Curcumol Inhibits Encephalomyocarditis Virus by Promoting IFN-β Secretion

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Research article

Keywords: EMCV, Curcumol, IFN-β, antivirus

DOI: https://doi.org/10.21203/rs.3.rs-71551/v1

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Abstract

Background: EMCV infection can cause reproductive failure in sows and acute myocarditis and sudden death in piglets. It has caused huge economic losses to the global pig industry. It is necessary to develop effective new compounds. Traditional Chinese medicine zedoary turmeric oil can be used for treating myocarditis caused by EMCV infection. Curcumol extracted from the roots of Curcuma is one of the main active ingredient of zedoary turmeric oil. To investigate whether curcumol has antiviral activity again the EMCV infection, and what are the molecular mechanisms of such antiviral activity with a focus on the IFN-β signaling pathway.

Method: MTT assay was used to determine the maximum non-toxic concentration (MNTC), 50% cytotoxic concentration (CC_{50}), maximum inhibition rate (MIR) and 50% effective concentration (EC_{50}) against EMCV. Through EMCV load, the anti-viral effect of curcumol was quantitatively determined using real-time quantitative PCR. The effect of curcumol on the expression of IFN-β was checked using real-time quantitative PCR and ELISA. Western blot was used to determine the amounts of MDA5, MAVS, TANK, IRF3 and P-IRF3 proteins in HEK-293T cells infected with EMCV.

Results: The results showed that compared with the positive drug ribavirin control, the MIR of curcumol was greater but the SI value was much smaller than that of ribavirin. The curcumol at 25 μg/ml has significant anti-EMCV activities and increased the expression of IFN-β (P < 0.05). The curcumol inhibited the degradation of TANK protein mediated by EMCV and promoted the expression of MDA5 and P-IRF3, while the protein expression level of MAVS and IRF3 remain unchanged.

Conclusion: Curcumol has biological activity against EMCV which we suggest that IFN-β signaling pathway is one of its mechanisms, providing new ideas for clinical treatment of EMCV infection.

Background

Interferon is a glycoprotein produced by viruses and other types of interferon inducers that stimulate macrophages, lymphocytes, epithelial cells and fibroblasts. Three types of IFN families are known type I (IFN-1, mainly IFN-α/β), type II (IFN-II or IFN-γ) and type III (IFN-III or IFN-λ) [1]. Interferon acts via autocrine and paracrine modes to induce an antiviral state in infected cells and also in neighboring cells containing interferon receptors. Activates JAK/STAT signaling pathway [2] and eventually induce OASs [3], PKR [4], and MX [5]. These antiviral proteins exert an antiviral effect by preventing cell infection and virus spread. The defense mechanism established by IFN can also have effects on cancer cells [6, 7] and inflammation [8]. In particular, the type I interferon has a strong antiviral effect. The rapid production of type I interferon in the early stage of viral infection is an important way of body immunity [9–11].

Encephalomyocarditis virus (EMCV) belongs to the Picornaviridae family and is a nonencapsulated single-stranded RNA virus. EMCV infection can cause reproductive failure in sows and acute myocarditis and sudden death in piglets [12–14]. Studies have shown that MDA5 is a cytoplasmic virus sensor that recognizes dsRNA produced during EMCV replication [15, 16] and activates TBK1 (TANK-binding kinase...
1) and IKKε (IκB kinase ε) through MAVS [17, 18]. These two activated kinases can phosphorylate IRF3, and the phosphorylated IRF3 is then transferred from the cytoplasm to the nucleus, promoting the expression of type I interferon [19, 20].

TANK, a TRAF-binding protein, can inhibit TRAF-2-mediated activation of NF-κB in the TNF-α and CD-40 signaling pathways [21]. TANK, TRAF2 and TBK1 form a trimer to activate NF-κB [22]. Studies have shown that TANK can link TBK1 and IKKε to synergistically activate NF-κB [23]. EMCV 3C protease can alleviate the inhibitory effect of TANK on TRAF6-mediated NF-κB activation by hydrolyzing TANK protein [24]. Others have also shown that EMCV 3C protease can hydrolyze TANK and interfere with TANK-TBK1-IKKε-IRF3 complex formation, thereby inhibiting the production of type I interferon [25].

Traditional Chinese medicine zedoary turmeric oil can be used for treating bruises; It can also help in prevent the influenza, pediatric viral encephalitis and myocarditis caused by EMCV infection [13, 14]. This might be due to the anti-cancer [26, 27] and curcumol extracted from the roots of Curcuma is one of the main active ingredient of zedoary turmeric oil [28]. We aimed to investigate whether zedoary alcohol has anti-EMCV effect and which hidden molecular mechanism is involved in its activity. We constructed an EMCV-infected HEK-293 cells model to observe the effects of curcumol on EMCV-infected HEK-293T cells. Further in vitro experiments were used to determine whether MDA5/IRF3/IFN-β signaling pathway is involved in the antiviral activity of the curcumol.

## Results

### Cytopathic effects

The MNTC and CC₅₀ of curcumol (Fig. 2A) and ribavirin (Fig. 2B) were determined using MTT assay. In a dose dependent manner, HEK-293T cells showed different degrees of lesions, cell shrinkage, rupture, and cytopathic effect. The MNTC of curcumol (25 µg/ml) was less than the MNTC of ribavirin (250 µg/ml) and the CC₅₀ value (124 µg/ml) was also less than that of ribavirin (2538 µg/ml) (Table 1).
Table 1
The cytotoxicity and antiviral activity of the curcumol used in the test

| Constituents | Dissolution | MNTC<sup>a</sup> (µg/ml) | CC<sub>50</sub><sup>b</sup> (µg/ml) | MIR (%)<sup>c</sup> | EC<sub>50</sub><sup>b</sup> (µg/ml) | SI |
|--------------|-------------|--------------------------|-----------------------|-----------------|------------------|---|
| Curcumol     | 1% DMSO     | 25                       | 124 ± 21              | 89              | 6 ± 1.5          | 20 |
| Ribavirin    | DMEM        | 250                      | 2538 ± 540            | 80              | 23 ± 12          | 110 |

<sup>a</sup>Maximum non-toxic concentration

<sup>b</sup>CC<sub>50</sub> and EC values represent mean standard deviation of the mean values in three different experiments.

<sup>c</sup>Maximum inhibition ratio

<sup>d</sup>Selection index

"-": Due to low inhibition ratio, EC<sub>50</sub> was not determined.

**Anti-EMCV activity of curcumol**

The MNTC of test compounds were selected to treat EMCV-infected HEK-293T cells for 24 h. The results showed that curcumol displayed MIR > 50% and SI > 3 (Table 1) and ribavirin showed an expected anti-EMCV activity. Both curcumol (Fig. 2C) and ribavirin (Fig. 2D) alleviated EMCV cytopathic effects in a dose-dependent manner. Microscopically, the virus group (Fig. 2F) showed an increase in cell gap, cell shrinkage and patches of cell death compared with the cell control group (Fig. 2E). The condition of cells in the curcumol treatment group was consistent with the cell control group as shown in Fig. 2G which effectively alleviated the cytopathic effect caused by virus and maintained the cell integrity. Although ribavirin reduced the death of EMCV-infected HEK-293T cells (Fig. 2H) but there is still a certain degree of cytopathic effect. The results showed that curcumol has a better anti-EMCV activity in-vitro than ribavirin.

**Antiviral efficacy of curcumol**

The antiviral efficacy of curcumol in the HEK-293T cells was measured using the viral load that was detected using real-time fluorescent quantitative PCR. Compared with the model group, curcumol (Fig. 3A) and ribavirin (Fig. 3B) significantly (P < 0.05) reduced viral load. These results demonstrated that curcumol and ribavirin significantly inhibited the infection of HEK-293T cells by EMCV.

**Effect of curcumol on the expression of IFN-β gene and protein**

At the 24 h after the HEK-293T cells infection with EMCV, the expression of IFN-β gene in the model group showed no difference from the cell control group. Compared with the model group, curcumol treatment has significantly (P < 0.05) increased IFN-β gene expression in the EMCV-infected HEK-293T cells.
(Fig. 4A) while ribavirin treatment did not (Fig. 4B). The curcumol has significantly increased the expression of IFN-β protein in EMCV-infected BHK-21 cells as detected with ELISA (Fig. 4C). This suggests that increased expression of IFN-β plays an important role in the curcumol's anti-EMCV activity.

**Curcumol relieves the cleavage of TANK by EMCV and promotes the formation of IRF3 phosphorylation**

The expression of MDA5, TANK and P-IRF3 protein decreased significantly ($P<0.05$) after HEK-293T cells infection with EMCV while the expression of MAVS and IRF3 protein showed no significant change. The expression of MAVS post 24 h of curcumol treatment had no significant change, which indicates that increase in IFN-β is not dependent on MAVS.

Compared with the model group, the expression levels of MDA5 and TANK protein were significantly increased after curcumol treatment, and the expression of P-IRF3 was increased while IRF3 expression remained unchanged. These results indicate that curcumol promotes IFN-β expression, possibly by alleviating the hydrolysis of TANK protein by EMCV (Fig. 5).

**Discussion**

Aim of this study was to investigate whether curcumol has anti-EMCV effects and associated molecular mechanisms. Compared with the model group (with virus, no constituent), at the MNTC of curcumol and ribavirin to the EMCV-infected HEK-293T cells, curcumol and ribavirin maintained cell integrity, reduced cell death and viral load ($P<0.05$). The results demonstrate that curcumol at 25 µg/ml has anti-EMCV activity at least equivalent to the potency of ribavirin. Evidence accumulates that curcumol may exert antiviral effects by improved immunity of the host. Pan et al. [29] showed that extracts from curcuma (Curcuma longa) using water, presumably with concentrated curcumol as it is the main ingredient of curcuma can increase the expression of NO in macrophages and improve immunity and telomerase activity. Choi et al. [30] showed that expanded zedoary (Curcuma zedoaria) can also significantly increase its antioxidant activity and reduce LPS-induced IL-6 and TNF-α expression. Therefore we speculate that curcumol improves host's immunity in defense of host from EMCV infection.

In term of the host response to the EMCV infections, this study has confirmed that viral infection causes the decreased expression of MDA5 in the EMCV infected host cells, the HEK-293T cells. MDA5 belongs to the RIG-I-like pattern recognition receptor recognizes dsRNA which is produced during EMCV replication and mediates the production of type I interferon [15, 16]. Li et al. [31] showed that EMCV 2C specifically inhibits the MDA5 mediated IFN-β signaling pathway by interacting with MDA5 and disrupting its normal function. This study showed that infection of HEK-293T cells by EMCV resulted in a significant decrease of the expression of TANK and P-IRF3 protein but did not change the expression of IRF3 and IFN-β.

In term of the curcumol treatment of HEK-293T cells infected by EMCV, this study showed that curcumol has significantly increased the expression of MDA5 although it did not cause any change in MAVS expression after 24 h treatment. The TANK forming complex with TBK1 and TRAF3 and other proteins
and this complex can phosphates IRF3 [18]. The P-IRF3 moves to nucleus, leading to production of IFN-α/β, RANTES, and ISGs. The present study found that protein expression of TANK, P-IRF3 and IFN-β were increased by the curcumol treatment of the EMCV infected cells. It is clear that curcumol has activated these major components of the IFN-β production pathway. It is however noted that the expression of the MAVS, the downstream protein of MDA5 was not changed upon the curcumol treatment. Thus the link between viral RNA sensor proteins, MAD5 to TANK/TBK1/TRAF3 complex is not affected by the curcumol treatment. Does this mean that curcumol can directly activate the TANK? We offer no explanation at this stage but protein binding assay of curcumol with TANK and other proteins may be performed. Increased level of TANK may also affect the pathway for the activation of NF-κB signaling pathway [22]. However we have no direct evidence of the increased production of NF-κB under curcumol treatment.

Conclusion

EMCV infected HEK-293T cells significantly decreased the expression of TANK and P-IRF3 protein in the IFN-β signaling pathway. Curcumol displays anti-EMCV activity by maintaining cell integrity, reducing viral load of EMCV infected HEK-293T cells. The anti-EMCV activity of curcumol is at least by increasing expression of IFN-β pathway. And we speculate that certain protein components of IFN-β signaling pathway might be the potential targets for the anti-EMCV activity of curcumol. This study provides a basis for clinical treatment of EMCV infection by curcumol, laying the foundation for new drug innovation.

Methods

Cell lines and viruses

BHK-21 (ZQ0130) cells were purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd (China). HEK-293T (CL-0005) cells were purchased from Wuhan procell Biotechnology Co., Ltd (China). EMCV NJ08 strain (a gift from Professor Jiang Ping of Nanjing Agricultural University) is preserved in our laboratory with a virulence of $10^{8.5}$ TCID$_{50}$/ml.

Reagents, plasmid, compounds and antibodies

Dulbecco’s modified eagle’s medium (DMEM, Hyclone, USA) supplemented with 10% or 2% heat-inactivated fetal bovine serum (FBS, BI, Israel), 100 IU/ml Penicillin G and 100 µg/ml Streptomycin was used for cell growth or maintenance medium. A 0.25% trypsin (Solarbio, China) was prepared in PBS (pH 7.2). The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT, Solarbio, China) was prepared in PBS (pH 7.2–7.4). Dimethyl sulfoxide (DMSO) was purchased from Solarbio (China). Reverse transcription kit was purchased from TaKaRa (Japan) and 2 × SYBR Green qPCR Master Mix was purchased from Bimake (Low ROX, USA). RIPA cell lysate, protease inhibitor and phosphatase inhibitor were purchased from Solarbio (China). LDS Sample buffer (4×) was purchased from Invitrogen (USA) and IFN-β ELISA kit was purchased from Cusabio (China). The recombinant plasmid for the EMCV 3D
gene was preserved in the laboratory. Curcumol and ribavirin were purchased from China Food and Drug Control Institute, with 99.9% and 100% purity, respectively. The chemical structure of the compounds are shown in Fig. 1.

MDA5 and MAVS Rabbit Polyclonal antibody, GAPDH Mouse Monoclonal antibody, Goat anti-Mouse and Goat anti-Rabbit secondary antibodies were purchased from Proteintech Biotechnology Co., Ltd. (China) TANK Ribbit Polyclonal antibody and IRF3 Rabbit Monoclonal antibody were purchased from Abcam (USA); Phospho-IRF3 Ribbit Polyclonal antibody was purchased from Bioss (China).

### Cytotoxicity assay

The compounds were dissolved as shown in Table 1. Curcumol and ribavirin were serially diluted (2-fold) with DMEM containing 2% FBS into 8 gradients and then added to a single layer of HEK-293T cells in the 96-well plates (100 µl/well) with 8 replicates per gradient. At the same time, cell control group was established. The plates were then incubated at 37 °C and 5% CO₂ for 72 h. The cytopathic condition of cells in the culture was observed every day and photographed. After 72 h of incubation, the supernatant was discarded, 20 µl of MTT was added to each well and incubated for 4 h. Subsequently, the supernatant was discarded and 150 µl of DMSO was added to each well, and incubated for 30 min. The optical density (OD value) was measured at 490 nm using the plate type multi-function analyzer (Tristar2 SLB 942, Berthold Technologies, Berthold Technologies). The cytotoxic ratio (CR) was expressed as CR = (OD control - OD test)/OD control. The CC₅₀ and MNTC values were calculated. CC₅₀ is the constituent concentration at which 50% of cells have developed lesions while MNTC is a maximal non-toxic concentration of a constituent that enables at least 80% of cells to survive.

### Antiviral assay

The HEK-293T cells were cultured in 96-well plates, infected with 100 TCID₅₀/ml EMCV (100 µl/well) and allowed 1.5 h for adsorption. The supernatant was discarded and MNTC of the test compound was serially diluted (2 folds) with DMEM containing 2% FBS into 8 gradients and added to 96-well plates (100 µl/well) with 8 replicates per gradient. Meanwhile, cell control group (no virus and no constituent), model group (with virus, no constituent) and ribavirin positive control group (with virus and ribavirin) were established. The plates were incubated at 37 °C and 5% CO₂ for 24 h. The inhibition ratio (IR) was calculated as IR = (OD test - OD model) / (OD control - OD model). EC₅₀ refers to a compound concentration that is effective in inhibiting 50% of cells infected with EMCV, while selection index (SI) is calculated as SI = CC₅₀/EC₅₀. The maximum inhibition ratio (MIR) of the compound was higher than 50% and SI > 3, and the antiviral effect was significant [32].

### RNA extraction and quantitative real-time PCR

The HEK-293T cells were cultured in 6-well plates, infected with 100 TCID₅₀/ml EMCV (2 mL/well) and allowed for adsorption for 1.5 h. The supernatant was then discarded and the MNTC of curcumol with 2 folds dilution (3 concentration gradients) were added to 6 well plates. Meanwhile, cell control group, model group and ribavirin positive control group were established and incubated for 24 h. One mL of
TRlzol was added to each well until the cells were completely lysed. RNA was extracted according to the standard method and reverse transcription was then completed. Viral load and IFN-β gene expression were detected by realtime PCR. The primer sequence for EMCV 3D gene is as: F 5’-TTAGGGCGGGTTTGTAT-3’, R 5’-TTTGTAGCGGGA GTTA-3’. IFN-β F 5’-ATGACCAACAAGTCTCCTCC-3’ R 5’-GCTCATGGAAAGAGCTGTAGTG-3’ And β-actin F 5’-CTGAGCTGCGTTTTACACC-3’ R 5’-CGCCTTCACCGTCCATTTT-3’.

**ELISA for detection of IFN-β production**

The amounts of IFN-β in cell supernatants were detected using enzyme-linked immunosorbent assay (ELISA). HEK-293T cells were cultured in 12-well plates, diluted virus stock solution (100 TCID_{50}/ml) was added (1 mL/well) and was allowed for adsorption for 1.5 h. The supernatant was discarded, MNTC of the test compounds with 2 folds dilution (3 concentration gradients) were added to 12 well plate. The cell control and model group were established and incubated at 37 °C and 5% CO₂ for 24 h. The rest of the process was carried out by following the manufacturer's instruction.

**Western blot analysis**

The HEK-293T cells were cultured in 6-well plates, infected with 100 TCID_{50}/ml EMCV (2 mL/well) and allowed for adsorption for 1.5 h. The supernatant was discarded and the MNTC of curcumol with 2 folds dilution (3 concentration gradients) were added to 6 well plates. Meanwhile, cell control group, model group were established and were incubated at 37 °C and 5% CO₂ for 24 h. The cells were lysed with RIPA buffer containing 1 mM protease inhibitor, 1 mM phosphatase inhibitor and cells were collected using a cell scraper. Equal amount of cell lysate was separated on a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane. After incubation with the above mentioned antibodies and chemiluminescence were used.

**Statistical analysis**

Both CC_{50} and EC_{50} were calculated using GraphPad PrismTM software (GraphPad Software, Inc. California, USA) and the data were presented as Mean ± SD. One-way analysis of variance (ANOVA) followed by a Dunnett's post-test was used to determine the difference between the groups of Real-time PCR results. The ELISA results were calculated by Curve Expert software and the results of Western blot were calculated by Image J (National Institutes of Health, USA) software. One-way ANOVA followed by a Dunnett's post-test was used to determine the difference between the groups. A star * indicates a significant difference (P< 0.05).

**Abbreviations**

EMCV: Encephalomyocarditis virus; BHK-21: Baby hamster syrian kidney; HEK-293T: Human embryonic kidney 293T; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; DMEM: Dulbecco's modified eagle's medium; DMSO: Dimethyl sulfoxide; CR: Cytotoxic ratio; MDA5: Melanoma differentiation-associated protein 5; MAVS: Mitochondrial antiviral signaling protein; TBK1: TANK-binding
Declarations

Ethics approval and consent to participate

The experiment was performed in accordance with the experiment operational guideline of Shanxi province, China.

Consent to publish

Not applicable.

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors have no conflict of interest in the research.

Funding

This work was supported by the National Key R&D Program of China (grant number 2017YFD0501500) and Shanxi Province Excellent Doctoral Work Award-Scientific Research Project (SXYBK2019051). The funding body had a role in the design of the study, collection, analysis, and interpretation of data or in the writing of this manuscript.

Authors’ Contribution

JZ, YX, NS and HL designed all the experiments. JZ and YX performed the MTT experiments, Western blot, ELISA assays. JZ, KF, WY and YS performed the qPCR assays. JZ, YX, NS, K, SW and PS wrote the manuscript. All authors read and approved the final manuscript.
**Acknowledgments**

We are very grateful to Prof. Jiang (Nanjing Agricultural University) for his assistance in the EMCV NJ08 strain.

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