Repurposing carrimycin as an antiviral agent against human coronaviruses, including the currently pandemic SARS-CoV-2

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\textbf{Abstract}  COVID-19 pandemic caused by SARS-CoV-2 infection severely threatens global health and economic development. No effective antiviral drug is currently available to treat COVID-19 and any other human coronavirus infections. We report herein that a macrolide antibiotic, carrimycin, potently inhibited the cytopathic effects (CPE) and reduced the levels of viral protein and RNA in multiple cell types
1. Introduction

Coronaviruses (CoVs) are a large family of enveloped, positive-sense, single-stranded RNA viruses with broad host ranges. Since the new millennium, cross species transmissions of CoVs from bats through intermediate mammalian hosts to humans have caused severe acute respiratory syndrome (SARS) in 2003, Middle East respiratory syndrome (MERS) in 2012, and current pandemic coronavirus disease 2019 (COVID-19). In addition, four human coronaviruses (HCoVs), including HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1, cause common cold and are speculated to be introduced into human population decades or even hundreds of years ago from unidentified animal hosts. It is anticipated that emergence and re-emergence of CoV infections via cross species transmission will be a continuing challenge for human health and development of broad-spectrum antiviral agents against HCoVs are essential to cope with the current COVID-19 and future CoV epidemics.

Drug repurposing is an effective strategy for urgent treatment of emerging viral diseases. In our efforts to search for the approved medicines that can suppress human CoV infections, an in-house collection of Chinese Food and Drug Administration (CFDA)-approved drugs including Chinese patent medicines, antibiotics, and antiviral agents were screened for their ability to protect the cytopathic effects (CPE) caused by HCoV-229E or HCoV-OC43 infection. We found a few macrolide antibiotics with antiviral activity against HCoV-229E and HCoV-OC43. Carrimycin, the most active one, was selected for further investigation of its antiviral activity against SARS-CoV-2 and determination of antiviral mechanism.

2. Materials and methods

2.1. Cells and viruses

Human hepatocellular carcinoma cell lines Huh7 and Huh7.5 and human lung cancer cell line H460 were kindly provided by Dr. Zonggen Peng and Dr. Zhen Wang, respectively, at Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College. Human hepatoblastoma cell line C3A was purchased from ATCC (Manassas, VA, USA). 293T-derived cell line expressing human recombinant angiotensin I converting enzyme 2 (293T-hACE2) was purchased from Delivectory Biosciences Inc. (Beijing, China). All cells cultured in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA, USA) or Minimum Essential Media (MEM, Invitrogen) supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) at 37 °C in a 5% CO2 incubator.

HCoV-229E (strain VR740) was purchased from ATCC. HCoV-OC43 (strain VR1558) was a kind gift from Dr. Xuesen Zhao at Beijing Ditan Hospital, Capital Medical University (Beijing, China). SARS-CoV-2 (GenBank: MT123290) for immunofluorescence (IF) assay was isolated from a throat swab of a COVID-19 patient and stored in biosafety level-3 laboratory (Guangzhou Customs Technology Center, Guangzhou, China). The vesicular stomatitis virus (VSV) and SARS-CoV-2 pseudotyped viral particles were obtained from Delivectory Biosciences Inc. (Beijing, China).

2.2. Compounds

Carrimycin was provided by Shenyang Tonglian Group Co., Ltd. (Shenyang, China). Clarithromycin, midecamycin, erythromycin, roxithromycin, acetylspiramycin, azithromycin, clindamycin, remdesivir (RDV), and ammonium chloride (NH4Cl) were purchased from MedChemExpress (Monmouth Junction, NJ, USA). Ribavirin (RBV) and chloroquine (CQ) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.3. Cell cytotoxicity assay

Cytotoxic effects of carrimycin on different cells were assayed by cell counting kit (CCK, TransGen Biotech, Beijing, China). Briefly, cells were seeded into 96-well culture plates and were incubated overnight. Then, the medium was removed and different concentrations of carrimycin were applied in triplicate. After 2 days’ incubation, the cytotoxicity of carrimycin was determined by CCK assay and then the 50% cytotoxic concentration (CC50) was calculated.

2.4. CPE inhibition assay

The anti-coronavirus activity of carrimycin was determined by a CPE inhibition assay. Briefly, cells were plated into 96-well culture plates and incubated for 24 h. The cells were infected with 100 times 50% tissue culture infective dose (TCID50) HCoV-229E or HCoV-OC43 and the indicated concentrations of compounds were added simultaneously. HCoV-229E infected Huh7 cells were treated for about 48 h and HCoV-OC43 infected H460 cells were treated for about 72 h. The 50% effective concentration (EC50) was determined by Reed & Muench method. The selectivity index (SI) was calculated as the ratio of CC50/EC50.
2.5. Immunofluorescence assay

C3A (2.0 × 10^5 cells/well), Huh7 (1.5 × 10^5 cells/well), or H460 (1.5 × 10^5 cells/well) cells grown on glass coverslips (Thermo Fisher Scientific, Waltham, MA, USA) were infected with coronavirus and treated with carrimycin at the same time of infection. At 48 h post infection, the culture medium was removed and the cells were washed and fixed. The cells were permeabilized in 0.5% Triton X-100 at room temperature for 15 min and blocked in phosphate buffer saline (PBS) containing 1% bovine serum albumin (BSA) for 60 min at room temperature. Cells were then incubated with an antiviral antibody (Millipore, Billerica, MA, USA) or dsRNA antibody (SCICONS, Szirák, Hungary) at a dilution of 1: 200 for 2 h at room temperature. After washing three times with PBS, the samples were reacted with Alexa Fluor 488-labeled goat anti-mouse secondary antibody (Beyotime Institute of Biotechnology, China) for 1 h at room temperature. After washing with PBS, images were taken using a fluorescence microscope (Olympus IX71, Olympus, Japan).

2.6. Western blot assays

For analysis of proteins, the cellular proteins were extracted using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) with halot protease inhibitor single-use cocktail. Immunoblotting for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Boston, MA, USA, 1:1000) and coronavirus NP (Millipore, Billerica, MA, USA, 1:1000) was performed as described previously.

| Table 1 | Primers used in qRT-PCR assay. |
|---------|--------------------------------|
| Name                | Primer Sequence (5′–3′)                           |
| HCoV-OC43 NP        | Sense CGATGAGGCTATCCGACTAGGT                       |
|                     | Antisense CCTTCCTGAGCCCTCAATATAGTAACC             |
|                     | Probe TAMRA-TCGCCCTGGACGCGTGACTCCCT-BHQ2         |
| GAPDH (human)       | Sense CGGAGTCACGGATTGGTCGTAT                      |
|                     | Antisense AGCCTTCCCATGTTGAGAAGAC                 |
|                     | Probe TAMRA-CCGTCAAGGCTGAGAACGG-BHQ2             |
| HCoV-229E NP        | Sense GACCRATCCCTGCCTCCTGAC                      |
| GAPDH (human)       | Sense GGGCATTYTGGACAAAKCGTCTACG                 |
|                     | Antisense CTCTGGAAAGCTGTGGTGCGTGATG             |
| 18S rRNA (human)    | Sense ATGCCAGTGAGCTCCCGTTCTAG                   |
|                     | Antisense TGGAGGAGAGCTTCCAGTGT                  |
|                     |                                      | GATCTGTCCAGGCAGTCCTT |

Figure 1  The chemical structures of macrolide antibiotics. (A) Carrimycin. (B) Acetylspiramycin. (C) Azithromycin.
Carrimycin inhibits coronaviruses replication.

### Table 2  Antiviral activity of tested macrolide antibiotics against HCoV-229E and HCoV-OC43 in vitro.

| Drug          | HCoV-229E | HCoV-OC43 |
|---------------|-----------|-----------|
|               | EC50 (µg/mL) | EC50 (µg/mL) | SI |
|               | CC50 (µg/mL) | CC50 (µg/mL) | SI |
| Clarithromycin| >78.87 ± 29.88 | >78.87 ± 29.88 | >100 ± 0 |
|               | 21.18 ± 2.73  | 21.18 ± 2.73 | >100 ± 0 |
| Midecamycin   | >100 ± 0     | >100 ± 0     | >100 ± 0 |
| Erythromycin  | >100 ± 0     | >100 ± 0     | >100 ± 0 |
| Roxithromycin | >78.87 ± 29.88 | >78.87 ± 29.88 | >100 ± 0 |
| Acetylsyriramycin | >100 ± 0 | >100 ± 0 | >100 ± 0 |
| Azithromycin  | 63.54 ± 8.20 | 63.54 ± 8.20 | >100 ± 0 |
| Ribavirin     | >100 ± 0     | >100 ± 0     | >100 ± 0 |
| Remdesivir    | >5.0 ± 0     | >5.0 ± 0     | >100 ± 0 |
| Carrimycin    | 45.53 ± 17.25 | 45.53 ± 17.25 | >100 ± 0 |

The cell cytotoxicity and antiviral activity assays presented in the table were tested by CPE assay.

* No antiviral activity at the maximal nontoxic concentration.
* The unit of remdesivir concentration is µmol/L.

### 2.7. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) assay

The total RNA of the infected cells was extracted using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. The one-step qRT-PCR was performed with TransScript Taqman One-Step qRT-PCR SuperMix (for HCoV-OC43 detection) and TransScript II Green One-Step qRT-PCR SuperMix (for HCoV-229E detection) (TransGen Biotech) using the ABI 7500 Fast Real-Time PCR system (Applied Biosystems). The applied primer sequences are shown in Table 1.

### 2.8. Time-of-addition assay

The viral replication steps targeted by carrimycin was mapped by determining the effect of sequentially delayed addition of the compounds on viral NP expression, i.e., time-of-addition experiment. Briefly, C3A cells (3 × 10⁵ cells/well) were infected with HCoV-OC43 at multiplicity of infection (MOI) of 0.5. Carrimycin (10 µg/mL) was added at the time of infection or at a different time post infection. All the cells were harvested at 24 h post infection and NP in the cell lysates were detected by Western blot assay.

### 2.9. Pseudovirus infection and luciferase assay

293T cells stably expressing human ACE2 were seeded into white wall and clear bottom 96-well plates and infected with lentiviruses pseudotyped with VSV glycoprotein (VSV-G) protein or SARS-CoV-2 spike protein in the absence or presence of carrimycin. NHE4-Cl² was used as a positive control. At 24 h post infection, the media were removed and cells were lysed with 20 µL/well of cell lysis buffer (Promega, Madison, WI, USA) for 15 min, followed by adding 50 µL/well of luciferase substrate (Promega). The firefly luciferase activities were measured by luminometry with an EnSparc instrument (PerkinElmer, Waltham, MA, USA).

### 2.10. Click-iT nascent RNA capture assay

C3A cells (3 × 10⁵ cells/well) were infected with HCoV-OC43 (MOI = 5) for 2 h. At 16 h post infection, the infected cells were mock-treated or treated with 10 µg/mL of carrimycin or 2 µmol/L of RDV for 3 h, and followed by continuing the treatment in the presence of 0.5 mmol/L 5-ethyluridine (EU) for 1 h. Total cellular RNA was extracted using the RNeasy Mini Kit (QIAGEN). EU-labeled nascent RNA was captured with Click-iT Nascent RNA Capture Kit according to the manufacturer’s instructions (Thermo Fisher Scientific). Then, the captured RNA was detected by qRT-PCR assay with primers specified in Table 1.

### 2.11. Statistics analysis

Statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad Software Inc., San Diego, CA, USA). Image J software (Rawak Software Inc., Stuttgart, Germany) was used for quantitative study on Immunofluorescence data. Results are expressed as mean ± standard deviation (SD). Data were analyzed by one-way ANOVA with Holm-Sidak’s multiple comparisons test. P < 0.05 was considered significant.

### 3. Results

#### 3.1. Carrimycin is a broad-spectrum antiviral agent against HCoVs

In order to identify the approved medicines that have a potential to be repurposed for the treatment of COVID-19, an in-house collection of more than 120 CFDA-approved drugs were screened for their ability to protect the CPE caused by HCoV-229E or HCoV-OC43 infection in Huh7 and H460 cells, respectively. Three macrolide antibiotics, acetylsyriramycin, azithromycin, and carrimycin (Fig. 1), were found to inhibit the infection of both viruses with an SI higher than 5 (Table 2). In particular, carrimycin demonstrated the highest antiviral potency (EC50 value of 2.5 g/mL) and selectivity (SI > 20) against both HCoV-229E and HCoV-OC43 (Table 2).

#### 3.2. Carrimycin reduced the levels of HCoV RNA and protein in infected cultures

To ascertain the antiviral effects of carrimycin against HCoVs, we further examined the effects of carrimycin on the levels of viral nucleocapsid protein and RNA in infected cultures, with RBV as a positive control. As shown in Fig. 2, carrimycin reduced the levels of HCoV-229E and HCoV-OC43 RNA in multiple cells lines in a concentration dependent manner. As a positive-strand RNA virus, double stranded RNA is a key intermediate of viral RNA replication.
replication and can be visualized in the cytoplasm of HCoV infected cells. As shown in Fig. 3, carrimycin treatment significantly reduced the levels of dsRNA in HCoV-229E-infected Hu7 cells and HCoV-OC43-infected C3A cells. Moreover, immunofluorescent staining of virally infected cultures demonstrated that carrimycin treatment dose-dependently reduced the protein levels of HCoV-OC43 and SARS-CoV-2 NPs in H460 and Vero-E6 cells, respectively (Fig. 4).

3.3. Carrimycin inhibited HCoV infection by targeting a post-entry replication event

To determine the HCoV replication step(s) targeted by carrimycin, we took the advantage of robust infection of C3A cells by HCoV-OC43 to perform a time-of-addition experiment. As shown in Fig. 5A, delayed addition of carrimycin at 6 h post infection still inhibited the expression of viral NP by approximate 90%, which is at the similar extent to that observed under the condition of treatment starting at 1 h before the infection (Fig. 4B). These results suggest that carrimycin most likely disrupted a post-entry replication step of the virus. In support of this notion, unlike NH4Cl that almost abolished the infection of lentiviral particles pseudotyped with SARS-CoV-2 envelope spike protein or VSV-G protein, carrimycin only modestly reduced the infection of both pseudoviruses at high concentration (Fig. 5B). Taken together, our results indicate that carrimycin efficiently inhibited the infection of multiple HCoVs by targeting one or multiple post-entry replication events.

3.4. Carrimycin inhibited the synthesis of HCoV RNA

As carrimycin dose-dependently reduced the amounts of HCoV dsRNA, an intermediate of viral RNA replication, in infected cells, we further investigated whether the antibiotic inhibited the viral RNA synthesis by using a click chemistry method to detect
the newly synthesized (nascent) viral RNA, with RDV, the HCoV RNA polymerase inhibitor, as a positive control. As depicted in Fig. 6A, HCoV-OC43 infected C3A cells were treated with carriamycin or RDV, starting at 16 h post infection for 3 h. The cells were then labeled with EU for 1 h. The EU-labeled nascent RNA was extracted from cell lysates by using a Click-iT Nascent RNA Capture Kit. HCoV-OC43 specific nascent RNA was quantified by a qRT-PCR assay. Similar to RDV, carriamycin also significantly reduced the amounts of HCoV-OC43 nascent RNA synthesis (Fig. 6B).

4. Discussion

Since the outbreak of COVID-19, there were no specific chemotherapeutic agents available to treat or prevent this disease. Currently, scientists around worldwide had focused on the repurposing of FDA approved drugs to treat COVID-19. Until now, more than 4000 clinical studies for COVID-19 were registered in the database of ClinicalTrials.gov (https://clinicaltrials.gov/ct2/results?cond=

Figure 3  Carrimycin reduced the amounts of double-stranded RNA in HCoV infected cells. Huh7 (1.5 × 10⁴ cells/well) or C3A (2.0 × 10⁴ cells/well) cells were plated into 96-well culture plates and incubated overnight. The cells were infected with HCoV-229E (MOI = 0.005, A) or HCoV-OC43 (MOI = 0.05, B), and various concentrations of carriamycin were added at the time of infection and treated for 24 h. The dsRNA was visualized by immunofluorescent staining assay. Scale bar: 200 μm. The quantitative study on immunofluorescence was tested by Image J software.
renders carrimycin more potent antibacterial activity, especially \textit{in vivo}, as a result of higher lipophilicity\textsuperscript{17,18}. Carrimycin was approved by CFDA for the treatment of acute tracheal-bronchitis caused by \textit{Haemophilus influenzae}, \textit{Streptococcus pneumoniae}, \textit{Moraxella catarrhalis}, and \textit{Staphylococcus}. In this study, it was found that carrimycin exhibited broad-spectrum antiviral activity against HCoVs in multiple cell lines. As shown in Table 1, carrimycin showed the higher antiviral potency than...
acetylsspiramycin. It remains to be determined whether the enhanced antiviral activity of carrimycin, as compared to acylspiramycin, is the result of higher lipophilicity and membrane permeability17,18.

Concerning the antiviral mechanism, the time-of-addition and pseudotyped lentiviral infection assays suggest that carrimycin efficiently inhibited the infection of multiple HCoVs by targeting one or multiple post-entry replication events. As positive strand RNA viruses, coronaviruses synthesize their RNA in the cytoplasmic replicase complexes consisting of viral nsp12–nsp7–nsp-8 core polymerase and cellular co-factors19,20. As shown in Fig. 6, similar to HCoV RNA polymerase inhibitor RDV, carrimycin significantly inhibited HCoV-OC43 RNA synthesis. Macrolide antibiotics inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit21. As a macrolide antibiotic, it is not clear whether carrimycin hinders the viral protein synthesis, and it remains to be further investigated whether carrimycin directly inhibits the synthesis of viral RNA or regulates the synthesis of viral RNA by affecting host targets.

Pneumonia caused by viral infections usually has secondary bacterial infection22. COVID-19 may also be associated with secondary bacterial infections23,24. Now, the demonstrated antiviral activity to HCoVs, including SARS-CoV-2, as well as the preferential distribution in lungs via oral administration warranted the clinical trials of carrimycin for the treatment of COVID-19 in China (ChiCTR2000029867 and ChiCTR2000032242). The clinical trial results will be reported elsewhere.

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Author contributions

Yuhuan Li and Jiandong Jiang designed experiments; Haiyan Yan, Jing Sun, Kun Wang, Shuo Wu, Linlin Bao, Airu Zhu, Tian Zhang, Rongmei Gao, Biao Dong, Jianrui Li, Qi Lv, Feifei Qin, Zhen Zhuang, and Xiaoafang Huang carried out the experiments; Huiqiang Wang, Lu Yang, and Dong Wang analyzed the data and provided advice on the interpretation of data; Huiqiang Wang, Jinyuan Li, Yongsheng Che, and Jiandong Jiang acquired funding; Yuhuan Li, Yongsheng Che, and Jiandong Jiang provided essential reagents; Haiyan Yan, Jing Sun, Kun Wang, and Shuo Wu wrote the original draft with input from co-authors; Weiqing He and Xinyi Yang provided essential reagents; Yuhuan Li, Yongsheng Che, and Jiandong Jiang acquired funding; Yuhuan Li, Yongsheng Che, and Jiandong Jiang wrote the final draft; all authors approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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