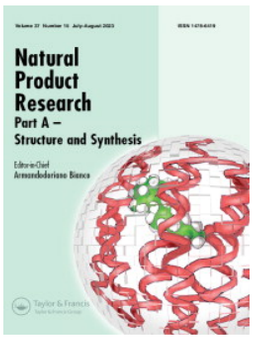

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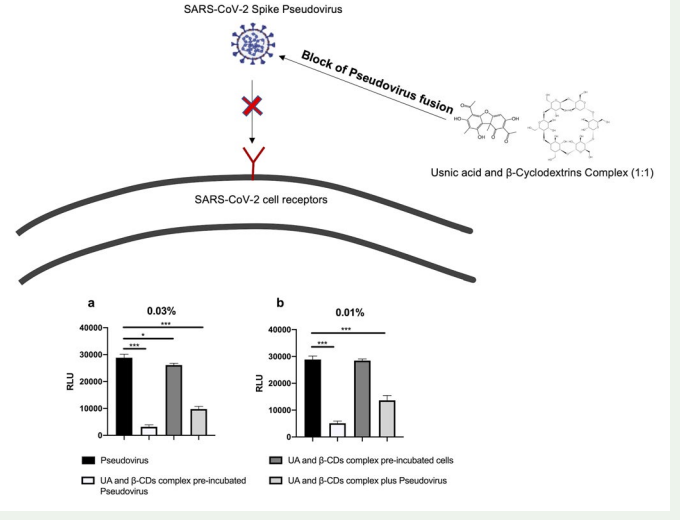
Neutralizing activity of Usnic acid and β-cyclodextrins complex against SARS-CoV-2 spike pseudovirus

Luigi Rosa[a], Antimo Cutone[b], Rebecca Galla[c,d], Francesca Uberti[c] and Piera Valenti[a]

[a]Department of Public Health and Infectious Diseases, University of Rome La Sapienza, Rome, Italy; [b]Department of Biosciences and Territory, University of Molise, Pesche, Italy; [c]Laboratory of Physiology, Department of Translational Medicine, University of Piemonte Orientale, Novara, Italy; [d]Noivita srls, spin-off, Novara, Italy

ABSTRACT

The rapid spread of SARS-CoV-2 and its infection severity require an urgent development of antiviral agents. In this respect, Usnic acid (UA), a natural dibenzofuran derivative, exerts antiviral activity against several viruses, though presenting very low solubility and high cytotoxicity. Here, UA was complexed with β-cyclodextrins (β-CDs), a pharmaceutical excipient used to improve drug solubility. The cytotoxic activity, tested on Vero E6 cells, revealed no effect for β-CDs alone whereas significant cytotoxicity for the UA/β-CDs complex was recorded at concentrations ≥ 0.05%. The neutralizing activity towards the fusion of SARS-CoV-2 Spike Pseudovirus showed no effects for β-CDs alone whereas the UA/β-CDs complex, when pre-incubated with the viral particles, efficiently inhibited the Pseudoviral fusion of about 90 and 82% at non-cytotoxic concentrations of 0.03 and 0.01%, respectively. In conclusion, although further evidences are needed to clarify the exact inhibition mechanism, UA/β-CDs complex could be useful in SARS-CoV-2 infection.



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CONTACT Piera Valenti piera.valenti@uniroma1.it
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1. Introduction

SARS-CoV-2 is a ssRNA virus with a genome encoding structural, nonstructural, and accessory proteins (Harrison et al. 2020). The structural proteins include spike (S) glycoprotein which mediates viral entry through host specific receptor ACE2 (Wang et al. 2020). In addition to the efficacy of vaccines, there is an urgent requirement of drugs able to interfere, without side effects, with the viral entry into the cells of the nasal and oral cavity to avoid infection and transmission. Usnic acid (UA) [2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H,9bH)-dibenzo-furandione], a dibenzofuran derivative biosynthesized by several species of lichens, such as *Usnea florida* (L.) F.H. Wigg., *Usnea barbata* (L.) F.H. Wigg. and *Usnea longissima* Ach. (Ingólfsdóttir 2002; Calcott et al. 2018), possesses different biological activities, including antibacterial, antifungal, antiparasitic, antiviral, immunostimulatory, antiinflammatory and antitumor (Araujo et al. 2015; Wang et al. 2022). UA, characterized as a yellow pigmented solid substance, is naturally found in two enantiomeric forms, such as (–) levogyrous and (+) dextrogyrous, depending on the projection of the angular methyl group. UA gets its acidic feature from the phenolic hydroxyl groups: the enolic group 3-OH shows the strongest acidic character (pKa 4.4) (Ingólfsdóttir 2002). UA isomers exhibit antiviral effects against Herpes simplex type 1 and polio type 1 viruses (Perry et al. 1999) as well as the pandemic influenza virus A(H1N1) (Sokolov et al. 2012). In 100 female patients suffering from genital papillomavirus, the intravaginal treatment of UA and zinc sulfate improved the re-epithelization of lesions and the recurrence of infection (Scirpa et al. 1999). However, due to 'triketone' moiety cytotoxicity, the Zn–UA treatment exerted local irritant effects in 8% of the patients (Kristmundsdóttir et al. 2005; Jin et al. 2013; Luzina and Salakhutdinov 2018). Recent investigation on cytotoxicity mechanism showed that UA causes cell cycle dysregulation, DNA damage, oxidative stress, and activation of Nrf2 signaling pathway (Chen et al. 2017). Another issue of UA is its low solubility in water, around 0.06 mg/mL (Nikolić et al. 2013). For this purpose, the use of a pharmacologically accessible form must be considered a priority to better address the balance of benefit/adverse effects. In this respect, β-cyclodextrins (β-CDs) could be a great candidate to improve the UA solubility (Nikolić et al. 2013). β-CDs are a pharmaceutical excipient able to improve the solubility of various drugs through the formation of water-soluble drug–CDs complexes. Toxicological studies have shown that orally administered β-CDs are non-toxic because of their low absorption into the circulation (Arima et al. 2011). Here, we investigated the *in vitro* cytotoxic activity of β-CDs alone or complexed with UA on Vero E6 cells. Successively, the non-cytotoxic concentrations have been tested to investigate, for the first time, the neutralizing activity of the β-CDs alone and in combination with UA against a Pseudovirus decorated with the S glycoprotein of SARS-CoV-2.

2. Results and discussion

2.1. Cytotoxic activity of β-cyclodextrins alone and Usnic acid and β-cyclodextrins complex on Vero E6 cells

All physico-chemical features of UA are reported in Table S1. Briefly, UA, extracted from *Usnea Longissima*, appears as a yellow crystalline powder with purity corresponding to 98.7% and presenting a specific rotation of +502° (Table S1).

Concerning the cytotoxicity assay, all the concentrations of the β-CDs alone (Table S2) were non-cytotoxic after either 8 and 24h of incubation with Vero E6 (Figure S1a and S1b, respectively). Of note, the cytotoxicity was considered significant for values of viability as lower than 70%, the threshold value to define a substance as cytotoxic (Cannella et al. 2019).

To preliminary explore the cytotoxic potential of the UA/β-CDs complex, different concentrations, ranging from 1 to 0.001% (Table S2), were tested.

Results reported in Table S3 indicated that: i) the UA/β-CDs complex was cytotoxic for Vero E6 cells at the concentrations of 1, 0.5, and 0.1% after 8 (cell viability < 55%) and 24h (cell viability < 42%) of incubation; ii) the concentration of 0.05% of the UA/β-CDs complex was at the threshold value of the cell viability (71%) at 8h, while at 24h of incubation the cell viability decreases (65%); iii) the concentration of 0.03% of the UA/β-CDs complex was non-cytotoxic, showing cell viability of 83 and 85% after 8 and 24h, respectively; iv) the concentrations of 0.01, 0.005 and 0.001% of the UA/β-CDs complex were non-cytotoxic for Vero E6 cells (cell viability > 90%) (Table S3).

To better set the range of cytotoxic vs non-cytotoxic concentrations, also in term of reproducibility and significance, the solutions of the UA/β-CDs complex at 0.05, 0.03 and 0.01%, representing the closest concentrations to the cytotoxic threshold, were tested by further independent experiments (Figure S2).

The results demonstrated that the UA/β-CDs complex at the concentration of 0.05% was cytotoxic for Vero E6 cells compared to the control after 8h (69.1 ± 3.1% vs 100%) and 24h (63.4 ± 2.7% vs 100%) (Figure S2a and S2b, respectively).

Conversely, the UA/β-CDs complex at the concentration of 0.03% was non-cytotoxic for the cell monolayers compared to the control after 8h (87.2 ± 3.8% vs 100%) and 24h (83.5 ± 0.9% vs 100%) (Figure S2a and S2b, respectively).

The cell viability with the UA/β-CDs complex at the concentration of 0.01% is similar to the control after 8h (94.5 ± 4.5% vs 100%) and 24 h (93.9 ± 2.7% vs 100%) (Figure S2a and S2b, respectively).

These results strongly encourage the use of β-CDs as cargo for UA to decrease its cytotoxicity, especially when compared to other carriers. Indeed, when UA was encapsulated with a copolymer of lactic and glycolic acid, the IC50 of encapsulated UA was 0.001% (Santos et al. 2005). Similarly, liposomes-loaded UA revealed a strong cytotoxic activity with a IC50 of about 0.002% (Lira et al. 2009).

2.2. Neutralizing activity of β-cyclodextrins alone and Usnic acid and β-cyclodextrins complex against SARS-CoV-2 Spike Pseudovirus

Any natural substance or drug, capable of inhibiting viral entry through a competitive binding with cell receptors and/or with viral component, such as S glycoprotein (Rosa et al. 2023; Cutone et al. 2022), can exert an antiviral action and act as a protective barrier against infections.

The neutralizing assay was carried out using SARS-CoV-2 S Pseudovirus, on Vero E6 cells, at multiplicity of infection (MOI) of 10, the optimal concentration to obtain an accurate luminescence test (Figure S3).

β-CDs alone, at all the concentrations tested (from 0.5 to 0.0005%), did not show any inhibitory effect (data not shown).

The inhibition of the Pseudovirus fusion by the UA/β-CDs complex at 0.03 and 0.01%, the highest concentrations non exerting cytotoxic activity, was tested according to the experimental scheme reported in supplementary material.

The results showed that the UA/β-CDs complex induced the highest neutralizing activity when pre-incubated with the Pseudovirus at a concentration of 0.03% (Figure S4a). When the complex is added at the moment of infection, its activity still appears significant even if at lower extent respect to the pre-incubation of the complex with the viral particles (Figure S4a). The inhibition of Pseudoviral fusion by the UA/β-CDs complex at the concentration of 0.03% significantly decreased when it was pre-incubated with the cells (Figure S4a).

The complex at the concentration of 0.01% showed the same inhibitory trend in all the experimental conditions, even if at lower extent (Figure S4b).

As reported by Nikolić et al. (2013), UA alone presents a very poor solubility (around 0.06 mg/mL), whereas the complex with β-CDs increases such solubility to 0.3 mg/mL (molar ratio 1:1). In our experiments, we have prepared the complex with a *w/w* ratio of 1:1, presenting a similar solubility of 0.5 mg/mL (Table S2). This increased solubility allows the UA to be applied at higher concentrations, without exerting significant cytotoxic activity (Figure S2), thus globally improving its neutralizing function against SARS-CoV-2 S decorated Pseudovirus (Figure S4).

Our results suggest that the UA/β-CDs complex does not bind to the host receptors exploited by the virus to enter into the host cells (Hu et al. 2021; Cutone et al. 2022). In the light of our results and of two recent works (Oh et al. 2022; Filimonov et al. 2022), the interaction occurring between UA and S glycoprotein could be considered as a possible mechanism of UA antiviral activity. *In silico* analysis showed that UA possesses high binding affinity (-6.02 kcal/mol) for the receptor binding domain (RBD) of S (Guthappa 2020). Therefore, it is possible to hypothesize that the UA/β-CDs complex interacts with S, the sole structure present on the surface of Pseudovirus able to mediate its fusion with host cell, however further studies are required to prove it.

At our knowledge, this is the first study demonstrating the efficacy of UA in complex with the β-CDs in inhibiting SARS-CoV-2 S-mediated cell fusion. Galla et al. (2023) have reported the efficacy of a new UA-based formulation (75 µg/mL UA + 150 µg/mL β-CD + 0.1% of tocopherol and tocotrienol + 1% hydroxypropyl methylcellulose) in hindering VSV-based SARS-CoV-2 pseudoviral entry of about 22.5% compared to the infected untreated control, indicating that it is also able to create a barrier that can trap viruses (Galla et al. 2023).

Overall, complexation of UA with β-CDs results in lowering cell cytotoxicity thus potentiating its antiviral activity against SARS-CoV-2 and their variants, similar to another antiviral compound as remdesivir (Oh et al. 2022).

3. Experimental

All details are provided in the supplementary material.

4. Conclusions

Even if further experiments are required to elucidate the molecular mechanism of action against SARS-CoV-2 and demonstrate the actual safety, the UA/β-CDs complex could be useful in SARS-CoV-2 infection.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Neutralizing Activity of Usnic Acid and β-Cyclodextrins Complex against SARS-CoV-2 Spike Pseudovirus

Luigi Rosa [a], Antimo Cutone [b], Rebecca Galla [c,d], Francesca Uberti [c] and Piera Valenti [a*]

[a] Department of Public Health and Infectious Diseases, University of Rome La Sapienza, 00185 Rome, Italy; [b] Department of Biosciences and Territory, University of Molise, 86090 Pesche, Italy; [c] Laboratory of Physiology, Department of Translational Medicine, University of Piemonte Orientale, 28100 Novara, Italy; [d] Noivita srls, spin-off, 28100 Novara, Italy

***** *Correspondence:*

Piera Valenti
Department of Public Health and Infectious Diseases, University of Rome La Sapienza, 00185 Rome, Italy
piera.valenti@uniroma1.it

The rapid spread of SARS-CoV-2 and its infection severity require an urgent development of antiviral agents. In this respect, Usnic acid (UA), a natural dibenzofuran derivative, exerts antiviral activity against several viruses, though presenting very low solubility and high cytotoxicity. Here, UA was complexed with β-cyclodextrins (β-CDs), a pharmaceutical excipient used to improve drug solubility.

The cytotoxic activity, tested on Vero E6 cells, revealed no effect for β-CDs alone whereas significant cytotoxicity for the UA/β-CDs complex was recorded at concentrations ≥ 0.05%. The neutralizing activity towards the fusion of SARS-CoV-2 Spike Pseudovirus showed no effects for β-CDs alone whereas the UA/β-CDs complex, when pre-incubated with the viral particles, efficiently inhibited the Pseudoviral fusion of about 90 and 82% at non-cytotoxic concentrations of 0.03 and 0.01%, respectively.

In conclusion, although further evidences are needed to clarify the exact inhibition mechanism, UA/β-CDs complex could be useful in SARS-CoV-2 infection.

Keywords: Usnic acid; Usnic acid and β-cyclodextrins complex; SARS-CoV-2; COVID-19; antiviral activity

SUPPLEMENTARY MATERIAL

3. Experimental

3.1. Characterization of Usnic acid and β-cyclodextrins

The UA (2,6-Diacetyl-7,9-dihydroxy-8,9b-dimethyldibenzo[b,d]furan -1,3(2H,9bH)-dione) was produced by Zhejiang Yixin Pharmaceutical Co., Ltd. (China) and supplied by Vivatis Pharma Italia s.r.l. The characteristics of (+)-UA, according to certificate of analysis, are reported in Table S1. The β-CDs, produced and supplied by Vivatis Pharma Italia s.r.l. (Code: 0598; Batch N.: 181204), appear as a white crystalline powder, odorless, slightly sweet, almost insoluble in methanol, ethanol, propanol or ether and soluble in water. The β-CDs derived from enzymatic conversion from starch and possess a specific optical rotation ranging from +160° to +164°. Their purity corresponded to 98% (HPLC method) with traces of heavy metals ≤ 1.0 ppm.

3.2. Preparation of the Usnic acid and β-cyclodextrins complex

The UA/β-CDs complex (1:1 *w/w*) were kindly supplied by Vivatis Pharma Italia s.r.l. In particular, dry extract of UA is selectively complexed with β-CDs to obtain an inclusion compound. The inclusion compound was obtained by a co-precipitation of (+)-UA and β-CDs, as reported by Nikolić et al. (2013). In brief, β-CDs were initially dissolved in water and, subsequently, the dry extract of UA was added to the solution under stirring. In the presence of appropriate concentration of β-CDs in water, precipitation of the inclusion compound began as the complexation reaction of the UA by β-CDs progressively proceed. The inclusion compound was collected after evaporation of the solution in a vacuum evaporator and, then, dried in a desiccator.

3.3. Solubilization of the β-cyclodextrins alone and the Usnic acid and β-cyclodextrins complex

Solubility assays of the β-CDs alone and of the UA/β-CDs complex (1:1 *w/w*) were performed by dissolving the compounds at different concentrations (ranging from 0.5 to 0.0005% for β-

CDs alone and 1 to 0.001% for the complex) in both sterile distilled water and cell culture medium D-MEM (Corning, Italy). The solubility of UA alone cannot be performed due to its poor solubility (Table S1).

For an excellent dispersion of the β-CDs alone and of the UA/β-CDs complex in D-MEM, an energetic mixing was required if the solution forms a visible precipitate. To further improve solubility, the different solutions have been immerged in water at 50°C for 20 min.

The β-CDs alone in D-MEM at the concentrations of 0.5, 0.25, 0.05, 0.025, 0.015, 0.005, 0.0025 and 0.0005% were completely dissolved. On the contrary, the UA/β-CDs complex solubilized in D-MEM at the concentrations of 1%, 0.5% and 0.1% showed a quantity of precipitate which is proportional to the concentration of the compound (Table S2). Therefore, for these latter solutions, the supernatants were employed for the cytotoxicity tests. For solutions of the UA/β-CDs complex solubilized in D-MEM at concentrations ≤ 0.05%, no precipitate was observed, hence, an aliquot of each solution was used for the cytotoxicity test.

Since identical solubility was observed for both β-CDs alone and the UA/β-CDs complex in distilled water and D-MEM, in vitro experiments were carried out by solubilizing the substances in D-MEM to avoid putative cell monolayer damage and maintain the same cell viability exerted in the absence of the compounds. The distilled water is known to compromise cell viability.

3.4. Vero E6 cells and SARS-CoV-2 Spike Pseudovirus

The African green monkey kidney–derived Vero E6 was purchased from American Type Culture Collection (ATCC, USA). Vero E6 cells were cultured in D-MEM supplemented with 1% of L-glutamine, 10% of fetal bovine serum (FBS) (Euroclone, Italy) and 1% of penicillin/streptomycin (Merck, Italy) and incubated at 37°C in humidified incubators with 5% CO_2. The cell line was propagated in 75 cm^2 cell culture flask (Corning, Italy) with 10 mL of complete D-MEM in order to obtain an adequate number of cells to inoculate 96-well plates.

Pseudovirus, an HIV-based luciferase lentivirus pseudotyped with SARS-CoV-2 full length S

glycoprotein of Wuhan strain, was purchased from Creative Biogene (USA) (SARS-CoV-2 S Pseudotyped Luciferase Lentivirus, cat. CoV-002).

The Pseudovirus presents SARS-CoV-2 S glycoprotein as the only surface protein that mediates viral fusion with host cells. The stock of Pseudovirus, harboring the SARS-CoV-2 S protein, contains 10^7 transduction units (TU)/mL, where the TU is the unit of measurement used to define the quantity of Pseudovirus. Of note, the Pseudovirus also contains the reporter gene for the firefly luciferase enzyme which allows to quantify the luminescence emitted by the intracellular viral particles.

3.5. Cytotoxicity assays

To establish the maximal non-cytotoxic dose of the β-CDs alone and of the UA/β-CDs complex, the previous described serial dilutions of compounds in D-MEM were incubated at 37°C with semi-confluent Vero E6 cells grown in 96-well tissue culture plates (Flow Laboratories) for 8 and 24h.

The MTT assay (Sigma Aldrich, Italy), colorimetric test based on the reduction of a yellow tetrazolium salt to formazan by metabolically active cells, was used to verify the possible cytotoxic activity of the compounds at different concentrations. More intensive violet color is related to a greater number of viable cells.

Briefly, for the cytotoxicity experiments, the cells previously detached from the 75 cm^2 flasks were seeded in 96-well plates at a final concentration of $1x10^4$ cells/well and incubated for 24h at 37°C in a humidified incubator with 5% of CO_2. After 24h of incubation, the culture medium was removed and 100 µl of the β-CDs alone and UA and β-CDs complex, previously dissolved in D-MEM, were added at different concentrations. The multiwells were incubated for 8 and 24h at 37°C in a humidified incubator with 5% of CO_2.

After 8 and 24h of incubation, the culture medium with the different concentrations of the β-CDs alone and UA and β-CDs complex was removed from the multiwells, and the cell monolayers were washed with 100 µl/well of phosphate buffered saline (PBS). Then, 100

µl/well of a 0.5 mg/mL solution of MTT dissolved in PBS were added and the multiwells were incubated in the dark for 3h at 37°C in a humidified incubator with a 5% of CO_2.

After 3h, the MTT solution was removed, the multiwells were washed with PBS and 100 µl/well of dimethyl sulfoxide (DMSO) were added. The multiwells were maintained at room temperature in the dark for further 15 min. Subsequently, the resulting solution was evaluated by spectrophotometric absorbance at the wavelength of 570 nm with correction at 690nm in order to define cell viability. For cytotoxic assay, a cell viability less than 70% classifies a substance or a complex as cytotoxic (Cannella et al. 2019).

3.6. Pseudovirus neutralization assay

For neutralization assays, Vero E6 cells were seeded in 96-well tissue culture plates (1×10^4 cells/well) for 24h at 37°C in a humidified incubator with 5% CO_2.

Cell confluence conditions were set following instructions provided by Pseudovirus manufacturer. Preliminarily, Pseudoviral fusion in Vero E6 cells, an epithelial cell line largely used in SARS-CoV-2 studies, was tested at a MOI of 100, 10, 1 and 0.1, corresponding to 10^6, 10^5, 10^4 and 10^3 TU of Pseudovirus, respectively. As reported in Figure S3, the optimal concentration of Pseudovirus luminescence is obtained with MOI 10, while MOI 100, 1 and 0.1 did not fall in the sensitivity limit of the luminescence method. Therefore, MOI 100, 1 and 0.1 were excluded.

To evaluate the inhibition of Pseudovirus fusion to host membrane, different concentrations of the β-CDs alone and of the UA/β-CDs complex were tested on Vero E6 cells. For studies on the interaction of the β-CDs alone and of the UA/β-CDs complex with pseudoviral particles and/or host cells, the neutralization assay was carried out at a MOI of 10, in the presence or absence of the β-CDs alone or of the UA/β-CDs complex, according to the following experimental plan: i) to evaluate the entry efficiency of the pseudoviral particles, cells were treated with the Pseudovirus for 8h at 37°C; ii) to evaluate whether the β-CDs alone or the UA/β-CDs complex interferes with the viral fusion by binding viral surface components, the

Pseudovirus was preincubated with the β-CDs alone or the UA/β-CDs complex for 1h at 37°C and then the cells were infected with these suspensions for 8h at 37°C; iii) to evaluate whether the β-CDs alone or the UA/β-CDs complex interferes with viral attachment to host cells, cells were preincubated with the β-CDs alone or the UA/β-CDs complex for 1h at 37°C. The cells were then washed with PBS and infected with the Pseudovirus for 8h at 37°C; iv) to assess whether β-CDs alone or the UA/β-CDs complex interferes with both viral and host cell components, β-CDs alone or the UA/β-CDs complex was added together with the Pseudovirus to cell monolayer for 8h at 37°C.

The MOI indicates how many viral particles are inoculated and, therefore, how many viral particles infect a known number of cells per well.

At the end of the incubation, cells were washed twice with PBS, covered with D-MEM supplemented with 2% of FBS and incubated for 48h at 37°C in a humidified incubator with 5% CO_2. After 48h, cells were washed, lysed with cell culture lysis reagent (Promega, Italy) and the transduction efficiency was determined by luminescence analysis using firefly luciferase assay kit (Promega, Italy). The relative luminescence unit (RLU) in each well was detected using Cytation 5 Cell Imaging Multi-Mode Reader (BioTek, Winooski, VT, USA).

3.7. Statistical analysis

For neutralization experiments, statistically significant differences were assessed by one-way ANOVA, with post-hoc Tukey test. All statistical analyses were run using Prism v9 software (GraphPad Software, USA).

All the results were expressed as the mean values ± standard deviation (SD) of three independent experiments. In each case, a p-value ≤ 0.05 was considered statistically significant.

Reference

Cannella V, Altomare R, Chiaramonte G, Di Bella S, Mira F, Russotto L, Pisano P, Guercio A. 2019. Cytotoxicity Evaluation of Endodontic Pins on L929 Cell Line. Biomed Res Int. 3469525. doi: 10.1155/2019/3469525

Nikolić V, Stanković M, Nikolić L, Nikolić G, Ilic´-Stojanović S, Popsavin M, Zlatković S, Kundaković T. 2013. Inclusion complexes with cyclodextrin and usnic acid. J Incl Phenom Macrocycl Chem. 76:173–182. doi: 10.1007/s10847-012-0187-8.

CHARACTERISTICS	(+)-UA
Description	Yellow crystalline powder
Solubility	Soluble in chloroform and Ethyl acetate, slightly soluble in ethanol, and not/slow soluble in water
Origin	*Usnea Longissima* Ach.
Product code	1405
Batch number	1405-1911-A001
Purity (HPLC assay)	98.7%
Granule size	10-20 microns
Particle distribution	0.2-15 μm
Loss on drying	0.1%
Specific rotation	+502°
Melting point	195-196°C
Heavy metals	≤ 10 mg/kg
Arsenic	≤ 1 mg/kg
Residual solvent (ethyl acetate)	64 mg/kg
Total plate count	≤ 1000 cfu/g
Yeast and mold	≤ 100 cfu/g
E. coli	Negative
Salmonella spp.	Negative

Table S1. Characteristics of (+)-Usnic acid (UA).

Concentrations of β-CDs alone in D-MEM	Concentrations of the UA/β-CDs complex (1:1 *w/w*) in D-MEM
0.5% (5 mg/mL)	1% (10 mg/mL)*
0.25% (2.5 mg/mL)	0.5% (5 mg/mL)*
0.05% (500 μg/mL)	0.1% (1 mg/mL)*
0.025% (250 μg/mL)	0.05% (500 μg/mL)
0.015% (150 μg/mL)	0.03% (300 μg/mL)
0.005% (50 μg/mL)	0.01% (100 μg/mL)
0.0025% (25 μg/mL)	0.005% (50 μg/mL)
0.0005% (5 μg/mL)	0.001% (10 μg/mL)

*Concentrations of the UA/β-CDs complex presenting precipitate at room temperature.

Table S2. Solubility test of different concentrations of both the β-cyclodextrins (β-CDs) alone and the Usnic acid (UA) and β-CDs complex (1:1 *w/w*) dissolved in Dulbecco's Modified Eagle Medium (D-MEM) at room temperature (the concentration values are expressed as percentage and as mg/mL ratio).

Conditions	% Vero E6 viability	
	8h	**24h**
Control	100	100
UA/β-CDs complex at 1%	15	7
UA/β-CDs complex at 0.5%	52	35
UA/β-CDs complex at 0.1%	54	41
UA/β-CDs complex at 0.05%	71	65
UA/β-CDs complex at 0.03%	83	85
UA/β-CDs complex at 0.01%	90	97
UA/β-CDs complex at 0.005%	90	98
UA/β-CDs complex at 0.001%	95	98

Table S3. Percentage of Vero E6 cell viability after 8 and 24 h of incubation with different concentrations of the UA/β-CDs complex.

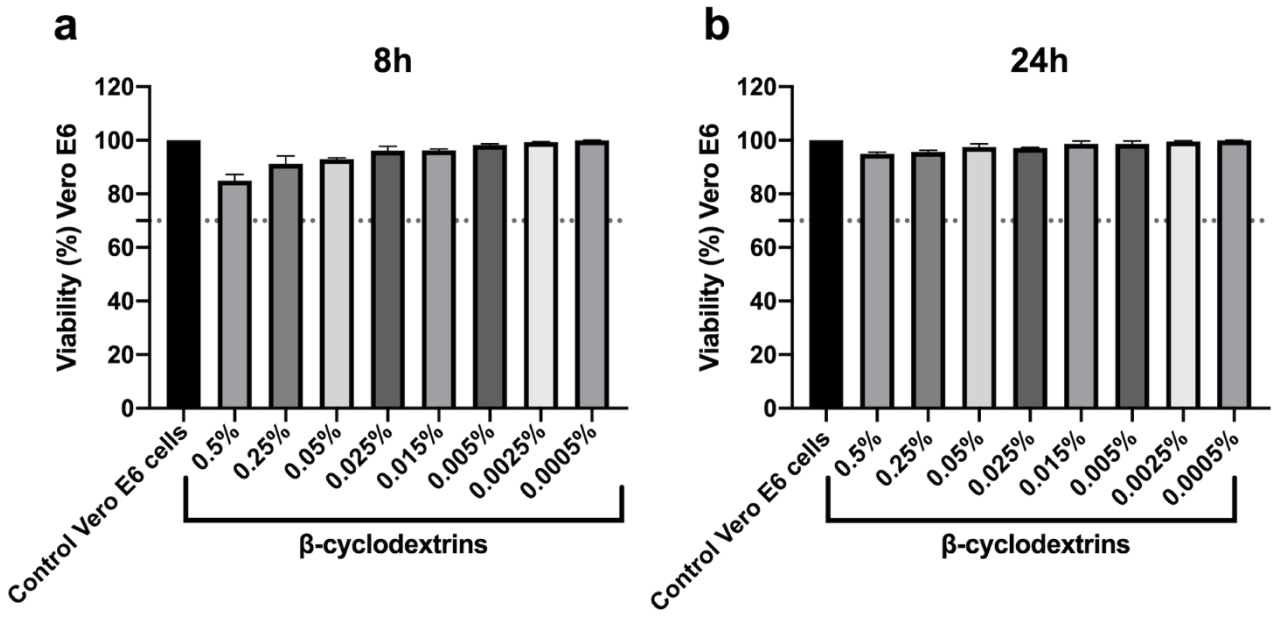

Figure S1. Percentage of Vero E6 cells viability after 8 (**a**) and 24h (**b**) of incubation with different concentrations of β-cyclodextrins alone. The dotted lines indicate the threshold value (70%) to define a substance as cytotoxic (Cannella et al. 2019).

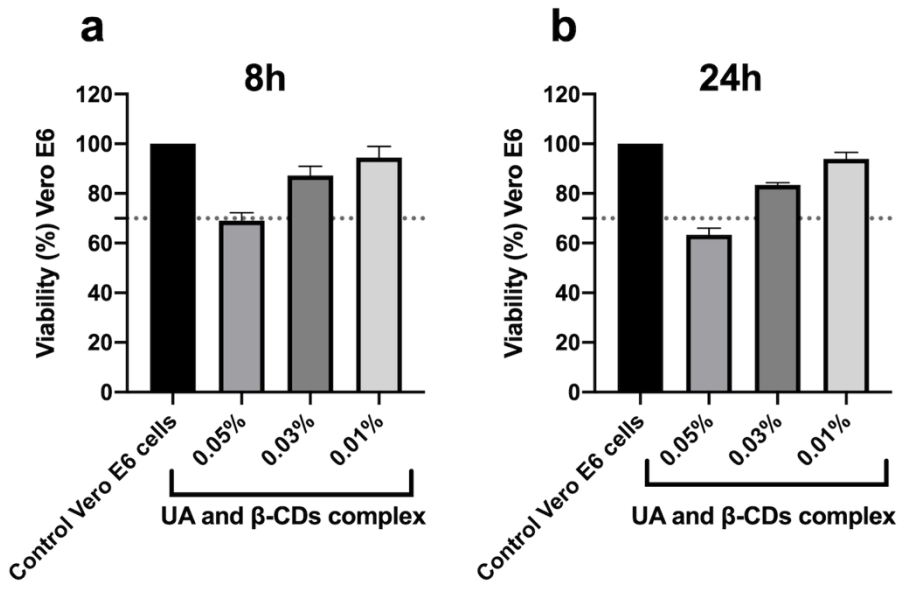

Figure S2. Percentage of Vero E6 cells viability after 8 (**a**) and 24 h (**b**) of incubation with different concentrations of the UA/β-CDs complex. The dotted lines indicate the threshold value (70%) to define a substance as cytotoxic (Cannella et al. 2019).

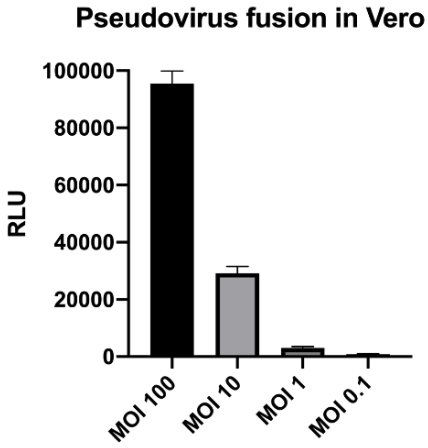

Figure S3. Luminescence of Pseudovirus observed in Vero E6 cells infected at multiplicity of infection (MOI) of 100, 10, 1, 0.1. RLU = Relative Luminescence Units.

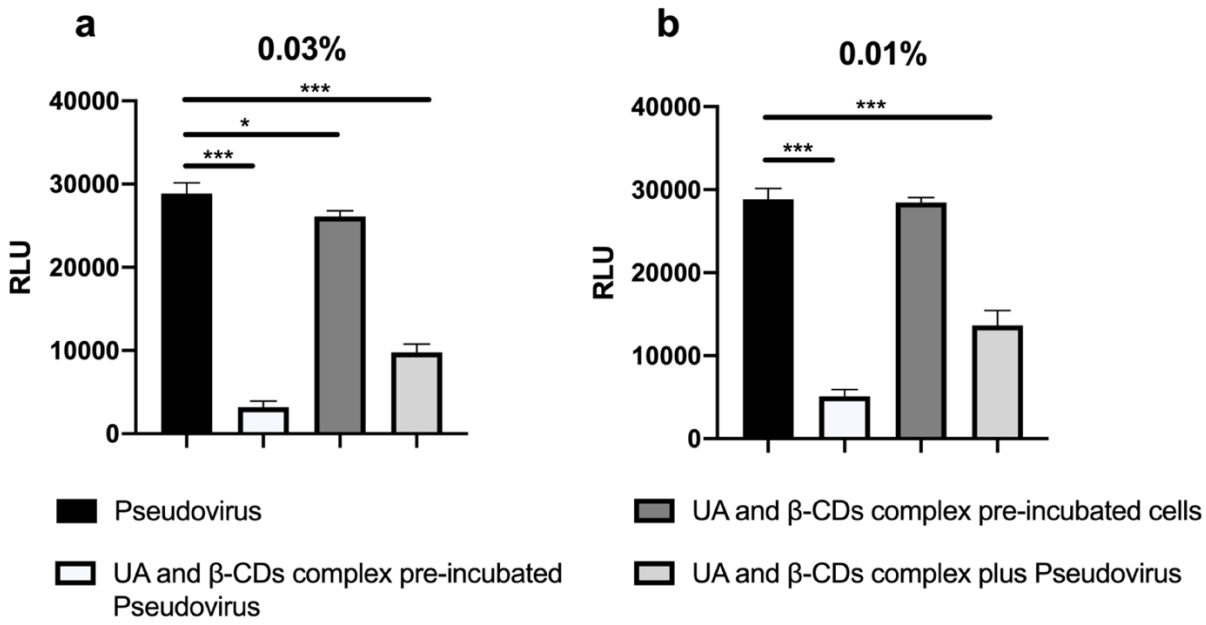

Figure S4. Luminescence of Pseudovirus observed in Vero E6 cells infected at MOI of 10 in the presence or absence of 0.03% (**a**) or 0.01% (**b**) of UA/β-CDs complex. See text for details. *: $p < 0.05$; ***: $p < 0.001$ (one-way ANOVA with post-hoc Tukey test). RLU = Relative Luminescence Units.