THE EMBO JOURNAL

Mechanism and inhibition of the papain-like protease, PLpro, of SARS-CoV-2

Theresa Klemm, Gregor Ebert, Dale Calleja, Cody Allison, Lachlan Richardson, Jonathan Bernardini, Bernadine Lu, Nathan Kuchel, Christoph Grohmann, Yuri Shibata, Zhong Yan Gan, James Cooney, Marcel Doerflinger, Amanda Au, Timothy Blackmore, Gerbrand van der Heden van Noort, Paul Geurink, Huib Ovaa, Janet Newman, Alan Riboldi-Tunnicliffe, Peter Czabotar, Jeffrey Mitchell, Rebecca Feltham, Bernhard Lechtenberg, Kym Lowes, Grant Dewson, Marc Pellegrini, Guillaume Lessene an d David Komander
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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 31st Jul 2020

Prof. David Komander
The Walter and Eliza Hall Institute of Medical Research
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1G Royal Parade
Parkville
Melbourne, Victoria 3052
Australia

31st Jul 2020

Re: EMBOJ-2020-106275
Mechanism and inhibition of SARS-CoV-2 PLpro

Dear David,

Thank you again for submitting your manuscript on SARS-CoV2 PLpro structure and mechanism to The EMBO Journal. It has now been seen by two referees with expertise in DUB structure/biochemistry and pathophysiological roles of viral proteases, and given their generally positive comments, we would be happy to offer publication in The EMBO Journal after addressing several specific concerns listed in the reports below. It will be particularly important to adequately respond to referee 2's major concerns, either with additional data or by toning down conclusions.

In addition, please make sure that a revised manuscript closely adheres to the instructions in our Guide to Authors (see link below), and in particular incorporates the following editorial changes:

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- Please make sure that ORCID identifier have been entered into the profiles of all (co-)corresponding authors (currently missing for Marc Pellegrini). This needs to be added by the personally and can unfortunately not be done by others on your behalf.

- With the current title being very terse, please consider making it (only slightly) more explicit, e.g. by adding more context information/spelling out "protease", mentioning ubiquitin, ...

- On the abstract page of the manuscript, please include general 4-5 keywords to enhance searchability.

- Please add a header "Results" to mark the beginning of the results section in the text.

- Please make sure to include references and ideally some brief discussion of/comparison to recent reports by Shin, Dikic et al (Nature) and by Rut, Olsen et al (our preprint citation style in the text is: " (preprint: NAME1 et al, YEAR)"; in the reference list: "Author NAME1, Author NAME2, ... (YEAR) article title. bioRxiv doi: XXX")

- In current Figure EV8A and B, please include in the legend a definition of the error bars and the number of replicates.

- In the "Data Availability", please include the spelt-out hyperlink to the referenced database (PDB in this case)

- please upload all main Figures and all Expanded View figures as individual files with sufficient resolution/quality for production.

- Please reorganize the main and supplementary data display in accordance with our author guide (www.embopress.org/page/journal/14602075/authorguide#expandedview) regarding "supplementary figures":
x The spreadsheet document with the "PLPro named compound results" should be renamed "Dataset EV1", and referenced in the main text at least once. Please also include a brief legend for this dataset, in a separate "Legend" tab of the spreadsheet.
x The "Supplementary Methods" PDF detailing compound synthesis and characterization should be renamed "Appendix" and headed by a brief Table of Contents. The included methods should be renamed "Appendix Supplementary Methods" and referenced as such at least once from the main text. Note that the Appendix may additionally contain Appendix Figures with legend, Appendix Tables, or Appendix references, which all should be mentioned in the ToC.
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x The uncropped blots and gels should be separated into several files and uploaded as "Source Data" files: one file (PDF) per main figure, one combined file for all Expanded View source data, and one combined file for any Appendix Figure source data

- Finally, please provide suggestions for a brief two-sentence summary statement and 3-5 one-sentence 'bullet points', containing brief factual statements that summarize key aspects of the paper - they will form the basis of an editor-written 'Synopsis' accompanying the online version of the article. Please see the latest research articles on our website (embojournal.org) for examples! Please also provide a simplified schematic image for the synopsis, restricted to (small) format of 550px in width and 400 px in height (landscape format).

I am herewith returning the manuscript to you, hoping you will be able to rapidly make these revisions and to upload modified files as early as possible. Once we will have received them, we should be ready to proceed with formal acceptance and production of the manuscript. Should you have any questions, please do not hesitate to contact me (or my colleage Karin in my absence).

With kind regards,

Hartmut

Hartmut Vodermaier, PhD
Senior Editor / The EMBO Journal
h.vodermaier@embojournal.org

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files, replacing Supplementary Information (Please see https://www.embopress.org/page/journal/14602075/authorguide#expandedview)

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors:
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Revision to The EMBO Journal should be submitted online within 90 days, unless an extension has been requested and approved by the editor; please click on the link below to submit the revision online before 29th Oct 2020:
link removed

Referee #1:

The manuscript by Klemm et al., describes the mechanisms by which SARS2 PLpro removes ubiquitin and ubiquitin-like modifications, and characterises non-covalent small molecule inhibitors that appear promising starting points for the development of antiviral compounds against SARS2.

The authors employ elegant biochemical and biophysical approaches to determine the specificity of SARS2 PLpro enzymatic activity towards Lys48-linked polyubiquitin chains and ISG15-mediated modifications, demonstrating how SARS2 PLpro catalytic sites have evolved to preferentially target

ISG15 post-translational marks while maintaining selective specificity for Lys48-linked polyubiquitin. The authors further expand on this aspect by solving the crystal structures of SARS2 PLpro in complex with ubiquitin and ISG15, and perform structure-guided mutagenesis analyses that provide the molecular basis for understanding how SARS2 PLpro targets ubiquitin- and ISG15-modified substrates.

These methodologies provide critical understanding of SARS2 PLpro functions and are fundamental to establish novel approaches for its inhibition. As a logical subsequent step, the authors focus on the inhibition of SARS2 PLpro and employ a high-throughput screening (HTS) approach to identify potential drug candidates to treat COVID-19. Despite this approach not yielding any successful drug candidates, the authors have set up a very robust HTS assay and have shown that this compound library is not likely to yield drug candidates against SARS2 PLpro. In light of the global pandemic and given that resources should be carefully focused and time is of the essence, the establishment of a robust drug screen like the one described in the current manuscript is paramount for the identification of "true" drug candidates.

Lastly, the authors exploit known SARS PLpro inhibitory compounds for the inhibition of SARS2 PLpro. Using cell-based approaches, the authors determine the specificity and antiviral efficacy of these compounds. While the cell-based data show that the Rac series have a modest effect as antivirals, this is consistent with the in vitro potency of these compounds.

Overall, the study provides important insights that enhances our understanding of SARS2 PLpro and aid in the development of future inhibitors of these enzymes. As such, the findings are of great interest and significance to a broad audience, from researchers studying ubiquitin and ubiquitin-like modifications to structural biologists, virologists and scientists involved in the development of drug candidates for Covid-19.

The following minor comments will need to be addressed before publication is granted.

1. Page 3, line 30 and Page 4, line 3 - Here the authors introduce the S2 and S1 ubiquitin binding sites, respectively. While this nomenclature is well documented by referencing up-to-date literature, and it is commonly used within the ubiquitin field, this reviewer feels that the broader audience will benefit if this terminology could be clarified in the current manuscript. A brief explanation of this nomenclature (or a schematic) will lead to an easier understanding of the results section, as the same terminology is used throughout.

2. Figure 1C - The schematic for the hydrolysed Ub-TAMRA substrate (under "Low FP" headings) needs to be changed to reflect the hydrolysis of both ubiquitin- and ISG15-TAMRA substrate variants.

3. Page 8, line 16 - The authors don't provide a rationale for why rac5c, and not the other two compounds previously tested (i.e. rac3j and rac3k), was taken forward in the analyses shown in Fig. 5D and EV7F-G. Can the authors comment on this?
4. Page 4, line 21-22 - M-1/s-1 is the wrong unit for kcat/Km.

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This paper investigates the structure of SARS-CoV-2 encoded PLpro protease and its substrate specificities towards ISG15 and ubiquitin chains. A preference for ISG15 as a substrate and a further preference for K48-linked ubiquitin chains was identified, which is in accordance with earlier studies on coronavirus PLpro's. The study also addresses possibilities for inhibition of this viral enzyme as a an antiviral strategy. A screen for repurposed drugs did not reveil useful candidates, however using inhibitors that were identified for SARS-CoV before, cross-inhibition of SARS-CoV-2 infection was claimed.

In view of the current SARS-CoV-2 outbreak and the urgent need for useful antiviral agents this work is of high importance. Most of the work looks sound and is presented clearly.
My major concern is with Fig. 6 and expanded fig 8 where the authors looked at the effect of inhibitor rac5c on SARS-CoV-2 infection. Although it is indicative of specific inhibition of PLpro by rac5C that the GFP fusion construct cleavage can be inhibited (Fig. 5) and the addition of the compound can rescue cells from virus-induced CPE (Fig 6B), expanded Fig. 8 shows that at concentrations that inhibit the protease and the virus (11, 33 uM) the compounds tested are toxic to cells. Therefore, Fig 6C is hard to interpret because the production of infectious virus will be influenced by cells dying of the compound alone: virus cannot replicate in dead cells. The effect of the antiviral activity of the compound can therefore not be clearly claimed from this. Toxicity at active concentrations furthermore means that there is low potential for this compound as an antiviral, as it will probably cause adverse side-effects when using the drug in humans. The authors should reconsider their conclusions here I think, and at least discuss this matter in the discussion as well.
Some minor comments:
In the introduction it sais: " ...viruses with blocked protease activity do not replicate efficiently in cells". The general idea among corona virologists is that a blocked protease will completely abolish replication of the virus in general, not only cause lower efficiency of infection.
Line 2 page 4: add " in vitro" to clarify that this preference was assayed in this way on purified proteins and does not necessarily reflect a real infection situation.

Response to the Editor and Reviewers

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In addition, please make sure that a revised manuscript closely adheres to the instructions in our Guide to Authors (see link below), and in particular incorporates the following editorial changes:

We adjusted the manuscript according to the EMBO J Guide to Authors.

- Please download and complete our author checklist (link also provided below)

The checklist has been completed and is going to be submitted alongside the revised manuscript.

- Please make sure that ORCID identifier have been entered into the profiles of all (co-)corresponding authors (currently missing for Marc Pellegrini). This needs to be added by the personally and can unfortunately not be done by others on your behalf.

We have reached out to all authors to update their profiles. Marc Pellegrini has already done this.

- With the current title being very terse, please consider making it (only slightly) more explicit, e.g. by adding more context information/spelling out "protease", mentioning ubiquitin, ...

We changed the title to: “Mechanism and inhibition of the papain-like protease PLpro of SARS-CoV-2”

- On the abstract page of the manuscript, please include general 4-5 keywords to enhance searchability.

A line with following keywords has been added:

Keywords: COVID-19/ SARS-CoV-2/ PLpro/ ubiquitin/ ISG15/ deubiquitinas/ deISGylase/ small molecule inhibitor/ high-throughput screening.

- Please add a header "Results" to mark the beginning of the results section in the text.

The header “RESULTS” has been added.

- Please make sure to include references and ideally some brief discussion of/comparison to recent reports by Shin, Dikic et al (Nature) and by Rut, Olsen et al (our preprint citation style in the text is: "(preprint: NAME1 et al, YEAR)"; in the reference list: "Author NAME1, Author NAME2, ... (YEAR) article title. bioRxiv doi: XXX")

We included these references in the Discussion.

- In current Figure EV8A and B, please include in the legend a definition of the error bars and the number of replicates.

The definition of the error bars (mean +/- SD) and the number of replicates are indicated in the Fig legend

- In the "Data Availability", please include the spelt-out hyperlink to the referenced database (PDB in this case)

The hyperlink is included for the pdb and for each of the structures.

- please upload all main Figures and all Expanded View figures as individual files with sufficient resolution/quality for production.

The Figures and Expanded View figures are uploaded as eps files

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The spreadsheet document with the compound results is renamed to Dataset EV1 and referenced in the text. A brief legend has been added.

x The "Supplementary Methods" PDF detailing compound synthesis and characterization should be renamed "Appendix" and headed by a brief Table of Contents. The included methods should be renamed "Appendix Supplementary Methods" and referenced as such at least once from the main text. Note that the Appendix may additionally contain Appendix

Figures with legend, Appendix Tables, or Appendix references, which all should be mentioned in the ToC.

The Supplementary Methods section is now part of the Appendix.
The Appendix contains a Table of Contents, including two Figures, Appendix Figure S1 and S2 (former EV Figures), Appendix Table S1 (HTS Table), the Appendix Supplementary Methods and Appendix References.

x While we can accommodate more than 6 main figures, EV Figures are usually limited to 5-6. Therefore, please consider re-arranging, either by promoting some of the currently 8 EV figures to main figures (if justified), or by moving some (or all) EV figures into the Appendix PDF (only in this case, their legends should go into the Appendix file as well. Figures and references to them would have to be renamed "Appendix Fig S1/2/3..."). Please make sure to correctly update all Figure panel call-outs in the text after rearrangement.

Expanded View Figures have been re-arranged, with two former EV figures (EV2 and EV3) added to the Appendix. The other figures have been re-numbered and also updated in the text.

x The uncropped blots and gels should be separated into several files and uploaded as "Source Data" files: one file (PDF) per main figure, one combined file for all Expanded View source data, and one combined file for any Appendix Figure source data

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We include this with the submission.

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The following minor comments will need to be addressed before publication is granted.

We thank the reviewer for the overall positive response to our work and are happy to address the minor comments in this letter and in the revised manuscript.

1. Page 3, line 30 and Page 4, line 3 - Here the authors introduce the S2 and S1 ubiquitin binding sites, respectively. While this nomenclature is well documented by referencing up-to-date literature, and it is commonly used within the ubiquitin field, this reviewer feels that the broader audience will benefit if this terminology could be clarified in the current manuscript. A brief explanation of this nomenclature (or a schematic) will lead to an easier understanding of the results section, as the same terminology is used throughout.

We thank the reviewer for this comment and added a brief explanation in the Introduction and a new schematic figure (**Fig 1B**), to clarify the ubiquitin binding sites on deubiquitinases / PLpro for the broader audience.

2. Figure 1C - The schematic for the hydrolysed Ub-TAMRA substrate (under "Low FP" headings) needs to be changed to reflect the hydrolysis of both ubiquitin- and ISG15-TAMRA substrate variants.

We changed the respective figure, which is **Fig 1D** in the revised manuscript and added the products of the other substrate variants to the schematic to reflect the hydrolysis of all utilised substrates.

3. Page 8, line 16 - The authors don't provide a rationale for why rac5c, and not the other two compounds previously tested (i.e. rac3j and rac3k), was taken forward in the analyses shown in Fig. 5D and EV7F-G. Can the authors comment on this?

We agree with the reviewer that our rationale to only proceed with rac5c is not clearly stated in the text. We edited the revised manuscript to indicate that we used the most promising inhibitor from our biochemical analysis, rac5c, displaying an IC50 value in the sub-µM range (**Fig 5C**) (compared to 1.1 / 1.4 µM for rac3k/j), and best inhibition in antiviral assays (Fig. 6), to proceed with the assays testing the inhibition of self-cleavage of PLpro and the processing of poly-ubiquitin chains in cells as shown in **Fig 5D** and **EV5F, G** (former EV7F, G).

4. Page 4, line 21-22 - M-1/s-1 is the wrong unit for kcat/Km.

We thank the reviewer for noticing this error and corrected the wrong unit for kcat/Km to $M^{-1}s^{-1}$ in the revised manuscript.

Referee #2:

This paper investigates the structure of SARS-CoV-2 encoded PLpro protease and its substrate specificities towards ISG15 and ubiquitin chains. A preference for ISG15 as a substrate and a further preference for K48-linked ubiquitin chains was identified, which is in accordance with earlier studies on coronavirus PLpro's. The study also addresses possibilities for inhibition of this viral enzyme as a an antiviral strategy. A screen for repurposed drugs did not reveil useful candidates, however using inhibitors that were identified for SARS-CoV before, cross-inhibition of SARS-CoV-2 infection was claimed.

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We thank the reviewer for this comment and agree that toxicity of rac5c at active concentrations would prevent the feasibility to progress the compound as a therapeutic antiviral candidate for the treatment of COVID-19 towards the clinic.

We also agree with the reviewer's notion that the inhibition of virus mediated CPE and drastically reduced release of infectious viral particles (TCID50) from Vero cells treated with rac5c at a dose of 33 µM (**Fig 6B** and **6C**) is likely attributed to toxicity of the compound to the cells at this highest concentration. Indeed, we found that rac5c causes cellular toxicity (around 25%) at a concentration of 33 µM in Vero cells, as shown in the toxicity titration of the compounds in **Expanded View Figure 6B**. At 33 µM of rac5c, this is at least in part caused by a toxic concentration of the vehicle (0.3% DMSO) required, causing some degree of cell death as demonstrated in **Expanded View Figure 6A**.
However, when we used rac5c at a concentration of 11 µM or lower, neither the compound nor the required DMSO concentration (0.1%) alone caused any toxicity in Vero cells as shown in **Expanded View Figure 6A** and **6B**. Thus, the statistically highly significant inhibition of CPE and reduced release of infectious SARS-CoV-2 from Vero cells (TCID50) treated with rac5c at a concentration of 11 µM is mediated through specific inhibition of PLpro in infected cells. This is supported by our finding that rac5c at concentration of 10 µM

(and lower) effectively inhibits the nsp3 protease mediated release of GFP from the nsp3-GFP fusion construct (**Fig 5D**). It is important to note that the antiviral activity of rac5c at 11 µM is on par with Remdesivir (RDV) at an antiviral active concentration of 12.5 µM (**Fig 6C**), which remains the only FDA approved compound for emergency use to treat hospitalised COVID-19 patients to date.

We agree with the reviewer that the compounds, rac5c, as presented is not fit to become a drug without medicinal chemistry optimisation, to improve solubility, stability and efficacy. We have added a comment to this regard in the Discussion.

Some minor comments:

In the introduction it sais: " ...viruses with blocked protease activity do not replicate efficiently in cells". The general idea among corona virologists is that a blocked protease will completely abolish replication of the virus in general, not only cause lower efficiency of infection.

We are grateful for reviewer2 sharing this insight with us and changed the wording in our revised manuscript.

Line 2 page 4: add " in vitro" to clarify that this preference was assayed in this way on purified proteins and does not necessarily reflect a real infection situation.

We added “in vitro” to the respective sentence, to clarify our findings.

1st Revision - Editorial Decision 4th Aug 2020

Dear David,

Thanks for sending us the revised version. As Hartmut is away this week, I am looking out for your manuscript. I have now had a chance to take a careful look at everything and all looks good.

I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a great manuscript!

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here: http://emboj.embopress.org/about#Transparent_Process

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If you have any questions, please do not hesitate to call or email the Editorial Office. Thank you for your contribution to The EMBO Journal.

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Corresponding Author Name: David Komander, Guillaume Lessene, Marc Pellegrini
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2020-106275

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For biochemical assays technical triplicates were measured in at least n=2 two independent experiments for PLpro mutants and at least n=4 independent experiments for PLpro wild-type to determine the catalytic efficiency against different substrates. With the exact number of replicates indicated in the respective figure legends. The high through put screen was performed with one measurement per compound in n=3 independent experiments. Counterscreen and confirmation screen were performed with 3-6 technical replicates in n=2 independent experiments. The cell based assay was repeated at least twice. For Infection studies, showing the cytopathic effect with rac5c, 6 biological replicates were performed in 3 independent experiments, HCQ and RDV control samples from n=2 and n=1 experiments using 6 biological repeats. TCID50 data represent one experiment with 6 technical replicates. Data for 3J and 3k cytopathic effect and TCID50 analysis represent 6 biological replicates from n=1 experiment. The sample sizes are indicated in the Fig legends.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	The HTS assay quality was monitored by calculation of robust Z' by the following formula where (+) denotes the positive controls (low signal), (-) denotes the negative controls (high signal) and MAD is the median absolute deviation: robust Z' = 1- (3*(MAD- + MAD+) / abs(median- - median+))
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	For Fig 6 B, P values were calculated using a one-way ANOVA, with regular Dunnet's post-hoc test ffor multiple comparisons between treatment arms and infected/vehicle treated control using a single pooled variance. We assumed normal distributions for the data.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jij.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is there an estimate of variation within each group of data?	Errorbars shown in Fig 6C and EV 6E, F correspond to standard deviation between replicates
Is the variance similar between the groups that are being statistically compared?	a single pooled variance was used for the test

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	PLpro antibody, chicken polyclonal, (Lifesensors, #AB-0602-0250); anti-Ubiquitin antibody Lys48-specific (Apu2), rabbit monoclonal, , (Sigma Aldrich, #05-1307); GAPDH mouse monoclonal antibody (6C5), (Invitrogen, #AM4300); anti-GFP antibody chicken polyclonal (Abcam, #ab13970)). IRDye 800CW goat anti-mouse IgG secondary (Li-Cor, #925-32210); goat anti-chicken IgY-HRP (SantaCruz, #sc-2428); rabbit IgG HRP (GE Healthcare, # NA934VS)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293T and Vero (CCL-81) cells displayed expected cell morphologies and were validated by the Garvan Molecular Genetics facility. Cell lines were screened on a monthly basis for mycoplasma contamination using the PlasmoTest kit (Invivogen) as per manufacturer's instructions. All used cells were mycoplasma free.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Structures have been deposited to the PDB. Accession codes (pdb 6xaa and 6xa9) are indicated with hyperlink under "Data availability and accession numbers".
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	HTS results are provided in Dataset EV1.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA