Peer Review File

Manuscript Title: Discovery of SARS-CoV-2 Antivirals through Large-scale Drug Repositioning

Editorial Notes:

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee #1:

In this manuscript, Riva et al describe an ambitious repositioning screen of a 12,000 drug/"in-human" library, seeking approved or investigational new drugs (INDs) that can be rapidly transitioned to use against the virus. To call this timely is an understatement, and there were encouraging findings of drugs that might be suited for translation into antiviral trials, so the manuscript likely will have substantial impact. I found the retinoid-like drugs, and the cationic drugs, particularly interesting. I do broadly support publication of this manuscript.

I have four suggestions for strengthening the work:

A. A weakness of the study is that the chemoinformatics was shallow, and so too the classification of drugs-by-targets. Certainly the authors capture the primary, MoA targets of the drugs they find, but they do not look beyond that, and thus perhaps miss some underlying themes not present in the assigned targets. For instance, while elopiperazole, SB-616234-A, MLN-3897, astemizole, Hanfangchin A, and N-tert-Butylisoquine have diverse and apparently unrelated primary targets, many or all of them may hit one or both of the Sigma receptors. Sigma receptors are dark horses of pharmacology, admittedly, but they have been mooted as mechanistically sensible in a recent paper in this journal (Gordon, 2020, which the authors cite), and the chaperone role of at least Sigma1 might make sense for viral recruitment. More generally, it would be great if the authors could look for shared themes among the targets, including the off-targets. This could be done in an afternoon, so it shouldn't be much of a burden.

B. A related comment is that the authors spent little time on biological mechanism. Why should retinoic acid receptor agonists be antiviral? How about the various GPCRs (or perhaps Sigma receptors)? Apart from speculation, the authors could test their targets, and their hypotheses, by either chemical or genetic epistasis. Chemically, the could challenge their agonists with antagonists, and their antagonists with agonists. Both sub-type selective and pan-active RAR antagonists are readily available, as are agonists for the dopamine and serotonin receptors (for elopiperazole), for CCR1...etc... The genetic epistasis would be even more powerful, but I don't insist on that, could be substantial work. But the chemical competition should be straight forward. I actually suspect that that agonists/antagonists for the putative MoA targets will not work for the GPCRs, suggesting a secondary, off-target. For the RAR receptors, this may work well. Either way, the result will be illuminating.

C. For many of the targets, one would expect multiple drugs to light up. For instance, astemizole hits the H1 receptor (I bet elopiperazole does too, btw). Were other H1 ligands active? If not, does this point to a secondary target? How about other 5HT1B antagonists?

D. The authors must publish all of their dose response curves and clearly identify all of their confirmed actives. If they have done this, it was not clear to me, certainly they all don't exist even in the supplements, certainly not all in one place. There is no reason not to do this, and every reason to do, especially for a paper like this whose major virtue is the potential discovery and communication of the drugs the planet desperately needs. What they are, and what their antiviral effects are, should be totally

and fully revealed. I have the impression that this is not really the case currently.

Notwithstanding these critiques, I do support publication of this work. I think three of these critiques can be addressed in a day, and even the harder one, on testing mechanism, shouldn't take more than a week.

Referee #2:

Discovery of SARS-CoV-2 Antivirals through Large-scale Drug Repositioning

The paper by Riva et al. describes a high throughput screening approach to identify existing drugs that could be repurposed as potential treatments of SARS-CoV-2/COVID-19. In discussing the results, the authors emphasize the characteristics and quality of the screen, which is important, but at the cost of insufficiently describing the promising hits from the screen. The dynamic range of the screens was quite small and the Z' seemed to vary and even dip into non-optimal ranges (ReFrame duplicate Z' 0.19). As written, the paper is a bit disjointed and lacks a clear narrative tying results of the screen to those compounds that were validated. This paper could be improved by clearly stating in the figures and in the text, what were the most promising molecules in the screens and how they ended up in Figure 3 in the dose response analysis. The pathway analysis in Figure 2 seems out of place and as written is not integrated into the other figures to be readily apparent how this analysis helped triage hits or give confidence in those described in Figure 3. In addition, it is unclear if the authors reanalyzed a publicly available data set gene expression data or generate the primary data themselves. If this was not generated by the authors, this should be crystal clear in the results and figure legend. The supposition that the reported EC50s herein would translate to doses achievable in people is premature without additional work (i.e. performing antiviral assays in primary human lung cells and PK/PD in an animal model). This is really exciting data and was done well given the oddities associated with SARS-CoV-2 in vitro systems. Studies like this should provide a rationale for more mechanistic preclinical development studies both in vitro and in vivo.

This paper could be improved by:

1) Creating a clearer narrative describing the journey of compounds from the screen to validation. This would help readers better understand the rationale for the progression of compounds from primary screen to validation.
2) The screening figures could be expanded and/or better explained to show the compounds that clear some threshold to become hits to follow up.
3) The authors mention potential issues with Vero cells as a vessel to understand antiviral activity for nucleoside analogs. But the fact that these are non-human primate cancer cells could also be affecting the activity seen by the compounds in this paper especially if they are targeting a host process. So, trying to make an argument that Vero EC50s fall within the range of human plasma drug levels seems premature. Validation (e.g. virus titer reduction) in another human cell line would be great and/or human primary lung cells would certainly strengthen your argument here.
4) The paper text could be refined. The discussion is very long and unfocused.

Specific Comments:

Line 190. Cells and Viruses. What is the passage history of both isolates used in these studies? Where and when were Huh-7 cells utilized? Where and when were Vero-hACE2-TMPRSS2 cells utilized?

Line 325. It is unclear where the 28 compounds chosen for follow up reside in the data plots. It would be helpful to label each panel rather than refer to "Figure 1B, top right middle."

Line 328-331 "which have all been shown to efficiently block either SARS-CoV-1 or 2 infection in vitro or

in vivo (46- 50)…". Which of the references 46-50 demonstrate efficient blocking of SARS-CoV-1 or 2 in vivo?

Figure 1: The data presentation and description of the results is confusing and should be reordered. Panel A shows the REFRAME library composition and ultimate assay flow but REFRAME Data is not discussed until Panel C. If 1B is LOPAC and 1C is REFRAME, 1A as displayed is confusing leading the reader to think that the entire figure is REFRAME.

Figure 1 Panel C: The assay performance for the HTS with REFRAME is discussed but the hits are not. How many hits were generated and what were they?

Figure 2A: This figure seems a bit out of place. The plots in panel A are not sufficiently described to understand what they are meant to show.

Figure 2B: Did the authors analyze publicly available data for the single-cell RNAseq or was new data generated using methods in Reference 41? As written this is hard to discern. If this was generated from primary data from other investigators, this needs to be much more clear in the description of the results and in the figure legend.

Line 395: Mention that the validation was performed with an independent isolate. The use of the Hong Kong isolate is not explicitly stated in the results for the primary screen. Also there is not a large amount of sequence variation among circulating strains. These are essentially the same viruses based on sequence? So, the rationale for this change is not all that clear.

Line 384-408: Orthogonal validation of selected anti-SARS-CoV-2 compounds This section describes the methods of the validation of hits from the primary screens. There is not much description of the data from this work and refer back to previously discussed figures S1 and 2A. There some mention of the results "Thirty-three percent of compounds (107 compounds) were found to reduce viral replication by at least 40 % at these concentrations (data not shown) when averaging data from at least two replicates" but the data is not shown. So, what is the purpose of this section? Did any of these hits in the validation screen by immunostaining reduce replication greater than 40%?

Line 410. Figure 3 and the Dose response analysis. This is great data but there is insufficient text describing the results. Apilomod has apparent toxicity issues but the others in the dose response section (Panel B) appear not to have this issue. This should be stressed but is not. The panel C is not discussed in the results and is quite small to see what is happening there.

Line 422. "…while 7 compounds harbored EC50s < 500 nM (6 of which reached > 80% inhibiton of the infection at the highest concentration (Figure 3B-C)), suggesting that effective antiviral potency could likely be achieved during therapeutic dosing of a COVID-19 patient." This statement has issues. Without information on human PK, penetration in the respiratory tract, the levels of non-protein bound compound vs. total compound in a person, etc. making claims based on EC50 here in Vero cells seems like a bit of an overstatement.

Line 472. The incongruency between replicate 1 and replicate 2 in the REFRAME is strange. What could be guiding this? Without a clear and upfront description of the unsatisfactory Z' for replicate 2, the decision to move forward with replicate 1 data primarily comes off as arbitrary and capricious. This should be more clearly discussed.

Referee #3:

This manuscript by Riva and co-workers describes the results of an extensive screen of 12,000 clinical-stage or FDA-approved small molecules for the ability to inhibit either the entry or replication of SARS-CoV-2 in VeroE6 cells. The experimental approach was to pretreated cells for 16 hours with the compounds, then infected the cells with SARS-CoV-2 at a low MOI of 0.01, and evaluate for cytopathic

effect at 72 hours post-infection. The results are clearly presented and documented, resulting in the identification of six compounds that demonstrate dose-dependent inhibition of CPE, as shown in Figure 3B. The authors describe that the second assay (evaluating detecting viral proteins by IF in virus-infected cells pre-treated with the compounds) was designed to independently interrogate the antiviral activity of the compounds. Again, the compounds were added prior to virus infection, so it is unclear if the compounds are inhibiting entry or a later step in virus replication (Figure 3C). Determining if any of these candidates block SARS-CoV-2 at a post-entry step, and if they inhibit replication in human airway epithelial cells would enhance the impact of this study. The ability to complete this screen of 12,000 compounds is impressive and the manuscript is well-written and referenced appropriately.
Comments for the authors' consideration:
1. Determining if any of these compounds act at a post-entry step is important. The authors could infect the cells with a high MOI (0.1 or 1.0), and then add the potential inhibitor at 1 or 2 hours post-infection. Any inhibition of virus replication could be evaluated by RT-qPCR or by CPE at 24 hours post-infection. Distinguishing inhibitors of entry versus other steps in virus replication would elevate this study.
2. The 6 final compounds should be evaluated for their ability to inhibit SARS-CoV-2 replication in either human airway epithelial cells or in another relevant cell type, not just Vero E6 cells. The compounds should be added at 1 or 2 hrs post-infection to determine if a post-entry step is affected. Remdesivir could be used as a control in these experiments.

Author Rebuttals to Initial Comments:

We thank the reviewers for taking the time to evaluate the manuscript and provide critical feedback. Based on their suggestions, we have conducted an extensive set of experiments which have significantly strengthened the study, including synergy and MOA studies, as well as validation in multiple orthogonal viral replication systems, including primary human pneumocytes and lung explant tissue. We hope these additional data address the concerns raised by the reviewers to enable publication of this manuscript.

Referees' comments:

Referee #1 (Remarks to the Author):

In this manuscript, Riva et al describe an ambitious repositioning screen of a 12,000 drug/"in-human" library, seeking approved or investigational new drugs (INDs) that can be rapidly transitioned to use against the virus. To call this timely is an understatement, and there were encouraging findings of drugs that might be suited for translation into antiviral trials, so the manuscript likely will have substantial impact. I found the retinoid-like drugs, and the cationic drugs, particularly interesting. I do broadly support publication of this manuscript.

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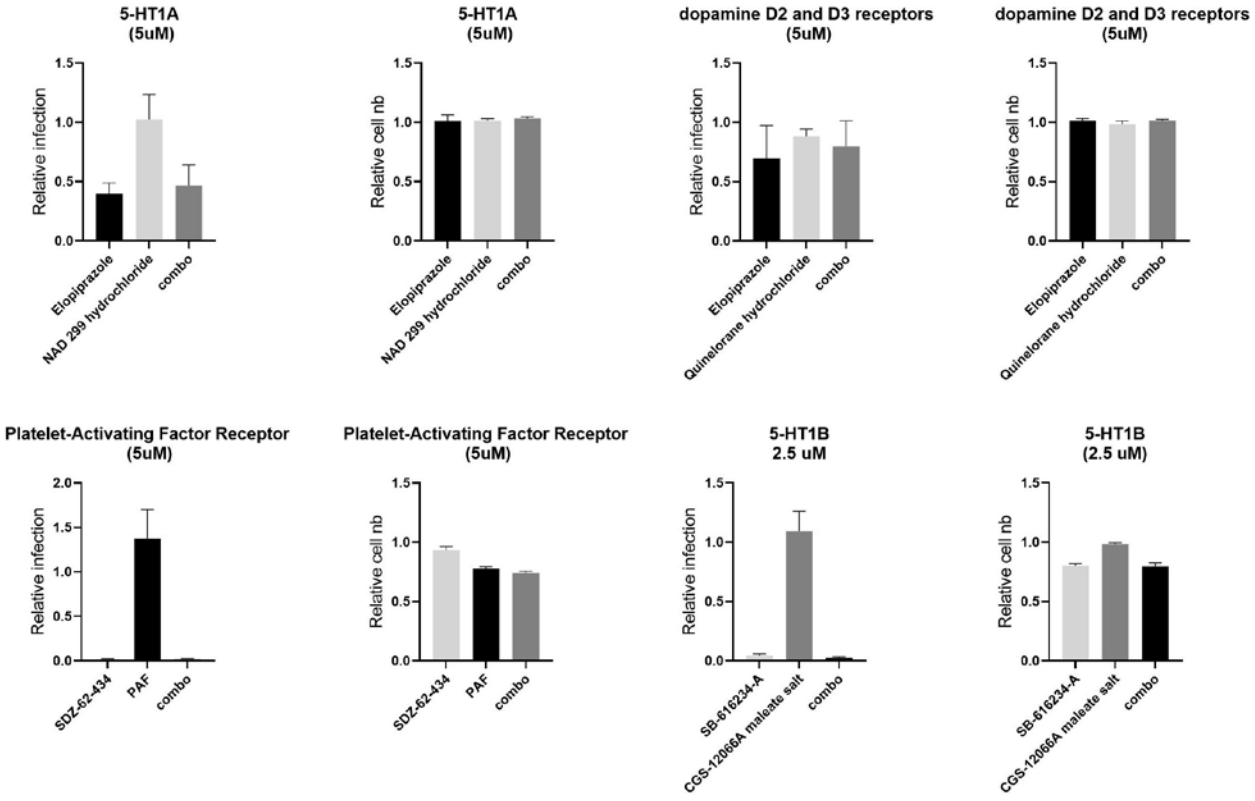
Sigma receptors are dark horses of pharmacology, admittedly, but they have been mooted as mechanistically sensible in a recent paper in this journal (Gordon, 2020, which the authors cite), and the chaperone role of at least Sigma1 might make sense for viral recruitment. More generally, it would be great if the authors could look for shared themes among the targets, including the off-targets. This could be done in an afternoon, so it shouldn't be much of a burden.

Author Response: No evident common chemical structures were identified among the validated hits based on in silico SAR analysis. For this reason, an in-depth chemoinformatic analysis is not included in the manuscript. This is perhaps not surprising, as this collection contains mainly clinical stage compounds that have unique composition of matter to enable IP protection, so structural similarities between molecules are expected to be limited.
Since there are not available comprehensive repositories of compound off-target activities (to our knowledge), we searched databases the literature for reported off-targets for elopiperazole, SB-616234-A, MLN-3897, astemizole, Hanfangchin A, and N-tert-Butylisoquine. Based on this analysis, we were not able to find over-representation of any off-target activities that could be shared between these molecules (although serotonin receptors were shared by a few). 60 compounds in the REFRAME collection have been annotated as targeting the sigma1 receptor, and no significant enrichment for these molecules was observed in the screening data, and none of the aforementioned molecules were reported to possess secondary activities that target sigma receptors. However, to provide the reader a visualization of target classes identified through this analysis, we have included a cell map reporting target clusters, subcellular localization, and potential mechanisms of viral inhibition (Figure S5).

Reviewer Query: *B. A related comment is that the authors spent little time on biological mechanism. Why should retinoic acid receptor agonists be antiviral? How about the various GPCRs (or perhaps Sigma receptors)? Apart from speculation, the authors could test their targets, and their hypotheses, by either chemical or genetic epistasis. Chemically, the could challenge their agonists with antagonists, and their antagonists with agonists. Both sub-type selective and pan-active RAR antagonists are readily available, as are agonists for the dopamine and serotonin receptors (for elopiperazole), for CCR1...etc... The genetic epistasis would be even more powerful, but I don't insist on that, could be substantial work. But the chemical competition should be straight forward. I actually suspect that that agonists/antagonists for the putative MoA targets will not work for the GPCRs, suggesting a secondary, off-target. For the RAR receptors, this may work well. Either way, the result will be illuminating.*

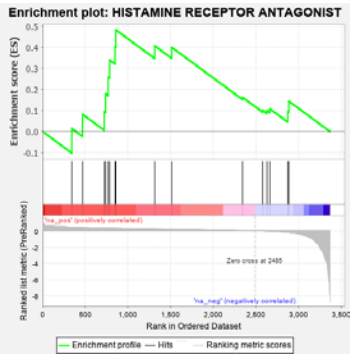
Author Response: We thank the reviewer for these comments and suggestions. As requested, we evaluated the biological mechanisms through determination of chemical epistasis. As predicted by this reviewer, challenging antagonists of dopamine and serotonin receptors with their agonists didn’t counteract their action. In addition, none of the antagonists enhanced the basal level of infection compared to the DMSO-control condition. This may suggest that the compounds are driving antiviral activity through off-target effects, that the selected agonists are not active in the assay, or the molecules are inhibiting replication in through the receptor in a ligand- independent manner. We have now noted this consideration in the discussion, and data are provided below:



Conversely, we find, as suggested by the reviewer, that the antiviral effects of retinoic acid receptor agonists could be reversed by an RAR antagonist. These results were included in the revision of the manuscript (Figure S3, results). Interestingly, when we performed gene expression analysis on Vero E6 cells challenged with the virus, we observed significant decrease (p= 0.0006) in genes associated with retinol metabolism (Table S2). Similar results were obtained by analyzing RNA-seq of nasopharyngeal swab specimens reported Butler et al. Taken together, these data suggest that SARS-CoV-2 may highjack retinoid metabolism and signaling, and reversing this host-pathogen interaction may have therapeutic benefits. We would like to thank again this referee for these suggestions, and we feel that the addition of these data to the manuscript strengthens the study.

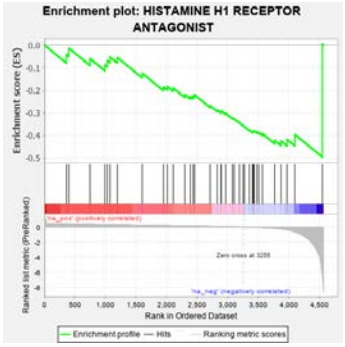
Reviewer Query: *C. For many of the targets, one would expect multiple drugs to light up. For instance, astemizole hits the H1 receptor (I bet elopiperazole does too, btw). Were other H1 ligands active? If not, does this point to a secondary target? How about other 5HT1B antagonists?*

Author Response: Molecules targeting histamine receptors were found to be slightly enriched, however the specific H1 antagonist class was not (please see graphs and tables below, including p-values and FDR q-values).



Upregulated in class	na_pos
GeneSet	HISTAMINE RECEPTOR ANTAGONIST
Enrichment Score (ES)	0.4826707
Normalized Enrichment Score (NES)	1.3899707
Nominal p-value	0.10081744
FDR q-value	0.33305207
FWER p-Value	0.972

Upregulated in class	na_neg
GeneSet	HISTAMINE H1 RECEPTOR ANTAGONIST
Enrichment Score (ES)	-0.49486402
Normalized Enrichment Score (NES)	-1.0899935
Nominal p-value	0.41426927
FDR q-value	0.87028843
FWER p-Value	1.0



In conjunction with data from the Gordon et al study that identified H1 antagonists cloperastine, clemastine, olanzapine, and haloperidol, this suggests that Astemizole may be functioning through inhibition of H1 receptors, or shared off-target activities, including dopamine D2 receptor, sigma non-opiod intracellular receptor, potassium channels, and serotonin receptor 1A (5HT1A). Similarly, 5HT1B antagonists were not found to be enriched in the screen, suggesting that off-target impact on related-GPCRs or other targets may be underlying the observed antiviral activities. This important caveat is now elaborated upon in the discussion.

Reviewer Query: *D. The authors must publish all of their dose response curves and clearly identify all of their confirmed actives. If they have done this, it was not clear to me, certainly they all don't exist even in the supplements, certainly not all in one place. There is no reason not to do this, and every reason to do, especially for a paper like this whose major virtue is the potential discovery and communication of the drugs the planet desperately needs. What they are, and what their antiviral effects are, should be totally and fully revealed. I have the impression that this is not really the case currently.*

Author Response: We have previously provided data for all 18 molecules that were confirmed to have a dose response relationship (Figure 3 and supplemental figure S7). Of note, since submission, we have confirmed an additional 3 molecules in dose response (Clofazimine, DS-6930 and R 82913) (Figure 3). Additionally, we now provide a list of 100 compounds that were confirmed in a secondary assay at a single dose (Table S3).

Notwithstanding these critiques, I do support publication of this work. I think three of these critiques can be addressed in a day, and even the harder one, on testing mechanism, shouldn't take more than a week.

Referee #2 (Remarks to the Author):

Discovery of SARS-CoV-2 Antivirals through Large-scale Drug Repositioning

The paper by Riva et al. describes a high throughput screening approach to identify existing drugs that could be repurposed as potential treatments of SARS-CoV-2/COVID-19. In discussing the results, the authors emphasize the characteristics and quality of the screen, which is important, but at the cost of insufficiently describing the promising hits from the screen. The dynamic range of the screens was quite small and the Z' seemed to vary and even dip into non-optimal ranges (ReFrame duplicate Z' 0.19). As written, the paper is a bit disjointed and lacks a clear narrative tying results of the screen to those compounds that were validated. This paper could be improved by clearly stating in the figures and in the text, what were the most promising molecules in the screens and how they ended up in Figure 3 in the dose response analysis. The pathway analysis in Figure 2 seems out of place and as written is not integrated into the other figures to be readily apparent how this

analysis helped triage hits or give confidence in those described in Figure 3. In addition, it is unclear if the authors reanalyzed a publicly available data set gene expression data or generate the primary data themselves. If this was not generated by the authors, this should be crystal clear in the results and figure legend. The supposition that the reported EC50s herein would translate to doses achievable in people is premature without additional work (i.e. performing antiviral assays in primary human lung cells and PK/PD in an animal model). This is really exciting data and was done well given the oddities associated with SARS-CoV-2 in vitro systems. Studies like this should provide a rationale for more mechanistic preclinical development studies both in vitro and in vivo.

This paper could be improved by:

Reviewer Query: *1) Creating a clearer narrative describing the journey of compounds from the screen to validation. This would help readers better understand the rationale for the progression of compounds from primary screen to validation.*

2) The screening figures could be expanded and/or better explained to show the compounds that clear some threshold to become hits to follow up.

Author Response: A supplemental figure (Figure S12) has been included in the manuscript, describing the complete workflow and indicating the selection criteria from primary screen to validation. The text in the results section was also modified to improve the clarity of the narrative.

Reviewer Query: *3) The authors mention potential issues with Vero cells as a vessel to understand antiviral activity for nucleoside analogs. But the fact that these are non-human primate cancer cells could also be affecting the activity seen by the compounds in this paper especially if they are targeting a host process. So, trying to make an argument that Vero EC50s fall within the range of human plasma drug levels seems premature. Validation (e.g. virus titer reduction) in another human cell line would be great and/or human primary lung cells would certainly strengthen your argument here.*

Author Response: We thank the reviewer for these comments. We have undertaken three strategies to address this concern. First, compounds were reprofiled in orthogonal *human* cell lines that support robust replication of SARS-CoV-2 (HEK293-ACE2 and Huh-7-ACE2). Nearly all tested compounds (18/21) confirmed in one of both cell types, indicating that most of these molecules are not acting in a cell-type specific manner (Figure 5A). In addition, dose response analysis of these molecules revealed EC50s consistent with those observed in Vero cells, further underscoring the observed antiviral potencies of these compounds can be recapitulated across multiple cell types (supplemental figures S10 and S11). Additionally, three compounds, ONO 5334, MDL 28170, and Apilimod, were evaluated for antiviral efficacy in iPS-derived pneumocytes, considered to be a primary cell type supporting SARS-CoV-2 replication *in vivo*, and observed significant reductions in viral infection after treatment with compound (Figure 5B-D). Finally, the impact of Apilimod on viral replication in *ex vivo* primary lung cultures was assessed, and found to significantly inhibit viral titers in this assay system (Figure 5E-F). Taken together, these data indicate that most of the known drugs identified through this approach harbor antiviral activity across multiple cell types, with several drugs tested and confirmed in disease relevant cells and tissues. These data support the need for further rapid preclinical and clinical evaluation of these molecules for the treatment of COVID-19.

Reviewer Query: *4) The paper text could be refined. The discussion is very long and unfocused.*

Author Response: The discussion has been streamlined and focused on major topics of consideration in the study.

Specific Comments:

Reviewer Query: *Line 190. Cells and Viruses. What is the passage history of both isolates*

used in these studies? Where and when were Huh-7 cells utilized? Where and when were Vero- hACE2-TMPRSS2 cells utilized?

Author Response: SARS-CoV-2 HKU-001a was isolated from a patient in Hong Kong and amplified in Vero E6 cells, and the virus was passaged three times before use in the screen. The SARS-CoV-2 USA-WA1/2020 strain was passaged once after obtaining samples from BEI. Vero-hACE2-TMPRSS2 was used to amplify the SARS-CoV-2 USA-WA1/2020, but was not used in the screen or validation studies. Appropriate sections in the materials and methods have been updated to underscore these points. Huh-7 were inadvertently referred to in the original version of the manuscript, as they were not utilized for any reported studies. However, we now report data using Huh-7 cells and have updated the methods section to reflect their use.

Reviewer Query: *Line 325. It is unclear where the 28 compounds chosen for follow up reside in the data plots. It would be helpful to label each panel rather than refer to "Figure 1B, top right middle."*

Author Response: Per the reviewer's suggestion, we have highlighted in red 'hit' compounds selected for follow-up in this figure, and have also re-labelled the figure.

Reviewer Query*: Line 328-331 "which have all been shown to efficiently block either SARS- CoV-1 or 2 infection in vitro or in vivo (46- 50)…". Which of the references 46-50 demonstrate efficient blocking of SARS-CoV-1 or 2 in vivo?*

Author Response: We regret this error, none of these compounds have been previously demonstrated to inhibit replication in vivo. We have amended the text to reflect this.

Reviewer Query*: Figure 1: The data presentation and description of the results is confusing and should be reordered. Panel A shows the REFRAME library composition and ultimate assay flow but REFRAME Data is not discussed until Panel C. If 1B is LOPAC and 1C is REFRAME, 1A as displayed is confusing leading the reader to think that the entire figure is REFRAME.*

Author Response: We appreciate the suggestion, and the order of these figures have been changed.

Reviewer Query*: Figure 1 Panel C: The assay performance for the HTS with REFRAME is discussed but the hits are not. How many hits were generated and what were they?*

Author Response: Confirmed hits are now presented in Table S3.

Reviewer Query: *Figure 2A: This figure seems a bit out of place. The plots in panel A are not sufficiently described to understand what they are meant to show.*

Author Response: We have provided clarification in both the text and the figure legend to

describe this figure. Briefly, these data depict the enrichment of drug targets found in the screen. The underlying hypothesis for this analysis is that the identification of multiple drugs with similar mechanisms of action/ cellular targets are more likely to be relevant for the control of viral replication.

Reviewer Query: *Figure 2B: Did the authors analyze publicly available data for the single-cell RNAseq or was new data generated using methods in Reference 41? As written this is hard to discern. If this was generated from primary data from other investigators, this needs to be much more clear in the description of the results and in the figure legend.*

Author Response: Publicly available single-cell RNA-seq dataset profiling samples from four macro-anatomical locations of human airway epithelium in healthy living (Deprez, M. *et al. (2019)*). Material and methods, results and the legend for Figure 2 have been modified to more clearly convey this point.

Reviewer Query: *Line 395: Mention that the validation was performed with an independent isolate. The use of the Hong Kong isolate is not explicitly stated in the results for the primary screen. Also there is not a large amount of sequence variation among circulating strains. These are essentially the same viruses based on sequence? So, the rationale for this change is not all that clear.*

Author Response: The high-throughput screen was conducted at the University of Hong Kong, where Dr. Yuen's lab had isolated the SARS-CoV-2 HKU-001a isolate and utilized it for the primary screen. Validation studies were done in the Chanda lab in La Jolla, CA, USA, and the HKU-001a could not be readily transferred from China to the USA due to regulatory issues. The Chanda lab obtained the CoV-2 USA-WA1/2020 isolate from BEI, and thus all subsequent validation and characterization studies were done with this virus. As pointed by the reviewer, the % of nucleotide similarity between the two strains is 99.95%. We have highlighted the use of two different isolates for clarity and transparency, but agree with the reviewer that these are essentially the same virus.

Reviewer Query: *Line 384-408: Orthogonal validation of selected anti-SARS-CoV-2 compounds This section describes the methods of the validation of hits from the primary screens. There is not much description of the data from this work and refer back to previously discussed figures S1 and 2A. There some mention of the results "Thirty-three percent of compounds (107 compounds) were found to reduce viral replication by at least 40 % at these concentrations (data not shown) when averaging data from at least two replicates" but the data is not shown. So, what is the purpose of this section? Did any of these hits in the validation screen by immunostaining reduce replication greater than 40%?*

Author Response: Of the 326 molecules selected for secondary confirmation studies, approximately 30% of compounds were confirmed at a single dose (i.e. reduction of viral replication by at least 40%). Of note, reanalysis of the confirmed hits disqualified 7 compounds, and the tally of the confirmed drugs stands at 100. These compounds and associated data are now provided in Table S3.

Reviewer Query: *Line 410. Figure 3 and the Dose response analysis. This is great data but there is insufficient text describing the results. Apilomod has apparent toxicity issues but the others in the dose*

response section (Panel B) appear not to have this issue. This should be stressed but is not. The panel C is not discussed in the results and is quite small to see what is happening there.

Author Response: Apilimod does induce cytotoxic/cytostatic effect at concentrations >100 nM. Near maximal inhibition of viral replication is observed at concentrations of 100 nM, thus we conclude that the antiviral efficacy is unrelated to the cytostatic effects of the compound. Consistently, the selectivity index for the compound is >100, and beyond a range wherein toxicity is expected to impact the biological readout. However, we have now noted this in the manuscript discussion. Importantly, systemically dosed Apilimod is well tolerated in patients (Sands et al., Inflamm Bowel Dis. 2010), and thus the observed cellular toxicity is likely not a concern in a clinical setting. Additionally, we have moved Panel C into the supplemental section to enable us to expand the images for easier visualization.

Reviewer Query: *Line 422. "…while 7 compounds harbored EC50s < 500 nM (6 of which reached > 80% inhibiton of the infection at the highest concentration (Figure 3B-C)), suggesting that effective antiviral potency could likely be achieved during therapeutic dosing of a COVID-19 patient." This statement has issues. Without information on human PK, penetration in the respiratory tract, the levels of non-protein bound compound vs. total compound in a person, etc. making claims based on EC50 here in Vero cells seems like a bit of an overstatement.*

Author Response: We agree with the reviewer's assertion and have modified the text to acknowledge these important stipulations. Of note, since the lung is a highly vascularized organ, and cell-based assays are done in the presence of serum, we believe that Cmax of the compound along with cellular EC50s is a reasonable guide to prioritizing candidate antivirals for further *in vivo* evaluation in preclinical and clinical settings. Towards this end, in Table 1, we provide human Cmax values from clinical trial data (if publicly available) which provides the reader, along with the aforementioned caveats, some guidance as to the potential for translating and/or prioritizing these data towards potential treatment options.

Reviewer Query: *Line 472. The incongruency between replicate 1 and replicate 2 in the REFRAME is strange. What could be guiding this? Without a clear and upfront description of the unsatisfactory Z' for replicate 2, the decision to move forward with replicate 1 data primarily comes off as arbitrary and capricious. This should be more clearly discussed.*

Author Response: We are uncertain of the reason for the degradation of the assay window between the first and second replicate screen, however, we suspect that it may be related to cell passage number. We have expanded the explanation in the text. In summary, data from replicate 1 was weighted more heavily due to the more robust Z' of this assay (0.51), with 100 compounds selected exclusively from this dataset. Additionally, 75 compounds were selected based on average scores between replicates 1 & 2, 75 compounds were selected that were only found to be highly active in set 2, while the remaining compounds were selected based on inclusion in one of the enriched GSEA categories. The text has been clarified to reflect this.

Referee #3 (Remarks to the Author):

This manuscript by Riva and co-workers describes the results of an extensive screen of 12,000 clinical-stage or FDA-approved small molecules for the ability to inhibit either the entry or replication of SARS-CoV-2 in VeroE6 cells. The experimental approach was to pretreated cells

for 16 hours with the compounds, then infected the cells with SARS-CoV-2 at a low MOI of 0.01, and evaluate for cytopathic effect at 72 hours post-infection. The results are clearly presented and documented, resulting in the identification of six compounds that demonstrate dose- dependent inhibition of CPE, as shown in Figure 3B. The authors describe that the second assay (evaluating detecting viral proteins by IF in virus-infected cells pre-treated with the compounds) was designed to independently interrogate the antiviral activity of the compounds. Again, the compounds were added prior to virus infection, so it is unclear if the compounds are inhibiting entry or a later step in virus replication

(Figure 3C). Determining if any of these candidates block SARS-CoV-2 at a post-entry step, and if they inhibit replication in human airway epithelial cells would enhance the impact of this study. The ability to complete this screen of 12,000 compounds is impressive and the manuscript is well-written and referenced appropriately.

Comments for the authors' consideration:

***Reviewer Query:** 1. Determining if any of these compounds act at a post-entry step is important. The authors could infect the cells with a high MOI (0.1 or 1.0), and then add the potential inhibitor at 1 or 2 hours post-infection. Any inhibition of virus replication could be evaluated by RT-qPCR or by CPE at 24 hours post-infection. Distinguishing inhibitors of entry versus other steps in virus replication would elevate this study.*

Author Response: We have conducted both time of addition and VLP assay studies to determine if the 5 most potent compounds function at an entry or post-entry step. Since most of these compounds are protease inhibitors, we evaluated if they could direct inhibit the function of viral proteases, and determined they were inactive in these assays (supplemental figure 8). Additionally, time of addition and VLP studies reveal that these molecules impact viral entry, consistent with known mechanisms of these molecules in targeting endosomal protease / endosomal function (Figure 4).

***Reviewer Query:** 2. The 6 final compounds should be evaluated for their ability to inhibit SARS- CoV-2 replication in either human airway epithelial cells or in another relevant cell type, not just Vero E6 cells. The compounds should be added at 1 or 2 hrs post-infection to determine if a post-entry step is affected. Remdesivir could be used as a control in these experiments.*

Author Response: To rule out cell-type specific effects, as suggested by the reviewer, the top 21 compounds were reprofiled in orthogonal *human* cell lines that support robust replication of SARS-CoV-2 (HEK293-ACE2 and Huh-7-ACE2). Nearly all compounds (18/21) confirmed in one of both cell types at efficacies comparable to those observed in Vero cells (Figure 5A and supplemental figures S10 and S11). We were able to profile three of the most potent compounds, ONO-5334, MDL 28170, and Apilimod, for antiviral efficacy in iPS-derived pneumocytes, and observed significant inhibition of viral replication with no cytotoxicity (Figure 5B-D and supplemental figure 9B-D). Additionally, we utilized an *ex vivo* primary lung cultures to

assess the antiviral activity of Apilimod. The PIKfyve inhibitor was found to strongly inhibit viral replication in this assay system (Figure 5E-F). Time of addition studies are addressed above. These data strongly suggest that a majority of the antivirals elucidated in this study can inhibit SARS-CoV-2 across multiple cell types, and the most potent molecules were found to harbor antiviral activity in disease relevant cells and tissues. Taken together, these data indicate that these compounds represent promising potential therapies for the treatment of COVID-19.

Reviewer Reports on the First Revision:

Referee #1:

The authors have answered most of my critiques and I do not want to further delay this manuscript. The one suggestion I'd make is to further strengthen their observation that the drugs targeting the GPCRs were not reversed by the chemical epistasis experiment (e.g., reversal by agonists). They show these data in the response letter but not in the manuscript, and while they say they go through this in the Discussion I did not see it. I'm not saying it isn't there, I'm sure I just missed it, but perhaps it isn't obvious since I was looking for it. Thus, I suggest they make it more prominent. It won't weaken the paper, far from it, it might make them look prophetic by suggesting that an off-target is involved.

In the interest of transparency, this is a signed review:

Brian Shoichet

Referee #2:

Nice JOB!

Overall, the revised paper is improved. There is a lot supplemental data. So much so that the results are often not adequately explained in the results section. In some cases, figures are not explained at all (e.g. Fig. S9). The “Discussion” often reads as a “Results” section with frequent call backs to figures and tables. At times, the discussion is the results section that I’d hoped to read. The authors did a great job incorporating the antiviral assays and IC50 curves from two human cell types. The new supplemental data is more powerful than some of that in the main text (i.e. single shot 5µM studies). This kind of paper has so much data every bit cannot be described in great detail. Nevertheless, the results section is lacking important detail and commentary that perhaps could be moved over from the Discussion. These are editorial comments but may help make this complex yet important story more digestible, accessible and best service the mountain of work performed.

Figure 5 and related figures. Figure 5 shows antiviral activity in Vero and human cells 293-hACE2 and Huh7-hACE2. The supplemental figure showing cell viability (cell number), Figure S9, is mentioned in the results and not explained. Some compounds show a reduction in viability or cell number more than 50% in one or more cell types but this is not mentioned in the results showing the antiviral activity data. It is important to show these data side by side to best interpret the antiviral data. This is especially important for SB-616234-A which diminishes cell number in all three cell types at 5µM (S9) by more that 50% so is this influencing the antiviral activity? The supplemental figures 10 and 11 help deal with these kinds of questions but Figures S10 and S11 are only described in passing and not in any great detail even though these data are more important, more impressive and better describe antiviral phenotypes over the single shot 5µM data. I would think that showing some of the curves in the main text would create a more powerful narrative than the single show bar graphs.

Line 700. Calling epithelial cells derived from iPS cells “primary human pneumocytes” is inaccurate. They may be “iPS derived pneumocytes” but they are not primary human pneumocytes. What was done to phenotype these cells to ensure that they were even pneumocyte-like cells?

Author Rebuttals to First Revision:

We thank the reviewers both for their original suggestions, which helped significantly strengthen the conclusions of the manuscript, and also for reconsidering the revised manuscript. Based on their suggestions, we have reorganized the figures, modified the presentation of the results, and redesigned the discussion, to enhance readability of the manuscript and facilitate the narrative flow.

Please find specific responses below

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have answered most of my critiques and I do not want to further delay this manuscript.

Reviewer Query: *The one suggestion I'd make is to further strengthen their observation that the* drugs targeting the GPCRs were not reversed by the chemical epistasis experiment (e.g., reversal by agonists). They show these data in the response letter but not in the manuscript, and while they say they go through this in the Discussion I did not see it. I'm not saying it isn't there, I'm sure I just missed it, but perhaps it isn't obvious since I was looking for it. Thus, I suggest they make it more prominent. It won't weaken the paper, far from it, it might make them look prophetic by suggesting that an off- target is involved.

In the interest of transparency, this is a signed

review: Brian Shoichet

Author Response: We thank Dr. Shoichet for his insightful comments and suggestions. As requested, we included the GPCR chemical epistasis experiment into the manuscript. Data are currently displayed in figure ED4b. We have highlighted those data and the potential off-target effect of GPCR in the discussion, agreeing this observation does not weaken the paper, but rather provide important context of the potential antiviral mechanisms of the identified compounds.

Referee #2 (Remarks to the

Author): Nice JOB!

Overall, the revised paper is improved. There is a lot supplemental data. So much so that the results are often not adequately explained in the results section. In some cases, figures are not explained at all (e.g. Fig. S9). The “Discussion” often reads as a “Results” section with frequent call backs to figures and tables. At times, the discussion is the results section that I’d hoped to read. The authors did a great job incorporating the antiviral assays and IC50 curves from two human cell types. The new supplemental data is more powerful than some of that in the main text (i.e. single shot 5µM studies).

Reviewer Query: *This kind of paper has so much data every bit cannot be described in great detail.* Nevertheless, the results section is lacking important detail and commentary that perhaps could be moved over from the Discussion. These are editorial comments but may help make this complex yet important story more digestible, accessible and best service the mountain of work performed.

Author Response: We would like to thank this referee for his/her feedback, critical comments, and support of publication of this study. Based on this last set of comments, we have significantly restructed the results and discussion sections to make the data clearer and more accessible to the reader, provide a better flow of the study narrative.

Reviewer Query: *Figure 5 and related figures. Figure 5 shows antiviral activity in Vero and human* cells 293-hACE2 and Huh7-hACE2. The supplemental figure showing cell viability (cell number), Figure S9, is mentioned in the results and not explained. Some compounds show a reduction in viability or cell number more than 50% in one or more cell types but this is not mentioned in the results showing the antiviral activity data. It is important to show these data side by side to best interpret the antiviral data. This is especially important for SB-616234-A which diminishes cell number in all three cell types at 5µM (S9) by more that 50% so is this influencing the antiviral activity? The supplemental figures 10 and 11 help deal with these kinds of questions but Figures S10 and S11 are only described in passing and not in any great detail even though these data are more important, more impressive and better describe antiviral phenotypes over the single shot 5µM data. I would think that showing some
of the curves in the main text would create a more powerful narrative than the single show bar graphs.

Author Response: We agree with this point, and we have removed the single point (5uM) data, and replaced with the more informative dose response data that were previously in supplemental figures 10 and 11. Additional dose response data are also presented in figure ED9. While we did observe toxicity for some compounds, including *SB-616234-A,* at high doses, the dose response data demonstrates a clear separation of antiviral activities and toxicity for all selected compound, and thus conclude that cytotoxicities are have negligible impact on observed antiviral activities of compounds.

Reviewer Query: *Line 700. Calling epithelial cells derived from iPS cells "primary human* pneumocytes" is inaccurate. They may be "iPS derived pneumocytes" but they are not primary human pneumocytes. What was done to phenotype these cells to ensure that they were even pneumocyte-like cells?

Author Response: We regret the error in mislabeling. We have corrected this as suggested. These pneumocytes we derived from iPSCs based on published protocols (Jacobs et al. (2017); Ghaedi et al (2013)), and validated for their ability to support SARS-CoV-2 replication.