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Repurposing old drugs as antiviral agents for coronaviruses

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ABSTRACT

Background: New therapeutic options to address the ongoing coronavirus disease 2019 (COVID-19) pandemic are urgently needed. One possible strategy is the repurposing of existing drugs approved for other indications as antiviral agents for severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). Due to the commercial unavailability of SARS-CoV-2 drugs for treating COVID-19, we screened approximately 250 existing drugs or pharmacologically active compounds for their inhibitory activities against feline infectious peritonitis coronavirus (FIPV) and human coronavirus OC43 (HCoV-OC43), a human coronavirus in the same genus (*Betacoronavirus*) as SARS-CoV-2.

Methods: FIPV was proliferated in feline Fcwf-4 cells and HCoV-OC43 in human HCT-8 cells. Viral proliferation was assayed by visualization of cytopathic effects on the infected Fcwf-4 cells and immunofluorescent assay for detection of the nucleocapsid proteins of HCoV-OC43 in the HCT-8 cells. The concentrations (EC_{50}) of each drug necessary to diminish viral activity to 50% of that for the untreated controls were determined. The viabilities of Fcwf-4 and HCT-8 cells were measured by crystal violet staining and MTS/PMS assay, respectively.

Results: Fifteen out of the 252 drugs or pharmacologically active compounds screened were found to be active against both FIPV and HCoV-OC43, with EC_{50} values ranging from 11 nM to 75 μM. They are all old drugs as follows, anisomycin, antimycin A, atovaquone, chloroquine, conivaptan, emetine, gemcitabine, homoharringtonine, niclosamide, nitazoxanide, oligomycin, salinomycin, tilorone, valinomycin, and vismodegib.

Conclusion: All of the old drugs identified as having activity against FIPV and HCoV-OC43 have seen clinical use in their respective indications and are associated with known dosing schedules and adverse effect or toxicity profiles in humans. Those, when later confirmed to have an anti-viral effect on SARS-CoV-2, should be considered for immediate uses in COVID-19 patients.

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At a glance commentary

Scientific background on the subject

Repurposing of existing drugs as antiviral agents for SARS-CoV-2 is a practical strategy for the urgent need in treating COVID-19. Thus, we screened 252 existing drugs or pharmacologically active compounds for their inhibitory activities against FIPV and HCoV-OC43, a human coronavirus in the same genus (Betacoronavirus) as SARS-CoV-2.

What this study adds to the field

All of the old drugs identified against FIPV and HCoV-OC43 have seen clinical use in their respective indications and are associated with known dosing schedules and adverse or toxicity profiles in humans. Those, when later confirmed to have an anti-SARS-CoV-2 effect, should be considered for immediate uses in COVID-19 patients.

Coronavirus disease 2019 (COVID-19), the disease caused by severe acute respiratory syndrome associated coronavirus-2 (SARS-CoV-2), has infected over 2,970,000 people and killed more than 206,000 in 213 countries since the first outbreak in WuHan, China [1] was reported in December 2019. To control this disease, effective treatments, including SARS-CoV-2 inhibitors, are being actively pursued [1]. One potentially efficient approach is the repurposing of drugs previously approved to treat other diseases, since they are immediately available for use in clinical trials with COVID-19 patients and have known safety profiles.

Coronavirus (CoV) has a large genome and its mutation rate is higher than that of other RNA viruses [2,3]. Animal CoVs cause persistent enzootic infections that inevitably infect new host species and become zoonotic, as happened for SARS-CoV, Middle East respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV-2. Due to the diversity and relentlessness of these viruses, their individual mitigation is difficult [4]; and therefore broad-spectrum inhibitors of emerging and endemic CoVs are needed, especially for those prone to be periodically cycling in and out of humans and livestock. Therefore, the small molecule inhibitors, either old drugs or new chemical entities, were actively pursued and developed aiming to prevent or cure the SARS-CoV and MERS-CoV infections [5–14]. Previous work established the clinical efficacy of pegylated interferon-α-2a, ribavirin, and lopinavir/ritonavir for the treatment of the MERS-CoV [15,16]. Therefore, the strategy of repurposing existing drugs to treat patients infected with novel or zoonotic CoV has been validated and merits consideration in the contest of tackling the SARS-CoV-2 and COVID-19 pandemic.

Two representative CoVs were selected for existing drugs to be tested against. Feline infectious peritonitis coronavirus (FIPV) belongs to the genus *Alphacoronavirus* and causes enteritis in domestic and wild cats. Approximately 5–15% of infected cats develop feline infectious peritonitis, which is usually fatal [17,18]. The pathogenesis, epidemiology, and pulmonary lesions associated with FIPV are similar to those associated with human SARS [19]. Human coronavirus OC43 (HCoV-OC43) belongs to the same viral genus (*Betacoronavirus*) as SARS-CoV and SARS-CoV-2, infects humans and cattle, and is one of the viruses responsible for the common cold [20,21].

252 old drugs or pharmacologically active compounds were assessed for their inhibitory activity of FIPV [5–16], of which 23 were found to exhibit some anti-FIPV activity and advanced to testing against HCoV-OC43. In total, 15 exerted an inhibitory effect on both FIPV and HCoV-OC43 and they turned out are old drugs. These included chloroquine, which was recently demonstrated to be capable of inhibiting SARS-CoV-2 *in vitro*, and exhibited some efficacy in the clinical treatment of COVID-19 patients [22]. Moreover, GS-441524, the active metabolite of remdesivir [23,24] which is currently being investigated in clinical trials for severe cases of COVID-19 [24,25], also exerted potent inhibitory activities against both FIPV and HCoV-OC43 herein.

Materials and methods

Cells, viruses, and antibodies

Felis catus whole fetus-4 (Fcwf-4) cells (ATCC®CRL-2787) were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone Laboratories, Logan, UT, USA) containing 10% fetal bovine serum (FBS) with 1% penicillin/streptomycin at 37 °C with 5% CO_2. The serotype II FIPV Taiwan isolate NTU156 strain, a kind gift from National Taiwan University, was propagated and titrated in Fcwf-4 cells [26]. Confluent Fcwf-4 cells were seeded in 96-well plates and treated with various concentrations of testing compounds of up to 100 μM at 37 °C under an atmosphere of 5% CO_2 for 48 h. Sixteen h post inoculation, cells were infected with FIPV NTU156 strain at 300 $TCID_{50}$ per well and incubated at 37 °C. After 1 h, the supernatant was discarded and a series of 7 concentrations at different dilution of testing compounds in DMEM containing 2% FBS added. Plates were incubated at 37 °C under an atmosphere of 5% CO_2 for additional 48 h; then, the cells were fixed with 10% formalin and stained with 0.1% crystal violet. The cytopathic effect (CPE) of the virus was assumed to correlate with the intensity of the crystal violet staining and measured visually for determination of the 50% effective concentrations (EC_{50}). Cell cytotoxicity was also measured by crystal violet staining. The 50% cytotoxicity concentration (CC_{50}) was calculated according to the Reed and Muench method [27].

HCT-8 colon epithelial cells (ATCC®CCL-244™) were grown as monolayers in a growth medium consisting of DMEM and 10% FBS, (Biological Industries, Cromwell, CT, USA). HCoV-OC43 (ATCC®VR1558™) was grown and propagated in HCT-8 cells cultured with DMEM and 2% FBS. EC_{50} was measured using an indirect immunofluorescent assay (IFA). HCT-8 cells

(5×10^4 cells/well) were deposited in 96-well plates, pre-treated with solutions of the compounds to be tested for 30 min, and then infected with HCoV-OC43 at a multiplicity of infection (MOI) of 0.05, and incubated at 37 °C (see above) for 72 h. For the IFA assay, HCT-8 cells were fixed with 80% acetone and subjected to IFA with (i) an antibody against nucleocapsid proteins of HCoV-OC43 (Mab9013; Merck Millipore, Burlington, MA, USA) and (ii) antibody fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin (#55499; MP Biomedicals, Irvine, CA, USA). After three washes with phosphate-buffered saline, cells were incubated with the FITC-conjugated anti-mouse immunoglobulin for 60 min at room temperature. The cells were washed three times with PBS and the fluorescence intensities measured using either a SpectraMax® Paradigm® system (Molecular Devices, San Jose, CA, USA) (excitation and emission wavelengths, 485 and 535 nm, respectively) to determine the EC_{50} for inhibiting nucleocapsid protein expression; or viewed by a fluorescence microscopy. Hoechst 33258 dye (H3569, Invitrogen™, Waltham, MA, USA) was used to stain the nuclear DNA of live cells. Images of the cells after IFA or Hoechst 33258 staining were captured using a charge-coupled device linked to a Nikon Image-Pro Express. The cells were treated with a series of 5 concentrations of the test compounds at 5-fold dilution; and the results of these assays used to obtain concentration–response curves from which EC_{50} values were determined. The % area of immunofluorescent staining of the cells was used to correct for EC_{50} values since the fluorescence intensity was disproportionately higher when only small portion of the cells were infected. For the cytotoxicity assay, HCT-8 cells cultured in DMEM and 10% FBS in 96-well plates were treated with a designed series of 5 concentrations at 5-fold dilution of the test compounds for 72 h. The results of these assays were used to obtain the concentration–response curves from which the CC_{50} concentrations were obtained.

Chemicals

Emetine (HY–B1479A, 99.81%, LCMS), salinomycin (HY-15597, >98%, NMR), tilorone (HY–B1080, 99.9%, LCMS), chloroquine (HY-17589, 99.9%, LCMS), homoharringtonine (HY-14944, 99.2%, LCMS), gemcitabine (HY–B0003, 99.9%, LCMS), vismodegib (HY-10440, 99.9%, LCMS), conivaptan (HY-18347A, 99.9%, LCMS), and atovaquone (HY-13832, 99.8%, LCMS) were purchased from MedChem Express (Monmouth Junction, NJ, USA); niclosamide (S3030, 99.8%, HPLC) and nitazoxanide (S1627, 99.3%, HPLC) were from Selleckchem (Houston, TX, USA); antimycin A (A8674, 97.64%, HPLC), anisomycin (A9789, >98%, HPLC) oligomycin (O4876, >90%, HPLC), valinomycin (V0627, ≧90%, HPLC) and crystal violet (C0775, Dye content ≥90%) were from Sigma–Aldrich (St. Louis, MO, USA); GS-441524 (AG167808, >98%, HPLC) were from Carbosynth (San Diego, CA, USA). All chemicals were used as supplied.

Results and discussion

252 drugs were collected and screened for their inhibitory activity against FIPV; this activity was ascertained by visual observation of their cytopathic effects. Of these 252, 23 were also tested for their inhibition of HCoV-OC43 by an IFA against HCoV-OC43 nucleocapsid protein (Table 1). Fifteen of these, all old drugs, exhibited inhibitory activity against HCoV-OC43 and exhibited - EC_{50} values ranging from 11 nM to 75 μM for FIPV and 62 nM to 48 μM for HCoV-OC43 (Table 1). Representative results from the cytopathy (FIPV) and IFA assays (HCoV-OC43) are depicted in Figs. 1 and 2,

Table 1 Inhibitory activities of 15 drugs and GS-441524 against FIPV and HCoVOC43 coronaviruses.

Compound Name	Feline infectious peritonitis virus[a]			Human coronavirus OC43[a]		
	EC_{50} (μM) (Visual assay)	CC_{50} (μM) (Visual assay)	Selectivity index	EC_{50} (μM) (IFA)	CC_{50} (μM) (MTS)	Selectivity index
Antimycin A	75.00 ± 0.00	81.94 ± 6.36	1.10	0.062 ± 0.003	>50	>806
Anisomycin	0.023 ± 0.00	0.047 ± 0.012	2.05	0.20 ± 0.03	22.8 ± 0.94	114
Oligomycin	17.78 ± 0.48	18.75 ± 0.00	1.06	5.22 ± 0.96	>50	>9.58
Valinomycin	1.63 ± 0.10	3.98 ± 0.39	2.45	0.41 ± 0.02	>50	>122
Emetine	0.011 ± 0.00	0.03 ± 0.00	3.00	0.21 ± 0.07	>50	>238
Homoharringtonine	0.031 ± 0.003	0.049 ± 0.002	1.58	0.29 ± 0.03	7.61 ± 0.60	26.7
Niclosamide	0.29 ± 0.02	0.23 ± 0.00	0.80	1.36 ± 0.56	>50	>36.8
Atovaquone	4.78 ± 0.51	>100	20.92	6.78 ± 0.73	>50	>7.37
Conivaptan	16.89 ± 1.51	>100	5.92	12.2 ± 4.20	>50	>4.10
Chloroquine (diphosphate)	27.92 ± 0.72	37.50 ± 0.00	1.35	27.4 ± 2.51	>50	>1.82
Salinomycin	0.70 ± 0.13	0.34 ± 0.10	0.49	5.78 ± 2.17	>50	>8.65
Tilorone (dihydrochloride)	11.25 ± 1.25	9.38 ± 0.00	0.84	26.0 ± 2.24	35.9 ± 2.97	1.38
Nitazoxanide	NA	4.69 ± 0.00	NA	28.6 ± 7.44	>50	>1.75
Gemcitabine (Hydrochloride)	1.08 ± 0.35	>100	92.60	38.6 ± 11.4	>50	>1.30
Vismodegib	32.5 ± 2.50	>100	3.08	47.6 ± 3.29	>50	>1.05
GS-441524	3.5 ± 0.0	>100	28.58	6.77 ± 0.71	>50	>7.39

[a] Data are means ± S.D. from three rounds of experiments, each in triplicate (FIPV); and means ± S.D. from three independent experiments, each in duplicate (HCoV-OC43).

Abbreviations: EC_{50}: The values of 50% maximal effective concentration; CC_{50}: The values of 50% maximal cytotoxic concentration; NA: Not available.

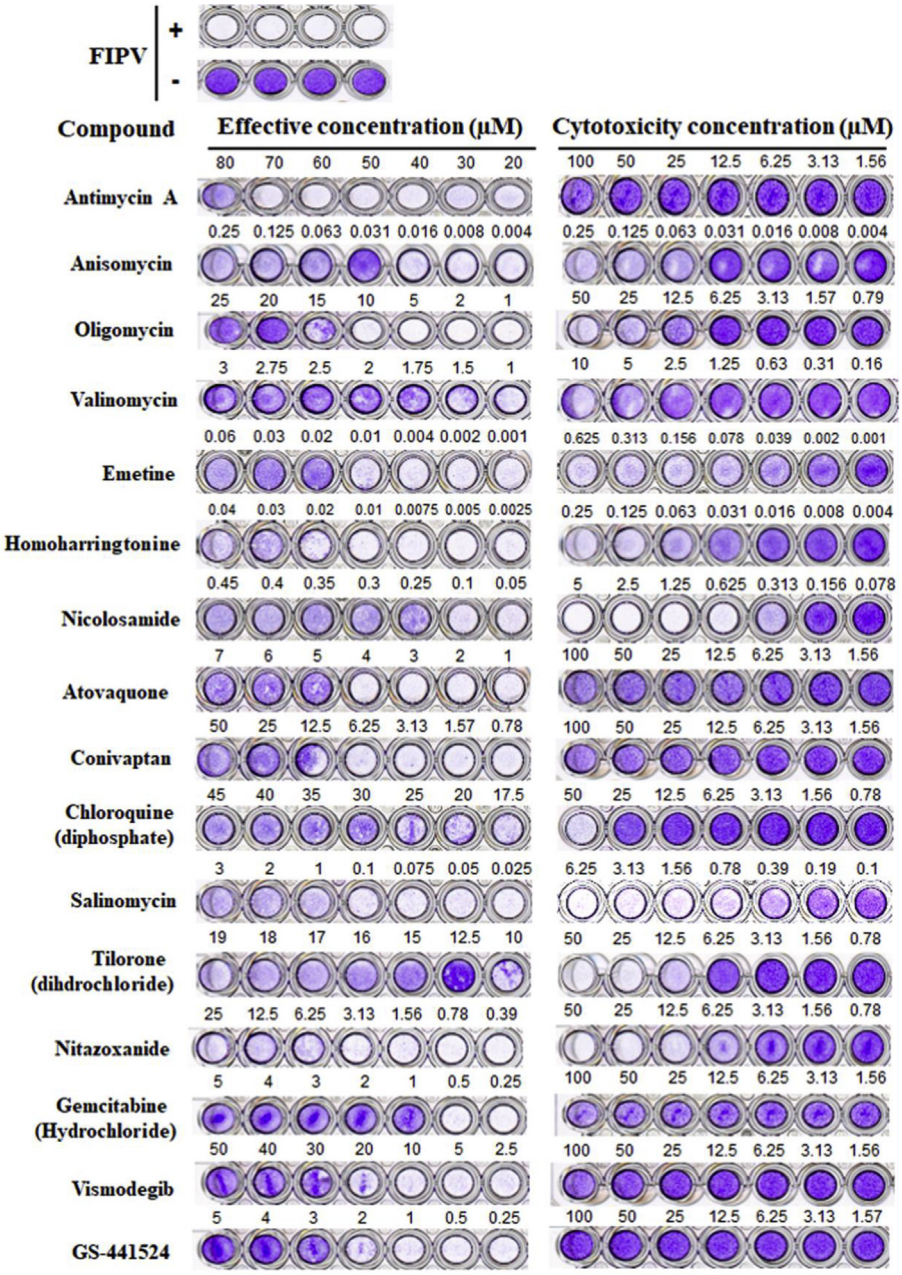

Fig. 1 The cytopathic effects of 15 drugs and GS-441524 against FIPV. Fcwf-4 cells infected with FIPV (NTU156) showed typical cytopathic effects by crystal violet staining compared to uninfected cells. FIPV infected cells were treated with a series of 7 concentrations at different dilution of the testing compounds. The cytotoxicity of the compounds being tested was also investigated. The 50% maximal effective concentration (EC_{50}) and cytotoxicity concentration (CC_{50}) of each compound were calculated by visual assays. Shown are means ± S.D. from three rounds of experiments, each in triplicate.

respectively. These 15 drugs include 4 antibiotics antimycin A, anisomycin, valinomycin, and oligomycin, with EC_{50} values of 75 μM, 23 nM, 1.63 μM, and 17.78 μM against FIPV and 62 nM, 0.2 μM, 0.4 μM, and 5.2 μM against HCoV-OC43 respectively.

Emetine, a drug used as an anti-protozoal and previously reported to have anti-coronaviral activity potential [28], exhibited EC_{50} values of 11 nM against FIPV and 0.21 μM against HCoV-OC43. Homoharringtonine, a natural plant alkaloid derived from *Cephalotaxus fortunei* and indicated for the treatment of chronic myeloid leukemia (CML) [29], exhibited EC_{50} values of 31 nM against FIPV and 0.29 μM against HCoV-OC43. Niclosamide, an anthelmintic indicated for the treatment of tapeworm infections, exhibited EC_{50} values of 0.29 μM against FIPV and 1.36 μM against HCoV-OC43. Interestingly, niclosamide also demonstrated efficacy against drug-resistant *Staphylococcus aureus* [30] and showed *in vitro* activity against SARS-CoV [11].

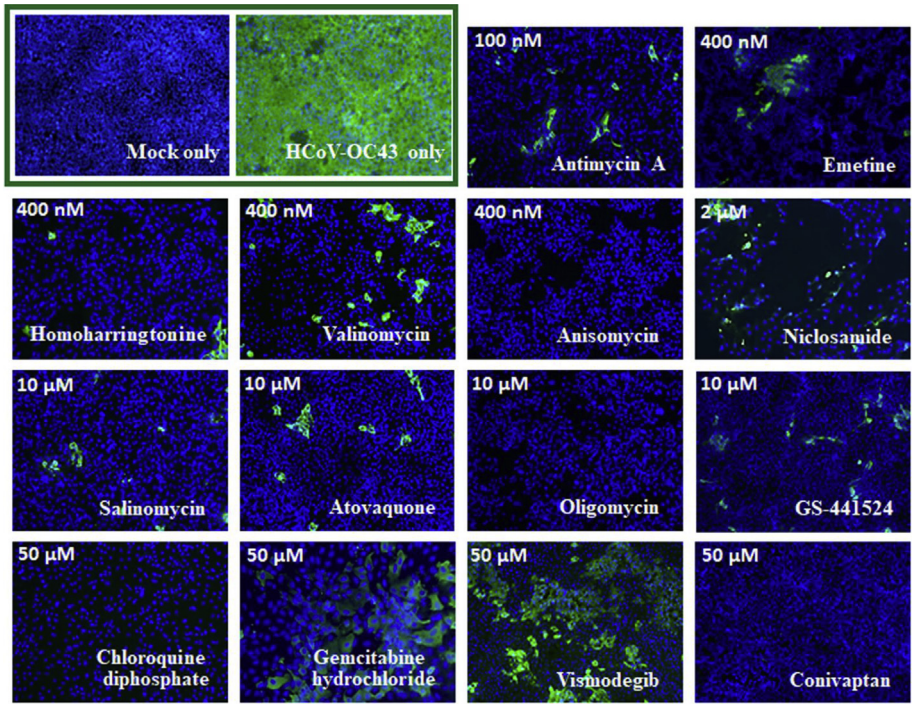

Fig. 2 Immunofluorescent assay of 15 drugs and GS-441524 against HCoV-OC43. Indirect immunofluorescent assay (IFAs) with the antibody against HCoV-OC43 nucleocapsid protein (in green) and Hoechst dye staining (in blue) for the DNA of the host live cells in HCoV-OC43 (0.05 MOI) infected HCT-8 cells at 72 h.p.i. were performed shown here are the representative images of the cells with mock infection (MOCK), the infected cells treated with vehicle (0.5% DMSO), and the infected cells treated with drugs as indicated from 3 independent experiments. Nuclei of live HCT-8 cells in blue were stained with Hoechst dye. The treated concentrations of each drug are labelled with the corresponding images (200×).

Atovaquone, a hydroxy-1,4-naphthoquinone with anti-pneumocystic activity [31], exhibited EC_{50} values of 4.78 μM against FIPV and 6.78 μM against HCoV-OC43. Conivaptan, a non-peptide inhibitor of the receptor vasopressin and originally approved for hyponatremia [32], exhibited EC_{50} values of 16.9 μM against FIPV and 12.2 μM against HCoV-OC43. Atovaquone is predicted to inhibit SARS-CoV-2 through targeting of the viral RNA-dependent RNA polymerase or 3C like protease [33].

Chloroquine is being intensively studied in the clinical setting for treating COVID-19 and in the preclinical setting for efficacies against SARS-CoV-2 both *in vitro* and *in vivo* [22], but its EC_{50} values against FIPV (27.9 μM) and HCoV-OC43 (27.4 μM) were higher than of the compounds mentioned above. Nonetheless, this identified EC_{50} of ~27 μM is comparable or better than the clinically used effective dosages of chloroquine, 200 ~ 1000 mg in qd or bid [34–36]. In addition, we also tested the nucleotide analogue GS-441524, the active metabolite of remdesivir [23,24], and it exhibited an EC_{50} of 3.5 μM against FIPV, compared to an EC_{50} of 6.77 μM against HCoV-OC43; GS-441524 has been reported to exhibit good *in vivo* efficacy against FIPV in cats [23] and against SARS-CoV *in vitro* [37], but it is not an FDA approved drug yet. Remdesivir (GS-5734), the prodrug of GS-441524, has also shown efficacy for the treatment of COVID-19 patients [24,25]. Moreover, this identified EC_{50} (3 ~7 μM) of GS-441524 is also equivalent or better than the clinically used effective dosages of remdesivir, 100 ~ 200 mg [24,25]. Thus the concentrations tested in this particular study are of clinical significance.

Other drugs which have already been repurposed in the context of COVID-19 include ivermectin, a type of avermectin used to treat many types of parasite infestations [38], and the combination of hydroxychloroquine and the antibiotic azithromycin [39]. Therefore, all of these above-mentioned drugs are therefore proposed as potential treatments for COVID-19. In conclusion, several existing drugs were identified as having good inhibitory activities against FIPV and HCoV-OC43, and the doses at which they are currently used for their respective disease indications could be referenced when contemplating their application against SARS-CoV-2 in patients of COVID-19.

Conflicts of Interest

The authors declare no conflict of interest.

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