK, treated with 6 PP, and infected with CCoV at MOI of 5 and at MOI of 1, respectively.

2.6. Immunofluorescence staining

A72 and CRFK cells, treated or not with 6 PP were infected CCoV at MOI of 0.05 and 1, respectively. Then, we assessed immunofluorescence staining for detecting AhR and viral N protein at 24 h post infection (p.i.) for CRFK cells and after 48 h of infection for A72 cells [16,36].

2.7. Virus production

Monolayers of A72 and CRFK cells, treated or not with 6 PP, were infected or not with CCoV, at MOI 5 for A72 and at MOI 10 for CRFK, and incubated at 37 °C. Real-time PCR was used for CCoV quantification in A72 cells [14,22]. In CRFK cells, the virus titer was tested by 50% tissue culture infective dose (TCID₅₀) titration [29,37]. Moreover, at 48 h p. i., cytopathic effect (CPE), produced by virus, was assessed by light microscope.

2.7.1. Viral nucleic acids extraction procedure

The extraction of nucleic acid was obtained as previously described [38].

2.7.2. CCoV viral load quantification by real-time reverse transcription PCR (RT-qPCR)

RT-qPCR was used for quantifying CCoV during infection in A72 cells [39]. Then, the quantification of CCoV was carried by the mean of a standard curve, making serial dilutions (from 3.5×10^9 to 3.5×10^2 TCID₅₀/mL) of the quantified canine coronavirus viral stock and plotting threshold cycle number (Ct) against the TCID₅₀/mL on a log scale [14,16,17,22,35].

2.8. Statistical analysis

GraphPad InStat software Version 3.00 for Windows 95 (GraphPad Software, San Diego, CA, USA) was employed to perform statistical analysis. To evaluate significant differences among samples, one-way ANOVA, using Tukey's post hoc test and Student's t-test at $p < 0.05$, were utilized.

3. Results

3.1. 6 Pentyl- α -pyrone (6 PP) characterization

6 PP (Fig. 1) was obtained by isolation from the strain P1 of *T. atroviride*. Characterization of 6 PP was performed by GC-MS analysis and comparison with an authentic standard identified in our labs. Moreover, mass spectrum of 6 PP was compared to those included in NIST 20 (National Institute of Standards and Technology) collection of known compounds. Chromatographic profile was also compared with pure commercial standard (Merck KGaA, Darmstadt, Germany). Chemical structure and fragmentation mass spectrum of 6 PP are reported in Fig. 1.

3.2. 6 PP enhances both cell viability and Proliferation in CCoV infection

To explore the effect of 6 PP in CCoV infection using A72 and CRFK cells, cell viability (% control) was assessed performing TB.

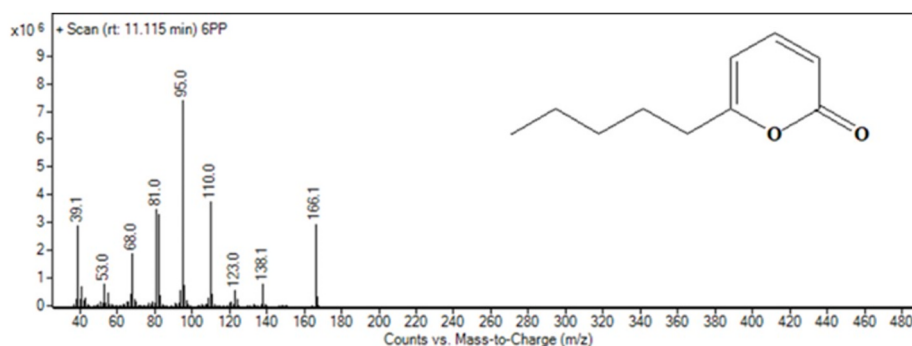


Fig. 1. Chemical structure of 6 PP and fragmentation mass spectrum obtained by GC-MS ESI analysis.

Hence, the effects of five different concentrations of 6 PP (0.01, 0.1, 1 and 10 $\mu\text{g/mL}$) on A72 and CRFK uninfected cells were examined, and a dose–response curve was developed (Fig. 2a). In both cell lines, the 6 PP response curve did not show a dose-dependency. After 48 h of treatment, IC_{50} was found with 0.9970 $\mu\text{g/mL}$ 6 PP in A72 cells, and with 0.7887 $\mu\text{g/mL}$ 6 PP in CRFK cells (Fig. 2a). 6 PP at 0.001 and 0.1 $\mu\text{g/mL}$ induced no significant changes in A72 and CRFK cell viability ($p > 0.05$); whereas cell toxicity was observed using 6 PP at 0.01, 1 and 10 $\mu\text{g/mL}$ in both cell lines (Fig. 2a and b). Interestingly, the cell toxicity at the lower concentration (0.01 $\mu\text{g/mL}$) of 6 PP induced some cytotoxicity while the higher (0.1 $\mu\text{g/mL}$) did not. A similar trend was detected in A72 and in bovine cells (MDBK) exposed to funicone-like compounds, other SMs [16,17,22]. The lack of a dose-dependent response has been noticed for different compounds which generally bind nuclear receptors [40].

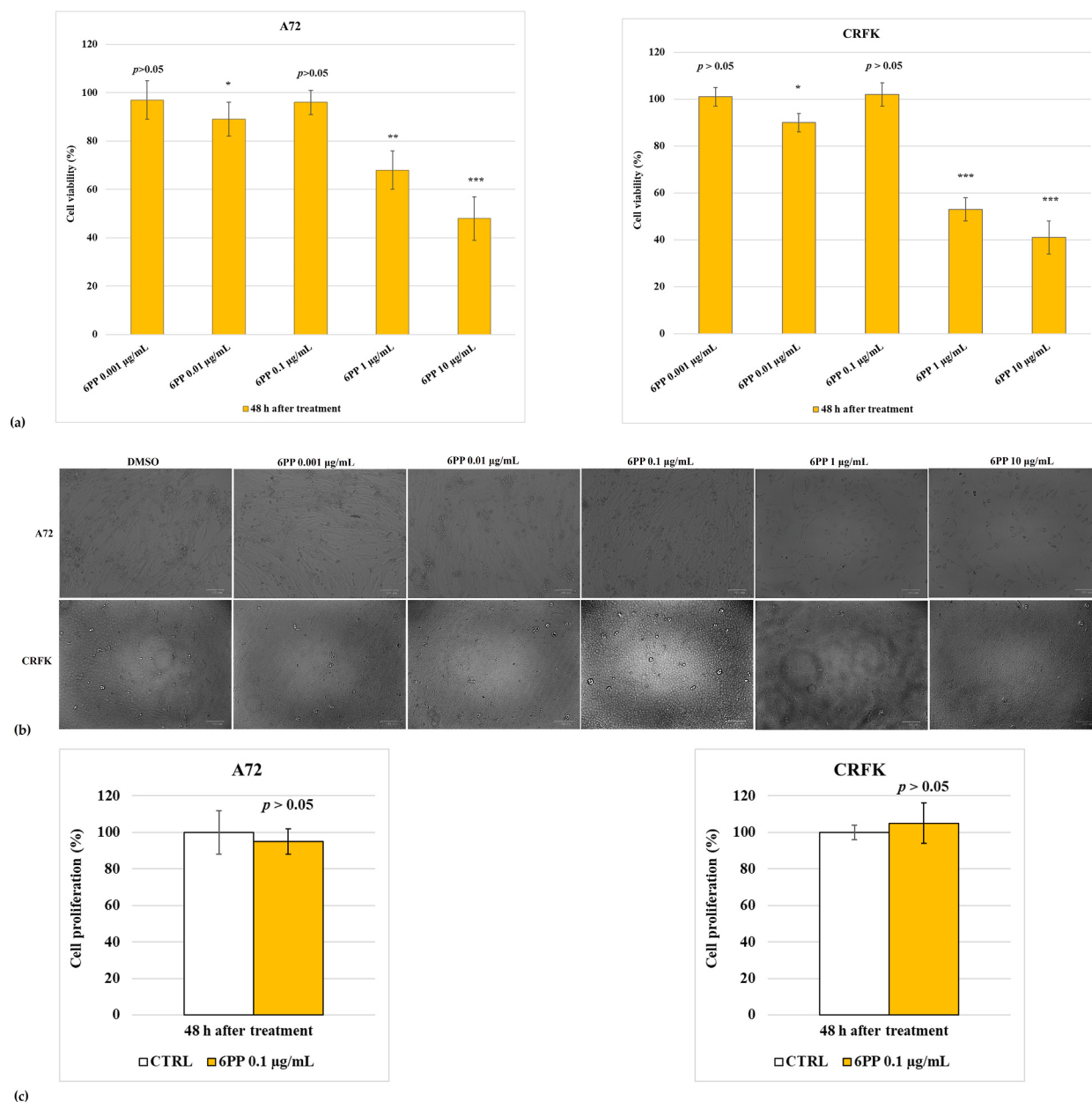


Fig. 2. Identification of CC_{50} of 6 PP at different concentrations and elaboration of dose–response curve in A72 and CRFK cells. (a) Dose–response curve of A72 and CRFK cells exposed to DMSO or 6 PP at different concentrations (0.01, 0.1, 1 and 10 $\mu\text{g/mL}$). After 48 h from treatment, cell viability (% control) was assessed using TB staining. (b) A72 and CRFK cells treated with control (DMSO) or 6 PP at different concentrations and observed by light microscope. Scale bar 100 μm . (c) Dose–response curve of A72 and CRFK cells treated with 6 PP (0.1 $\mu\text{g/mL}$) for 48 h and analysed by MTT assay. To indicate significant differences between control vehicle (DMSO) and 6 PP-treated cells probability p was used. ** $p < 0.01$ and *** $p < 0.001$ in A72 cells; * $p < 0.05$ and *** $p < 0.001$ in CRFK cells.

To further check that 6 PP at the concentration of 0.1 $\mu\text{g/mL}$ was biocompatible as well as not cytotoxic, MTT assay was performed in A72 and CRFK cells. After 48 h of treatment, 6 PP at 0.1 $\mu\text{g/mL}$ vs. DMSO control groups caused no substantial ($p > 0.05$) alterations in the action of dehydrogenase in mitochondria of both cell lines (Fig. 2c).

After that, CCoV-A72 and CCoV-CRHK infected cells, at MOI of 5 and at MOI of 1, respectively, were exposed to no cytotoxic doses of 6 PP (0.001 and 0.1 $\mu\text{g/mL}$). During infection, we found a significant reduction in both CCoV-infected cell lines, treating them with the non-toxic dose of 0.1 $\mu\text{g/mL}$ 6 PP (Fig. 3a), which was chosen to be utilized for further experiments. At the concentration of 0.1 $\mu\text{g/mL}$, 6 PP also enhanced cell proliferation ($p < 0.001$) in A72 cells as well as ($p < 0.01$) in CRFK cells (Fig. 3c).

3.3. 6 PP Provokes a reduction in signs of morphological cell death in CCoV infection

To investigate the effects of 6 PP on cell morphology following CCoV infection, light microscopy examination of A72 and CRFK cells was achieved by using Giemsa staining and acridine orange staining, which are known methods to detect morphological features of cell death [41,42]. Herein, at 48 h p. i., in Giemsa stained CCoV-infected cells, 6 PP decreases signs of morphological cell death, while in unexposed infected cells, an improvement of spaces between the cells because of lack of cell adhesion to plate was detected in both cell

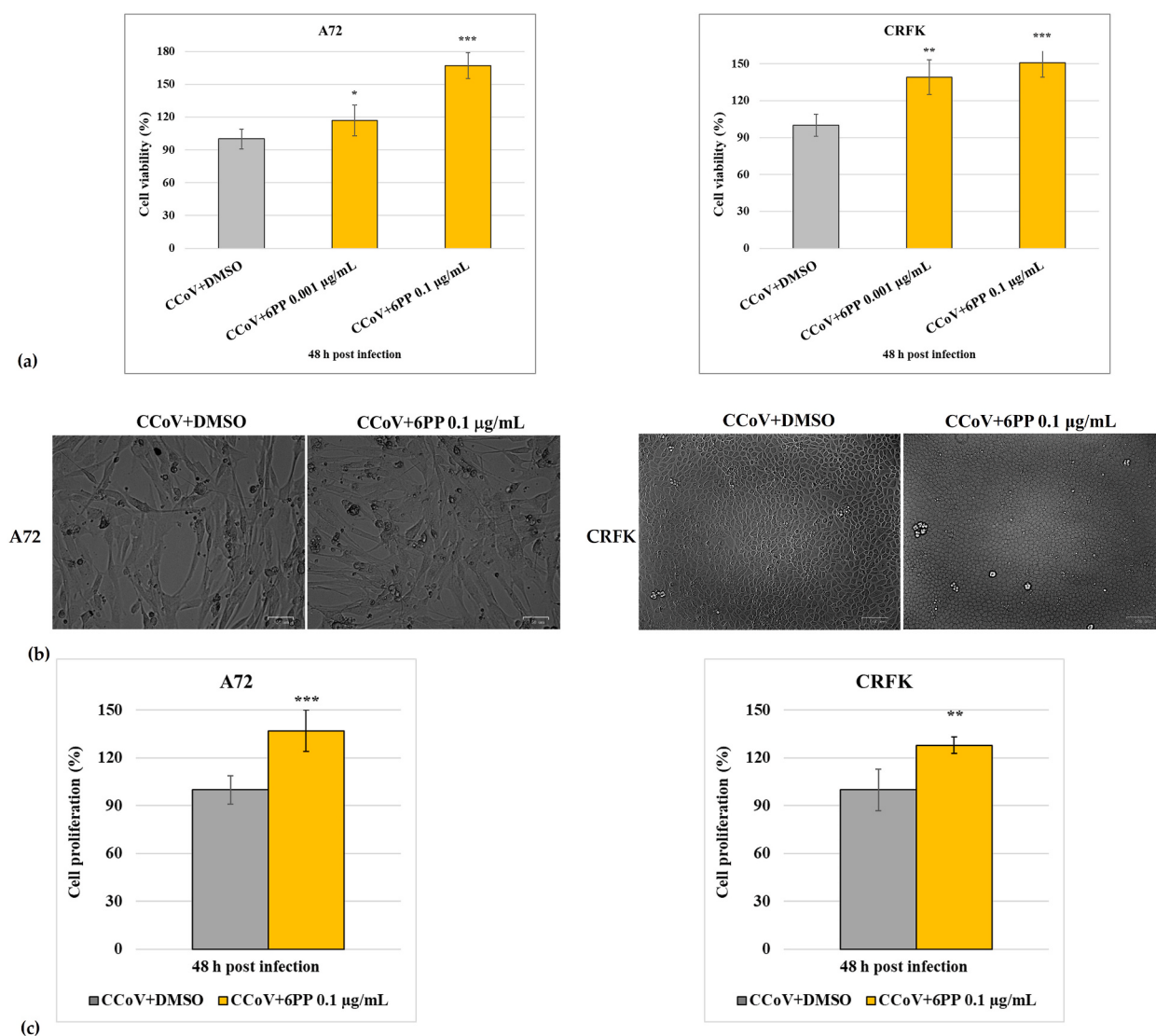


Fig. 3. 6 PP enhances cell viability during CCoV infection. (a) Dose–response curve of A72 and CRFK cells infected with CCoV and treated with 6 PP (0.1 $\mu\text{g/mL}$). At 48 h of exposure, to determine cell viability, TB staining was used, and cells were counted by light microscope. (b) Cells infected with CCoV, and treated or untreated with 6 PP (0.1 $\mu\text{g/mL}$). At 48 h p. i., cells were observed by light microscope. Scale bar 50 μm . (c) Dose–response curve of A72 and CRFK cells infected with CCoV, exposed to 6 PP (0.1 $\mu\text{g/mL}$) for 48 h and analysed by MTT assay. Significant differences between CCoV and CCoV+6 PP-treated cells are pointed by probability p . *** $p < 0.001$ and ** $p < 0.01$.

lines (Fig. 4a and b), in which pyknosis, chromatin condensation (Fig. 4, circle), and cell shrinkage (Fig. 4, arrowhead) were found. All these features of morphological cell death were remarkably reduced by 6 PP in infected cells (Fig. 4).

3.4. 6 PP diminishes virus yield in CCoV infection

In order to examine the activity of 6 PP in CCoV infection, RT-qPCR as well as TCID₅₀ titration were performed, and CPE was examined.

3.4.1. Real time PCR quantification of CCoV during infection in A72 cells

Following infection in A72 cells, RT-qPCR was achieved to assess the effect of 6 PP on CCoV yield. Viral load (TCID₅₀/mL) in the presence or not of 6 PP was analysed at various times post infection (0, 1, 12, 24, 48, and 72 h) by running all the samples (in triplicate) with the standard curve protocol. Virus titer in each sample was then calculated using the standard curve equation using from the C_t values (Fig. 5). In the presence of 6 PP, a significant decrease in virus titer at 48 h and 72 h p. i. ($p < 0.001$ and $p < 0.05$, respectively) (Fig. 6a) was found.

3.4.2. CCoV titration following infection in CRFK cells

Following infection in CRFK cells for 24 h, in the presence of 6 PP, a reduction of virus yield (TCID₅₀) was found (Fig. 6b).

3.4.3. CPE evaluation following CCoV infection in A72 and CRFK cells

Additionally, after 48 h of infection, in A72 cells marks of CPE, detected through syncytia (Fig. 4, arrowhead) and detachment of cells from culture plates (Figs. 3b and 4, arrow), were wide in infected groups [14,22]. In CRFK cells, CCoV developed a typical CPE [43], with granular (Fig. 4, arrow) and small multinucleated cells (Figs. 3b and 4, arrowhead). In both cell lines, virus yield was markedly reduced by the presence of 6 PP (Figs. 3 and 4).

Overall, 6 PP strongly decreased virus yield and CPE during CCoV infection in both A72 and CRFK cells.

3.5. 6 PP decreased the expression of AhR and NP in CCoV infection

To study whether 6 PP affected the expression of AhR and NP in CCoV infection, immunofluorescence staining was made. Firstly, A72 and CRFK cells were treated with 6 PP 0.1 µg/mL and, at 24 h post treatment a decrease of AhR expression, compared to DMSO, was noted in both cell lines used (Fig. 7a–c).

Both A72 and CRFK cells were then subjected to CCoV infection and exposure to 6 PP 0.1 µg/mL. At 24 h p. i. for CRFK, and at 48 h p. i. for A72, results of immunofluorescence staining demonstrated a reduction of AhR in infected cells treated with 6 PP (8a). Moreover, in both cell lines, a significant reduction of the viral protein NP in 6 PP infected groups was found (Fig. 8a). Integrated measurement of density fluorescence confirmed these results obtained (Fig. 8b–e).

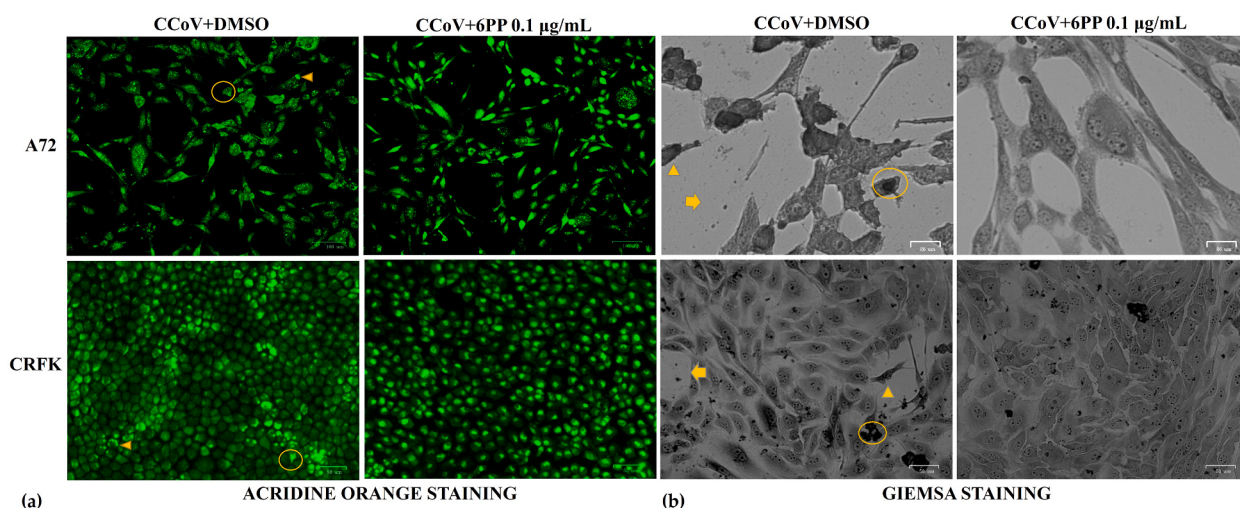


Fig. 4. 6 PP decreases features of morphological cell death during CCoV infection. A72 cells, infected with CCoV, in the presence or absence of 6 PP, were stained by (a): acridine orange and observed by fluorescence microscope after 24 h of infection; (b): or by Giemsa and observed by light microscope after 48 h of infection. In unexposed infected cells, photomicrographs indicate an enhancement of intercellular spaces because of detachment from culture plate (arrow). Furthermore, morphological signs of cell death, like pyknosis and chromatin condensation (circle), as well as cell shrinkage (arrowhead), were identified in A72 as well as in CRFK cells. These signs of morphological cell death were noticeably reduced by the presence of 6 PP in infected cells. Scale bar 100 µm and 25 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

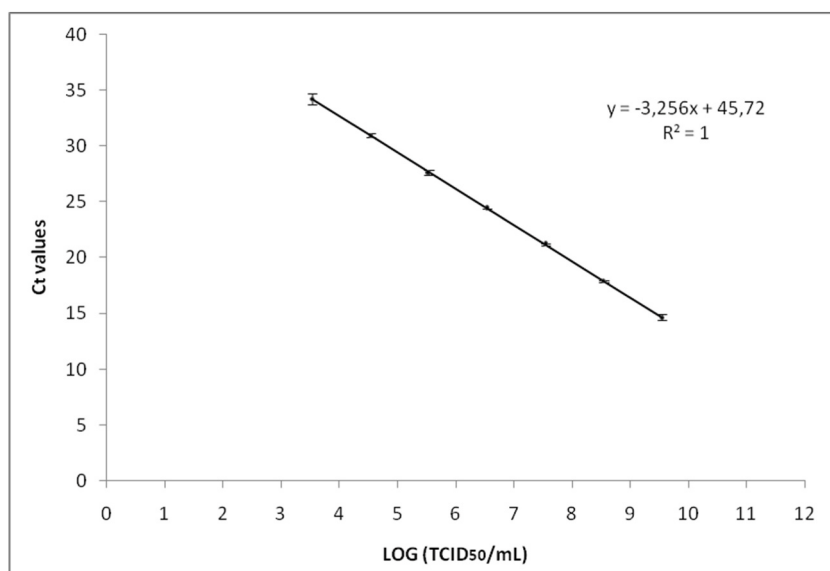


Fig. 5. Standard curve obtained by qRT-PCR analyzing serial dilutions (from 3.5×10^9 to 3.5×10^2 TCID₅₀/mL) of canine coronavirus viral stock and plotting the C_t obtained against TCID₅₀/mL. C_t values are presented as the meaning of six replicates for each dilution $\pm$ SD.

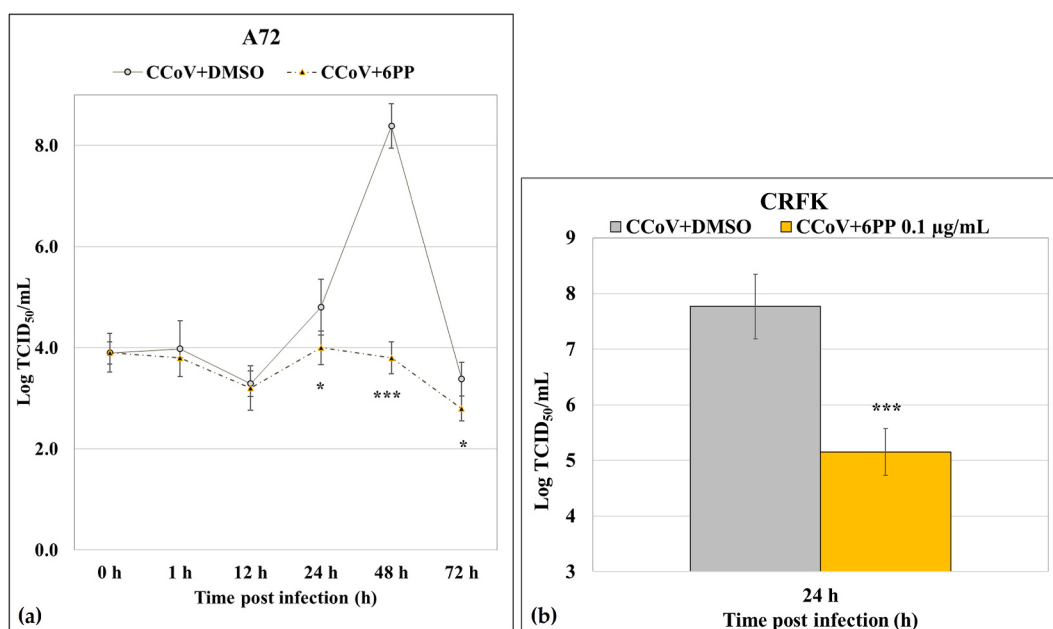


Fig. 6. 6 PP diminishes virus titer during CCoV infection. (a) For viral growth curves, following CCoV infection in A72 cells, exposed or not to 6 PP, virus titers were evaluated by RT-qPCR, at indicated times post infection. (b) During CCoV infection in CRFK, virus titer was assessed by TCID₅₀. Comparing CCoV-infected cells to 6 PP-treated infected cells, significant differences were indicated by probability p . *** $p < 0.001$ and * $p < 0.05$ in A72 cells. *** $p < 0.001$ in CRFK cells.

Results shown in Fig. 7 indicated that 6 PP reduced the expression of NP and induced a markable down-regulation of AhR in A72 and in CRFK cells.

4. Discussion

Coronaviruses, like CCoV, can rapidly mutate their genome, leading to the formation of new variants with increased infectivity and transmissibility, sometimes able to cross interspecies barriers. For example, recombinant canine–feline–porcine strains were isolated

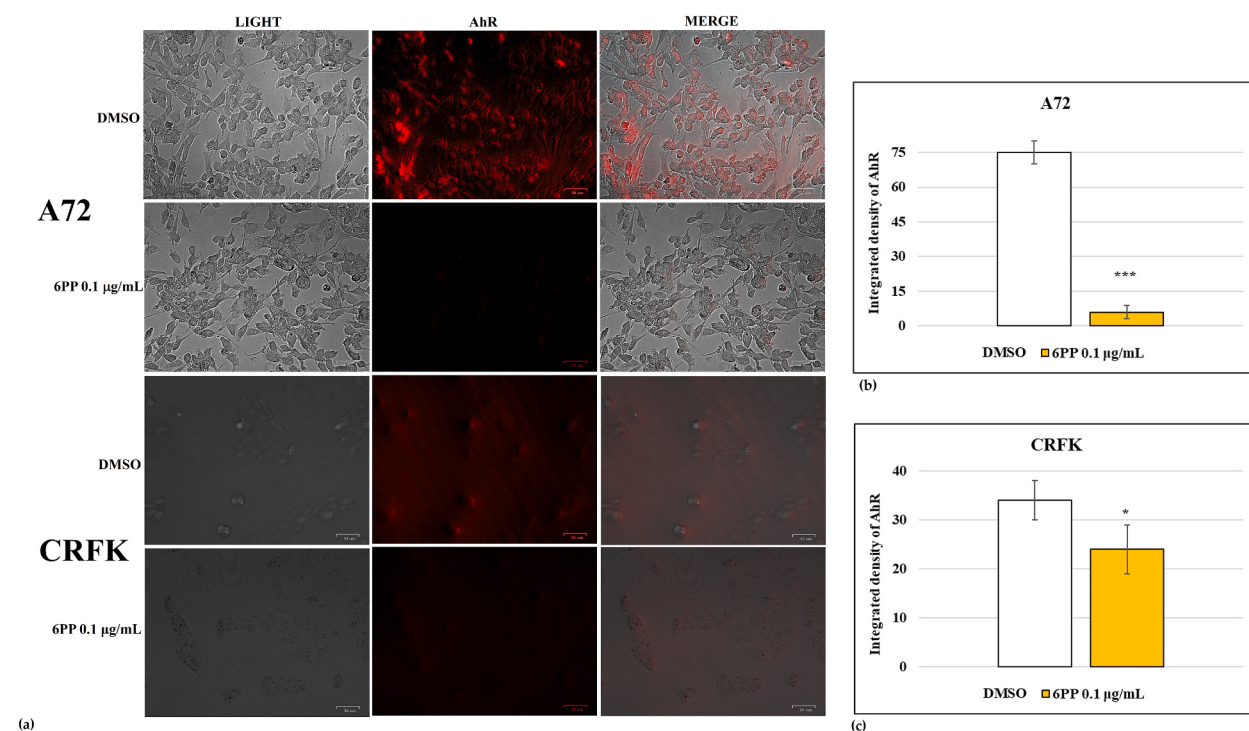


Fig. 7. 6 PP reduces the expression of AhR in A72 and in CRFK cells. A72 and CRFK cells were exposed to 6 PP, and after 24 h of treatment, immunofluorescence staining for AhR was carried out. (a) In 6 PP exposed cells a significant down-regulation of AhR expression was observed. Scale bar 50 µm. (b) Bars represent the mean ratio obtained from the integration of density (product of the area and mean intensity of fluorescence) of the expression of AhR in A72 and in (c) CRFK treated cells calculated by ImageJ. Error bars correspond to standard deviation measurement. Significant differences between 6 PP-treated cells and DMSO are indicated by probability p . *** $p < 0.001$ in A72 cells; * $p < 0.05$ in CRFK cells.

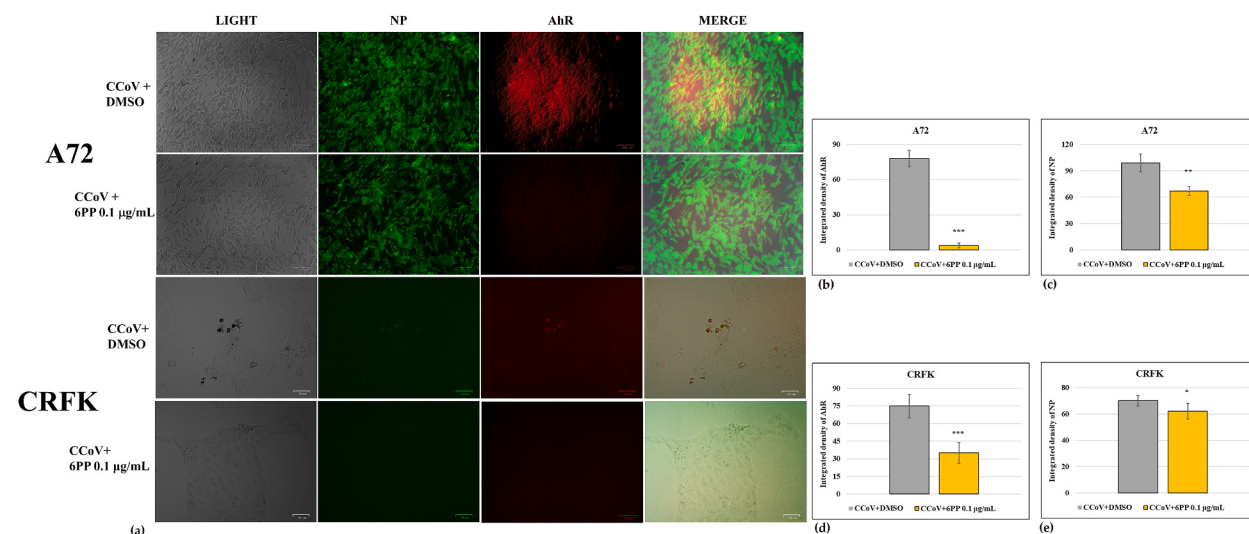


Fig. 8. 6 PP down-regulates the expression of AhR and NP following CCoV infection in A72 and in CRFK cells. A72 and CRFK cells, infected with CCoV, in the presence or not of 6 PP, and at 48 h and 24 h p. i., respectively, immunofluorescence staining for AhR (red fluorescence) and NP (green fluorescence) was carried out. (a) 6 PP induced a significant down-regulation of the expression of AhR and reduced the expression of NP during CCoV infection in both cell lines. Scale bar 50 µm. (b) Bars are the mean ratio generated from the integrated density of the AhR expression in A72 cells or in (d) CRFK, and (c) of the NP expression in A72 and (e) in CRFK, evaluated by ImageJ. Error bars are standard deviation measurement. Significant differences between unexposed infected groups and 6 PP-treated infected cells are indicated by probability p . *** $p < 0.001$ for AhR in both cell lines; and * $p < 0.05$ for NP in A72 and CRFK cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

from dogs and humans in various countries of the world [1,6,44,45]. The emergence of remarkably virulent variants needs for continuous monitoring of CCoV, and also suggests the necessity for discovering novel antiviral agents. So far, it has been proved that the anti-inflammatory drug indomethacin has effective antiviral features to fight against CCoV infection, through action both on viral replication and block of viral RNA synthesis [46–48]. Recent advances show as a recombinant adenovirus, which expresses canine interferon lambda 3, has an antiviral activity against CCoV [49]. Due to high toxicity of the conventional antiviral drugs, numerous natural substances are being recently tested to conceive new therapeutics against viral infections. On this regard, natural products, such as bufotenine, an alkaloid obtained by skin secretions of amphibians and plant extracts, did not show potential antiviral activity against CCoV infection [50].

Due to their attitude to live in extreme conditions for other organisms, fungi produce different secondary metabolites belonged to several chemical classes such as alkaloids, peptides, quinones, terpenes, acyclic compounds, steroids, as well as pyrones. They have been investigated for different uses, from antibiotics to fungicides, plant growth regulators and hormones. In addition, previous studies on compounds derived from fungi have highlighted potential antiviral properties [11–13,51]. Herein, during CCoV infection, the harmless concentration of 0.1 µg/mL 6 PP significantly increased cell viability, cell proliferation and reduced the typical marks of death in cell morphology, both in A72 and in CRFK [41–43,52]. In addition, 6 PP produced a considerable lessening in virus yield, together with a diminishment of NP expression and a strong reduction of AhR expression, according to previous reports about SMs. Indeed, funicones, like OMF, as well as funicones-like PEN and PS, can reduce virus yield during *in vitro* infection by CCoV. They are able to induce a significant reducing in virus yield, downregulation in the expression of NP, and a relevant reduction in the expression of AhR [14,17,22]. AhR is a well-known transcription factor that can be activated by various ligands, both endogenous (bilirubin, biliverdin, tryptophan metabolites) and exogenous (environmental pollutants and microbial metabolites) [53,54]. Several studies indicate that inflammatory response as well as immunity are modulated by AhR, emphasizing its involvement in the host response to alpha and beta-CoVs as well as to no-CoVs (Zika and Dengue). For example, the pharmacological block of AhR by chemicals (CH223191, FICZ) as well as by natural products (fungal SMs, bioflavonoids) that causes anti-CoVs activity *in vitro*, proves the relevance of AhR in CoVs infection, as well as indicates AhR as a druggable target for introducing new antivirals [14,17,22,55–65]. Hypothetically, due to the chemical structure of 6 PP (Fig. 1), its aromatic nature may explain the interaction with AhR, as detected for OMF and funicones-like compounds during CCoV *in vitro* infection [14,17]. These findings highlight the importance to investigate the modulation of AhR signaling pathway to fight CoVs off infection. Herein, we identified valid *in vitro* models to select new potential therapeutics against CoVs, employing animal CoV, such as a reference strain of CCoV, not pathogen for humans.

In conclusion, due to the diversity of their chemical structures and biological properties, fungal SMs are able to inhibit the replication of CoVs. Thus, this study findings align with an important goal indicated in the 2030 Agenda of United Nations about good health and well-being.

Data availability

Data will be made available on request.

CRediT authorship contribution statement

Claudia Cerracchio: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation. **Luca Del Sorbo:** Investigation. **Francesco Serra:** Methodology, Investigation, Data curation. **Alessia Staropoli:** Writing – review & editing, Writing – original draft, Methodology. **Maria Grazia Amoroso:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization. **Francesco Vinale:** Writing – review & editing, Writing – original draft, Conceptualization. **Filomena Fiorito:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

No competing financial interests were declared by authors. No role for designing of the study, collecting, and analyzing of data, writing of the manuscript was made by funders.

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