A screen of the NIH Clinical Collection small molecule library identifies potential anti-coronavirus drugs

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Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

ARTICLE INFO

Article history:
Received 1 July 2014
Revised 8 October 2014
Accepted 20 November 2014
Available online xxxx

Keywords:
Coronavirus
Small molecule
NCC library
Screening

ABSTRACT

With the recent emergence of Middle East Respiratory Syndrome coronavirus in humans and the outbreak of devastating porcine epidemic diarrhea coronavirus in swine, therapeutic intervention is urgently needed. However, anti-coronavirus drugs currently are not available. In an effort to assist rapid development of anti-coronavirus drugs, here we screened the NIH Clinical Collection in cell culture using a luciferase reporter-expressing recombinant murine coronavirus. Of the 727 compounds screened, 84 were found to have a significant anti-coronavirus effect. Further experiments revealed that 51 compounds blocked virus entry while 19 others inhibited viral replication. Additional validation studies with the top 3 inhibitors (hexachlorophene, nitazoxanide and homoharringtonine) demonstrated robust antiviral activity (a reduction of 6 to 8 log10 in virus titer) with an IC50 ranging from 11 nM to 1.2 μM. Furthermore, homoharringtonine and hexachlorophene exhibited broad antiviral activity against diverse species of human and animal coronaviruses. Since the NIH Clinical Collection consists of compounds that have already been through clinical trials, these small molecule inhibitors have a great potential for rapid development as anti-coronavirus drugs.

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In an effort to identify potential drugs capable of inhibiting coronavirus infection, in the present study, we performed an in vitro screen of a small molecule library from the National Institutes of Health Clinical Collections (NCC). Because both SARS and MERS coronaviruses belong to the same biologically and genetically closely related Betacoronavirus subgroup as murine coronavirus, we used a recombinant murine coronavirus expressing a luciferase reporter gene as a safe surrogate to evaluate the anti-coronavirus efficacy of the drugs. Our screen identified 84 compounds with anti-coronavirus properties. Importantly, several compounds exhibited robust anti-coronavirus activity at micromolar or nanomolar concentrations, without overt cytotoxicity to host cells. Thus, these compounds can be advanced to animal and clinical trials, having the potential to be developed as effective anti-coronavirus drugs.

### Materials and methods

#### 2.1. Cells, virus, and reagents

Mouse astrocytoma DBT and fibroblast 17Cl-1 cells were cultured at 37 °C in DMEM containing 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/ml). A recombinant murine coronavirus mouse hepatitis virus (MHV) strain A59 expressing firefly luciferase, termed MHV-2aFLS (de Haan et al., 2003), was used for screening throughout the study. Wild-type MHV-A59, MHV-A59GFP (Das Sarma et al., 2002), MHV-1, MHV-2, and MHV-JHM were also used for some experiments. Virus titer was determined by standard plaque assay. Bovine coronavirus strain L9 (BoCV-L9) (Zhang et al., 1991), and human enteric coronavirus strain 4408 (HECoV-4408) (Zhang et al., 1994) were grown in human rectal tumor (HRT)-18 cells. Monoclonal antibody (mAb) J3.3 was used for detecting MHV N protein, and mAb#46 for the N protein of BCoV-L9 and HECV-4408 (Zhang et al., 1994) were grown in human rectal tumor (HRT)-18 cells. Monoclonal antibody (mAb) J3.3 was used for detecting MHV N protein, and mAb#46 for the N protein of BCoV-L9 and HECV-4408 (Zhang et al., 1994). Antibody to β-actin was purchased from Invitrogen. Goat anti-mouse

#### Table 1

Top anti-coronavirus drug candidates identified in DBT and 17Cl-1 cells.

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Note: Data showing here were obtained from validation study. Drug candidates are ranked from strongest to weakest antiviral efficacy according to SSMD score. Only the candidates with an SSMD score of less than –9 are listed. Mean, average luciferase activity of triplicate expressed as percentage of the control (DMSO), which is 100%. SD, standard deviation of the mean. SSMD, strictly standardized mean difference.
IgG conjugated with horseradish peroxidase (HRP) or with FITC was purchased from Sigma–Aldrich.

2.2. Screening of small molecule drug library

The NCC library contains a total of 727 small molecule drugs (compounds) supplied in 96-well plates that are prepared in DMSO at 1 mM (http://www.nihclinicalcollection.com). For screening, 10 µl of each drug was first transferred to a new 96-well plate and diluted to 100 µl with Opti-MEM 1 serum-free medium to make stock plates. Then, 10 µl of the stock was transferred to a well in another 96-well plate and mixed with 90 µl of MHV-2aFLS in DMEM/TPB10 to give a final concentration of 10 µM for each drug. The drug/virus mixture (45 µl) was delivered to each well and the infection was carried out for 8 h. The vehicle control contained 1% DMSO. For primary screening, duplicate plates were used. For validation screening, experiments were conducted in triplicate plates. At the end of the infection, culture medium was removed and cells were stored at −80°C overnight. The plates were then allowed to thaw at room temperature and 50 µl of culture medium was harvested.

Fig. 2. Correlation of inhibition on luciferase reporter expression and virus titer. DBT cells were treated with various drugs as indicated (10 µM) or DMSO (1%) as a control for 1 h and then infected with MHV-2aFLS at MOI of 1 for 8 h. The medium was harvested for determining viral titer (TCID50) and cells were lysed for determining luciferase activity. Data indicate the mean of 3 replicates (independent treatments) and standard deviation of the mean. Statistical significance of the inhibitory effect of the drugs on luciferase activity (A) or virus titer (B) as compared to those of DMSO control is indicated by the number of asterisks (*p < 0.05; **p < 0.01; ***p < 0.001).

Fig. 3. Inhibitory effect of hexachlorophene on MHV infection. (A) Chemical structure of hexachlorophene. (B) Determination of IC50. DBT cells were treated with hexachlorophene at various concentrations as indicated or 1% DMSO (vehicle control) for 1 h, and were infected with MHV-2aFLS at MOI of 1 in the presence of the drug for 8 h. Cells were then lysed for luciferase assay. Inhibition of MHV infection was expressed as percent reduction in luciferase activity following drug treatment compared to the control, and the IC50 was then calculated as indicated by the solid lines. (C) Inhibition of viral titer. DBT cells were treated with hexachlorophene (10 µM) or DMSO (1%) as a control for 1 h and then infected with MHV-2aFLS at MOI of 1 for 12 h. The medium was harvested for determining viral titer (TCID50). Data indicate the mean of 3 replicates and standard deviation of the mean. (D) Inhibition of viral N protein expression. The experiments were performed identically to (C), except that different concentrations of the drugs were used. Following drug treatment and viral infection, cells were lysed to evaluate viral N protein expression levels by Western blotting. Beta-actin serves as a loading control.
2.3. Cell viability assay

Cells grown in 96-well plates were incubated for 16 h with each drug at 10 µM and then cell viability was determined using the XTT assay kit TOX2-1KT according to the manufacturer’s instruction (Sigma–Aldrich). DMSO at 1% served as vehicle control.

2.4. Western blot analysis and immunofluorescence assay (IFA)

For detecting proteins, either Western blot analysis or IFA was performed as previously described (Cao and Zhang, 2012).

2.5. Determination of virus titer (TCID_{50})

Virus titer was determined by the standard 50% tissue culture infectious dose (TCID_{50}) in DBT cells in a 96-well plate.

2.6. Statistics analysis

 Luciferase data from each library screening plate were combined and used for statistical analysis. Mean luciferase activity for replicates and standard deviation (SD) of the mean were calculated by standard statistics methods and were expressed as a percentage of the negative control (DMSO), which was set as 100%. Student’s t-test was used to calculate p-values for statistical significance. Strictly standardized mean difference (SSMD) (Zhang, 2007) was used to select the candidates with a score of −2 or less for inhibitors.

3. Results

3.1. Screening of the NCC drug library for anti-coronavirus activity

Primary screening of the entire NCC library was performed in DBT cells infected with MHV-2aFLS. Duplicate plates were used for the screening and SSMD was used for hit selection and ranking. Recently, SSMD has been widely used for hit selection in high-throughput screening assay (HTS) such as siRNA and small molecule screenings as well as antiviral drug selection (Andruska et al., 2012; Aulner et al., 2013; Gough et al., 2014; Rachidi et al., 2014; Zhang, 2007). A negative value of SSMD suggests inhibitory effect while a positive value indicates enhanced effect of the compound. An SSMD score of <−2 suggests strong inhibitory effect (Zhang, 2007). Thus, we used this score as a cut-off threshold for hit selection. Results showed that 84 drugs had an SSMD score of less than −2, indicating that these drugs likely have anti-MHV activities. Of the 84 drug candidates, 37 exhibited very strong inhibition of MHV infection with an SSMD score of <−5 (Supplemental Table 1). All candidate anti-coronavirus drugs were subjected to further validation (see below).

3.2. Validation of the candidate drugs

To verify the antiviral effect of the candidate drugs, secondary screening was carried out in both DBT and 17Cl-1 cells. We found that 70 and 69 of the 84 drugs, respectively, inhibited MHV infection in DBT and 17Cl-1 cells (Supplemental Table 1). Specifically, in DBT cells, 11 drugs strongly inhibited viral infection with an SSMD score of less than −9 (Table 1). In 17Cl-1 cells, viral infection was strongly inhibited by 14 drugs, which exhibited SSMD scores of less than −9 (Table 1). Cell viability assays revealed that all the candidate drugs did not significantly decrease cell viability at the

**Fig. 4.** Inhibitory effect of nitazoxanide on MHV infection. (A) Chemical structure of nitazoxanide. (B) Determination of IC_{50}. DBT cells were treated with nitazoxanide at various concentrations or 1% DMSO (vehicle control) for 1 h, and were infected with MHV-2aFLS at MOI of 1 in the presence of the drug for 8 h. Cells were then lysed for luciferase assay. Inhibition of drug on MHV infection was expressed as percent reduction on luciferase activity to the control and the IC_{50} was then calculated as indicated by the solid lines. (C) Inhibition of viral titer. DBT cells were treated with nitazoxanide (10 µM) or DMSO (1%) as a control for 1 h and then infected with MHV-2aFLS at MOI of 1 for 12 h. The medium was harvested for determining viral titer (TCID_{50}). Data indicate the mean of 3 replicates and standard deviation of the mean. (D) Inhibition of viral N protein expression. The experiments were performed identically to (C), except that different concentrations of the drugs were used. Following drug treatment and viral infection, cells were lysed to evaluate viral N protein expression levels by Western blotting. Beta-actin serves as a loading control.
concentrations tested (Supplemental Table 2). Comparative analysis revealed that 10 candidate drugs exhibited antiviral effects only in DBT cells, indicating that some differential cellular targets may play a role in viral infection (Supplemental Table 1). Importantly, 61 of the candidate drugs were commonly effective in inhibiting viral infection of both DBT and 17Cl-1 cells (Supplemental Table 1), suggesting that the cellular targets for these drugs are conserved between the two cell types. Interestingly, many of the anti-coronavirus candidate drugs could be grouped by clinical application. The 3 most abundant groups of anti-coronavirus candidate drugs are those used for cancer treatment or as antidepressant and antipsychotic (Supplemental Table 3). To validate the statistical approach employed for identifying candidate drugs, we selected two representative drugs from each of the three groups based on their SSMD score (low, median, and high), and determined their inhibitory effects on virus infection. Indeed, two drugs (homoharringtonine and hexachlorophene) with the lowest SSMD score (≈C25/C0) almost completely inhibited coronavirus infection (a reduction of >95% in luciferase activity and >8log10 in virus titer) while ribavirin and Minoxidil with the highest SSMD score (≈C25) had the least inhibitory effect on virus infection (a reduction of ≈30% in luciferase activity and ≈1log10 in virus titer); the other two drugs (Paroxetine and Sertraline) with a median SSMD score (≈C5) reduced luciferase activity by about 50% and virus titer about 2log10 (Fig. 2). Thus, the inhibitory effect of the drugs correlated inversely and proportionally with the SSMD score. These data demonstrate a general applicability of the SSMD scoring system for selecting candidate drugs (Zhang, 2007). To further confirm the anti-coronavirus activity of the candidate drugs, we selected 3 top-ranked drugs for additional studies. First, we determined the IC50. DBT cells were infected with MHV-2aFLS at MOI of 1, and then treated with hexachlorophene, nitazoxanide, and homoharringtonine (panel A in Figs. 3–5) at various

Fig. 5. Inhibitory effect of homoharringtonine on MHV infection. (A) Chemical structure of homoharringtonine. (B) Determination of IC50. DBT cells were treated with homoharringtonine at various concentrations or 1% DMSO (vehicle control) for 1 h, and were infected with MHV-2aFLS at MOI of 1 in the presence of the drug for 8 h. Cells were then lysed for luciferase assay. Inhibition of drug on MHV infection was expressed as percent reduction on luciferase activity to the control and the IC50 was then calculated as indicated by the solid lines. (C–E) Cells were pretreated with homoharringtonine at various concentrations as indicated or 1% DMSO for 1 h. Cells were then infected with MHV-A59 for 12 h (C and D in DBT cells) or MHV-A59GFP for 16 h (E in 17Cl-1 cells) at MOI of 1 in the presence of the drug. (C) Virus titer in the medium was determined by TCID50. Data represent the mean of 3 replicates and standard deviation of the mean. (D) Viral N protein expression in cell lysates was detected by Western blotting using mAb J.3.3. Beta-actin serves as a loading control. (E) Expression of GFP was directly observed using a fluorescence microscope (Olympus IX-70), and images were captured using a digital camera (Zeiss).
concentrations. Results showed that while the IC50 varied widely from about 11 nM for homoharringtonine to about 1 μM for hexachlorophene and nitazoxanide, the antiviral effect for each drug was clearly dose-dependent (panel B in Figs. 3–5).

As complementary alternative approaches to luciferase reporter assays, we also performed TCID50 and Western blot. DBT cells were treated with the 3 drugs at indicated concentrations for 1 h and then infected with MHV-2aFLS at MOI of 1. At 12 h p.i., viral titer in the medium was determined by TCID50 and viral N protein in the cells was assessed by Western blot. As expected from the luciferase reporter screen, all 3 drugs had a robust inhibitory effect on virus titer (a reduction of >8 log10 for hexachlorophene and homoharringtonine (Figs. 2B, 3C and 5C) and >6 log10 for nitazoxanide (Fig. 4C). Consistent with the inhibition of virus production, viral N protein expression was undetectable following treatment with hexachlorophene at 2.5 μM, nitazoxanide at 5 μM and homoharringtonine at 31 nM (panel D in Figs. 3–5). A dose-dependent inhibition of viral N protein and EGFP reporter gene expression was also evident (panel D in Figs. 3–5 and Fig. 5E). Although there were slight variations in viral inhibition measured by the 3 methods (compare data in Supplemental Table 1 with those in Figs. 3–5), the overall inhibitory effect of the selected drugs on MHV infection can be firmly established.

3.3. Identification of candidate drugs that exert anti-coronavirus effect at different stages of the virus life cycle

To gain insight into steps in the virus life cycle targeted by candidate drugs, we sought to define whether candidate drugs were inhibitory when administered before or after infection of host cells. Since the original screen involved simultaneously treating and infecting target cells, we evaluated post-entry effects by treating cells with the 70 candidate drugs identified from the previous screenings at 3 h p.i. for 5 h, and determined luciferase activity at 8 h p.i. Results showed that 19 of the drugs significantly reduced luciferase activity (SSMD < −2), 9 of which (homoharringtonine, duloxetine, chloroxine, hexachlorophene, ebselen, nitazoxanide, mitoxantrone, disulfiram, and 6-azauridine) had an SSMD score of less than −9 (Supplemental Table 4). It is important to note that even the well-known anti-RNA virus inhibitor ribavirin in the library had only a relatively weak inhibitory effect on MHV replication with an SSMD score of −1.7 and a reduction of virus titer of ≈1 log10 (see Supplemental Table 1 and Fig. 2), which suggests that several of the drugs identified here may be more potent than ribavirin. To further corroborate these findings, in a second set of experiments, cells were treated with 11 selected drugs at 1 h prior to, or 3 h after, virus infection. It was found that all 11 drugs strongly inhibited luciferase activity at either time point (Fig. 6A). In agreement with results obtained using the luciferase reporter virus, EGFP expression from MHV-A59GFP also was drastically inhibited when the drugs were added at 3 h p.i. (Fig. 6B; further data not shown). These data indicate that these drugs inhibited virus infection at post entry stages (most likely at the step of viral replication), because most, if not all, infectious viral particles have entered into cells during the first 3 h of infection, with MHV biosynthesis commencing by 1 h p.i. in DBT cells (Zhu et al., 2009). In contrast, 51 other drugs did not inhibit luciferase activity (SSMD > −2) when the drugs were added 3 h p.i. (Supplemental Table 5). This indicates that these drugs most likely blocked viral entry only, because their antiviral activity was established during primary and secondary screenings when drug treatment and virus infection were carried out at the same time. To further support this conclusion, cells were treated with selected candidate drugs at either 1 h before, or 3 h after, virus infection and luciferase activity was determined at 8 h p.i. As expected, all 7 candidate drugs inhibited luciferase activity by more than 50% when the

Fig. 6. Identification of candidate drugs that inhibit MHV infection during viral replication. (A) DBT cells were either treated with drugs (10 μM) as indicated or DMSO (1%) at 1 h before or 3 h after infection with MHV-2aFLS at MOI of 1. The cells were lysed at 8 h p.i. and luciferase activity was measured and expressed as a percentage of DMSO control. Data represent the mean of triplicate experiments and standard deviation from the mean. (B) 17Cl-1 cells were either treated with drugs (10 μM) as indicated or DMSO (1%) At 1 h before or 3 h after infection with MHV-A59GFP at MOI of 1. At 16 h p.i. EGFP expression was observed using a fluorescence microscope (Olympus IX-70), and images were captured using a digital camera (Zeiss). Representative images for 3 h post infection are shown.

Please cite this article in press as: Cao, J., et al. A screen of the NIH Clinical Collection small molecule library identifies potential anti-coronavirus drugs. Antiviral Res. (2014), http://dx.doi.org/10.1016/j.antiviral.2014.11.010
drugs were added 1 h before infection but had no inhibitory effect when added at 3 h p.i. (Fig. 7A). Interestingly, some of the drugs (clomid, oxaprozin, and azathioprine) instead enhanced luciferase activity when added at 3 h p.i. The reason for this enhancement is not currently clear. Consistent with the results from luciferase assay, viral gene expression as measured by EGFP reporter expression was strongly inhibited only when the drugs were added 1 h prior to infection (Fig. 7B).

3.4. Potential broad-spectrum anti-coronavirus activity of candidate drugs

To extend our findings from MHV strain A59, we utilized several different MHV strains, which possess various pathogenic phenotypes in cell culture and animals. For example, JHM strain causes more extensive cell fusion in DBT cell and more severe encephalitis and demyelination in mice than does A59 strain, while MHV-2 does not induce cell fusion or cause encephalitis/demyelination in mice (Das Sarma et al., 2000; Hirano et al., 1974; Phillips et al., 1999). Thus, DBT cells were treated with homoharringtonine at 60 nM for 1 h, and then infected with MHV-1, MHV-2, and MHV-JHM at MOI of 1 for 12 h. Viral N protein was then detected by Western blot. As shown in Fig. 8A, the N protein for all 3 MHV strains was undetectable in the presence of the drugs, while in control untreated samples, expression of the N protein was robust. These data suggest that homoharringtonine is capable of inhibiting infection by various MHV strains. Furthermore, treatment of human HRT-18 cells with homoharringtonine or hexachlorophene prior to infection with bovine coronavirus (BCoV-L9) or human enteric coronavirus (HECoV-4408) also resulted in potent inhibition of viral N protein expression as judged by immunofluorescence analysis (Fig. 8B and C). Thus, by extrapolating from these results, we postulate that a great number of candidate anti-coronavirus drugs identified through our screen of the NCC library likely have broad antiviral activity against both human and animal coronaviruses.

4. Discussion

In this study we have identified a substantial number of candidate drugs that exhibited anti-coronavirus activity. It is worth noting that some of the same candidate drugs identified in this study have been previously shown to inhibit infections by other viruses. For example, nitazoxanide was initially discovered to have antiparasitic activity (White, 2003), but it also inhibits infection by Influenza A virus (Rossignol et al., 2009), hepatitis B virus (HBV) (Korba et al., 2008), hepatitis C virus (Keeffe and Rossignol, 2009), Japanese encephalitis virus (Shi et al., 2014), and Norovirus

Fig. 7. Identification of candidate drugs that inhibit MHV infection during cell entry. (A) DBT cells were either treated with drugs (10 μM) as indicated or DMSO (1%) at 1 h before or 3 h after infection with MHV-2aFLS at MOI of 1. The cells were lysed at 8 h p.i. and luciferase activity was measured and expressed as a percentage of DMSO control. Data represent the mean of triplicate experiments and standard deviation from the mean. (B) 17Cl-1 cells were either treated with drugs (10 μM) as indicated or DMSO (1%) at 1 h before or 3 h after infection with MHV-A59GFP at MOI of 1. At 16 h p.i. EGFP expression was observed using a fluorescence microscope (Olympus IX-70), and images were captured using a digital camera (Zeiss).
A screen of the NIH Clinical Collection small molecule library identifies potential anti-coronavirus drugs.

In summary, identification of candidate anti-coronavirus drugs from the NCC library in the current study will advance the discovery and development process, thereby allowing us to focus on a collection of drugs that have undergone multiple selections (screens) from numerous chemical libraries. However, the high hit rate is not particularly surprising, considering that the NCC library is a collection of drugs that have exhibited potent biologic activities against various diseases and that have advanced from pre-clinical to clinical trials. Thus, the NCC library consists primarily of screen “winners”. Another possible explanation for the high hit rate is that clusters of the drugs that are selected may target the same cellular pathways.

The overall hit rate for the library screen is approximately 10%. This rate is indeed very high compared to previous screens of raw chemical libraries. However, the high hit rate is not particularly surprising, considering that the NCC library is a collection of drugs that have undergone multiple selections (screens) from numerous different libraries. Collected in the library are only those that have exhibited potent biologic activities against various diseases and that have advanced from pre-clinical to clinical trials. Thus, the NCC library consists primarily of screen “winners”. Another possible explanation for the high hit rate is that clusters of the drugs that are selected may target the same cellular pathways.

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Fig. 8. Candidate drugs are capable of inhibiting infection with diverse coronaviruses. (A) DBT cells were infected with various MHV strains (MHV-1, MHV-2, and MHV-JHM) in the presence of the indicated drugs for 12 h, and the viral N protein was detected by Western blot using mAb J.3.3. Beta-actin serves as loading control. (B and C) HRT-18 cells were infected with either BCoV-L9 (B) or HECoV-4408 (C) in the presence of the drugs for 36 h, and the viral N protein was detected by IFA using specific mAb#46. The concentration of homoharringtonine is 1 μM and hexachlorophene is 5 μM. The control is 1% DMSO.
few potent inhibitors to rapidly prioritize for preclinical and clinical trials. This is particularly urgent as the newly emergent MERSCoV continues to spread from the Middle East to the rest of the world.

5. Conclusion

Of the 727 small molecules in the NCC drug library screened, 84 were found to have a significant antiviral coronavirus effect, of which 51 blocked virus entry while 19 others inhibited viral replication. Several candidate drugs exhibited robust antiviral activity against human and diverse animal coronaviruses at micromolar or nanomolar concentrations without any cytotoxicity.

Acknowledgments

We thank Drs. P. Rottier and C. de Haan (Utrecht University, The Netherlands) for kindly providing the recombinant MHV-2aFLS. We also thank James A. Stahl (UAMS) for his technical assistance. This work was supported by the Department of Microbiology and Immunology, UAMS to XZ, start-up funds from the UAMS College of Medicine and Arkansas Biosciences Institute to Jiren Charity Foundation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.11.010.

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