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Original Article

Cytidine nucleoside analog is an effective antiviral drug against Trichomonasvirus

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Abstract *Background: Trichomonas vaginalis* is the causative agent of a sexually transmitted disease in humans. The virulence of the parasite depends on multiple factors, including the presence of endosymbiotic dsRNA viruses. The presence of Trichomonasviruses (TVV) was associated with more severe genital symptoms, increased proinflammatory host reactions, and modulated parasite sensitivity to metronidazole. However, no efficient antiviral drugs are available against TVV to derive isogenic TVV-positive and TVV-negative cell lines that are essential for investigations of the TVV impact on *T. vaginalis* biology.
Methods: 7-Deaza-2′-C-methyladenosine (7d2CMA) and 2′-C-methylcytidine (2CMC) were used for TVV inhibitory assay. TVV replication was monitored using quantitative reverse transcription PCR (RT qPCR) and western blotting. Modeling of TVV1 RNA-dependent RNA polymerase (RdRp) was performed to visualize the inhibitor-RdRp interaction. Susceptibility to metronidazole was performed under aerobic and anaerobic conditions.
Results: We demonstrated that 2CMC but not 7d2CMA is a potent inhibitor of TVV replication. Molecular modeling suggested that the RdRp active site can accommodate 2CMC in the active triphosphate nucleotide form. The effect of 2CMC was shown on strains infected with a single and multiple TVV species. The optimal 2CMC concentration (10 μM) demonstrated strong selectivity for TVVs over trichomonad growth. The presence of TVV has no effect on *T. vaginalis* metronidazole susceptibility in derived isogenic cell lines.
Conclusions: 2CMC acts against TVVs and represents a new inhibitor against Totiviridae viruses. Our isogenic clones are now available for further studies of various aspects of *T. vaginalis* biology related to TVV infection.

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Introduction

Trichomonas vaginalis, a causative agent of human trichomoniasis, carry dsRNA viruses (TVV) of Totiviridae family in approximately 40—75% clinical isolates.[1—3] The TVV genome consists of a 4.3 kb—5.5 kb dsRNA that encodes capsid protein (Cp) and RNA-dependent RNA polymerase (RdRp). The latter is expressed as a fusion protein (Cp/RdRp) through −2 ribosomal frameshifting mechanisms.[4] To date, four TVV species (TVV1, TVV2, TVV3, and TVV4) have been categorized with some sequence variability within each species.[3] TVV1 is the most prevalent species, while TVV4 is the least frequent.[5,6] Multiple coexistence of these viruses in different combinations in several strains has been reported.[5—8] The possible role of TVV in *T. vaginalis* virulence and sensitivity to metronidazole has been proposed. TVV was shown to modulate *T. vaginalis* protein expression,[9] and TVV-positive trichomonads expressed higher numbers and greater amounts of proteases, which are important virulence factors, than TVV-negative strains.[10] Moreover, TVV2 and TVV3 increased the expression of immunogen P270 of the parasite surface while TVV1 and TVV2 were associated with more severe genital symptoms.[11—13] The release of TVV virions from *T. vaginalis* appeared to be sensed by cells of the cervicovaginal epithelium and caused proinflammatory reactions,[14] whereas extracellular vesicles released from TVV-positive trichomonads appeared to be immunosuppressive in *vitro* experiments.[15] On the other hand, a significant relationship between the clinical symptoms of infected patients and the presence or absence of TVV was not observed by others.[1,2,16]

To provide a new tool to study TVV impact, we decided to derive isogenic TVV-positive and TVV-negative *T. vaginalis* lines. To eliminate TVV in *T. vaginalis* we used antivirals based on nucleoside analogs.[17,18] These inhibitors enter the infected cell and then are converted to the 5′-triphosphate form, which is essential for their recognition by RdRp.[19—21] Upon incorporation into the RNA chain, activated inhibitors behave as terminators of chain synthesis.[22] The activity of nucleoside analogs against Totiviridae RdRp was reported in *Leishmania*,[20,23] and here, we found that 2CMC was an efficient (and currently only known) inhibitor against TVV1-3 in *T. vaginalis*.

Methods

Strains and cell cloning

TVV-positive *T. vaginalis* strains T1[24] and TV79-49[25] were cloned, and derived clones T1c1^{+} and TV79-49c1^{+} were used for the experiments. TV17-2MI was isolated at the General Hospital, Prague, Czech Republic, axenized as described,[26] and used as a TVV-negative strain; TV10-02 was used as a metronidazole-susceptible and HL-2MT as a metronidazole-resistant strain.[25,26] Cells were cultivated in tryptone-yeast extract—maltose medium (TYM) as described.[27] Single-cell cloning was performed by limiting dilution using 96-well plates that were incubated under anaerobic conditions using AnaeroGen (Oxoid, Thermo Fisher Scientific, Waltham, USA).

Compounds

7-Deaza-2′-C-methyladenosine (7d2CMA) and 2′-C-methylcytidine (2CMC) were purchased from Carbosynth (Berkshire, UK). Stock solutions were prepared in 20 mM dimethyl sulfoxide (DMSO) and diluted 1:1000 in experiments.

TVV inhibition assay

To test the concentration-dependent drug effect, 5×10^4 trichomonads in 10 ml of TYM with the indicated drug concentrations and incubated for 24 h under anaerobiosis. After incubation, the cells were pelleted and total RNA extracted. Cell counting was performed using a Bürker counting chamber. A time-dependent test was performed with 10 μM 2CMC in TYM, and the cells were sampled at different time intervals for total RNA isolation. After the indicated period of treatment, the cell population was cloned and grown without drug selection pressure.

RNA extraction and RT-qPCR

Total RNA was extracted using a High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) and 100 ng RNA was used for cDNA synthesis. The RT-PCR was performed as previously described.[3] The RT-qPCR conditions were as follows: cDNA synthesis at 42 °C for 10 min, initial denaturation at 95 °C for 3 min, and 40 cycles of 95 °C/5 s, 58 °C/30 s, and 72 °C/20 s. The melt curve analysis was performed following the default settings for the Rotor-Gene 3000 (Corbett Research/Qiagen GmbH, Hilden, Germany). DNA topoisomerase II (TVAG_038,880) was used for normalization.[28] RT-qPCR was performed with the Kapa SYBR FAST Universal One-step RT-qPCR kit (Sigma—Aldrich, Missouri, USA). The relative gene expression level was calculated using equation $2^{-\Delta\Delta Ct}$.[29] The primers (Table S1) were designed based on sequences: TVV1, JF436869; TVV2, NC_003873; and TVV3, NC_004034. The significant *p-value* ($p < 0.05$) was calculated using the two sample t test algorithm in Graph Pad Prism 7 (La Jolla, California, USA). All experiments were biologically triplicated.

Immunoblotting

The protein concentrations in all samples were determined by Lowry assay and 50 μg was loaded to each well for separation using 8% SDS PAGE and transferred onto nitrocellulose membranes. Rabbit polyclonal antibodies against TVV1 and TVV3 capsid proteins were a kind gift from Dr. J-H Tai (Academia Sinica, Taiwan). The dilution of antibodies was 1:1000. The proteins were visualized using the goat anti-rabbit StarBright Blue 700 (Bio-Rad, Hercules, USA) fluorescent secondary antibody (1:5000).

Modeling of the TVV1 RdRp active site

The *T. vaginalis* TVV1 RdRp protein sequence (AAA62868.1) was used for the structural modeling of the nucleotide binding pocket. The RDRP_4 (PF02123) domain was identified using Pfam (http://pfam.xfam.org/), and the T301-

R560 domain was submitted for structural prediction using the PHYRE2 v2.0 web service (intensive method).[30] The model was refined using locPREFMD.[31] Nucleotide binding pocket prediction was performed using P2Rank.[32] CTP was superimposed on the binding pocket using PyMOL version 2.0 (Schrödinger, LLC).

Metronidazole susceptibility test

The susceptibility of the *T. vaginalis* strain to metronidazole was assayed under aerobic and anaerobic conditions as previously described.[33]

Results

Characteristics of *T. vaginalis* clones

Two TVV harboring strains, TV79—49 and T1, were chosen for antiviral treatment. Both strains were cloned to obtain T1c1$^+$ and TV79-49c1$^+$ clones that were tested for TVV using PCR. T1c1$^+$ was positive only for TVV1 (Fig. 1A), whereas TV79-49c1$^+$ possessed TVV1, TVV2 and TVV3 (Fig. 1B, negative control in Fig. S1). To estimate the ratio between TVV species in Tv79-49c1$^+$, we used RT qPCR with primers specific for TVV1, TVV2 and TVV3 RdRp. TVV1 showed an approximately 11 and 17-fold higher viral load (-ΔCq 6.65 ± 0.22) than TVV2 (-ΔCq 0.6 ± 0.13), and TVV3 (-ΔCq 0.38 ± 24), respectively. Because dsRNA viruses are randomly distributed during cell division into daughter cells,[23,34] we determined the generation time (G) for each clone (4.11 h for T1c1$^+$ and 5.03 h for Tv79-49c1$^+$, Figs. S2A and B) to estimate initial conditions for trichomonad treatment.

2CMC inhibits TVV replication

Modeling of TVV1 RdRp using the crystal structure of (+) ssRNA Norwalk virus RdRp (PDB entry 2b43) as the closest template allowed visualization of amino acid residues that are predicted to interact with nucleotide triphosphates within the nucleotide-binding pocket (Fig. 2A—C). All interacting amino acid residues within motifs A (Y391, N395), B (S446, N455), C (D478) and F (R335) were identical in TVV1 and LRV1 RdRp except Q320,[23] which corresponded to LRV1 T387 in the F motif (Fig. 2C) (motif nomenclature according to[35]). Thus, we expected similar inhibitor preference for both RdRps. To our surprise, the treatment of T1c1$^+$ cells with 100 μM 7d2CMA for 24 h (5.8 cell doublings) did not lead to any change in the TVV1 load and there was also no effect on the cell growth (Fig. S3). In contrast, treatment with 100 μM 2CMC resulted in a loss of the signal for RdRp; however, it also decreased cell growth (Fig. S3). Therefore, 2CMC was used for further experiments.

To determine the lowest effective concentration of 2CMC at which cell growth will not be significantly affected, we treated T1c1 with 0.1 μM—100 μM 2CMC for 24 h (Fig. 3). According to the RdRp RNA level, the replication of TVV1 gradually decreased from 0.1 to 10 μM 2CMC and had minimal effect on cell growth (Fig. 3A). We can estimate that the half maximal effective concentration (EC_{50}) of 2CMC is approximately 0.75 μM for TVV1 inhibition. At concentrations ≥10 μM 2CMC, the RdRp RNA was at the limit of detection (Fig. 3B). Cell growth was affected at concentrations of 2CMC ≥25 μM. Thus, 10 μM appeared to be an optimal 2CMC concentration and had strong selectivity for TVV1 over trichomonad growth that was not affected even after an extended time period (Fig. S4).

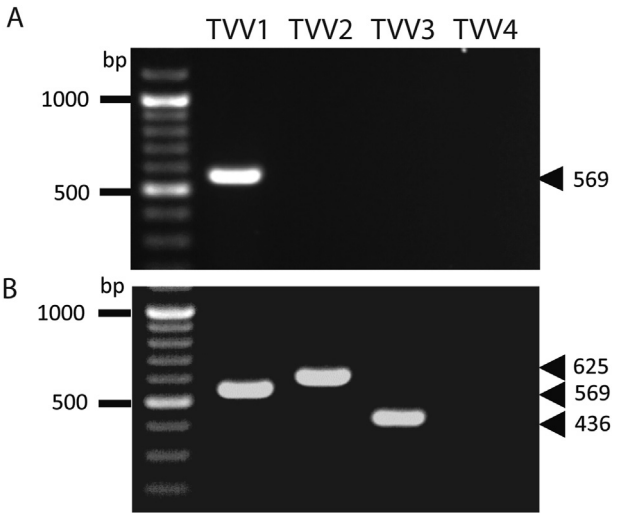

Figure 1. Detection of TVV species in T1c1$^+$ and TV79-49c1$^+$. RdRp of TVV1, TVV2, TVV3, and TVV4 was amplified by PCR in T1c1$^+$ (A) and TV79-49c1$^+$ (B) using specific primers (Table S1). TV17-2MI was used as a negative control (NC).

Trichomonad cell doubling-dependent viral loss

To determine the number of cell doublings that are needed for the loss of TVV1, we monitored the RNA levels of RdRp and the capsid protein after 12, 18, 24 and 48 h of treatment with T1c1$^+$ cells using 10 μM 2CMC. At shorter time points (12 and 18 h), the RNA level for both RdRp (Fig. 4A) and capsid gene (Fig. 4B) declined to less than 10% after 18 h of treatment. A very low level close to that of the TVV1-free control was observed after 24 h; however, the complete loss was reached after 48 h of treatment (Fig. 4A and B; Fig. S5A). Similarly, the gradual decrease in the level of the capsid protein was detected by a TVV1-specific antibody that recognized the 72 kDa protein of the expected size.[4] Additional 60 kDa protein disappeared together with 72 kDa protein, suggesting that this protein was most likely a cleavage product of the 72 kDa protein. The capsid protein completely disappeared after 48 h treatment, which corresponded to 11.6 cell doublings (Fig. 4C).

TVV1 does not recover after 48 h 2CMC treatment

Next, we tested whether TVV1 replication recovers after 24 h of 2CMC treatment, after which the RdRp and capsid RNA levels were less than 1% if the drug pressure was released and whether the absence of detectable RdRp observed after 48 h of treatment is a stable phenomenon. Therefore, the cells were subjected to cloning after 24 and 48 h of treatment and

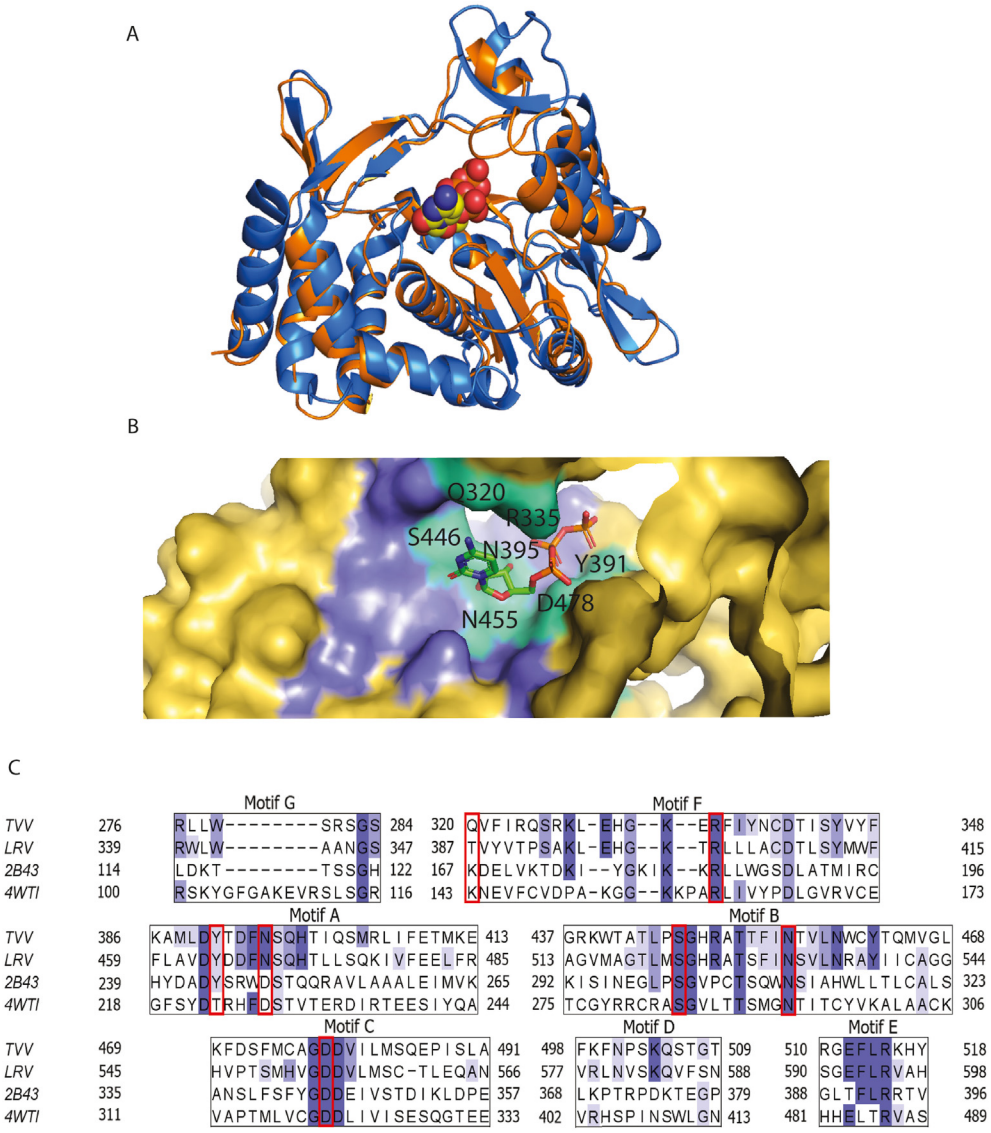

Figure 2. ***T. vaginalis*** **TVV1 RdRp active site model.** (A) Structural alignment of the predicted TVV1 RdRp active site domain (T301-R560, orange) to the crystal structure of the active site domain (T137-L449, orange) of the Norwalk virus RdRp (PDB entry 2B43, blue). The CTP is shown within the NTP binding pocket (red, blue, and yellow indicate O, N, and C, respectively). The predicted RdRp domain showed over 90% confidence for 98% amino acids. The resulting model was refined using locPREFMD,[36] which led to a MolProbity score of 1.7.[39] (B) The predicted nucleotide-binding pocket is in blue, and amino acid residues within the binding pocket predicted to interact with CTP in the vicinity of 4 Å are indicated (turquoise).

two passages of subculture (each for 24 h) in medium without the drug. TVV1 recovered in all ten clones derived after 24 h of drug treatment (Fig. 5A). In contrast, TVV1 RdRp was not detected in any of the clones derived after 48 h of 2CMC treatment (hereafter, T1c1$^-$); thus, this treatment apparently led to virus elimination (Fig. 5B).

2CMC is effective to treat multiple TVV infections

T. vaginalis clinical isolates often contain multiple TVV species that may differ in sensitivity to 2CMC and the dynamics of elimination from *T. vaginalis* cells. Therefore, we treated TV 79-49c1$^+$ with 10 μM 2CMC. After 18 h of treatment, the level of TVV1 RdRp RNA was decreased to only 47% while TVV2 and TVV3 RdRp were reduced to 11% and 5%, respectively, which corresponded to differences in the viral load in TV79-49c1$^+$ cells. The replication of all TVVs gradually decreased at 24 h and 48 h, and RdRp became undetectable for all three TVVs at 72 and 96 h (Fig. 6A, Fig. S5B). Accordingly, the immunoblotting analysis revealed that the level of TVV1 (72 kDa) (Fig. 6B)[4] and TVV3 (90 kDa) (Fig. 6C)[13] capsid proteins gradually decreased during the treatment.

To test the recovery of TVVs after 2CMC treatment of TV79-49c1$^+$ cells for 18 h, 48 h, and 96 h, clones were derived as described above. TVV1 recovered to approximately the original level in 18 h clones (Fig. 7A). Interestingly, the level of

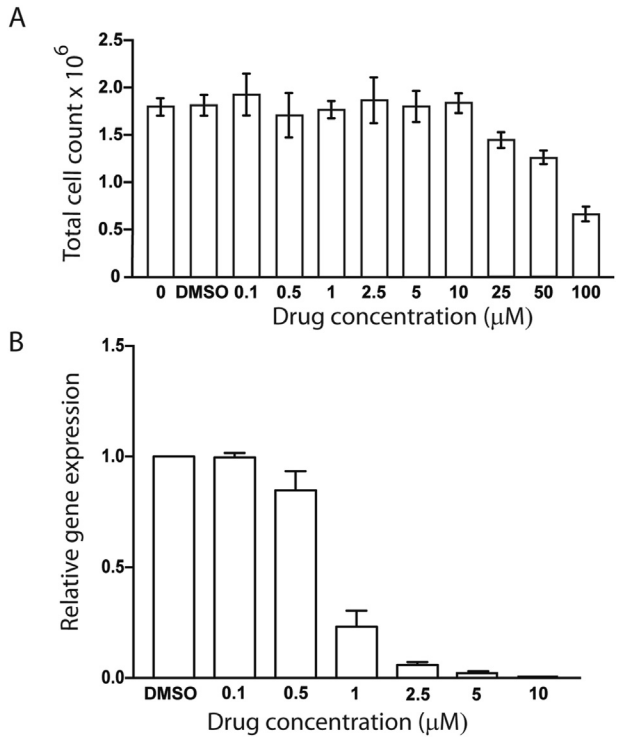

Figure 3. Effect of 2CMC on T1c1$^+$ growth and level of TVV1 RdRp RNA. (A) T1c1$^+$ cell number was determined after 24 h incubation with 2CMC. DMSO, a control without a drug with 20 μM DMSO. (B) Relative gene expression analysis for TVV1 RdRp. Error bars represent standard deviation.

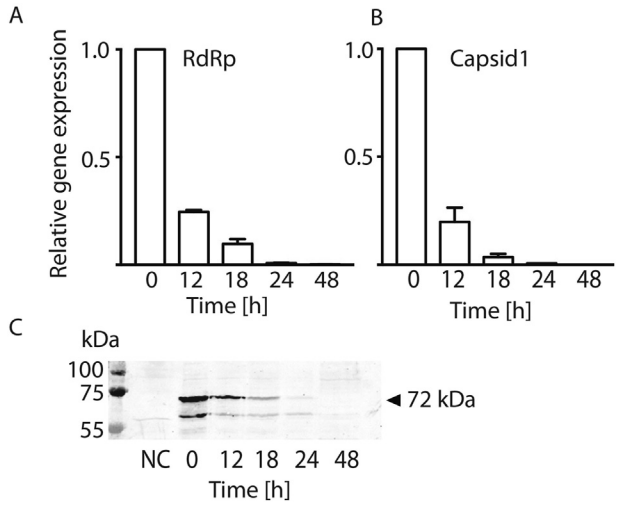

Figure 4. Time-dependent effect of 2CMC treatment on the level of TVV1 RdRp and capsid gene. Relative gene expression was determined for TVV1 RdRp (A) and capsid1 (B) in T1c1$^+$ cells treated with 10 μM 2CMC. Error bars indicate a standard deviation. (C) Immunoblot detection of capsid1 protein.

RdRp in the clones derived after 48 h of drug treatment did not increase upon drug pressure release (Fig. 7B). As clone 4 contained detectable RdRp for all three TVV species, we cultivated this clone for another 20 passages without the drug;

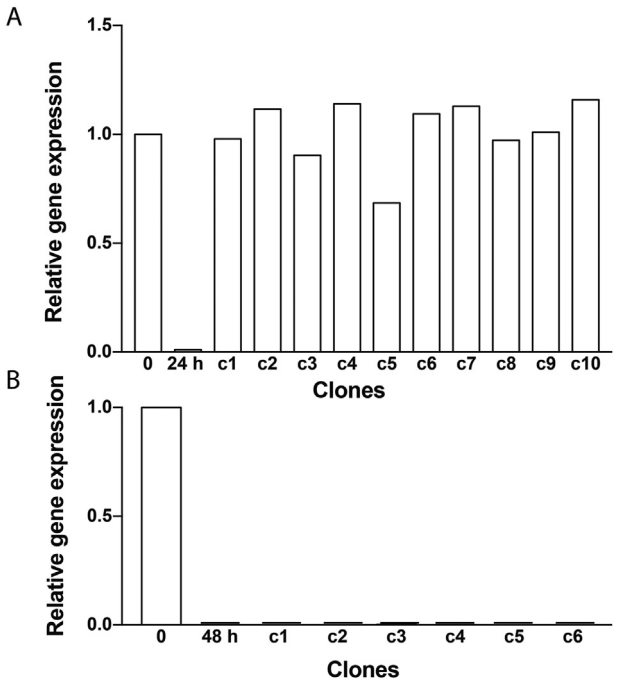

Figure 5. 2CMC treatment for 48 h resulted in virus-free T1c1 clones. The cells were treated with 10 μM 2CMC for 24 h (A) and 48 h (B), and after the treatment, individual cells were isolated and grown for two passages without the drug. The RdRp RNA level was then determined by RT-qPCR for each clone (c1-c10). 24 h and 48 h indicate the RdRp RNA level before (0 h) and after drug treatment.

however, the RdRp level did not increase (Table S2). None of the TVV species was detected in any clones after 96 h of treatment with 10 μM 2CMC (Fig. 7C). These cells (hereafter named TV79-49c1$^-$) were considered TVV-free isogenic clones of the TV79-49c1$^+$ parent clone.

Presence of TVV has no effect on *T. vaginalis* susceptibility to metronidazole

Previous studies suggested a possible relationship between TVV presence and *T. vaginalis* susceptibility to metronidazole.[16] However, no difference in metronidazole susceptibility was observed for T1c1$^+$/T1c1$^-$, and MLCs determined for these clones were identical to the metronidazole-susceptible control TV10-02 strain (3.13 and 0.78 μg/ml metronidazole under aerobic and anaerobic conditions, respectively) (Table S3). Similar results were obtained with TV79-49$^+$/TV79-49$^-$ (Table S3). These results demonstrated that the presence of TVVs had no significant effect on *T. vaginalis* susceptibility to metronidazole under our experimental conditions.

Discussion

Nucleoside analogs are broadly utilized as RdRp inhibitors for the treatment of viral infections. Here, we reported successful inhibition of dsRNA TVV replication in *T. vaginalis* that led to virus-free progeny of TVV-infected

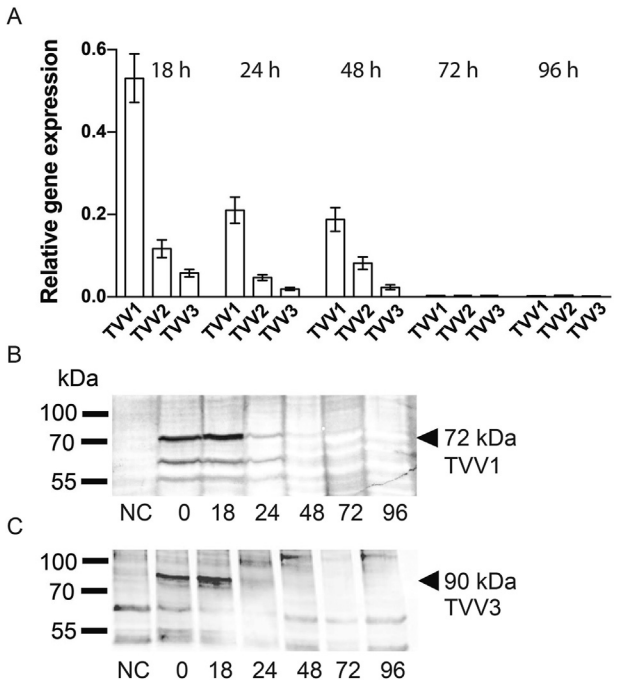

Figure 6. Effect of 2CMC on multiple TVV infections in TV79-49c1^{+}. (A) RdRp RNA levels corresponding to TVV1, TVV2, and TVV3 in TV79-49c1^{+} cells were monitored during 18—96 h of treatment with 10 μM 2CMC. The levels of TVV1 (B) and TVV3 (C) capsid proteins were visualized using immunoblotting. TV17-2MI was used as a negative control (NC).

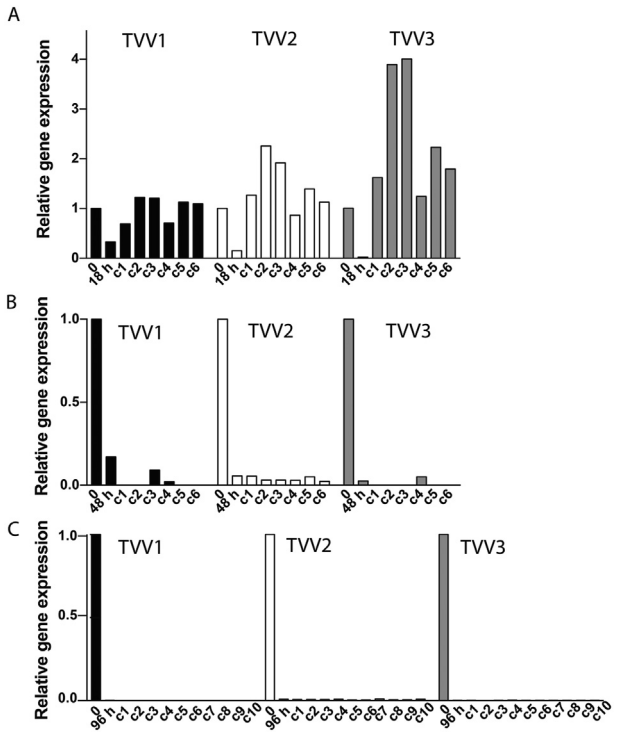

Figure 7. TVV recovery after TV79-49c1^{+} treatment with 2CMC. TV79-49c1^{+} cells were treated for 18 h (A), 48 h (B), and 96 h (C) with 10 μM 2CMC, and individual cells were subsequently isolated and grown without the drug for two passages. The relative RdRp RNA level was determined for each clone (c1-c10). 18, 48, and 96 h indicate relative RdRp RNA levels before (0 h) and after the treatment.

clones. The treatment was efficient against multiple TVV species (TVV1, TVV2, and TVV3) using 2CMC, whereas no inhibitory effect was observed with 7d2CMA. In parasitic protists, an inhibitory effect of nucleoside analogs was studied in *Leishmania guyanensis*, which harbors dsRNA LRV1 virus. Screening of 81 compounds against LRV1 revealed effective viral inhibition using 7d2CMA and 2′-C-methyladenosine (2CMA) but little effect on LRV1 using 2CMC and other 2′ position modifications. Although the reason for such a different efficiency of inhibitors against TVVs and LRV1 is not known, we can expect differences in their interaction with RdRp active sites. Molecular modeling of the nucleotide binding pocket of TVV1 RdRp suggested that the inhibitor is accommodated in the expected form of the activated triphosphate nucleotide, as in the case of LRV1.[23] However, future structural studies of TVVs and LRV1 RdRps are required to understand the observed highly specific effect. The number of TVV virions per cell was not reported, although numerous spots uniformly distributed in the cytoplasm of the TVV-positive strain have been observed using an anti-dsRNA antibody.[6] In our experiments, upon inhibition of TVV1 replication in the T1c1^{+} clone by 2CMC, the majority of RdRp and capsid protein-coding RNA disappeared after 24 h, which corresponded to almost 6 cell doublings (G = 4.1 h). Therefore, we can roughly estimate the presence of over 2^6 (~64) TVV copies per T1c1^{+} cell considering random binary spreading during mitosis. This copy number is close to the estimation of LRV1 load in *L. guaynensis* (8—64 copies).[23] However, elimination of the TVV RdRp signal in TV79-49c1^{+} infected with TVV1, TVV2 and TVVV3 required at least 72 h, which is over 13 doublings with G = 5 h. This result suggests a considerably higher TVV load (thousands of copies) or less efficient inhibition of TVV replication or both.

Successful preparation of isogenic TVV-positive and isogenic TVV-negative clones provides a new tool for evaluating the impact of TVV on host *T. vaginalis*. Previous attempts to derive isogenic strains were performed using prolonged *in vitro* cultivation for six months to two years, which were used for various comparative studies.[15,16,36,37] However, prolonged trichomonad cultivation has been shown to cause changes in parasite features, particularly a decrease in virulence.[38] As proof of principle, we tested the effect of TVV on *T. vaginalis* metronidazole sensitivity, which is a controversial issue.[1,16] Using T1c1$^{+/-}$ and TV79-49c1$^{+/-}$, we demonstrated in our experimental condition that the presence of TVV had no effect on *T. vaginalis* metronidazole sensitivity (aerobic MLC for all clones ranged between 0.7 and 3.3 μg/ml metronidazole). In a previous report, sensitivity to metronidazole was tested in 30 fresh clinical *T. vaginalis* isolates, and it revealed aerobic MLCs ranging from 16 to 31 μg/ml metronidazole. After prolonged *in vitro* cultivation, aerobic MLCs increased in 24 strains to ≥50 μg/ml metronidazole, and the increase in MLCs was correlated with the loss of TVV.[16] On the contrary, no association of metronidazole resistance with TVV

infection of *T. vaginalis* isolates was found in a recent clinical study,[1] a view that also supports our data.

In conclusion, 2CMC is the only known inhibitor that acts against TVVs and represents a new inhibitor against Totiviridae viruses. Our model isogenic clones are now available for further studies of various aspects of *T. vaginalis* biology, particularly its virulence that might be related to TVV infection.

Declaration of competing interest

The authors declare no conflict of statement.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2021.08.008.