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Structure–activity relationship analysis of curcumin analogues on anti-influenza virus activity

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Curcumin (Cur) is a commonly used colouring agent and spice in food. Previously, we reported that Cur inhibits type A influenza virus (IAV) infection by interfering with viral haemagglutination (HA) activity. To search for a stable Cur analogue with potent anti-IAV activity and to investigate the structure contributing to its anti-IAV activity, a comparative analysis of structural and functional analogues of Cur, such as tetrahydrocurcumin (THC) and petasiphenol (Pet), was performed. The result of time-of-drug addition tests indicated that these curcuminoids were able to inhibit IAV production in cell cultures. Noticeably, Pet and THC inhibit IAV to a lesser extent than Cur, which is in line with their effect on reducing plaque formation when IAV was treated with Cur analogues before infection. Unexpectedly, both THC and Pet did not harbour any HA inhibitory effect. It should be noted that the structure of Pet and THC differs from Cur with respect to the number of double bonds present in the central seven-carbon chain, and structure modelling of Cur analogues indicates that the conformations of THC and Pet are distinct from that of Cur. Moreover, simulation docking of Cur with the HA structure revealed that Cur binds to the region constituting sialic acid anchoring residues, supporting the results obtained by the inhibition of HA activity assay. Collectively, structure–activity relationship analyses indicate that the presence of the double bonds in the central seven-carbon chain enhanced the Cur -dependent anti-IAV activity and also that Cur might interfere with IAV entry by its interaction with the receptor binding region of viral HA protein.

Introduction

Cur has generated considerable interest as a result of its versatile medicinally beneficial properties, including anti-carcinogenesis and anti-inflammation [1–3]. Furthermore, a number of studies indicate curcumin (Cur) is a potent antiviral agent as a result of its ability to interfere with the cellular signalling pathways or with proteins required for viral replication [4–8].

Abbreviations
BDMC, bisdemethoxycurcumin; Cur, curcumin; DMC, demethoxycurcumin; GSH, glutathione; GSSG, oxidized glutathione; HA, haemagglutination; HI effect, inhibition of HA activity; hpi, hours post infection; IAV, type A influenza virus; MAE, Michael acceptor electrophile; MDCK, Madin–Darby canine kidney; NF-κB, nuclear factor kappa B; Pet, petasiphenol; pfu, plaque-formnig units; SA, sialic acid; SH, sulfhydryl; THC, tetrahydrocurcumin.

Upon administration, Cur rapidly transforms into several compounds. Among these, tetrahydrocurcumin (THC), which lacks double bonds in the central seven-carbon chain, is one of the major metabolites, and is more stable than Cur in 0.1 M phosphate buffer, pH 7.2 [9]. Demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC) are two natural congeners of Cur isolated from turmeric and present in the commercially available Cur mix [3]. These compounds differ only with respect to the number of methoxy groups on the aromatic rings. Cur contains two methoxy groups: DMC has one and BDMC has none. Studies of Cur metabolism in the rat revealed that BDMC is chemically more stable than Cur and DMC; nevertheless, the reductive metabolite THC is the most stable [10]. It is well recognized that suppression of cellular signalling pathways, such as nuclear factor kappa B (NF-κB), is responsible for the biological effects modulated by Cur and its derivative compounds [1,11,12]. Sandur *et al.* [13] demonstrated that the relative potency for the suppression of NF-κB activation induced by tumour necrosis factor-α was Cur > DMC > BDMC, whereas THC was unable to suppress the transcription factor [13], indicating that Cur analogues differentially modulate the anti-proliferative and anti-inflammatory responses.

Petasiphenol (Pet) is a compound isolated from *Petasites japonicus*, a higher plant of Japanese vegetable. The structure of Pet differs from BDMC only by the double bonds in the central chain (Fig. 1). It selectively inhibits the activities of mammalian pol λ *in vitro* [14]; however, little is known about whether Pet shares biological properties with Cur.

Our previous study demonstrated Cur acts as a novel agent to inactivate infectivity of the type A influenza viruses (IAV) via multiple mechanisms, such as the inhibition of viral haemagglutination (HA) function, and to affect envelop integrity [4,15]. Moreover, the anti-influenza activity of Cur and other two curcuminoids, DMC and BDMC, is indistinguishable, indicating that the methoxyl group on the phenyl rings was not the crucial portion contributing to the anti-IAV activity and the inhibition of viral HA function mediated by Cur [4]. At present, the effect of Cur derivatives on anti-IAV has not yet been studied. Hence, the present study aimed, first, to determine whether the stable Cur metabolite exerts anti-IAV activity and, second, to compare the effect of Cur analogues on IAV infection to map out the structure of Cur crucial for anti-viral activities.

Results

Structure and cytotoxicity of Cur analogues

The structure of various curcuminoids analyzed is shown in Fig. 1A. Cur contains two conjugated/double bonds in the central seven-carbon chain that constitutes unsaturated diketone (enone groups). Unlike Cur, Pet has one enone group and THC contains none. Because Cur, THC and Pet were analyzed in the cell based system, the cytotoxicity of these compounds was initially determined and the concentration below CC_{50} (i.e. drug concentration inhibiting cell growth by 50% relative to the dimethylsulfoxide solvent control) was used for the anti-viral assays (Fig. 1B).

THC inhibits IAV infection and plaque formation but to a lesser extent than Cur

To evaluate the effect on influenza virus replication, THC, the major metabolite of Cur, or Cur was included in cell culture medium at 8 h before infection and then maintained for the duration of the experiment. The yield of virus was determined at 12, 24 and 36 h post infection (hpi). As shown in Fig. 2A, in contrast to Cur

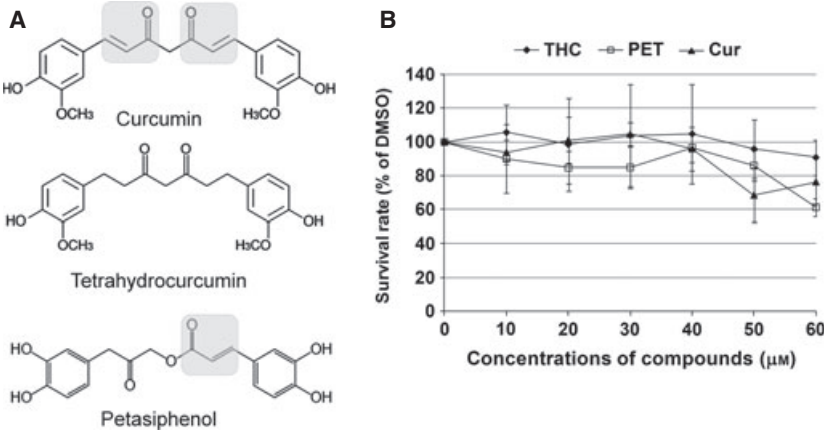

Fig. 1. The chemical structures and cytotoxicity of Cur and Cur analogues. Pet and THC are structure analogues of Cur (A). Unlike Cur, Pet and THC contain either one or no enone group. The cytotoxicity of the analogues of Cur was tested on the canine MDCK cell line (B). The survival rate was plotted as the percentage of cell number relative to that of cells treated with dimethylsulfoxide, the solvent control.

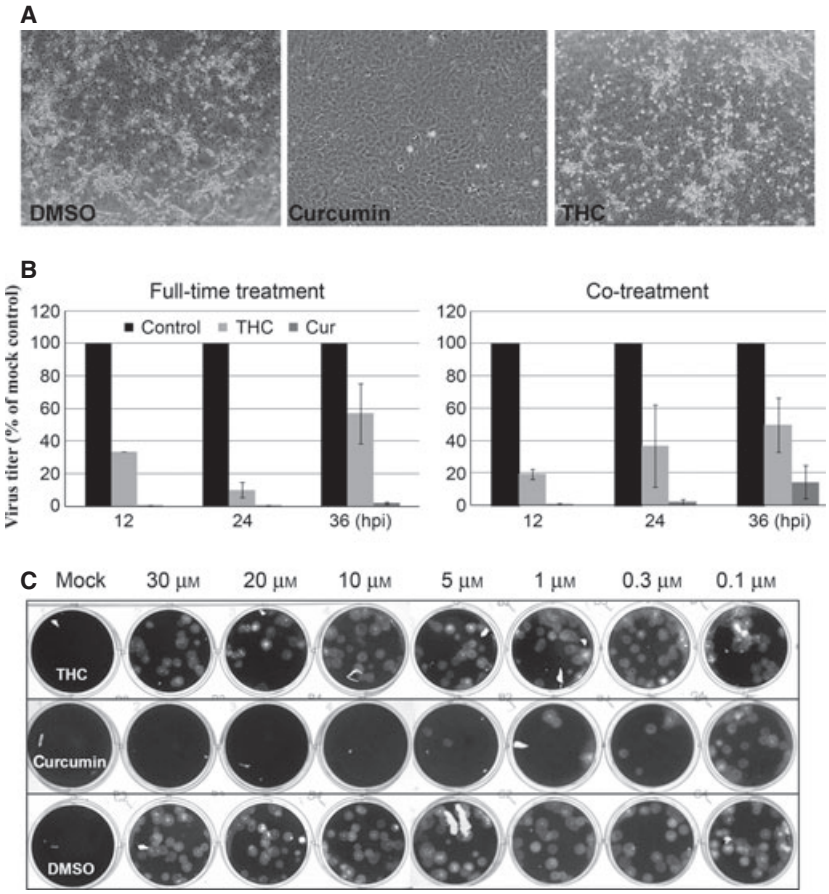

Fig. 2. Treatment of THC reduces influenza A virus replication to a lesser extent than Cur. (A) MDCK cells were pre-incubated with 30 μM of Cur or THC at 8 h before and throughout the time of IAV (strain PR8) (2000 pfu). The cytopathogenic effect was recorded. (B) The effect of THC on different stages of virus infection. 30 μM Cur or THC was added to cells at two distinct time points: 8 h before and throughout the time of infection (full-time treatment) or at the same time as virus infection (co-treatment). The yield of progeny viruses in the supernatant was determined at 12, 18, 24 and 30 hpi. Experiments were conducted with three independent repeats. The effect of THC on the yield of virus progeny was plotted as a percentage of the untreated control (dimethylsulfoxide). (C) THC did not completely inactivate the plaque formation activity. PR8 viruses were pre-treated with a serial dilution of Cur or THC for 1 h and the remaining viral infectivity was measured by the plaque assay.

treatment, THC moderately protected cells from IAV infection at 36 hpi, as demonstrated by the severe cytopathic effect. Nevertheless, the production of virus progenies was significantly reduced upon treatment with THC at the early time points of infection; in the presence of 30 μM THC, the titre of virus was < 40%, and 20% of that in mock-treated cells at 12 and 24 hpi, respectively (Fig. 2B,left panel). Similarly, when THC was added to cells simultaneously during virus treatment (co-treatment), inhibition of IAV replication was observed at all time points of infection (Fig. 2B, right panel), albeit at a lower strength compared to Cur.

Because co-treatment of THC exhibits a similar inhibitory effect to that of full-time treatment, it is possible that, as with Cur, THC may interfere with IAV at a very early stage or directly interacts with the virus particle, thereby preventing infection. We then conducted plaque reduction assays to estimate the plaque formation ability of influenza virus particles pre-treated with THC. Unexpectedly, pre-incubation of virus with THC did not affect viral infectivity; even treatment with the highest concentration (30 μM) only moderately reduced the plaque number. This is distinctly different from the effects of Cur; at concentration higher than 5 μM, Cur treatment completely abrogated the plaque formation ability of IAV (Fig. 2C).

Cur structure analogues differ in their ability to inhibit IAV

The structures of Cur and THC only differ by the unsaturated diketone (Fig. 1A). To explore whether the unsaturated diketone is indeed responsible for anti-IAV activity, Pet with one enone group was included in the comparative analysis.

Time-of-drug addition experiments were conducted to determine the effect of Cur analogues at different stages during infection. Test compounds, each at a concentration of 30 μM, were added to cells at three time points: before infection (pre-treatment), simultaneously with virus particles (co-treatment) or at 2 hpi (after-entry). At 12 and 24 hpi, treatment of the analogues significantly inhibited the amplification of IAV progenies (Fig. 3). Overall, full-time treatment of Cur analogues provided the highest protection. The inhibition strength of co-treatment of THC or Pet with IAV was significantly different; at 12 and 24 hpi, the yields of viral progenies were greatly reduced by Pet than

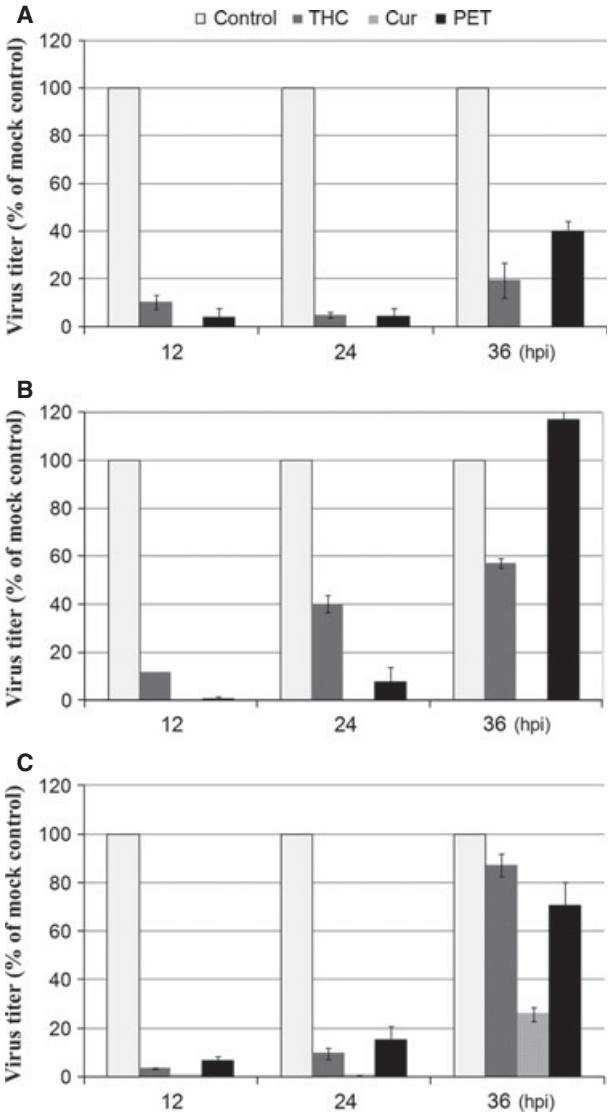

Fig. 3. The effect of Cur analogues on different stages of virus infection. 30 μM Cur, THC, Pet or dimethylsulfoxide (solvent control) was added to cells at three distinct time points: 8 h before and throughout the time of PR8 influenza virus infection (A; as full-time treatment), at the same time as virus infection (B; as co-treatment) or at 2 hpi (2 h post infection) (C; as post-entry treatment). The yield of progeny viruses in the supernatant was determined at 12, 24 and 36 hpi. Experiments were conducted with three independent repeats. The effect of tested Cur analogues on the yield of virus progeny was plotted as a percentage of the mock control (dimethylsulfoxide).

THC (Fig. 3B). Nevertheless, the inhibitory effect of Pet on IAV was lost or obviously diminished at a later time of infection (36 hpi) in both co- and after entry treatments. Collectively, Cur with two enone functionalities (account for Michael acceptor electrophile; MAE) harbours a stronger inhibitory effect of virus production than those with one or none (Pet and THC, respectively).

The two double bonds in the central carbon chain correlate with their ability to inhibit IAV plaque formation and viral HA activity

Next, we conducted plaque reduction assay and HA inhibition test to evaluate the effect of analogues on viral infectivity and the entry machinery. Consistent with our previous finding, pre-incubation of IAV with Cur strongly blocked the IAV plaque formation ability; the EC_{50} (i.e. the concentration of Cur that reduced the plaque formation by 50% relative to the control without test compound) of Cur was 0.17 μM. Notably, Pet and THC had much lower inhibitory effect than Cur (Fig. 4A). At the highest concentration tested, THC failed to reduce 50% of plaque formation, and the EC_{50} of Pet was 14.65 μM. This finding implicates that the enone functionalities of Cur attribute to the differential inhibition of IAV activity.

Our previous study indicated that inhibition of viral HA function, leading to interference with viral entry, participates in anti-IAV effect mediated by Cur [4]. It is important to investigate whether Cur analogues share this effect with Cur. Consistently, pre-incubation of IAV with Cur (≥ 31.2 μM) prevented the binding of influenza virus to chicken red blood cells, as indicated by the spot-like appearance of non-haemagglutinated cells. However, unexpectedly, none of the tested Cur analogues interrupted the engagement of viral HA with cells (Fig. 4B).

Pre-incubation of glutathione (GSH) but not oxidized glutathione (GSSG) attenuates the inhibitory effect of curcumin on anti-IAV

It has been shown that MAEs (molecules containing enone functionalities, such as Cur) potentially form covalent Michael adducts by interacting with the cysteine sulfhydryls (SH) of certain proteins or of GSH acting as a strong electron donor [16–19]. We then further tested whether addition of glutathione attenuates Cur-dependent anti-IAV response, including plaque reduction and inhibition of HA activity (HI effect) (Fig. 5A). In this assay, GSSG, comprising dimerized glutathione lacking electrons, serves as a negative competitor. Neither cell viability (data not shown), nor viral infectivity (marked as Mock in Fig. 5B) was affected by the treatment of GSH and GSSG. However, the inhibitory effect of Cur on viral plaque formation was partially attenuated by pre-incubation of Cur with GSH before adding to IAV (Fig. 5B). By

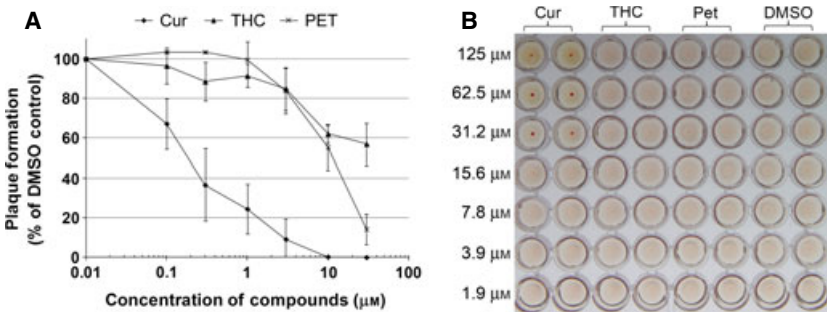

Fig. 4. Curcumin analogues displayed differential anti-influenza virus activities. (A) The effect of Cur analogues on IAV plaque formation. 2000 pfu of PR8 viruses were pre-incubated with various concentrations of Cur, three analogues or dimethylsulfoxide for 1 h. The remaining viral infectivity was then measured by the plaque assay and plotted as a percentage of the dimethylsulfoxide controls (A). Data were obtained from three independent experiments. (B) The effect of Cur analogues on HA function. Four HA units of PR8 viruses were incubated with two-fold serially diluted Cur, analogues or dimethylsulfoxide (negative control) and HA activity was tested by incubation with chicken red blood cells.

contrast, pre-incubation with GSSG did not interfere with the Cur-dependent anti-IAV effect. This result supports the hypothesis that Michael addition conjugation participates in the inhibitory effect of Cur on influenza virus infection. Nevertheless, pre-incubation of Cur with GSH did not reverse the inhibitory effect of Cur on IAV HA activity (Fig. 5C).

Not all phytocompounds with enone moiety possess anti-IAV activity

Because the differential inhibitory effect on IAV is correlated (at least in part) with the presence of enone moiety on Cur, the anti-IAV effect mediated by a collection of phytocompounds with the enone moiety, including flavokawain B, cinnamaldehyde, caffeic acid, lucidone and rosmarinic acid, was tested. The results from both plaque reduction and HA inhibition assays indicated that none of these compounds exerted anti-IAV activities (Fig. 6).

Three-dimensional structures of Cur and analogues

To obtain information about the molecular basis of the inhibitory properties of Cur and its five analogues, such as DMC, BDMC, Pet and THC, computational analyses of curcuminoid structure were undertaken using molecular simulation and surface analysis software. Figure 7A shows the energy-minimized three-dimensional structures by comparing the electrostatic potential surfaces of these compounds. The electrostatic potential at each point on a constant electronic density surface (approximating the van der Waals surface for each arrangement) is represented graphically in red and corresponds to regions where the electrostatic potential is most negative; blue corresponds to the most positive regions. Notably, the distribution of the electrostatic potential is similar in those with two double bonds, such as Cur, DMC and BDMC, although it is distinct from that of Pet and THC. Moreover, because of the presence of double bonds in the central chain, a striking conformational difference was found in THC and Pet compared to Cur and other curcuminoids; the three-dimensional structures of Cur, DMC and BDMC are more rigid, whereas Pet and THC are twisted.

Docking simulation of Cur interaction interface on HA

Among the curcuminoids tested, only Cur harbours HA inhibition activity. We then aimed to investigate whether Cur blocks HA function via a direct interaction. To address this, a docking simulation of the Cur interaction interface on HA was conducted. In the energy-minimized docking simulation of Cur and HA using AUTODOCK, version 4.2, we scanned for the Cur binding surface of HA and found that Cur binds to the region constituting the sialic acid (SA) anchoring residues. This binding of Cur would abrogate the interaction of HA with the cellular receptor (Fig. 7B). The molecular size of SA and Cur was almost the same, in both length and width; therefore, Cur fitted the SA binding groove (Fig. 7C). As shown in Fig. 7D, the ten amino acid residues, which possibly interact with Cur, were indicated, and the distances between Cur and these residues were within 5.0 Å. In particular, Cur could conjugate to the residues of Asn133 and Gln226 by the formation of one and two hydrogen bonds, respectively, and the respective binding distances are in the range 3.1–3.1 Å (the yellow dotted lines of Fig. 7D). The estimated binding energy

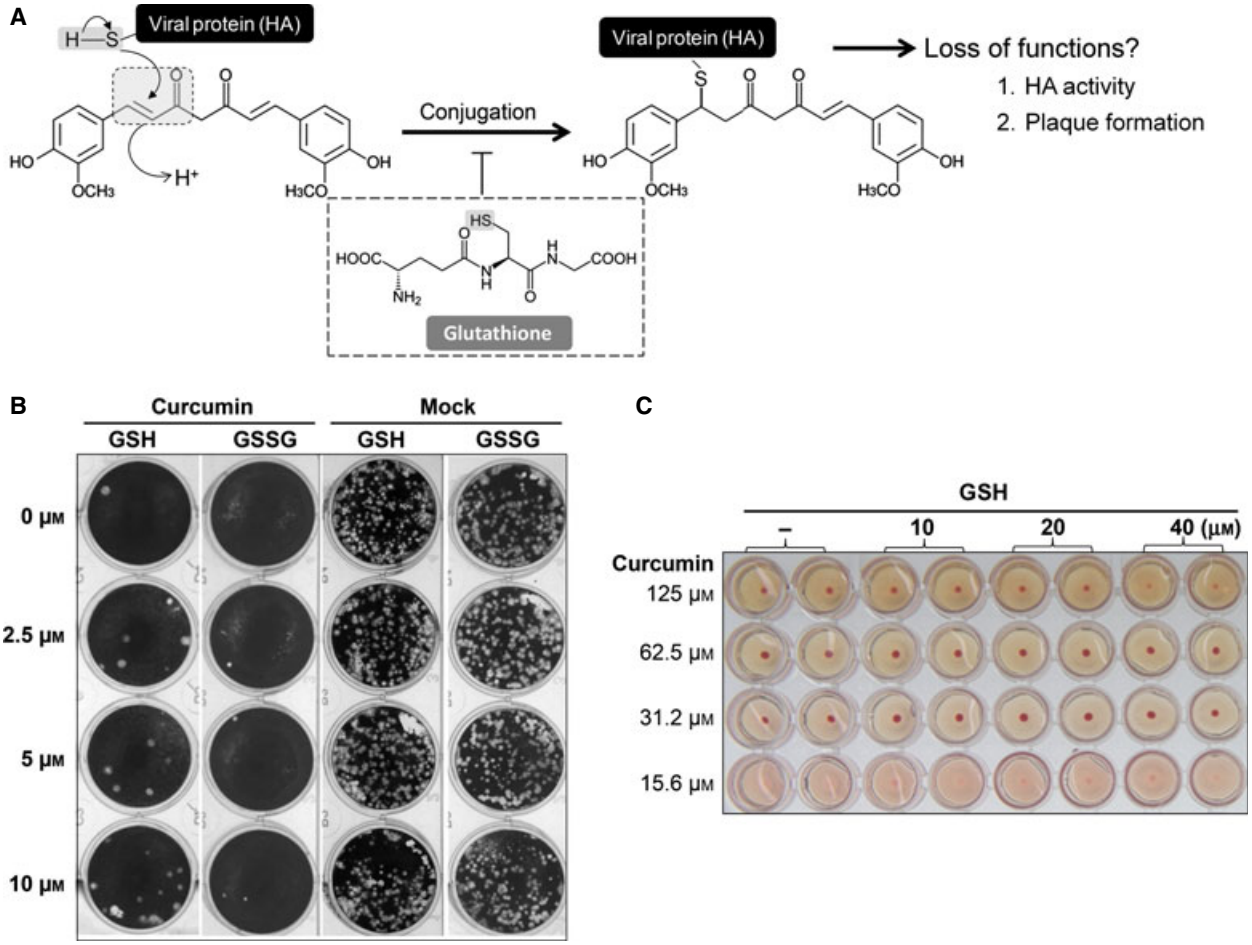

Fig. 5. Addition of GSH attenuated the effect of Cur on anti-IAV activity. (A) The acting model of modification of viral protein by Cur. The enone functionalities (highlighted in the grey box by a dashed line) of Cur potentially conjugate with protein(s) containing the SH-group with an electron donating property. In the presence of GSH, as a competitor that preferentially conjugated with Cur, the anti-IAV activity mediated by Cur would be attenuated. (B, C) The effect of GSH on Cur-dependent anti-IAV activity. Curcumin was incubated with various concentrations of GSH (as a competitor) or GSSG (as a negative competitor control) at room temperature for 1 h. The Cur-dependent IAV activity was evaluated with respect to plaque reduction (B) and HA inhibition (C) using the methods described previously. The images in (B) indicate the titre from 10^{-1} diluted virus-Cur mix.

between Cur and HA was -4.89 kcal·mol^{-1} and the inhibition constant (K_i) was 26.21 μM at 298.15 K. These results suggested that *in silico* docking simulation data correlates with the *in vitro* experiments (i.e. inhibition of HA activity assay), as well as the time-of-drug addition test (such as co-treatment).

Discussion

In the present study, to gain better understanding of the antiviral mechanism of Cur, and to search for stable derivatives exhibiting a higher biological activity than Cur, aspects regarding the anti-IAV activity of curcuminoids were investigated: (a) does the stable Cur metabolite THC exert anti-IAV function and (b) extending from our previous study, what is the critical structure responsible for the anti-IAV activity mediated by Cur?

Curcumin is rapidly degraded or bio-transformed at neutral or basic pH conditions unstable at physiological conditions [9,20]. Thus, it is important to investigate whether Cur could exert its anti-viral activity through its metabolites. Despite the lesser potency than Cur, treatment of THC inhibited the yields of virus progenies, especially at 12 and 24 hpi, indicating that THC, one of the major metabolites of Cur, can serve as a potential anti-influenza viral agent.

Antiviral agents would be developed to target viral factors or/and cellular factors or pathways required for efficient viral replication. As for the curcuminoids

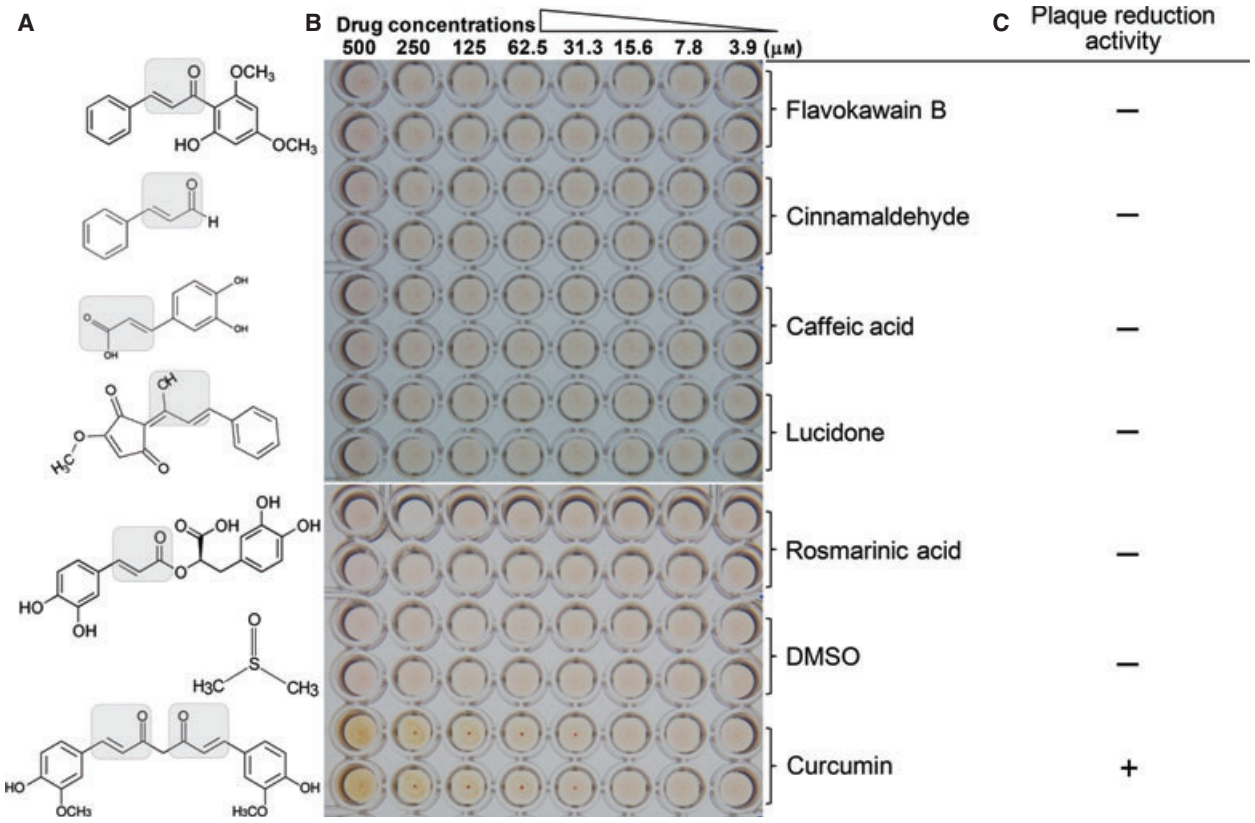

Fig. 6. The effect of compounds with enone moiety on viral HA activity. (A) The structure of the tested phytocompounds. Five compounds, including flavokawain B, cinnamaldehyde, caffeic acid, lucidone and rosmarinic acid, contain enone moiety as highlighted in the grey box. (B) The effect of phytocompounds on HA functions. Four HA units of influenza virus strain PR8 were incubated with two-fold serially diluted Cur, other phytocompounds or dimethylsulfoxide (negative control) and HA activity was tested by incubation with chicken red blood cells at room temperature for 1 h. (C) The effect of phytocompounds on IAV plaque formation. 2000 pfu of PR8 were pre-treated with various concentrations of the tested compound for 1 h. The remaining viral infectivity was measured by the plaque assay.

analyzed in the present study, these compounds differentially inhibit influenza virus propagation that is likely through multiple mechanisms. First, upon addition of THC to cell culture after viral entry, the yields of IAV decreased to < 20% at 12 hpi, and 24 hpi of that in mock treatment (Fig. 3C), implying that THC could possibly target either the certain step(s) of viral replication machinery or suppress the cellular signalling pathways essential for viral replication. Three pathways, Raf/MEK/ERK module belonging to mitogen-activated protein kinase cascades [21–23], NF-κB signalling [24,25] and phosphatidylinositol 3-kinase/protein kinase B [26,27], have been shown to be activated during infection and are required for efficient influenza virus propagation. A recent study indicated that THC significantly suppressed activation of phosphatidylinositol 3-kinase/protein kinase B and mitogen-activated protein kinase signalling in human HL-60 cells [28]. Because incubation of viral particles with THC only moderately reduced its infectivity (Fig. 2C and 4A) and did not block the viral HA function (Fig. 4B), it is possible that down regulation of these signalling pathways greatly contributes to the anti-IAV activity mediated by THC.

In addition to interfering with the cellular signalling pathway required for viral replication, curcuminoids might exert inhibitory effects via blocking the function of viral protein(s) and/or interacting with viral particles. Our previous study showed that Cur treatment of IAV before cell adsorption caused the loss of viral infectivity and the HI effect [4]. Moreover, two Cur analogues, DMC and BDMC, containing either only one or no methoxy groups, respectively, harbour the same inhibition potency as Cur, indicating that the methoxy groups are not responsible for the HI effect. Curcumin has a symmetric structure with two aromatic rings that are bridged by two α,β-unsaturated carbonyl (enone) groups (as indicated by the grey block in Fig. 1). The comparative analysis of Cur analogues described in the present study demonstrated

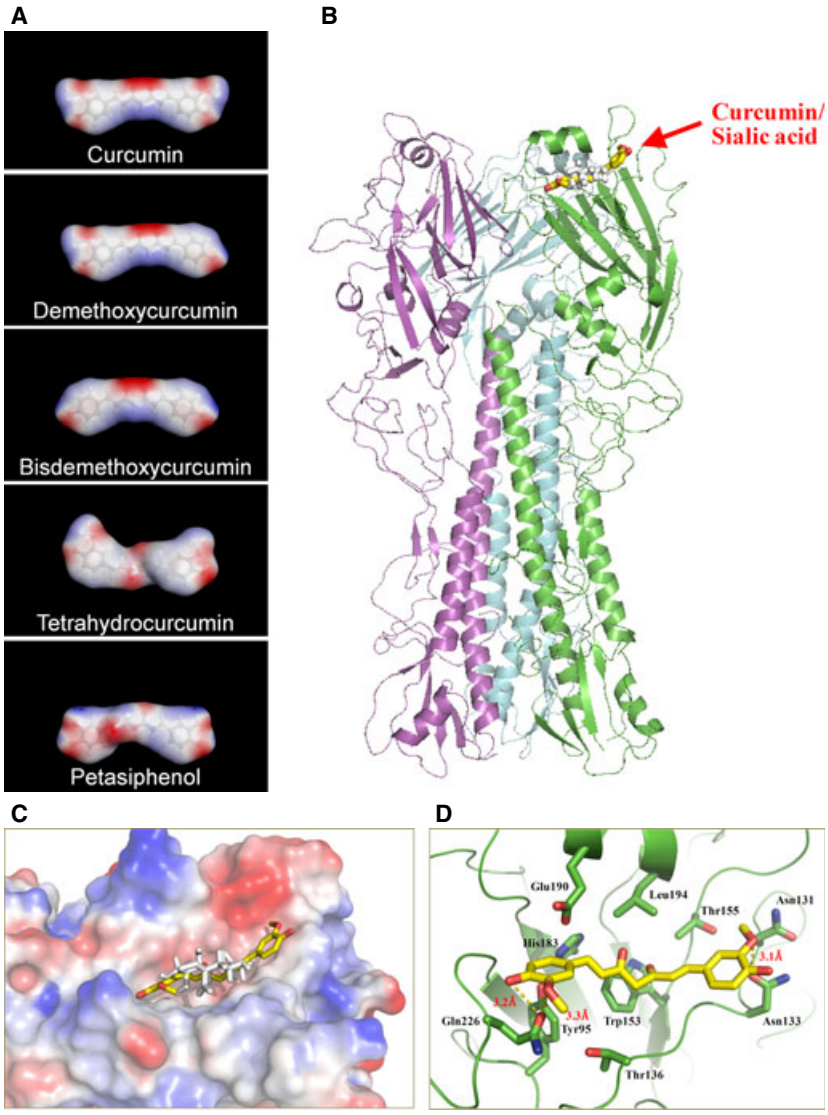

Fig. 7. Simulation of curcuminoid structure and the docking analysis of the Cur interaction interface on HA. (A) Three-dimensional structures of Cur and analogues. The energy-minimized three-dimensional structures of Cur, DMC, Pet, BDMC and THC were analyzed computationally. The electrostatic potential at each point on a constant electronic density surface is represented graphically in red and corresponds to regions where the electrostatic potential is most negative; blue corresponds to the most positive regions. Docking simulation of Cur interaction interface on HA. (B) Ribbon diagram of the trimer of PR8 HA. The overall crystal structure of A/puerto rico/8/1934 HA was downloaded from the Protein Data Bank (Protein Data Bank code: 1RU7). HA is homotrimer, and each subunit structure is indicated in green, pink and light cyan. The carbon and oxygen of stick structures of Cur are indicated in yellow and red, respectively. The stick structure of SA is indicated in white. (C) Electrostatic potential surface near the putative Cur binding pocket in HA. Blue indicates a positive charge, red indicates a negative charge and white indicates neutral. The carbons and oxygens of the stick structure of Cur are indicated in yellow and red, respectively. The stick structure of SA is indicated in white. (D) Curcumin interactive amino acid residues of HA. The carbons, nitrogens and oxygens of the stick structures of amino acid residues are indicated in green, blue and red, respectively. The carbons and oxygens of the stick structure of Cur are indicated in yellow and red, respectively. The dotted lines indicate potential hydrogen-bond interactions between Cur and the residues of HA protein. These structural images were prepared using PYMOL.

that the relative potency of curcuminoids on plaque reduction was Cur > pet > THC, indicating that the number of enone group correlated with the inhibitory potency of curcuminoids on plaque formation ability.

The enone group acts as the acceptor of the Michael addition reaction that is involved in the intermolecular conjugation with certain proteins. Previous studies demonstrated that the presence of Michael acceptor groups

at critical positions contributes to several triterpenoid analogues of oleanolic acid-induced cellular activities, such as anti-inflammation, inhibition of cell proliferation, promotion of cell differentiation and induction of apoptosis in various tumour cell lines [29–31]. Interestingly, this portion of Cur structure is responsible for activating transcription factor NF-E2-related factor 2 through covalent modification of its repressor Keap1 [32]. Similarly, the α,β-unsaturated carbonyl functional group of chalcone is essential for the inhibition of NF-κB via covalent modification of inhibitor of NF-κB kinase subunit β [17]. It is very possible that, via Michael addition reaction, Cur conjugates with viral surface protein(s) and interferes with their function, thereby inactivating virus infectivity. With respect to HA inhibition, in contrast to Cur, both THC and Pet failed to block viral HA activity. Because each double bond conjugated to the carbonyl moiety of Cur was saturated, losing the property of MAE, the resulting THC cannot act as a Michael reaction acceptor. Similarly Pet bearing one enone functional group has much weaker effect on inhibition of IAV infectivity, and did not inhibit HA activity. As indicated in Fig. 5, addition of GSH attenuated the inhibition effects of Cur. This implies that incubation of Cur with a protein or a peptide (i.e. GSH) having exposed electron donating functional groups (i.e. SH group of cysteine in GSH) precludes the accessibility of the MAE portion in Cur, which in turn would diminish the opportunity of Cur to modify viral surface proteins. It is important to note that the HI effect was not affected by GSH. We suspect that either the Michael addition reaction is not a sufficient factor (other Cur-dependent effects contribute to its anti-IAV activity) or GSH is not an appropriate molecule to compete with the IAV surface protein (i.e. HA) for the conjugation to Cur. A number of phytocompounds with MAE properties failed to inhibit the HA activity of IAV (Fig. 6), indicating that Michael addition was not the sole contributor to the anti-IAV activity, thus favouring the first assumption. For the second assumption, the docking simulation demonstrated that Cur could interact with the Asn133 and Gln226 residues on the receptor binding region of viral HA protein by formation of one and two hydrogen bonds, respectively. This explains the inefficient competition of GSH because cysteine is unlikely to be the main residue on the RBS region of the HA protein that interacted with Cur.

W153, T155, G134, T136, H183, E190, L194, Y98, Q226 and G228 on PR8 HA protein are responsible for anchoring the SA monosaccharide, the host receptor of influenza virus [33]. Incidentally, most of these (seven out of ten) SA anchoring residues also potentially interact with Cur, as analyzed by docking simulation (Fig. 7C), suggesting that conjugation of Cur with RBS residues on HA is likely to decrease the possibility of IAV engaging with its cellular receptor; thereby abrogating viral entry. This is in line with our experimental evidence showing that incubation of virus particles with Cur before cell attachment blocks IAV agglutinating red blood cells (HI effect), inhibits plaque formation on Madin–Darby canine kidney (MDCK) cells and also reduces the yield of viruses.

Although, by interaction with the viral HA protein, Cur efficiently blocks viral entry, based on the experience of NA inhibitors, it might be suspected that resistant virus variants could be developed in response to selection with Cur. Nevertheless, the emergence of IAV variants against Cur was not observed after five rounds of blind passage under Cur treatment [4]. This would be explained by the fact that Cur-dependent anti-viral ability also occurs via the targeting of cellular factors [4,5,7] and accumulated evidence indicates that anti-viral compounds targeting cellular factors appear to be a high barrier for development of resistant virus variants [25,34].

Conclusions

Several novel findings are highlighted in the present study. (a) The two Cur structure analogues (Pet and THC) exhibit anti-influenza virus activity. This also implies, that after Cur undergoes bio-transformation, the resulting THC, a stable metabolite of Cur, is able to inhibit influenza virus propagation. (b) Structure–activity relationship analysis revealed that the two-enone functional groups, as an acceptor of Michael addition conjugation, contribute to the differential inhibitory effect of curcuminoids. (c) The results from an *in vitro* assay (HI test), cell-based measurements and *in silico* docking simulation indicated that Cur effectively blocks influenza entry by interrupting the interaction of HA protein with the cellular receptor.

Materials and methods

Cells and virus

MDCK cells were cultured in MEM with 10% fetal bovine serum, penicillin 100 $U \cdot mL^{-1}$ and streptomycin 10 $\mu g \cdot mL^{-1}$. For infection, cells were washed with $NaCl/P_i$ and cultured in infection medium (MEM without fetal bovine serum) supplemented with antibiotics and 1 $\mu g \cdot mL^{-1}$ of trypsin (Worthington, Freehold, NJ, USA).

Human IAV strain PR8, A/Puerto Rico/8/34 (H1N1), kindly provided by Paul Digard (University of Cambridge, Cambridge, UK) was propagated in MDCK cells.

Compounds

Curcumin, dimethylsulfoxide, GSH and GSSG were purchased from Sigma-Aldrich (St Louis, MO, USA). THC was a gift kindly provided by Sabinsa Corp. (Esat Windsor, NJ, USA). Pet was prepared as described previously [14]. Phytocompounds, including flavokawain B [35], cinnamaldehyde [36], caffeic acid [37], rosmarinic acid [38] and lucidone [39], were isolated as described in our previous studies. All of the tested compounds were dissolved in dimethylsulfoxide and stored as small aliquots at −80 °C.

Cytotoxicity test

Proliferation of cells treated with Cur analogues was determined as described previously [4]. Cytotoxicity of the compounds was estimated by comparison of the cell survival rate of drug-treated cells with that of dimethylsulfoxide-treated cells. The dimethylsulfoxide-treatment control was arbitrarily set as 100%.

Time-of-drug addition test

The indicated concentrations of Cur, its analogues or dimethylsulfoxide (as solvent control) were added to the medium at different time points of infection. In brief, (a) full-time treatment: the test compound was included in the cell culture medium for 8 h and throughout the course of infection; (b) co-treatment: the test compound mixed with virus in the infection medium was added simultaneously to the cells and left with the cells throughout the course of the experiment; (c) post-entry treatment: Cur and its analogues were added to cells at 2 hpi) and left with the cells throughout the course of infection.

Plaque assay

MDCK cells seeded in 12-well plates (1.2×10^5 cells per well) were washed twice with $NaCl/P_i$ followed by infection with serial dilutions of IAV at 37 °C. Two hours after adsorption, unbound virus particles were removed and cells were then cultured with 1 mL per well MEM supplemented with 0.6% agarose at 37 °C, 5% CO_2 for 2 days or until the plaques were visible. Viral plaques were then fixed with 100% methanol for 10 min and stained with crystal violet (Sigma-Aldrich).

Plaque reduction assay

This method was used to evaluate the effect of curcuminoids on viral particles. After treatment with Cur analogues, the remaining infectivity of IAV was determined by the plaque reduction assay [4]. Two-thousand plaque-forming units (pfu) of IAV were pre-incubated with 30 μM (unless otherwise stated) of Cur, Cur analogues or related compounds for 1 h. To obtain countable plaque numbers, the virus-test drug mixture was ten-fold serially diluted (i.e. 10^{-1}, 10^{-2}, 10^{-3}) and then added onto cell monolayers followed by the standard protocol of the plaque assay.

GSH competition test

The effect of GSH on Cur-dependent anti-IAV activity was evaluated in two systems: plaque reduction and HI. For plaque reduction ability, Cur (40 μM) or dimethylsulfoxide (as solvent control) was mixed with an equal volume (150 μL) of four-fold serially diluted (i.e. 40, 20, 10 μM) competitors, such as GSH or GSSG for 1 h at room temperature. Then, the mixture of Cur/competitor was incubated with 2000 pfu of IAV for 1 h followed by the plaque formation assay. For the HA inhibition effect, various concentrations of Cur were mixed with an equal volume of GSH (i.e. 160, 80, 40 μM) in round-bottomed 96-well microplates for 1 h at room temperature. Subsequently, 4 HA units of IAV stock was added into each well and incubated at room temperature. After 1 h of incubation, chicken erythrocytes (diluted to 0.75% v/v in $NaCl/P_i$) were added to each well. The haemagglutination reaction was observed after 30 min of incubation.

Structure modelling of Cur analogues

The molecular structures of compounds were constructed using DISCOVERY STUDIO, version 2.5 (Accelrys Inc., San Diego, CA, USA) modelling software. Energy minimization was achieved by using a solvation model and calculated by the GBSW parameter and minimization and dynamics protocols within DISCOVERY STUDIO. The calculation used a CHARMm (Chemistry at HARvard Macromolecular Mechanics) force-field.

Docking simulation of influenza HA and Cur

Docking simulation was carried out using AUTODOCK, version 4.2 (The Scripps Research Institute, La Jolla, CA, USA) [40]. The structural template of influenza HA (Protein Data Bank code: 1RU7) was obtained from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). To define the Grid box, we used a set of predefined binding site determined from the structure of HA complex with SA (Protein Data Bank code: 1HGE). The flexible residues of protein were defined as His183 and Lys222. The docking simulation was run using the Lamarckian genetic algorithm. All structural images were created with PYMOL (http://www.pymol.org).

Statistical analysis

All data were calculated with EXCEL (Microsoft Corp., Redmond, WA, USA) and analyzed with SAS (SAS Insitute, Cary, NC, USA). The results are reported as the mean ± SD.

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