Bisbenzimide compounds inhibit replication of prototype and pandemic potential poxviruses

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Abstract

We previously identified the bisbenzimide Hoechst 33342 (H42) as potent multi-stage inhibitor of the prototypic poxvirus, vaccinia virus (VACV) and several parapoxviruses. A recent report showed that novel bisbenzimide compounds similar in structure to H42 could prevent human cytomegalovirus replication. Here we assessed whether these compounds could also serve as poxvirus inhibitors. Using virological assays, we show that these bisbenzimide compounds inhibit VACV spread, plaque formation and the production of infectious progeny VACV with relatively low cell toxicity. Further analysis of the VACV lifecycle indicated that the effective bisbenzimide compounds had little impact on VACV early gene expression, but inhibited VACV late gene expression and truncated the formation of VACV replication sites. Additionally, we found that bisbenzimide compounds including H42 can inhibit both mpox (monkeypox) and a VACV mutant resistant to the widely used anti-poxvirus drug TPOXX (Tecovirimat). Therefore, the tested bisbenzimide compounds were inhibitors of both prototypic and pandemic potential poxviruses and could be developed for use in situations where anti-poxvirus drug resistance may occur. Additionally, these data suggest that bisbenzimide compounds may serve as broad activity antiviral compounds, targeting diverse DNA viruses such as poxviruses and betaherpesviruses.

Importance

The 2022 mpox (monkeypox) outbreak served as a stark reminder that due to the cessation of smallpox vaccination over 40 years ago, most of the human population remains susceptible to poxvirus infection. With only two antivirals approved for treatment of smallpox infection in humans, the need for additional anti-poxvirus compounds is evident. Having shown that the bisbenzimide H33342 is a potent inhibitor of poxvirus gene expression and DNA replication, here we extend these findings to include a set of novel bisbenzimide compounds that show anti-viral activity against mpox and a drug-resistant prototype poxvirus mutant. These results suggest that further development of bisbenzimides for treatment of pandemic potential poxviruses is warranted.
Introduction

The 2022 mpox (monkeypox) outbreak served as a potent reminder of the pandemic potential of poxviruses (1). While existing smallpox vaccines (Imvanex and ACAM2000) provide good protection against mpox infection (2), the cessation of smallpox vaccination has left the global population susceptible to infection by many existing poxviruses for which vaccine efficacy is unknown.

To supplement vaccination, novel drug strategies are required to treat poxvirus infection. In the United States there are only two drugs approved for human treatment of smallpox: Tembexa (also known as brincidofovir) and TPOXX (also known as Tecovirimat or ST-246) (3–6). In the United Kingdom and European Union TPOXX is the only drug approved for the treatment of orthopoxvirus infections including smallpox, mpox, vaccinia and cowpox. In 2022, TPOXX was approved for treatment of mpox under the US CDCs expanded access investigational new drug protocol (CDC 2023). Despite its efficacy against many poxviruses including mpox (7), a single point mutation within the poxviral genome was sufficient to give rise to TPOXX resistance (4). Therefore, the search for additional anti-poxviral compounds is required.

We have shown that the bisbenzimide Hoechst 33342 (H42) is an effective inhibitor of human and animal poxviruses in vitro. H42 was found to inhibit VACV DNA replication and late gene expression of the prototype poxvirus, vaccinia virus (VACV), at low micromolar concentrations (8).

H42 is a fluorescent dye that binds within the minor groove of double-stranded DNA, preferentially to AT-rich regions (9–13). As the anti-poxvirus efficacy of H42 correlated with its membrane permeability and accessibility to the VACV DNA, our data suggested a model in which H42 blocked DNA replication by coating cytoplasmic VACV DNA genomes (8).

A recent report by Finardi and co-workers showed that another bisbenzimide compound, RO-90-7501 (2’-(4-aminophenyl)-[2,5’-bi-1H-benzimidazol]-5-amine) (referred to here as R90) and several of its analogues produced by MRC-Technology (MRT, now LifeArc), could inhibit replication
of human cytomegalovirus (HCMV). This was likely to occur by binding to the HCMV DNA genome and inhibiting production of HCMV capsids containing genomes (14). These compounds included MRT00210423, MRT00210424, MRT00210425, MRT00210426 and MRT00210427 (14) (referred to here as M23, M24, M25, M26, and M27). Given the potential broad-spectrum antiviral activity of this extended class of bisbenzimide compounds against viruses with DNA genomes, we set out to determine if R90 or the MRT compounds were effective inhibitors of both prototype and pandemic potential poxvirus replication.

Results

MRT compounds M23-M26 display anti-poxvirus activity. R90 and the MRT compounds are structurally similar to H42, with differences in the terminal groups of the compounds and/or substitution of methyl groups for amine groups (Fig. 1A). In previous studies, the compounds R90, M23, M24 and M25 which retained DNA binding activity inhibited HCMV replication (14). It was noted that M23 was a more effective inhibitor of HCMV replication than R90, which may be due to its greater ability to interact with DNA (14). M26 and M27 were ineffective inhibitors of HCMV replication, likely due to their inability to interact with DNA (14).

To investigate their potential as anti-poxvirus agents we first tested the ability of R90 and the MRT compounds to block VACV cell-to-cell spread. During infection poxviruses produce two forms of infectious particles: mature virions (MVs) and extracellular virions (EVs). MVs are more abundant and mediate host-to-host transmission, while EVs contribute to intra-host and cell-to-cell virus spread (15). The VACV replication cycle is efficient, newly assembled EVs are released by 8 hpi and intracellular replication is complete within 24 h (16). With this mind HeLa cells were infected with VACV L-EGFP, a VACV recombinant expressing EGFP from a late viral promoter, at MOI 0.1 to obtain <30% primary infection of the monolayer. To allow sufficient time for virus cell-to-cell spread infection was allowed to proceed for 24 h in the absence or presence of R90 or the MRT compounds.
at increasing concentrations. H42 and TPOXX, a tricyclononene carboxamide that inhibits the production of EVs, were included as controls (4). At 24 hpi samples were analysed by flow cytometry for the number of EGFP expressing cells (Fig. 1B; black lines).

As expected, TPOXX and H42 controls prevented virus spread at low to sub-micromolar concentrations. They lowered the percentage of EGFP expressing cells by 70% and 80% respectively at concentrations as low as 0.4 µM. At low micromolar concentrations R90 showed limited activity, only blocking VACV spread by 20% at 40 µM. M23, M24 and M25 showed concentration dependent inhibition of VACV spread. While M23 inhibitory activity plateaued between 10 µM and 40 µM, M24 and M25 completely blocked infection at 40 µM. M26 and M27, which potentially lack DNA binding activity (14) had limited or no obvious anti-VACV activity. M26 displayed modest dose-dependent inhibition of VACV spread at higher concentrations (20-40 µM) while M27 showed no anti-VACV activity.

To ensure the observed anti-viral effects were not due to cellular cytotoxicity caused by the compounds, we measured uninfected cell viability in the presence of each by assaying the ability of cells to metabolise the salt WST-1. Consistent with previous reports, R90 and the MRT compounds had no impact on cell viability at the concentrations used, while H42 displayed toxicity at higher concentrations outside of the effective range of VACV inhibition (Fig. 1B; red lines) (8, 14).

The half maximal inhibitory concentration (IC50) and half maximal cell cytotoxicity concentration (CC50) measurements indicate that H42 and the MRT compounds M23-M25 display anti-poxvirus activity, without obvious cellular cytotoxicity (Fig. 1). Compounds R90, M26 and M27 displayed poor or no anti-VACV activity, which may be due to factors that include their poor ability to associate with DNA.

**MRT compounds M23, M24 and M25 reduce VACV yield and block plaque formation.** Having determined that R90 and the MRT compounds have varying effects on VACV spread, we next assessed their ability to prevent virus production. The effective concentration against VACV for each
compound was determined from the cell-to-cell spread assays shown in Figure 1. These compound concentrations were used in subsequent experiments, including virus production assays (see Figures and Figure Legends). HeLa cells were infected with WT VACV at an MOI of 1 in the presence of R90, M23, M24, M25, M26 or M27. Infection in the presence of H42 served as a control for inhibition of virus production. At 24 hpi cells were harvested and the infectious virus yield determined by plaque assay (Fig. 2A). Similar to the results of the cell-to-cell spread assay (Fig. 1), M27 was ineffective and did not impact VACV yield at 24 h while R90 and M26 showed only a modest (≤1 log) reduction in viral yield. M23, M24 and M25 all reduced virus yield by 3-3.5 logs, confirming the inhibitory effects of these three MRT compounds.

We next assayed plaque formation in the presence of the effective compounds. Focusing on M23, M24 and M25 we infected confluent human retinal pigment epithelia cells (A-RPE-19s) with WT VACV in the presence of the MRT compounds, DMSO, TPOXX or H42. At 48 hpi, monolayers were stained and assessed for VACV plaque formation (Fig. 2B). M23, M24 and M25 were found to effectively inhibit VACV plaque formation. These results indicate that M23, M24, and M25 inhibit VACV production and that they maintain anti-poxvirus activity for at least 48 hours. This is consistent with our finding that H42 retained anti-poxvirus activity for at least 72 h (8).

**M23, M24, and M25 inhibit VACV gene expression.** We have shown that H42 inhibits poxvirus gene expression (8). To ascertain if the MRT compounds act in a similar fashion we infected HeLa cells in the presence of M23, M24 or M25 at an MOI of 20 with VACV E-EGFP or VACV L-EGFP (recombinant VACVs expressing EGFP from an early or late viral promoter, respectively). As VACV early gene expression (EGE) occurs before DNA replication and late gene expression (LGE) after DNA replication, we included cycloheximide (CHX) and Cytosine Arabinoside (AraC) controls which inhibit EGE and LGE, respectively. A TPOXX control was also included as a late-stage block that does not affect viral gene expression (8). Cells were harvested at 8 hpi and the number of E-EGFP and L-EGFP expressing cells were quantified by flow cytometry (Fig. 3A). Compared to CHX, H42, M23, M24 and
M25 had very modest effects on EGE. Conversely, all bisbenzimide compounds effectively diminished LGE to the levels seen in the presence of AraC. As expected TPOXX had no effect on either EGE or LGE.

**M23, M24 and M25 reduce the size of VACV replication sites.** To corroborate the gene expression results we infected cells with VACV EGFP-A5 (a VACV recombinant that expresses an EGFP-tagged version of the VACV late core protein A5) in the presence of the various compounds. At 24 hpi cells were fixed and immunostained for the early VACV protein I3, which is found on uncoated genomes and within VACV replication sites (Fig. 3B). As expected, I3- and A5 co-localised in large replication sites in the presence of either DMSO or TPOXX. In the presence of CHX, which prevents VACV genome uncoating (17), stabilized A5-positive virus cores, but no I3- or A5-positive replication sites were observed. In the presence of AraC, I3-positive uncoated genomes and A5-positive incoming cores were observed, but no VACV replication sites were seen. In the presence of bisbenzimide compounds, small I3-positive, A5-negative replication sites were observed. The replication sites were similar to those seen in the presence of H42, being far smaller and “more compact” than those seen in infected, DMSO-treated controls (8). A5 was not robustly expressed in the presence of any bisbenzimide compound, therefore, no replication sites in which I3 and A5 co-localised were observed. Overall, this data demonstrated that all bisbenzimide compounds inhibit LGE (Fig. 3A), which in turn blocked the development of VACV replication sites (Fig. 4B).

**Bisbenzimides H42, M23, M24 and M25 inhibit mpox infection.** We have shown that H42 is effective against orthopox- and parapox- viruses, suggesting that the bisbenzimides display broad anti-poxviral activity (8). Given the recent worldwide mpox outbreak we wanted to assess if H42 and MRT compounds could inhibit mpox. Human foetal foreskin fibroblast (Hft) cells were infected with a WT mpox strain, isolated during the recent pandemic, in presence of H42 or the MRT compounds at various concentrations. At 48 hpi cells were assessed, in parallel, for cytopathic effect (CPE) and cell cytotoxicity (Fig. 4).
We employed TPOXX as a control for inhibition of mpx spread (4). As expected, TPOXX displayed potent anti-mpx activity with no apparent toxicity (Fig. 4). H42 and the three MRT compounds were also found to be effective mpx inhibitors with IC50s of 0.075 µM for H42, 4.0 µM for M23, 1.9 µM for M24 and 6.1 µM for M25. H42, M23 and M24 were most effective, completely blocking mpx CPE at 0.22 µM, 13.3 µM, and 4.4 µM, respectively (Fig. 4; blue lines). M25, at its most effective concentration (4.4 µM), reduced CPE to <40%. At these concentrations, H42 caused 50%, M23 48%, M24 27% and M25 21% cell cytotoxicity, with CC50s of 0.19 µM for H42, 24 µM for M23, 15 µM for M24 and 18 µM for M25. While the Hft cells used for the mpx assay appear to be more sensitive to the compounds than HeLa, A-RPE-19 and BSC cells, overall, these data indicated that bisbenzimides were effective inhibitors of mpx replication.

**Bisbenzimides effectively inhibit TPOXX resistant VACV.** TPOXX was approved for treatment of mpx under the US CDCs expanded access investigational new drug protocol in 2022. It remains the only drug approved for the treatment of both smallpox and mpx. TPOXX targets the viral envelope wrapping protein F13, which is conserved in all poxviruses. Single point mutations in the gene encoding F13 are known to confer TPOXX resistance to poxviruses *in vitro* and *in vivo* (18, 19). Thus, we were curious to see if the bisbenzimides could inhibit spread of a VACV that is partially resistant to TPOXX. For this, we used a VACV expressing the F13 mutant (G277C), which has been described to lower the antiviral efficacy of TPOXX both *in vitro* and *in vivo* (4, 20). To assure that any phenotypes observed were due to the presence of the G277C mutation and not any other unknown mutations, we generated a control virus - RevG277C- in which the G277C mutant virus was repaired. To examine the effect on virus spread, HeLa cells were infected at a low MOI (0.01) with WT, G277C or RevG277C. Infections were performed in the presence of DMSO, TPOXX, H42 or M23. Cells were harvested at 24 hpi and the virus production quantified by plaque assay (Fig. 5A). TPOXX effectively lowered WT and RevG277C control virus production by >95% compared to the DMSO control. The G277C mutant virus, as expected, showed some resistance to TPOXX (70% reduction).
Both H42 and M23 effectively blocked production of VACV WT, G277C mutant, and RevG277C control viruses, in each case decreasing virus yield by >3.5 log.

To assess the effect of the compounds on plaque formation, A-RPE-19 cells were infected with VACV WT, G277C or RevG277C in the presence of DMSO, TPOXX [0.4µM], H42 [0.4µM] or M23 [10µM] (Fig. 5B). As expected, in the presence of DMSO all viruses formed plaques. Both WT and VACV RevG277C control virus were sensitive to TPOXX, while the G277C mutant virus was resistant (albeit, forming somewhat smaller plaques). Consistent with the 24 h yield results (Fig. 5A), H42 and M23 both completely abrogated VACV WT, G277C and RevG277C virus plaque formation.

To confirm that the mechanism of H42 and M23 inhibition remained the same, HeLa cells were infected with G277C or the RevG277C control virus in the presence of DMSO, H42 or M23. At 24 hpi cells were fixed and viral replication sites visualized by immunostaining for I3 (Fig. 5C). In the presence of DMSO, infection with both VACV viruses produced large I3-positive replication sites. In the presence of H42 or M23 I3-positive replication sites were reduced in number and size (Fig. 5C).

Collectively, these results indicate that H42 and M23 are effective inhibitors of a TPOXX-resistant mutant VACV.

Discussion

We have previously shown that bisbenzimidines are potent inhibitors of poxvirus infection (8). We found that these compounds, which preferentially bind the minor groove of double-stranded DNA, inhibit infection by blocking DNA replication and post-replicative gene transcription. The bisbenzimide H42 was found to inhibit a range of human and animal poxviruses but was ineffective against several other DNA and RNA viruses, including herpes simplex virus-1 (HSV-1) and influenza A.

Here, we tested a series of novel bisbenzimide analogues, with reported activity against HCMV (14), against VACV and mpox. Three of these compounds M23, M24 and M25 proved to be effective...
inhibitors of prototype (VACV) and pandemic potential (mpox) poxviruses with low cytotoxicity. We show that, like H42 (8), these compounds block poxvirus replication and subsequent LGE.

Poxviruses with varied genomic AT content, ranging from VACV (67% AT) to squirrel pox SQPV (33% AT) (21) showed similar sensitivity to H42. Thus, we concluded that the inhibitory efficacy of the bisbenzimidines did not correlate with their preferential binding to adenosine-threonine (AT)-rich regions of DNA; but with the cytoplasmic accessibility of replicating poxvirus genomes and the lipophilicity of the bisbenzimide compounds, which largely dictates their binding to double-stranded DNA via hydrophobic interactions with adenosine/threonine-rich regions (9, 22, 23). Consistent with this model, in the presence of the MRT compounds VACV DNA replication sites were small and condensed, and the IC50s of R90, H42, M23, M24, M25 in HeLa cells largely correlated with their predicted lipophilicity (LogP): H42>M23>M24=M25>R90 (Fig. 1A). This of course does not preclude other properties of these compounds that might affect their efficacy, such as membrane permeability and toxicity.

We further show that H42, M23, M24, and M25 were all effective at blocking mpox infection. When assaying mpox replication, TPOXX showed high efficacy and low cytotoxicity compared to the MRT compounds. Despite its in vitro potency, as TPOXX targets a viral protein it is subject to mutational resistance (4, 24). During the 2022 mpox pandemic, TPOXX resistant mutants were in fact isolated from mpox patients undergoing TPOXX treatment (18, 19). We found that H42 and the MRTs were still effective against a virus that shows resistance to TPOXX. This is not surprising as the bisbenzimidines target a different stage in VACV replication than TPOXX (late gene expression and virus assembly/release, respectively) and interact with different factors required for VACV replication (protein F13 and the VACV DNA genome, respectively). As yet we have been unable to isolate a H42-resistant mutant virus in more than 20 passages of VACV in vitro (data not shown) (Similar observations have been made passaging HCMV in the presence of a bisbenzimide (RO) (14).

1 (8)
Thus, the bisbenzimide compounds do not appear to be subject to the development of VACV resistance. This suggests that TPOXX/bisbenzimide co-inhibition studies could be further explored for additive or synergistic effects against poxvirus infection.

It is interesting to compare the mechanisms of action of bisbenzimide compounds on poxviruses and HCMV. At low concentrations, bisbenzimides inhibit VACV gene expression but prevent the formation of HCMV genome-containing capsids (14). While at higher concentrations both VACV and HCMV genome replication is blocked (8, 14). Given that the anti-viral effects correlate with low concentration bisbenzimide treatment it may be worth examining the relationship between HCMV gene expression and genome packaging.

Collectively, this work supports further exploration of bisbenzimides as anti-viral agents. This is supported by the long-standing observations that some bisbenzimide compounds have no obvious adverse effects in mice and have been used with no serious adverse effects in human clinical trials (25). Using the compounds tested here as a platform to generate modified bisbenzimide analogues, in the future we hope to identify new bisbenzimides with increased potency against poxviruses and perhaps other DNA virus families.

Materials and Methods

Cells, viruses, and compounds

HeLa (ATCC), BSC-40, A-RPE-19 (kind gift from Frickel lab, UoB), Vero E6, and Primary Human foetal foreskin fibroblasts immortalized by retrovirus transduction to express the catalytic subunit of human telomerase (Hft) (26) were maintained at 37.0 °C and 5.0% CO₂ in Dulbecco’s modified Eagle's medium (DMEM; Gibco, Life Technologies) with the addition of 10% foetal bovine serum (FBS; Sigma), and 1% penicillin-streptomycin (Pen-Strep; Sigma).
Vaccinia virus strain Western Reserve (VACV) was used throughout. VACVs used were either wild type (WT) or transgenic, containing EGFP under early VACV gene promoter (VACV E-EGFP), late VACV gene promoter EGFP (VACV L-EGFP), or A5-tagged EGFP inserted into the endogenous A5 locus (VACV EGFP-A5). WT, E-EGFP (27), L-EGFP (27) and EGFP-A5 (28) were previously published. All VACV mature virions (MVs) were purified from BSC40 cytoplasmic lysates by being pelleted through a 36% sucrose cushion for 90 min at 18,000 × g. The virus pellet was resuspended in 1 mM Tris (pH 9.0).

The titre (PFU per millilitre) was determined in BSC40 cells as previously described. MPox virus (accession number: ON808413; strain designation MPXV CVR-S1) was isolated from a clinical sample in Glasgow in 2022 (29). Vero cells were used to propagate mpox. HFT cells were used in antiviral activity and toxicity assays.

Cycloheximide (CHX; Sigma), cytosine arabinoside (AraC, Sigma), and TPOXX (generously provided by Dennis Hruby and Douglas Grosenbach, SIGA Technologies, Inc.) were diluted in DMSO and used as indicated in the text and figures and figure legends. Bisbenzimides Hoechst 33342 (H42, Sigma), RO-90-7501 (R90; Sigma); MRT00210423, MRT00210424, MRT00210425, MRT00210426, MRT00210427 (all generously provided by Andy Merritt, LifeArc (formerly MRC Technology)) were dissolved in DMSO and used at concentrations indicated in the text and figures and figure legends. DMSO was used as a drug carrier control at the same volume as drug or compound diluted in DMSO.

**Bisbenzimide predicted lipophilicity**

To determine the non-ionic consensus partitioning coefficient (LogP) of all structures we used AxonChem Marvin cheminformatics suite. Calculations for all structures assumed Cl- and Na+ K+ concentrations of 0.1 mol/dm3 each. Tautomerization or resonance were not considered.

**Flow cytometry**
HeLa cells in 96 well plates were infected with VACV L-EGFP at MOI 0.5 for 24 h (Spread Assay); or VACV E-EGFP or L-EGFP at MOI 20 for 8h (EGE or LGE assay). After 30 min at room temperature (RT) the inoculant was replaced with DMEM containing compounds at indicated concentrations. For Spread Assay: TPOXX, H42, AraC: 40-40.4-0.04-0.004-0.0004 μM; R90, M23-27: 40-20-10-5-2.5-1.25 μM. For EGE or LGE, effective concentration (EC) with acceptable cytotoxicity derived from the Spread Assay was used. (Effective concentration was the compound concentration where at least 90% of cells in an infected well were not expressing GFP.) After incubation at 37 °C wells were aspirated and cells detached with trypsin, followed by addition of 5% BSA in PBS and fixation with 9% formaldehyde in PBS (for a final 3% FA concentration). The percentage of green fluorescent cells out of all cells was then counted using a Guava® easyCyte™ flow cytometer. Gating was done using “live cells” gate first, and then a “<99% of uninfected cells are below threshold” gate. The results – % of cells expressing GFP – were then normalised to infected, DMSO-treated controls (DMSO = 1).

IC50/CC50 concentrations were determined in GraphPad Prism using a four-parameter logistic nonlinear regression model (Inhibitor) vs response – four parameters).

**Cytotoxicity**

Cytotoxicity was assessed using Abcam’s Quick Cell Proliferation Assay Kit II (WST-1), following manufacturers’ instructions. Briefly, HeLa cells in 96-well plates were incubated for 24 h at 37 °C in the compound concentrations mirroring those concentrations used in the flow cytometry Spread Assay. WST solution was then added to each well and incubated for 3 h, followed by absorbance measurement at 460nm, corrected by subtracting absorbance in wells without cells but with media. Values were then normalized to cells incubated without any compounds.

**Virus yield and spread assays**

HeLa cell monolayers in 6-well plates were infected with VACV WT at MOI 1 (24 h yield) or MOI 0.01 (24 h spread) in presence of specified compound. At 24 hpi, cells were collected and centrifuged, and the pellet was resuspended in 100 μl 1 mM Tris (pH 9.0). Cells were then freeze-thawed three
times to lyse the cells, and the lysate solution was serially diluted to determine the PFU per millilitre
by plaque assay on BSC40 cell monolayers.

**Plaque inhibition assays**

A-RPE-19 cells grown in 12-wells were infected with 200 pfu of VACV WT, G277C, or G277C-rev in
the presence of specified compound at 37 °C. 48 hpi cells were fixed and stained with 0.1% crystal
violet in 4% formaldehyde. Plate images were digitally captured using a desktop scanner (Cannon).

**Immunofluorescence microscopy**

HeLa were cells seeded on CellView slides (Greiner Bio-One). They were infected with VACV EGFP-A5
for 30 min at RT. The inoculant was then replaced with indicated compounds in the text, figures and
figure legends. After 20 h at 37 °C cells were washed and fixed with 4% EM grade FA in PBS. They
were permeabilized and blocked simultaneously in 0.5% Triton-X 1000 in 5% BSA in PBS. Anti-I3
antibody (generously provided by Jakomine Krijnse Locker; Institute Pasteur) was used at 1:1,000. All
secondary antibodies (goat anti-mouse-AF488 and goat anti-rabbit-AF647; Invitrogen) were used at
1:1,000. Primary I3 antibody was added for 60 min at RT, followed by a wash and 60 min RT staining
with secondary antibody and DAPI. Images were captured using a 100x oil immersion objective (NA
1.45) on a VT-iSIM microscope (Visitech; Nikon Eclipse Ti), using 488 nm and 640 nm laser
frequencies for excitation.

**Mpox antiviral activity and toxicity assays.**

HFt cells were seeded in 96-well plates (Costar) at a density of 1x10^4 cells per well and incubated for
24 hours. Three hours prior to infection, the cells were incubated with three-fold serial dilutions of
each compound prepared in infection medium (DMEM containing 2% FBS). For mpox antiviral
assays, the plates were transferred to a CL3 facility before each well was infected with an equal
volume of infection medium containing mpox virus at an MOI of 0.1 (1.4x10^3 plaque forming units
(PFU)) per well. Following incubation for 48 hours, cells were fixed in 8% formaldehyde in PBS and
stained with Coomassie Blue. The dried plates were scanned using a Pherastar SFX plate reader (BMG) at an optical density of 595 nm to quantitate the level of cytopathic effect (CPE). For toxicity assays, an equal volume of infection medium without virus was added to each well. Following 48 hours, 10 µl of resazurin (Sigma R7017) prepared at a concentration of 0.5 mM in PBS was added to each well. After a 2h incubation period, resofurin was quantified by measuring fluorescence intensity (Ex530/Em560) using a Varioskan LUX microplate reader (Thermo Scientific). Percentage virus replication was calculated by normalising well clearance to infected and uninfected DMSO controls, while percentage cell viability was determined by normalising values to untreated cells and our high toxicity control (50% DMSO). IC50/CC50 concentrations were determined in GraphPad Prism using a four-parameter logistic nonlinear regression model (Inhibitor) vs normalized response – four parameters).


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Author Contributions

JS: Data Analysis and Curation, Methodology, Supervision, Writing-Original Draft Preparation, Writing-Review and Editing. DCM: Conceptualization, Methodology, Investigation, Data Analysis and Curation and Writing-Review and Editing. NU: Conceptualization, Methodology, Investigation, Data Analysis and Curation and Writing-Review and Editing. MMcE: Investigation. ML: Investigation. AY: Investigation, Writing-Review and Editing. AHP: Supervision, Writing-Review and Editing and Funding.

Conflict of Interest

The authors declare no conflicts of interest.

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Research (BMBF) and by the Saxon Ministry for Science, Culture, and Tourism (SMWK) with tax funds on the basis of the budget approved by the Saxon State Parliament (AY).
**Figure Legends**

**Fig 1. MRT compounds inhibit cell-to-cell spread of VACV.**

A) Structures of Bisbenzemides and MRT compounds. H42 (Hoechst 33342), R90 (RO-90-7501), M23 (MRT00210423), M24 (MRT00210424), M25 (MRT00210425), M26 (MRT00210426) and M27 (MRT00210427). Respective partitioning coefficient (LogP) and chemical abstract number (CAS) where applicable are provided below. B) HeLa cells were infected at MOI 0.1 with VACV L-EGFP in presence of H42, R90 or the MRT compounds. 24h post infection (hpi) cells were quantified by flow cytometry for EGFP and displayed as normalised to infected + DMSO (black lines). Cytotoxicity was assessed using a WST-1 assay and displayed as normalised to DMSO (red dashed lines). Data represents biological triplicates and error bars represent the standard deviations of those data.

**Fig 2. M23, M24, and M25 reduce virus 24h yield and inhibit plaque formation.**

A) HeLa cells were infected with WT VACV at MOI 1 in presence of the indicated compounds. At 24 hpi cells were harvested and VACV progeny quantified by a titre on BSC40 cells (plaque forming units (pfu)/ml). Data represents biological triplicates and error bars represent the standard deviations of those data. B) A-RPE-19 cells were infected with WT VACV (200 pfu) in the presence of indicated compounds. At 48 hpi cells were subjected to fixation and staining to visualize plaques. Experiments were performed in biological duplicate and representative wells of those experiments are shown.

**Fig 3. M23, M24 and M25 block LGE and reduce replication site size.**

A) HeLa cells were infected with either VACV E-EGFP or VACV L-EGFP at MOI 20 in the presence of the indicated compounds. At 8 hpi cells were harvested and EGFP expressing cells quantified by flow cytometry. Data displayed as normalised to infected + DMSO = 1. Data represents biological triplicates and error bars represent the standard deviations of those data. B) HeLa cells were
infected with VACV A5-EGFP (green) at MOI 20 in presence of indicated compounds, concentrations as in A. At 24 hpi fixed cells were immunostained for I3 (magenta), stained with DAPI (blue) and imaged. Scale bar = 20 μm. Experiments were performed in biological duplicate and representative images of those experiments are shown.

**Fig 4. Inhibitory activity of Bisbenzemides and MRT compounds against Monkeypox virus.**

Hft cells in 96-well plates were infected with mpox virus (MOI 0.1) in the presence of 3-fold serial dilutions of TPOXX, H42, M23, M24 or M25. At 48 hpi, virus induced CPE was quantified from fixed and Coomassie-stained plates. Cell viability was quantified from compound-treated, uninfected cells by measuring the conversion of resazurin to fluorescent resofurin. Percentage CPE was normalised to infected and uninfected controls, while percentage cell viability was normalised to untreated cells and high toxicity control (50% DMSO). For both CPE and toxicity measurements, data represents biological triplicates and error bars represent the standard deviations of those data.

**Fig 5. Bisbenzemides are effective against a TPOXX resistant VACV recombinant.**

A) HeLa cells were infected with VACV WT, G277C, or RevG277C viruses at MOI 0.01, to measure virus spread, in the presence of indicated compounds. Cells were harvested 24 hpi and the virus yield determined by plaque assay. Experiments are biological triplicates and error bars represent the standard deviations of those data. B) ARPE-19 cells were infected with 200 pfu of VACV WT, G277C, or RevG277C viruses, in presence of indicated compounds at concentration as in A. At 48 hpi wells were fixed and stained to visualize virus plaques. Experiments were performed in biological duplicate and representative wells shown. C) HeLa cells were infected with G277C or RevG277C virus in presence of DMSO, H42 or M23 (concentrations like in A). At 24 hpi cells were fixed and immunostained for I3 and stained
with DAPI. Experiments were performed in biological duplicate and representative wells of those experiments are shown. Scale bar = 20 μm.
Figure 1

A

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<th>Short Name</th>
<th>H42</th>
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<th>M23</th>
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B

Graphs showing the normalized % EGFP expression and % viability against various concentrations of compounds. IC50 and CC50 values are indicated for each compound.
Figure 3

A

Normalized EGFP expression

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<th>Treatment</th>
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<th>0.6</th>
<th>0.8</th>
<th>1.0</th>
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<td>AraC [5 μM]</td>
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<td>TPOXX [0.4 μM]</td>
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<td>H42 [0.4 μM]</td>
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</tbody>
</table>

B

DMSO

AS-GFP

DAPI

CHX

AraC

TPOXX

H42

M23

M24

M25
Figure 4
Figure 5

A

![Graph showing the Pfu/mL values for different conditions: TPOXX [0.4 μM], H42 [0.4 μM], M23 [10 μM]. The graph compares WT, G277C, and RevG277C conditions.](image)

B

![Images of cell cultures under different treatments: DMSO, TPOXX, H42, M23 for WT, G277C, and RevG277C conditions.](image)

C

![Images of cellular staining with DAPI and different treatments: DMSO, H42, M23 for G277C and RevG277C conditions.](image)