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Potent antiviral activity of brequinar against the emerging Cantagalo virus in cell culture

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ABSTRACT

In the present work, the antiviral activity of brequinar (BQR) against the replication of Cantagalo virus was evaluated. BQR is a potent inhibitor of cellular dihydroorotate dehydrogenase, an enzyme of the de novo pyrimidine biosynthetic pathway. Infection in the presence of 0.5 μM BQR reduced virus progeny production by >90%, revealing an EC_{50} (drug concentration required to inhibit 50% of virus replication) of 0.017 μM. Replication of other orthopoxviruses was also inhibited by BQR at similar levels. In the presence of the drug, virus early proteins accumulated to control levels, whereas late gene expression was severely impaired. This result was confirmed by indirect immunofluorescence assays and analysis of time-regulated expression of a reporter gene under the control of a virus promoter. Both assays revealed nearly 90% inhibition of late gene expression. BQR also blocked virus DNA replication, which accounted for the subsequent inhibition of virus late gene expression. The ablation of virus DNA replication, late gene expression and infectious progeny production was restored to control levels when infected cells were co-treated with uridine (URD) and BQR. These data demonstrated that BQR targeted virus DNA synthesis by depleting the cellular pyrimidine pool, which was bypassed by the salvage pathway when URD was added to the cell cultures.

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1. Introduction

Cantagalo virus (CTGV) is a strain of vaccinia virus (VACV) that was originally isolated in 1999 from lesions on cows and dairy workers during an outbreak of a pustular skin disorder in dairy farms in Rio de Janeiro State, Brazil [1]. This zoonosis was initially detected in southeastern states of the country, but its spread to and establishment in different regions, including the Brazilian Amazon, have been reported over the last decade [2–4]. The uncontrolled dissemination of CTGV infection in Brazil is worrisome because important economic issues may arise in affected farms, which have their income based mostly on dairy activities [4]. In addition, no antiviral therapy is currently available for CTGV or other poxvirus-related infections.

Poxviruses are large, double-stranded DNA-containing viruses that replicate exclusively in the cell cytoplasm. The replicative cycle is complex and involves a tight control of virus gene expression in a cascade fashion. Therefore, virus DNA replication occurs after the early stage of gene expression has succeeded and, on the other hand, the production of new genome molecules is an essential pre-requisite for completion of the pos-replicative phases of the virus cycle [5].

Over the last two decades there have been numerous studies focusing on the identification of anti-poxvirus agents. The relevance of these efforts is based on the concern of a possible use of variola virus as a bioweapon, the elevated number of cowpox virus infections in pet and zoo animals in Europe, the outbreaks of monkeypox virus in Africa, the occurrence of severe adverse effects following smallpox vaccination [6], and the widespread dissemination of CTGV zoonosis in Brazil [2].

Our group has reported the antiviral activity of several immunosuppressants, such as cyclosporine A (CsA), FK-506 and azathioprine, as well as non-immunosuppressive drugs against the replication of distinct VACV strains, including CTGV [7–12]. Brequinar (BQR) is an immunosuppressive and antiproliferative drug effective against allograft and xenograft rejection following transplantation [13,14]. It inhibits the proliferation of T-cells, production of antibodies and tumour growth [15]. The mechanism of action of BQR probably relies on inhibition of dihydroorotate dehydrogenase (DHODH) activity, the fourth enzyme and a rate-limiting step in the de novo biosynthesis of pyrimidines [16]. Therefore, BQR probably exerts its effect by depleting the cellular pyrimidine pool, which is needed for RNA and DNA synthesis. Nevertheless, inhibition of tyrosine phosphorylation has been reported as another activity of BQR [17]. Here we have investigated the antiviral efficacy of BQR on the replication of CTGV. We show that BQR severely inhibited the production of infectious virus and the late stages of gene expression. The drug specifically targeted virus DNA synthesis since the

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effect was reversed by the addition of uridine (URD), which leads to the bypass of the de novo synthesis of pyrimidines.

2. Materials and methods

2.1. Brequinar

BQR was provided by DuPont Merck Pharmaceutical Company (Wilmington, DE). The drug was dissolved in 100% dimethyl sulphoxide (DMSO) and was stored at −20 °C.

2.2. Cells and viruses

Monolayer cultures of BSC-40 (African green monkey kidney) cells were propagated in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA) supplemented with 5% heat-inactivated foetal bovine serum, 100 μg/mL streptomycin and 500 U/mL penicillin at 37 °C [12]. In this study, the following viruses were used: CTGV isolate CM-01 [1]; VACV strains IOC clone A1-1-1 [1], WR and Wyeth; cowpox virus (CPXV) strain Brighton-Red (originally obtained from Richard Moyer, University of Florida, FL); and recombinant CTGV expressing the β-galactosidase gene of *Escherichia coli* (CTGV-βGal) under the control of an early/late VACV promoter (Reis and Damaso, unpublished data). All viruses were available in the laboratory's collection and were routinely propagated and titered by plaque assay in BSC-40 cells [12].

2.3. Virus infection and yield analysis

For analysis of intracellular virus yield, cells were infected with 1000 plaque-forming units (PFU) of CTGV (1×10^6 cells per plate) for 2 h (adsorption period). The inocula were removed and the cells were treated with either 0.1% DMSO (control) or BQR concentrations ranging from 0.005 μM to 10 μM. After 24 h, the monolayers were harvested and were processed for virus titration by plaque assay [12]. In experiments where a multiplicity of infection (MOI) of 1 PFU/cell was used, the adsorption period was 30 min. In assays to determine the effect of BQR on the replication of VACV-IOC, VACV-WR, VACV-Wyeth and CPXV, the infected cells were treated with 0.1% DMSO or 5 μM BQR for 24 h. For one-step growth assays, 30 μM BQR was used and CTGV-infected cells were harvested at 3, 6, 8, 16, 18, 20 and 24 h. In some assays, URD was added at 50, 100 or 300 μM. The procedures for obtaining cell lysates and determining virus titres were carried out essentially as described above. To evaluate the virucidal effect of BQR, purified CTGV (4×10^6 PFU/mL) [1] was incubated with BQR at 0.1, 1 and 5 μM or with 0.1% DMSO (control) for 1 h at room temperature with occasional shaking, followed by titration as described above. All measurements of virus production are expressed as the average of at least two experiments performed in duplicate.

2.4. Toxicity assays

The neutral-red uptake assay was used to evaluate cell viability essentially as described [7]. BSC-40 cells were seeded in 96-well plates in the presence of concentrations of BQR ranging from 0.01 μM to 75 μM for 24 h. Control cells were incubated with 0.1% DMSO. Neutral red was methanol/acetic acid-extracted from cells and was quantitated at an absorbance of 490 nm (A_{490}) [7]. All measurements expressed the average of four independent assays.

2.5. Western blot assay

BSC-40 cells were infected with CTGV at a MOI of 1 and were treated with either 0.1% DMSO (control) or 30 μM BQR. In some assays, URD was added at 100 μM or 300 μM. At different times post infection, cells were harvested in sodium dodecyl sulphate (SDS)-containing sample buffer and were subjected to electrophoresis in 11.5% SDS polyacrylamide gel electrophoresis (PAGE) [7,12]. Proteins were transferred to nitrocellulose membranes and Western blot analysis was carried out essentially as described [7,10]. Blots were probed with rabbit anti-VACV structural proteins [10], mouse anti-α-tubulin (Sigma-Aldrich, St Louis, MO) or rabbit anti-F11 antibody [10]. The bound antibodies were detected using anti-rabbit or anti-mouse antibody conjugated to horseradish peroxidase IgG (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with Super Signal West Pico (Thermo Scientific, Rockford, IL).

2.6. Measurement of β-galactosidase activity

BSC-40 cells were infected with CTGV-βGal at a MOI of 1 and were treated with 0.1% DMSO, 30 μM BQR or 40 μg/mL cytosine arabinoside (Ara-C). At 6 h and 24 h post infection, cells were harvested in Milli-Q water and were processed for determination of β-galactosidase activity essentially as described [12]. For each duplicate sample, 250 μg/mL of the substrate *O*-nitrophenyl-β-D-galactopyranoside (Sigma-Aldrich) was added and reactions were stopped with 230 μM Na_2CO_3 when a light yellow colour was developed. Absorbance was measured at A_{420}. All measurements expressed the average of two independent assays performed in eight replicates.

2.7. Indirect immunofluorescence assays

BSC-40 cells were grown on 13-mm round glass coverslips in 24-well plates and were infected with CTGV at a MOI of 1. After 20 h in the presence of 30 μM BQR or 0.1% DMSO (control), the monolayers were fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) and were permeabilised using 0.5% Triton X-100 for 5 min as described [18]. The coverslips were then incubated with 50 mM NH_4Cl for 30 min, followed by 1% bovine serum albumin/PBS for 1 h. Cells were stained with rabbit anti-VACV structural proteins and mouse anti-D8 (kindly provided by Geoffrey Smith, Imperial College, London, UK) for 1 h, followed by species-specific fluorescent secondary antibodies (rabbit anti-IgG Alexa488 and mouse anti-IgG Alexa555; Invitrogen). DNA was stained with 1 μg/mL 4′-6-diamidino-2-phenylindole (DAPI) for 5 min. Samples were analysed with a Zeiss Axio Observer Z1 microscope (Carl Zeiss Inc., Thornwood, NY) using Axiovision software. For quantitation of infected cells, 1095 control cells and 1065 BQR-treated cells were counted in three independent assays.

2.8. Analysis of DNA replication by slot-blot assay

Cells were infected with CTGV at a MOI of 1, incubated or not with 30 μM BQR, and harvested at different times post infection. In some assays, URD was added at 100 μM or 300 μM. Cell extracts were prepared in 10× saline–sodium citrate (SSC) buffer, 1 M NH_4Ac as described [12], and samples were applied to nylon membranes in triplicate, under vacuum, using a Minifold® II Slot-Blot apparatus. DNA was denatured in situ. Blots were hybridised to the *Hind*III D fragment of the VACV genome after nick-translation labelling with [α-^{32}P]dCTP (Perkin-Elmer Life Sciences, Waltham, MA), followed by exposure to X-ray films [10,12]. At least two independent assays were performed and applied onto nylon membranes in three replicates.

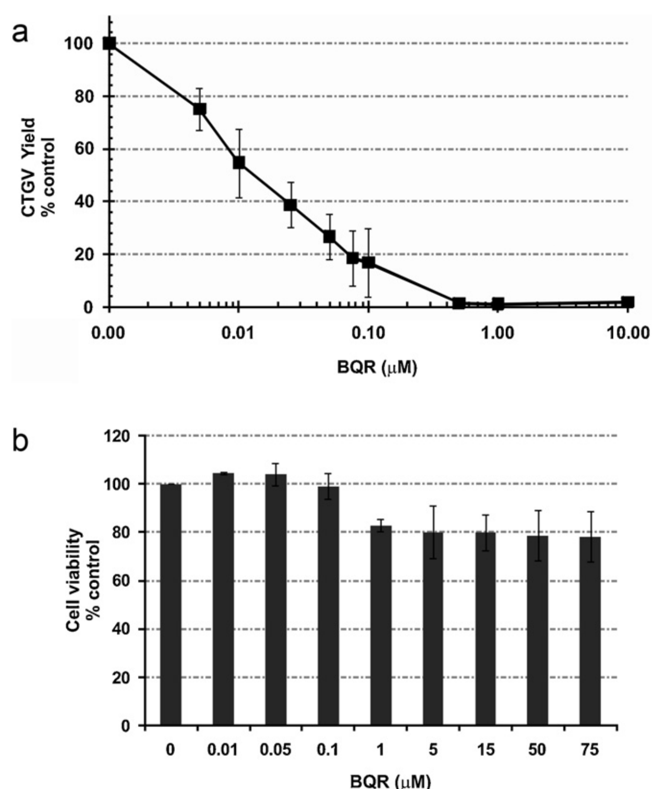

Fig. 1. Antiviral effect and cytotoxicity of brequinar (BQR). (a) Dose-dependent inhibition of virus progeny production. Cells were infected with 1000 plaque-forming units (PFU) of Cantagalo virus (CTGV) and were incubated with the indicated concentrations of BQR. Virus yield was determined at 24 h post infection. (b) The indicated concentrations of BQR were added to BSC-40 monolayers for 24 h. Cell viability was determined by neutral-red uptake assay. Values represent the mean of at least two independent assays.

2.9. Densitometric analysis

Autoradiographs were scanned and densitometry analysis was performed using the program Scion Image (Beta release 4; Scion Corporation, Frederick, MD) as described [10].

3. Results

3.1. Effect of brequinar on virus yield

As a first approach to evaluate the antiviral potential of BQR, production of infectious particles in infected cells treated with increasing concentrations of the drug for 24 h was investigated. BQR led to severe inhibition of CTGV yield in a dose-dependent manner, reaching nearly 98% at 0.5 μM (Fig. 1a). Similar levels of inhibition were also observed when virus plaque formation was evaluated in the presence of BQR (data not shown). As shown in Fig. 1b, the antiviral activity was not due to cytotoxic effects of BQR since cell viability remained nearly 80% at concentrations up to 75 μM BQR [50% cytotoxic concentration (CC_{50}) > 75 μM]. Based on these data, the EC_{50} was calculated to be 0.017 ± 0.05 μM, and the selective index (CC_{50}/EC_{50}) was estimated to be >4410.

The effect of BQR on the replication of other orthopoxviruses was also analysed. The MOI was elevated to 1 and infected cells were incubated for 24 h with 5 μM BQR. All VACV strains tested as well as CPXV had the production of infectious particles severely inhibited by BQR (Fig. 2a). A similar inhibitory effect was observed at 1 μM BQR (data not shown). Whether BQR could affect the infectivity of CTGV particles was also tested. For that, purified CTGV particles were incubated with different concentrations of BQR, followed by titration in BSC-40 cells. As shown in Fig. 2b, BQR had no effect on the infectivity of CTGV particles (virucidal effect).

3.2. Effect of brequinar during one-step replication cycles

3.2.1. Analysis of the production of infectious particles and protein accumulation

To evaluate better the stages of the virus replicative cycle targeted by BQR, the next set of assays was performed using a MOI of 1 and 30 μM BQR. Analysis of one-step growth curves demonstrated that BQR completely abolished the formation of infectious CTGV particles since the initial hours of infection under these conditions. On the other hand, virus yields progressively increased after 8 h post infection in untreated cells (Fig. 3a). To map the stage of the virus cycle inhibited by BQR, accumulation of virus early and late proteins was evaluated by Western blot. As a marker for the early stage, F11 was detected, which is synthesised at the early phase of the cycle but accumulates until the late stages [10]. Detection of CTGV structural proteins (anti-VACV), which are primarily expressed at the late stages after 8 h post infection, was taken as a marker of post-replicative gene expression. As observed in Fig. 3b, BQR did not affect F11 accumulation in BQR-treated cells compared with untreated cells. On the other hand, accumulation of CTGV structural proteins was completely abrogated during infection in the presence of BQR (Fig. 3b). This result indicated that BQR did not affect virus early gene expression, but had a severe effect on the late stage of the virus cycle.

To confirm this result, the effect of BQR on the expression of a reporter gene under the control of a VACV early/late promoter was evaluated. Cells infected with a recombinant CTGV expressing the β-galactosidase gene of *E. coli* were either left untreated or were treated with 30 μM BQR or 40 μg/mL Ara-C. This drug is a well-known inhibitor of DNA synthesis and, consequently, it does not affect virus early gene expression, whereas late gene expression is shut off as a result of the inhibition of DNA replication [12]. Cells were collected at 6 h or 24 h post infection and the activity of β-galactosidase was measured as described in Section 2.6. As shown in Fig. 3c, BQR had minor effects on β-galactosidase activity at 6 h post infection, similar to the effect of Ara-C. On the other hand, both drugs strongly inhibited β-galactosidase activity at 24 h post infection. These results confirmed the Western blot data shown in Fig. 3b, demonstrating that early gene expression remained unaffected by BQR whereas post-replicative gene expression was severely inhibited in the presence of the drug. In addition, because BQR acted similarly to Ara-C, this result also suggested that virus DNA synthesis may be the target of BQR.

To analyse this possibility further, BSC-40 cells were infected with CTGV and were treated with 30 μM BQR for 20 h, followed by immunofluorescence assay. Analysis of DAPI staining revealed the presence of virosomes spread in the cytoplasm of untreated infected cells, as would be expected for cells in late stage of the cycle (Fig. 4, arrows). On the other hand, BQR treatment drastically reduced the formation of virosomes by 82.9%, and the few that were observed were mostly in cells co-stained for virus late proteins (Fig. 4, arrows). As a marker for virus late polypeptides, the post-replicative envelope protein D8 as well as CTGV structural proteins (anti-VACV) were detected. Severe inhibition of D8 expression in the presence of BQR was observed in different fields in distinct assays, similar to the results obtained for the detection of CTGV structural proteins (Fig. 4). Cells positive for D8 and virus structural proteins were occasionally visualised in the presence of BQR, and cell counting revealed an 89.12% inhibition of positive cells under this situation. These results confirmed the data from the Western blot and reporter gene analyses, revealing a strong

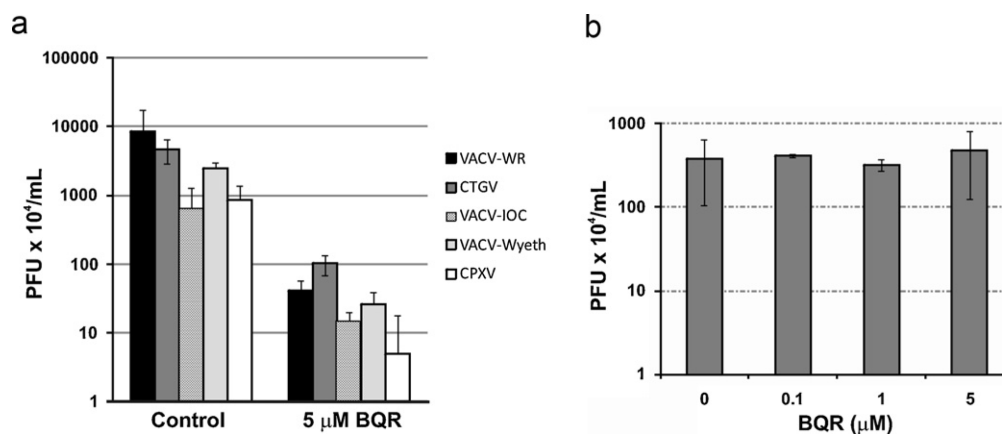

Fig. 2. Effect of brequinar (BQR) on the replication of distinct orthopoxviruses and on Cantagalo virus (CTGV) infectivity. (a) Cells were infected (multiplicity of infection = 1) with vaccinia virus (VACV) strains WR, CTGV, IOC and Wyeth or cowpox virus (CPXV) and were treated with either 0.1% dimethyl sulphoxide (DMSO) (control) or 5 μM BQR for 24 h. Virus yields were determined by plaque assay of the cell lysates. (b) Effect of BQR on CTGV infectivity. Purified CTGV particles (4×10^6 PFU/mL) were incubated with the indicated concentrations of BQR for 1 h, followed by titration in BSC-40 cells. Values represent the mean of at least two independent assays titered in duplicate. PFU, plaque-forming units.

inhibition of post-replicative gene expression in the presence of BQR, and reinforced the suggestion that the drug probably targeted virus DNA replication.

3.2.2. Virus DNA replication is inhibited by brequinar

Replication of virus DNA in the presence of BQR was evaluated next. Extracts of cells infected in the presence of 30 μM BQR were collected at the indicated times of infection and were hybridised to the ^{32}P-*Hind*III D fragment of the VACV genome. As shown in Fig. 5a, in untreated cells the accumulation of virus DNA was initially detected at 6–8 h post infection and increased with the progression of the cycle. On the other hand, when infection proceeded in the presence of BQR, replication of virus genome was severely repressed.

3.2.3. Uridine counteracts the antiviral effect of brequinar

To investigate whether viral DNA replication was in effect the primary target for BQR, infected cells were co-treated with 30 μM BQR and an excess of URD. The rationale of the assay was to generate pyrimidines through the salvage pathway, thus bypassing the effect of BQR on de novo biosynthesis. Addition of URD together with BQR for 24 h completely restored normal levels of virus DNA accumulation during infection (Fig. 5b). This was accompanied by the recovery of virus late gene expression measured by the detec-

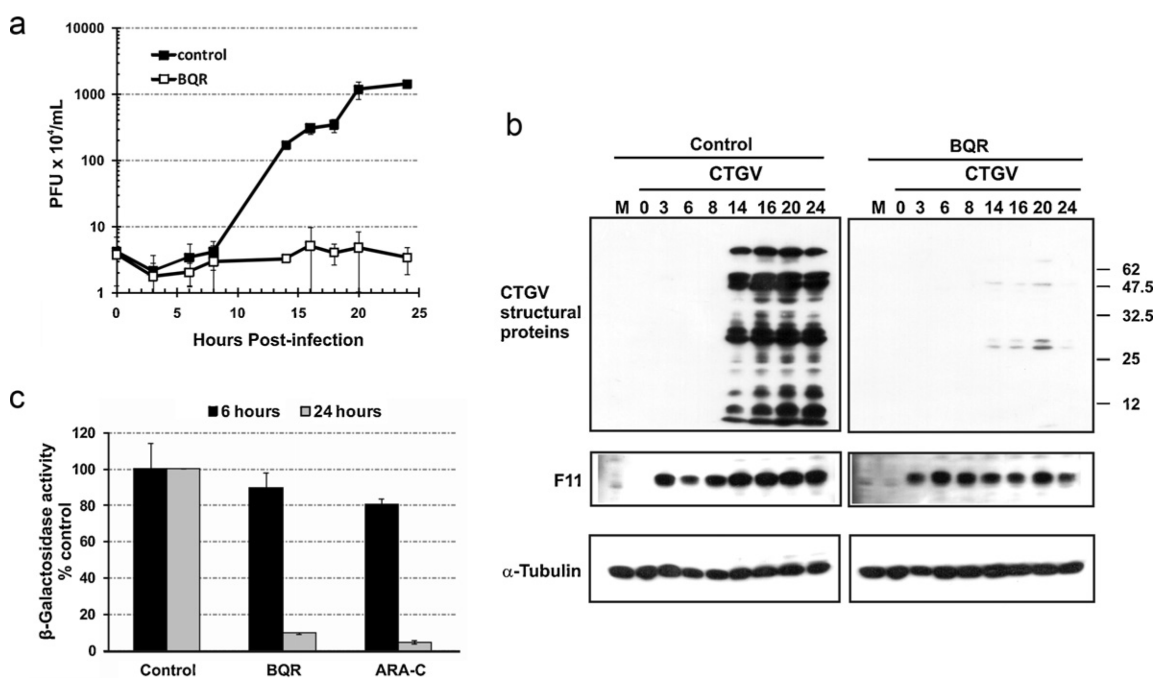

Fig. 3. Effect of brequinar (BQR) on Cantagalo virus (CTGV) replication during one-step replicative cycles. (a) Time-course analysis of virus yield. Cells were infected with CTGV [multiplicity of infection (MOI) = 1] and were treated with either 0.1% dimethyl sulphoxide (DMSO) (control) or 30 μM BQR. At the indicated hours post infection, cell lysates were prepared for determination of virus yield by plaque assay. Values represent the mean of two independent assays titered in duplicate. (b) Effect of BQR on virus protein accumulation. Cells were infected and treated as described in (a), and at the indicated hours post infection cell lysates were prepared for Western blot analysis using antibodies against CTGV structural proteins or F11. α-Tubulin was also detected as a control for protein loading. M, mock-infected cells. Molecular weight markers (kDa) are shown on the right. A representative experiment is shown. (c) Inhibition of late expression of β-galactosidase by BQR. Cells were infected with recombinant CTGV-βGal (MOI = 1) and were treated with either 0.1% DMSO (control), 30 μM BQR or 40 μg/mL cytosine arabinoside (ARA-C). At 6 h and 24 h post infection, cell lysates were prepared for determination of β-galactosidase activity as described in Section 2.6. Values represent the mean of at least two independent assays. PFU, plaque-forming units.

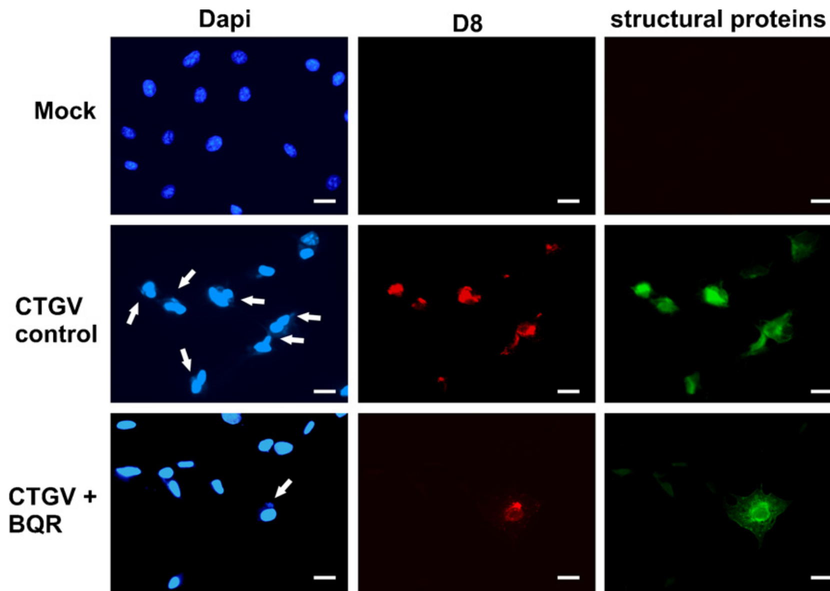

Fig. 4. Detection of virus DNA and late proteins by immunofluorescence assay. Cells were infected with Cantagalo virus (CTGV) (multiplicity of infection = 1) and were incubated with 0.1% dimethyl sulphoxide (DMSO) (control) or 30 μM brequinar (BQR). At 20 h post infection, cells were fixed, permeabilised and stained with antibodies against virus late protein D8 or CTGV structural proteins. Cell nuclei and viral DNA were stained with 4′-6-diamidino-2-phenylindole (DAPI). Mock, mock-infected cells. Arrows point to virosomes in the cell cytoplasm. Representative images are shown. Scale bars, 20 μm.

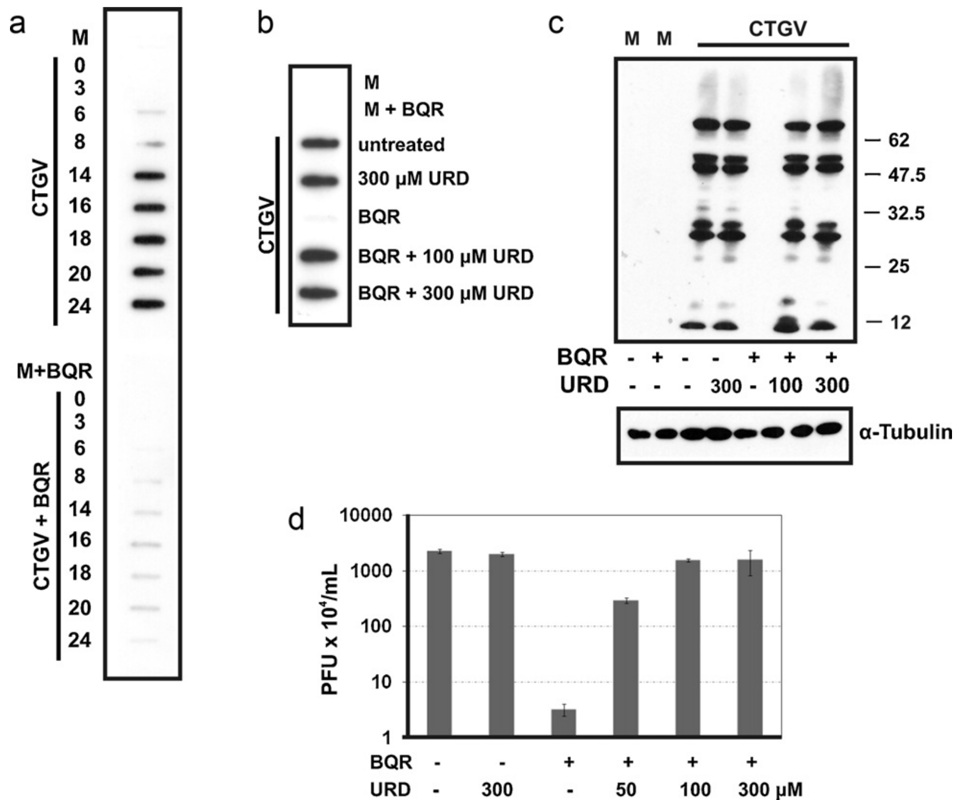

Fig. 5. Effect of brequinar (BQR) on virus DNA accumulation and rescue of virus replication by uridine (URD). (a) Cells were infected with Cantagalo virus (CTGV) (multiplicity of infection = 1) and were treated with either 0.1% dimethyl sulphoxide (DMSO) (control) or 30 μM BQR. At the indicated hours post infection, cell lysates were prepared for slot-blot assay. Viral DNA was detected by using the ^{32}P-*Hind*III D fragment of the vaccinia virus (VACV) genome as probe. (b–d) URD restores normal levels of viral DNA replication, late protein accumulation and virus progeny production. Experiments were performed as described in (a), except that URD, at the indicated concentrations, was added to the cells together with 30 μM BQR or separately. At 24 h post infection, cell lysates were collected for (b) detection of viral DNA by slot-blot assay, (c) Western blot analysis of CTGV structural proteins and α-tubulin (protein loading control) and (d) determination of virus yield by plaque assay. In (c), the molecular weight markers (kDa) are shown on the right. M, mock-infected cells; untreated, infected cells not treated with BQR or URD. (a–c) show representative images. PFU, plaque-forming units.

tion of CTGV structural proteins by Western blot (Fig. 5c). Most importantly, URD rescued normal production of CTGV infectious particles during infection in the presence of BQR (Fig. 5d). Taken together, these data demonstrated that the stage of virus DNA replication was the main target of the antiviral effect of BQR during CTGV infection.

4. Discussion

CTGV is an emerging pathogen that causes a pustulovesicular disease in dairy cows and dairy workers in Brazil [1]. Actions to restrain the spread of the disease have been scarce, and numerous outbreaks have been reported in different states over the last decade [2–4]. No specific antiviral therapy is commercially available, encouraging the search for natural and synthetic drugs effective against CTGV replication in vitro [7–9]. Here we demonstrate that BQR severely inhibited CTGV production when added during infection, but no direct effect on the infectivity of the virus particles was observed. A similar inhibitory effect of virus yield was detected when other strains of VACV and CPXV were investigated.

A detailed analysis of the CTGV replicative cycle revealed that virus early gene expression was not inhibited in the presence of BQR, as shown by the accumulation of the early protein F11 to control levels. In addition, the expression of β-galactosidase under control of a viral early/late promoter was not affected at early times. On the other hand, BQR clearly silenced virus late gene expression as demonstrated by Western blot assays, immunofluorescence assays and the inhibition of β-galactosidase activity at 24 h post infection. Further analysis revealed that a blockage in virus DNA synthesis accounted for the ablation of post-replicative gene expression. This conclusion was supported by the complete rescue of CTGV DNA synthesis and subsequent restoration of late protein accumulation and virus yield when URD was added to infected cells together with BQR. These data suggest that BQR targeted CTGV DNA synthesis by inhibiting the de novo pathway, leading to depletion of the intracellular pyrimidine pool. Therefore, these data are in agreement with previous work showing that BQR inhibits the activity of cellular DHODH, a rate-limiting enzyme in the de novo synthesis of pyrimidines [16]. A similar mechanism of action was recently reported for the antiviral effect of BQR against the replication of dengue virus [19]. Based on the EC_{50} values, CTGV appears to be nearly 4.5 times more sensitive to the effect of BQR than dengue virus. However, it is worth noting that the experiments were performed using different cell lines and distinct methods to evaluate virus production. Other inhibitors of de novo pyrimidine biosynthesis have also been shown to be potent antiviral drugs against RNA and DNA viruses in vitro [20–26].

However, in vivo studies have not been successful in demonstrating the antiviral efficacy of drugs involved in the inhibition of de novo biosynthesis. Attempts to address the antiviral activity of inhibitors of orotidylate decarboxylase against distinct viruses, including vaccinia virus inoculated intranasally in hamsters, have failed to show significant reductions in virus load [26]. Similarly, recent efficacy studies using novel DHODH inhibitors against dengue virus and respiratory syncytial virus (RSV) in different animal models were not able to show reductions in virus yield [20,25]. The authors have raised the possibility that the high bioavailability of extracellular pyrimidines for cell uptake would restore normal rates of virus replication, therefore annulling the antiviral effect of the drugs in vivo. For RSV infection, it was reported that increased doses of the inhibitors were cytotoxic, and their immunosuppressive activity did not favour the host to mount an immune response [25]. The authors suggested that the cellular de novo pathway may not be the target of choice for antiviral drugs. Nevertheless, Dunn et al. [24] have recently demonstrated the in vivo efficacy of another DHODH inhibitor, leflunomide, which reduced RSV load in rat lungs by 3 log. However, the mechanism by which this drug restrained RSV replication in vivo is still unknown and did not appear to result from its ability to block the de novo biosynthesis of pyrimidines, although this pathway seemed to be the target of leflunomide in in vitro infections [24].

Several immunosuppressants have been proven effective against distinct VACV strains in cell culture, but they have not been tested in animal models, thus no data are currently available to discuss their antiviral efficacy in vivo [7,10–12,18,27]. Their immunosuppressive features would probably be an important negative aspect in animal models but, on the other hand, these compounds have proven to be excellent tools to study the involvement of cellular pathways in the virus replicative cycle in vitro. CsA, for example, had its antiviral activity against VACV correlated with its ability to bind to and suppress the prolyl-peptidyl isomerase activity of cellular cyclophilin A [11,12]. This protein was later reported to be recruited to virosomes during VACV infection and be encapsidated into the core of mature virions [18].

Therefore, immunosuppressive drugs with antiviral activity may turn out to be an interesting source for lead structures for developing novel compounds with varied activities. Perhaps this may not apply to BQR because its immunosuppressive action appears to be exerted through the same pathway by which its antiviral activity is accomplished. However, it is worth noting that CTGV infection in animals and humans produces a self-limited disease that generates localised pustular lesions on the skin, and topical antiviral therapy may be indicated without the need for systemic administration of the drug. In these cases, the putative cytotoxic activity of the drug may not compromise the antiviral therapy. For instance, the off-label use of topical trifluridine has been reported as the best option for treating VACV-associated keratitis resultant from autoinoculation following exposure to smallpox vaccine [28,29]. Similarly, topical cidofovir has been more effective than parenteral cidofovir in treating progressive vaccinia lesions on the skin of immunocompromised mice [30]. This is particularly important in view of the poor oral bioavailability and limited intravenous use of cidofovir owing to nephrotoxicity [6]. Therefore, the current data on the antiviral effect of BQR against CTGV replication are highly promising and it may be considered for future assays as a topical antiviral therapy.

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Ethical approval: Not required.

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