UNITED STATES
SECURITIES AND EXCHANGE COMMISSION
Washington, D.C. 20549

FORM 8-K

CURRENT REPORT

Pursuant to Section 13 or 15(d) of the Securities Exchange Act of 1934

Date of report (date of earliest event reported): January 24, 2023

TONIX PHARMACEUTICALS HOLDING CORP.

(Exact name of registrant as specified in its charter)

Nevada	**001-36019**	**26-1434750**
(State or Other Jurisdiction of Incorporation)	**(Commission File Number)**	**(IRS Employer Identification No.)**

26 Main Street, Chatham, New Jersey 07928
(Address of principal executive offices) (Zip Code)

Registrant's telephone number, including area code: (862) 904-8182

Check the appropriate box below if the Form 8-K filing is intended to simultaneously satisfy the filing obligation of the registrant under any of the following provisions (see General Instruction A.2. below):

☐ Written communications pursuant to Rule 425 under the Securities Act (17 CFR 230.425)
☐ Soliciting material pursuant to Rule 14a-12 under the Exchange Act (17 CFR 240.14a-12)
☐ Pre-commencement communications pursuant to Rule 14d-2(b) under the Exchange Act (17 CFR 240.14d-2(b))
☐ Pre-commencement communications pursuant to Rule 13e-4(c) under the Exchange Act (17 CFR 240.13e-4(c))

Securities registered pursuant to Section 12(b) of the Act:

Title of each class	Trading Symbol(s)	Name of each exchange on which registered
Common Stock	TNXP	The NASDAQ Capital Market

Indicate by check mark whether the registrant is an emerging growth company as defined in Rule 405 of the Securities Act of 1933 (§ 230.405 of this chapter) or Rule 12b-2 of the Securities Exchange Act of 1934 (§ 240.12b-2 of this chapter).

Emerging growth company ☐

If an emerging growth company, indicate by check mark if the registrant has elected not to use the extended transition period for complying with any new or revised financial accounting standards provided pursuant to Section 13(a) of the Exchange Act. ☐

Item 7.01 Regulation FD Disclosure.

On January 24, 2023, Tonix Pharmaceuticals Holding Corp. (the "Company") announced the publication of a paper entitled, "***Development of a rapid image-based high-content imaging screening assay to evaluate therapeutic antibodies against the monkeypox virus***," in the journal *Antiviral Research* (the "Paper"). A copy of the press release which discusses this matter is furnished hereto as Exhibit 99.01, and incorporated herein by reference. The Paper, which may contain nonpublic information, is filed as Exhibit 99.02 hereto and incorporated herein by reference.

The information in this Item 7.01 of this Current Report on Form 8-K, including Exhibits 99.01 and 99.02 attached hereto, shall not be deemed "filed" for purposes of Section 18 of the United States Securities Exchange Act of 1934 (the "Exchange Act") or otherwise subject to the liabilities of that section, nor shall they be deemed incorporated by reference in any filing under the United States Securities Act of 1933 or the Exchange Act, except as shall be expressly set forth by specific reference in such a filing.

Item 8.01 Other Events.

On January 24, 2023, the Company announced the publication of the Paper, which describes the development and optimization of two high-content image-based assays that were employed to screen for potential therapeutic antibodies against the monkeypox virus using surrogate poxviruses such as vaccinia virus, and highlights the Company's TNX-3400 product candidate, which includes antibodies to potentially prevent or treat monkeypox and smallpox. One assay detected viral proteins of vaccinia virus and was used to screen a large library of antibodies made against pox viruses. This assay identified several antibodies with the capacity to protect against vaccinia virus infection. The second assay specifically detected phenotype changes in cells, called syncytia, after infection and protection by therapeutics. The Company believes this technology may allow it to identify combination therapies for monkey pox and smallpox viruses. Data suggest that applying this technology has the potential to increase the throughput of screening novel antivirals to shorten the discovery time for antivirals..

Forward- Looking Statements

This Current Report on Form 8-K contains certain forward-looking statements within the meaning of Section 27A of the Securities Act of 1933 and Section 21E of the Securities Exchange Act of 1934 and Private Securities Litigation Reform Act, as amended, including those relating to the Company's product development, clinical trials, clinical and regulatory timelines, market opportunity, competitive position, possible or assumed future results of operations, business strategies, potential growth opportunities and other statement that are predictive in nature. These forward-looking statements are based on current expectations, estimates, forecasts and projections about the industry and markets in which we operate and management's current beliefs and assumptions.

These statements may be identified by the use of forward-looking expressions, including, but not limited to, "expect," "anticipate," "intend," "plan," "believe," "estimate," "potential," "predict," "project," "should," "would" and similar expressions and the negatives of those terms. These statements relate to future events or our financial performance and involve known and unknown risks, uncertainties, and other factors which may cause actual results, performance or achievements to be materially different from any future results, performance or achievements expressed or implied by the forward-looking statements. Such factors include those set forth in the Company's filings with the SEC. Prospective investors are cautioned not to place undue reliance on such forward-looking statements, which speak only as of the date of this press release. The Company undertakes no obligation to publicly update any forward-looking statement, whether as a result of new information, future events or otherwise.

Item 9.01 Financial Statements and Exhibits.

(d)

Exhibit No.	Description.
99.01	Press release of the Company, dated January 24, 2023
99.02	Development of a rapid image-based high-content imaging screening assay to evaluate therapeutic antibodies against the monkeypox virus
104	Cover Page Interactive Data File (embedded within the Inline XBRL document)

SIGNATURE

Pursuant to the requirement of the Securities Exchange Act of 1934, the registrant has duly caused this report to be signed on its behalf by the undersigned thereunto duly authorized.

TONIX PHARMACEUTICALS HOLDING CORP.

Date: January 24, 2023

By: /s/ Bradley Saenger
Bradley Saenger
Chief Financial Officer

Exhibit 99.01

Tonix Pharmaceuticals Announces Publication of Paper in *Antiviral Research* Highlighting the Company's Development of Monoclonal Antibody Therapeutics for Monkeypox and Smallpox

Data Represent Research and Development Work Being Conducted at Tonix's Infectious Disease R&D Center (RDC) in Frederick, Md.

CHATHAM, N.J., January 24, 2023 – Tonix Pharmaceuticals Holding Corp. (Nasdaq: TNXP) (Tonix or the Company), a clinical-stage biopharmaceutical company, today announced the publication of a paper entitled, "***Development of a rapid image-based high-content imaging screening assay to evaluate therapeutic antibodies against the monkeypox virus***," in the journal *Antiviral Research.* The publication describes the development and optimization of two high-content image-based assays that were employed to screen for potential therapeutic antibodies against the monkeypox virus using surrogate poxviruses such as vaccinia virus. The article highlights Tonix's TNX-3400 platform, which includes antibodies to potentially prevent or treat monkeypox and smallpox. The article can be accessed online at https://pubmed.ncbi.nlm.nih.gov/36592670/.

"These data represent the first wave of research and development conducted at our Infectious Disease R&D Center (RDC) in Frederick, Md," said Seth Lederman, M.D., Chief Executive Officer of Tonix Pharmaceuticals. "The RDC greatly enhances our ability to advance development of our pipeline of vaccines and therapeutics for infectious diseases. The TNX-3400 platform has promise for preventative and therapeutic monoclonal antibodies to treat monkeypox and smallpox."

"The article describes how we optimized and standardized two high-content high-throughput image-based assays. The first assay was a neutralizing assay which detected viral proteins of vaccinia virus and used the assay to screen a large library of antibodies made against pox viruses. The second assay specifically detected phenotype changes in cells, called syncytia, after infection and protection by therapeutics." said Sina Bavari, Ph.D., Executive Vice President of Tonix Pharmaceuticals and site director of the RDC. A critical component in assessing antibodies during pandemics requires the development of rapid but detailed methods to detect and quantitate the neutralization activity. "The neutralizing assay identified several antibodies with the capacity to protect against vaccinia virus infection. We believe this technology will allow us to identify combination therapies for monkeypox and smallpox viruses which was difficult to achieve before. Furthermore, our data suggest that applying this technology has the potential to increase the throughput of screening novel antivirals to shorten the discovery time for antivirals."

About TNX-3400

TNX-3400 is the term for series of monoclonal antibodies which bind to key components of pox viruses such as monkeypox and smallpox and protect cells and tissues from infection with these pandemic causing viruses. These antibodies are being developed as broad-spectrum antipox virus and to potentially prevent or treat monkeypox and smallpox infection.

Tonix Pharmaceuticals Holding Corp.*

Tonix is a clinical-stage biopharmaceutical company focused on discovering, licensing, acquiring and developing therapeutics to treat and prevent human disease and alleviate suffering. Tonix's portfolio is composed of central nervous system (CNS), rare disease, immunology and infectious disease product candidates. Tonix's CNS portfolio includes both small molecules and biologics to treat pain, neurologic, psychiatric and addiction conditions. Tonix's lead CNS candidate, TNX-102 SL (cyclobenzaprine HCl sublingual tablet), is in mid-Phase 3 development for the management of fibromyalgia with a new Phase 3 study launched in the second quarter of 2022 and interim data expected in the second quarter of 2023. TNX-102 SL is also being developed to treat Long COVID, a chronic post-acute COVID-19 condition. Tonix initiated a Phase 2 study in Long COVID in the third quarter of 2022 and expects interim data in the third quarter of 2023. TNX-1300 (cocaine esterase) is a biologic designed to treat cocaine intoxication and has been granted Breakthrough Therapy designation by the FDA. A Phase 2 study of TNX-1300 is expected to be initiated in the first quarter of 2023. TNX-1900 (intranasal potentiated oxytocin), a small molecule in development for chronic migraine, is expected to enter the clinic with a Phase 2 study in the first quarter of 2023. TNX-601 ER (tianeptine hemioxalate extended-release tablets) is a once-daily formulation of tianeptine being developed as a potential treatment for major depressive disorder (MDD) with a Phase 2 study expected to be initiated in the first quarter of 2023. Tonix's rare disease portfolio includes TNX-2900 (intranasal potentiated oxytocin) for the treatment of Prader-Willi syndrome. TNX-2900 has been granted Orphan Drug designation by the FDA. Tonix's immunology portfolio includes biologics to address organ transplant rejection, autoimmunity and cancer, including TNX-1500, which is a humanized monoclonal antibody targeting CD40-ligand (CD40L or CD154) being developed for the prevention of allograft and xenograft rejection and for the treatment of autoimmune diseases. A Phase 1 study of TNX-1500 is expected to be initiated in the first half of 2023. Tonix's infectious disease pipeline includes a vaccine in development to prevent smallpox and monkeypox, TNX-801, a next-generation vaccine to prevent COVID-19, TNX-1850, a platform to make fully human monoclonal antibodies to treat COVID-19, TNX-3600, and humanized anti-SARS-CoV-2 monoclonal antibodies, TNX-3800, recently licensed from Curia. TNX-801, Tonix's vaccine in development to prevent smallpox and monkeypox, also serves as the live virus vaccine platform or recombinant pox vaccine (RPV) platform for other infectious diseases. A Phase 1 study of TNX-801 is expected to be initiated in Kenya in the second half of 2023.

** All of Tonix's product candidates are investigational new drugs or biologics and have not been approved for any indication.*

This press release and further information about Tonix can be found atwww.tonixpharma.com.

Forward Looking Statements

Certain statements in this press release are forward-looking within the meaning of the Private Securities Litigation Reform Act of 1995. These statements may be identified by the use of forward-looking words such as "anticipate," "believe," "forecast," "estimate," "expect," and "intend," among others. These forward-looking statements are based on Tonix's current expectations and actual results could differ materially. There are a number of factors that could cause actual events to differ materially from those indicated by such forward-looking statements. These factors include, but are not limited to, risks related to the failure to obtain FDA clearances or approvals and noncompliance with FDA regulations; delays and uncertainties caused by the global COVID-19 pandemic; risks related to the timing and progress of clinical development of our product candidates; our need for additional financing; uncertainties of patent protection and litigation; uncertainties of government or third party payor reimbursement; limited research and development efforts and dependence upon third parties; and substantial competition. As with any pharmaceutical under development, there are significant risks in the development, regulatory approval and commercialization of new products. Tonix does not undertake an obligation to update or revise any forward-looking statement. Investors should read the risk factors set forth in the Annual Report on Form 10-K for the year ended December 31, 2021, as filed with the Securities and Exchange Commission (the "SEC") on March 14, 2022, and periodic reports filed with the SEC on or after the date thereof. All of Tonix's forward-looking statements are expressly qualified by all such risk factors and other cautionary statements. The information set forth herein speaks only as of the date thereof.

Contacts

Jessica Morris (corporate)

Tonix Pharmaceuticals
investor.relations@tonixpharma.com
(862) 904-8182

Olipriya Das, Ph.D. (media)
Russo Partners
Olipriya.Das@russopartnersllc.com
(646) 942-5588

Peter Vozzo (investors)
ICR Westwicke
peter.vozzo@westwicke.com
(443) 213-0505

Exhibit 99.02

Elsevier has created a Monkeypox Information Center in response to the declared public health emergency of international concern, with free information in English on the monkeypox virus. The Monkeypox Information Center is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its monkeypox related research that is available on the Monkeypox Information Center - including this research content - immediately available in publicly funded repositories, with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the Monkeypox Information Center remains active.

ARTICLE IN PRESS

Antiviral Research xxx (xxxx) xxx

Contents lists available at ScienceDirect

Antiviral Research

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

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

Krishna P. Kota [a], Natasza E. Ziółkowska [a], Jiayi Wei [a], Junzhong Peng [a], David Ordonez [a], Christy Raney [a], Jon Prigge [c], Jay W. Hooper [b], Mayanka Awasthi [a], Scott J. Goebel [a], Brian A. Zabel [d], Farooq Nasar [a], Seth Lederman [a], Sina Bavari [a,*]

[a] Tonix Pharmaceuticals Inc, Frederick, MD, USA
[b] United States Army Medical Institute of Infectious Diseases, Frederick, MD, USA
[c] Bioqual Inc., Rockville, MD, USA
[d] Curia Global Inc., Albany, NY, USA

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.

* Corresponding author.
E-mail address: sina.bavari@tonixpharma.com (S. Bavari).

https://doi.org/10.1016/j.antiviral.2022.105513
Received 9 December 2022; Received in revised form 21 December 2022; Accepted 22 December 2022
Available online 30 December 2022
0166-3542/© 2022 Elsevier B.V. All rights reserved.

Please cite this article as: Krishna P. Kota, *Antiviral Research*, https://doi.org/10.1016/j.antiviral.2022.105513

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,

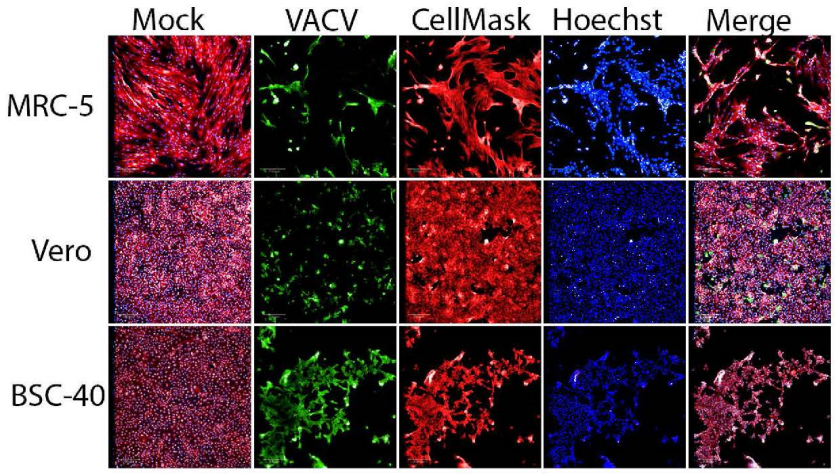


Fig. 1. High Content Imaging Assay Development for Vaccinia Virus
MRC-5, Vero, and BSC-40 cells were either mock-infected or infected with 0.05 moi of the VACV virus. After 2hr., cells were fixed and stained with VACV-specific polyclonal antibody conjugated with FITC to detect the virus-infected cells (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.

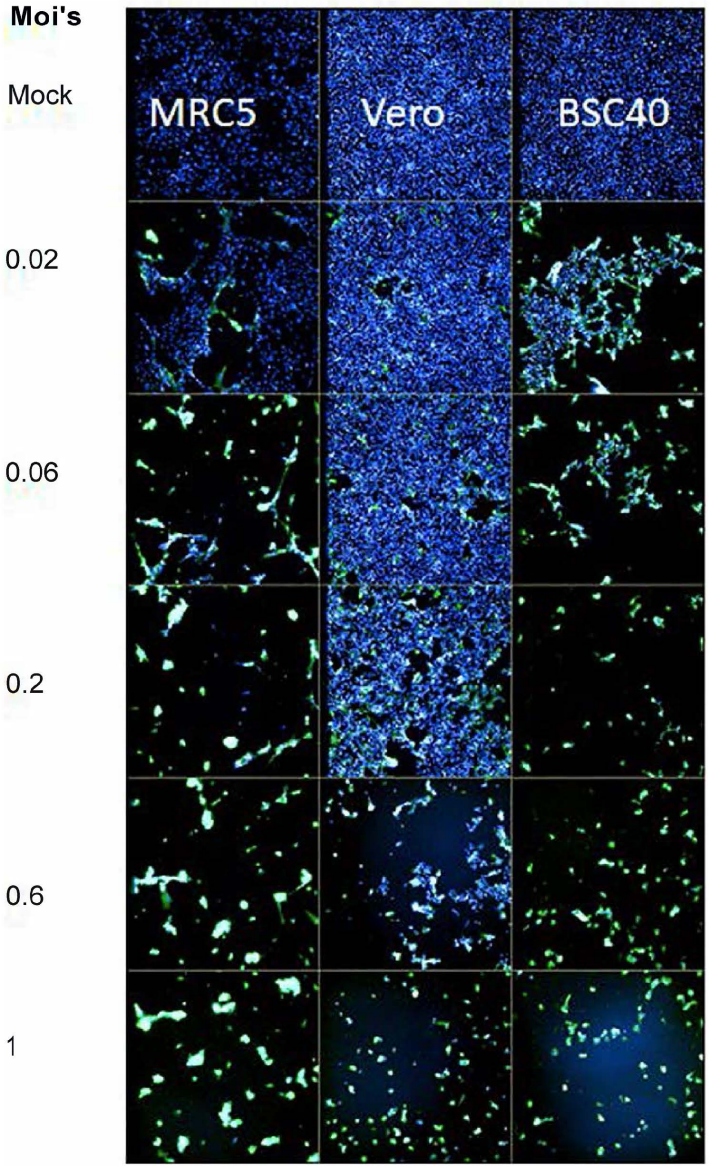


Fig. 2. Determining optimal moi for Vaccinia High Content Imaging Assay
MRC-5, Vero, and BSC-40 cells were either mock-infected or infected with multiple moi's of the virus. After 22 hr, cells were fixed and stained with VACV-specific polyclonal antibody conjugated with FITC to detect the virus (green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

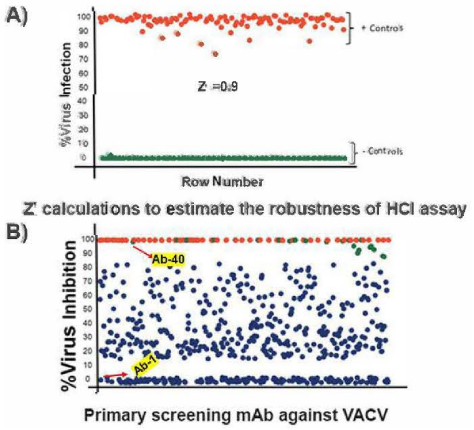


Fig. 3. Primary screening of neutralizing antibodies against VACV
BSC-40 cells were either mock-infected or infected with 0.05 moi of the VACV IHD-W strain of the virus. After 22 hr, cells were fixed and stained with VACV-specific polyclonal antibody conjugated with FITC to detect the virus. (a) Z′ was 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

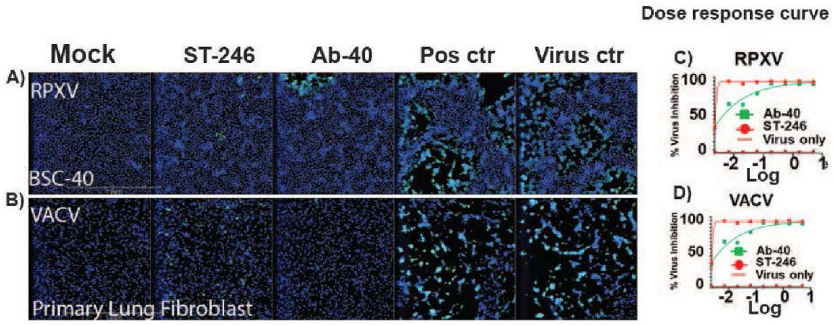


Fig. 4. Dose-response studies of Ab-40 against RPXV (BSC-40 cells) and VACV in primary lung fibroblast cells
BSC-40 cells or primary lung fibroblast cells were either mock-infected or infected with 0.05 moi of the RPXV or VACV (Virus ctr, respectively. Before cell infection, RPXV or VACV was incubated 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

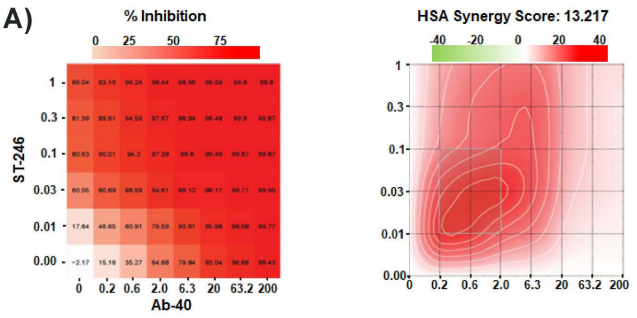
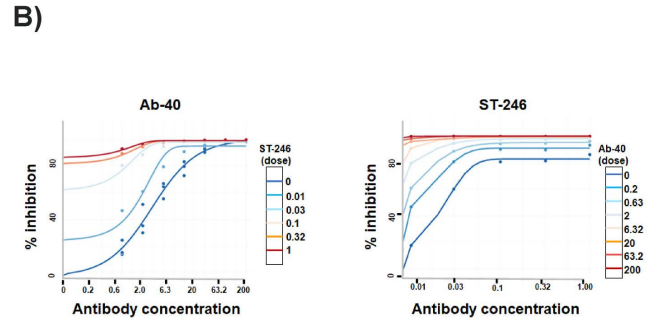
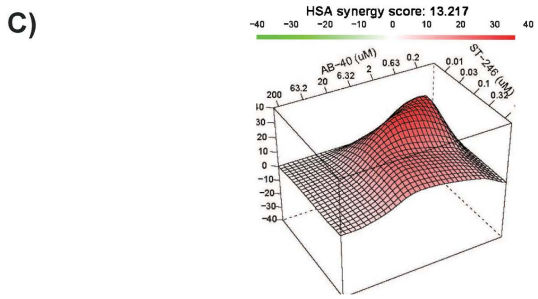


Fig. 5. Ab-40 and ST-246 drug combinations have synergistic effects on VACV infections in BSC-40 cells

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.

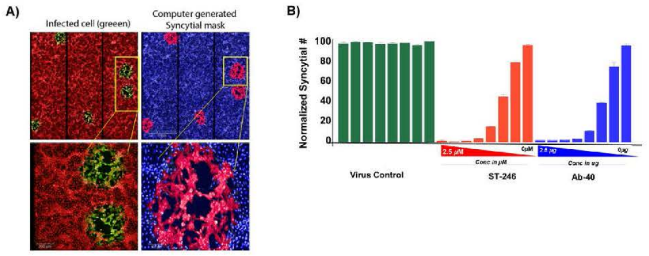


Fig. 6. Identification of syncytia using computer scripts in BSC40 cells infected with VACV
BSC-40 cells were treated with various doses of ST-246 or Ab-40, and mock-infected, infected with 0.05 moi of VACV. (a) After 22 hr of infection, cells were fixed and stained with VACV-specific polyclonal antibody conjugated with FITC to detect the virus (green staining), nuclear stain Hoechst (blue), and whole cell stain HCS CellMask™ 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.

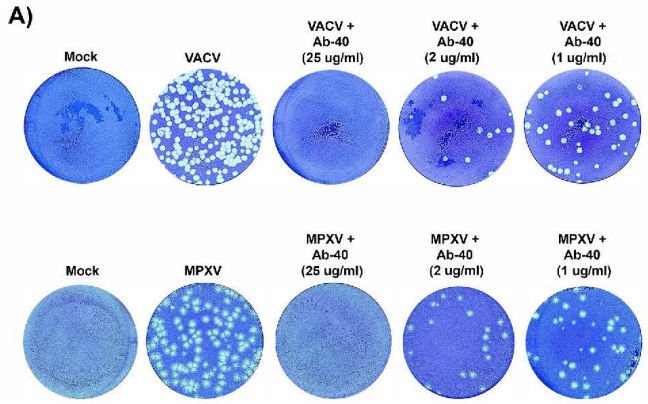


Fig. 7. Neutralization activity of Ab-40 against VACV and MPXV isolates. Micrographs of VACV or MPXV treated with Ab-40 (25, 2, 1 μg/mL) or untreated (mock) are shown (Fig. 7a).

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.

References

Albrecht, T., et al., 1996. Effects on cells. In: Baron, S. (Ed.), Medical Microbiology, Th. Galveston (TX).

Berthet, N., et al., 2021. Genomic history of human monkey pox infections in the Central African Republic between 2001 and 2018. Sci. Rep. 11 (1), 13085.

Bunge, E.M., et al., 2022. The changing epidemiology of human monkeypox-A potential threat? A systematic review. PLoS Neglected Trop. Dis. 16 (2), e0010141.

Crozier, I., et al., 2022. The evolution of medical countermeasures for Ebola virus disease: lessons learned and next steps. Vaccines 10 (8).

Gates, I., et al., 2015. Development of a high-content orthopoxvirus infectivity and neutralization assays. PLoS One 10 (10), e0138836.

Gu, X., et al., 2022. Protective human anti-poxvirus monoclonal antibodies are generated from rare memory B cells isolated by multicolor antigen tetramers. Vaccines 10 (7).

Inigo Martinez, J., et al., 2022. *Monkeypox outbreak predominantly affecting men who have sex with men*, Madrid, Spain, 26 April to 16 June 2022. Euro Surveill. 27 (27).

Jessie, B., Dobrovolny, H.M., 2021. The role of syncytia during viral infections. J. Theor. Biol. 525, 110749.

Kaler, J., et al., 2022. Monkeypox: *a comprehensive Review of transmission, pathogenesis, and manifestation*. Cureus 14 (7), e26531.

Kielian, M., 2014. Mechanisms of virus membrane fusion proteins. Annu Rev Virol 1 (1), 171–189.

Kombe Kombe, A.J., et al., 2021. Potent molecular feature-based neutralizing monoclonal antibodies as promising therapeutics against SARS-CoV-2 infection. Front. Mol. Biosci. 8, 670815.

Lefkowitz, E.J., Wang, C., Upton, C., 2006. Poxviruses: past, present and future. Virus Res. 117 (1), 105–118.

Mas, V., Melero, J.A., 2013. Entry of enveloped viruses into host cells: membrane fusion. Subcell. Biochem. 68, 467–487.

Mudhasani, R., et al., 2013. IFITM-2 and IFITM-3 but not IFITM-1 restrict Rift Valley fever virus. J. Virol. 87 (15), 8451–8464.

Mudhasani, R., et al., 2015. High-content image-based screening of a signal transduction pathway inhibitor small-molecule library against highly pathogenic RNA viruses. J. Biomol. Screen 20 (1), 141–152.

Nalca, A., et al., 2008. Evaluation of orally delivered ST-246 as postexposure prophylactic and antiviral therapeutic in an aerosolized rabbitpox rabbit model. Antivir. Res. 79 (2), 121–127.

Panchal, R.G., et al., 2010. Development of high-content imaging assays for lethal viral pathogens. J. Biomol. Screen 15 (7), 755–765.

Pelegrin, M., Naranjo-Gomez, M., Piechaczyk, M., 2015. Antiviral monoclonal antibodies: can they Be more than simple neutralizing agents? Trends Microbiol. 23 (10), 653–665.

Sherwat, A., et al., 2022. Tecovirimat and the treatment of monkeypox - past, present, and future considerations. N. Engl. J. Med. 387 (7), 579–581.

Silva, N.I.O., et al., 2020. Here, there, and everywhere: the wide host range and geographic distribution of zoonotic orthopoxviruses. Viruses 13 (1).

Ward, D.J., et al., 2015. Trends in clinical development timeframes for antiviral drugs launched in the UK, 1981-2014: a retrospective observational study. BMJ Open 5 (11), e009333.

Zaeck, LM, Lamers, MM, Verstrepen, BE, Bestebroer, TM, van Royen, ME, Götz, H, Shamier, MC, van Leeuwen, LPM, Schmitz, KS, Alblas, K, van Efferen, S, Bogers, S, Scherbeijn, S, Rimmelzwaan, GF, van Gorp, ECM, Kooprmans, MPG, Haagmans, BL, GeurtsvanKessel, CH, de Vries, RD, 2022 Oct 18. Low levels of monkeypox virus-neutralizing antibodies after MVA-BN vaccination in healthy individuals. Nat Med. https://doi.org/10.1038/s41591-022-02090-w. Epub ahead of print. PMID: 36257333.

Radoshitzky, SR, Pegoraro, G, Chi, XO, D Ng, L, Chiang, CY, Jozwick, L, Clester, JC, Cooper, CL, Courier, D, Langan, DP, Underwood, K, Kuehl, KA, Sun, MG, Cai, Y, Yi, SQ, Buck, R, Zamani, R, Kota, K, Kuhn, JH, Bavari, S, 2016 Mar 31. siRNA Screen Identifies Trafficking Host Factors that Modulate Alphavirus Infection. PLoS Pathog 12 (3), e1005466. https://doi.org/10.1371/journal.ppat.1005466. PMID: 27031835; PMCID: PMC4816540.