Antiviral Activity of Carbenoxolone Disodium Against Dengue Virus Infection

Jieying Pu,1,2 Li He,1,2,3 Heping Xie,4 Siyu Wu,1,2 Yuye Li,1,2 Ping Zhang,2 Zhicong Yang,5* and Xi Huang1,2**

1Program of Immunology, Affiliated Guangzhou Women and Children’s Medical Center, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China
2Key Laboratory of Tropical Diseases Control (Sun Yat-sen University), Ministry of Education, Guangzhou, China
3The First College of Clinical Medical Science, China Three Gorges University, Yichang, China
4Department of Traditional Chinese Medicine, Third Affiliated Hospital, Sun Yat-sen University, Guangzhou, China
5Guangzhou Center for Disease Control and Prevention, Guangzhou, China

As one of the most important mosquito-borne viral diseases, dengue infection is now becoming a global concern due to its rapid spread and rise in incidence. Currently, there is no approved vaccine or effective antiviral drug for dengue virus (DENV) infection. Glycyrrhetinic acid (GNa) and its related derivatives have been reported to inhibit a broad spectrum of viruses. However, it is unknown whether Carbenoxolone disodium (CBX), one of the GNa derivatives, affects DENV infection. Here, we found that the production of infectious DENV particles was significantly decreased by CBX treatment in DENV-permissive cells, while the viral RNA and viral protein synthesis were not affected. Moreover, results from time-of-addition study showed that the inhibitory effect of CBX on DENV was exhibited by targeting the virus itself, not the host cells. Directly incubating DENV with CBX resulted in a remarkable reduction of virus titer and virus infectivity. Furthermore, DENV RNA from progeny virions in the supernatants was significantly decreased by CBX treatment in a dose-dependent manner. Taken together, these data indicate that the antiviral activity of CBX against DENV may be mainly due to a virucidal effect exerted by the compound itself. Our work, for the first time, demonstrates that CBX has antiviral activity against DENV infection, providing useful information for development of potential therapeutic interventions against dengue. J. Med. Virol. 89:571–581, 2017.

INTRODUCTION

Dengue virus (DENV), a member of the genus Flavivirus in the family Flaviviridae, causes one of the most widespread mosquito-borne diseases in human. There are four serotypes of dengue virus, types 1 (DENV-1) to 4 (DENV-4), which have similar clinical manifestations and epidemiology in tropical and subtropical regions of the world [Wang et al., 2000].

KEY WORDS: dengue virus; carbenoxolone; antiviral compound; virucidal effect
DENV attacks a set of target cells, including dendritic cells, monocytes/macrophages, endothelial cells, and disseminates in other non-immune cells [Pagni and Fernandez-Sesma, 2012]. Once DENV enters cytoplasm, its single-stranded, positive-sense RNA genome is directly translated into a single polyprotein which is eventually cleaved into the 10 individual viral proteins, including three structural proteins (C, prM, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [Chambers et al., 1990; Filomatori et al., 2006; Zybert et al., 2008; Idrees and Ashfaq, 2012].

Dengue infection either results in a mild illness known as dengue fever (DF) or the more severe and even life-threatening diseases, dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) [Guzman and Kouri, 2002]. Currently, it is estimated that over 50 million cases of dengue infection occur annually, which have swept through more than 100 countries, with potential for further spread [Simmons et al., 2012]. From September to December 2014, an unexpected large outbreak of dengue infection with a significant proportion of severe cases was reported in Pearl River Delta of Guangdong Province, China. It has been the most severe dengue epidemic over the years, with more than 40,000 cases was diagnosed, including five deaths (http://www.cdcpc.org.cn). The emergence of the unprecedented magnitude of dengue outbreak has aroused widespread social concern. In this situation, the WHO considers the development of an effective dengue vaccine to be a high priority. Due to great endeavor of scientific researchers from around the world, Dengvaxia (Sanofi Pasteur, Lyon, Rh) has become the first licensed vaccine available to around the world, Dengvaxia (Sanofi Pasteur, Lyon, Rh) has become the first licensed vaccine available to prevent DF. However, for now there is no available antiviral agent approving for combating DENV [Idrees and Ashfaq, 2012]. Therefore, under current circumstance, the pressing task is to develop safe and effective antiviral drugs against dengue.

Currently, a series of natural compounds and their derivatives are reported as important source of antiviral drugs [Pompei et al., 1979, 2009; Chen et al., 2004; Fiore et al., 2008]. For example, triterpenic compounds, such as glycyrrhizic acid (GRA), glycyrrhetinic acid (GNA), and some of their derivatives have been demonstrated to block the replication of a broad spectrum of viruses, including human immunodeficiency virus (HIV), SARS related coronavirus, Epstein–Barr virus, flaviviruses, vesicular stomatitis virus (VSV), hepatitis B and C virus, influenza virus, rotavirus, adenovirus, etc. [Dargan et al., 1992a,b; Takahara et al., 1994; Matsuo et al., 2001; Cinatl et al., 2003; Crance et al., 2003; Lin, 2003; Orlent et al., 2006; Cos et al., 2008; Ashfaq et al., 2011; Hardy et al., 2012; Smirnov et al., 2012]. Carbenoxolone disodium (CBX), one of the GNA derivatives, is similar to substances found in the root of licorice plant. With a steroid-like structure, CBX has been clinically applied in the treatment of gastric ulcers and other types of inflammation [Connors, 2012]. Besides, CBX is best known in cellular physiology as an effective, water-soluble blocker of gap junctions [Rozental et al., 2001]. In earlier studies, CBX was reported to exhibit complex activities against herpes simplex virus (HSV), including synthesis, processing, and transportation of viral gene products [Dargan and Subak-Sharpe, 1985, 1986; Dargan et al., 1988]. Nonetheless, whether CBX has an inhibitory effect on DENV remains unknown.

In this study, we investigated whether CBX treatment inhibits dengue infection by measuring the production of progeny virions and viral RNA as well as viral protein expression. Our data showed a dose-dependent inhibition of DENV-1 and DENV-2 progeny virions following treatment with indicated concentration of CBX. Moreover, pre-incubating DENV-2 with CBX remarkably abolished virus infectivity and decreased virus titer. Furthermore, treatment with CBX significantly decreased DENV RNA from progeny virions in the supernatants. Our results for the first time identify the old drug CBX as a novel anti-DENV compound, and it might be developed as a potential treatment for dengue infection.

MATERIALS AND METHODS

Cells and Virus

A549 cells (ATCC; CCL-185) were cultured in DMEM (GIBCO, Carlsbad, CA) complemented with 5% FBS at 37°C. THP-1 cells (ATCC; TIB-202) were cultured in RPMI-1640 (GIBCO) with 10% fetal bovine serum (FBS) (HyClone, Logan, UT) and 1% 2-mercaptoethanol (Invitrogen, Carlsbad, CA) at 37°C. HUVEC cells (Clonetics Corp., San Diego, CA) were cultured in M199 medium (JRH Biosciences, Lenexa, KS) containing 20% FBS, 50 mg/ml endothelial cell growth supplement (Collaborative Biomedical Products, Bedford, MA), and 50 mg/ml heparin (Sigma, St. Louis, MO) at 37°C. Mosquito cell line C6/36 cells (ATCC; CRL-1660) were cultured in RPMI-1640 with 5% FBS at 28°C. All media were supplemented with 1% sodium pyruvate, 100 μg/ml of penicillin and 100 U/ml streptomycin (Invitrogen).

The DENV-1 Hawaii strain and DENV-2 New Guinea C strain were provided by Guangzhou Centers for Disease Control, and propagated in C6/36 cells. C6/36 cells were inoculated with DENV at a multiplicity of infection (MOI) of 0.01, and incubated at 35°C for 5–6 days (DENV-1) and 2–3 days (DENV-2). The supernatants were collected and clarified by centrifugation. Virus titer was measured by TCID50 assay in C6/36 cells and viral stocks were stored at –80°C. Vesicular stomatitis virus (VSV) was kindly provided by Dongyan Jin [Kok et al., 2011], and propagated in Vero cells.

Cell Viability Assay

MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) was purchased from

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Sigma–Aldrich (St Louis, MO). A549 cells were cultured in 96-well plates with different concentrations of CBX for 24 hr. Then 10 μl of PBS containing MTT (0.5 mg/ml) was added to each well. After 4 hr of incubation at 37˚C, the supernatant was removed and 150 μl of DMSO was added to each well to solubilize the formazan crystals. Absorbance was measured in a microplate reader at 490 nm after vigorous shaking.

**Infection of Cells**

Adherent A549 and HUVEC cells were seeded on cell culture plates 1 day prior to infection. For the suspension cell line, THP-1 cells were seeded on the day of infection before being infected by DENV. Virions were diluted in DMEM (A549 and HUVEC) or RPMI-1640 (THP-1) without FBS, and inoculated to cell monolayers or suspensions at MOI of 3 (DENV-1) or MOI of 1 (DENV-2). After 1 hr incubation at 37˚C to allow virus attachment, supernatants were removed and cells were washed three times with PBS to remove residual virus. Infected cells were then incubated at 37˚C in culture medium containing 2% FBS for indicated time.

**Time-of-Addition Study**

CBX (3β-hydroxy-11-oxoolean-12-en-30-oic acid 3-hemisuccinate) was purchased from Sigma–Aldrich and dissolved according to product instruction. Appropriate volumes of medium containing specific concentration of CBX were added to cells at different time frames: starting at 1 hr prior to DENV infection (pre-treatment); 0 hr concurrently with viral infection till 24 hr post-infection (post-treatment); from 1 hr prior to viral infection till 24 hr post-infection (continuous-treatment); from 1 hr prior to viral infection till 16 hr post-infection (short-treatment). The cells were then incubated at 37˚C for indicated time of infection before they were harvested.

**Quantitative RT-PCR**

Total RNAs were extracted and purified from cells using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was carried out by using 1 μg of RNA. For quantitative PCR, the reactions were performed in triplicate with each cDNA template by using IQ SYBR green Supermix (Bio-Rad, Hercules, CA) and performed on Bio-Rad CFX96 real-time Detection System (Bio-Rad). The mRNA expression levels were normalized to GAPDH expression. Primer pairs used were as follows: GAPDH (glyceraldehyde-3-phosphate dehydrogenase), forward primer GCTTCCGTGTCCCACCTGC; reverse primer CCGCTGTCACCACTTCT; DENV-C gene, forward primer TCCTAACAATCCCACC AACAGCA, reverse primer AGTTCTGCGTCTCCTGT TCAAGA.

**Immunofluorescence Assay**

Cells were seeded onto coverslips (30–50% confluency) and infected with DENV-2 (MOI of 1) or DENV-1 (MOI of 3) for 24 hr, or left uninfected. Then the cells were fixed with 4% paraformaldehyde at 4˚C for 30 min, and permeabilized with PBS containing 0.1% Triton X-100. The virus protein was detected by using a monoclonal antibody against DENV pM glycoprotein (Abcam, Cambridge, MA) and a secondary anti-mouse antibody (Invitrogen). Cell nuclei were stained with DAPI. Stained samples were visualized using Zeiss fluorescence microscope.

**Virus Titration**

DENV titer in harvested supernatants was determined by TCID50 assay. Samples were serially diluted and inoculated into C6/36 cells in 96-well plates. After 5-day (DENV-2) or 7-day (DENV-1) incubation at 35˚C with 5% CO2, cells were examined for cytopathic effects (CPE) under a light microscope. The virus titer (TCID50/ml) was calculated using the Reed–Muench method [Reed and Muench, 1938]. One TCID50/ml was equivalent to 0.69 PFU/ml. The titer of VSV was quantified using plaque assay. Ninety percent confluent Vero cells in 12-well plates were infected with optimally diluted VSV and then covered with low melting temperature agarose (Sangon Biotech, Shanghai, China) after rinsing with phosphate buffered saline (PBS). At 48 hr post-infection, 1% crystal violet was used to stain the Vero cells and plaques were quantified.

**Virucidal Activity Assay**

DENV-2 suspension containing 1 × 10⁶ PFU/ml or DENV-1 suspension containing 1 × 10⁷ PFU/ml was incubated with an equal volume of DMEM with or without indicated concentrations of CBX for indicated time at 37˚C. The mixtures were then diluted in DMEM, and were used for infection in C6/36 cells to test viral titer or in A549 cells to test residual infectivity by TCID50 assay, immunofluorescence assay, or quantitative RT-PCR.

**Statistical Analysis**

For each separate set of assays, at least three independent experiments were evaluated. Data are represented as mean ± SD. Statistical significance was determined by two-tailed, unpaired Student’s t-test; P < 0.05 was considered statistically significant.

**RESULTS**

**Inhibitory Effect of CBX on DENV Infection**

Chemical structure of CBX is shown in Figure 1a. Cytotoxicity of CBX was first determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method in A549 cells, one of the...
dengue-permissive cells, to establish suitable dosages for further studies. As shown in Figure 1b, cell viability was close to control in treatments with 10, 25, 50, and 100 μM CBX, whereas only 80% cells survived in the presence of 200 μM CBX. Therefore, CBX concentrations ranging from 10 to 100 μM were considered for the following assay. To examine the effect of CBX on DENV infection, A549 cells were infected with DENV-1 Hawaii strain and DENV-2 New Guinea C strain, respectively, and incubated for 24 hr in the presence or absence of CBX. As shown in Figure 1c and d, the production of infectious DENV particles was significantly decreased by CBX treatment in a dose-dependent manner. At concentrations of 50 and 100 μM, CBX treatment led to 500- and 1500-fold reduction of DENV-1 titer, respectively (Fig. 1c). Likewise, at 25, 50, and 100 μM CBX, around 20-, 2000-, and 5000-fold lower levels of DENV-2 titer were observed than control, respectively (Fig. 1d). Taken together, these results suggest that CBX has a dose-dependent inhibition effect on progeny DENV production in A549 cells, and the anti-DENV effect of CBX is not limited to a specific DENV serotype.

To determine whether CBX inhibits the production of infectious progeny DENV in other dengue-permissive cells, TCID_{50} assay was performed to measure the virus titer in CBX-treated THP-1 and HUVEC cells, both of which are widely used in DENV studies [Halstead, 1988; Wu et al., 2000]. As shown in Figure 2a and b, both THP-1 and HUVEC cells were well infected with DENV-2, with about 30% of cells being infected. In THP-1 cells, treated with 25, 50, and 100 μM CBX resulted in 100-, 400-, and 4000-fold reduction of virus titer, respectively (Fig. 2c). In HUVEC cells, treated with 25, 50, and 100 μM CBX led to 15-, 400-, and 1000-fold reduction of virus titer, respectively (Fig. 2d). These results indicate that the anti-DENV activity of CBX is not cell-specific.

**Influence of CBX on DENV Viral RNA Synthesis and Protein Expression**

To examine the effect of CBX on the synthesis of DENV viral RNA, quantitative RT-PCR was performed. As shown in Figure 3a and b, both DENV-1 and DENV-2 viral RNA (C protein) synthesis were not affected by CBX treatment even at the highest
concentration (100 μM). Meanwhile, immunofluorescence assay was performed to analyze the effect of CBX on DENV viral protein expression upon infection. Antibody specific to the DENV structural prM protein was used in this part of the study. The percentages of prM containing cells were calculated to determine the expression of viral protein (Fig. 3d and f). As shown in Figure 3c and e, CBX treatment at the highest concentration (100 μM) did not reduce DENV-1 or DENV-2 viral protein expression. These results suggest that both DENV viral RNA synthesis and protein expression are not regulated by CBX treatment.

**Time-of-Addition Study of CBX on DENV Infection**

To further identify the step(s) of progeny DENV production inhibited by CBX, time-course of the inhibitory effect was analyzed by a time-of-addition experiment in A549 cells. 100 μM CBX was added into the media at different time frames (Fig. 4a). Starting at 1 hr prior to DENV infection (pre-treatment); 0 hr concurrently with viral infection (post-treatment); from 1 hr prior to viral infection to 24 hr post-infection (continuous-treatment), or till 16 hr post-infection (short-treatment). Cell supernatants were harvested for TCID₅₀ assay. As shown in Figure 4b, both post-treatment and continuous-treatment of cells by CBX led to approximately 3000-fold reduction of progeny virus titer. By contrast, pre-treatment with CBX did not have a significant impact on DENV-2 yields (Fig. 4b). As CBX can freely shuttle into cytoplasm by all three treatments mentioned above [Parke, 1983; Connors, 2012], this result indicates that the inhibitory effect of CBX on DENV is not exhibited by targeting the host cells. When eliminating CBX from the media at 16 hr post-infection (short-treatment), the titer of progeny DENV-2 particles also was not affected (Fig. 4b). In our DENV-2-infected system, newly formed virions began to secret from A549 cells at 16 hr post-infection (data not shown). So at this point, short-treatment as mentioned can prevent progeny virions from contacting with CBX. Therefore, these results from time-of-addition study suggest that CBX inhibits DENV by directly targeting the infectious virions extracellularly.
Pre-Incubation With CBX Decreases DENV Titer and Infectivity

To further study the direct effects of CBX on DENV particles, DENV-1 and DENV-2 suspensions were pre-incubated with different concentrations of CBX, respectively for indicated time, and then the viral titer was determined by TCID₅₀ assay. As shown in Figure 5a, DENV-1 titer was significantly decreased by 10- and 1500-fold when incubated with 50 or 100 μM CBX, respectively, but not with 25 μM CBX. When DENV-1 suspensions were incubated with 50 or 100 μM CBX for 1, 6, and 12 hr, respectively, virus titer at each time point was significantly decreased (Fig. 5b). Likewise, CBX exhibited a similar behavior on DENV-2. As shown in Figure 5c, treating DENV-2 suspensions with 25, 50, and 100 μM CBX resulted in 10-, 80-, and 1000-fold reduction of virus titer, respectively. Moreover, longer incubation of CBX correspondingly led to a lower titer of residue DENV-2, peaking at 12 hr incubation (Fig. 5d). Surprisingly, the titer of DENV-2 was almost undetectable when incubating with 100 μM CBX for 12 hr (Fig. 5d). Taken together, these data indicate that incubation with CBX effectively decreases DENV titer in a dose- and time-dependent manner.

Fig. 3. Roles of CBX on DENV viral RNA and viral protein synthesis. A549 cells were treated with 100 μM CBX from 1 hr prior to DENV-1 or DENV-2 infection till 24 hr post-infection. a and b: The total RNA was isolated from DENV-1(a)- or DENV-2(b)-infected cells and analyzed by quantitative RT-PCR. c and d: The expression of DENV-1(c) or DENV-2(e) viral protein was determined by immunofluorescence assay using DENV prM glycoprotein antibody, and the percentages of DENV-1(d) or DENV-2(f) prM containing cells were determined by PicCnt 100+. Data are shown as mean ± SEM of at least three independent experiments. NS, non-significant by t-test (n = 3).

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Furthermore, we directly incubated DENV-2 with 100 \( \mu \)M CBX at 37˚C for 1 hr, and then followed by an infection in A549 cells for indicated time. The viral RNA synthesis, viral protein expression and progeny virus production were compared as indicated above. As shown in Figure 6a, the levels of viral RNA at 12 and 24 hr were barely detected in cells infected with CBX-incubated DENV-2. While in control cells, viral RNA synthesis was gradually increased post infection (Fig. 6a). Immunofluorescence microscopy images showed that no or just few cells were positively stained with DENV prM glycoprotein antibody in CBX-incubating group, compared to approximate 52% of control cells were positively stained at 24 hr post DENV-2 infection (Fig. 6b and c). Likewise, the production of progeny virions from DENV-2 in CBX-incubating group was significantly decreased compared to control group (Fig. 6b).
CBX-incubating group was dramatically decreased (Fig. 6d). Therefore, DENV infectivity is significantly inhibited by CBX pre-incubation.

The Virucidal Activity of CBX Against DENV Infection

Data from Figures 5 and 6 suggested that the antiviral activity of CBX against DENV may be mainly due to a virucidal effect exerted by the compound itself. To test this assumption, A549 cells were infected with DENV-2 and then incubated for 24 hr with different concentrations of CBX. The supernatants were collected for virus RNA detection. As shown in Figure 7a, DENV-2 RNA from progeny virions in the supernatants was significantly decreased by CBX treatment in a dose-dependent manner. At concentrations of 50 and 100 μM, CBX treatment led to 5- and 10-fold reduction of DENV-2 mRNA expression, respectively (Fig. 7a). It suggests that CBX has a virucidal activity to inactivate the progeny DENV particles. To further corroborate the virucidal effect of CBX, A549 cells were infected with a low MOI of DENV-2 for 48 hr to complete at least two replication cycles, and incubated with 100 μM CBX till the end of infection. Quantitative RT-PCR result showed that DENV-2 viral RNA synthesis was significantly decreased by 50% in CBX-treated group (Fig. 7b). Similarly, result from immunofluorescence assay showed that CBX treatment led to effective reduction of viral protein expression (Fig. 7c). The percentage of prM containing cells was half less than control group (Fig. 7d). Taken together, these results indicate that CBX inhibits the replication of progeny DENV particles through its virucidal activity.

Antiviral Effect of CBX Against Other Enveloped Virus

To confirm the specificity of the antiviral activity of CBX, VSV, another enveloped virus was evaluated. VSV suspensions were pre-incubated with different concentrations of CBXm, respectively for indicated time, and then the viral titer was determined by plaque assay. As shown in Figure 7e, VSV titer was significantly decreased by 10- and 40-fold when incubated with 50 or 100 μM CBX, respectively, but not with 25 μM CBX. When VSV suspensions were incubated with 50 or 100 μM CBX for 1, 6, and 12 hr, respectively, virus titer was significantly decreased in
a time-dependent manner (Fig. 7f). Potent inhibition against another enveloped virus (VSV) indicates that the antiviral effect of CBX is not specific to DENV.

**DISCUSSION**

The current epidemic of dengue infection poses a threat to public health worldwide, making it clear that safe and effective countermeasures are urgently needed against this infection. Unfortunately, there is almost no specific antiviral treatment currently available for dengue diseases. Given the complex pathology of the illness and the need to control four virus serotypes simultaneously, dengue vaccine development has been challenging too. In response to the urgent need for effective anti-DENV drugs, our work for the first time identifies CBX, one of the glycyrrhetinic acid (GNa) derivatives, as a potential natural compound against different serotypes of DENV.

CBX treatment did not inhibit DENV-1 or DENV-2 RNA synthesis and protein expression in one replication cycle, but markedly reduced progeny virus...
production, suggesting that post-translation stages of DENV lifecycle as possible targets. Time-of-addition experiment was also performed to narrow down the effective period of this compound. It is reported that CBX can freely shuttle into cytoplasm [Parke, 1983; Connors, 2012]. However, progeny DENV-2 was inhibited only when CBX was added after virus attachment till 24 hr post-infection (post-treatment) or throughout the course of infection (continuous-treatment), but not with pre-treatment of cells (pre-treatment) or till 16 hr post-infection (short-treatment), indicating that the inhibition of CBX is not exhibited by targeting the host cells. From these results, it was deduced that DENV itself is the target of CBX. To test this assumption, virucidal activity assay was performed. Both the titer and residual infectivity of DENV were affected as assumed by pre-incubating with CBX, which manifested in the reduction of viral RNA and viral protein, and the drop in viral titer. Further study found that the level of viral RNA in the supernatants was significantly down-regulated in CBX-treated group, demonstrating CBX directly inactivates the progeny DENV particles via its virucidal activity. The virucidal effect of CBX on DENV might be exerted by irreversibly disrupting viral envelope intactness so that virus loses its protection and results in degradation.

In this study, we used three different cell lines (A549, THP-1, and HUVEC) to investigate the effect of CBX on DENV. Given their high susceptibility to DENV, A549 cells were the main cell line used. THP-1 cell line substituted peripheral blood mononuclear cells (PBMC) to mimic in vivo infection [Clyde et al., 2006]. HUVEC cells, a cell line of human umbilical vein endotheliocyte, were also tested as they are a representative of non-cancerous, primary cell type. As a result, DENV-2 was inhibited dose-dependently with increasing CBX in all three cell lines, demonstrating that CBX effectively inhibits DENV-2 in the various cell types targeted by the virus. Beside DENV-2, DENV-1 viruses were also used to verify the inhibitory effect of CBX. All the results have demonstrated that the antiviral activity of CBX has no specificity on cell type or DENV serotype, which might predict CBX as a new anti-DENV compound to exhibit favorable application perspectives.

Previous study has showed that, glycyrrhizin, another effective compound derived from glycyrrhiza, inhibited DENV infection in Vero cells [Crance et al., 2003]. This promoted us to suppose that CBX, one of the derivatives of glycyrrhetic acid (GNa), may play an effective role in anti-DENV infections. As observed in our study, CBX treatment significantly reduced the progeny virus yield of different serotype of DENV (1 and 2). Further study found that CBX inhibits DENV infection through its virucidal activity. Beside DENV, CBX has been previously found to inhibit another virus, HSV [Partride and Poswillo, 1984; Dargan and Subak-Sharpe, 1986]. However, the antiviral mechanisms of CBX on these two viruses are quite distinct. In regarding to HSV, CBX appeared to be active throughout the replication cycle: by reducing the synthesis of some polypeptides, affecting the transportation of certain proteins, and inhibiting the post-translational processing. As a result, CBX treatment greatly diminished progeny HSV quality, and it is thought to inhibit HSV infection in a non-specific manner, by altering cellular activities that can affect virus replication or activate host antiviral mechanisms. While in our model, CBX directly inhibited the infectivity of DENV particles without targeting the host cells. Likewise, further study found that pre-incubation with CBX significantly decreased VSV titer. Taken together, it suggests that the antiviral activity of CBX was not limited to a specific type of virus, and the mechanisms might be something different.

To be pointed out, CBX is a widely used compound as an inhibitor of gap junctions [Connors, 2012]. Gap junctions are fast-track intercellular communication routes. Genetic studies have revealed that connexin (Cx) protein is the molecular component of vertebrate gap junction channels [Paul, 1986]. Gap junctions comprise clusters of transcellular channels that allow small signaling molecules and inorganic ions to transfer rapidly and directly from one cell to another. Recent studies have reported that gap junctions play key roles in the physiological processes and pathological conditions of several viruses, such as HIV, cytomegalovirus (CMV), Borna disease virus, and Rous sarcoma virus [Eugenin, 2014]. Importantly, in order to increase virus production, infection with the latter two viruses above results in down-regulation of Cx expression. As a non-specific gap junction blocker, CBX inhibits various Cx proteins including Cx26, Cx32, Cx36, and Cx43 [Tonkin et al., 2015]. Therefore, the possibility that CBX inhibits DENV infection through blocking Cx proteins could not be ruled out.

To this end, much more work would be needed to elucidate exactly how CBX inactivates DENV through its virucidal activity. Further studies in suitable animal models would also be required to evaluate the reproducibility of the inhibitory effects of CBX in vivo. In conclusion, our work provides compelling data that CBX has inhibitory effect on DENV infection. Importantly, the antiviral activity of CBX is not limited to a specific cell type or a specific DENV serotype. Combined with its history as a clinical treatment for gastric ulcers or inflammation with a good safety profile [Turpie and Thomson, 1965; Ram et al., 2009], CBX might potentially be developed as a promising antiviral drug for dengue infection.

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