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Curcumin inhibits influenza virus infection and haemagglutination activity

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ABSTRACT

Curcumin (diferuloylmethane) is a widely used spice and colouring agent in food. Accumulated evidence indicates that curcumin is associated with a great variety of pharmacological activities, including an anti-microbial effect. In this study, the anti-influenza activity of curcumin was evaluated. Our results demonstrated that treatment with 30 μM curcumin reduced the yield of virus by over 90% in cell culture. The EC_{50} determined using plaque reduction assays was approximately 0.47 μM (with a selective index of 92.5). Time of drug addition experiments demonstrated curcumin had a direct effect on viral particle infectivity that was reflected by the inhibition of haemagglutination; this effect was observed in H1N1 as well as in H6N1 subtype. In contrast to amantadine, viruses did not develop resistance to curcumin. Furthermore, by comparison of the antiviral activity of structural analogues, the methoxyl groups of curcumin do not play a significant role in the haemagglutinin interaction.

1. Introduction

Curcumin (diferuloylmethane), a nature polyphenolic compound derived from turmeric (*Curcuma longa*), is a widely used spice and colouring agent in food (Goel, Kunnumakkara, & Aggarwal, 2007). Traditionally, curcumin is commonly applied in many therapeutic remedies, either alone or in conjunction with other natural substances (Araujo & Leon, 2001). Accumulated evidence indicates that curcumin is associated with a great variety of pharmacological activities, such as anti-inflammatory (Brouet & Ohshima, 1995), antioxidant (Sreejayan & Rao, 1997), and anti-microbial (Jagannath & Radhika, 2006; Kutluay, Doroghazi, Roemer, & Triezenberg, 2008; Si et al., 2007). Curcumin also inhibits a number of tumours *in vitro* and in animal models (Anand, Kunnumakkara, Newman, & Aggarwal, 2007; Maheshwari, Singh, Gaddipati, & Srimal, 2006). Such effects have been attributed to the interaction of curcumin with a diverse range of molecular targets involved in cell growth, metastasis, tumourangiogenesis and apoptosis; for instance, nuclear factor κB (NF-κB), cyclooxygenase-2, matrix metalloproteinase, vascular cell adhesion molecule-1, and p53 (Goel et al., 2007). By inhibiting IκB phosphorylation by IκB kinase, curcumin effectively suppressed NF-κB signalling, which regulates the expression of genes contributing to tumourigenesis and cell survival (Aggarwal & Shishodia, 2004; Bharti, Donato, Singh, & Aggarwal, 2003; Kumar, Dhawan, Hardegen, & Aggarwal, 1998).

Influenza A virus (IAV) caused three pandemics in the 20th century. In 1997, a highly pathogenic strain, H5N1, emerged in Hong Kong. Worldwide attention was drawn to avian influenza for the first time, due to the devastating outbreaks in domestic poultry and sporadic human infections with a high fatality rate (Webster & Govorkova, 2006). The IAV genome consists of eight negative-stranded RNA segments encoding 11 viral proteins; among those, the major glycoproteins on the viral surface, haemagglutinin (HA) and neuraminidase (NA), are two of the main target antigens of the host immune system (Fiers, De Filette, Birkett, Neirynck, & Min Jou, 2004; Nicholls, 2006). Outbreaks of avian H5N1 pose a public health risk of potentially pandemic proportions; however, the pre-existing antiviral resistance to amantadine and the emergence of H5N1 variants resistant to oseltamivir and zanamivir, highlight the need for developing new antiviral therapeutic strategies.

One of the hallmark cellular responses to influenza virus infection is the activation of transcription factor NF-κB signalling (Ludwig, Planz, Pleschka, & Wolff, 2003; Ludwig, Pleschka, Planz, & Wolff, 2006; Shin, Liu, Tikoo, Babiuk, & Zhou, 2007) by the action of double-stranded viral RNA, and viral proteins (Bernasconi, Amici, La Frazia, Ianaro, & Santoro, 2005; Wurzer et al., 2004; Zhirnov & Klenk, 2007). Recently, several reports demonstrated that NF-κB inhibitors efficiently blocked propagation of influenza, suggesting

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that modulation of NF-κB signalling may be a target for anti-influenza intervention (Mazur et al., 2007; Nimmerjahn et al., 2004).

Our study is based on the observation that curcumin is a strong inhibitor of NF-κB signalling and may therefore impact upon IAV propagation. We demonstrated that treatment of cells with curcumin greatly reduced the yield of IAV at sub-cytotoxic doses. Pre-incubation of virus with curcumin pronouncedly inhibited influenza virus plaque formation. Thus, in addition to its potential effects on cellular function, curcumin also acts through direct interaction with viral particles that interrupts an early stage(s) of IAV infection. In addition, we confirmed that curcumin interferes with HA receptor binding activity. To our knowledge, this is the first report demonstrating that curcumin exerts anti-influenza activity, and the anti-influenza effect is via a mechanism that abolishes virus-cell attachment.

2. Materials and methods

2.1. Cell culture and virus infection

Madin-Darby canine kidney (MDCK) cells were passed in minimal-essential medium (MEM) with 10% foetal bovine serum (FBS), penicillin 100 U/ml, and streptomycin 10 μg/ml. Before infection, cells were washed with PBS and cultured in infection medium (MEM without FBS) supplemented with antibiotics and 1 mg/ml of trypsin (Gibco; Invitrogen, Carlsbad, CA).

Human influenza virus PR8, A/Puerto Rico/8/34 (H1N1), and avian influenza virus A/chicken/Taiwan/NCHU0507/99 (H6N1), kindly provided by Paul Digard (Cambridge) and H.-K. Shieh (Lee et al., 2006), were propagated in MDCK cells.

2.2. Compounds

Curcumin, obtained from Sigma–Aldrich, was dissolved in DMSO at a stock concentration of 100 mM and stored at −80 °C.

2.3. Antiserum

The PR8 antiserum used in western blot was prepared from two six-week-old BALB/c mice initially immunised with PR8 virus (2^{10} HA units, HAU) followed by two boosters (same dose) at two-week intervals. Two weeks after the second booster, the serum was collected.

2.4. Cytotoxicity test

MDCK cells (1×10^5) grown in 24-well plates for 24 h were washed twice with PBS and then were treated with curcumin at the indicated concentrations or mock control solutions (DMSO) at 37 °C and 5% CO_2 for 24 h. Proliferation of cells was measured directly by total cell counts and the survival rate was estimated as the ratio of living cells/total cell counts after staining with 0.4% trypan blue. Cytotoxicity of the compounds was estimated by comparison of the cell survival rate of curcumin-treated cells with that of mock-treated. The mock-treatment control was arbitrarily set as 100%.

2.5. Viral infections and curcumin treatment

MDCK cells (4×10^4/well) were seeded in 48-well plates 16 h before infection. Cell monolayers were infected with 2000 pfu (plaque forming units) of A/PR/8/34 virus. Supernatant from infected cells was collected at 12, 18, 24, and 30 h post-infection (hpi) and the yield of virus progeny was measured by plaque assay.

For time of addition experiments, the indicated concentrations of curcumin or mock treatment (DMSO) were added to the medium at various times of infection. Briefly, (1) pre-treatment: curcumin was included in the cell culture medium for 8 h and was removed prior to virus infection; (2) simultaneous: curcumin mixed with virus in the infection medium was added simultaneously to the cells and left on the cells throughout; (3) post-infection: curcumin was added to cells at 2 hpi and remained throughout the time of infection.

2.6. Plaque assay

MDCK cell monolayers in 12-well plates (2×10^5 cells/well) were washed twice with PBS followed by infection with serial dilutions of virus. After 2 h absorption at 37 °C, unbound viruses were removed and cells were then cultured for 2 days with 1 ml/well MEM supplemented with 0.6% agarose at 37 °C and 5% CO_2. Viral plaques were visualised by staining with Giemsa (Sigma, St. Louis, MO).

2.7. Plaque reduction assay

Five thousand pfu of virus were pre-incubated with 30 μM (unless otherwise stated) of curcumin or various concentrations of related compounds for one hour. MDCK cells seeded in 12-well plates were washed twice with PBS followed by infection with serial dilutions of the curcumin-treated viruses. After 2 h absorption at 37 °C, the virus inoculum was removed and cells were then cultured for 2 days with 1 ml/well MEM supplemented with 0.6% agarose at 37 °C and 5% CO_2. Viral plaques were visualised by staining with crystal violet (Sigma).

2.8. Haemagglutination inhibition (HI) test

The haemagglutination (HA) titres of virus stocks were initially determined by standard HA assay. HI tests were subsequently performed using 4 HA units (HAU) of virus per reaction. Twofold serial dilutions of curcumin were prepared in round-bottomed 96-well micro-plates. An equal volume (25 μl/well containing 4 HAU) of virus stock was added into each well and incubated at room temperature for 1 h. Subsequently, 50 μl of chicken erythrocytes (diluted to 0.75% v/v in Hank's buffered saline) were added to each well. The haemagglutination reaction was observed after 30 min incubation.

2.9. High performance liquid chromatography (HPLC)

HPLC was employed to isolate the curcuminoid components of curcumin. The HPLC system consisted of an Agilent quaternary HPLC, Model 1100 series (Agilent, Waldbronn, Germany), fitted with a COSMOSIL 5SL-II Waters (Milford, MA) silica column (10 × 250 mm i.d.). An Intelligent UV–Vis detector (Agilent 1100) used at a wavelength of 280 nm was used for detection. Curcumin prepared as a 5 mg/ml stock dissolved in ethyl acetate (EA) was applied to the column and the three distinct fractions of curcuminoids were eluted individually with EA/Hexane (50/50 v/v). The solvent from HPLC elutes was then removed using a rotatory vacuum evaporator. For identification, the purified compounds were subjected to ^{1}H NMR spectral analysis. ^{1}H NMR spectra were recorded at 200 MHz on an INOVA 200 instrument (Varian, Palo Alto, CA).

2.10. Western blot analysis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE; 10%) was performed with a MINI-PROTEAN III appara-

tus (Bio-Rad; Hercules, CA), and then the proteins were electrophoretically transferred to PVDF membranes according to the manufacturer's recommendations. After a blocking step in PBS containing 0.1% Tween-20 (PBS-T) and 5% dried milk for 1 h at room temperature, the filter was incubated with the primary antibody (mouse anti-PR8 serum) diluted in PBS-T containing 5% dried milk at room temperature for 2–3 h. Subsequently, the filter was washed with PBS-T at least four times, followed by incubation with 1:5000 diluted secondary antibody conjugated with alkaline phosphatase (goat anti-mouse antibody; Sigma) for 1 h. After extensive washes with PBS-T, the signal was revealed using BCIP/NBT reagent (Sigma).

2.11. Drug resistance test

Amantadine was used as a control for the drug resistance test. In detail, 10 μM of curcumin or amantadine were included in medium of MDCK cell monolayers infected with 5000 pfu of PR8 viruses in 6-well plates. Supernatants were collected at 18 hpi and the titre of progeny virions was determined by standard plaque assay. Subsequently, 5000 pfu of the passaged PR8 viruses were taken for the next round of infection. Procedures were repeated for five rounds. The yield of progeny virus was monitored.

2.12. Statistical analysis

All data were calculated by Microsoft Excel and analysed with SAS statistical software (Cary, NC). Results were reported as mean values ± standard deviation (SD). For the anti-influenza efficacy study, the *chi*-square test was used and *p*-values less than 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Treatment of curcumin reduces influenza A viruses replication

The initial goal of our study was to determine whether curcumin (also designated curcumin I elsewhere) has anti-influenza virus activity in cell culture. Firstly, cytotoxicity to MDCK cells was measured based on cell proliferation and viability. The CC_{50} (drug concentration inhibiting cell growth by 50% relative to untreated control) was approximately 43 μM and no significant cellular toxic effect was observed below 30 μM (Supplementary Fig. S1).

To evaluate the effect of curcumin on influenza virus replication, cell culture medium was supplemented with various concentrations of curcumin at 8 h prior to infection and then maintained for the duration of the experiment. The yield of virus was determined at 12, 18, 24 and 30 hpi. As shown in Fig. 1A, the production of virus was significantly reduced upon treatment with curcumin in a dose-dependent manner; in the presence of 30 μM curcumin, the titre of virus was less than 5% of that in mock-treated cells at all time points of infection analysed (Fig. 1A). Noticeably, the synthesis of viral proteins, such as haemagglutinin (HA), neuraminidase (NA), and matrix protein (M1) was affected by curcumin treatment (Fig. 1B). However, virus protein production was delayed rather than abrogated; substantial amounts of virus protein were produced between 24 and 30 hpi, although virus yields were reduced by over 95% at these time points (compare with Fig. 1A, 30 hpi/30 μM). This phenomenon is consistent with a previous study showing that inactivation of NF-κB signalling by aspirin impaired viral RNP export and subsequent virus multiplication, but did not significantly affect viral protein accumulation (Mazur et al., 2007).

3.2. Curcumin affects an early stage of virus infection

Time of addition experiments were performed to determine the stage(s) at which curcumin exerted its inhibitory effects. Curcumin was added to MDCK cells at three distinct time points: prior to infection (pre-treatment), at the same time as virus infection (simultaneously), or at 2 hpi (after entry). As shown in Fig. 2, MDCK cells pre-treated with curcumin 8 h prior to infection (but removed just before virus infection) reduced the production of virus to 20% at 12, 18, and 24 hpi (possibly through effects on NF-κB, although this is not addressed by this study). The addition of curcumin simultaneously with virus resulted in a much stronger inhibition than that of cells pre-treated with curcumin at 18 and 24 hpi (Fig. 2, with significance $p < 0.05$). Importantly, addition of curcumin at 2 h after infection reduced the degree of inhibition (in the

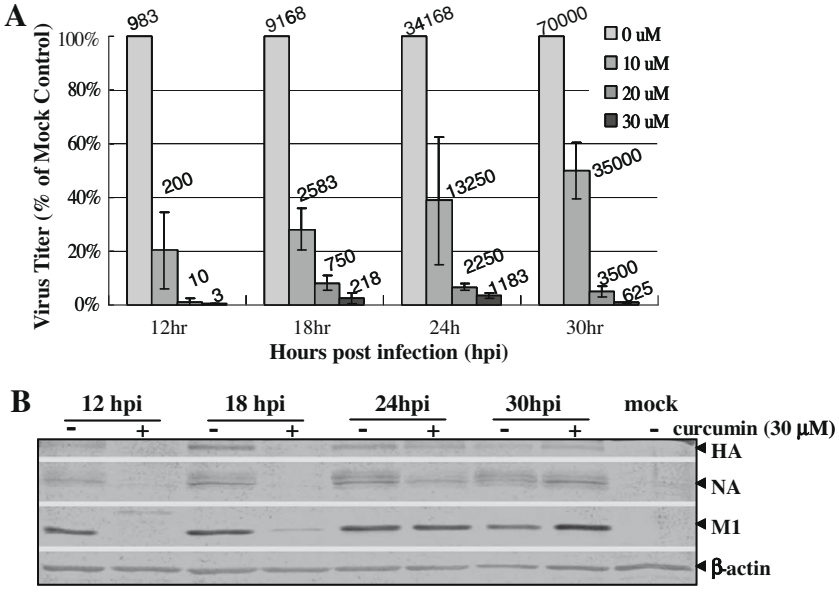

Fig. 1. Treatment of curcumin reduces influenza A viruses replication. (A) MDCK cells were pre-incubated with curcumin 8 h prior to and throughout the time of PR8 influenza virus infection (MOI = 0.05). The yield of virus progeny was determined by plaque assay as shown in the top of each column and plotted as a percentage of the untreated control. (B) Accumulation of viral proteins as determined by Western Blot of infected cell extracts taken at 12, 18, 24, and 30 h post-infection (hpi).

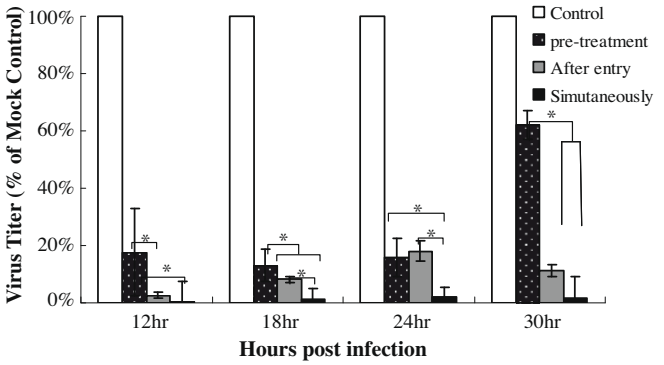

Fig. 2. The effect of curcumin on different stages of virus infection. About 30 μM curcumin was added to cells at three distinct time points: 8-h prior to infection (pre-treatment), at the same time as virus infection (simultaneously), or at 2 hpi (after entry). The yield of progeny viruses in supernatant was determined at 12, 18, 24, and 30 hpi. * indicates the *p*-value < 0.05.

case of the 18 and 24 h time points back to the pre-treatment levels). This suggested that curcumin may directly interfere with a very early stage (possibly directly with the virus particle), to prevent infection. We therefore performed plaque reduction assays to measure the plaque formation ability of IAV particles pre-incubated with curcumin. As indicated in Fig. 3A, the minimal concentration for complete inhibition was 6 μM (Fig. 3A) and the EC_{50} (i.e. the concentration of curcumin that reduced the plaque formation by 50%, relative to the control without test compound) was 0.47 μM. Given the CC_{50} of curcumin was 43 μM, the selectivity index (SI) value (CC_{50}/EC_{50}) of curcumin is approximately 92.5, higher than several anti-influenza agents published elsewhere (Song et al., 2007).

Since the inhibitory effect was observed when virions were pre-exposed to curcumin prior to infection, whereas when it was introduced into the cell culture medium after virus attachment, a moderate inhibitory effect was observed in the yield of progeny viruses (Fig. 2), these results led us to suspect that the main target of curcumin is at the early stage of virus infection, most likely virus attachment. Therefore, we used a plaque reduction assay to evaluate whether curcumin affected attachment or not. Binding of IAV was carried out at 4 °C, the temperature that permits attachment but not endocytosis and membrane fusion, for 1 h in the presence of curcumin. Unbound viruses were then removed by cold buffer wash, and the quantity of bound virus was determined by counting the subsequent formation of plaques. The results indicate that incubation of curcumin with virus prior to (Fig. 3B-I), or upon (Fig. 3B-II) virus attachment completely abolished plaque formation.

To assess the effect of curcumin on penetration, viral attachment was synchronised at 4 °C without curcumin, unbound viruses were removed by washing and virus penetration was carried out at 37 °C for 30 min with curcumin treatment, after which the curcumin was removed. Noticeably, the plaque formation in cells treated with curcumin after virus attachment (Fig. 3B-III) displayed a similar infection rate to that of mock-treated cells (Fig. 3B-IV), indicating the curcumin-mediated antiviral activity acts on viral attachment but not penetration.

3.3. Curcumin blocks haemagglutinating activity of IAV virus particles

Previous results indicated that treatment with curcumin, prior to, or upon virus entry completely abrogated virus infectivity (Figs. 2 and 3B); hence, it is likely that the action of curcumin may be through the interference with binding of virus particles to the sialic acid receptor at the cell surface. To determine whether this was the case, a HA inhibition (HI) assay was employed to evaluate whether curcumin is able to inhibit haemagglutination by IAV. Four HAU of IAV were incubated with various concentrations of curcumin for 60 min at room temperature, followed by detection of RBC agglutination. Results demonstrated that curcumin pre-treatment (31.2 μM) prevented the binding of PR8 virus to chicken RBCs, as indicated by the spot-like appearance of non-haemagglutinated cells (Fig. 4A). This concentration is markedly higher than the EC_{50} against virus in the plaque reduction assay, but this may reflect the different assay parameters (4 HAU is many orders of magnitude more virus than is used in any of the tissue culture assays). The development of effective compounds that block virus infectivity by inhibition of the receptor binding or membrane fusion activities of HA has been limited due to the lead compounds acting against only certain subtypes of HA. Interestingly, curcumin also prevented the binding of another subtype of influenza virus (strain H6N1) to RBC; a concentration as low as 15.6 μM was sufficient to interfere with HA activity (Fig. 4A).

Loss of the HA activity of curcumin-treated influenza viruses suggests curcumin interrupts the link between the viral HA molecule and its cellular receptor by preoccupying the binding site on HA protein or by modification of the virus envelope. Increasing evidence indicates many proteins are influenced by curcumin, for instance, epidermal growth factor receptor (Chen, Xu, & Johnson, 2006), P-glycoprotein (Anuchapreeda, Leechanachai, Smith, Ambudkar, & Limtrakul, 2002), etc. Nevertheless, to date no direct

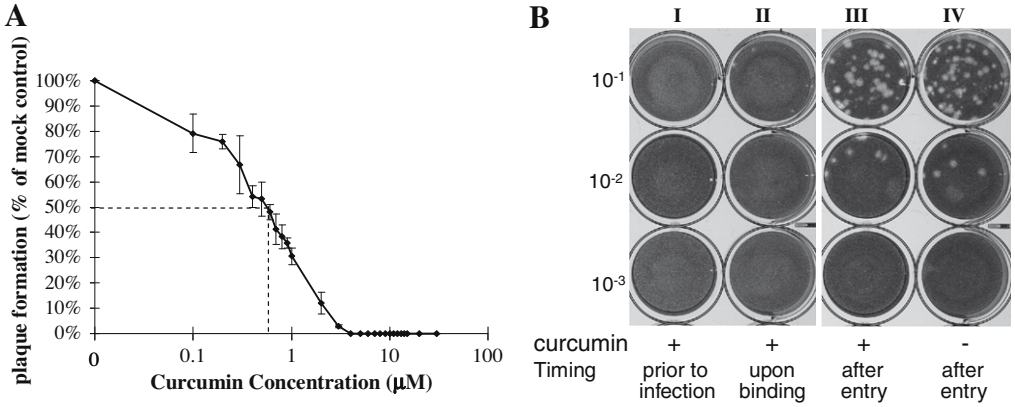

Fig. 3. Curcumin reduces plaque formation activity. Data are from three independent experiments. The dose-dependent effect of curcumin treatment was observed and the dotted line shows the EC_{50} of 0.47 ± 0.05 μM (A). (B) Evaluation of the effect of curcumin on various stages, such as virus attachment (I, II as labelled on top of panel B) or on penetration (III, IV).

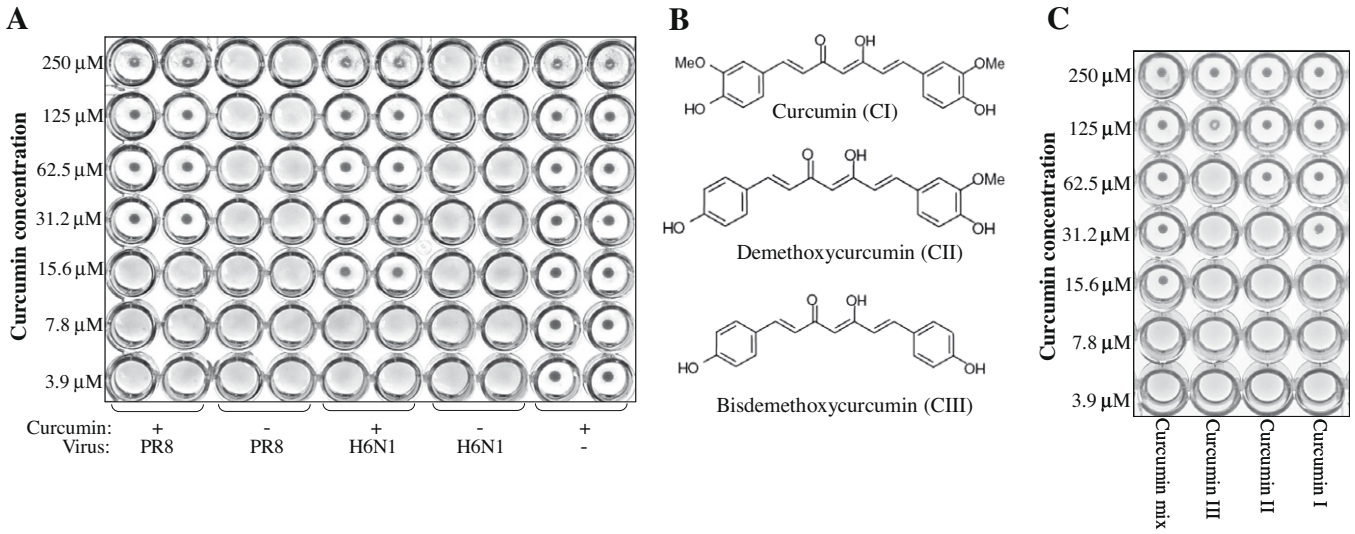

Fig. 4. Haemagglutination inhibitory activity of curcumin and other structure analogues. (A) 4 HA units of influenza viruses strain PR8 or H6N1 were incubated with twofold diluted curcumin or PBS (negative control) and the HA activity tested by incubation with chicken RBC (cRBC). The concentrations of curcumin necessary to completely inhibit haemagglutination (MIC) were approximately 26.04 and 15.63 μM for H1N1 and H6N1, respectively. The HI activities of three curcuminoids (B), isolated by HPLC (C), were analysed by same protocol as described in experiment A.

binding interaction with curcumin has been identified for any of these proteins. It was proposed that curcumin associates with membranes and high concentration of curcumin (>100 μM) leads to the alteration of erythrocyte cell membrane integrity (Jaruga, Sokal, Chrul, & Bartosz, 1998). However, the HA inhibitory effect is not an artifact resulting from curcumin-induced disruption of RBC because the minimal concentration required for HA inhibition was under 15 μM, which is not toxic to MDCK cells (Supplementary Fig. S1) and haemolysis was not observed at a concentration as high as 250 μM in the HA test (Fig. 4A). In addition, pre-treatment of RBC cells with curcumin followed by its removal did not affect the HA activity of influenza viruses (data not shown), indicating the membranes of RBC were intact at the concentrations used. Taken together, the HA inhibitory effect is primarily due to the interaction of curcumin with virus particles, not via an effect on RBC cells.

3.4. Characterising the pharmacophore of curcumin involved in HA interference

Commercially available curcumin consists of three major components: curcumin (curcumin I; ~77%), demethoxycurcumin (curcumin II; ~17%), and bisdemethoxycurcumin (curcumin III; ~3%) (Goel et al., 2007). The structure of curcuminoids differs only by the number of methoxyl groups (Fig. 4B). Curcumin has been shown to exert various biological effects; bisdemethoxycurcumin appeared to be the most active scavenger of superoxide radicals and inhibition of Ehrlich ascites tumour in mice (Ruby, Kuttan, Babu, Rajasekharan, & Kuttan, 1995). Another goal of this study was to define the structure/activity relationship (SAR) of curcumin; more specifically, to determine whether the methoxyl groups contribute to the antiviral effect by comparing the anti-influenza activities among three curcuminoids. Consequently, the curcuminoid components were separated by HPLC (Fig. 4C) and the authenticity of the three individual constituents was confirmed by NMR (Supplementary Fig. S2).

The antiviral activity of the three purified curcuminoids was initially confirmed by plaque reduction assay (data not shown). The structure of curcumin is symmetric with two phenolic groups, two methoxyl groups and two adjacent carbonyl/enol groups that give rise to an active methylene, which act as potential active sites for chemical modification and covalent linking with biomolecules. As indicated in Fig. 4D, in the HA interference assays the compounds lacking one methoxyl group (curcumin II), or both methoxyl groups (curcumin III) exhibited similar potency to curcumin I. This indicates that the presence of the methoxyl groups does not play a significant role in the HAI interaction. However, chemical synthesis of a series of curcumin analogues is required for a more detailed SAR assessment of the functional groups involved in its anti-influenza activity.

3.5. Curcumin treatment does not elicit viral resistance

Currently, two classes of antiviral drugs are available to treat influenza A infection: the inhibitors of M2, amantadine and rimantadine, and the neuraminidase inhibitors, zanamivir and oseltamivir (Monto, 2003). In light of the recent evidence for the emergence

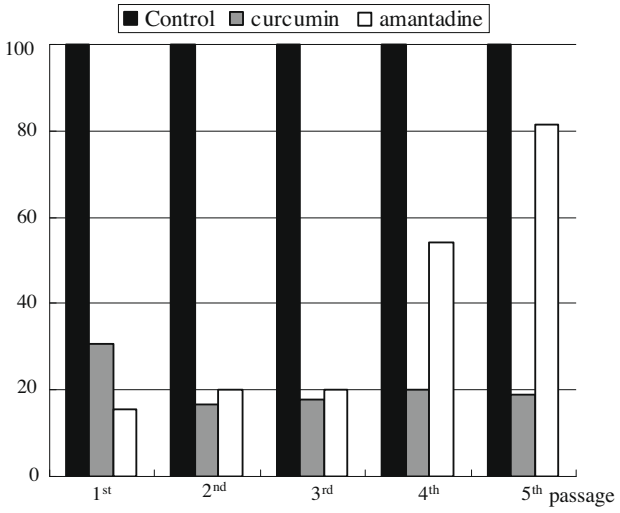

Fig. 5. Curcumin treatment does not elicit viral resistance. Virus yield of mock-treated cells was arbitrarily set as 100%.

of resistance to both classes of drugs, it is of importance to evaluate whether curcumin has the potential to induce viral resistance. To do so, a multi-passage experiment was performed, in which MDCK-passaged viruses that lacked the drug pressure were used as sensitive controls and amantadine treatment as the parallel control for resistant virus development. As shown in Fig. 5, the titre of progeny virions from cells treated with amantadine increased significantly after the fourth passage. In contrast to amantadine, the inhibition of virus passage remained throughout the time of the experiment and the vial yield did not rise even after the fifth passage, indicating treatment of curcumin is not prone to emerging of resistant viruses (Fig. 4).

4. Conclusions

Results from the plaque reduction test and HI test clearly show that curcumin interrupts virus-cell attachment, which leads to inhibition of influenza virus propagation, although it is not known yet whether curcumin directly interacts with the viral HA protein or with other viral surface components. With an established safety profile and high SI value of 92.5, curcumin has promising potential for using as an anti-influenza drug.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2009.09.011.

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