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Development of a rapid image-based high-content imaging screening assay to evaluate therapeutic antibodies against the monkeypox virus

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A B S T R A C T

Antibody-based therapy is emerging as a critical therapeutic countermeasure to treat acute viral infections by offering rapid protection against clinical disease. The advancements in structural biology made it feasible to rationalize monoclonal antibodies (mAbs) by identifying key and, possibly, neutralizing epitopes of viral proteins for therapeutic purposes. A critical component in assessing mAbs during pandemics requires the development of rapid but detailed methods to detect and quantitate the neutralization activity. In this study, we developed and optimized two high-content image (HCI)-based assays: one to detect viral proteins by staining and the second to quantify cytopathic viral effects by a label-free phenotypic assay. These assays were employed to screen for therapeutic antibodies against the monkeypox virus (MPXV) using surrogate poxviruses such as vaccinia virus (VACV). Plaque-based neutralization results confirmed the HCI data. The phenotypic assay found pox virus-induced syncytia formation in various cells, and we were able to quantitate and use this phenotype to screen mAbs. The HCI identified several potent VACV-neutralizing antibodies that showed in vitro efficacy against both clades of MPXV. In addition, a combination study of ST-246/tecovirimat/TPOXX a single neutralizing antibody Ab-40, showed synergistic activity against VACV in an *in-vitro* neutralization assay. This rapid high-content method utilizing state-of-the-art technologies enabled the evaluation of hundreds of mAbs quickly to identify several potent anti-MPXV neutralizing mAbs for further development.

1. Introduction

The subfamily *Chordopoxvirinae* in the family *Poxviridae* comprises enveloped viruses with large genomes containing a linear double-stranded DNA molecule ~128–270 kilobase pairs (kbp) (Lefkowitz et al., 2006). Two members of the family, variola virus (VARV) and monkeypox virus (MPXV), are important human pathogens that can cause fatal human diseases. VARV is the causative agent of human smallpox with 10–30% mortality rates. Although smallpox was declared eradicated in 1980 following a global immunization campaign, monkeypox is emerging as a significant global threat (Bunge et al., 2022)

Monkeypox virus (MPXV) is a zoonotic disease historically limited to parts of Africa. Since the discovery of MPXV in 1958, MPXV has caused sporadic outbreaks in Central and West Africa (Silva et al., 2020). The full-length sequencing of isolates from these regions has shown that MPXV comprises clade I (Central Africa) and clade II (West Africa) (Berthet et al., 2021). Viruses from both clades can cause human disease. However, they differ in the severity of the clinical condition. Clade I is more virulent, with a case fatality rate of ~10%; in contrast, clade II is rarely lethal. The current 2022 outbreak of MPXV is caused by a clade II virus and has generated nearly 100,000 confirmed cases (Bunge et al., 2022).

The human-to-human transmission of MPXV is via respiratory droplets or contact with skin lesions. However, the 2022 outbreak has demonstrated that the virus can also spread via sexual transmission. Multiple studies have shown that predominate cases are in men who have sex with men (Inigo Martinez et al., 2022). Despite the narrow transmission route, the outbreak rapidly spread to over 100 countries. Regardless of the transmission route, the global outbreak has highlighted the potential for the rapid spread of MPXV and the critical need to develop additional countermeasures.

The current strategy to combat MPXV infection is to utilize

Abbreviations: MPXV, Monkeypox virus; RPXV, Rabbitpox virus; VACV, Vaccinia virus; HCI, High Content Imaging; PRNT, Plaque reduction neutralization tests; IFA, Immunofluorescence assay.

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therapeutics developed for smallpox, and during the development path, these therapeutics were tested against MPXV. The two antivirals designed to treat smallpox were recently approved by the FDA under the emergency use authorization (EUA) to treat human MPXV infections; Tecovirimat (TPOXX) from Siga Technologies targets viral VP37 to stop the viral spread, and brincidofovir (TEMBEXA) from Chimerix inhibits VARV polymerase (Kaler et al., 2022; Sherwat et al., 2022). Screening large sets of small-molecule libraries are laborious, expensive, and time-consuming (Ward et al., 2015). Hence, alternative focus approaches may provide a more fruitful path forward against pandemic-causing viruses such as MPXV (Gu et al., 2022).

The development of biologics such as monoclonal antibodies (mAbs) is an attractive alternative as the initial screening process is directed (higher success rate), cost-effective, lower tox profile, and in most cases, shorter development timelines. mAbs are a versatile class of antiviral countermeasures that can bind to and neutralize/inactivate the virus in infected patients to reduce/prevent clinical disease. The advancement of structural biology made it feasible for the rational design of neutralizing monoclonal antibodies by identifying potent vulnerable regions of viral proteins for therapeutic purposes (Kombe et al., 2021). In addition, recent studies have shown that mAb therapy can induce long-lasting antiviral immunity in infected individuals (Pelegrin et al., 2015). Due to these advantages, multiple mAbs (Inmazeb and Ebanga) to treat Ebola virus infection were developed and approved by the FDA as first-line therapies (Crozier et al., 2022). The success of these mAb therapies warrants the development of such therapies against other high-consequence deadly pathogens, including MPXV (Zaeck et al., 2022; Gates et al., 2015)

In response to the current MPXV outbreak, Tonix Pharmaceuticals developed a pathogen-agnostic screening platform utilizing high-content imaging (HCI) to rapidly screen a library comprising 900 antibodies for MPXV specificity and neutralizing activity. The antibodies were screened at Biosafety Level-2(BSL-2) Laboratory to identify lead candidates with surrogate poxviruses. These selected candidates were then screened against multiple MPXV isolates in Biosafety Level-3(BS-3) laboratory. This HCI platform enabled rapid library screening in days and identified several neutralizing highly potent antibodies against clade I and II MPXV isolates.

2. Results

2.1. High content image-based assay development and standardization

MRC-5, BSC-40, and Vero-E6 cells were tested in HCI assay development for VACV infections. Multiple seeding densities of BSC-40, Vero E6, and MRC-5 were evaluated, and a cell density of 16,000/well was found to be optimal (data not shown). Because the spatial distribution of the cells is essential for phenotypic assay, and this cell density produced a reliable viral infection, we selected 16,000 cells/well as the cell density for subsequent infections. Minimal variations among replicates were noted without compromising the proper segmentation of cells (data not shown). VACV-infected BSC-40 cells were visualized by Immunofluorescence assay (IFA)detection of the VACV using the Fluorescein isothiocyanate (FITC) conjugated Vaccinia Virus Polyclonal Antibody (PA1-73191, Thermo Fisher, CA) and by confocal imaging using the Opera Phenix high-content imager (PerkinElmer, MA). (Fig. 1). To identify the ideal moi and cells for HCI screening, we infected MRC-5, Vero, and BSC-40 cells with increasing moi from 0.02 to 1 (Fig. 2). Based on previous experiments, we found 22 hr post-infection to be the optimal time to evaluate infection rate (data not shown). VACV at the moi of 0.02 at 22 hr after infection achieved an ideal infection rate of approx. 60% (Fig. 2). This percentage of infection allowed us to screen for antibodies that not only inhibited viral infection but also enhanced the viral infection in BSC-40 cells. These conditions allowed for multiple rounds of virus replication and facilitated the screening of therapeutic antibodies targeting/reducing virus entry into cells. Finally, the VACV-infected cells were classified as cells with sufficient green signal within the defined boundaries of a cell compared with the background signal (in mock-infected cells). Many additional parameters (>50; data not shown) were acquired and evaluated from the images, including the nuclear and cytoplasmic intensities of the viral signal, nuclear size, nuclear intensities, syncytia formation, number of nuclei in syncytia,

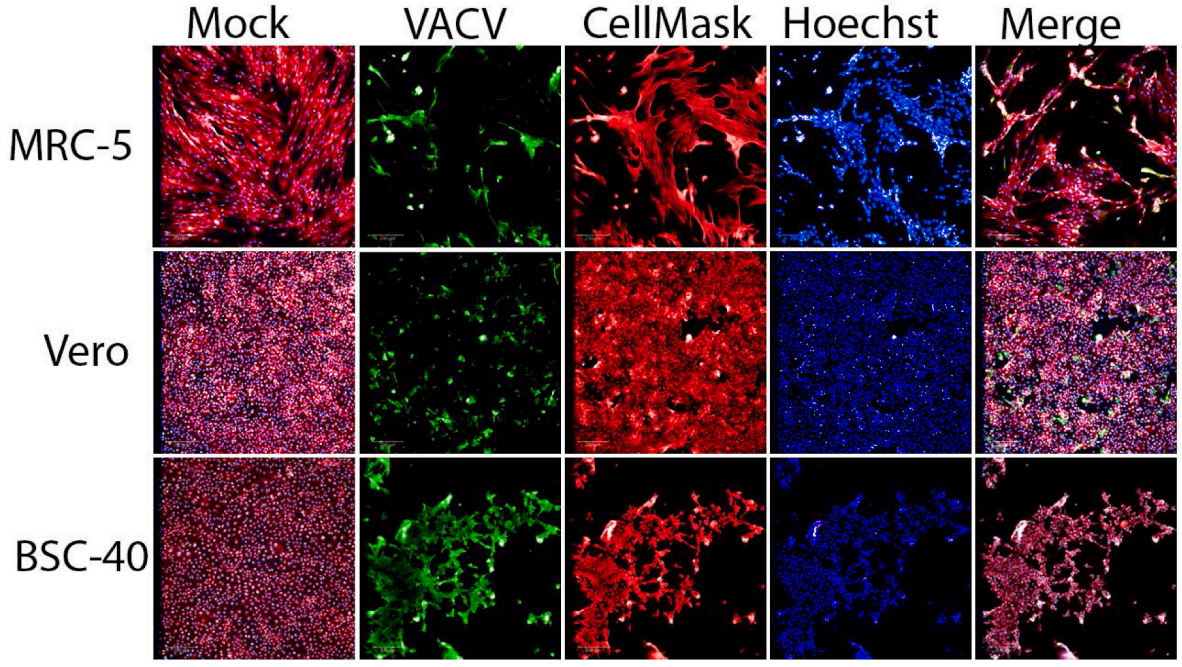


Fig. 1. High Content Imaging Assay Development for Vaccinia Virus
MRC-5, Vero, and BSC-40 cells were either mock-infected or infected with 0.05 moi of the VACV virus. After 2hr., cells were fixed and stained with VACV-specific polyclonal antibody conjugated with FITC to detect the virus-infected cells (green staining). The whole cell stain HCS CellMask™ Deep Red Stain (red cells) and nuclear stain Hoechst (blue cells) were used to detect cells and nuclei, respectively.

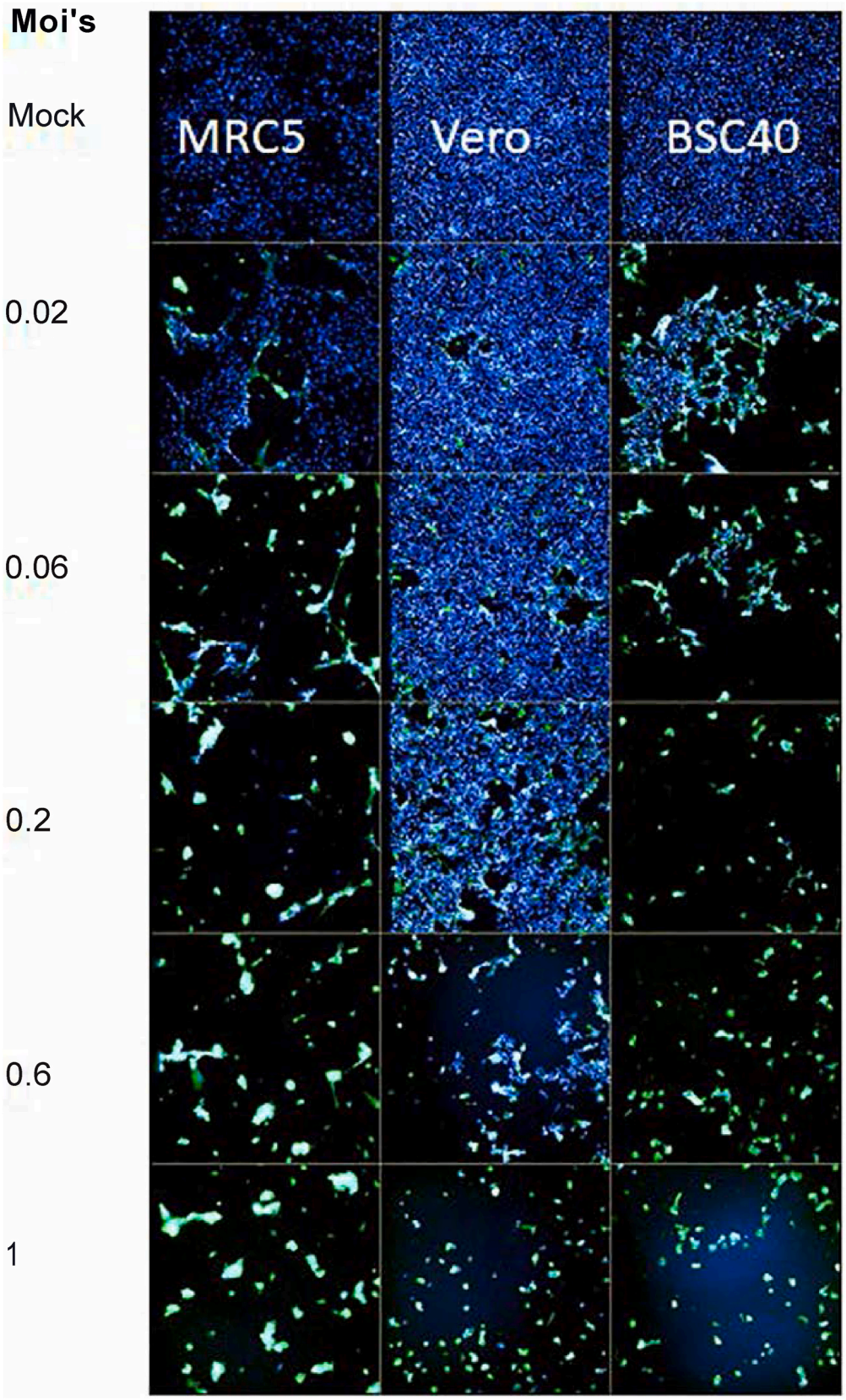


Fig. 2. Determining optimal moi for Vaccinia High Content Imaging Assay
MRC-5, Vero, and BSC-40 cells were either mock-infected or infected with multiple moi's of the virus. After 22 hr, cells were fixed and stained with VACV-specific polyclonal antibody conjugated with FITC to detect the virus (green staining). The nuclear stain, Hoechst (blue staining), was used to detect nuclei.

and syncytia size. Other parameters besides spatial distribution, such as cell viability, ease of infection, morphological aspects of the cells, and ease of imaging for pox infection, were also considered in establishing the phenotypic assays. We selected BSC-40 cells for the HCI screen based on our aggregate observations.

2.2. Primary mAb screening against VACV infection

We set up a primary screening assay using mock, VACV-infected cells, and cell numbers were used to determine the percentage of infected cells. The Z′-factor was evaluated to test the robustness of the assay in 96- and 384-well formats (Fig. 3a). The Z′-factor was calculated using the average and standard deviations of the percent infection of the positive and negative controls described in the methods section. The experiment was performed in triplicate on three separate days, and the calculated Z′-factor was 0.9. A Z′-factor >0.5 indicates a statistically reliable separation between positive and negative controls (Fig. 3a).

A poxvirus-specific mAb library comprising 900 antibodies from internal and external sources was used for antiviral screening. The library was screened at a single four ug/well concentration against VACV. Primary hits were selected based on the following criteria: >50% inhibition and >80% cell viability compared to positive controls. A scatter plot of the primary screening data was generated by normalizing infected cells with mock-treated VACV-infected cells to determine the percent infection inhibition (Fig. 3b). Uninfected control wells are depicted in red (Fig. 3b). We elected to call any mAb with >85% inhibition of infection a "hit" (Fig. 3b, green). The scatter plot shows 15 antibodies were "hits" (Fig. 3b). The percent infection inhibition of these antibodies ranged from 85 to 100% without any apparent cytotoxicity.

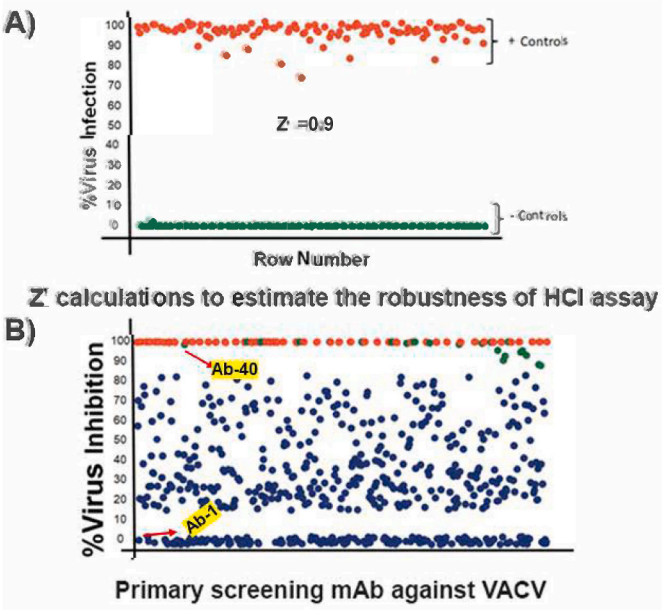


Fig. 3. Primary screening of neutralizing antibodies against VACV
BSC-40 cells were either mock-infected or infected with 0.05 moi of the VACV IHD-W strain of the virus. After 22 hr, cells were fixed and stained with VACV-specific polyclonal antibody conjugated with FITC to detect the virus. (a) Z′ was calculated to determine the robustness of the screening assay. Y-axis shows a % virus infection. + control, virus-infected cells (red); - control, mock-infected cells (green). The values for the treatment wells are evenly distributed across the x-axis (b). Before cell infections, VACV was incubated with neutralizing antibodies from the Tonix monoclonal antibody library raised against the VACV virus for 1 hr at 37°C. Each antibody was tested in triplicate wells in the same assay plates. Y-axis shows % virus inhibition based on syncytial formation normalized to infected control wells. Mock-infected cells (red). The antibodies that inhibited viral infection and syncytia formation are shown in green.

2.3. Secondary screening of mAb candidates via dose-response studies

We selected Ab-40 from among the top hits to advance to the next stage of testing based on its HCI data suggesting this antibody was able to improve characteristics such as cell number, cell morphology, and cell growth kinetics. We next examined the activity of Ab-40 against another orthopox virus, the rabbitpox virus (RPXV). In these assays, as a positive control, we used ST-246. This antiviral small molecule prevents virus envelopment and cell-cell spread at a concentration of 30 nM, which was previously reported to inhibit RPXV and many other pox viruses (Nalca et al., 2008). ST-246 inhibited 80% RPXV infection at this concentration with no apparent cytotoxicity in BSC-40 cells (Fig. 4a). We also used polyclonal antibody generated against VACV at four μg/well as additional control and observed little efficacy against RPXV. In contrast, at four μg/well, Ab-40 inhibited RPXV similarly to ST-246, diminishing infection by over 90%. This data suggests that Ab-40 can neutralize multiple pox viruses.

To ask if Ab-40 inhibits pox infection in primary cells, we next studied its effects in a primary lung fibroblast cell infection model using VACV. ST-246 at 30 nM inhibited the infection by about 85%, while the VACV polyclonal Ab again showed little protection (Fig. 4b). In contrast, Ab-40 inhibited primary lung fibroblast cell infection by over 90%. These data suggest that Ab-40 protects primary cells from pox infection with an efficacy similar to ST-246 (Fig. 4b). To further examine Ab-40 and obtain IC50 values, we evaluated various antibody and compound doses for efficacy against VACV (Fig. 4c) and RPXV (Fig. 4d) using BSC-40 cells. The IC50 values for Ab-40 against RPXV and VACV were 20 ng/mL and 0.7 ng/mL, respectively (Fig. 4c and d). ST-246 had an IC50 of about 2 nM, which is in line with the reported activity of ST-246 against these pox viruses. The CC50, which measures the cytotoxicity of compounds and antibodies, is above the highest tested concentration (>25 μg/mL).

2.4. Ab-40 has an additive and possibly synergistic effect with ST-246

Next, we wanted to know if combinations of targeting a surface protein with Ab-40 and ST-246, which targets a different pathway during virion formation, can act in concert and be synergistic. The antiviral activity of Ab-40 and ST-246 was evaluated separately, and then the two antivirals were combined in 0.5 log dose responses starting at 1 μM or 200 ng/ml for ST-246 and Ab-40, respectively (Fig. 5A). The readout for this assay was VACV infection as measured by staining for viral proteins. We used a high-single agent (HSA) reference model to analyze our combination data. The HSA modeling could distinguish between additive and synergistic responses (Fig. 5B). Using this reference model, a score of >10 suggests synergistic responses, and the model provided a score of 13.217. A clear synergistic response was observed at 0.2 and 0.63 ng/well for Ab-40 when combined with 0.01 μg of ST-246. The immediate higher concentrations of both antivirals were at least additive; beyond this, combining the two antivirals had no additional benefit. This is most likely because these two antivirals are very potent and quickly reach their respective EC90. It is important to note that the maximum inhibition by ST-246 was about to 86%. Ab-40 at concentrations between 0.6 and 6.3 ng/ml elicited 35–79% inhibition. However, the combination of the two antivirals at concentrations of Ab-40 0.6–6.3 ng/ml and ST-246 at concentrations of 0.1 and 0.3 μM produced nearly 100% infection inhibition. In future studies, we will examine if other weaker mAbs can synergize with ST-246.

2.5. Syncytia formation as a no-label phenotypic assay

Viral infection begins at the cellular level when a viral particle binds to cellular receptors or cofactors on the target cell surface, initiating the fusion process (Mas and Melero, 2013). Viral and host cell membrane fusion reactions introduce viral genetic material into the cytoplasm of infected cells. Once inside the cell, the virus replicates, and newly

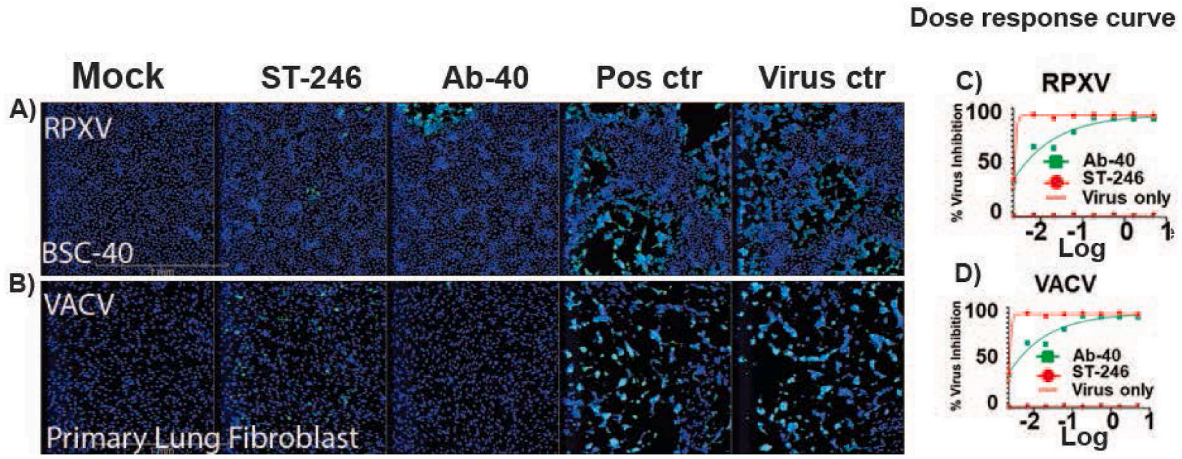


Fig. 4. Dose-response studies of Ab-40 against RPXV (BSC-40 cells) and VACV in primary lung fibroblast cells
BSC-40 cells or primary lung fibroblast cells were either mock-infected or infected with 0.05 moi of the RPXV or VACV (Virus ctr, respectively. Before cell infection, RPXV or VACV was incubated with Ab-40 or polyclonal antibody (Pos ctr) for 1 hr at 37°C. Reference compound ST-246 was added to cells 2 hr before viral infections. After 22 hr, cells were fixed and stained with VACV-specific polyclonal antibody conjugated with FITC to detect the virus (Green staining). As shown in the graphs, Ab-40 inhibited both RPXV (Fig. 4C) and VACV (Fig. 4D) infections in a dose-dependent manner.

replicated viruses infect the nearby cells. The infected cells either make more viral particles or fuse with nearby cells via fusion proteins. The cellular structures formed by fusions of multi-cell fusion of uninuclear cells are termed Syncytia (Kielian, 2014). Syncytia formation indicates cytopathic effects, which are common during viral infection and facilitate viral spread (Albrecht et al., 1996). Syncytia formation can be visualized by light microscopy, and when the infection is severe enough, cellular morphological changes can be quantitated by phenotypic HCI. These changes, such as the size or number of fused cells, may reflect the severity of the infection and may enable drug efficacy testing.

Imaging technologies have enabled phenotypic analyses of enveloped viruses and host cellular receptor interactions (Mudhasani et al., 2013, 2015; Panchal et al., 2010; Radoshitzky et al., 2016). Exploiting such approaches requires quantifiable, reproducible, reliable assay systems to monitor virus-cell or cell-cell fusion. In the present article, we describe a novel vaccinia virus-based assay to measure the fusogenic activities of enveloped viruses. We demonstrate this assay's specificity, sensitivity, simplicity, and versatility by measuring syncytia formation elicited by VACV infection without using any label or Abs. We examined syncytia formation by VACV-infected cells over 48 hr. Video-microscopy analysis (supplement fig 1) and high-content image analysis showed that syncytia appeared rapidly, starting at 6 hr post-infection, and grew as bystander cells were incorporated in fused cells (Fig. 6 and Movie 1). We quantified syncytia formation by acquiring and measuring the fused cell area (syncytia) with a high-content imager and specialized computer algorithms. As expected, syncytia formation and the size of syncytia were directly correlated with moi (data not shown (Jessie and Dobrovolny, 2021). Syncytia formation was due to VACV infection because we observed no syncytia or clumping in the uninfected cells (Fig. 6a). We used a computer-generated depiction of syncytia formation to allow the phenotypic analysis and quantitation to occur in a label-free system. This approach provided a means to quantify the inhibition of syncytia over the large dose range of inhibitors. As quantified in Fig. 6b, there is a clear dose-dependent increase in the syncytial number with decreasing amounts of ST-246 (IC50 = 22 nM and Ab-40 (IC50 = 15 ng). The number of syncytia is normalized to mock and infected control wells. The advantage of using this method compared to traditional IFA is that the phenotypic quantification generated is independent of antibody-based virus detection. Script writing to calculate syncytia number is solely based on an aggregation of nuclei due to viral infections. This method has broad application for antiviral drug efficacy testing and may be applied to many other viruses that elicit syncytia formation.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.antiviral.2022.105513

2.6. *Plaque reduction neutralization tests (PRNT) assay*

To further assess the broad-spectrum activity of Ab-40 and confirm the HCI data, we performed PRNT assays with VACV, MPXV comprising of Clade I and Clade II including MPXV-Zaire-79, MPXV WR 7–61, MPXV US 2003, and MPXV-MA22 isolates. We calculated $PRNT_{80}$ and $PRNT_{50}$ values (Table 1). We used VACV polyclonal Ab and a non-neutralizing mAb Ab-1 (See Fig. 3) as controls. ST-246 was incorporated into these studies as another control to allow us to bridge these data sets to all the other experiments. VACV and MPXV MA-2022 showed no visible plaques in the presence of 25 μg/ml Ab-40 (Fig. 7). A few plaques were observed at 1 and 2 μg/ml. The neutralizing ability of Ab-40 was similar against VACV and MPXV MA-2022. To further delineate the broad-spectrum activity of Ab-40, we tested the antibody against multiple Clade I and 2 MPXVs, including the highly pathogenic Zaire-79. The polyclonal antibody yielded $PRNT_{80}$ and $PRNT_{50}$ values of 4 μg/ml and 2 μg/ml, respectively, against VACV. The $PRNT_{80}$ of ST-246 was about >1 μM in the assay (data not shown). As expected, Ab-1 showed no observable PRNT up to 25 μg/ml. More importantly, $PRNT_{80}$ and $PRNT_{50}$ values of Ab-40 were similar for VACV and all Clade I and II MPXVs.

3. Conclusions

In these studies, we optimized and standardized a HCI neutralizing assay using 96- and 384- well plates which detected viral proteins of VACV and used the assay to screen a large library of mAbs made against pox viruses. The assay identified several mAbs with the capacity to neutralize VACV infection fully. We selected a single mAb, Ab-40, for further testing. Syncytia formation, a key hallmark of pox infection of cell lines, was identified as a pure phenotypic no-label assay that can be implemented to determine the efficacy of ST-246 and Ab-40 against VACV. The syncytia assay results correlated well with neutralizing HCI assay based on detecting VACV proteins. The in vitro efficacy data against VACV was confirmed using primary lung fibroblast cells. The synergistic combination of antiviral drugs is typically highly efficacious, especially those targeting different steps in the virus life cycle, as this strategy often prevents viral-resistant adoption. We tested the potential for synergy between Ab-40 and ST-246. The HSA score suggested that at low concentrations, these two antivirals can act synergistically, and this

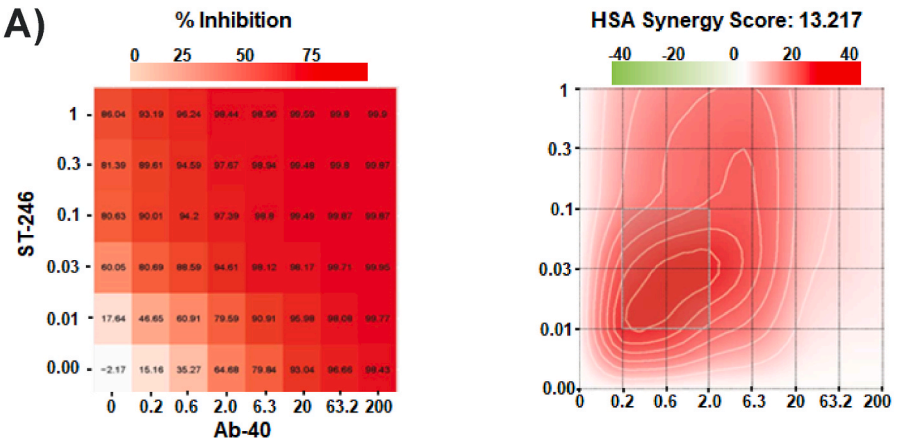
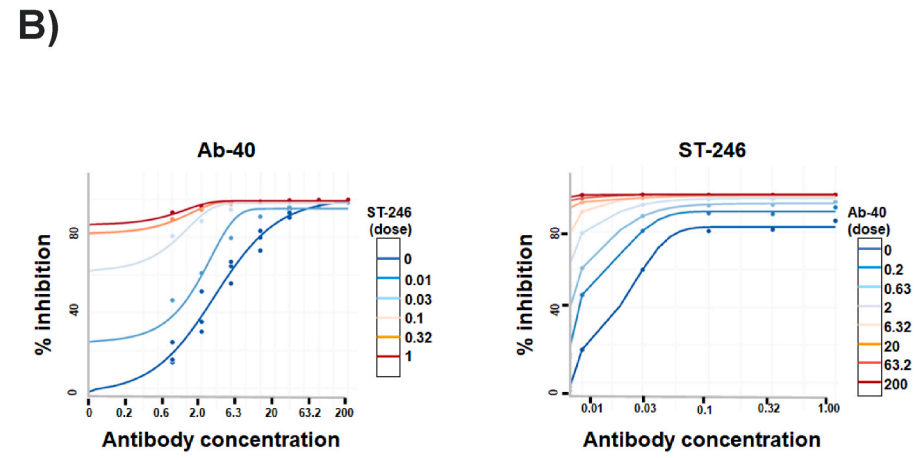
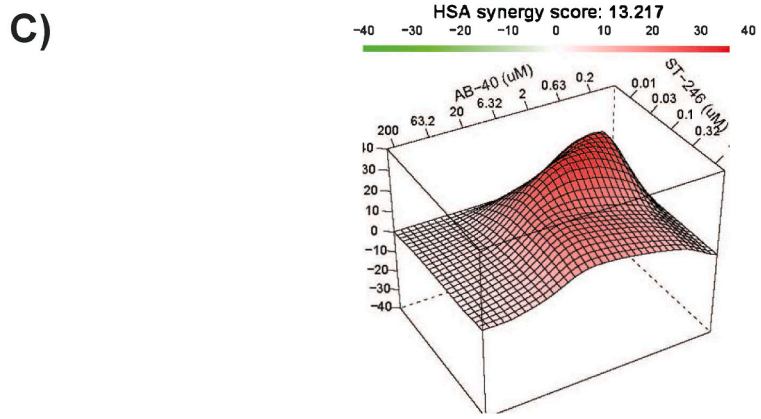


Fig. 5. Ab-40 and ST-246 drug combinations have synergistic effects on VACV infections in BSC-40 cells

To evaluate the potential combined effects of Ab-40 and ST-246 against VACV infections, an 8 × 8 matrix study was performed. The X-axis shows the concentrations of Ab-40, and the Y-axis shows the drug ST-246. The highest single agent (HSA) method quantifies the degree of combination synergy Fig. 5a left plot shows the % virus infection inhibition, and the right plot shows the 2d topography with areas of synergy. Fig. 5b shows the dose-response curves shift of ST-246 with varying concentrations of Ab-40 (left) and the dose-response shift of Ab-40 with varying concentrations of ST-246 (right) Fig. 5C: Dose-response surface interaction combinations for Ab-40 and ST-246 in 3D.





combination may increase the efficacy of each drug and reduce resistance. Interestingly, Ab-40 synergized the maximum inhibitory values of ST-246 from a maximum of 86% to nearly 100%. Further studies are needed to tease out the dose responses, the average EC/IC reduction for each antiviral, and the mechanism(s) of synergy. We confirmed the HCI data with PRNT assay and showed that Ab-40 neutralized all Clade I, and II MPXVs tested, including the highly pathogenic Zaire strain. We are in the process of defining how Ab-40 neutralizes all pox viruses tested and identify additional efficacious mAbs against MPXV. Finally, our data suggests applying HCI technology, such as no-label phenotypic syncytia detection and other phenotypic-based assays may increase the throughput of screening novel antivirals to shorten the discovery time for antivirals.

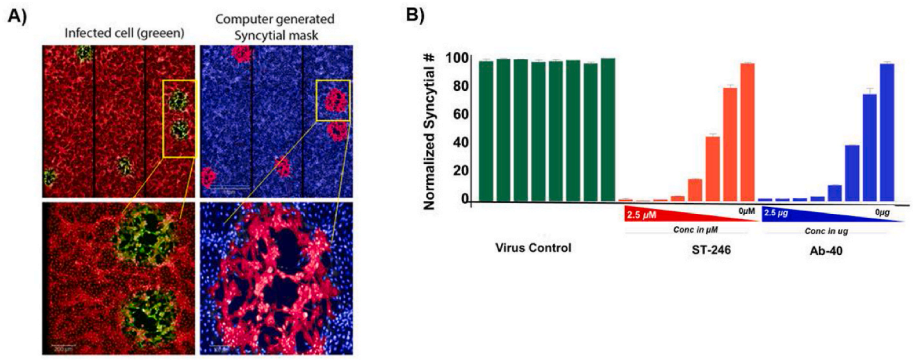


Fig. 6. Identification of syncytia using computer scripts in BSC40 cells infected with VACV

BSC-40 cells were treated with various doses of ST-246 or Ab-40, and mock-infected, infected with 0.05 moi of VACV. (a) After 22 hr of infection, cells were fixed and stained with VACV-specific polyclonal antibody conjugated with FITC to detect the virus (green staining), nuclear stain Hoechst (blue), and whole cell stain HCS CellMask^{TM} deep blue (red). Syncytia was detected using computer scripts written in the Harmony environment. A computer-generated Mask (red coloring) was superimposed on the nuclei of the cells inside the syncytia.

(b) **Quantitation of syncytia in presence or absence of various concentrations of ST-246 or Ab-40.** The figure shows dose-dependent inhibition of syncytial formation by ST-246 or Ab-40. The values on the Y-axis are normalized to the syncytial number of untreated virus-infected wells.

Table 1
Plaque Reduction Neutralization Test (PRNT) results in the table.

Antibody	Anti-VACV		Anti-MPXV Zaire-79		Anti-MPXV WR 7-61		Anti-MPXV US-2003		Anti-MPXV MA-2022	
	$PRNT_{80}$	$PRNT_{50}$	$PRNT_{80}$	$PRNT_{50}$	$PRNT_{80}$	$PRNT_{50}$	$PRNT_{80}$	$PRNT_{50}$	$PRNT_{80}$	$PRNT_{50}$
Ab-Polyclonal	4 μg/ml	2 μg/ml	4 μg/ml	2 μg/ml	4 μg/ml	2 μg/ml	4 μg/ml	2 μg/ml	4 μg/ml	2 μg/ml
Ab-40	2 μg/ml	1 μg/ml	2 μg/ml	1 μg/ml	2 μg/ml	1 μg/ml	2 μg/ml	1 μg/ml	2 μg/ml	1 μg/ml
Ab-1	>25 μg/ml	>25 μg/ml	>25 μg/ml	>25 μg/ml	>25 μg/ml	>25 μg/ml	>25 μg/ml	>25 μg/ml	>25 μg/ml	>25 μg/ml

PRNT 50/80 values for polyclonal antibody, Ab-40 or Ab-1are shown against VACV and MPXV.

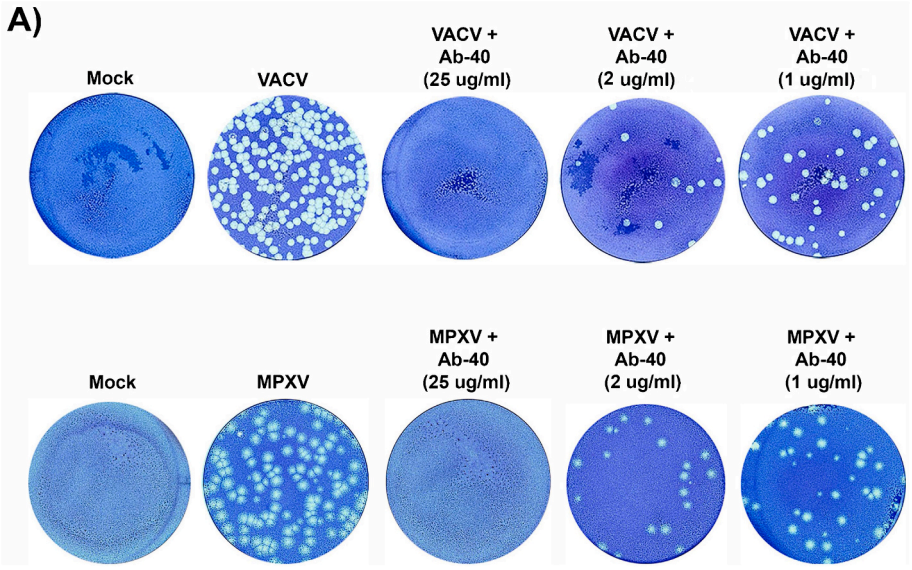


Fig. 7. Neutralization activity of Ab-40 against VACV and MPXV isolates. Micrographs of VACV or MPXV treated with Ab-40 (25, 2, 1 μg/mL) or untreated (mock) are shown (Fig. 7a).

Disclaimer

Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data including the Material and Methods section of this article can be found online at https://doi.org/10.1016/j.antiviral.2022.105513.

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