JOURNAL OF
GENERAL VIROLOGY

SHORT COMMUNICATION
Li *et al.*, *Journal of General Virology*
DOI 10.1099/jgv.0.001466

MICROBIOLOGY SOCIETY

Antiviral and virucidal effects of curcumin on transmissible gastroenteritis virus *in vitro*

Yaoming Li†, Jing Wang†, Yinchuan Liu, Xiang Luo, Weiqiang Lei and Lilan Xie*

Abstract

Emerging coronaviruses represent serious threats to human and animal health worldwide, and no approved therapeutics are currently available. Here, we used Transmissible gastroenteritis virus (TGEV) as the alpha-coronavirus model, and investigated the antiviral properties of curcumin against TGEV. Our results demonstrated that curcumin strongly inhibited TGEV proliferation and viral protein expression in a dose-dependent manner. We also observed that curcumin exhibited direct virucidal abilities in a dose-, temperature- and time-dependent manner. Furthermore, time-of-addition assays showed that curcumin mainly acted in the early phase of TGEV replication. Notably, in an adsorption assay, curcumin at 40 µM resulted in a reduction in viral titres of 3.55 log $TCID_{50}$ ml^{-1}, indicating that curcumin possesses excellent inhibitory effects on the adsorption of TGEV. Collectively, we demonstrate for the first time that curcumin has virucidal activity and virtual inhibition against TGEV, suggesting that curcumin might be a candidate drug for effective control of TGEV infection.

Coronaviruses (CoVs) are enveloped RNA viruses that consist of a positive-sense, ssRNA genome of about 28.5 kb, and belong to the family Coronaviridae [1]. According to features of the genome and antigenicity, the members of the family Coronaviridae have been divided into four genera: Alphacoronavirus, Betacoronavirus, Gammacoronavirus and Deltacoronavirus [2]. Transmissible gastroenteritis virus (TGEV), a member of the alpha-genus Coronavirus, is the causative agent of transmissible gastroenteritis (TGE) [3]. TGEV infection mainly causes acute enteric disease characterized by lethal watery diarrhoea, severe dehydration and high mortality in seronegative neonatal piglets. Since the virus was first isolated by Doyle and Hutchings in 1946, TGEV infection has been a serious threat to the global swine industry [4, 5]. At present, vaccination is the primary means of preventing TGEV infection. However, TGEV infection in piglets remains widespread, suggesting that current commercial vaccines are not sufficient to provide complete protection [6, 7]. Therefore, an alternative strategy to control TGEV, such as pharmacotherapy, should be given attention.

Curcumin, the main polyphenolic compound of the food flavouring turmeric, has attracted significant attention due to its versatile biological effects such as anti-tumour, anti-inflammatory, immunomodulating, antioxidant, antibacterial, antifungal, antiparasitic and antiviral activities [8, 9]. Notably, curcumin exhibits inhibitory ability against the proliferation of diverse viruses, such as dengue virus [10], hepatitis B virus [11], Zika virus (ZIKV) and chikungunya virus (CHIKV) [12]. However, no potency of curcumin against TGEV has yet been reported. In this study, we investigated the antiviral properties of curcumin against TGEV and elucidate its mode of action.

We first evaluated the cytotoxicity of curcumin (Sigma-Aldrich) to porcine kidney (PK-15) cells, permissive cells for TGEV infection *in vitro*, by using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide] assays. Briefly, PK-15 cells were seeded at 1×10^4 per well in 96-well cell culture plates and were incubated at 37 °C in 5% CO_2. When cells had grown to approximately 95% confluence, they were washed three times with PBS and incubated with different concentrations of curcumin (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µM) for 36 h. DMSO-treated cells served as controls (0 µM). After washing with PBS, the cells were analysed by an MTT assay as described previously [13]. Cell survival rate

Received 14 May 2020; Accepted 19 June 2020; Published 17 July 2020
Author affiliations: [1]Hubei Engineering Research Center of Viral Vector, Applied Biotechnology Research Center, Wuhan University of Bioengineering, Wuhan 30415, PR China.
***Correspondence:** Lilan Xie, xielilan@webmail.hzau.edu.cn
Keywords: transmissible gastroenteritis virus; curcumin; inhibition assay; virucidal assay.
Abbreviations: CC50, 50 % cytotoxic concentration; CHIKV, chikungunya virus; CoV, coronavirus; hpi, hours post-infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PK-15 cells, porcine kidney cells; TGEV, transmissible gastroenteritis virus; ZIKV, zika virus.
†These authors contributed equally to this work

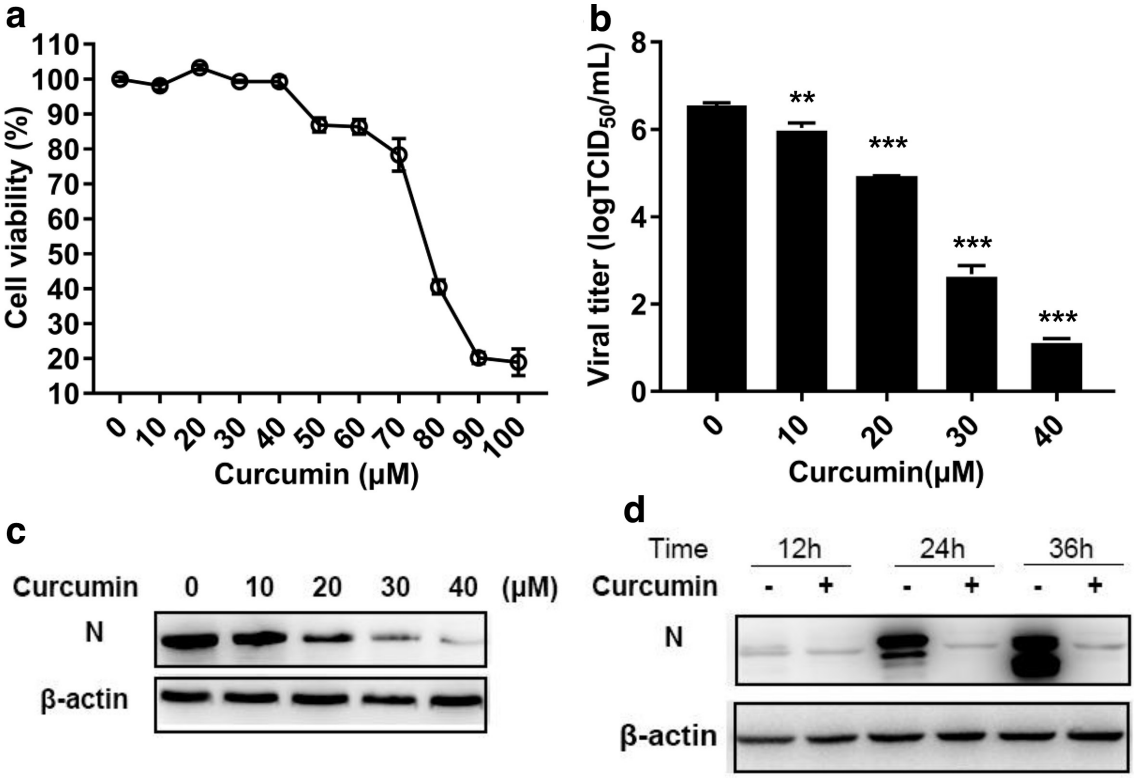

Fig. 1. Curcumin has antiviral activity against TGEV. (a) Cytotoxic effect of curcumin on PK-15 cells. The cells were treated with different concentrations of curcumin (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μM) or DMSO (control) for 36 h before the MTT assay. (b, c) PK-15 cells were pretreated with curcumin (0, 10, 20, 30 and 40 μM) at 37°C for 1 h before infection with TGEV (0.1 m.o.i.). After infection for 1 h, cells were replaced with fresh medium containing the indicated concentrations of curcumin. At 24 h post-infection, the viral titres (b), and expression of N protein (c) were determined by a $TCID_{50}$ assay and western blot, respectively. (d) PK-15 cells were pretreated with curcumin (40 μM) for 1 h before infection with TGEV (0.1 m.o.i.). Then, cells were cultured in the presence of curcumin (40 μM). N protein was analysed at the indicated times. Results in (a) and (b) are the mean±SD of three independent experiments. Results in (c) and (d) are representative of three independent experiments. Differences between curcumin-treated groups and the control group were assessed using Student's *t*-test (**P<0.01, ***P<0.001).

was determined as drug average OD value/control average OD value. As shown in Fig. 1a, relative cell viability was above 95% after treatment with curcumin at concentrations of 10, 20, 30 and 40 μM, whereas viability showed a gradual decreasing trend after treatment with increasing concentrations of curcumin (50, 60, 70, 80, 90 and 100 μM) (Fig. 1a). The 50% cytotoxic concentration (CC_{50}) value was calculated as 77.96±1.005 μM by GraphPad PRISM software. Therefore, a concentration of 40 μM was the maximum concentration of curcumin we adopted for the following antiviral assay in this study.

To investigate whether curcumin has antiviral activity against TGEV, PK-15 cells were seeded at 5×10^5 per well in six-well plates and were cultured at 37 °C until approximately 95% confluence. Cells were then pretreated with different concentrations of curcumin (10, 20, 30 and 40 μM) at 37 °C for 1 h, and subsequently infected with TGEV strain WH-1 (0.1 m.o.i.) at 37 °C for 1 h. Then, the infected cells were washed with cold PBS three times to remove the non-infected viral particles and cultured in DMEM culture media with the indicated concentrations of curcumin for 24 h. The viral titres in the cell culture supernatants were valuated according to the method of Reed and Muench and expressed as $TCID_{50}$ [14]. Briefly, virus samples were diluted 10-fold serially in cell culture media. A 100 μl aliquot of each virus dilution was added to monolayer PK-15 cells (96-well plate) with eight-well repeats. Cells incubated in virus-free medium served as a negative control. The plate was observed twice daily for cytopathic effect (CPE). As shown in Fig. 1b, treatment with curcumin caused a notable decrease in TGEV titre in a dose-dependent manner, with a 5.34 log $TCID_{50}$ ml^{-1} reduction of the virus yield at 40 μM, and reduction of 0.48, 1.64 and 3.82 log $TCID_{50}$ ml^{-1} at 10, 20 and 30 μM, respectively. A concentration of 8.573±0.1723 μM was identified as the IC_{50} by GraphPad PRISM. This gave a selectivity index of curcumin of 9.09. Meanwhile, the expression of TGEV nucleocapsid (N) protein in the cell lysates was also determined by western blot. Briefly, cell protein lysates were subjected to SDS-PAGE and electroblotted onto a PVDF membrane (Millipore). Protein expressions were analysed by western blot with anti-TGEV N protein mAb (1:1000;

kindly provided by Professor Liurong Fang of Huazhong Agricultural University) and anti-β-actin mAb (1:2000; Beyotime) [15]. HRP-conjugated goat anti-mouse antibody were used as a secondary antibody (1:2000; Beyotime). As shown in Fig. 1c, expression of N protein decreased in parallel with the increase in the concentration of curcumin compared to the control group. We also performed a time course of infection study. PK-15 cells were treated as above with minor modifications. At 12, 24 and 36 h post-infection (hpi), expression of N protein in cell lysates was analysed. We observed reduced N protein expression at all selected time points (12, 24 and 36 h) after curcumin treatment (Fig. 1d). Collectively, the above results show that treatment with curcumin decreased cell infection of TGEV.

Current studies have shown that curcumin has various antiviral properties, by interfering with viral adsorption, entry, replication and even budding [9]. To determine how curcumin could affect TGEV replication, subconfluent monolayers were prepared by seeding PK-15 cells in 24-well plates at a density of 1×10^5 cells per well, followed by curcumin treatment at different times during infection (Fig. 2a). PK-15 cells were infected with TGEV (0.1 m.o.i.) at 37 °C (set as 0 h). Curcumin at 40 µM was added to the cells at different time intervals: 0–12 h, 12–24 h and 24–36 h (Fig. 2a). At 36 hpi, viral yields in cell supernatants were determined by a $TCID_{50}$ assay. We found that the TGEV titre was markedly decreased only at 0–12 h (Fig. 2b), suggesting that curcumin primary inhibits the early stages of TGEV replication. To further decipher the key viral infection cycle step affected by curcumin, a time-of-addition assay that included the most drug-sensitive phase of virus replication was conducted (Fig. 2a, 0, 1, 3, 6, 9 and 12 h). Briefly, PK-15 cells were infected with TGEV (0.1 m.o.i.) at 37 °C (set as 0 h). Curcumin at 40 µM was added to the cells at 0, 1, 3, 6, 9 and 12 hpi. At 36 hpi, $TCID_{50}$ assays were performed to valuate the viral titre in the cell supernatants.

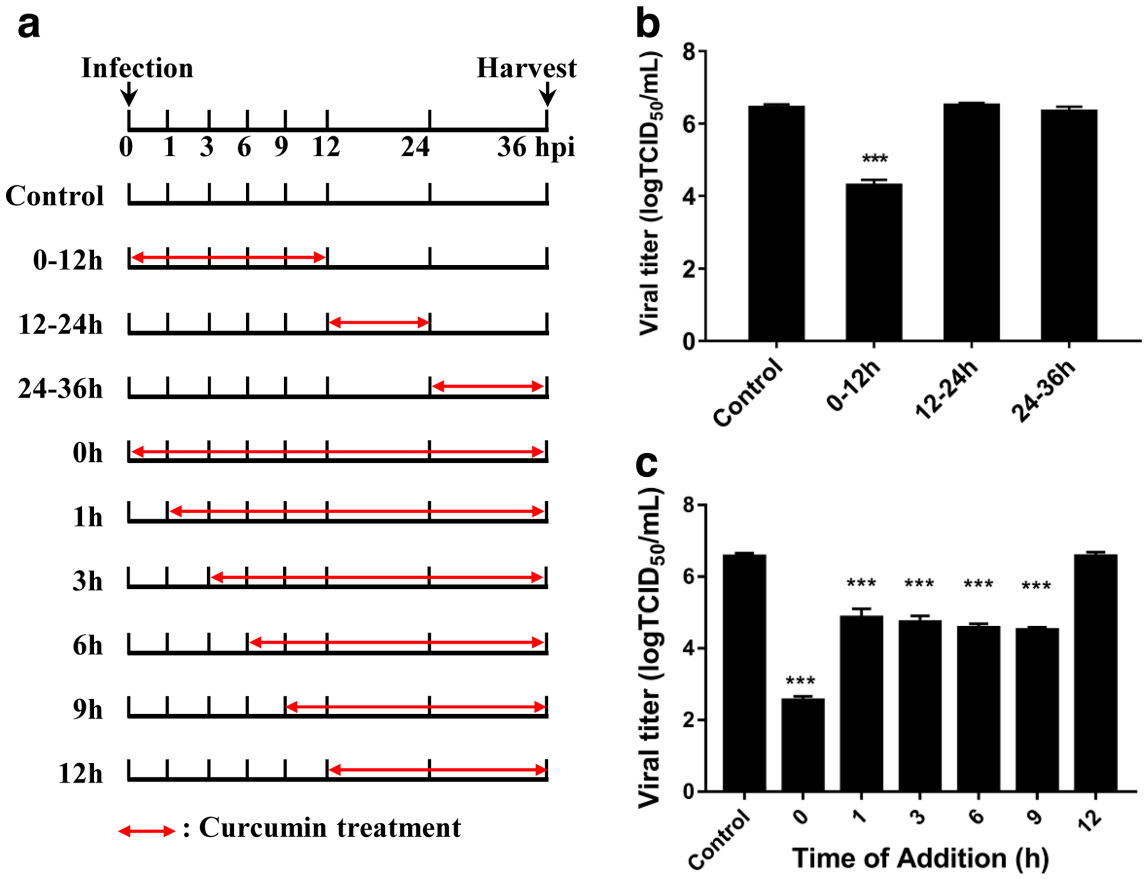

Fig. 2. Curcumin inhibits TGEV infection at the early stages of infection. (a) Schematic diagram of time-of-addition experiments to explore the stage(s) at which curcumin exerts the anti-TGEV effect. PK-15 cells were infected with TGEV (0.1 m.o.i.) at 37 °C (set as 0 h). Curcumin was added to the cells at different times during infection. 'Control' indicates that cells that were not treated with curcumin. Samples (cell culture supernatants) were harvest at 36 hpi. Double-headed red arrows indicate the presence of curcumin. (b, c) PK-15 cell monolayers in 24-well plates infected with TGEV (0.1 m.o.i.) were treated with 40 µM curcumin at different time intervals of 0–12, 12–24 and 24–36 h (b), or at the indicated times (0, 1, 3, 6, 9 and 12 h) (c). Viral litres were analysed at 36 hpi. The results represent the mean±SD from three independent experiments. Differences between curcumin-treated groups and the control group were assessed using Student's *t*-test (***P<0.001).

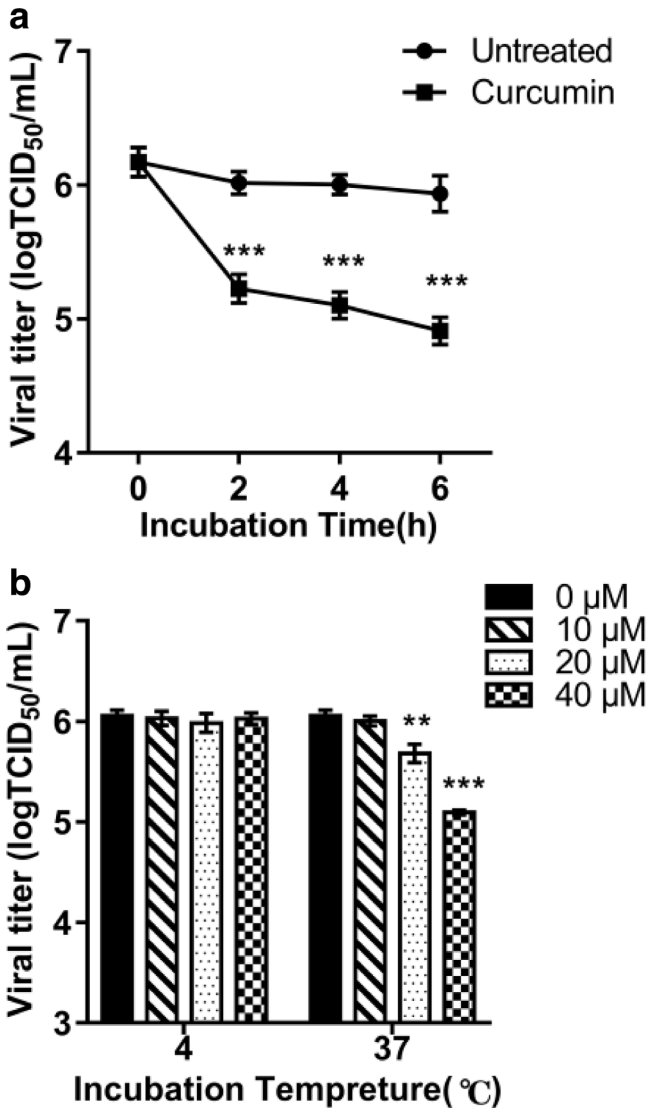

Fig. 3. Virucidal effect of curcumin on TGEV. (a) TGEV was incubated with curcumin (40 μM) or DMSO at 37 °C for 0, 2, 4 and 6 h. (b) Virus was incubated with different concentrations of curcumin (0, 10, 20 and 40 μM) at 4 or 37 °C for 2 h. After the indicated treatments, the mixtures of viruses and curcumin were diluted 10-fold serially immediately and viral titres were tested by a $TCID_{50}$ assay. The results represent the mean±SD from three independent experiments. Differences between curcumin-treated groups and the untreated group (0 μM) were assessed using Student's *t*-test (***P*<0.01, ****P*<0.001).

As shown in Fig. 2c, compared to control cells, the titres were significantly reduced with addition of curcumin at 0, 1, 3, 6 and 9 h, but there was no distinguishable decrease at 12 hpi, which is consistent with the our previous findings (Fig. 2b). Notably, when the drug was added at the same time of viral infection (0 h), the titre of the virus showed a sharp decrease, with a 4.02 log $TCID_{50}$ ml^{-1} reduction in virus yield, indicating that curcumin has its most important inhibitory affect before viral entry.

Virus infection of cells can be divided into adsorption, penetration, replication and release [3]. The greatest reduction in TGEV titres was found when the drug was added at the time of TGEV infection, suggesting that curcumin impacts TGEV infection at the time of adsorption/penetration. Moreover, because curcumin is a lipophilic compound [16], when curcumin is co-incubated with TGEV, an envelope virus, curcumin may also directly affect the infectivity of the virus. To further investigate these possibilities, an extracellular virucidal assay, viral attachment assay and penetration assay were performed. First, extracellular virucidal assays were performed as follows. TGEV was directly incubated with curcumin (final concentration of 40 μM) at 37 °C in a 1.5 ml Eppendorf tube for either 0, 2, 4 or 6 h. At the indicated time after incubation, the mixtures of virus and curcumin were diluted 10-fold serially immediately and viral titres were tested by a $TCID_{50}$ assay. As shown in Fig. 3a, compared to the untreated control, direct curcumin treatment at 37 °C for 2, 4 and 6 h neutralized TGEV by 0.79, 0.90 and 0.98 log $TCID_{50}$ ml^{-1}, respectively. To test whether a suitable temperature is required for curcumin-induced virus inactivation, TGEV was incubated with various final concentrations of curcumin (0, 10, 20 and 40 μM) at 37 °C, or at 4 °C for 2 h, followed by 10-fold serial dilution for the $TCID_{50}$ assay. When the virus was incubated with curcumin at 37 °C, the virus titre decreased significantly with an increase in curcumin concentration. However, TGEV was insensitive to direct curcumin treatment at 4 °C, even at 40 μM (Fig. 3b). The results showed that curumin had direct virucidal effects on TGEV in a time-, temperature- and dose-dependent manner. Given the direct inactivation effect of curcumin on the virus, we speculate that one possible reason why there is no significant difference in the decrease in virus titres when curcumin treatment was at different times (1, 3, 6 and 9 h) after virus infection is that inactivation of curcumin was on the new virions (Fig. 2b).

Subsequently, the impact of curcumin on the adsorption stage of the virus was further investigated as follows. Briefly, PK-15 cells were cultured in the 24-well plate at a seeding density of 1×10^5 per well for 18–24 h; cells were then pre-cooled at 4 °C for 1 h, followed by TGEV (0.1 m.o.i.) incubation in the presence of curcumin (10, 20 and 40 μM) at 4°C for another 1 h. Subquently, the cells were washed with ice-cold PBS three times and cultured with fresh medium without curcumin at 37 °C. Following 24 h of virus infection, the cell supernatants were harvested to determine the viral titres by a $TCID_{50}$ assay. As shown in Fig. 4a, decreased titres of 0.39, 1.74 and 3.55 log $TCID_{50}$ ml^{-1} were observed when cells were treated with 10, 20 and 40 μM curcumin, respectively. Therefore, curcumin treatment can significantly reduce TGEV adsorption in a dose-dependent manner. We were also interested in evaluating the vitality of PK-15 cells, which also reflected the degree of cell damage upon virus infection. PK-15 cells were seeded at 1×10^4 per well in 96-well cell plates and treated as above with minor modifications. Following 24 h of virus infection, an MTT assay was used to determine the vitality. As shown

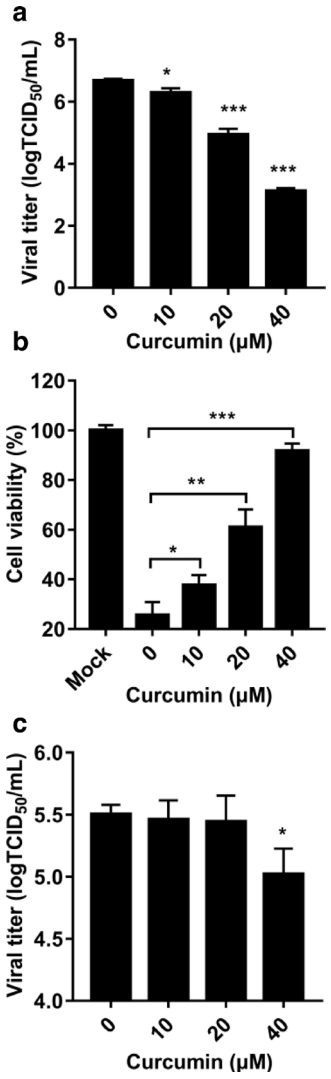

Fig. 4. Curcumin strongly inhibits TGEV replication at the adsorption step. (a, b) Adsorption assay. PK-15 cells were pre-cooled at 4 °C, followed by TGEV (0.1 m.o.i.) incubation in the presence of curcumin (0, 10, 20 and 40 μM) at 4 °C for another 1 h. Then, cells were washed with PBS three times and incubated at 37 °C. At 24 hpi, the vitality of PK-15 cells were measured by an MTT assay (b), presented as experimental cell viability relative to viability of mock-treated cells (100%, uninfected). The viral titres in cell supernatants were detected by a $TCID_{50}$ assay (a). (c) Penetration assay. PK-15 cells were prechilled, followed by TGEV infection (10 m.o.i.) at 4 °C for 1 h. After virus attachment, the cells were washed with cold PBS three times and cultured with medium containing curcumin (0, 10, 20 and 40 μM) at 37°C for 2 h. Cells were then replaced with fresh medium without any curcumin and were further incubated for another 8 h. Whole cells and supernatant were harvested and lysed. Each sample was used to determine the viral titre by a $TCID_{50}$ assay. Data represent the mean±SD from three independent experiments. Differences between curcumin-treated groups and the control group (0 μM) were assessed using Student's *t*-test (**P*<0.05; ***P*<0.01, ****P*<0.001).

in Fig. 4b, compared with the control group (0 μM), the cell viabilities of curcumin-treated groups were significantly increased in a dose-dependent manner. Together, these results suggest that curcumin has a strong inhibitory effect on the adsorption process of TGEV. Previous reports have demonstrated that curcumin interferes with cell binding of several viruses such as CHIKV, ZIKV and hepatitis C virus. Studies have shown that hepatitis C virus can alter the fluidity of the cell membrane, thus influencing the binding of virus to cells [12, 17]. It has also been reported that aminopeptidase N (APN) and epidermal growth factor receptor (EGFR), the receptors for TGEV, can be affected at the activity or expression levels after curcumin treatment in certain cell types [18, 19]. Thus, curcumin may affect TGEV adsorption by changing cell membrane fluidity or surface receptors, or both. Further studies will be necessary to confirm this hypothesis.

To evaluate whether curcumin impacted TGEV at the penetration step, subconfluent monolayers of PK-15 cells cultured in the 24-well plate at a seeding density of 1×10^5 per well were prechilled at 4 °C for 1 h and then infected with TGEV (10 m.o.i., to ensure all cells were infected and to prevent multiple rounds of infection) at 4 °C for 1 h. After virus attachment, the cells were washed with cold PBS three times and cultured with medium containing curcumin (0, 10, 20 and 40 μM), and the temperature was set to 37 °C to permit TGEV internalization. Two hours later, cells were replaced with fresh medium without any curcumin and were further incubated for another 8 h at 37 °C according to the one-step growth curve of TGEV [20]. Whole cells and supernatant were harvested and lysed, followed by viral titre analysis by a $TCID_{50}$ assay. As shown in Fig. 4c, compared with the control group, the virus titres did not change significantly when cells were treated with low concentrations of curcumin (10 and 20 μM). Inhibitory effects were seen only when cells were treated with curcumin at 40 μM, with a 0.48 log $TCID_{50}$ ml^{-1} decrease. The results indicated that curcumin had some inhibitory effect on TGEV internalization.

Collectively, our work has shown that curcumin has direct inactivation effects on TGEV, as well as robust antiviral abilities that mainly interfere with the adsorption stage in the TGEV life cycle. There are multiple possible mechanisms underlying the antiviral effects of curcumin [9]. In general, curcumin mediates the antiviral effects by directly targeting viral components or by altering cell metabolism. For example, curcumin inhibits human immunodeficiency virus proliferation by promoting proteasomal degradation of tat. In influenza A virus-infected cells, curcumin activates the Nrf2 signalling pathway to regulate virus replication [21, 22]. Besides the direct inactivation and significant inhibition of adsorption against TGEV, curcumin also has an effect on the penetration step of the virus replication cycle at a high concentration of 40 μM. These results indicate that there are complex mechanisms for curcumin regulating TGEV replication. Furthermore, increasing evidence indicates that utilization of curcumin in animals and humans

can be safe in various doses [11]. Thus, curcumin could potentially act as an effective drug for preventing TGEV infection. Further studies will be required to evaluate its inhibitory abilities *in vivo*.

Funding information
This work was supported by the National Science Foundation of China (Nos. 31972692, 31402181), the Hubei Provincial Natural Science Foundation of China (No. 2018CFB662), and the science and technology research project of Hubei Provincial Department of Education of China (No.B2016303).

Acknowledgements
We gratefully acknowledge Professor Liurong Fang of Huazhong Agricultural University for providing the anti-N monoclonal antibody.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Perlman S. Research driven by curiosity: the journey from basic molecular biology and virology to studies of human pathogenic coronaviruses. *PLoS Pathog* 2015;11:e1005023.
2. Payne S. *Chapter 17 - Family Coronaviridae*. Viruses, Academic Press; 2017. pp. 149–158.
3. Brian DA, Baric RS. Coronavirus genome structure and replication. *Curr Top Microbiol Immunol* 2005;287:1–30.
4. Mullan BP, Davies GT, Cutler RS. Simulation of the economic impact of transmissible gastroenteritis on commercial pig production in Australia. *Aust Vet J* 1994;71:151–154.
5. Xia L, Yang Y, Wang J, Jing Y, Yang Q. Impact of TGEV infection on the pig small intestine. *Virol J* 2018;15:102.
6. Ren X, Meng F, Yin J, Li G, Li X *et al*. Action mechanisms of lithium chloride on cell infection by transmissible gastroenteritis coronavirus. *PLoS One* 2011;6:e18669.
7. Zou H, Zarlenga DS, Sestak K, Suo S, Ren X. Transmissible gastroenteritis virus: identification of M protein-binding peptide ligands with antiviral and diagnostic potential. *Antiviral Res* 2013;99:383–390.
8. Moghadamtousi SZ, Kadir HA, Hassandarvish P, Tajik H, Abubakar S *et al*. A review on antibacterial, antiviral, and antifungal activity of curcumin. *Biomed Res Int* 2014;2014:186864.
9. Praditya D, Kirchhoff L, Brüning J, Rachmawati H, Steinmann J *et al*. Anti-infective properties of the golden spice curcumin. *Front Microbiol* 2019;10:912.
10. Balasubramanian A, Pilankatta R, Teramoto T, Sajith AM, Nwulia E *et al*. Inhibition of dengue virus by curcuminoids. *Antiviral Res* 2019;162:71–78.
11. Hesari A, Ghasemi F, Salarinia R, Biglari H, Tabar Molla Hassan A *et al*. Effects of curcumin on NF-κB, AP-1, and Wnt/β-catenin signaling pathway in hepatitis B virus infection. *J Cell Biochem* 2018;119:7898–7904.
12. Mounce BC, Cesaro T, Carrau L, Vallet T, Vignuzzi M. Curcumin inhibits Zika and Chikungunya virus infection by inhibiting cell binding. *Antiviral Res* 2017;142:148–157.
13. Zouharova D, Lipenska I, Fojtikova M, Kulich P, Neca J *et al*. Antiviral activities of 2,6-diaminopurine-based acyclic nucleoside phosphonates against herpesviruses: *in vitro* study results with pseudorabies virus (PrV, SuHV-1). *Vet Microbiol* 2016;184:84–93.
14. Wei Z, Burwinkel M, Palissa C, Ephraim E, Schmidt MFG. Antiviral activity of zinc salts against transmissible gastroenteritis virus in vitro. *Vet Microbiol* 2012;160:468–472.
15. Zhou Y, Wu W, Xie L, Wang D, Ke Q *et al*. Cellular RNA helicase DDX1 is involved in transmissible gastroenteritis virus nsp14-Induced interferon-beta production. *Front Immunol* 2017;8:940.
16. Maheshwari RK, Singh AK, Gaddipati J, Srimal RC. Multiple biological activities of curcumin: a short review. *Life Sci* 2006;78:2081–2087.
17. Colpitts CC, Schang LM, Rachmawati H, Frentzen A, Pfaender S *et al*. Turmeric curcumin inhibits entry of all hepatitis C virus genotypes into human liver cells. *Gut* 2014;63:1137–1149.
18. Xu W, Li Q. Progress in the development of aminopeptidase N (APN/CD13) inhibitors. *Curr Med Chem Anticancer Agents* 2005;5:281–301.
19. Yang J, Zhu D, Liu S, Shao M, Liu Y *et al*. Curcumin enhances radiosensitization of nasopharyngeal carcinoma by regulating circRNA network. *Mol Carcinog* 2020;59:202–214.
20. An K, Fang L, Luo R, Wang D, Xie L *et al*. Quantitative proteomic analysis reveals that transmissible gastroenteritis virus activates the Jak-STAT1 signaling pathway. *J Proteome Res* 2014;13:5376–5390.
21. Ali A, Banerjea AC. Curcumin inhibits HIV-1 by promoting Tat protein degradation. *Sci Rep* 2016;6:27539.
22. Dai J, Gu L, Su Y, Wang Q, Zhao Y *et al*. Inhibition of curcumin on influenza A virus infection and influenzal pneumonia via oxidative stress, TLR2/4, p38/JNK MAPK and NF-κB pathways. *Int Immunopharmacol* 2018;54:177–187.