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Cyclopiazonic acid, an inhibitor of calcium-dependent ATPases with antiviral activity against human respiratory syncytial virus

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ABSTRACT

Human respiratory syncytial virus (RSV) is a common cause of lower respiratory tract infections in infants and young children worldwide, yet no vaccine or effective antiviral treatment is available. To search for new anti-RSV agents, we developed a cell-based assay that measures inhibition of RSV-induced cytopathic effect (CPE) and identified cyclopiazonic acid (CPA), an intracellular calcium ATPase inhibitor as a RSV inhibitor (EC_{50} values 4.13 μM) by screening of natural product library. CPA inhibited the replication of RSV strains belonging to both A and B subgroups and human parainfluenza virus type 3, but not Enterovirus 71. Mechanism of action study by time-of-addition assay and minigenome assay revealed that CPA acts at the step of virus genome replication and/or transcription. Moreover, two other calcium ATPase inhibitors (Thapsigargin and BHQ) and calcium ionophores (A23187 and ionomycin), but not calcium channel blockers (nifedipine, nimodipine, and tetrandrine), also had similar effect. These results indicate that an increase in intracellular calcium concentration is detrimental to RSV replication. Thus, our findings provide a new strategy for anti-RSV therapy via increasing intracellular calcium concentration.

1. Introduction

Human respiratory syncytial virus (RSV) is the major causative agent of lower respiratory tract infections, such as bronchiolitis and pneumonia, in young children worldwide, and a leading cause of morbidity and mortality in infants, elderly and immunocompromised individuals (Falsey et al., 2005; Nair et al., 2010). Premature infants and children with congenital heart disease or bronchopulmonary dysplasia are at high risk for severe RSV disease (Meissner et al., 2004). However, healthy infants with no known risk factors constitute most RSV hospital admissions (Boyce et al., 2000). RSV causes disease globally, with an estimated 3.4 million hospitalizations and 66,000 to 199,000 deaths (99% in developing countries) in 2005 in children younger than 5 years of age (Nair et al., 2010). Attempts to develop a safe and effective vaccine against RSV have been unsuccessful to date (Collins and Melero, 2011; Krilov, 2011). Nucleoside analog ribavirin is the only licensed antiviral treatment, but it has very limited use due to uncertain efficacy and toxicity concerns as well as prolonged aerosol administration (Ohmit et al., 1996). Palivizumab (Synagis), a humanized monoclonal antibody against the viral fusion protein, is costly and effective only if administered prophylactically and used in the small percentage of infants who are born prematurely or who have serious underlying conditions (American Academy of Pediatrics Committee on Infectious and American Academy of Pediatrics Bronchiolitis Guidelines, 2014a, 2014b; Chu and Englund, 2013). Thus, there is an urgent

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and unmet medical need for additional effective and safe treatments for RSV.

RSV is an enveloped virus with a negative-sense, single-stranded RNA genome belonging to the genus *Pneumovirus* within the *Paramyxoviridae* family. RSV strains have been classified into two major subgroups (designated RSV-A and RSV-B) based on antigenic and sequencing studies (Anderson et al., 1985; Coates et al., 1963, 1966; Johnson et al., 1987; Mufson et al., 1985; Sullender et al., 1993). Strains of both groups can circulate separately or concurrently during RSV epidemics, with RSV-A more frequently predominating over RSV-B (Akerlind and Norrby, 1986; Hendry et al., 1989; Mufson et al., 1988). The viral genome is 15.2 kb long and is transcribed by a sequential stop-restart mechanism to yield 10 mRNAs encoding 11 proteins, including 3 surface glycoproteins (F, G, and SH) and 4 proteins that comprise the viral RNA polymerase complex (N, P, L, and M2-1) (Collins and Crowe, 2007). Some of these proteins have been the targets of drug discovery efforts, e.g. RSV604 targets the nucleocapsid protein at postentry steps (Chapman et al., 2007); ALS-008176 and GS-5806, the two promising compounds currently in clinical development, target the RNA-dependent RNA polymerase and F protein, respectively (Deval et al., 2015; DeVincenzo et al., 2014, 2015; Perron et al., 2015).

Aiming to discover new anti-RSV compounds, we screened natural product library containing 502 compounds and identified cyclopiazonic acid (CPA), an intracellular calcium ATPase inhibitor (Goeger et al., 1988; Seidler et al., 1989) as a RSV inhibitor. CPA inhibited RSV strains of subgroups A and B and human parainfluenza virus type 3; however it did not inhibit the replication of Enterovirus 71. Moreover, pharmacological inhibitors capable of elevating intracellular calcium concentration could also inhibit RSV replication. These findings reveal essential roles of intracellular calcium during the RSV life cycle and open new possibilities to control infections by this virus.

2. Materials and methods

2.1. Cells, viruses, and compounds

HEp-2 cells (ATCC CCL-23), LLC-MK2 cells (ATCC CCL-7), and Vero (African green monkey kidney) cells were cultured in Dulbecco modified Eagle medium (DMEM) (Invitrogen) with 10% fetal bovine serum (FBS) (Thermo Scientific HyClone) and 100 U/ml penicillin/streptomycin (PS) (Invitrogen) at 37 °C with 5% CO_2. BSR T7/5 cells stably expressing the T7 RNA polymerase (kindly provided by Prof. Dr. Karl-Klaus Conzelmann from Max-von-Pettenkofer Institut, Germany) were maintained in Glasgow minimal essential medium (GMEM) (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, and 2% MEM amino acids (Invitrogen), and the medium supplemented with 1 mg/ml G418 (Invitrogen) was changed every second passage. Human respiratory syncytial viruses (strain Long, subgroup A, ATCC VR-26, and strain 9320, subgroup B, ATCC VR-955) were cultured on HEp-2 cells. Human parainfluenza virus type 3 (PIV-3, strain C243, ATCC VR-93) was cultured on LLC-MK2 cell. Enterovirus 71 (EV-A71, strain G082) derived from an infectious cDNA clone was cultured on Vero cells (Gao et al., 2015). Natural product library containing 502 compounds was purchased from Enzo Life Sciences. Cyclopiazonic acid (CPA), Thapsigargin (TG), BHQ, 4-Aminopyridine (4-AP), disopyramide phosphate salt (DP), and A23187 were purchased from Sigma. Lidocaine, nimodipine, nifedipine, tetrandrine, and ouabain were purchased from Selleck. Ionomycin was purchased from Santa Cruz. All compounds were dissolved in dimethyl sulfoxide (DMSO) except DP salt which was dissolved in water.

2.2. Primary screening of natural product library

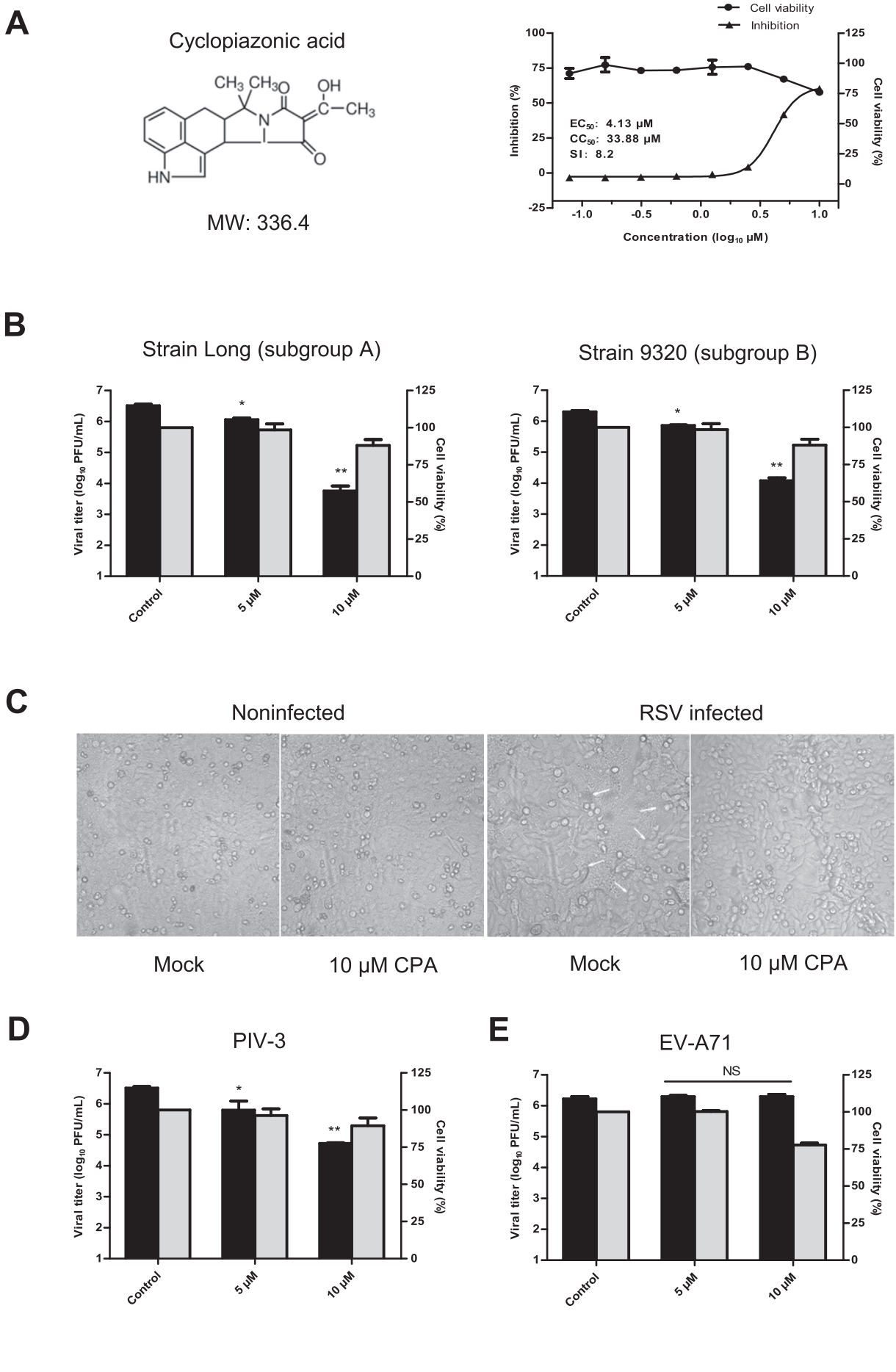
The inhibition of the CPE caused by RSV infection in HEp-2 cells was used as the primary assay to identify inhibitors. HEp-2 cells (5,000 cells in 50 μl of DMEM) were seeded into each well of a white 96-well plate (Corning Costar) for 24 h prior to infection. Five microliters of each test compound at a final concentration of 2.5 μg/ml (diluted in assay media with a final DMSO concentration of 0.25%) were added to the plates (one compound per well). In cell control and virus control wells, 0.25% DMSO alone was added. Within 10 min of compound addition, 45 μl of diluted virus (2500 PFU, which corresponds to a multiplicity of infection [MOI] of 0.5 based on initial cell plating density of 5,000 cells/well) was added to each well. In cell control wells, 45 μl of assay medium was added. The final assay volume was 100 μl/well. Plates were incubated at 37 °C for 96 h and then allowed to equilibrate to room temperature for 30 min. Afterward, 50 μl of CellTiter-Glo (Promega) reagent was added to each plate well, and the plates were incubated at room temperature for 10–30 min before being read by Veritas Microplate Luminometer (Turner BioSystems). A total of 502 natural product were screened in single dose.

2.3. Cytotoxicity assay

Cytotoxicity of each compound in different cell lines was assessed. Briefly, 5,000 HEp-2 cells in a volume of 50 μl were seeded into each well of a white 96-well plate. After 24 h incubation at 37 °C, 5 μl of 2-fold serial dilutions of compounds and 45 μl of medium were added and incubated for 2 days (same incubation period as virus yield reduction assay) or 4 days (same incubation period as CPE assay). Cell viability was determined by the addition of 50 μl of CellTiter-Glo (Promega) reagent. For Vero and LLC-MK2 cells, 5,000 cells were seeded and the incubation period with compound was 2 days.

2.4. Immunoplaque assay and plaque assay

For RSV, approximately 1.5×10^5 HEp-2 cells per well were seeded in 24-well plates (Corning Costar) one day in advance. Cells were infected by serial of 10-fold dilution of virus at 37 °C for 1.5 h with shaking every 15 min, and then virus was removed and cells were overlaid with 500 μl of 0.8% methylcellulose (Aquacide II, Calbiochem) and 2% FBS. After 3 days of incubation at 37 °C with 5% CO_2, the cells were fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature. The fixed cells were washed twice with PBS, incubated with 0.25% Triton X-100 in PBS for 20 min, washed twice with PBS, and incubated with 200 μl of 1:1,000-diluted mouse anti-RSV F monoclonal antibody (Fitzgerald, MA, USA) for 1 h at room temperature. The cells then were washed three times with PBS-T, incubated with 200 μl of 1:6000-diluted HRP-conjugated goat anti-mouse IgG antibody (Bethyl, TX, USA) for 1 h at room temperature, washed three times with PBS-T, and color was developed by using TrueBlue Peroxidase Substrate (KPL, MD, USA) according to the manufacturer's instructions. For PIV-3, there are two modifications in the assay condition: (i) LLC-MK2 cell was used for infection, and (ii) anti-PIV-3 monoclonal antibody (Fitzgerald, MA, USA) was used as primary antibody. For EV-A71, plaque assay was performed as described previously (Gao et al., 2015).


A
Cyclopiazonic acid
MW: 336.4
Cell viability
Inhibition
EC50: 4.13 μM
CC50: 33.88 μM
SI: 8.2
Inhibition (%)
Cell viability (%)
Concentration (log10 μM)
B
Strain Long (subgroup A)
Strain 9320 (subgroup B)
Viral titer (log10 PFU/mL)
Cell viability (%)
Control
5 μM
10 μM
C
Noninfected
RSV infected
Mock
10 μM CPA
Mock
10 μM CPA
D
PIV-3
E
EV-A71
NS

2.5. Virus yield reduction assay

For RSV, HEp-2 cells were seeded in 12 well plate at a density of 3×10^5 cells per well. After 24 h incubation at 37 °C with 5% CO_2, medium was removed and cells were infected with RSV at an MOI of 0.1, and treated with serially diluted compound. The tested concentrations were chosen based on cytotoxicity assay and the highest concentration should not exceed 50% cytotoxicity. Supernatants were collected at 48 h post-infection (p.i.), flash-frozen under liquid nitrogen and stored at −80 °C. For PIV-3 and EV-A71, LLC-MK2 and Vero cells were used for infection, respectively. Virus titers were determined using the assay described above.

2.6. Time-of-addition assay

To study the anti-RSV mechanism of CPA, a time-of-addition assay was performed. HEp-2 cells were seeded at 3×10^5 cells/well in a 12-well plate 24 h before the experiment and infected with RSV at an MOI of 3 by adsorbing the virus for 1 h at 4 °C. The infected cells were then washed thrice with cold medium, and then 1 ml of medium was added to the cells. CPA (10 μM) was added at −2, 0, 2, 4, 6, 8, 12, 16 h, and supernatants were collected at 20 h p.i.. The titers of the virus were then determined by immunoplaque assay as described above.

2.7. Minigenome assay

BSR T7/5 cells were seeded in 24 well at 2×10^5 cells/well 24 h in advance. Cells were transfected with plasmids encoding for RSV N (200 ng), P (200 ng), M2-1 (200 ng), L (100 ng), and minigenome (200 ng) containing a firefly luciferase reporter gene using Lipofectamine 2000 (Invitrogen) according to the manufacturer's directions. Plasmid pRL-SV40 (10 ng) encoding renilla luciferase was co-transfected as an internal control to normalize transfection efficiency, and transfection without plasmid expressing L protein served as negative control. CPA was added after transfection, and left to incubate with the cells. After 24 h of transfection, cells were lysed in 250 μl of luciferase lysis buffer and firefly luciferase as well as renilla luciferase signals were measured for each well with dual-luciferase reporter assay system (Promega) by Veritas Microplate Luminometer (Turner BioSystems).

2.8. Data analysis and statistics

Signal-to-background (S/B) and Z-factor were calculated using Microsoft Excel 2010 to assess the screening assay quality. $S/B = \mu_c/\mu_v$, where μ_c is the mean cell control signal and μ_v is the mean virus control signal; $Z = 1 - (3\sigma_c + 3\sigma_v)/|\mu_c - \mu_v|$ where σ_c is the standard deviation of the cell control signal, and σ_v is the standard deviation of the virus control signal, Z-factor between 0.5 and 1 indicates an excellent assay with good separation between controls (Zhang et al., 1999). Antiviral activity is described as percent CPE inhibition $= (\mu_{cpd} - \mu_v)/(\mu_c - \mu_v) \times 100\%$, where μ_{cpd} is the mean test compound well signal. The 50% effective concentration (EC_{50}) was defined as the compound concentration required to achieve 50% of maximal CPE inhibition, the 50% cytotoxic concentration (CC_{50}) was defined as 50% reduction in luminescence compared to control wells. EC_{50} value was calculated by nonlinear regression using Prism software (GraphPadPrism5, CA, USA). The selectivity index (SI) was calculated as $SI = CC_{50}/EC_{50}$. Statistical significance was analyzed by 2-tailed Student's test. Differences with a *P* value of less than 0.05 were considered statistically significant.

3. Results

3.1. Identification of CPA as an inhibitor of RSV replication

In the search of new anti-RSV agents, we developed a cell-based assay measuring CPE induced by RSV infection. A natural product library containing 502 compounds was screened in 96-well format. The average S/B was 21.73, and Z factors of screening plates were in the range of 0.53 to 0.78 with a median of 0.72, indicative of robust assay performance. CPA, an inhibitor of calcium ATPases, showed greater than 40% inhibition of virus-induced CPE. CPA was then reordered and dose-response analysis was performed with the same CPE assay as for primary screening and a parallel cytotoxicity assay. As shown in Fig. 1A, CPA inhibited the RSV-induced CPE in a dose-responsive manner with an EC_{50} of 4.13 μM and CC_{50} of 33.88 μM, yielding an SI of 8.2.

3.2. Selectivity of CPA against various viruses

In order to examine the spectrum of activity of CPA, representative strains of both RSV subgroups A and B, closely related PIV-3 (also a member of the paramyxovirus family), and unrelated EV-A71 (a nonenveloped, positive-stranded RNA virus of the *picornaviridae* family) were selected and analyzed in the virus yield reduction assay. CPA was efficacious against strains of both RSV subgroups A and B, and PIV-3 (Fig. 1B&D). No associated toxicity was observed in the cytotoxicity assay with concentrations of up to 10 μM. CPA reduced viral titers of RSV strain Long (subgroup A), strain 9320 (subgroup B), and PIV-3 by 2.7, 2.2, and 1.8 $\log_{10}$ units at 10 μM, respectively. Moreover, CPA treatment did not cause an adverse effect on HEp-2 cells since no changes in morphology or density were observed compared to untreated cells. In RSV-infected, untreated HEp-2 cells, large syncytia were visible under microscope at 48 h p.i.. In contrast, CPA reduced the number of syncytia and the level of CPE (Fig. 1C). These results further confirmed that CPA inhibits RSV infection at low cytotoxic concentrations. However, no activity was observed against EV-A71 (Fig. 1E), suggesting CPA is a selective inhibitor of RSV and PIV-3. It should be noted that PIV-3 and EV-A71 were conducted on LLC-MK2 and Vero cells, respectively, cytotoxicity values for CPA in these cells were also measured. CPA did not exhibit significant

Fig. 1. Identification of CPA as a RSV inhibitor. (A) left, chemical structure of CPA; right, validation of CPA by CPE assay. HEp-2 cells were infected with RSV at an MOI of 0.5 and treated with CPA at the indicated concentrations. After 96 h incubation at 37 °C, luminescence was measured. CPE inhibition was calculated as described in Materials and methods. Cytotoxicity of CPA on HEp-2 cells was assessed in parallel. Average results from two independent experiments are shown. (B) Inhibition of RSV infection by CPA. HEp-2 cells were infected with RSV strain long (subgroup A, left) or strain 9320 (subgroup B, right) at an MOI of 0.1, and treated with 5 or 10 μM CPA. Supernatants were collected at 48 h post-infection and viral titers were determined by immunoplaque assay. (C) Morphology of HEp-2 cells treated with CPA. HEp-2 cells were infected with RSV at an MOI of 0.1, and 10 μM CPA was added immediately after infection. Cell images were taken at 48 h p.i.. From left to right are: Noninfected, untreated HEp-2 cells; Noninfected, CPA-treated HEp-2 cells; RSV-infected, untreated HEp-2 cells; and RSV-infected, CPA-treated HEp-2 cells. Large multinucleated cells (syncytia) are clearly seen and indicated by the white arrow. (D) Effect of CPA on PIV-3. LLC-MK2 were infected with PIV-3 at an MOI of 0.1, and treated with 5 or 10 μM CPA. Supernatants were collected at 48 h postinfection and viral titers were determined by immunoplaque assay. (E) Effect of CPA on EV-A71. Vero cells were infected with EV-A71 at an MOI of 0.1, and treated with 5 or 10 μM CPA. Supernatants were collected at 42 h postinfection and viral titers were determined by plaque assay. For (B), (D), and (E), cytotoxicity of CPA on corresponding cell lines was assessed as described in Materials and methods. Grey bars represent cytotoxicity of CPA. The data presented were obtained from two independent experiments. Error bars represent the standard deviations from two independent experiments. Statistical significance between CPA-treated and control group were analyzed by t-test (ns, not significant, *, $p < 0.05$, **, $p < 0.01$).

cytotoxicity at 10 μM (the highest test concentration) in any of the cell lines.

3.3. CPA acts at the step of virus genome replication and/or transcription

To investigate the inhibition mechanism of CPA, we performed a time-of-addition assay to identify the step(s) at which CPA suppress RSV infection. HEp-2 cell were synchronously infected with RSV at an MOI of 3. CPA (10 μM) was added to the infected cells at various time points. Viral titers in the culture medium were determined at 20 h p.i.. As controls, 0.25% DMSO was added to infected cells at 0, 8, and 16 h p.i., for estimation of its effect on viral yield. As shown in Fig. 2A, CPA was able to inhibit virus replication at similar levels when given prior to infection and at up to 8 h postinfection and the inhibitory effect gradually diminished when the compound was added later than 8 h after infection. Unlike nucleoside inhibitors which need to be converted into other active form, CPA was a fast-acting inhibitor. Thus, our time-of-addition assay results suggest that CPA acts at a step subsequent to adsorption and viral fusion. Using a minigenome assay, we showed that CPA reduced luciferase activity by 23% compared to the control at 10 μM concentration (Fig. 2B), confirming that CPA acts at the step of virus genome replication and/or transcription. Since the virus genome replication and/or transcription is essential for viral replication, a more than 20% decrease in the activity may account for the antiviral effect observed in virus yield reduction assay.

3.4. Effect of other ATPase inhibitors and calcium ionophores

CPA is an inhibitor of endogenous calcium-dependent ATPases, we then used another two calcium ATPases inhibitors (Thapsigargin [TG] (Thastrup et al., 1990) and BHQ (Kass et al., 1989)) and a specific Na^+/K^+-ATPase inhibitor ouabain (Blaustein et al., 1998) to test whether the observed anti-RSV effect was associated with inhibition of Ca^{2+}-ATPase. Both TG and BHQ showed a concentration-dependent inhibition of RSV infection similar to CPA (Fig. 3A). TG (10 nM) and BHQ (10 μM) reduced the viral titer of RSV by 124.5- and 9.9-fold, respectively. However, ouabain also showed inhibition with 25.5-fold reduction in viral titer at 10 nM (Fig. 3B). Because inhibition of Ca^{2+}-ATPase by pharmacological agents such as CPA, TG, and BHQ will cause an increase in intracellular calcium concentration and ouabain could also induce an elevation of intracellular calcium levels via sodium-calcium exchange (Roevens and de Chaffoy de Courcelles, 1990), we then generate a hypothesis that increase in intracellular calcium concentration is detrimental to RSV replication. To test this hypothesis, we evaluated two calcium ionophores (A23187 and ionomycin) that could also raise intracellular calcium using virus yield reduction assay. As shown in Fig. 3C, inhibition of RSV by both A23187 and ionomycin was observed. These data suggest that the anti-RSV effects of both calcium ATPases inhibitors and calcium ionophores are mediated by their ability to elevate intracellular calcium concentration.

3.5. Inhibition of Ca^{2+}, Na^+, and K^+ channels does not have an impact on RSV infection

Since elevating intracellular calcium concentration by pharmacological agents was detrimental to RSV replication, we then questioned whether a decrease in intracellular calcium concentration may also result in inhibition of RSV replication. We tested inhibitors of plasma membrane Ca^{2+} channel such as nifedipine and nimodipine, and two-pore channel blocker tetrandrine using virus yield reduction assay. Treatment with these pharmacological inhibitors did not impact RSV infection (Fig. 4A). This could indicate that RSV infection is independent on calcium channel activity. Tetrandrine which could decrease intracellular calcium concentration on HEp-2 cells (Cui et al., 2015) was recently identified as a highly effective inhibitor of Ebola virus (also an enveloped virus with a negative-sense, single-stranded RNA genome) (Sakurai et al., 2015), suggesting calcium channels play distinct role in virus infection for these two viruses. We also assessed pharmacological agents previously characterized as specific modulator of Na^+ and

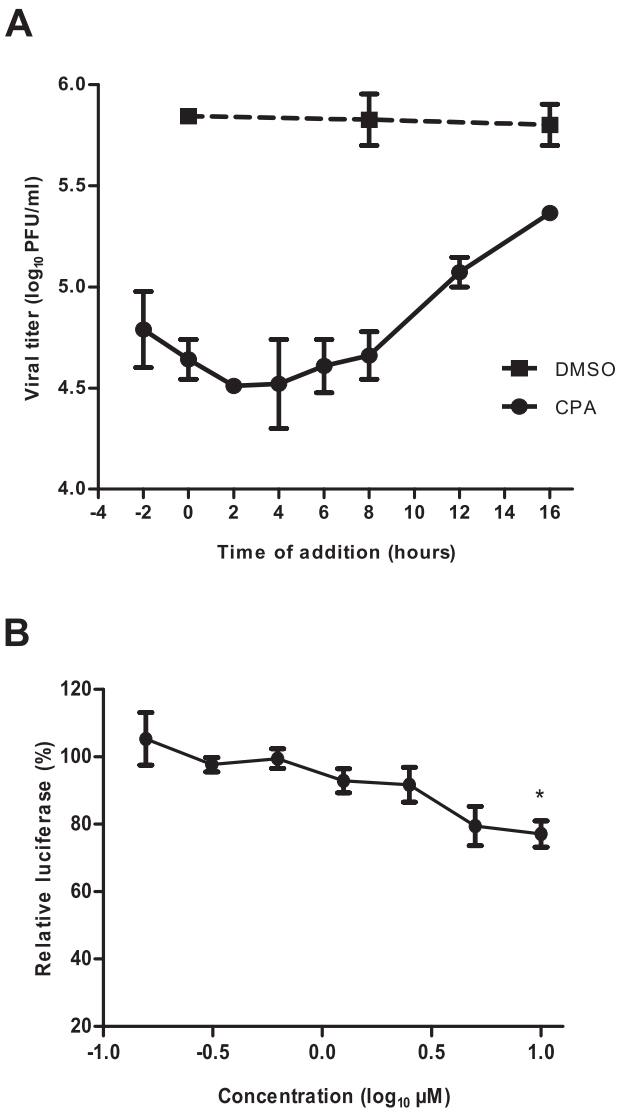


Fig. 2. Mechanism of CPA on RSV inhibition. (A) Time-of-addition analysis of CPA in RSV infection. HEp-2 cells were infected with RSV at an MOI of 3 at 4 °C for 1.5 h. The infected cells were washed three times with cold medium. CPA (10 μM) was added to the cells at the indicated time points. Supernatants were harvested at 20 h post infection and assayed for determination of viral titers. The results shown are representative of one of two independent experiments. (B) Minigenome assay with CPA. BSR T7/5 cells were co-transfected with RSV minigenome and plasmids encoding for N, P, L, and M2-1 proteins as well as pRL-SV40, cells were treated with CPA at indicated concentrations. At 24 h post transfection, firefly luciferase and renilla luciferase activities were measured with dual-luciferase reporter assay system (Promega). The effects of CPA were presented as a percentage of firefly luciferase (normalized against renilla luciferase) derived from the compound-treated cells compared with that the mock-treated cells. The data presented were obtained from two independent experiments. Error bars represent the standard deviations from two independent experiments. Statistical significance between treated and control group were analyzed by t-test (*, $p < 0.05$).

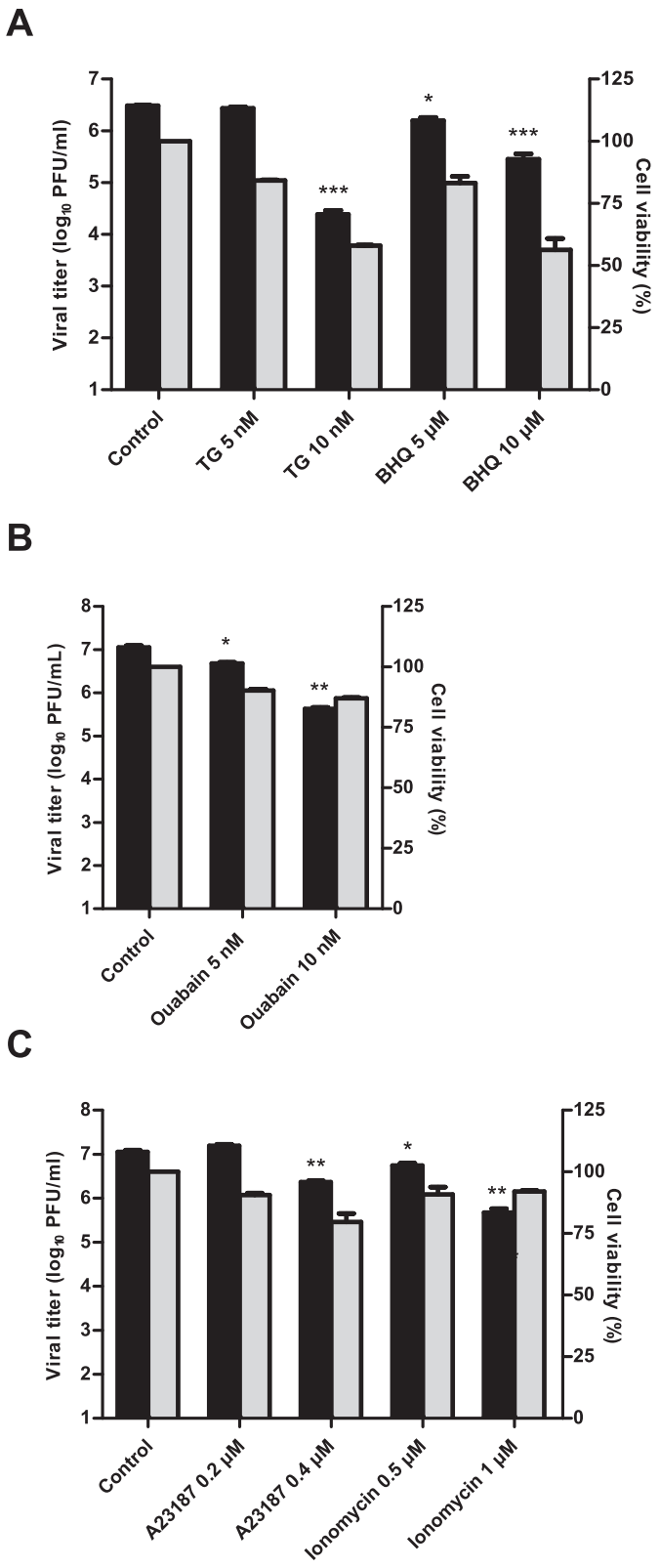


Fig. 3. Effect of pharmacological agents capable of increasing intracellular calcium concentration on RSV. HEp-2 cells were infected with RSV strain long at an MOI of 0.1, and treated with calcium ATPases inhibitors TG and BHQ (A), a specific Na^+/K^+-ATPase inhibitor ouabain (B), or calcium ionophores A23187 and ionomycin (C) at indicated concentrations. Supernatants were collected at 48 h postinfection and viral titers were determined by immunoplaque assay. Grey bars represent cytotoxicity of the compounds. The data presented were obtained from two independent experiments. Error bars represent the standard deviations from two independent experiments. Statistical significance between treated and control group were analyzed by t-test (*, $p < 0.05$, **, $p < 0.01$).

K^+ channel families. When cells were treated with voltage gated Na^+ channel blocker DP salt or lidocaine, no reduction in viral titer occurred (Fig. 4B). Similar result was observed for 4-AP, a blocker of voltage-gated K^+ channels (Fig. 4C). We thus concluded that inhibition of Ca^{2+}, Na^+, and K^+ channels does not have an impact on RSV infection.

4. Discussion

RSV was first discovered in 1956 as an agent causing coryza in chimpanzees (Blount et al., 1956). Despite almost 60 years of effort, there remains a need for a safe vaccine and more effective antiviral drugs. In an effort to identify new small molecule inhibitors of RSV, we initiated a screen of natural product library containing 502 compounds in CPE assay. Natural products have been an important source of lead compounds for drug discovery. Historically, natural products have been the most successful source of new drugs, particularly as anticancer and antimicrobial agents (Dias et al., 2012; Harvey, 2008; Harvey et al., 2015). The indole alkaloid CPA was identified by high-throughput screening and subsequently shown to have activity in the low micromolar range against RSV strains belonging to both A and B subgroups and PIV-3. In contrast, CPA did not inhibit the replication of EV-A71, indicating CPA is a specific inhibitor of RSV and PIV-3.

CPA, a secondary metabolite produced by certain fungi of the *Penicillium* and *Aspergillus* genera (Holzapfel, 1968), has been reported to inhibit Sendai virus (Ono and Kawakita, 1994), hepatitis B virus (Xia et al., 2006), and rotavirus (Ruiz et al., 2007) through different mechanisms. CPA has been shown to block the transport of envelope proteins (HN and F0) of Sendai virus at different steps (Ono and Kawakita, 1994) and induce intracellular disassembly of infectious rotavirus particles by depletion of Ca^{2+} sequestered in the endoplasmic reticulum at the end of virus cycle (Ruiz et al., 2007). The molecular mechanism of antiviral effect of CPA on hepatitis B virus is not very clear (Xia et al., 2006). Here, we found that CPA inhibits RSV at the step of virus genome replication and/or transcription, providing another mechanism of inhibition. It will be of interest to elucidate the differences in mechanisms of action of CPA for these viruses in future studies. CPA, TG, and BHQ are three structurally unrelated specific inhibitors of sarco-endoplasmic reticulum-type calcium transport ATPases (SERCA) (Inesi and Sagara, 1994). These agents induce leakage of stored calcium from ER into the cytosol, resulting in an increase in intracellular calcium concentration. We therefore speculate that higher concentration of intracellular calcium would impair virus genome replication and/or transcription, thereby reducing viral yield.

The viral transcription and replication often rely on host cell machinery. Strategies of targeting conserved host cellular proteins and mechanisms required by viruses could reduce the development of resistance, particularly for RNA viruses with high mutation rates. In our study, resistant viruses were not able to be generated in the presence of CPA (data not shown). Thus, our findings provide a new strategy for antiviral therapy via increasing intracellular calcium concentration to combat RSV and potentially other viruses that also require similar mechanisms.

In conclusion, we have identified pharmacological inhibitors capable of elevating intracellular calcium concentration as a new class of RSV inhibitors. Although these inhibitors are toxic in high concentration and therefore will be difficult to use them as new therapeutic treatment against RSV, they are useful as potential leads for developing antiviral drugs and can also be served as tool compounds to dissect the role of intracellular calcium during virus infection which in turn will shed light on RSV life cycle.

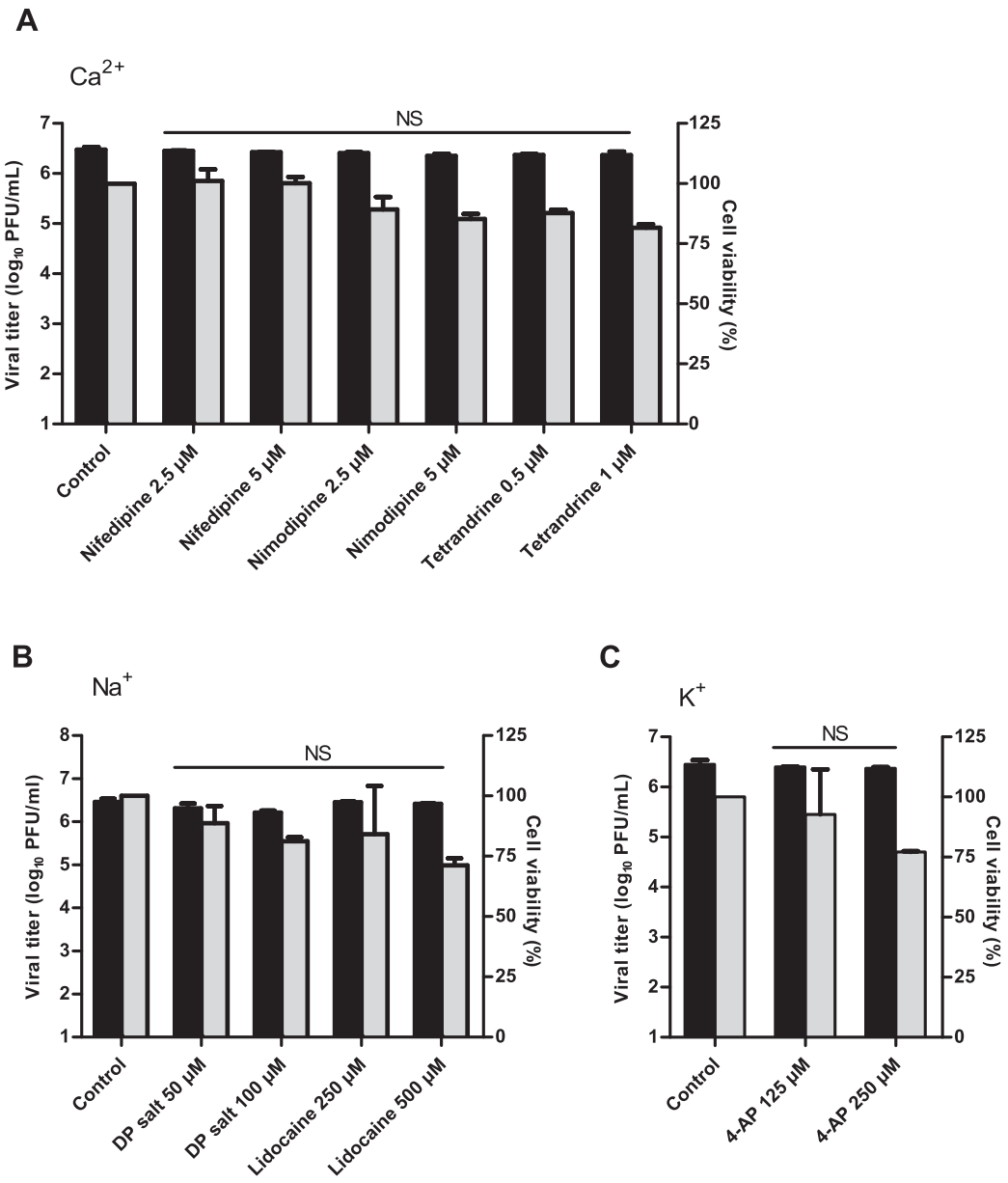


Fig. 4. Assessment of the role of Ca^{2+}, Na^{+}, and K^{+} channels during RSV replication. HEp-2 cells were infected with RSV strain long at an MOI of 0.1, and treated with calcium channel blockers nifedipine, nimodipine, and tetrandrine (A), Na^{+} channel blocker DP salt and lidocaine (B), or K^{+} channels blocker 4-AP (C) at indicated concentrations. Supernatants were collected at 48 h postinfection and viral titers were determined by immunoplaque assay. Grey bars represent cytotoxicity of the compounds. The data presented were obtained from two independent experiments. Error bars represent the standard deviations from two independent experiments. NS, no differences at a significance level of 0.05.

Acknowledgments

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