

# **Development of a replication-defective monkeypox virus platform for fundamental and therapeutic research**

Corresponding Author: Dr Rong Zhang

**This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.**

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Authors present a timely and extensive work. Here authors have developed a non-replicative Mpox viral system using CRISPR-Cas9 and recombinant methods, resulting in a platform technology with reduced the safety rating for Mpox work from BLS-3 to BSL-2. Further, authors utilized the platform to screen for anti-Mpox molecules and successfully identified two molecules and provided. majority of the work that is developing the replication-defective Mpox system was done meticulous with proper controls. I believe the final biophysical characterization of OPG57 protein-ligand interactions lacked a similar rigor. Nonetheless, I recommend the publication of this article upon careful consideration on other reviews and corrections mentioned below

1. A brief description in the introduction for protein encoded by Mpox genome and highlight the function of relevant protein function for the study. Also, IMV and EEV are introduced in the results sections; it will readers if IMV and EEV stages of Mpox cycle are highlighted briefly in introduction to contextualize Cidofovir and tecovirimat mode of action.

2. OPG85 aslo seems to strongly mutate in both cell lines apart from OPG57, are any experiments carried out eliminate as a target for G243-1720, please comment on this

2.Mass photometry is highly sensitive and niche techniques, if the antiviral molecules can induce dimerization of sOPG57, straight forward experiments with size exclusion chromatography or Ultracentrifugation or even native-PAGE may have been used.

4. For mapping the interaction site of G243-1720 methods such as amide-hydrogen deuterium exchange mass spectrometry (HDXMS) can be used to conclusively show the binding interface.

Minor issues

1.Line 151 "Transmission electron microscopy was used to study the assembly of virions." Please rephrase it gives the impression that mechanistic study using TEM was carried out instead authors used TEM imaging to observe whether the system produced intact particles or not. Line 205 also

2. Showing a table comparing the IC50 values of antivirals and antibodies (7D11) from known studies for Mpox virus and current study with replication defective particles (lines 235-240) will be helpful for readers, as supplementary data

3. Panel 2E , should have mock data too ?

4. "(PDB code = 9FHS)" to PDB ID: 9FHS

5.plasmid availability for the research community should be addressed as per the journal requirements.

6.Title should be modified to emphasize the replication-defective Mpox platform alone

Reviewer #2

(Remarks to the Author)

Very interesting work. The authors have developed a BAC-MPXV system with dual gene (OPG96 and OPG158) deletions, which under helper FPXV infection produced replication-defective MPXV (rdMPXV) to be used as a tool for MPXV virology studies and drug screening under standard BSL-2 lab settings, as compared to the WT MPXV which requires BSL-3 containment. The new virus system was confirmed for safety and applied to screening a TargetMol library of 3185 compounds in trans-complementing cells using a high-content imaging assay. From this pilot screen, one of the 3 initial hits, compound G243-1720, showed sub-micromolar potency without cytotoxicity, and hence was further studied in a wide range of rigorous experiments, and was characterized as a potent, non-toxic and bioavailable, broad-spectrum antiviral against orthopox viruses, by mechanistically promoting the dimerization of p37 and blocking IMV wrapping. The paper is very well written, and the technical quality is very high. However, it's less clear how significant the work really is.

Major points

- 1) Although the extremely well-characterized new drug hit G243-1720 showed sub-micromolar potency in infected cells and demonstrated efficacy in mice, it's substantially much less potent than tecovirimat: a) in CV-1-96,158 cells infected with rdMPXVΔ96,158, G243-1720 (EC<sub>50</sub> = 140 nM) was 47-fold less potent than tecovirimat (EC<sub>50</sub> = 3 nM); b) in vivo efficacy: G243-1720 twice daily i.g. at 45 mg / kg produced much less viral titer reduction than tecovirimat once daily i.g. at 50 mg / kg (1.56 log for G243-1720 vs 2.7 log for tecovirimat).
- 2) G243-1720 is mechanistically identical to tecovirimat with overlapping resistance profile, diminishing its development value.
- 3) One purported value of the rdMPXV is the safety when screening compounds for inhibitors impacting replication in wt cells, which amounts to a single replication cycle assay. It's unclear why only a limited screen in trans-complementing cells was conducted. The latter is a multiple rounds replication assay which increases the risk of producing MPXV via recombination.
- 4) It's unclear what advantages this system offers over VACV as a surrogate virus for compound screening and drug MOA characterization, as was the case in the development of tecovirimat. The assay is cumbersome, especially when the trans-complementing cells are used.

Minor points

- 5) IC<sub>50</sub> should be EC<sub>50</sub>
- 6) G243-1720 should be properly authenticated with <sup>1</sup>H and <sup>13</sup>C NMR, HRMS and HPLC. These analytical data should be included in the supplemental file.
- 7) References: for many citations only the first author is listed—this is not appropriate.

Reviewer #3

(Remarks to the Author)

In this communication, the authors describe the construction of a replication-defective MPXV and use it to screen a small-compound library for replication inhibitors. The most potent inhibitor, G243-1720, has similar biological activity and the same OPG57 target as Tecovirimat, though a different chemical structure. Like Tecovirimat, G243-1720 inhibited multiple orthopoxviruses including VACV. The work was very well done, exceptionally complete and the paper clearly written. Nevertheless, the screening could have been done with VACV instead of the defective MPXV as a broad inhibitor is most advantageous and G243-1720 does not seem to offer any advantage in potency over Tecovirimat. Additionally, spontaneous mutants that resist the latter are also resistant to G243-1720.

Specific comments

1. The deletion of two essential genes from MPXV, which increases safety, and establishment of a complementing cell line were well described.
2. Is there an identifier or catalog number for the TargetMol compound library?
3. Does G243-1720 inhibit members of other poxvirus genera? For example, inhibitors of parapoxviruses would be useful.

Reviewer #4

(Remarks to the Author)

The generation of a replication defective model of MPXV infection is noteworthy and will likely prove to be a valuable tool to support the identification of antiviral compounds. This will be an important addition as it will allow additional laboratories to complete research against MPXV infections.

Overall, the work that has been completed is sound and the conclusions are well-supported by the data.

The statistical analyses appear to be appropriate and enough replicates have been completed to instill confidence in the results.

The methodology is clear and meets the standards in the field. There is enough information provided to accurately allow other researchers to reproduce this work.

There are a few small things which could be clarified to benefit the readers.

1. The authors demonstrate efficient infection of rdMPXV in several different cell lines, however, screening of antiviral compounds was only completed in HaCat-96,158 cells. The rationale for choosing this cell line over the others is not clear and a rationale would help the readers understand why other cell lines were not utilized.

2. The authors identified Merimepodib as an inhibitor of MPXV during the screening assays. It is not clear whether this compound was an intentional positive control or whether it was detected randomly in the compounds that were tested.
3. The legend for Figure 6A does not specify what statistical analysis was used to compare the dimerization activity of F243-1720.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Authors have addressed all the queries. I recommend the manuscript for publication

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## RESPONSE TO REFEREES

### Reviewer #1 (Remarks to the Author):

Authors present a timely and extensive work. Here authors have developed a non-replicative Mpox viral system using CRISPR-Cas9 and recombinant methods, resulting in a platform technology with reduced the safety rating for Mpox work from BLS-3 to BSL-2. Further, authors utilized the platform to screen for anti-Mpox molecules and successfully identified two molecules and provided. majority of the work that is developing the replication-defective Mpox system was done meticulous with proper controls. I believe the final biophysical characterization of OPG57 protein-ligand interactions lacked a similar rigor. Nonetheless, I recommend the publication of this article upon careful consideration on other reviews and corrections mentioned below.

We sincerely thank the Reviewer's evaluation and feedback of our manuscript.

1. A brief description in the introduction for protein encoded by Mpox genome and highlight the function of relevant protein function for the study. Also, IMV and EEV are introduced in the results sections; it will readers if IMV and EEV stages of Mpox cycle are highlighted briefly in introduction to contextualize Cidofovir and tecovirimat mode of action.

We agree with the Reviewer that providing this background information will improve the clarity. We have added the following description in the “Introduction” section of the revised manuscript.

“Poxviruses enter host cells via fusion with either the plasma membrane or the membrane of an intracellular acidified vesicle<sup>1</sup>. Following fusion of the viral envelope with the host membrane, the core—containing the ~200 kb viral genome (encoding nearly 200 proteins) and structural components (e.g., RNA polymerase)—is released into the cytoplasm<sup>2</sup>. The transcription system of the core then drives the expression of early genes, which facilitate immune evasion, genome release, and subsequent DNA replication. Newly synthesized genomes serve as templates for sequential expression of intermediate and late genes, initiating viral particle assembly<sup>3</sup>.

The earliest hallmark of particle assembly is the formation of rigid, crescent-shaped structures within viral factories<sup>4, 5, 6, 7</sup>. These crescents require at least nine essential proteins, comprising three structural and six regulatory (eg., L2 (now renamed orthopoxvirus gene 96, OPG96), A30.5 (OPG158)) factors, collectively termed viral membrane assembly proteins (VMAPs). Among these, OPG96 and OPG158 are the smallest, and critical for regulating crescent formation. The crescent extends with constant curvature and eventually closes to form immature virions (IV), which then matures into the infectious intracellular mature virus (IMV), also called mature virus (MV). IMV particles mediates cell-to-cell transmission but are released primarily upon cell lysis. A subset of IMV acquires a double-layer envelope from the trans-Golgi network or early endosomes, forming intracellular enveloped virus (IEV), also called wrapped virus (WV). IEV particles are transported along microtubules to the cell periphery, where they fuse with the plasma membrane to become cell-associated enveloped virus (CEV) on the cell

surface. CEV can induce actin tail formation to propel itself towards neighboring cells for direct spread; alternatively, it may be released into extracellular space as extracellular enveloped virus (EEV), also called enveloped virus (EV), to mediate systemic dissemination<sup>4, 5, 6, 7</sup>.”

2. OPG85 also seems to strongly mutate in both cell lines apart from OPG57, are any experiments carried out eliminate as a target for G243-1720, please comment on this.

We thank the Reviewer for raising this question. Indeed, our sequencing data showed mutations in OPG85 (G1L in VACV) in addition to OPG57 (F13L in VACV) in both cell lines. We excluded it from the investigation based on the following information: a. OPG85, a reported viral metalloprotease, is essential for viral particle transition from IV to IMV via its proteolytic activity, acting after core cleavage but before condensation and infectivity acquisition. Deletion of OPG85 arrests viral morphogenesis at the IV stage and reduces infectious progeny by 98.9%<sup>8, 9</sup>; b. Electron microscopy revealed that G243-1720 does not affect IMV morphogenesis (Figs. 4G–4I); c. Subsequent IMV titration in cells also showed G243-1720 does not impair IMV infectivity (Figs. 4J–4K); d. Although OPG85 F215S mutation emerged in viruses passaged in CV-1-96,158 and HaCaT-96,158 cells under G243-1720 treatment, the mutation also occurred in DMSO-treated controls during resistance screening.

Based on these observations, we conclude that the OPG85 F215S mutation arises from viral passaging (adaptive changes) rather than specific resistance to G243-1720, and exclude OPG85 as a candidate target for G243-1720.

3. Mass photometry is highly sensitive and niche techniques, if the antiviral molecules can induce dimerization of sOPG57, straight forward experiments with size exclusion chromatography or Ultracentrifugation or even native-PAGE may have been used.

We thank the Reviewer for the suggestion. To complement the mass photometry experiments, we have now incorporated analytical ultracentrifugation (AUC) and SEC-SAXS data, which demonstrate that G243-1720 induces the dimerization of OPG57. In the AUC experiments performed in the absence and presence of 10  $\mu$ M G243-1720 and tecovirimat (as a positive control), we found that G243-1720 shifted the equilibrium from monomer to homodimers, as tecovirimat does. In the same line, SEC-SAXS data confirmed that G243-1720 promotes the dimerization of sOPG057, as the experimental curve aligned well with the theoretical SAXS profile calculated using the crystallographic dimer. Overall, consistent with the results from the mass photometry experiment, we observed that G243-1720 shifted the equilibrium in solution of OPG057 to the dimeric form in both the AUC and SEC-SAXS experiments. These findings are now presented in Figure 6 and Supplementary Figure 8, along with SAXS data and collection parameters included in Supplementary Tables 4 and 5.

4. For mapping the interaction site of G243-1720 methods such as amide-hydrogen deuterium exchange mass spectrometry (HDXMS) can be used to conclusively show the binding interface.

Thanks for the suggestion. We are sorry that we are currently do not have access to HDXMS technique. To complement this, we have conducted crystallization, mass photometry, as well as analytical ultracentrifugation and SEC-SAXS to assess the interaction of G243-1720 with OPG57 protein, showing the compound-induced dimerization.

#### Minor issues

1.Line 151 “Transmission electron microscopy was used to study the assembly of virions.” Please rephrase it gives the impression that mechanistic study using TEM was carried out instead authors used TEM imaging to observe whether the system produced intact particles or not. Line 205 also

As requested, we have rephrased these sentences for accuracy: “Transmission electron microscopy revealed structurally intact virions in CV-1-96 cells...” shown in line 186 of the revised manuscript.

2. Showing a table comparing the IC<sub>50</sub> values of antivirals and antibodies (7D11) from known studies for Mpox virus and current study with replication defective particles (lines 235-240) will be helpful for readers, as supplementary data

This is a helpful suggestion. We include a new Supplementary Table 1 that compiles the EC<sub>50</sub> values for tecovirimat, cidofovir, and the antibody 7D11 from published studies using wild-type MPXV, alongside the values we obtained with our replication-defective MPXV particles.

Compound	Strains	EC <sub>50</sub> values (μM)	Cell lines	Reference
Tecovirimat	Zaire	0.01	Vero	<sup>10</sup>
	Zaire	0.008±0.0014	Vero E6	<sup>11</sup>
	MPXV-V78-I-3945	0.023±0.0026	BSC-40	<sup>12</sup>
	MPXV-V81-I-179	0.032±0.0061	BSC-40	<sup>12</sup>
	MPXV-2003-USA-039	0.036±0.0045	BSC-40	<sup>12</sup>
	MPXV-V77-I-823	0.030±0.0114	BSC-40	<sup>12</sup>
	MPXV-V1979-I-005	0.039±0.0016	BSC-40	<sup>12</sup>
	12 MPXV isolates from the outbreak in 2022	0.004-0.02	HFF and HFK	<sup>13</sup>
	SP2833	0.006±0.0002	Vero E6	<sup>11</sup>
	rdMPXV <sup>Δ96,158</sup>	0.003	CV-1-96,158	this study
	rdMPXV <sup>Δ96,158</sup>	0.007	HaCat-96,158	this study
Cidofovir	Zaire	27±11	Vero 76	<sup>14</sup>
	Zaire	5.722±0.2059	Vero E6	<sup>11</sup>

	12 MPXV isolates from the outbreak in 2022	5-32	HFF and HFK	<sup>13</sup>
	SP2833	1.980±0.0579	Vero E6	<sup>11</sup>
	rdMPXV <sup>Δ96,158</sup>	10.45	CV-1-96,158	this study
	rdMPXV <sup>Δ96,158</sup>	52.45	HaCat-96,158	this study

Antibody	Strain	EC <sub>50</sub> value (μg/mL)	Cell line	referenc
7D11	IIb.c.1	1.8	Vero-E6	<sup>15</sup>
	rdMPXV <sup>Δ96,158</sup>	0.83	CV-1-96,158	this study

3. Panel 2E, should have mock data too?

We apologize for this oversight. We did not include mock infection in Panel 2E when comparing the replication of rdMPXV and authentic MPXV in dormice.

4. (PDB code = 9FHS)” to PDB ID: 9FHS

We have corrected it.

5. plasmid availability for the research community should be addressed as per the journal requirements.

Thanks for the suggestion. We have added the statement in Data Availability section: "Materials generated in this study are available upon request."

6. Title should be modified to emphasize the replication-defective Mpox platform alone

Thanks for the suggestion. We agree and have modified the title as "Development of a replication-defective monkeypox virus platform for fundamental and therapeutic research".

## Reviewer #2 (Remarks to the Author):

Very interesting work. The authors have developed a BAC-MPXV system with dual gene (OPG96 and OPG158) deletions, which under helper FPXV infection produced replication-defective MPXV (rdMPXV) to be used as a tool for MPXV virology studies and drug screening under standard BSL-2 lab settings, as compared to the WT MPXV which requires BSL-3 containment. The new virus system was confirmed for safety and applied to screening a TargetMol library of 3185 compounds in trans-complementing cells using a high-content imaging assay. From this pilot screen, one of the 3 initial hits, compound G243-1720, showed sub-micromolar potency without cytotoxicity, and hence was further studied in a wide range of rigorous experiments, and was characterized as a potent, non-toxic and bioavailable, broad-spectrum antiviral against orthopox viruses, by mechanistically promoting the dimerization of p37 and blocking IMV wrapping. The paper

is very well written, and the technical quality is very high. However, it's less clear how significant the work really is.

We sincerely thank the Reviewer for the thoughtful and constructive feedback.

#### Major points

1) Although the extremely well-characterized new drug hit G243-1720 showed sub-micromolar potency in infected cells and demonstrated efficacy in mice, it's substantially much less potent than tecovirimat: a) in CV-1-96,158 cells infected with rdMPXV $\Delta$ 96,158, G243-1720 (EC<sub>50</sub> = 140 nM) was 47-fold less potent than tecovirimat (EC<sub>50</sub> = 3 nM); b) in vivo efficacy: G243-1720 twice daily i.g. at 45 mg / kg produced much less viral titer reduction than tecovirimat once daily i.g. at 50 mg / kg (1.56 log for G243-1720 vs 2.7 log for tecovirimat).

2) G243-1720 is mechanistically identical to tecovirimat with overlapping resistance profile, diminishing its development value.

We sincerely thank the Reviewer for the insightful comments of 1 and 2 regarding the comparative potency of G243-1720 and its shared target with tecovirimat.

We acknowledge that G243-1720 is less potent than the FDA-approved drug tecovirimat, both *in vitro* and *in vivo*. We also acknowledge that its shared mechanism and resistance profile with tecovirimat present a challenge for its direct clinical development.

Tecovirimat was the result of a massive screening (>350,000 compounds) followed by extensive medicinal chemistry optimization. In contrast, G243-1720 was identified from a much smaller, proof-of-concept screen of ~3,000 compounds using our novel rdMPXV platform and has not undergone any chemical optimization. Despite this, its distinct chemical scaffold—different from both tecovirimat and IMCBH—and its sub-micromolar potency position it as a lead compound, not a final drug candidate. It provides a new chemical starting point for a future optimization to improve its bioavailability and efficacy.

The clinical performance of tecovirimat against MPXV has been more variable than anticipated, with one trial (PALM007) showing no significant benefit, potentially due to suboptimal drug exposure. This underscores that the full therapeutic potential of F13L as a target may not yet be realized. As only the third F13L-targeting compound ever identified, G243-1720 serves as a valuable chemical probe. Its unique structure and the resistant mutations it selects (e.g., I372N, A290V, D294V) provide new tools to study the structure and function of F13L, which could inform the development of next-generation inhibitors.

Finally, the primary contribution of this work is the validation of the rdMPXV platform itself. The efficient discovery of G243-1720 from a modest library demonstrates the power and reliability of our platform for high-throughput antiviral discovery in a clinically relevant, BSL-2 setting. In accordance with other Reviewers' suggestions, we have revised the manuscript title to better highlight the establishment of this platform for fundamental and therapeutic studies.



3) One purported value of the rdMPXV is the safety when screening compounds for inhibitors impacting replication in wt cells, which amounts to a single replication cycle assay. It's unclear why only a limited screen in trans-complementing cells was conducted. The latter is a multiple rounds replication assay which increases the risk of producing MPXV via recombination.

We thank the Reviewer for raising this important regarding our screening strategy. We agree that using the rdMPXV particles in wild-type cells represents the safest single-cycle system. However, we chose to conduct the primary high-throughput screen in complementing cells (HaCaT-96,158) for the following reasons:

- a. Our reporter system uses a viral late promoter to drive expression. In a single-cycle assay, this system cannot detect inhibitors of late-stage processes like virion assembly and egress—a class of compounds with therapeutic potential. A multi-cycle replication assay in complementing cells is essential to identify these valuable inhibitors.
- b. A single-cycle assay presents challenges for a large-scale screen. A readout based on abolition of signal is overly stringent, as even potent drugs like Cidofovir cannot achieve ideal inhibition in one cycle (Fig. 4D). While a readout based on reduced fluorescence intensity is possible, it is less robust and more susceptible to technical variation. The multi-cycle assay, with its wider dynamic range and signal amplification, provides a much more reliable and reproducible platform for screening thousands of compounds.

Following the primary screen, we specifically employ the rdMPXV particles in wild-type cells for secondary assays to characterize hit compounds and differentiate their stage of action (e.g., entry/replication vs. post-replication).

We acknowledge the Reviewer's concern regarding recombination. The safety of our system is supported by our data demonstrating no replication in wild-type cells over multiple passages (Supplementary Figure 2A, 2B, and 3D) and is consistent with the extensive established safety record of similar replication-defective vaccinia virus systems<sup>16, 17, 18</sup>. We are confident the risk of generating replication-competent MPXV is minimal and effectively mitigated.

4) It's unclear what advantages this system offers over VACV as a surrogate virus for compound screening and drug MOA characterization, as was the case in the development of tecovirimat. The assay is cumbersome, especially when the trans-complementing cells are used.

We thank the Reviewer for this question regarding the advantages of our rdMPXV platform over the well-established VACV model.

While VACV has been an invaluable surrogate model, it has undergone extensive laboratory passaging, resulting in significant biological and pathogenic differences from the circulating MPXV. Specifically, there are genes and proteins encoded by MPXV that are absent from VACV and which are potential targets for drug development. Given that

the ongoing global health threat is driven specifically by MPXV, our replication-defective MPXV system offers a direct and clinically relevant tool to study the authentic pathogen responsible for the current outbreaks. This allows for a more accurate understanding of virus-host interactions and therapeutic efficacy against the virus of primary concern.

Our system is engineered to be both safe and representative. The dual-gene deletion ensures a high safety profile, enabling work at a reduced biosafety level (BSL-2). Importantly, it faithfully recapitulates the entire viral lifecycle: it undergoes single-cycle infection in wild-type cells and multi-round replication in complementing cells. As a proof of concept, the system accurately reproduced the known efficacy profiles of established anti-poxvirus agents (tecovirimat, cidofovir, 7D11 Ab), yielding EC<sub>50</sub> values consistent with those reported for authentic viruses (we summarized in a table above, as suggested by Reviewer #1). This validates its reliability for high-throughput screening and mechanistic studies.

Thus, this platform does not seek to replace VACV but rather provides a specialized, powerful, and necessary tool for the research community to study MPXV biology and accelerate antiviral discovery with direct translational relevance to the ongoing mpox outbreak.

Minor points

5) IC50 should be EC50

Thank you for the suggestion. We have corrected “IC50” to “EC50” throughout the manuscript.

6) G243-1720 should be properly authenticated with <sup>1</sup>H and <sup>13</sup>C NMR, HRMS and HPLC. These analytical data should be included in the supplemental file.

Thanks for the suggestion. We provided the synthesis route and analytical report as Supplementary Table 2, and stated this in lines 298-299. The structure of G243-1720 was verified using <sup>1</sup>H nuclear magnetic resonance (<sup>1</sup>H-NMR) and electrospray ionization liquid chromatography-mass spectrometry (ESI-LCMS).

7) References: for many citations only the first author is listed—this is not appropriate.

We apologize for this error. We have checked the citations in the text and corrected it.

As to reference list, it is automatically generated using Endnote software following the journal's formatting guidelines.

### **Reviewer #3 (Remarks to the Author):**

In this communication, the authors describe the construction of a replication-defective MPXV and use it to screen a small-compound library for replication inhibitors. The most

potent inhibitor, G243-1720, has similar biological activity and the same OPG57 target as Tecovirimat, though a different chemical structure. Like Tecovirimat, G243-1720 inhibited multiple orthopoxviruses including VACV. The work was very well done, exceptionally complete and the paper clearly written. Nevertheless, the screening could have been done with VACV instead of the defective MPXV as a broad inhibitor is most advantageous and G243-1720 does not seem to offer any advantage in potency over Tecovirimat. Additionally, spontaneous mutants that resist the latter are also resistant to G243-1720.

We thank the Reviewer for the comments and concerns which was also raised by reviewer #2. We are pleased to provide the following clarifications.

As to the screening model, the primary goal of our study was to discover therapeutics specifically against MPXV. While VACV is a valuable model virus, biological differences—such as host range and virulence—can limit the translational relevance of findings. Our replication-defective MPXV platform allows for screening in a more clinically relevant context, enabling the direct identification of lead compounds against MPXV. We fully agree that VACV remains essential for validating broad-spectrum activity. Consequently, as part of our characterization of the lead compound G243-1720, we evaluated its efficacy against VACV and confirmed its potent, broad-spectrum anti-orthopoxviral activity (see Figure 3I and 3J).

As to the developmental prospects of G243-1720, we have provided a detailed response regarding the potential of G243-1720 to Reviewer #2. We agree that its shared mechanism with tecovirimat and current lower efficacy present challenges. However, we believe G243-1720 still retains value for three reasons: a. it represents a lead compound for further medicinal chemistry optimization to improve its bioavailability and potency; b. it serves as a successful proof-of-concept for our rdMPXV platform in identifying antivirals through high-throughput screening; c. its distinct chemical scaffold makes it a valuable biological probe for studying OPG57 / F13 protein structure and function.

#### Specific comments

1. The deletion of two essential genes from MPXV, which increases safety, and establishment of a complementing cell line were well described.

We thank the Reviewer for the positive feedback on our development of the replication-defective MPXV platform.

2. Is there an identifier or catalog number for the TargetMol compound library?

The compound library used was the TargetMol #LC00. We have added the catalog number to the Methods section in the revised manuscript.

3. Does G243-1720 inhibit members of other poxvirus genera? For example, inhibitors of parapoxviruses would be useful.

We thank the Reviewer for this question. To evaluate the broad-spectrum potential of G243-1720, we have tested its efficacy against a panel of orthopoxviruses, including VACV, CPXV-BR, CPXV-E, CMLV, and RPXV, as presented in Figure 3I and 3J of the manuscript. G243-1720 effectively inhibited all of these viruses.

In response to the reviewer's point regarding specificity, we also sought to test its activity against a more distantly related poxvirus. While parapoxviruses were unfortunately unavailable for testing, we evaluated fowlpox virus (FPV), a member of the distantly related *Avipoxvirus* genus. As expected for a F13-targeting compound, neither G243-1720 nor tecovirimat inhibited FPV replication, confirming the specificity of their mechanism for orthopoxviruses. In contrast, the viral DNA polymerase inhibitor cidofovir showed activity at high concentrations ( $>10\ \mu\text{M}$ ), serving as a positive control. These new data have been added to Supplementary Fig.5B in the revised manuscript.

#### **Reviewer #4 (Remarks to the Author):**

The generation of a replication defective model of MPXV infection is noteworthy and will likely prove to be a valuable tool to support the identification of antiviral compounds. This will be an important addition as it will allow additional laboratories to complete research against MPXV infections. Overall, the work that has been completed is sound and the conclusions are well-supported by the data. The statistical analyses appear to be appropriate and enough replicates have been completed to instill confidence in the results. The methodology is clear and meets the standards in the field. There is enough information provided to accurately allow other researchers to reproduce this work.

We sincerely thank the Reviewer for the thorough and positive assessment of our manuscript. We are particularly grateful for the Reviewer's recognition of the potential value that our replication-defective MPXV platform holds the research community.

There are a few small things which could be clarified to benefit the readers.

1. The authors demonstrate efficient infection of rdMPXV in several different cell lines, however, screening of antiviral compounds was only completed in HaCat-96,158 cells. The rationale for choosing this cell line over the others is not clear and a rationale would help the readers understand why other cell lines were not utilized.

We thank the Reviewer for this question. Our decision to use HaCaT-96,158 cells for the primary high-throughput screen was based on two rationales. First, HaCaT is an immortalized human keratinocyte line, representing a physiologically relevant target for MPXV infection, which enhances the translational relevance of our findings. Second, as shown in Fig. 2H, parental HaCaT cells exhibited high susceptibility to rdMPXV <sup>$\Delta$ 96,158</sup> infection. The derived HaCaT-96,158 cell line thus provided a robust platform with a high signal-to-noise ratio, supporting efficient multi-round viral replication, which is essential for a reliable large-scale imaging-based screen. We have clarified this in the revised manuscript (line 258-262, and 286).

2. The authors identified Merimepodib as an inhibitor of MPXV during the screening assays. It is not clear whether this compound was an intentional positive control or whether it was detected randomly in the compounds that were tested.

We thank the Reviewer for requesting this clarification. Merimepodib was not included as an intentional positive control; rather, it was identified as a hit from the unbiased compound library screen. Its known mechanism of action (inhibition of Inosine-5'-monophosphate dehydrogenase, IMPDH) along with its previously reported anti-MPXV activity established it as a validating hit from our screening platform.

3. The legend for Figure 6A does not specify what statistical analysis was used to compare the dimerization activity of F243-1720.

We thank the Reviewer for pointing this out. The statistical analysis in Figure 6A (now Figure 6B in the revised manuscript) was an unpaired, two-tailed Student's t-test. We have updated the figure legend in the revised manuscript.

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