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Decoding the antiviral potential of eugenol, thymol and vanillin against human cytomegalovirus infection

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Abstract

Human cytomegalovirus (HCMV) poses serious health risks, particularly for immunocompromised individuals. However, the current FDA-approved anti-HCMV drugs face challenges such as drug resistance and significant side effects, underscoring the need for alternative treatment options. Essential oil components (EOCs), including eugenol, thymol and vanillin, are recognized for their therapeutic potential. This study evaluates their antiviral effects against HCMV in epithelial (ARPE-19) and fibroblast (MRC-5) cell lines. Among the EOCs, vanillin demonstrated the highest efficacy, characterized by low toxicity and a high selectivity index in both cell types. Mechanistic differences were noted between the cell lines. In ARPE-19 cells, eugenol showed virucidal activity, inhibited viral entry and suppressed early gene expression (IE-1). Conversely, in MRC-5 cells, eugenol mainly blocked viral entry and exhibited virucidal effects. Thymol was most effective in ARPE-19 cells, where it completely suppressed IE-1 expression as a result of both inhibition of viral entry and a direct disruptive effect on IE-1 expression. In addition, thymol showed an effect on viral replication. In MRC-5 cells, thymol primarily inhibited viral entry and attachment. Vanillin exhibited dual inhibitory activity in both cell lines, blocking viral attachment and entry. In MRC-5, vanillin also appears to affect intermediate processes. Notably, combining EOCs with ganciclovir resulted in synergistic effects. The eugenol/ganciclovir combination was particularly effective in ARPE-19 cells, while thymol/ganciclovir showed enhanced efficacy in MRC-5 cells. These findings suggest that EOCs have significant potential as adjunct therapies to improve antiviral outcomes and address drug-resistant HCMV strains.

INTRODUCTION

Human cytomegalovirus (HCMV) infection is highly prevalent worldwide, ranging from nearly 100% prevalence in developing countries to up to 60% in developed regions such as Europe or the USA [1–3]. While HCMV infection typically exhibits none or mild symptoms in healthy individuals [4], immunocompromised individuals, such as solid organ transplant recipients, HIV-infected patients and newborns, are at high risk of severe symptoms, leading to increased morbidity and mortality [5, 1]. Additionally, HCMV is a major cause of congenital infections, producing birth defects in neonates, including mental retardation, motor disabilities and hearing loss [6–9].

Efforts to develop an HCMV vaccine have been ongoing for decades [10, 11]; however, none of the candidates tested in clinical trials [11–14] have received approval due to insufficient efficacy or safety concerns [11].

Although antiviral treatments for HCMV infection have improved over the years [15], currently available drugs, while effective, present some limitations. These include adverse side effects such as nephrotoxicity, myelosuppression, the emergence of resistant viral strains, limited efficacy in immunocompromised individuals and the inability to completely eradicate latent viral reservoirs in

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Abbreviations: DMEM, Dulbecco's Modified Eagle Medium; dpi, day post-infection; EC_{50}, effective concentration 50; EOCs, essential oil compounds; EOs, essential oils; EU, eugenol; gB, glycoprotein B; GCV, ganciclovir; HCMV, human cytomegalovirus; hpi, hours post-infection; MOI, multiplicity of infection; PFA, paraformaldehyde; TC_{50}, toxic concentration 50; TH, thymol; VA, vanillin.

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One supplementary table and six supplementary figures are available with the online version of this article.

addition to the fact that they cannot be administered to prevent maternal–foetal transmission [16–18]. In this context, continued efforts are essential to develop novel therapeutic strategies and antiviral agents against HCMV [19–23].

Essential oils (EOs) are volatile, natural, liquid and complex mixtures of low-molecular-weight compounds biosynthesized by certain plant families, e.g. *Alliaceae*, *Apiaceae*, *Asteraceae*, *Brassicaceae*, *Lamiaceae*, *Myrtaceae* and *Rutaceae* [24, 25]. They are produced as secondary metabolites in response to contact with insects, herbivores and other organisms [26]. While EO compounds (EOCs) have traditionally been used as flavouring agents in food products and perfumery [25], recent studies have highlighted their significant bioactive properties, both as individual agents and in combination [24]. In fact, EOs have attracted increasing interest for their antimicrobial potential. Derived from plants, EOCs offer several advantages: they are generally considered non-toxic when used appropriately and pose a lower risk of promoting microbial resistance due to their complex chemical compositions, and they are biodegradable with minimal environmental impact. Moreover, they can be relatively cost-effective compared to conventional pharmaceutical agents [27]. Previous studies have demonstrated the antimicrobial activity of EOCs against pathogenic bacteria and yeasts, primarily through disruption of the cell membrane, ultimately leading to cell death [28–32]. More recently, EO derived from the *Labiatae* family, as well as individual compounds such as eugenol (EU) and carvacrol, has shown antiviral activity against enveloped viruses such as HSV-1, influenza virus and SARS-CoV-2 [33–37]. Given that HCMV is also an enveloped virus, EOCs may offer a promising alternative for antiviral intervention. However, to date, no studies have specifically evaluated the antiviral activity of EOCs against HCMV infection. EU, the major constituent of clove EO (*Syzygium aromaticum*), has been used in dental practice for centuries due to its analgesic and antiseptic properties [38]. Thymol (TH; 2-isopropyl-5-methylphenol), a principal component of thyme EO (*Thymus vulgaris*) [39], belongs to the phenolic monoterpenes and is widely used for treating respiratory tract conditions, as well as disorders of the digestive, cardiovascular and nervous systems [40]. Vanillin (VA; 4-hydroxy-3-methoxybenzaldehyde), a phenolic compound derived from vanilla beans of some plants of the *Vanilla* genus, is primarily used in the food and fragrance industries [41].

In the present study, we aim to investigate the antiviral activity of three EOCs, EU, TH and VA, against HCMV. Our findings demonstrate that these compounds inhibit HCMV infection through two distinct mechanisms: direct antiviral (anti-virion) activity and interference with early stages of infection.

METHODS

Cell lines, viruses and chemicals

The MRC-5 human lung fibroblasts (ATCC, CCL-171) and the ARPE-19 human retinal epithelial cells (ATCC, CRL-2302) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, 11765–054) supplemented with 10% FBS, 20 mM glutamine (Lonza), 10 units of penicillin and 10 µg of streptomycin (Lonza).

The HCMV AD169 virus strain derived from BADrUL131-Y4, in which the UL131 sequence was repaired [42] (kindly provided by Dr. Shenk (Princeton University), was propagated in MRC-5 fibroblast cells as previously described [43]. Infectious virus yield was assayed on MRC-5 by median tissue culture infective dose (TCID50).

EU (99% w/w) and TH (≥99% w/w) were commercially available (E51791 and T0501, Sigma-Aldrich, Madrid, Spain). VA (99% w/w) was provided by Ernesto Ventós S.A. (121-33-5, Barcelona, Spain). Ganciclovir (GCV) was purchased from MedChem Express (HY-13637).

In vitro cytotoxicity assay

EOC's cytotoxic effect was determined in ARPE-19 and MRC-5 cells using the AlamarBlue (AB) assay as previously described [38, 39, 44]. Briefly, the day before the assay, 100 µl of cells were plated on 96-well culture plates (30,000 and 15,000 ARPE-19 and MRC-5 cells, respectively). Increasing concentrations of EU, TH and VA were tested. All the solutions were prepared immediately prior to use. Plates were incubated for 48 h, and during this time, neither the medium nor the tested compounds were replenished. After incubation, the medium was removed, and wells were washed with 100 µl of PBS. Then, 100 µl of DMEM medium with 10% AB solution (v/v) was added. Plates were incubated (37°C, 5% CO_2, 3 h), and AB absorbance was measured ($\lambda_{excitation}$=570nm, $\lambda_{emission}$=600nm) in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany). Cell viability was expressed as a percentage related to the solvent control (0.1% DMSO). The experiment was run in triplicate, and data were presented as the mean (SEM) of the three independent experiments. The mean toxic concentration (toxic concentration 50, TC_{50}) values were calculated whenever possible by a nonlinear fit log (inhibitor)-versus-response (three parameters) test, using GraphPad Prism software (version 9.5.1, USA).

Antiviral activity assay

In order to determine the antiviral activity of EOCs, HCMV infection inhibition assays were carried out. ARPE-19 and MRC-5 cells were plated in each well of a 96-well plate (Greiner, Monroe, NC). The following day, the medium was replaced with DMEM with increasing concentrations of the compounds in sextuplicate experiments (2 h at 37°C), and cells were infected with MOI

(multiplicity of infection)=1. At 48 h post-infection (hpi), cells were fixed with 1% paraformaldehyde (PFA) 1X for 10 min at room temperature (RT), and nuclei were stained with 5 µg ml^{-1} of Hoechst in PBS for 30 min at RT. The plates were analysed with a fluorescence microscope (Cytell). The percentage of infected cells (green-GFP signal) was calculated based on the total number of cells stained with the Hoechst nucleus marker (blue). The software Cytell™ Cell Imaging System (v3.6.7.19) was used for the analysis. For each well, 6 images were taken with the 10X objective to homogeneously cover the whole surface of the well. Using cells treated with 0.1% DMSO equivalent to the highest dose of EOCs and infected with HCMV as 100% infection, the percent infection of cells pre-treated with increasing amounts of compound was determined. The half maximal effective concentrations (effective concentration 50, EC_{50}) were calculated using GraphPad Prism software (version 9.5.1, USA) using the average nonlinear fit log (inhibitor)-versus-response (three parameters) analysis and extrapolating to the concentration that would produce 50% infection relative to DMSO treatment. +Selectivity index (SI) was calculated as the ratio of TC_{50}/EC_{50}.

Virion neutralization assay

The day before the assay, 100 µl of ARPE-19 and MRC-5 cells (30,000 and 15,000, respectively) was plated in each well of a 96-well plate (Greiner, Monroe, NC). The following day, 1 MOI of HCMV was treated with the EC_{50} of each compound in DMEM, and the mixture was incubated for either 30 min, 1 and 2 h at 37°C, after which the different mixtures of HCMV+EOCs were added to the cells. Experiments were run in quadruplicate. At 48 hpi, the plates were analysed with a fluorescence microscope (Cytell) to count the number of GFP-positive cells as described above. Quadruplicate wells containing HCMV treated with 0.1% DMSO in DMEM (30 min, 1 and 2 h at 37°C) were used as a control to define 100% infection.

Virus attachment and entry assays

To determine if the antiviral activity of the EOCs affects blocking the virus–membrane interaction or virus cell entry, two separate experiments were performed. (i) The day before the assay, 100 µl of ARPE-19 and MRC-5 cells (30,000 and 15,000, respectively) was plated in each well of a 96-well plate (Greiner, Monroe, NC) and incubated at 37°C for 24 h. After incubation, cells were cooled down at 4°C for 15 min and treated with increasing concentrations of EOCs and incubated for an additional 2 h at 4°C, after which cells were infected at 1 MOI and incubated for an additional 2 h at 4°C. After this time, cell supernatant was discarded, and 100 µl of DMEM was added. Cells were incubated for 2 days at 37°C, and the percentage of infection was determined as described in the previous sections. (ii) The day before the assay, 100 µl of ARPE-19 and MRC-5 cells (30,000 and 15,000, respectively) was plated in each well of a 96-well plate (Greiner, Monroe, NC). The next day, increasing concentrations of EOCs were added to the cells and incubated for 1 h at 37°C. Cells were then infected with 1 MOI of HCMV and placed at 4°C for 2 h to allow viral attachment, and then, the cultures were shifted to 37°C for 1 h to initiate viral entry. Lastly, cells were washed with citrate buffer (pH 3) to inactivate any remaining extracellular virus. After incubating at 37°C for 48 h, the cell monolayer was fixed using 1X PFA, and the percentage of infection was quantified.

Time-of-addition experiments

The day before the assay, 100 µl of ARPE-19 and MRC-5 cells (30,000 and 15,000, respectively) was plated in each well of a 96-well plate (Greiner, Monroe, NC). The following day, cells were infected with MOI=1, and EOCs were added to the wells at the designated time points relative to virus infection (−2, 0, 1, 2, 4 and 6 hpi). Experiments were run in sextuplicate. The concentrations of the EOCs used were those that inhibited 50% of the infection (EC_{50}). At 48 hpi, the plates were analysed with a fluorescence microscope (Cytell) to count the number of GFP-positive cells. The per cent EOCs treated infected cells at the different time points was normalized, considering DMSO pretreated HCMV-infected cells as 100% infection.

Quantifying viral levels of mRNA and protein

The day before the assay, 2 ml of ARPE-19 and MRC-5 cells (500,000 and 250,000, respectively) was plated in each well of a 6-well plate. The following day, cells were treated either with EC_{50} or EC_{90} of the different compounds prepared in DMEM or 5 µM of flavopiridol (a known mRNA synthesis inhibitor, Sigma-Aldrich), and then, treated cells were infected with MOI=1 of HCMV and incubated at 37°C for 48 h. Cells were then washed with PBS and removed from the plate using a scraper. Total RNA was extracted from cells using the NZY Total RNA Isolation kit (Nzytech, Portugal). For the RNA analysis, 1 µg of total RNA was treated for 30 min at 37°C with RNase-free DNase I (Roche) according to the manufacturer's protocol. Maxima Reverse Transcriptase (Thermo Scientific) was used to synthesize cDNA from DNase I-treated RNA according to the manufacturer's recommendations. Quantitative real-time PCR was performed in a Light Cycler 480 II (Roche) using the SYBR Premix Ex Taq kit (TaKaRa) for fluorescent labelling. For this purpose, 2.5 µl cDNA was added to each reaction in a final volume of 10 µl. Real-time PCRs using 5 µM of the corresponding oligonucleotides (Table S1, available in the online Supplementary Material) were performed under the following conditions: 95°C for 10 s, followed by 40 cycles of 10 s at 95°C and 15 s at 55°C. At the end of the amplification cycles, a melting curve analysis was conducted to verify the specificity of the reaction. A standard curve was made with serial dilutions of the cDNA sample. The cycle threshold value for each sample was calculated by the LightCycler 480 Abs-Quant second-derivative function, and using those values, the amount of IE-1 and glycoprotein B (gB) cDNA in each sample relative to GAPDH cDNA was calculated, and viral mRNA was expressed relative to DMSO treatment. The data and error bars

represent the average and SD of three independent biological samples. In parallel experiments, total protein lysates were obtained by adding lysis buffer to the cells and quantified. IE-1 protein levels were analysed by Western blot. For the analysis, 2 µg (or 3 µg in ARPE-19 cells with EC90) of protein lysates was separated on a 10% precast SDS gel (Bio-Rad). Additionally, proteins were transferred to nitrocellulose membranes (0.45 µm) and probed with mouse monoclonal antibody 8B1.2 to HCMV IE-1 protein (MAB810R, Sigma-Aldrich) in blocking buffer (1X PBS+0.1% Tween 20+5% skim milk) and incubated at 4°C overnight followed by an HRP-labelled anti-mouse IgG (diluted 1 : 10000; 7076S, Cell Signaling), and detected using chemiluminescence (SuperSignal West Pico Detection Kit, Thermo Scientific) following the manufacturer's recommendations. Images were captured on a ChemiDoc Touch Imaging System (Bio-Rad Laboratories). GAPDH protein level was used in parallel as a loading control, using GA1R monoclonal antibody at a diluted 1:10000 dilution (MA5-15738, Invitrogen) and the HRP-conjugated anti-mouse IgG secondary antibody at a 1:10000 dilution (7076S, Cell Signaling).

Post-infection assessment of EOCs

To evaluate the direct effect of EOCs on IE-1 expression independently of viral entry, ARPE-19 and MRC-5 cells (500,000 and 250,000 cells per well, respectively) were plated in 6-well plates. The following day, cells were HCMV-infected at an MOI of 1 and incubated for 2 h at 4°C. Cells were then shifted to 37°C and incubated for 6 h (MRC-5) or 9 h (ARPE-19). After this period, the supernatant was removed, and cells were treated with the EC_{50} of each compound and incubated at 37°C for 48 h. IE-1 mRNA and protein levels were subsequently assessed by RT-qPCR and Western blot, respectively, as described in the previous section. This approach bypassed the viral entry stage, allowing direct quantification of IE-1 transcript and protein levels.

Inhibition of multi-cycle viral proliferation

The day before the assay, 100 µl of ARPE-19 and MRC-5 cells (30,000 and 15,000, respectively) was plated in each well of a 96-well plate (Greiner, Monroe, NC). The following day, cells were infected with 1 MOI HCMV. HCMV-infected cells were incubated at 37°C for 12 h in the case of MRC-5 and 24 h in the case of ARPE-19 and then treated with either an EC_{50} concentration of EOCs or an EC_{50} concentration of GCV (used as a positive control). At 3 and 6 days post-infection (dpi), media was replaced with fresh media. At 7 dpi, cells were fixed in 1X PFA and analysed with a fluorescence microscope (Cytell) to determine the number of GFP-positive cells relative to the positive control (DMSO 0.1%).

Drug combination studies

Six-by-six checkerboard experiments were performed. The day before the assay, 100 µl of ARPE-19 and MRC-5 cells (15,000 and 30,000 in 100 µl, respectively) was plated in a 96-well plate (Greiner, Monroe, NC). The following day, cells were infected with 1 MOI HCMV. HCMV-infected cells were incubated at 37°C for 12 h in the case of MRC-5 and 24 h in the case of ARPE-19. After this time, all possible two-drug combinations and concentrations of EOCs and GCV were performed in U-bottom 96-well plates and subsequently added to the cell monolayers. In parallel, a viability study was conducted using the AlamarBlue assay, as previously mentioned. Each combination was performed in triplicate experiments. Media was replaced at 3 and 6 dpi, and infection was analysed at 7 dpi using a Cytell microscope.

Synergy scores were calculated using SynergyFinder 3.0 based on the Loewe additivity model. The Loewe additivity model defines the expected effect as if a drug was combined with itself, and it considers the dose-response curves of individual drugs. The relative percentage of infection was calculated.

Statistical analysis

Statistical tests were performed using GraphPad Prism 9. The results are expressed as mean and SD of at least three independent biological replicates. Significance was determined by performing a tailed t-student test with comparisons to infected DMSO-treated cells as a control. The asterisk (*) indicates statistically significant differences with P value≤0.05. To investigate differences between ARPE-19 virions and MRC-5 virions, a nested t-test was performed using GraphPad Prism. Bars labelled with different letters (e.g. a and b) indicate significant differences between groups (P<0.05).

RESULTS

Antiviral activity of EOCs

Relative cell viability and antiviral activity of EOCs were tested. The results are summarized in Fig. 1 and Table 1. All tested compounds exhibited a dose-dependent effect on cell viability, with the exception of VA, which showed no cytotoxicity in ARPE-19 and MRC-5 cells at the concentrations tested (Fig. 1d, g).

EU exhibited greater cytotoxicity in MRC-5 cells compared to ARPE-19 cells, with TC_{50} values of 440 and 1,990 µM, respectively. Notably, the lower EC_{50} observed in MRC-5 cells suggests a more potent antiviral effect of EU in fibroblasts. This enhanced antiviral activity was further reflected in the SI, which was approximately threefold higher in MRC-5 cells (SI=22) than in ARPE-19 cells

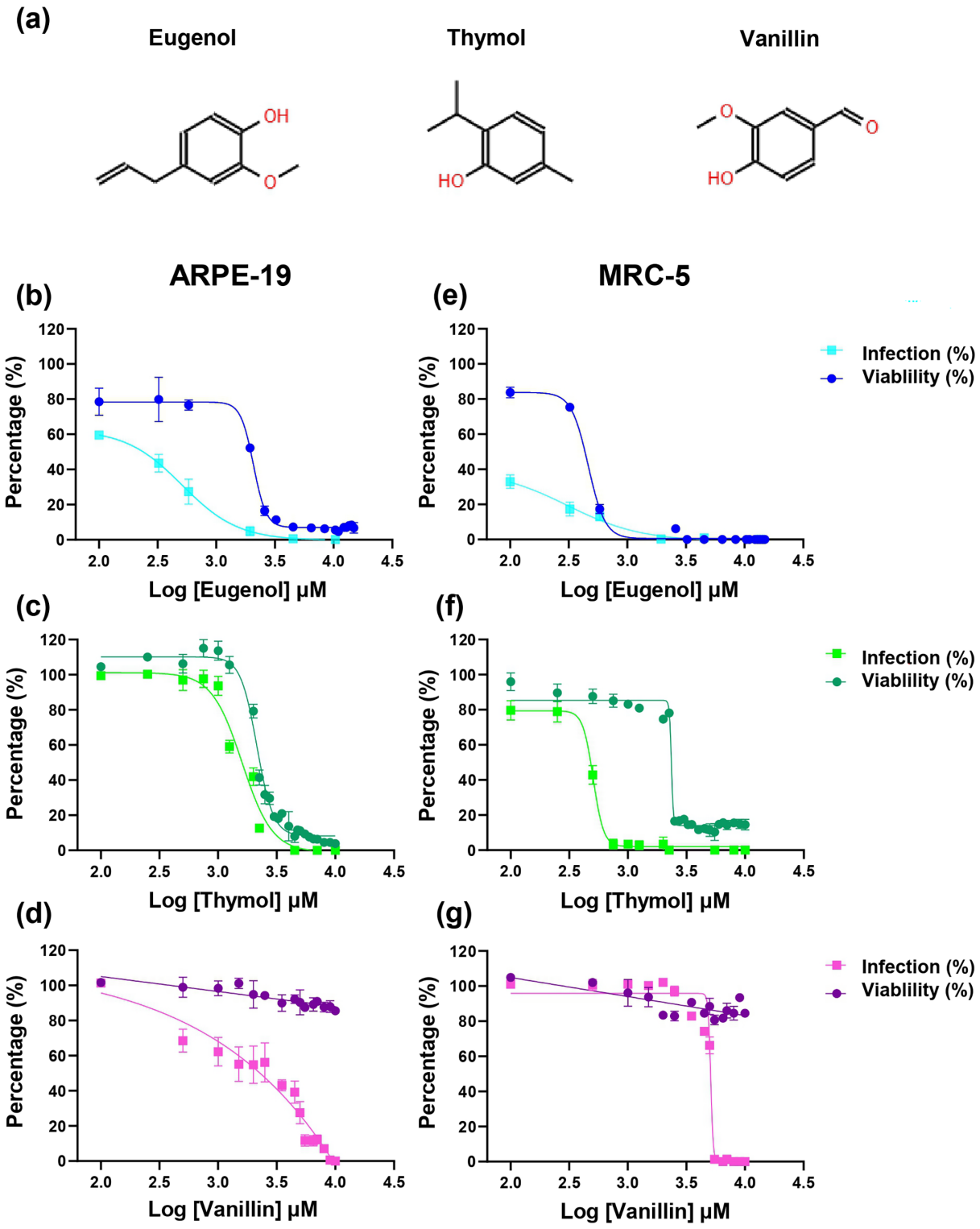

Fig. 1. (a) Chemical structure of the three used EOC: EU, TH and VA. (b–d) Percentage of ARPE-19 cell viability and cell infection after incubating with EU, TH and VA. (e–g) Percentage of MRC-5 cell viability and cell infection after incubating with EU, TH and VA. Results are expressed as logarithmic concentrations. DMSO-treated cells were used as negative controls. Data represent the mean±SD of six biological replicates.

Table 1. TC_{50}, EC_{50} and SI values for the indicated compounds in HCMV-infected ARPE-19 and MRC-5 cells. The TC_{50} and EC_{50} values were calculated from at least four independent experiments

	ARPE-19			MRC-5		
	TC_{50}	EC_{50}	SI	TC_{50}	EC_{50}	SI
EU	1990	320	6.22	440	20	22
TH	2150	1220	1.76	2350	500	4.70
VA	2.3×10^{7}	1550	1.5×10^{4}	8.9×10^{6}	5090	1.7×10^{3}
GCV*	>20	7.8	>2.56	>20	11.2	>1.79

TC_{50}, 50% toxic concentration (μM); EC_{50}, 50% inhibitory concentration of HCMV-GFP infection (μM); SI, selectivity index ($SI=TC_{50}/EC_{50}$) (μM). EU, eugenol; TH, thymol; VA, vanillin; GCV, ganciclovir.

When no significant toxicity was observed at the highest concentration tested (20 μM), TC_{50} and SI values are reported as '>' relative to this experimental limit. Extremely high extrapolated values are not shown, as they lack biological relevance.

*Regarding GCV, the values shown in Table 1 derive from another experimental approach. Because GCV acts as an inhibitor of viral DNA replication and does not interfere with virus infection, these values were determined using a 7-day replication assay to assess its antiviral effect.

(SI=6.22, Table 1). In contrast, TH demonstrated reduced cytotoxicity compared to EU in both cell types, with higher TC_{50} values (2,350 μM for MRC-5 and 2,150 μM for ARPE-19). TH also showed greater antiviral efficacy in fibroblasts with an SI of 4.7 compared to an SI of 1.76 in epithelial cells (Table 1). These differences may reflect distinct mechanisms of action in each cell type, underscoring the importance of evaluating candidate antivirals across multiple cell models.

In contrast, as previously noted, cells treated with VA showed minimal cytotoxic effects, with exceptionally high TC_{50} values of 2.3×10^{7} μM in ARPE-19 cells and 8.9×10^{6} μM in MRC-5 cells. Among the three EOCs tested, VA exhibited the highest EC_{50} value in both cell lines, indicating lower antiviral potency relative to EU and TH. However, due to its extremely low cytotoxicity, VA demonstrated substantial antiviral activity, reflected in its high SI, particularly in ARPE-19 epithelial cells which are primary targets during natural HCMV infection ($SI=1.5 \times 10^{4}$ in ARPE-19, $SI=1.7 \times 10^{3}$ in MRC-5; Table 1).

Anti-virion activity

To investigate the potential anti-virion activity of the three tested compounds against extracellular HCMV particles, we conducted a three-time-point experiment using the EC_{50} values previously determined in the antiviral activity assay. The results are presented in Fig. 2a. All three EOCs demonstrated comparable anti-virion effects in both cell lines, with TH being the least effective. EU exhibited the most potent activity, reducing infection by ~30–40% after 30 min, which increased significantly after 2 h, reaching 85% inhibition in ARPE-19 cells and 70% inhibition in MRC-5 cells. VA, although less potent, still achieved a 50% reduction in infectivity after 2 h. Considering that virions produced in different host cells may vary in tegument and membrane composition, an additional experiment was conducted to compare the anti-virion activity of the EOCs on virions derived from either ARPE-19 or MRC-5 cells. Fig. S1 displays infection percentages in both cell types after both types of virions were exposed to the EC_{50} concentrations of each compound at different time points. No significant differences in infection rates were observed in ARPE-19 cells, regardless of the origin of the virions. However, in MRC-5 fibroblasts, notable differences were observed with TH: virions produced in epithelial cells were more susceptible to TH, showing greater inhibition in MRC-5 cells compared to the same virions infecting ARPE-19 cells.

Blocking initial stages of the viral cycle

To determine which step of the early infection process is targeted by EOCs, we performed two distinct experiments to assess the EOCs' effect on viral attachment to the cell surface and subsequent entry into the cell cytoplasm (Fig. 2b). The attachment assay was designed to evaluate the impact of EOCs on the initial interaction between HCMV particles and cellular receptors. This experiment was conducted at 4°C, to prevent viral entry, thereby isolating the attachment phase. In contrast, the entry assay focused on the next step of infection. In this case, cells were first incubated with viruses to allow attachment, then shifted to 37°C to permit entry. Following this, a citrate buffer at acidic pH was applied to remove weakly bound virions and inactivate any uninternalized particles, thereby ensuring that only successfully internalized virus was measured.

In the virus attachment assay, VA in both cell lines and TH in MRC-5 showed a reduction in the infection rate. In contrast, all three EOCs markedly interfered with viral entry across both cell types, resulting in infection reductions ranging from 20 to 85%. Among them, TH demonstrated the strongest inhibitory effect, reducing viral infection by more than 80% in both ARPE-19 and MRC-5 cells compared to the control.

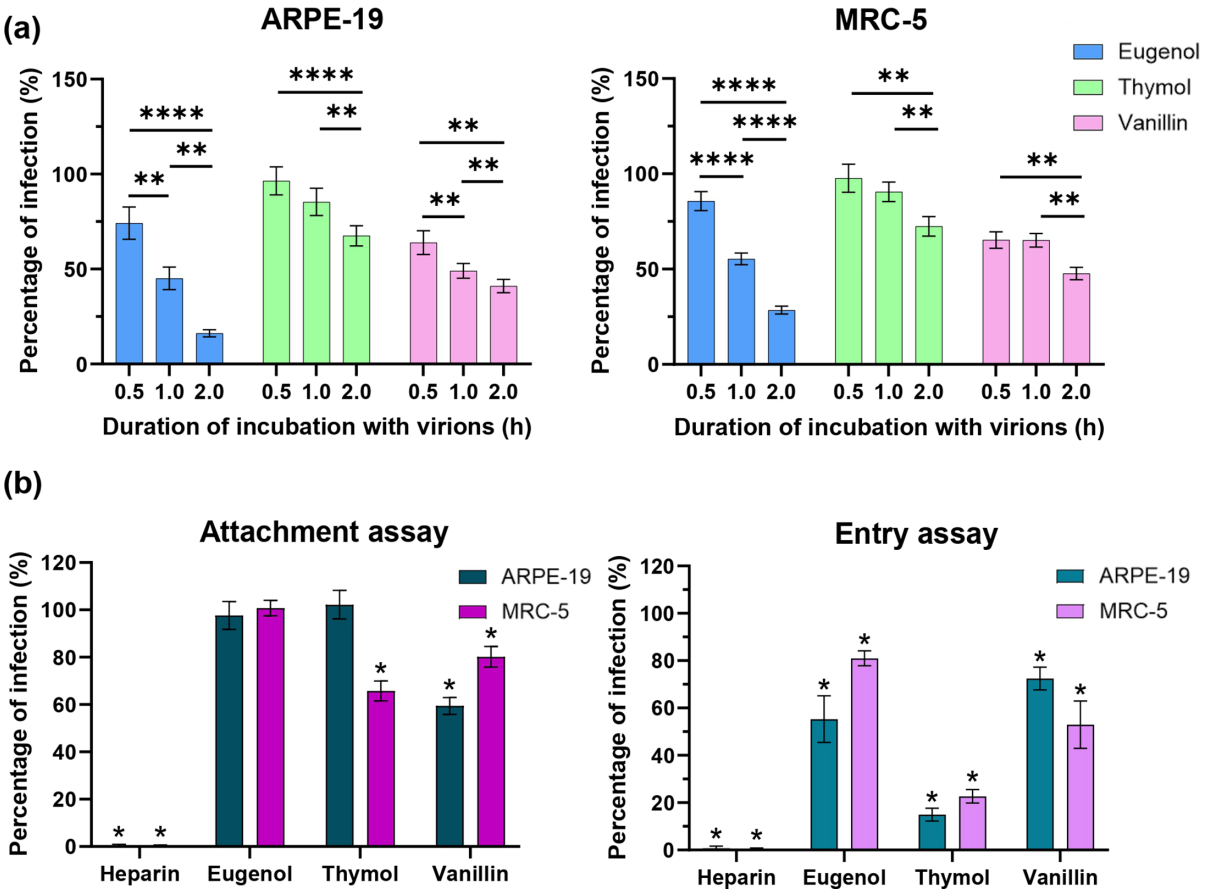

Fig. 2. (a) Anti-virion activity of EOCs measured as percentage of HCMV-infected ARPE-19 or MRC-5 cells. Viruses were incubated at 37°C for 0.5, 1 or 2 h with DMEM containing EC_{50} of the EOCs before virus inoculation. The percentage of infection was relative to DMSO treatment. Statistical significance was determined by a paired t-test comparing between time points within the same EOC (*P* value, *<0.05, **<0.01, ****<0.0001). Data represent the mean±SD of four biological replicates. (b) Effects of EOCs on virus attachment (i) and penetration assay (ii). (i) Before infecting the cells with HCMV for 2h at 4°C, prechilled (4°C) cells were treated with EC_{50} values in each case or 10 µg heparin for 2h at 4°C. (ii) ARPE-19 and MRC-5 cells pretreated with EOCs or 10 µg heparin were infected with HCMV at 4°C, shifted to 37°C for 1 h and then washed with citrate buffer (pH=3). The percentage of infection was relative to DMSO treatment. *P* values of <0.05 were considered significant with respect to the DMSO control. Data represent the mean±SD of six biological replicates.

Temporal analysis of antiviral activity by time-of-addition assay

To evaluate whether the compound exerts antiviral activity beyond the initial stages of HCMV infection, such as during the uncoating process or early replication, we conducted a time-of-addition assay, in which each compound was added at −2, 0, 1, 2, 4 and 6 hpi using its respective EC_{50} concentration (Fig. S2). When applied at 1 hpi in ARPE-19 cells, TH was the only compound to achieve a substantial reduction in infection (~70%), maintaining ~50% inhibition at later time points. In contrast, EU and VA displayed minimal inhibitory effects throughout the course of infection, with reductions ranging from 0 to 20%. Notably, from 2 to 6 hpi, only TH continued to exert a moderate antiviral effect, achieving up to 40% inhibition, suggesting its potential action during post-entry stages of the viral replication cycle.

In fibroblasts, the EOCs also appeared to target early stages of HCMV infection. However, the response profile differed from that observed in ARPE-19 cells. Notably, the addition of EU and VA at 1 hpi resulted in a more pronounced inhibitory effect in fibroblasts. In contrast, although TH inhibited ~80% of infection when added at 0 hpi, this effect diminished at later time points. Interestingly, VA was the only compound that maintained inhibitory activity during the 2–6 hpi window, with infection reductions ranging from 70 to 40%, suggesting a potential role in interfering with post-entry stages in fibroblasts.

EOC inhibition of HCMV Immediate Early gene expression and protein levels

To further investigate the mechanism of action of the EOCs at the translation level, expression and abundance of the Immediate Early (IE-1) protein were assessed by RT-qPCR and Western blot, respectively (Fig. 3a). In ARPE-19 cells, RT-qPCR analysis

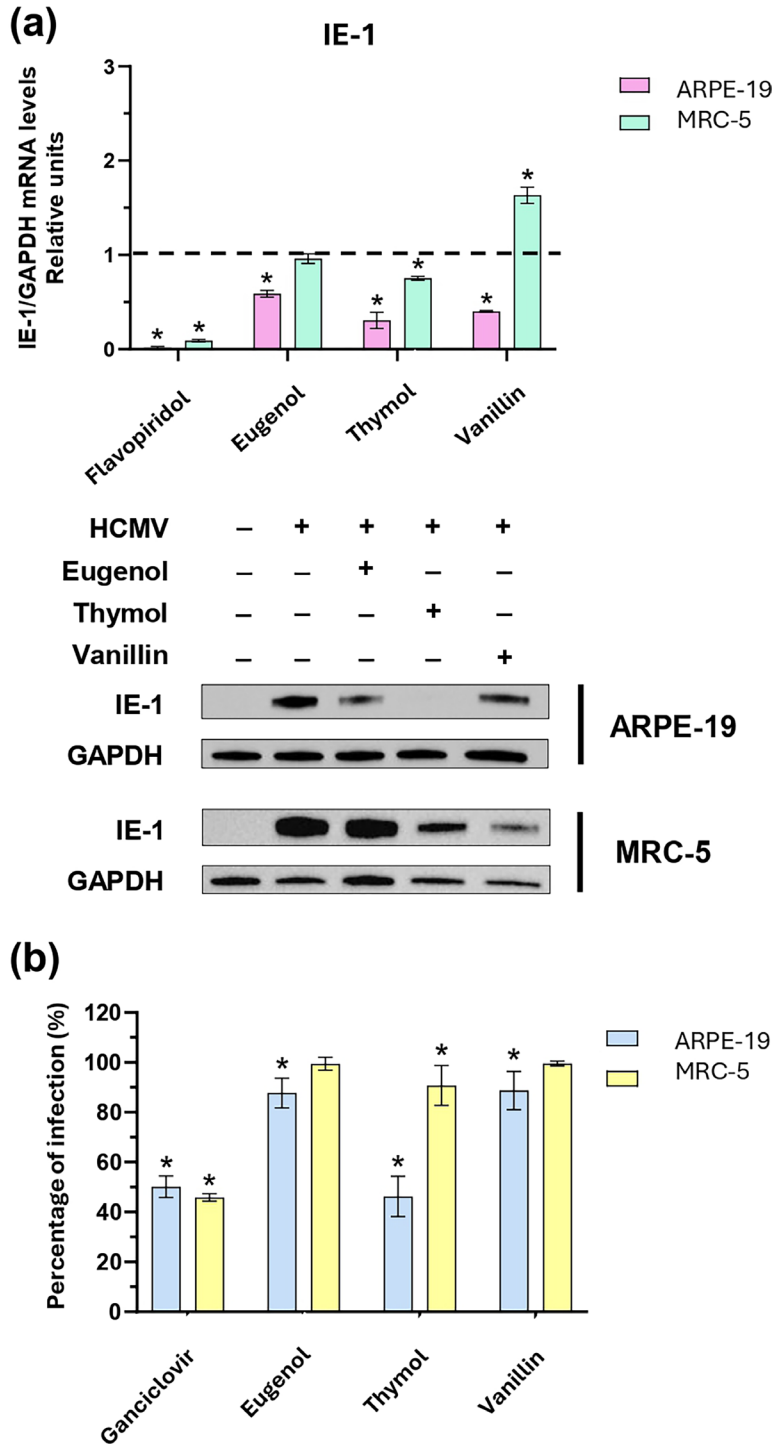

Fig. 3. (a) ARPE-19 and MRC-5 cells were treated with the EC_{50} of EOCs, followed by infection. Subsequently, the cultures were incubated for 48 h at 37°C, after which the cells were harvested by scraping. IE-1 and GAPDH mRNA levels determined by qPCR and Western blot are represented. Flavopiridol was used as a positive control for mRNA synthesis inhibition. Data represent mean relative values (±SD) of three biological replicates. A dashed line indicates the expression of the control condition. The asterisk (*) denotes significant differences compared to DMSO-treated cells. For Western blot analysis, cells were lysed after 48 h, and protein lysate was analysed using the indicated antibodies. GAPDH level was used as a loading control. (b) Effect of EOCs in late stages of HCMV life cycle. The percentage of infection was determined by using DMSO-treated cells as 100%. As a control, ARPE-19 and MRC-5 cells were continuously treated with GCV (7.8 and 11.2 µM, respectively). The error bars represent the SD from six replicates. *P* values of <0.05 were considered significant with respect to the DMSO-treated control.

revealed a reduction in IE-1 gene expression following treatment with all three EOCs. Consistent with these findings, Western blot analysis showed a moderate decrease in IE-1 protein levels, with TH treatment resulting in undetectable levels of protein.

In contrast, EOC treatment in MRC-5 fibroblasts displayed a different inhibition profile. TH again caused a marked reduction in both IE-1 transcript and protein levels. However, EU showed no significant effect on IE-1 expression at the mRNA or protein level. Interestingly, while VA treatment led to an apparent overexpression of IE-1 transcripts, IE-1 protein levels were significantly reduced, suggesting post-transcriptional regulation or translation inhibition.

In addition, the expression of the late gene UL55 (encoding gB) was also analysed by RT-qPCR (Fig. S3). In both cell types, treatment with all three EOCs led to significant downregulation of gB mRNA, except for VA-treated MRC-5 cells, where gB expression was significantly upregulated. Furthermore, to evaluate whether EOCs have a stronger antiviral effect at higher concentrations, EC_{90} values were also tested (Fig. S4). Under these conditions, IE-1 expression was more markedly inhibited at both the mRNA and protein levels in ARPE-19 and MRC-5 cells. The only exception was EU treatment in fibroblasts, where neither mRNA nor protein levels were affected. Consistent with the results obtained at EC_{50}, VA treatment in MRC-5 cells induced overexpression of the IE-1 gene, which was even more pronounced than at the lower dose (EC50). Interestingly, despite this transcriptional increase, IE-1 protein levels were reduced.

HCMV proliferation analysis

To assess whether the tested EOCs could inhibit multi-cycle HCMV replication, a 7-day viral proliferation assay was conducted (Fig. 3b). Among the compounds tested, only TH achieved ~50% inhibition of infection in ARPE-19 cells after 7 days, a level comparable to that observed for the antiviral control GCV.

These findings indicate that the early-stage anti-virion activity of TH in epithelial cells is sustained throughout the entire viral replication cycle.

Impact of EOCs on IE-1 expression independently of viral entry

To determine whether EOCs restrict IE-1 expression by blocking viral entry or by directly affecting early viral transcription, cells were treated with the compounds at 6 h (MRC-5) and 9 h (ARPE-19) post-infection (Fig. 4), time points at which viral entry had already occurred. In ARPE-19 cells, RT-qPCR analysis revealed a reduction in IE-1 gene expression following treatment with EU and TH, whereas no significant changes were observed with VA. Consistent with these results, Western blot analysis showed a moderate decrease in IE-1 protein levels in response to EU and TH, but not to VA.

In contrast, in MRC-5 cells, the compounds did not appear to exert a direct inhibitory effect on IE-1 expression. The only exception was VA, which induced upregulation of IE-1 transcript levels (Fig. 4a). However, IE-1 protein levels remained unchanged across all three treatments, as determined by Western blot analysis (Fig. 4b).

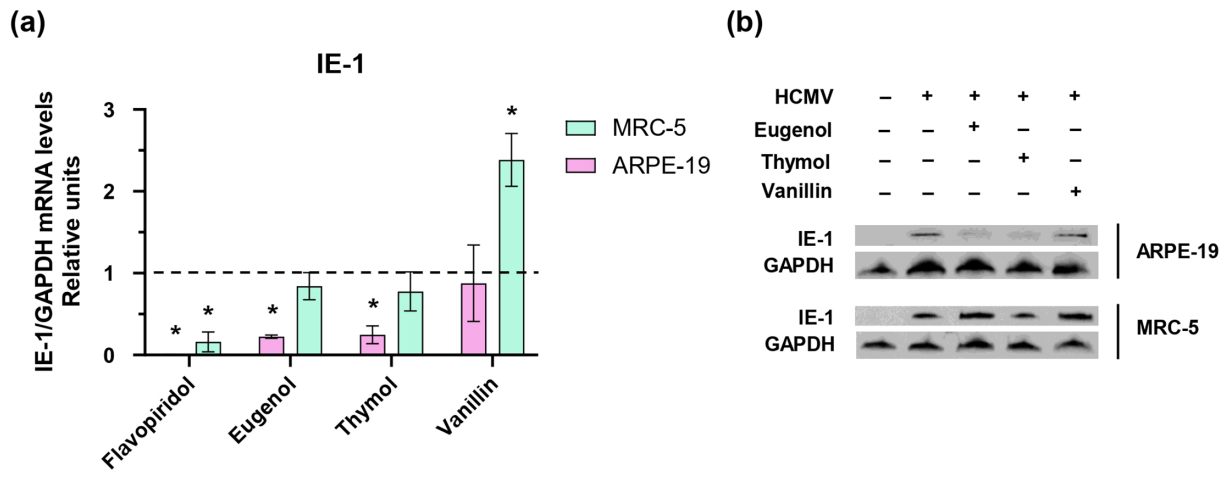

Fig. 4. (a) ARPE-19 and MRC-5 cells were treated with the EC_{50} of EOCs, after 9 and 6 hpi, respectively. Subsequently, the cultures were incubated for 48 h at 37°C. IE-1 and GAPDH mRNA levels quantified by qPCR are represented. Flavopiridol was used as a positive control for mRNA synthesis inhibition. A dashed line indicates the expression of the control condition. The asterisk (*) denotes significant differences compared to DMSO-treated cells. (b) HCMV protein levels in EOC-treated cells were determined by Western blot analysis. Cells were lysed after 48 h, and protein lysate was analysed using the indicated antibodies. GAPDH level was used as a loading control.

Synergy assays in combination with GCV

Drug combination therapy is a widely used and effective strategy that involves the simultaneous administration of two or more drug agents to enhance therapeutic efficacy, reduce toxicity and minimize the selection of resistance mutations. To the potential of EOCs as combinatorial agents, we assessed their antiviral effects against HCMV in combination with GCV in both ARPE-19 and MRC-5 cells.

In ARPE-19 epithelial cells, the combination of EU/GCV demonstrated a highly synergistic effect, reaching a maximum synergy score of 27.3 at 290.7 µM EU and 15 µM GCV (Table 2). This combination reduced infection to 16% (Fig. 5a) while maintaining 98% cell viability (Fig. S6). Although synergistic interactions were also observed with TH/GCV and VA/GCV treatments, the predominant effect was additive (Table 2). Notably, while the highest concentration of TH completely suppressed infection (0%), this was accompanied by a sharp drop in cell viability to ~5%.

In MRC-5 fibroblasts, a similar cytotoxic effect was observed at high concentrations of TH/GCV. However, lower concentrations of TH exhibited notable synergy. The most effective combination (563 µM TH and 3.75 µM GCV) achieved the highest synergy score of 30.36 (Table 2), resulting in complete inhibition of infection (0%) with 100% cell viability (Figs 5e and S6). For the EU/GCV combination, the highest synergy score was 9.8 at 36.2 µM EU and 7.5 µM GCV, corresponding to 41% infection and 96% cell viability (Table 2). In contrast, the VA/GCV combination showed a maximal synergy score of 1.8 (9,000 µM VA/30 µM GCV), indicating an additive effect. Despite achieving a low infection rate (2%), this combination significantly compromised cell viability, which dropped to 18%.

Table 2. Synergy scores for each EOC-GCV combination tested. Combinations with a synergy score greater than +10 are considered synergistic, those between −10 and +10 are considered additive, and those with scores below −10 are considered antagonistic

		ARPE-19							MRC-5					
EU (µM)	**581**	0	−7.9	2.2	23.4	13.1	16	**581**	0	−49.4	−17.4	−6.5	−1.1	0.6
	290.7	0	−2.8	22.4	17.4	27.3	12.7	**290.7**	0	−51	−24.8	−0.8	−2.4	0.5
	148.6	0	−1.8	−5.4	24.8	21.7	23.3	**148.6**	0	−34.8	−43.3	−10.8	−1.8	0.27
	71.1	0	−21.3	4.5	13.8	16	23.2	**71.1**	0	−1.7	−5.2	−16.7	−2.9	0.5
	36.2	0	2.8	−2.6	-1	10.1	21.7	**36.2**	0	2.3	−5.6	9.8	−3.8	0.71
	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0
		0	**1.8**	**3.75**	**7.5**	**15**	**30**		**0**	**1.8**	**3.75**	**7.5**	**15**	**30**
				GCV (µM)							***GCV (µM)***			
TH (µM)	**2250**	0	22	22	22	22	22	**2250**	0	30	30	30	30	30
	1125	0	21.9	21.9	21.9	21.8	21.8	**1125**	0	29.9	29.8	29.5	28.9	25.3
	563	0	6.6	−1.1	−1.5	1.8	−1.1	**563**	0	29.8	30.4	30.1	29.4	25.3
	281	0	−5.4	−19.7	−4.2	6.8	12.3	**281**	0	23.9	27.9	29.5	29	24.3
	141	0	−31.7	−43.7	−10.4	6	10.5	**141**	0	−7.6	-1	22	24.1	23.1
	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0
		0	**1.8**	**3.75**	**7.5**	**15**	**30**		**0**	**1.8**	**3.75**	**7.5**	**15**	**30**
				GCV (µM)							***GCV (µM)***			
VA (µM)	**9000**	0	4.1	3.8	4.4	5	5.4	**9000**	0	-8	−4.7	−1.1	0.2	1.8
	4500	0	7.1	8.2	9.5	8.3	12	**4500**	0	−31.8	−23.9	−9.7	−2.9	0.8
	2250	0	−4.6	4	6.2	9.4	13.6	**2250**	0	−60.9	−56.4	−28.9	−4.4	0.5
	1125	0	−7.2	4.4	8.9	9.8	14.3	**1125**	0	−60.6	−62.3	−46.5	−13.3	−1.4
	562	0	−10	1.4	4	10.8	14.5	**562**	0	−60	−60.4	−48.8	−13.9	0.3
	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0
		0	**1.8**	**3.75**	**7.5**	**15**	**30**		**0**	**1.8**	**3.75**	**7.5**	**15**	**30**
				GCV (µM)							***GCV (µM)***			

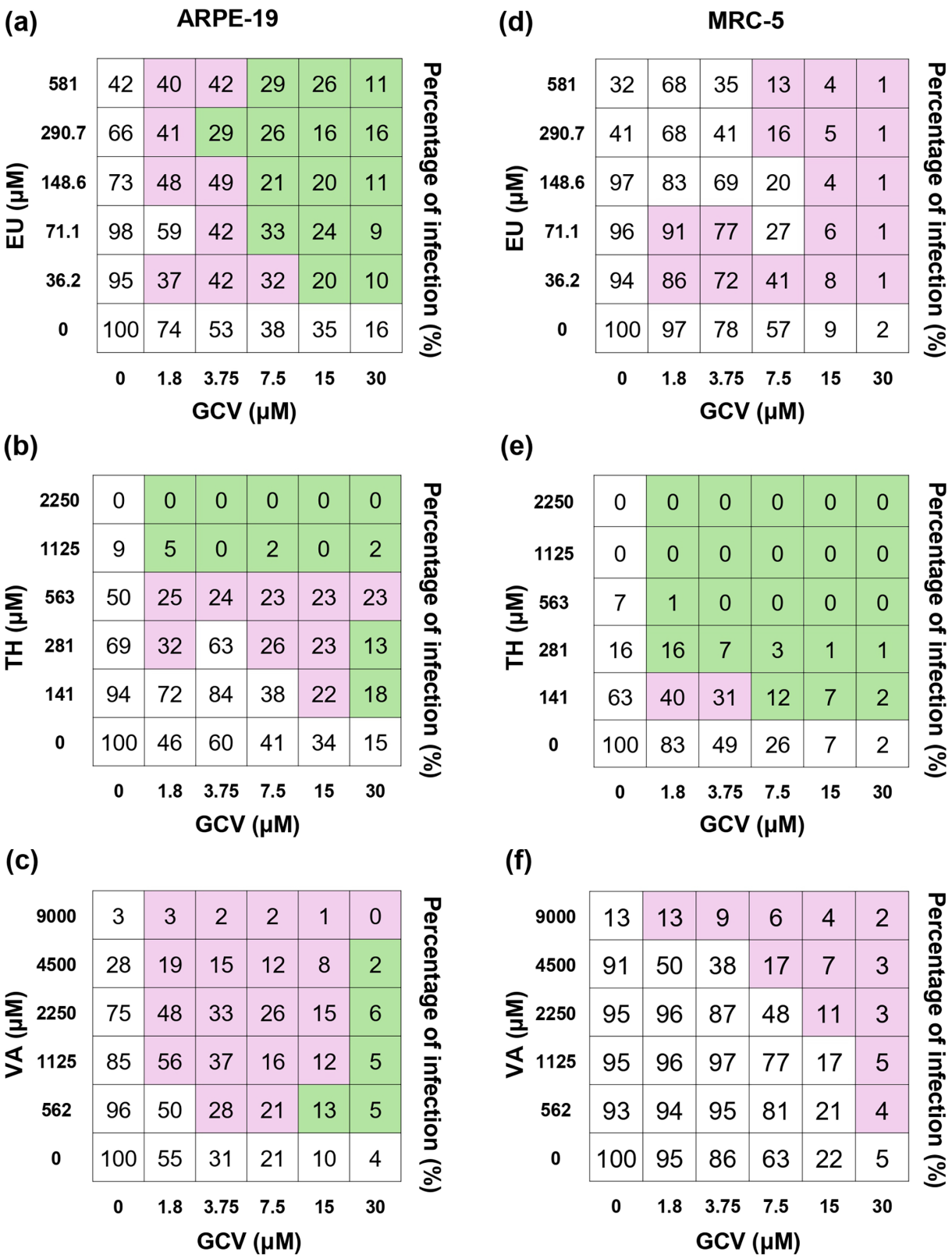

Fig. 5. Percentage of infection obtained in the checkerboard assay for ARPE-19 cells (a–c) and MRC-5 cells (d–f). Synergistic combinations are shown in green, while additive combinations are shown in pink.

DISCUSSION

The limitations of current anti-HCMV therapies (including toxicity, emergence of resistance and their unsuitability for use during pregnancy) underscore the urgent need for novel antiviral agents with novel mechanisms of action [40, 41]. EOCs have been widely used for decades in cosmetic and culinary applications, as well as for their broad-spectrum biological and therapeutic properties [27, 45]. In recent years, increasing attention has been given to the antiviral potential of EOCs, with demonstrated efficacy against both enveloped viruses (e.g. influenza virus, herpes simplex virus-1/2 or HIV) and non-enveloped viruses (e.g. coxsackievirus, adenovirus and rhinovirus) being highlighted [46–48]. However, to our knowledge, this is the first study to investigate the antiviral activity of EOCs against HCMV infection.

In this study, we evaluated the *in vitro* cytotoxicity and antiviral activity of three EOCs, EU, TH and VA, against HCMV in two major target cell types of natural infection (epithelial and fibroblast cells). Among the tested EOCs, VA demonstrated the most favourable profile, exhibiting the lowest toxicity and the highest SI in both cell lines. This low cytotoxicity is consistent with previous findings in HepG2 cells [49, 32]. Both EU and TH exhibited stronger antiviral activity in fibroblasts compared to epithelial cells, as reflected by their higher SI in fibroblasts, although neither matched the performance of VA. These observations align with previous studies demonstrating the antiviral properties of EU and TH against viruses such as herpes simplex type-1, hepatitis A and HIV [50–53].

To explore the most plausible mechanisms of action for the three tested compounds, it is essential to consider the differences between the two cell types studied. Among the three compounds tested, EU demonstrated the most potent antiviral activity in epithelial cells by directly interacting with the virion and interfering with viral entry, as well as by directly inhibiting the expression of the viral genes IE-1 and B. These findings suggest that EU disrupts HCMV infection at early stages by blocking both entry and the initiation of viral gene expression by inhibiting IE-1 expression. This is consistent with previous reports demonstrating EU's anti-virion effects against other enveloped viruses such as herpes simplex virus types 1 and 2, influenza virus and SARS-CoV-2, where EU was shown to inhibit the interaction between viral proteins and host cell receptors, thereby preventing viral entry [54, 55].

In epithelial cells, the inhibitory effect of TH was evident during viral entry and within the first 2 hpi. The reduction in IE-1 observed upon treatment with TH (Fig. 3a) was also detected when cells were treated post-infection, indicating an effect on IE-1 expression beyond viral entry (Fig. 4). In addition, TH continued to inhibit infection throughout the replicative cycle, suggesting that TH may interfere not only with viral entry and expression of IE-1, but also with post-entry processes such as uncoating or viral genome replication. These findings are supported by previous results highlighting TH's broad pharmacological properties, including its use in treating respiratory tract infections, further supporting its potential as a therapeutic agent for viral diseases [56].

The bioactive properties of VA, including its anti-inflammatory, antimicrobial and antioxidant effects, have been largely underexplored [57, 58]. While previous studies have reported antiviral activity of VA against viruses, such as SARS-CoV-2 and influenza virus [59, 60], this is the first report demonstrating a significant anti-virion effect of VA against HCMV. VA achieved inhibition rates of ~65%, comparable to those observed with EU, which may be attributed to their chemical structures' similarity. In epithelial cells, VA primarily interfered with viral attachment and, to a lesser extent, entry. At the molecular level, VA did not directly affect the expression of early and late viral transcripts or early viral protein levels (IE-1). The slight reduction observed may be likely attributable to decreased viral entry, which consequently limits IE-1 expression.

Thus, our results in epithelial cells suggest that the EOCs primarily target the early stages of the HCMV infectious cycle in epithelial cells. This early inhibition may result from either (a) a direct interaction with IE viral proteins, thereby blocking viral entry, or (b) interaction with host regulatory proteins that subsequently suppress the expression of HCMV-encoded early genes.

A closer examination of the three compounds in MRC-5 fibroblasts reveals distinct differences in their antiviral mechanisms compared to epithelial cells, highlighting the importance of considering cell type–specific entry pathways when designing antiviral strategies [61]. EU exhibited anti-virion activity in fibroblasts by interacting with HCMV particles and preventing infection, although its effect was less pronounced than in ARPE-19 cells. Time-of-addition assays indicated modest entry inhibition, with ~50% reduction in infection at 1 hpi. Unlike in epithelial cells, EU did not affect IE-1 protein expression in fibroblasts, suggesting a shift in its antiviral mechanism, potentially acting at later stages of the viral replication cycle in this cell type.

In fibroblasts, TH exhibited anti-viral activity similar to that observed in epithelial cells, though with some notable differences. While TH demonstrated modest anti-virion activity (~35% inhibition at 2 h), this effect was lower compared to the other two EOCs. TH effectively interfered with both viral attachment and entry stages. Interestingly, although TH achieved 80% infection inhibition when administered at 0 hpi, this effect diminished over time, suggesting a possible virostatic rather than anti-virion mechanism. Unlike in epithelial cells, where TH completely suppressed IE-1 expression, its impact in fibroblasts became more apparent during attachment and entry stages. This suggests a distinct antiviral mode of action in fibroblasts in which inhibition of attachment and entry produces a slight decrease in IE-1 and gB genes. It has been shown that the proteomic composition of virions varies significantly depending on the propagating cell type [62]. These differences, particularly in tegument proteins,

influence the virus's capacity to initiate infection and replicate in new host cells [63–66]. Such structural heterogeneity may also affect sensitivity to inhibitors, as the presence or absence of specific virion-associated proteins can modulate drug efficacy [67]. This suggests that the therapeutic response may be directly influenced by the cellular origin of the viral inoculum.

Regarding VA, a comparable level of antiviral activity (~50%) was observed in fibroblasts, similar to that seen in epithelial cells. This suggests that VA primarily acts by inhibiting HCMV attachment and entry in both cell types. However, its effectiveness appears to vary by stage and cell type: VA showed greater efficacy during the entry stage in fibroblasts and during the attachment stage in epithelial cells. This is supported by the time-of-addition assay results, where the highest inhibition in fibroblasts (70%) occurred between 1 and 2 hpi, while in epithelial cells, the greatest inhibition (60%) was seen at 0 hpi.

Further analysis of gene expression revealed that VA modulates different targets (IE-1 and gB) differently in the two cell lines. In epithelial cells, both IE-1 and gB mRNA levels were reduced due to the effect on entry, consistent with transcriptional suppression. In contrast, in MRC-5 fibroblasts, both transcripts were upregulated, but protein levels were reduced, suggesting that VA may interfere with post-transcriptional events, such as mRNA stability or translation or a mechanism of negative feedback. However, when VA was added at 6 hpi, IE-1 protein levels were not reduced, whereas transcript levels remained upregulated. These findings suggest that VA exerts a temporally dependent effect on IE-1 expression: it may interfere with the initial production of the protein but does not affect protein that has already been synthesized. Although IE-1 mRNA levels were elevated under all conditions, possibly due to VA-mediated alteration in promoter regulation, translation appeared to be impaired only when the compound was present during the immediate-early phase. Consequently, the apparent loss of negative feedback was observed only when VA was added prior to infection, indicating an indirect, timing-dependent effect rather than a direct disruption of IE-1 autoregulation. This broader and cell-type-specific inhibitory profile indicates that VA may disrupt multiple stages of the HCMV replication cycle, offering potential as a versatile antiviral agent.

Given the strong pharmacological activity of some current antivirals, one potential application of EOCs may be for combination therapies that target multiple viral pathways and stages. However, further research is required to evaluate and optimize such combinations, particularly to ensure efficacy at non-toxic, well-tolerated doses. In small clinical trials addressing persistent infections with drug-resistant strains, combining existing drugs has already shown potential [68–71]. Strategically using combination regimens may offer several advantages over monotherapy, especially when targeting multiple steps of the viral life cycle. In our study, we evaluated the combination of GCV with EOCs to assess possible synergistic effects, laying the groundwork for future investigations into their therapeutic potential and tolerability.

Our results demonstrated synergistic interactions between EOCs and GCV in both cell lines. Notably, the EU/GCV combination exhibited the highest synergistic effect in epithelial cells, while TH/GCV showed the strongest synergy in fibroblasts. These findings highlight the potential of EOCs to enhance the efficacy of existing antivirals, particularly against drug-resistant HCMV strains. Nonetheless, further studies are needed to optimize these combinations and evaluate their clinical applicability.

It is important to note that in MRC-5 cells, the most synergistic combinations of TH/GCV and VA/GCV were associated with higher toxicity compared to those observed in ARPE-19 cells. Interestingly, in the TH/GCV assay performed in fibroblasts, increasing GCV concentrations led to a reduction in TH-induced toxicity (Fig. S5), even in uninfected cells. This unexpected observation may suggest a protective or modulatory effect of GCV on cellular pathways affected by high concentrations of TH, or a direct chemical interaction between the two compounds that attenuates TH's cytotoxicity. Further studies are warranted to elucidate the underlying mechanisms and assess the therapeutic implications of this interaction.

However, it is important to acknowledge the significant limitations associated with the pharmacological application of EU, TH and VA, particularly in terms of tolerability and the feasibility of achieving effective *in vivo* concentrations. Previous studies have shown that all three compounds exhibit low oral bioavailability and undergo rapid, extensive metabolism. For instance, EU is characterized by low oral bioavailability and rapid metabolic clearance. For example, EU demonstrates poor oral bioavailability (~4.25% in rats) and undergoes extensive first-pass metabolism, with less than 1% excreted unchanged [72]. TH is often undetectable in human plasma, primarily circulating in its sulphate form, and has a reported bioavailability of ~16% [73]. Similarly, VA exhibits limited bioavailability (~7.6% in rats) and is largely metabolized to vanillic acid in humans [74].

Furthermore, achieving systemic therapeutic concentrations of EU, TH and VA often requires doses that exceed levels considered safe or tolerable. At such elevated doses, all three compounds have been associated with notable toxicities, including hepatotoxicity, central nervous system disturbances and chemical burns [75]. While these compounds are generally recognized as safe for use in food applications, this designation does not extend to the significantly higher concentrations required for pharmacological efficacy. Their narrow therapeutic window represents a major barrier to clinical translation. Overcoming these pharmacokinetic and tolerability limitations will require the development of advanced drug delivery strategies, such as nanoparticle-based systems or self-emulsifying drug delivery systems [76–78]. Furthermore, rigorous and targeted human clinical studies will be essential to bridge the gap between their *in vitro* antiviral potential and achievable *in vivo* therapeutic outcomes.

Our findings underscore the *in vitro* antiviral potential of the three EO components, EU, TH and VA, each demonstrating distinct antiviral profiles in epithelial and fibroblast cell lines. These differences reflect the complex interplay between

cell-type-specific factors, cellular susceptibility (viral receptors, cellular signalling pathways), viral replication kinetics, intracellular pharmacodynamics or pharmacokinetics and compound-specific cellular toxicity. Therefore, further studies should focus on optimizing the application of these compounds, particularly through combination with existing antivirals. Such efforts could enhance therapeutic efficacy while minimizing toxicity, paving the way for potential clinical use of EOCs in HCMV infection management.

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Conflicts of interest
P.P.-R. is the founder and shareholder of Vaxdyn, S.L., a biotechnology company developing vaccines. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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