Virology Journal

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Curcumin-like diarylpentanoid analogues exhibit antiviral and anti-inflammatory activities in influenza A virus-infected A549 lung epithelial cells by inhibiting multiple retinoic acid-inducible gene I (RIG-I)-mediated pathways

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Abstract

Influenza A virus (IAV) causes seasonal epidemics and occasionally pandemics. Curcumin, a well-known phytochemical, has been reported to exhibit anti-IAV effects; however, most studies were not comprehensive and utilized Mardin-Darby canine kidney (MDCK) cells. Curcumin-like diarylpentanoid analogues, namely 2-benzoyl-6-(3,4-dihydroxybenzylidene)cyclohexen-1-ol (BDHBC) and 5-(3,4-dihydroxyphenyl)-3-hydroxy-1-(2-hydroxyphenyl)penta-2,4-dien-1-one (DHHPD), exhibit improved drug-like properties, including increased solubility and stability, along with stronger antiviral effects against rhinovirus (RV). Thus, this study evaluates the antiviral and anti-inflammatory activities of BDHBC and DHHPD using A549 lung epithelial cells infected with influenza A/Puerto Rico/8/34 (H1N1), and compared their antiviral effects with curcumin in different treatment modes (pre-, co- and post-treatment). Like curcumin, BDHBC and DHHPD significantly reduced virus yield in the post-treatment mode and exhibited direct virucidal activity. Post-treatment with BDHBC and DHHPD also significantly suppressed viral replication as well as pro-inflammatory cytokines (IL-6, IL-8 and IP-10) and interferons (IFN-β and IFN-λ1) at both the gene and protein levels. The stronger antiviral and anti-inflammatory effects of DHHPD were attributed to its inhibition of multiple retinoic acid-inducible gene I (RIG-I)-mediated signalling pathways, including NF-κB, AP-1, MAPKs (p38 and Erk1/2), IRF3 and Akt. Only some of these pathways were affected by BDHBC treatment. Neither compound affected RIG-I or IRF7 expression, which is consistent with their lack of impact on Stat1 activation. Interestingly, DHHPD also demonstrated significant prophylactic effect against IAV infection, which was not observed for BDHBC and curcumin. In conclusion, BDHBC and DHHPD inhibit IAV replication and cytokine responses by targeting host signalling pathways. Further investigation in vivo is required.

Keywords Influenza A virus, H1N1, Curcumin, Diarylpentanoid analogue, Antiviral, Cytokine, Retinoic acid-inducible gene I (RIG-I)

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Introduction

Influenza A virus (IAV) is one of the most common respiratory pathogens that causes upper respiratory tract infections. Young children, elderly and people with chronic diseases, such as diabetes, cancer, heart diseases, asthma and chronic obstructive pulmonary disease (COPD), are prone to severe influenza and susceptible to complications like pneumonia [1]. Seasonal epidemics and pandemics due to the spread of IAV are a significant cause of hospitalisations and deaths [2]. While there are a few classes of antiviral drugs available for influenza treatment, increasing resistance to M2 ion channel blockers and neuraminidase (NA) inhibitors highlights the need for new anti-influenza drugs [3].

IAV primarily infects epithelial cells in the airways and triggers innate defense mechanisms, mainly the production of cytokines, chemokines and interferons (IFNs). While these mediators recruit immune cells to combat pathogens, excessive production of pro-inflammatory cytokines can cause damage to the lungs [4]. Besides that, acute respiratory distress syndrome (ARDS) may develop due to infiltration of inflammatory cells and fluid accumulation in the lungs. Cytokine storm also leads to systemic inflammation, organ dysfunction and death [5]. These contribute to increased morbidity and mortality during severe influenza. As such, novel therapeutic agents that suppress both viral replication and exaggerated inflammatory responses during infection may offer better therapeutic benefits for severe influenza [6].

Upon infection, pattern recognition receptors (PRRs) such as retinoic acid-inducible gene-I (RIG-I) and Toll-like receptors (TLRs) recognise viral nucleic acids and initiate a myriad of signalling cascades [7]. Antiviral IFNs are mainly regulated by interferon regulatory factors (IRFs) (IRF3 and IRF7), whereas the inflammatory responses are controlled by different pathways, including nuclear factor-kappa B (NF-κB), activator protein-1 (AP-1) and mitogen-activated protein kinases (MAPKs) [8]. As a result, infected airway epithelial cells secrete type I (IFN-α and -β) and type III (IFN-λs) IFNs as well as pro-inflammatory cytokines/chemokines, such as interleukin-6 (IL-6), IL-8, interferon γ-induced protein 10 (IP-10) and monocyte chemoattractant protein-1 (MCP-1) [9–11]. Innate immune signalling pathways play a crucial role in cytokine production and IAV replication and thus could be promising antiviral host targets to reduce viral load and diminish pro-inflammatory cytokines production [12].

Since the discovery of curcumin in the rhizome of turmeric (*Curcuma longa*), several studies have reported its diverse pharmacological activities, including anticancer, anti-inflammatory and antiviral effects [13]. Notably, it has been shown to inhibit viral replication and inflammatory responses in different infection models of respiratory viruses, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [14], respiratory syncytial virus (RSV) [15] and influenza viruses [16–19]. However, curcumin is only available as supplements but not clinically used for diseases because its poor stability and solubility make it less bioavailable in human body after consumption [20, 21]. Curcumin-like diarylpentanoid analogues, particularly 2-benzoyl-6-(3,4-dihydroxybenzylidene)cyclohexen-1-ol (BDHBC) and 5-(3,4-dihydroxyphenyl)−3-hydroxy-1-(2-hydroxyphenyl)penta-2,4-dien-1-one (DHHPD), are more superior than curcumin in terms of stability and solubility [22, 23]. Studies have also demonstrated that BDHBC and DHHPD have potent anti-inflammatory and antinociceptive activities [22–26]. Besides that, BDHBC and DHHPD were found to have stronger antiviral effects on rhinovirus (RV) compared to curcumin [27]. Thus, this study aims to investigate the inhibitory effects of BDHBC and DHHPD on IAV infection as well as cytokine responses using A549 cells as an in vitro epithelial cell infection model. Additionally, signalling pathways that may contribute to their antiviral and anti-inflammatory effects are identified.

Materials and methods

Materials

A549 cells (Cat No.: CRM-CCL-185™), influenza A/Puerto Rico/8/34 (H1N1) (IAV PR8) (Cat No.: VR-1469™) and Kaighn's Modification of Ham's F-12 Medium (F-12K) were purchased from the American Type Culture Collection (ATCC). Madin-Darby canine kidney (MDCK) cell line was a kind gift of Assoc. Prof. Dr. Li-Yen Chang (Universiti Malaya, Malaysia). Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4500 mg/L) (Cat No.: D1152), curcumin (Cat No.: C1386), dexamethasone (DEX) (Cat No.: D4902) and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Cat No.: T1426) were obtained from Sigma-Aldrich. Oseltamivir carboxylate (OC) (Cat No.: 15779) was purchased from Cayman Chemical. 3-(4,5-Dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) (Cat No.: Amresco 0793) was obtained from VWR Life Science.

Synthesis of curcumin-like diarylpentanoid analogues

Curcumin-like diarylpentanoid analogues (BDHBC and DHHPD) (Fig. 1) were produced using previously described methods [24, 25]. Nuclear magnetic resonance (NMR) was used to ensure that the purity of the compounds was more than 99%. The ^{1}H-NMR and ^{13}C-NMR

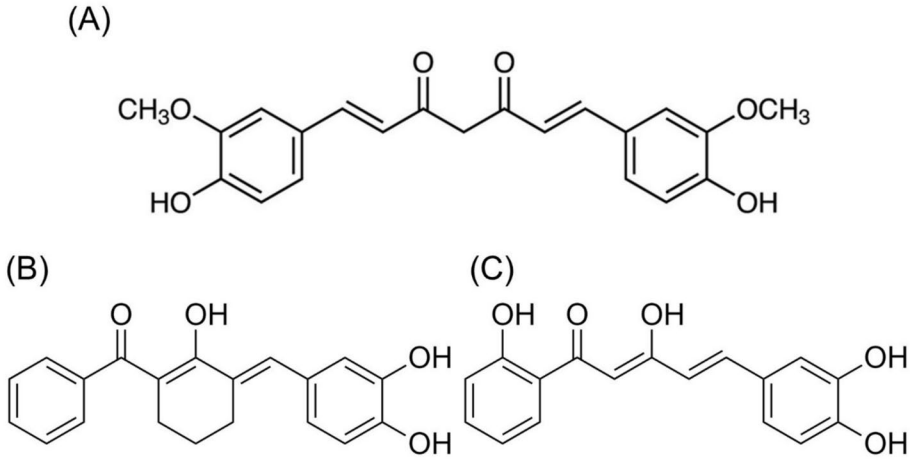


Fig. 1 Chemical structures of (**A**) curcumin, (**B**) 2-benzoyl-6-(3,4-dihydroxybenzylidene)cyclohexen-1-ol (BDHBC) and (**C**) 5-(3,4-dihydroxyphenyl)−3 -hydroxy-1-(2-hydroxyphenyl)penta-2,4-dien-1-one (DHHPD)

results of both compounds are provided in the Supplementary Materials (Fig. S1 and S2).

Compounds stock solutions

Curcumin, BDHBC and DHHPD were dissolved in dimethyl sulfoxide (DMSO) to prepare the stock solutions (100 mM). OC (20 mM) stock solution was prepared in phosphate-buffed saline (PBS), whereas DEX (10 mM) stock solution was prepared in DMSO. They were aliquoted and stored at −20℃ to minimise freeze–thaw cycles.

Cell culture

MDCK and A549 cells were grown in DMEM and F-12K, respectively, at 37℃ in a 5% CO_2 incubator. Both culture media were supplemented with 10% FBS. Media were changed every two to three days. When cell confluency was more than 80%, the cells were detached using trypsin and passaged or seeded for assays.

Propagation of IAV

IAV PR8 was propagated in MDCK cells according to the ATCC's protocol with slight modifications. When MDCK cells in T-25 flasks were more than 80% confluent, they were infected with 0.5 $TCID_{50}$/cell of ATCC IAV PR8 stock. Serum-free DMEM supplemented with 0.5 µg/mL of TPCK-treated trypsin was used for virus growth. The flasks were incubated at 37℃ until more than 80% cytopathic effect (CPE) was observed. The virus was harvested by centrifuging the culture supernatants at 2000 × g, 4℃ for 20 min to remove dead cells.

Median tissue culture infective dose ($TCID_{50}$) assay

The virus stock titre, virus yield of infected A549 cells and infectivity of compound-treated virus were measured using $TCID_{50}$ assay. After overnight incubation, MDCK cells in 96-well plates (2×10^4 cells/well) were inoculated with ten-fold serial dilutions (100 µL/well) of virus or culture supernatant in eight replicates. After 1 h of adsorption at 37℃, the virus inoculum was removed. The virus growth medium for IAV PR8 (serum-free DMEM with 0.5 µg/mL of TPCK-treated trypsin) was then added into each well. The cells were incubated at 37℃ for 4 days. Based on the number of CPE-positive wells for each dilution, the virus titre ($TCID_{50}$/mL) was calculated using the Reed-Muench method [28]. The viral titre of the IAV PR8 stock used in this study is 2.15×10^7 $TCID_{50}$/mL.

Cytotoxicity assay

MTT cytotoxicity assay was used to assess the effects of curcumin, BDHBC and DHHPD on A549 cell viability. After 24 h of seeding, A549 cells in 96-well plates (2×10^4 cells/well) were treated with different concentrations of the compounds for 72 h. Fresh medium (100 µL) and MTT solution (5 mg/mL) (20 µL) were then added and incubated at 37℃ for 4 h. After discarding the supernatants, 100 µL of DMSO was added to dissolve the purple formazan crystals. The absorbance readings were read at 570 nm using Tecan Infinite® F50 microplate reader.

Infection of A549 cells

A549 cells in 6- (1×10^6 cells/well) or 12-well (4×10^4 cells/well) plates were infected with 0.01 $TCID_{50}$/cell of

IAV PR8 in serum-free F-12K when they were around 90% confluent. After 1 h of adsorption at 37℃, the cells were washed twice with PBS, followed by the addition of serum-free F-12K containing 0.1 µg/mL of TPCK-treated trypsin. The cells were incubated at 37℃ until the desired time points (24, 48 or 72 h).

Treatment of A549 cells

To assess the antiviral effects in different treatment modes, cell treatment was performed before (pre-treatment), during (co-treatment) or after (post-treatment) infection [29] (Fig. 2). (A) Pre-treatment: A549 cells were treated with the compounds at 37℃ for 1 h, followed by IAV PR8 infection. (B) Co-treatment: A549 cells were simultaneously treated with the compounds and infected with IAV PR8 at 37℃ for 1 h. (C) Post-treatment: A549 cells were treated with the compounds right after the 1 h-adsorption period.

Virus yield reduction assay

Treatment and infection of A549 cells in 12-well plates were performed as described. Culture supernatants were harvested at 72-h post-infection to assay for virus yield.

Virucidal assay

IAV PR8 (2×10^4 $TCID_{50}$/mL) was treated with the compounds at 37℃ for 1 h. After that, the infectivity of the compound-treated virus was determined using $TCID_{50}$ assay [30]. Virus treated with serum-free DMEM alone at 37℃ for 1 h serves as the negative control group, whereas virus treated with serum-free DMEM alone at 60℃ for 1 h serves as the positive control group as high temperature abolishes IAV infectivity [31].

Reverse transcription-real time polymerase chain reaction (RT-qPCR)

At 48-h post-infection, the total RNA of A549 cells in 12-well plates was extracted using Favorgen FavorPrep™ Blood/Cultured Cell Total RNA Mini Kit (Cat No.: FABRK 001–1) according to the manufacturer's protocol. After that, 1 µg of total RNA was used for reverse transcription using biotechrabbit™ cDNA Synthesis Kit (Cat No.: BR400404). qPCR for viral nucleoprotein (NP), cytokines and IFNs (Table S1) was carried out using Roche LightCycler® 480 Real-Time PCR System according to the recommended cycling conditions of Qiagen QuantiNova™ SYBR® Green PCR Kit (Cat No.: 208052). 18S ribosomal RNA (rRNA) was used as the

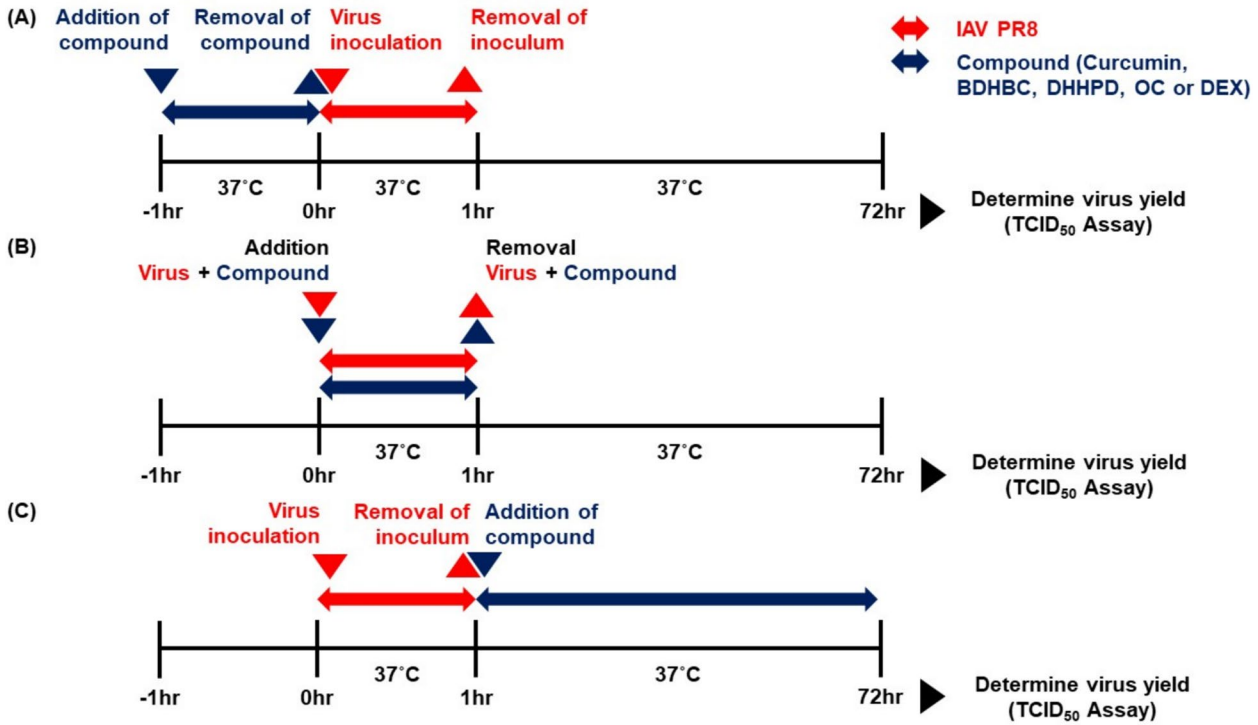


Fig. 2 Schematic diagram of IAV infection and different treatment modes of A549 cells. **A** Pre-treatment: A549 cells were treated with the compounds at 37℃ for 1 h, followed by IAV PR8 infection. **B** Co-treatment: A549 cells were simultaneously treated with the compounds and infected with IAV PR8 at 37℃ for 1 h. **C** Post-treatment: A549 cells were treated with the compounds right after the 1 h-adsorption period. At 72-h post-infection, virus yield was determined via $TCID_{50}$ assay

housekeeping gene as GAPDH expression was altered by IAV infection (Fig. S3). Fold change relative to the negative control group was calculated using the formula $2^{-\Delta\Delta Ct}$.

Western blot

At 24- or 48-h post-infection, the protein of A549 cells in 6-well plates was extracted using radioimmunoprecipitation (RIPA) lysis buffer in the presence of protease and phosphatase inhibitor cocktail. After that, equal amounts of protein samples (20 μg) were subjected to Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE), wet transfer and subsequently blocking (1 h) using 5% bovine serum albumin (BSA) in Tris-buffered saline with Tween-20 (TBST). Primary antibodies incubation was done at 4℃ overnight, followed by secondary antibodies incubation at room temperature for 1 h (Table S2). The protein bands were detected using Advansta WesternBright Sirius HRP substrate (Cat No.: K-12043) in Vilber Lourmat Fusion FX gel documentation system. The intensity of protein bands was determined using the software ImageJ version 150 (National Institutes of Health, USA). β-actin was used as the loading control.

Enzyme-linked immunosorbent assay (ELISA)

The levels of pro-inflammatory cytokines (IL-6, IL-8 and IP-10) and IFNs (IFN-β and IFN-λ1) were measured using R&D Systems DuoSet ELISA kits according to the manufacturer's protocol.

Statistical analysis

Results are presented as mean ± standard deviation (SD) of three independent experiments. One-way analysis of variance (ANOVA) and post hoc Dunnett's test were performed using GraphPad Prism 8.0. A p value < 0.05 was considered statistically significant.

Results

BDHBC and DHHPD have similar cytotoxicity profiles like curcumin in A549 cells

The cytotoxic effects of curcumin, BDHBC and DHHPD on A549 cells were evaluated using MTT. As shown in Fig. 3, the compounds had no significant effect on A549 cell viability at 20 μM and lower concentrations. Conversely, significant cytotoxic effects were observed at 40 and 80 μM. However, their median cytotoxic concentrations (CC_{50}) differ slightly. DHHPD has the highest CC_{50} (52.75 μM), followed by curcumin (41.50 μM) and BDHBC (36.80 μM) (Fig. S4). In subsequent assays, the non-cytotoxic concentrations of the compounds at 5, 10 and 20 μM were used.

Antiviral effects of curcumin, BDHBC and DHHPD on IAV in different modes of treatment

As curcumin has been shown to inhibit IAV infection in different modes of treatment in MDCK cells [16], we investigated whether the same was observed for BDHBC and DHHPD in A549 cells. As shown in Fig. 4A, DHHPD, but not curcumin and BDHBC, showed significant prophylactic antiviral effect on IAV. Pre-treatment of A549 cells with 10 and 20 μM DHHPD significantly decreased virus titres by 0.79 and 0.95 $\log_{10}$ $TCID_{50}$/mL compared to the NC group. However, when used as a co-treatment, all three compounds had no significant effect on progeny virus level (Fig. 4B). Conversely, post-treatment of IAV-infected A549 cells with 5, 10 and 20 μM curcumin significantly inhibited progeny virus release by 1.19, 1.24 and 1.68 $\log_{10}$ $TCID_{50}$/mL, respectively (Fig. 4C). Virus titres were also significantly reduced by 0.74 and 1.93 $\log_{10}$ $TCID_{50}$/mL in 10 and 20 μM DHHPD post-treatment groups. On the other hand, significant reduction of progeny virus level was only observed at 20 μM of BDHBC but not lower concentrations. Collectively, curcumin showed the strongest antiviral effect on IAV in the post-treatment mode, followed by DHHPD and BDHBC. In addition to prophylactic and/or therapeutic antiviral effects, all three compounds at the highest non-cytotoxic concentration (20 μM) also significantly reduced IAV infectivity, suggesting that they have virucidal effect on IAV (Fig. 4D). Consistent with its function as a neuraminidase inhibitor, OC (100 μM) demonstrated significant inhibitory effect on progeny virus release in the post-treatment mode, with about 3.0 $\log_{10}$ $TCID_{50}$/mL reduction (Fig. 4C). No significant effect was recorded in other modes of treatment. By contrast, the potent anti-inflammatory drug DEX (10 μM) had no effect on IAV titres in all treatment modes.

BDHBC and DHHPD inhibit IAV replication in A549 cells

As the compounds demonstrated the strongest antiviral effect in the post-treatment mode, it was postulated that they target IAV replication. Thus, the effects of curcumin, BDHBC and DHHPD on viral NP expression were further evaluated. As shown in Fig. 5A and 5B, curcumin significantly suppressed IAV NP expression by at least 78.7% and 80.0% at both gene and protein levels, respectively. Significant inhibition was also observed for all three concentrations of DHHPD; however, the inhibitory effect at 5 μM was relatively weaker (34.4% inhibition for gene and 63.9% inhibition for protein) compared to curcumin. On the other hand, only 20 μM BDHBC significantly inhibited NP gene and protein expression by 55.0% and 60.2%, respectively. OC significantly reduced NP expression at both gene and protein levels by 63.1% and 53.5%, respectively. By contrast, DEX had no effect

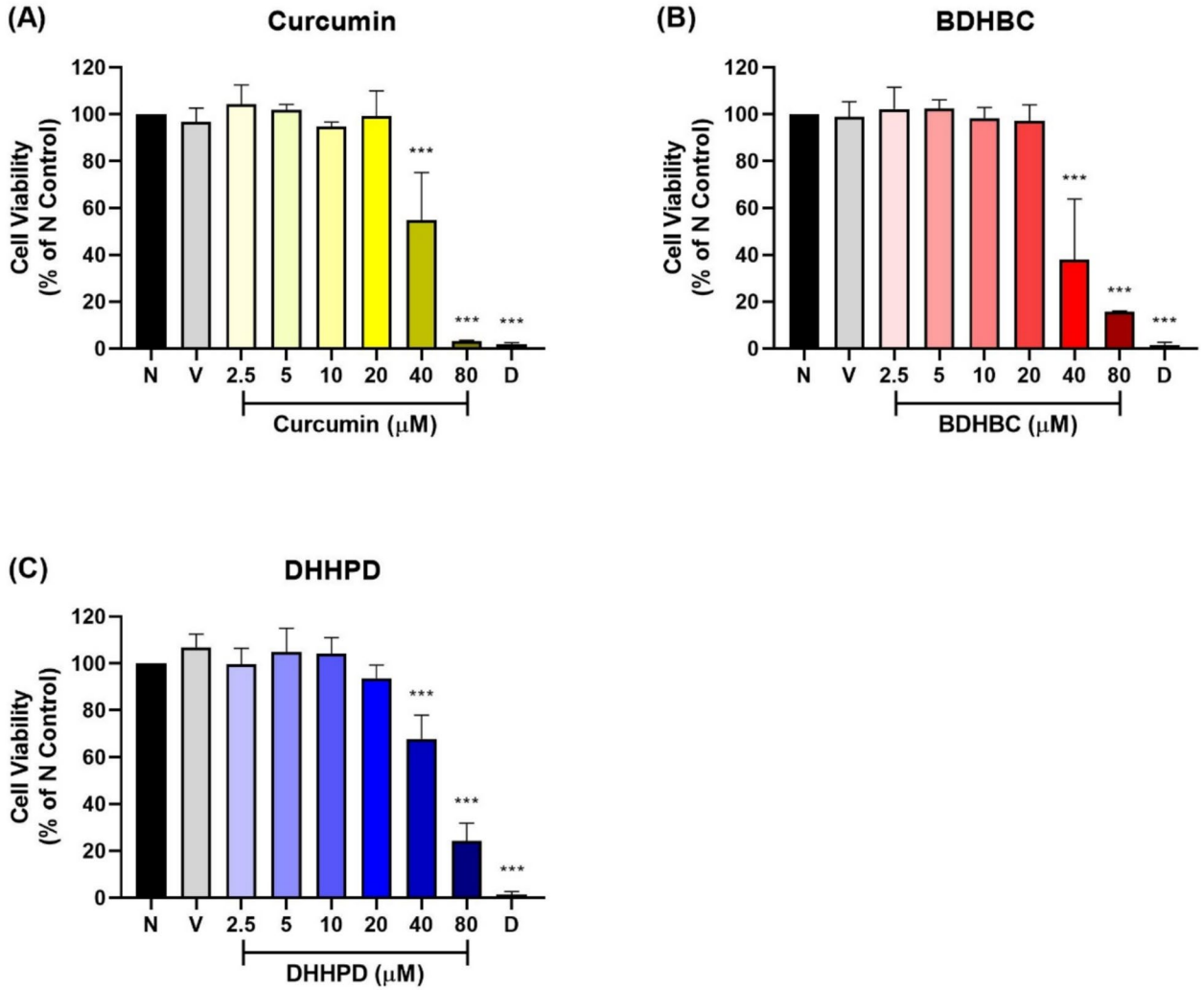


Fig. 3 BDHBC and DHHPD have similar cytotoxicity profiles in A549 cells as curcumin. A549 cells were treated with increasing concentrations of (**A**) curcumin, (**B**) BDHBC and (**C**) DHHPD for 72 h, followed by the assessment of cell viability using MTT assay. The cell viability was expressed as a percentage of the normal control group. N: Normal control group; V: Vehicle control group – 0.1% DMSO; D: 100% DMSO group (positive control). Data are presented as mean ± SD of three independent experiments. *** $p < 0.001$ represents significant difference compared to V (ANOVA, followed by post hoc Dunnett's test)

on NP protein level, but significantly increased NP gene by 51.7%.

BDHBC and DHHPD inhibit pro-inflammatory cytokines expression at gene and protein levels in IAV-infected A549 cells

Pro-inflammatory cytokines play a crucial role in the pathogenesis of severe influenza [4]. To assess whether the compounds suppress pro-inflammatory cytokines in the best antiviral treatment mode, IAV-infected A549 cells were treated right after the adsorption period (post-treatment). Consistent with its strong inhibitory effect on viral replication, curcumin significantly suppressed IL-6, IL-8 and IP-10 expression at both gene and protein levels compared to the NC group (Fig. 6A and 6B). DHHPD at 10 and 20 μM also demonstrated significant inhibitory effect on these cytokines, which was comparable to curcumin at respective concentrations. However, the effect of 5 μM DHHPD was largely insignificant. On the other hand, a weaker inhibitory effect on all cytokines was recorded for BDHBC at 20 μM. Generally, curcumin as a post-treatment showed the strongest inhibitory effect on pro-inflammatory cytokines, followed by DHHPD and BDHBC. The antiviral drug, OC, significantly inhibited IL-6 and IL-8 expression at both gene and protein levels but had no effect on IP-10. At protein level, DEX showed very strong inhibition on

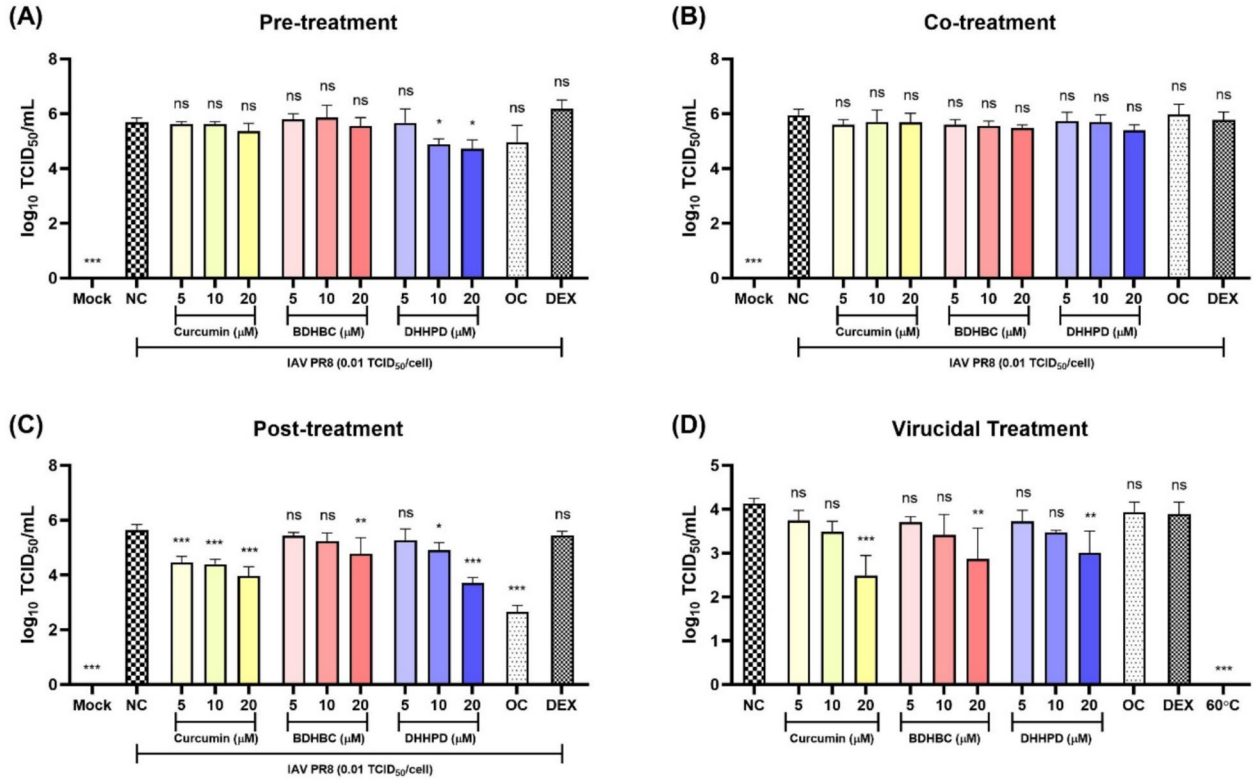


Fig. 4 Antiviral effects of curcumin, BDHBC and DHHPD on IAV in different modes of treatment. **A** Pre-treatment: A549 cells were treated with the compounds at 37℃ for 1 h, followed by IAV PR8 infection. **B** Co-treatment: A549 cells were simultaneously treated with the compounds and infected with IAV PR8 at 37℃ for 1 h. **C** Post treatment: A549 cells were treated with the compounds right after the 1 h-adsorption period. **A**-**C** At 72-h post-infection, virus yield was determined via $TCID_{50}$ assay. **D** Virucidal treatment: IAV PR8 was treated with the compounds at 37℃ for 1 h, followed by the measurement of infectivity via $TCID_{50}$ assay. Mock: Non-infected control group; NC: Negative control group – (**A**-**C**) infected cells without any treatment and (**D**) IAV PR8 treated with medium alone at 37℃ for 1 h; OC: Oseltamivir carboxylate (100 μM); DEX: Dexamethasone (10 μM); 60℃: Virus treated with medium alone at 60℃ for 1 h (positive control). Data are presented as mean ± SD of three independent experiments. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ represent significant difference compared to NC (ANOVA, followed by post hoc Dunnett's test). "ns" represents no significant difference

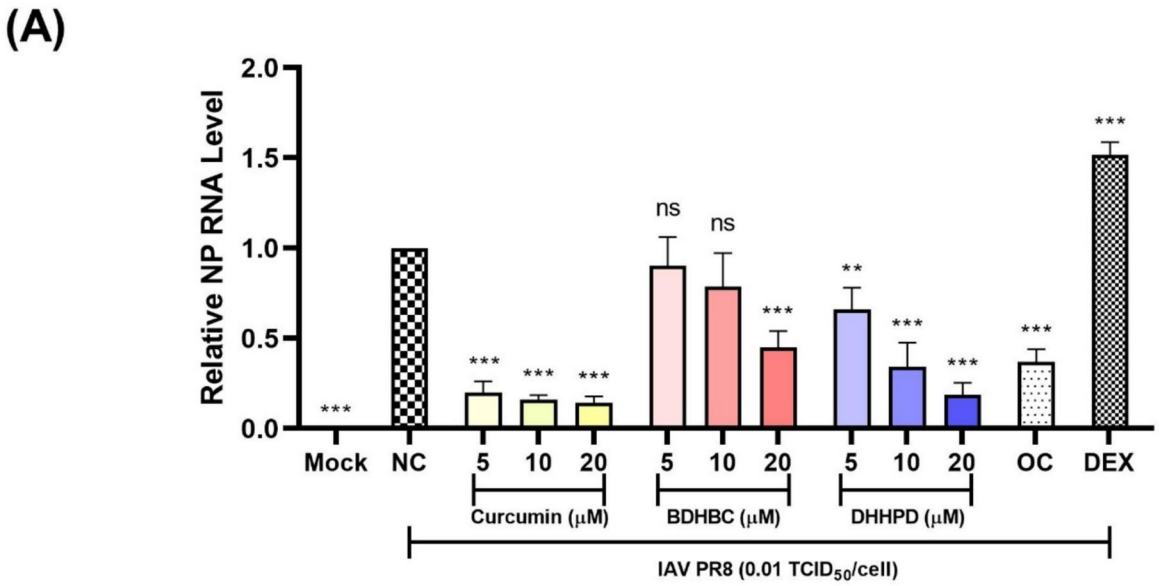
IL-8, moderate inhibition on IL-6, but no effect on IP-10. However, surprisingly DEX only significantly suppressed IL-8 gene level but significantly increased IL-6 and IP-10 gene levels.

BDHBC and DHHPD inhibit IFNs expression at gene and protein levels in IAV-infected A549 cells

Other than pro-inflammatory cytokines, IFNs secreted in response to infection also contribute to disease severity [32]. As shown in Fig. 6C and 6D, all three concentrations of curcumin significantly inhibited IAV-induced IFN-β secretion, but significant inhibition of IFN-β gene level was only observed at 20 μM curcumin. IFN-β secretion was also significantly reduced by BDHBC at 20 μM as well as DHHPD at 10 and 20 μM. Like curcumin, their inhibitory effects on IFN-β gene level were weaker as significant inhibition was only observed for 20 μM DHHPD. On the other hand, IFN-λ1 gene and protein levels were significantly reduced by curcumin (5, 10 and 20 μM), BDHBC (5 μM) and DHHPD (10 and 20 μM). OC demonstrated a moderate inhibitory effect on IFN-β and IFN-λ1, whereas DEX had no effect on IFN-β but significantly increased IFN-λ1 gene without affecting its protein level.

(See figure on next page.)

Fig. 5 BDHBC and DHHPD inhibit IAV NP expression. A549 cells were infected with IAV PR8 and subsequently treated with the compounds for 48 h. IAV NP (**A**) gene and (**B**) protein levels were determined via RT-qPCR and Western blot, respectively. Mock: Non-infected control group; NC: Negative control group – infected cells without any treatment; OC: Oseltamivir carboxylate (100 μM); DEX: Dexamethasone (10 μM). Data are presented as mean ± SD of three independent experiments. *** $p < 0.001$ and ** $p < 0.01$ represent significant difference compared to NC (ANOVA, followed by post hoc Dunnett's test). "ns" represents no significant difference



(B)

Curcumin (μM)	-	-	5	10	20	-	-	-	-	-	-	-	-
BDHBC (μM)	-	-	-	-	-	5	10	20	-	-	-	-	-
DHHPD (μM)	-	-	-	-	-	-	-	-	5	10	20	-	-
OC (μM)	-	-	-	-	-	-	-	-	-	-	-	100	-
DEX (μM)	-	-	-	-	-	-	-	-	-	-	-	-	10
IAV PR8	-	+	+	+	+	+	+	+	+	+	+	+	+

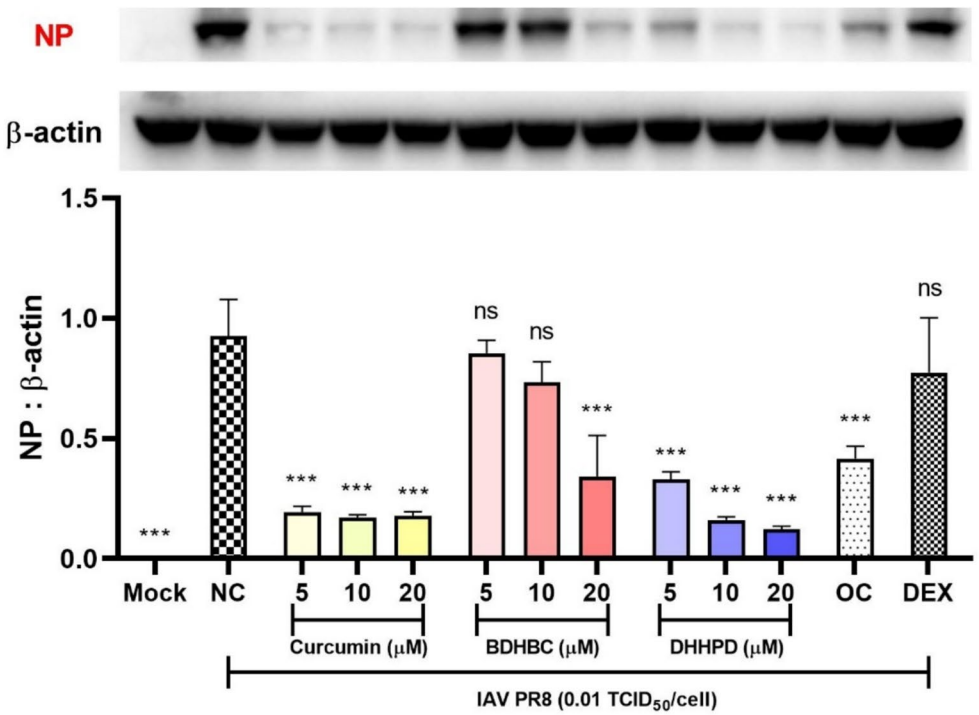


Fig. 5 (See legend on previous page.)

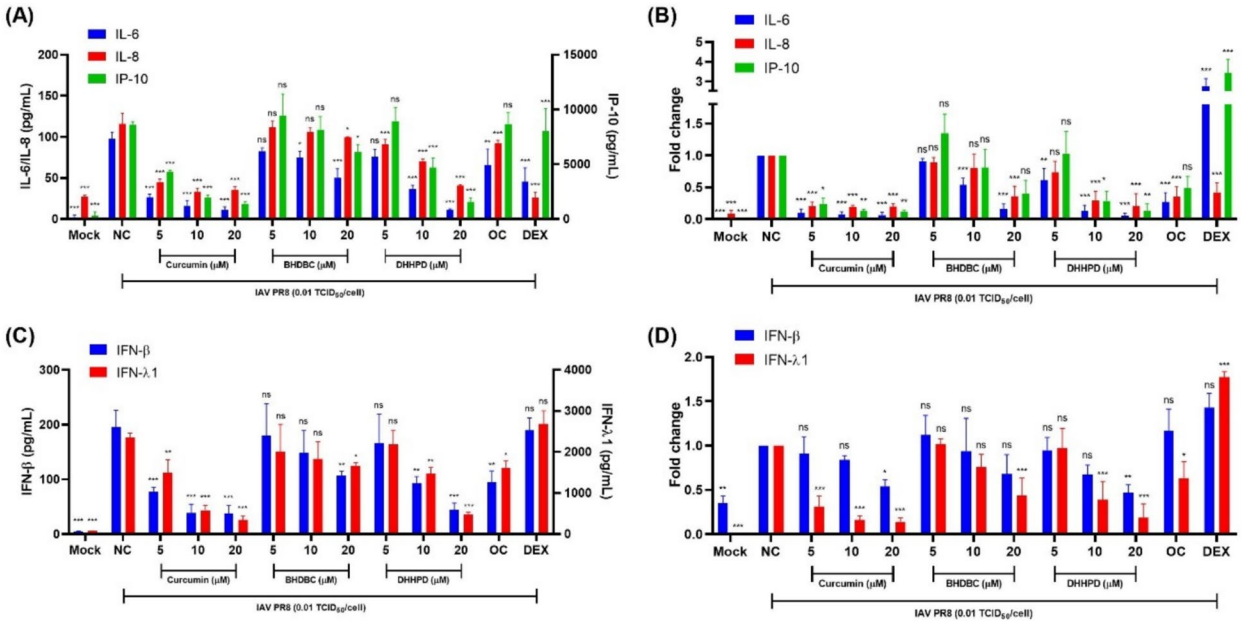


Fig. 6 BDHBC and DHHPD inhibit IAV-induced pro-inflammatory cytokines and IFNs. A549 cells were infected with IAV PR8 and subsequently treated with the compounds. Culture supernatants harvested at 72-h post-infection were assayed for (**A**) pro-inflammatory cytokines (IL-6, IL-8 and IP-10) and (**C**) IFNs (IFN-β and IFN-λ1) protein levels via ELISA. mRNA levels of (**B**) pro-inflammatory cytokines (IL-6, IL-8 and IP-10) and (D) IFNs (IFN-β and IFN-λ1) at 48-h post-infection were determined via RT-qPCR. Mock: Non-infected control group; NC: Negative control group – infected cells without any treatment; OC: Oseltamivir carboxylate (100 μM); DEX: Dexamethasone (10 μM). Data are presented as mean ± SD of three independent experiments. For each cytokine, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ represent significant difference compared to NC (ANOVA, followed by post hoc Dunnett's test). "ns" represents no significant difference

BDHBC and DHHPD inhibit multiple signalling pathways mediated by RIG-I

During infection, the recognition of viral nucleic acids by PRRs such as RIG-I triggers the activation of different signalling pathways, including NF-κB, AP-1 and IRFs, which lead to the production of pro-inflammatory cytokines and IFNs [8]. Additionally, NF-κB, c-JUN, Erk1/2 MAPK and Akt are required for IAV replication [12]. Consistent with their strong inhibitory effects on IAV replication (Fig. 5) as well as pro-inflammatory cytokines and IFNs production (Fig. 6), curcumin and DHHPD were found to significantly inhibit the activation of NF-κB p65, MAPKs (p38 and Erk1/2), AP-1 (c-JUN and ATF-2), IRF3 and Akt (Fig. 7). The weaker antiviral and anti-inflammatory effect of BDHBC during IAV infection was reflected by the selective inhibition of P-p65, c-JUN, P-Erk1/2 and P-Akt^{S473} but not other signalling pathways. Notably, the inhibition of these pathways by BDHBC was also weaker compared to curcumin and DHHPD. On the other hand, OC and DEX significantly inhibited IAV-induced activation of NF-κB p65, Erk1/2 MAPK, AP-1 and IRF3 without affecting the levels of P-p38 and P-Akt^{S473}.

BDHBC and DHHPD do not affect Stat1 activation

As antiviral and pro-inflammatory responses in IAV-infected airway epithelial cells are regulated by the cytosolic RNA sensor RIG-I [7], the expression of RIG-I was next determined. As shown in Fig. 8, curcumin, BDHBC and DHHPD did not inhibit IAV-induced RIG-I expression in A549 cells. Furthermore, they also had no significant effect on P-Stat1 levels that regulate RIG-I expression [33], indicating that they had no effect on Stat1 activation during IAV infection. In support of this idea, the activation of IRF7, which is another downstream target of Stat1 activation [34], was found to be unaffected by BDHBC and DHHPD. However, curcumin significantly increased IRF7 protein level compared to the NC group. OC also had no significant effect on RIG-I expression, Stat1 activation and IRF7 expression. On the other hand, DEX did not affect RIG-I and IRF7 expression but significantly suppressed IAV-induced Stat1 activation.

Discussion

Most of the studies which reported the anti-IAV effects of curcumin used MDCK cells as an in vitro model [16, 18, 35, 36]. As MDCK cells originated from the kidney tissue of an adult cocker spaniel [37], this study used A549 lung epithelial cell line to assess the antiviral effects

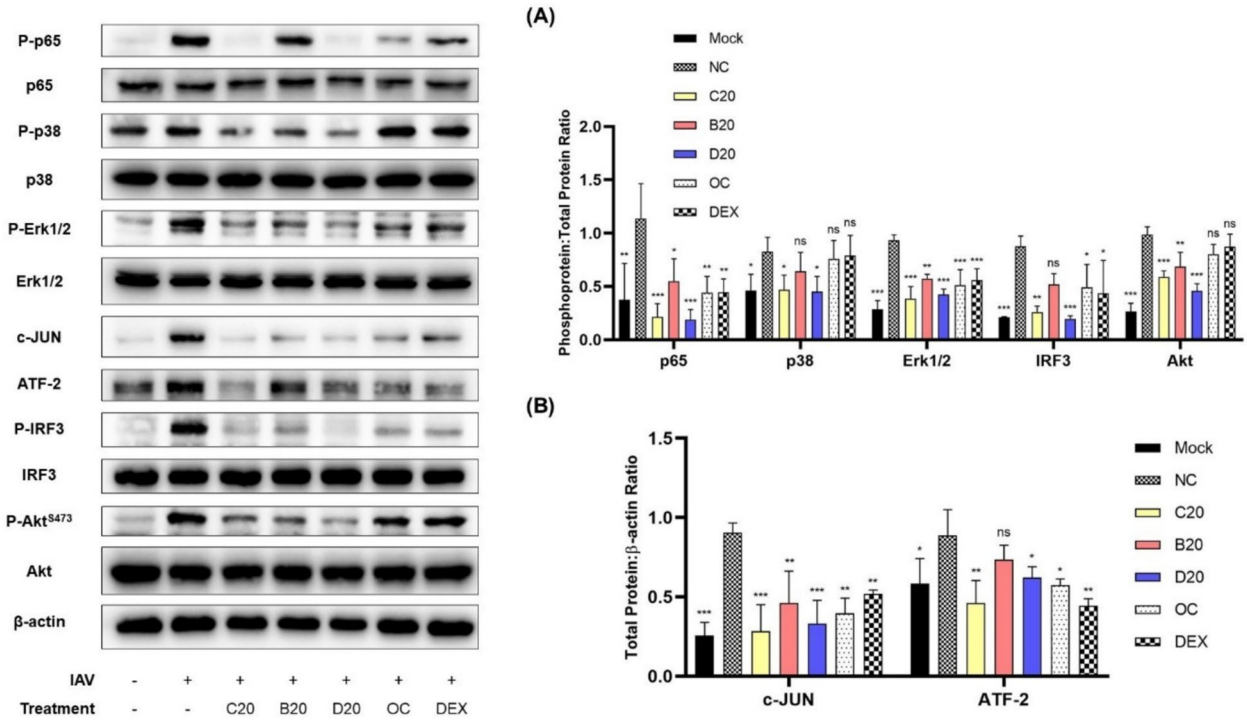


Fig. 7 BDHBC and DHHPD inhibit multiple RIG-I-mediated signalling pathways. A549 cells were infected with IAV PR8 and subsequently treated with the compounds for 24 h (for p38, IRF3 and Akt) or 48 h (for p65, Erk1/2, c-JUN and ATF-2). The levels of (**A**) phosphoprotein relative to total protein or (**B**) total protein relative to β-actin in the whole cell lysates were determined via Western blot. Mock: Non-infected control group; NC: Negative control group – infected cells without any treatment. Data are presented as mean ± SD of three independent experiments. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ represent significant difference compared to NC (ANOVA, followed by post hoc Dunnett's test). "ns" represents no significant difference

of curcumin-like diarylpentanoid analogues on IAV. Curcumin has been shown to inhibit IAV infection in MDCK cells in multiple modes of treatment, but it had no effect in IAV-infected human macrophages when used as a post-treatment [19]. Besides that, a study by Han et al. [17] which demonstrated the anti-IAV effect of curcumin in A549 cells only used it as a post-treatment. The antiviral effects of curcumin in other modes of treatment have not been evaluated in A549 cells. Thus, this study compares the antiviral effects of curcumin, BDHBC and DHHPD on IAV in the pre-, co- and post-treatment modes in A549 cells. Here we demonstrate that curcumin inhibited IAV infection in the post-treatment, but not pre- and co-treatment modes (Table 1), suggesting that its antiviral effect might be cell type-dependent. Chen et al. [35] has shown that 8 h of curcumin pre-treatment (30 μM) significantly inhibited progeny virus release in IAV-infected MDCK cells, whereas Dai et al. [16] has demonstrated that 3 h of curcumin pre-treatment (25 μg/mL or ~ 67.9 μM) did not affect virus yield. These findings indicate that the prophylactic antiviral effect of curcumin against IAV may also depend on its pre-treatment duration. Interestingly, DHHPD pre-treatment for only 1 h showed significant antiviral effect, suggesting that it has prophylactic effect against IAV infection. This could be due to a higher cellular uptake of DHHPD in A549 cells within the 1-h pre-treatment compared to curcumin. A previous study by Chang et al. [38] has shown that a higher cellular uptake of other curcumin-like analogues, such as demethoxycurcumin and bisdemethoxycurcumin, was associated with stronger anticancer effects in breast cancer cells. Future study should correlate their cellular uptake levels with the prophylactic antiviral effects against IAV infection. Therefore, DHHPD may have the potential to be further developed as an antiviral drug for IAV with dual therapeutic and prophylactic properties. This unique characteristic of DHHPD, which is not observed for curcumin and BDHBC, may also be beneficial for the prevention of IAV infection among people who are susceptible to severe influenza complications, such as those with chronic diseases.

The absence of antiviral effect of curcumin in the co-treatment mode might be due to lower concentrations used (≤ 20 μM) compared to previous studies [35, 36]. While molecular docking suggests that curcumin may block the receptor binding region on IAV haemagglutinin

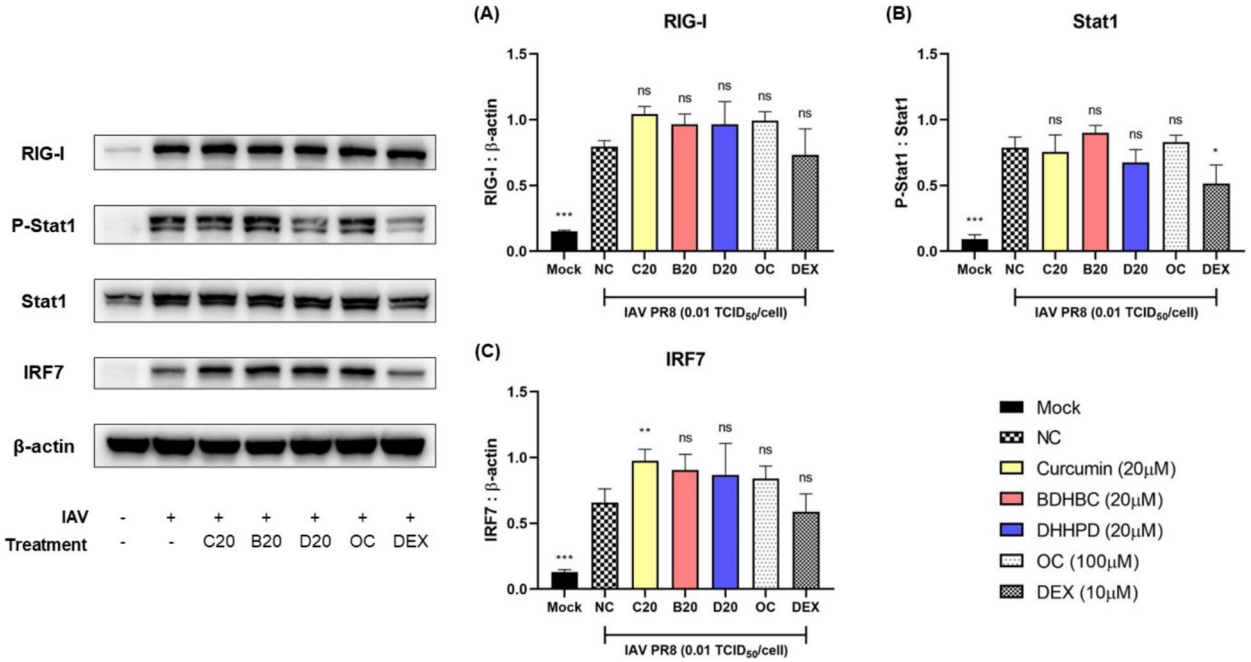


Fig. 8 BDHBC and DHHPD do not affect Stat1 activation. A549 cells were infected with IAV PR8 and subsequently treated with the compounds for 48 h. The levels of (**A**) RIG-I, (**B**) P-Stat1/Stat1 and (**C**) IRF7 in the whole cell lysates were determined via Western blot. β-actin was used as the loading control. Mock: Non-infected control group; NC: Negative control group – infected cells without any treatment. Data are presented as mean ± SD of three independent experiments. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ represent significant difference compared to NC (ANOVA, followed by post hoc Dunnett's test). "ns" represents no significant difference

Table 1 Summary of antiviral effects of curcumin, BDHBC and DHHPD on IAV. "+" and "-" indicate the presence and absence of antiviral effect on IAV PR8, respectively

Compounds	Pre-treatment	Co-treatment	Post-treatment	Virucidal Treatment
Curcumin	-	-	+	+
BDHBC	-	-	+	+
DHHPD	+	-	+	+
OC	-	-	+	-
DEX	-	-	-	-

(HA) to prevent viral entry [36], curcumin was found to suppress haemagglutination activity at 31.2 μM [35]. Thus, it is unlikely that curcumin, as well as BDHBC and DHHPD, inhibits HA at the concentrations used in this study. Apart from that, curcumin has been previously shown to irreversibly inhibit the infectivity of enveloped viruses, including IAV, by disrupting their viral envelope [39]. Like curcumin, BDHBC and DHHPD at the highest non-cytotoxic concentration (20 μM) also demonstrated virucidal effect on IAV PR8. Therefore, it is possible that BDHBC and DHHPD could also disrupt the viral envelope of IAV. As the compounds directly reduced IAV infectivity in virucidal assay, it is intriguing to note that the compounds had no antiviral effect during co-treatment. One potential reason could be that IAV binds to receptor rapidly, thereby preventing the compounds from affecting the virions.

Overall, all three compounds showed the strongest antiviral effects on IAV in the post-treatment mode, indicating that they may target viral replication. This is substantiated by their inhibition of viral NP expression at both gene and protein levels. NP is the most abundant viral protein that regulates transcription and replication of vRNA as well as nuclear trafficking of viral ribonucleoproteins (vRNPs) [40]. Reduction of NP expression consequently attenuates virus yield by infected cells. When used as a post-treatment, curcumin, BDHBC and DHHPD also significantly suppressed pro-inflammatory cytokines (IL-6, IL-8 and IP-10) and IFNs (IFN-β and IFN-λ1) in IAV-infected A549 cells, suggesting that they also exhibit anti-inflammatory effects during IAV infection. Notably, the strongest inhibitory effect was shown by curcumin, followed by DHHPD and BDHBC. While both RIG-I and TLR3 regulate IAV-induced cytokine responses, we found that A549 cells do not constitutively express TLR3 gene (data not shown). Hence, this study further investigated the effects of curcumin-like diarylpentanoid analogues on RIG-I-mediated pathways that regulate viral replication and cytokine responses.

In line with their strong antiviral and anti-inflammatory effects, curcumin and DHHPD were found to suppress several pathways, including NF-κB, AP-1, p38 and Erk1/2 MAPKs, IRF3 and Akt (Fig. 9). On the other hand, BDHBC selectively inhibits NF-κB, c-JUN, Erk1/2 and Akt. While the compounds suppressed the expression of IFN-β and IFN-λ1, the unchanged IRF7 expression suggests that the IFN-Stat1-IRF7 signalling axis was not inhibited. Wu et al. [41] previously demonstrated that a second phase IFN induction in IAV-infected A549 cells is mediated primarily by IRF7, rather than IRF3. Specifically, siRNA-mediated knockdown of IRF7, but not IRF3, significantly reduced RIG-I mRNA expression and IFN induction following IAV infection. As the compounds did not completely abolish IFN-β and IFN-λ1 production (Fig. 6), the remaining IFN levels may be still sufficient to stimulate IRF7 and RIG-I induction via the JAK-STAT1 pathway. Without disrupting Stat1 activation, these compounds would therefore have no effect on the IRF7-mediated positive feedback loop of IFN responses. Taken together, our results suggest that curcumin and DHHPD inhibit RIG-I-mediated signalling pathways without impairing IAV-induced Stat1 activation.

This study included OC and DEX as antiviral and anti-inflammatory drug controls, respectively, to relate viral replication and cytokine responses. Interestingly, OC, which demonstrated strong inhibitory effect on progeny virus release during post-treatment (Fig. 4C), only showed modest inhibition on IAV-induced cytokine responses (Fig. 6). By contrast, the anti-inflammatory drug DEX, which had no antiviral effect in all treatment modes (Fig. 4), only significantly suppressed IL-6 and IL-8. Some discrepancies between RT-qPCR and ELISA results could be due to different time points used to determine gene (i.e. 48-h post-infection) and protein (i.e. 72-h post-infection) levels. Nevertheless, these findings indicate that the suppression of viral replication does not necessarily result in cytokine inhibition and vice versa. While influenza antiviral therapy has been shown to significantly reduce the duration of flu symptoms and virus shedding, it may not be effective for the indirect damage caused by cytokine storm during later stages of infection [6, 42]. As such, curcumin-like diarylpentanoid analogues with both antiviral and anti-inflammatory properties, particularly DHHPD, may offer better therapeutic benefits for severe influenza compared to the antiviral monotherapy.

Nevertheless, this study has some limitations. Firstly, PR8 was selected as the IAV strain to assess the antiviral effects of the compounds. Their effects on other subtypes or lineages of influenza viruses, including IAV H3N2 and IBV (Victoria and Yamagata lineages), should also be evaluated. As A549 is a continuous cell line, a more physiologically relevant model like primary airway

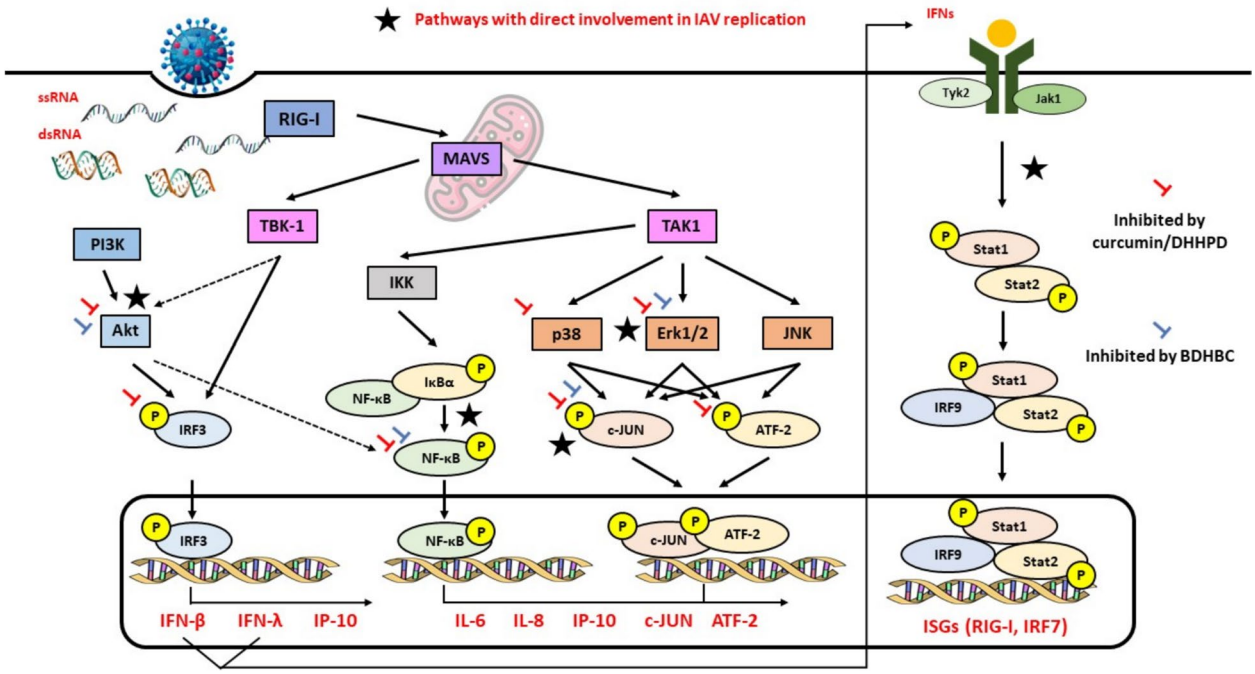


Fig. 9 Overview of IAV-activated signalling pathways inhibited by curcumin, BDHBC and DHHPD. Curcumin and DHHPD inhibit the same signalling pathways, including NF-κB, AP-1, p38 and Erk1/2 MAPKs, IRF3 and Akt, without affecting Stat1 activation that regulates RIG-I expression. On the other hand, BDHBC only suppresses the activation of certain signalling molecules, including NF-κB, c-JUN, Erk1/2 and Akt

epithelial cells could be used to validate the inhibitory effects on viral replication and cytokines. Besides that, a specific molecular target was not identified due to the involvement of many intermediate signalling molecules in the RIG-I-mediated signalling pathways investigated in this study. While curcumin demonstrated stronger antiviral effects against IAV infection in the post-treatment mode, DHHPD has several advantages compared to curcumin, including its prophylactic antiviral effect and better drug-like properties, such as increased solubility and stability. Other than that, the stronger antiviral effect of DHHPD against RV infection may also give it an edge for viral-induced exacerbations of chronic respiratory diseases, which are commonly triggered by influenza viruses and RV. Numerous studies have reported the limited clinical efficacy of curcumin for different diseases, largely attributed to its low bioavailability [43]. Although the in vivo bioavailability of DHHPD has not yet been characterized, it has previously demonstrated pharmacological activity in animal models of nociception [25], suggesting systemic bioactivity. Intraperitoneal injection of DHHPD significantly reduced formalin-induced paw licking in both the early/neurogenic phase and late/inflammatory phases in ICR mice at doses as low as 0.1 mg/kg and 1 mg/kg, respectively. Although this study did not include curcumin for a direct head-to-head comparison, a previous report by El Nebrisi et al. [44] showed that curcumin inhibited paw licking in ICR mice only during the late/inflammatory phase and required a much higher dose (10 mg/kg), with no significant effect observed in the early phase even at doses up to 30 mg/kg. Therefore, future studies utilizing animal models of influenza virus infection are worthwhile to evaluate both the antiviral efficacy and pharmacokinetic properties of DHHPD. Given the well-established temporal dynamics of influenza A virus infection in vivo where early viral replication is followed by a delayed hyperinflammatory response [45], it is also essential to assess the therapeutic potential of DHHPD across both phases, including its ability to suppress viral replication and mitigate cytokine-driven pathology during the late phase.

Abbreviations

ANOVA	One-way analysis of variance
AP-1	Activator protein-1
ARDS	Acute respiratory distress syndrome
BCA	Bicinchoninic acid
BDHBC	2-Benzoyl-6-(3,4-dihydroxybenzylidene)cyclohexen-1-ol
BSA	Bovine serum albumin
CC_{50}	Median cytotoxic concentration
cDNA	Complementary deoxyribonucleic acid
COPD	Chronic obstructive pulmonary disease
CPE	Cytopathic effect
DEX	Dexamethasone
DHHPD	5-(3,4-Dihydroxyphenyl)-3-hydroxy-1-(2-hydroxyphenyl) penta-2,4-dien-1-one
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
EC_{50}	Median effective concentration
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
HA	Haemagglutinin
HRP	Horseradish peroxidase
IAV	Influenza A virus
IFN	Interferon
IL	Interleukin
IP-10	Interferon γ-induced protein 10
IRF	Interferon regulatory factor
MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemoattractant protein-1
MDCK	Mardin-Darby canine kidney
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	Neuraminidase
NF-κB	Nuclear factor-kappa B
OC	Oseltamivir carboxylate
PBS	Phosphate buffered saline
PRR	Pattern recognition receptor
PVDF	Polyvinylidene fluoride
RIG-I	Retinoic acid-inducible gene I
RIPA	Radioimmunoprecipitation
rRNA	Ribosomal RNA
RSV	Respiratory syncytial virus
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
RV	Rhinovirus
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SD	Standard deviation
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
TBST	Tris-buffered saline with Tween-20
$TCID_{50}$	Median tissue culture infective dose
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone
vRNA	Viral ribonucleic acid
vRNP	Viral ribonucleoprotein

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12985-025-02914-4.

Supplementary Material 1.

Supplementary Material 2.

Acknowledgements

The authors would like to thank the members of Cell Signalling Lab and Virology Lab, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia for providing technical support for this study.

Authors' contributions

Conceptualization: KYL and CLT; Data curation: KYL, HY-C, and CLT; Formal analysis: KYL, FA, and SWL; Funding acquisition: CLT; Investigation: KYL, FA, and SWL; Methodology: KYL, HY-C, FA, and CLT; Project administration: CLT; Resources: HY-C, FA, SWL, MRS, and CLT; Software: KYL, FA, and SWL; Supervision: HY-C, HHH, MTL and CLT; Validation: KYL, HY-C and CLT; Visualization: KYL; Writing – original draft: KYL and CLT; Writing – review: KYL, HY-C, FA, SWL, HHH, MTL, MRS and CLT.

Funding

The study was funded by the Ministry of Higher Education Malaysia under Fundamental Research Grant Scheme (FRGS/1/2023/SKK15/UPM/02/3). KYL was a recipient of Universiti Putra Malaysia Graduate Research Assistantship (GRA) and Special Graduate Research Allowance (SGRA). The funder has no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 5 May 2025 Accepted: 13 August 2025
Published online: 27 October 2025

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