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SHORT COMMUNICATION

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Trifluridine for treatment of mpox infection in drug combinations in ophthalmic cell models

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Abstract

The Mpox virus can cause severe disease in the susceptible population with dermatologic and systemic manifestations. Furthermore, ophthalmic manifestations of mpox infection are well documented. Topical trifluridine (TFT) eye drops have been used for therapy of ophthalmic mpox infection in patients, however, its efficacy against mpox virus infection in this scenario has not been previously shown. In the present study, we have established ophthalmic cell models suitable for the infection with mpox virus. We show, that TFT is effective against a broad range of mpox isolates in conjunctival epithelial cells and keratocytes. Further, TFT remained effective against a tecovirimat-resistant virus strain. In the context of drug combinations, a nearly additive effect was observed for TFT combinations with brincidofovir and tecovirimat in conjunctival epithelial cells, while a slight antagonism was observed for both combinations in keratocytes. Altogether, our findings demonstrate TFT as a promising drug for treatment of ophthalmic mpox infection able to overcome tecovirimat resistance. However, conflicting results regarding the effect of drug combinations with approved compounds warrant close monitoring of such use in patients.

KEYWORDS

drug combination, monkeypox, ophthalmic infection, tecovirimat resistance, trifluridine

1 | INTRODUCTION

Most patients infected with mpox (formerly known as monkeypox) virus experience a self limiting illness with a case-fatality rate of less than 0.1% for clade IIa and IIb, while infection with clade I results in higher case fatality rates of roughly 1%–12%. Children, pregnant women and immunocompromised individuals, including people with uncontrolled HIV infection, are at higher risk of severe disease.[1]

Although mpox virus causes mainly dermatologic and systemic manifestations, a broad spectrum of ophthalmic manifestations such as conjunctivitis, keratitis, corneal scarring or even visual impairment have been documented.[2] In endemic countries, conjunctivitis was reported to occur in 23.1% of cases and keratitis was documented in 3%–4% of patients.[3] In the 2022–2023 outbreak, ocular involvement was reported between 0.8% and 2.9% of cases. Importantly, the eye is considered a "special hazard" anatomic site by the centers for

disease control and prevention; therefore, ophthalmic manifestations of mpox infection are an indication for antiviral therapy.[2]

Topical trifluridine (TFT) has been used in number of mpox cases during 2022–2023 outbreak. Furthermore, TFT was used in combination with tecovirimat.[4,5] Drug combinations have the potential to significantly decrease the risk of the development of drug resistance and can act synergistically, thereby increasing their antiviral efficacy. For this matter, preclinical testing of drug combinations might be helpful to select useful combinations for clinical use.[6]

Here, we have investigated TFT efficacy against mpox virus in the context of cell types involved in ophthalmic presentation of mpox infection, tecovirimat resistance and drug combinations.

2 | MATERIALS AND METHODS CELLS

Human dermal fibroblasts (DF) were cultured in Dulbecco's Modified Eagle Medium with 4.5 g/mL glucose supplemented with 5% fetal bovine serum (FBS) and 100 IU/mL penicillin. Human primary keratocytes were obtained from ProVitro and cultured in Iscove's Modified Dulbecco's Medium supplemented with 100 µg/mL streptomycin, 100 IU/mL penicillin, 4 mM glutamine and 10% FBS. Immortalized human conjunctival epithelial cells were obtained from BioCat cultured in epithelial cell medium (ScienCell #4101) with 5% FBS. All cell lines were regularly authenticated by short tandem repeat analysis and tested for mycoplasma contamination.

2.1 | Virus isolates

Mpox virus clinical isolates were obtained as previously described.[7] Virus stocks were prepared by infecting HaCaT cells for 72 h and subsequently frozen at −80°C until further processing. After thawing, supernatants were centrifuged at 150 g for 10 min and virus stocks stored at −80°C. Virus titers were determined as TCID50/mL using confluent DF in 96-well microtiter plates. The tecovirimat-resistant virus strain, MPXV1^{R}Teco, was acquired and characterized as previously described.[8]

2.2 | Virus growth kinetics

Confluent cell layers in 96-well plates were infected with the MPXV1 isolate at mulitplicity of infection (MOI) 0.01 and incubated at 37°C. The number of mpox-positive cells was assessed by immunofluorescent labeling after 24, 48 and 72 h postinfection. Briefly, fixed cells were blocked with 2% BSA and 5% goat serum. Staining was performed using antivaccinia virus antibody (1:4000 dilution, #ab35219 Abeam) and secondary Alexa Fluor™ 647 antibody (1:1000 dilution, #A-21246 Thermo Fisher Scientific) and DAPI (1:1000 dilution). Cells were imaged and analyzed using Operetta CLS High Content Analysis System (PerkinElmer) and Harmony 4.9 software.

2.3 | Dose response antiviral assay

Confluent cells in 96-well plates were treated with drug dilutions followed by infection with mpox virus isolates at MOI 0.01 for 48 h. Subsequently, cells were fixed with acetone:methanol (40:60) solution and immunostaining was performed using an antiVaccinia Virus antibody (1:4000 dilution, #ab35219 Abeam), which was detected with a peroxidase-conjugated anti-rabbit secondary antibody (1:1000, Dianova), followed by addition of AEC substrate. The MPVX positive area was scanned and quantified by the Bioreader® 7000-F-Z-I microplate reader (Biosys). The results are expressed as the percentage of inhibition relative to virus control which received no drug.

2.4 | Drug combination assay

To evaluate the antiviral activity of TFT in combination with tecovirimat or brincidofovir, the compounds were tested alone and in fixed combinations at 1:2 dilutions as previously described.[8] Briefly, the calculation of combination indexes (CIs) was performed using the software CalcuSyn (Biosoft) based on the method of Chou and Talalay.[9] The weighted average CI value (CI_{wt}:) was calculated according to the formula: CI_{wt}: [CI_{50} + 2 CI1s + 3Cloo + 4Clgs]/10. CI_{wt}: values were calculated for mutually exclusive interactions where CI_{wt}: <0.8 indicates synergism, CI_{wt}: between 0.8 and 1.2 indicates additive effects, and CI_{wt}: >1.2 suggest antagonism.

2.5 | Statistics

The results are expressed as the mean ± standard deviation of the number of biological replicates indicated in figure legends. The statistical significance is depicted directly in graphs and the statistical test used for calculation of p values is indicated in the figure legends. GraphPad Prism 9 was used to determine the IC_{50} values.

3 | RESULTS

3.1 | TFT efficacy in ophthalmic cell models

First, we assessed the infection rate of mpox virus in two ophthalmic cell types: primary human keratocytes and immortalized human conjunctival epithelial cells (HConEpiC), in comparison to primary human DF. Immunostaining of mpox virus in DF, keratocytes and HConEpiC has shown comparable numbers of infected cells over 72 h (Figure 1A). No decrease in cell number (DAPI+ cells) has been observed for DF and keratocytes during mpox infection, whereas a cytopathic effect could be observed in HConEpiC at 48 and 72 h with a significant decrease in cell number at both time points. These

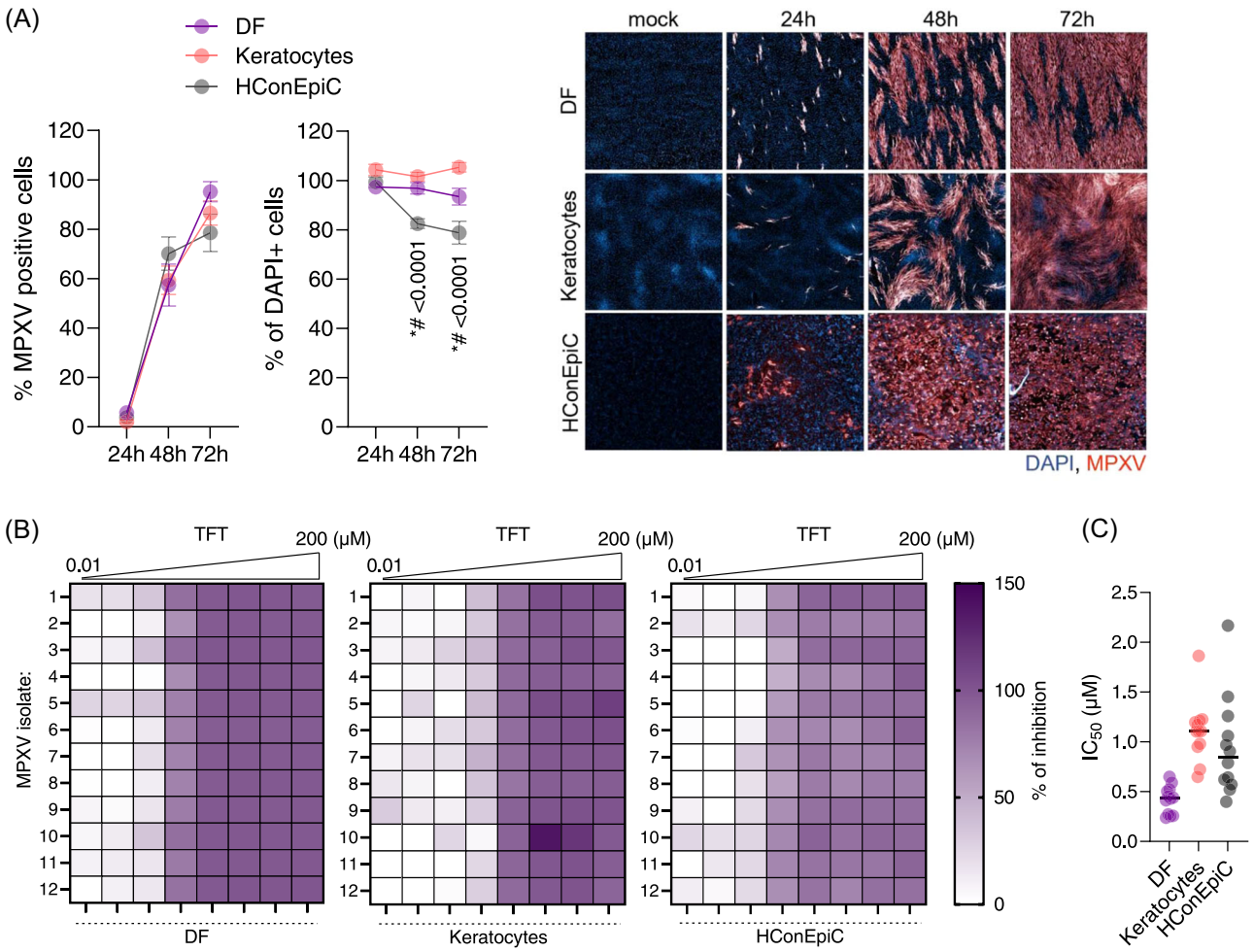

FIGURE 1 Trifluridine efficacy against mpox in ophthalmologically relevant cell models. (A) Quantification of virus growth kinetic (left) and cell number (right) in DF, keratocytes and HConEpiC. (B) Heat-map of dose–response of TFT against 12 different mpox isolates. (C) Comparison of IC_{50} values. DF, dermal fibroblasts; HConEpiC, human conjunctival epithelial cells. *DF versus HConEpiC, #Keratocytes versus HConEpiC.

results are in line with previously published data showing that the mpox virus causes more pronounced cytopathogenic effect in epithelial cells than in DF,[7] most likely due to differences in the host cell response.

Next, we evaluated the antiviral efficacy of TFT against 12 well-characterized mpox virus isolates (from the 2022 outbreak)[7] in DF, keratocytes and HConEpiC (Figure 1B). TFT dose dependently inhibited all mpox isolates in all three cell types. However, an intercell type variation in efficacy of TFT could be observed (Figure 1C). In DF, TFT inhibited mpox virus with IC_{50} values comparable to previously described TFT efficacy in Vero cells[10] as well as against other orthopoxviruses.[11] In contrast, TFT displayed twofold lower potency in keratocytes (mean IC_{50} values 1.11 µM) and HConEpiC (mean IC_{50} values 0.95 µM) in comparison to DF (mean IC_{50} values 0.42 µM). Possible explanations for this lower activity include different levels of TFT activating enzymes, such as thymidine kinase, in these cell types.[12] and differences in the metabolic inactivation of TFT by thymidine phosphorylase.[13] No cytotoxicity of TFT was observed for any of the tested cell types.

3.2 | TFT efficacy against tecovirimat-resistant strains

Rapid emergence of tecovirimat resistance in mpox patients has been documented.[14] The resistance has been attributed to several mutations located in the *F13L* gene, a target of tecovirimat (Figure 2A). We have recently selected a tecovirimat-resistant mpox strain, MPXV1^{R}Teco, by one-round selection in cell culture in the presence of 4 µM of tecovirimat.[8] Importantly, the two mutations N267D and I372N detected in MPXV1^{R}Teco were also present in patients from the 2022 outbreak that received tecovirimat treatment and had shown phenotypically confirmed resistance[15] (Figure 2A).

We have tested the sensitivity of MPXV1^{R}Teco to TFT therapy. Whereas tecovirimat failed to inhibit MPXV1^{R}Teco, TFT prevented

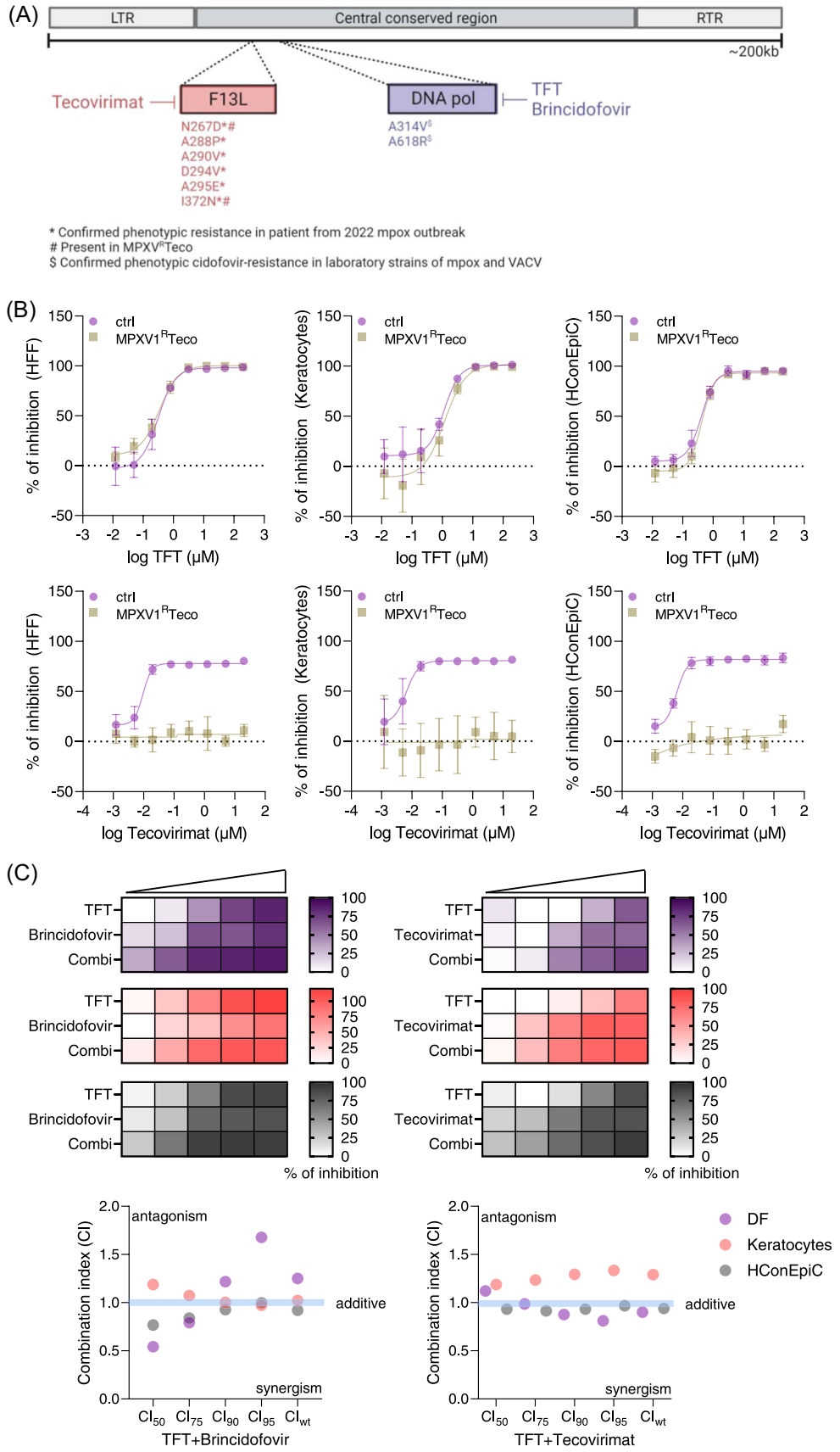

FIGURE 2 (See caption on next page).

MPXV1^{R}Teco infection to the same extent as control virus (passaging control) in all three cell types (Figure 2B).

3.3 | Evaluation of drug combinations with TFT

However, studies to identify synergistic combinations and to flag antagonistic combinations are necessary before their application in a clinical setting.[6] Due to a lack of preclinical evaluation studies for the antiviral efficacy of TFT in combination with tecovirimat/brincidofovir, we have performed drug combination assays of these compounds in different cell types (Figure 2C). TFT displayed a slight antagonism in combination with brincidofovir both in DF (CI_{wt}: 1.25) and keratocytes (CI_{wt}: 1.19). In HConEpiC, this combination showed a nearly additive effect (CI_{wt}: 0.92). Combination of TFT and tecovirimat displayed a nearly additive effect in DF (CI_{wt}: 0.9) and HConEpic (CI_{wt}: 0.94), whereas it was found moderately antagonistic in keratocytes (CI_{wt}: 1.29).

We have also tested the combination of tecovirimat and brincidofovir in DF (Suppl. Figure 1). Although, this combination has been previously reported to display synergistic activity against for vaccinia and cowpox viruses,[16] we observed additive effects against mpox virus. These discrepancies highlight that drug effects can differ among experimental systems, emphasizing the importance of using clinical virus isolates in physiologically relevant cellular models.

4 | DISCUSSION

A global mpox virus outbreak in 2022–2023 brought attention to a neglected zoonotic pathogen, which has been almost exclusively circulating in regions of West and Central Africa over the past decades.[1] Mounting evidence indicates that this outbreak caused by mpox clade IIb was sustained through human-to-human transmission, including sexual contact. The rapid spread of mpox virus among men having sex with men uncovered a global susceptible population, in which a high proportion of people are living with HIV.[1,17] In this regard, 38%–50% of people with confirmed mpox infection in 2022–2023 outbreak had diagnosed HIV. Higher disease severity, worse clinical outcomes and higher mortality was observed in mpox-infected patients with more advanced HIV.[17]

Two drugs that received emergency use authorization by the FDA against smallpox virus, tecovirimat and brincidofovir, have been used for the treatment of mpox infection.[1] Worryingly, an increasing number of studies have reported on the development of tecovirimat resistance in mpox-infected patients during the recent outbreak.[14,15] In addition, TFT eye drops have been applied in a clinical setting for therapy of ophthalmic mpox infection.[4] TFT is a nucleoside analog, which has been approved for other ophthalmic conditions like herpes simplex keratitis.[2] A recent in vitro study demonstrated the antiviral efficacy of TFT against mpox infection in Vero cells.[10] However, the efficacy of antiviral drugs may differ between laboratory cell lines and primary cells derived from relevant tissues.[17,18] Here, we show that TFT inhibits infection of a broad range of mpox isolates in cell models relevant for ophthalmic manifestations, albeit with lower efficacy than in dermal cells.

For the treatment of ophthalmic manifestations of mpox virus, a combination of TFT and tecovirimat has been applied.[4] Simultaneous use of TFT with tecovirimat and brincidofovir resulted in additive to slightly antagonistic effects. In DF, the combination of TFT with tecovirimat was nearly additive while combination with brincdofovir was slightly antagonistic. Whereas a nearly additive effect for both combinations was observed in HConEpic cells, a slight to moderate antagonism was detected for these combinations in keratocytes. Since synergy is expected rather for drugs with different targets,[6] these results are not surprising for combination of TFT and brincidofovir, which both inhibit DNA synthesis (Figure 2A). In contrast, TFT and tecovirimat target different parts of the viral life cycle. Hence, other processes such interactions modifying drug metabolism may contribute to the observed phenotype.[19] Altogether, the slightly antagonistic effects detected for both TFT combinations in certain cell types warrant further preclinical evaluation in animal models and close monitoring of such combinations in clinical trials.

Although drug antagonism is not desirable, the drug combinations might remain beneficial in preventing the selection of drug resistant mutations.[20] In the context of mpox therapy, mounting evidence shows emergence of tecovirimat-resistant mpox strains accompanied by therapy failure in patients.[15] Here we show that TFT remains highly effective against a tecovirimat-resistant mpox strain.

In conclusion, TFT is a potent inhibitor of mpox virus infection in both dermal and ophthalmic cell models, which also displayed activity against tecovirimat-resistant virus strains.

AUTHOR CONTRIBUTIONS

Conceptualization: Jindrich Cinatl, Denisa Bojkova. *Methodology/Investigation*: Marco Bechtel, Philipp Reus, Melanie Ott, Florian Rothweiler, Jindrich Cinatl, and Denisa Bojkova. *Formal analysis*: Marco Bechtel, Philipp Reus, Jindrich Cinatl, and Denisa Bojkova. *Visualization*: Denisa Bojkova. *Resources*: Jindrich Cinatl, Sandra Ciesek, and Denisa Bojkova. *Writing—original draft*: Jindrich Cinatl, Martin Michaelis, and Denisa Bojkova. *Writing—reviewing*: All authors. *Funding*: Jindrich Cinatl, Sandra Ciesek, and Denisa Bojkova.

FIGURE 2 Drug resistance and drug combinations of TFT in ophthalmic cell models. (A) Schematic depiction of mpox genome with drug targets and respective mutations related to the drug resistance. (B) Dose–response graph of antiviral efficacy of TFT (upper panel) and tecovirimat (lower panel) against tecovirimat resistant variant MPXV1^{R}Teco and ctrl (passaging control) in different cell types. (C) Drug combination assay in different cell types. Left panel: Heat-map of inhibition rate of TFT in combination with brincidofovir and respective combination indexes. Right panel: Heat-map of inhibition rate of TFT in combination with tecovirimat and respective combination indexes.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data are available upon reasonable request from the corresponding author.

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REFERENCES

1. Mitjà O, Ogoina D, Titanji BK, et al. Monkeypox. *Lancet*. 2023;401:60-74.
2. Kaufman AR, Chodosh J, Pineda R. Monkeypox virus and ophthalmology - a primer on the 2022 monkeypox outbreak and monkeypox-related ophthalmic disease. *JAMA Ophthalmol*. 2023;141:78-83.
3. Hughes C, McCollum A, Pukuta E, et al. Ocular complications associated with acute monkeypox virus infection DRC. *Int J Infect Dis*. 2014;21:276-277.
4. Perzia B, Theotoka D, Li K, et al. Treatment of ocular-involving monkeypox virus with topical trifluridine and oral tecovirimat in the 2022 monkeypox virus outbreak. *Am J Ophthalmol Case Rep*. 2023;29:101779.
5. Abdelaal A, Serhan HA, Mahmoud MA, Rodriguez-Morales AJ, Sah R. Ophthalmic manifestations of monkeypox virus. *Eye*. 2023;37:383-385.
6. Shyr ZA, Cheng YS, Lo DC, Zheng W. Drug combination therapy for emerging viral diseases. *Drug Discov Today*. 2021;26:2367-2376.
7. Boikova D, Marco B, Tamara R. Drug sensitivity of currently circulating Mpox viruses. *N Engl J Med*. 2023;388:279-281.
8. Boikova D, Zöller N, Tietgen M, et al. Repurposing of the antibiotic nitroxoline for the treatment of mpox. *J Med Virol*. 2023;95:e28652.
9. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev*. 2006;58:621-681.
10. Hishiki T, Morita T, Akazawa D, et al. Identification of IMP dehydrogenase as a potential target for anti-Mpox virus agents. *Microbiol Spectr*. 2023;11(4):e0056623.
11. Kern ER. In vitro activity of potential anti-poxvirus agents. *Antiviral Res*. 2003;57(1-2):35-40.
12. Kataoka Y, Iimori M, Niimi S, et al. Cytotoxicity of trifluridine correlates with the thymidine kinase 1 expression level. *Sci Rep*. 2019;9:7964.
13. Lyseng-Williamson KA, Burness CB, Duggan ST. Trifluridine/tipiracil in metastatic colorectal cancer: a guide to its use. *Drugs Ther Perspect*. 2017;33:110-118.
14. Alarcón J, Kim M, Terashita D, et al. An Mpox-related death in the United States. *N Engl J Med*. 2023;388:1246-1247.
15. Garrigues JM, Hemarajata P, Karan A, et al. Identification of tecovirimat resistance-associated mutations in human monkeypox virus - Los Angeles county. *Antimicrob Agents Chemother*. 2023;67(7):e0056823.
16. Quenelle DC, Prichard MN, Keith KA, et al. Synergistic efficacy of the combination of ST-246 with CMX001 against orthopoxviruses. *Antimicrob Agents Chemother*. 2007;51:4118-4124.
17. Mitjà O, Alemany A, Marks M, et al. Mpox in people with advanced HIV infection: a global case series. *Lancet*. 2023;401:939-949.
18. Mumtaz N, Jimmerson LC, Bushman LR, et al. Cell-line dependent antiviral activity of sofosbuvir against Zika virus. *Antiviral Res*. 2017;146:161-163.
19. Jia J, Zhu F, Ma X, Cao ZW, Li YX, Chen YZ. Mechanisms of drug combinations: interaction and network perspectives. *Nat Rev Drug Discov*. 2009;8:111-128.
20. Chait R, Craney A, Kishony R. Antibiotic interactions that select against resistance. *Nature*. 2007;446(7136):668-671.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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