ABSTRACT

**Background:** Fenbendazole, a dewormer drug, is used widely in the clinical treatment of parasite infections in animals. Recent studies have shown that fenbendazole has substantial effects on tumor growth, immune responses, and inflammatory responses, suggesting that fenbendazole is a pluripotent drug. Nevertheless, the antiviral effects have not been reported. Fenbendazole can disrupt microtubules, which are essential for multiple viruses infections, suggesting that fenbendazole might have antiviral effects.

**Objectives:** This study examined whether fenbendazole could inhibit bovine herpesvirus 1 (BoHV-1) productive infection in cell cultures.

**Methods:** The effects of fenbendazole on viral production, transcription of the immediate early (IE) genes, viron-associated protein expression, and the cellular signaling PLC-γ1/Akt pathway were assessed using distinct methods.

**Results:** Fenbendazole could inhibit BoHV-1 productive infections significantly in MDBK cells in a dose-dependent manner. A time-of-addition assay indicated that fenbendazole affected both the early and late stages in the virus replication cycles. The transcription of IE genes, including BoHV-1 infected cell protein 0 (bICP0), bICP4, and bICP22, as well as the synthesis of viron-associated proteins, were disrupted differentially by the fenbendazole treatment. The treatment did not affect the cellular signaling pathway of PLC-γ1/Akt, a known cascade playing important roles in virus infection.

**Conclusions:** Overall, fenbendazole has antiviral effects on BoHV-1 replication.

**Keywords:** Bovine herpesvirus 1; immediate early gene; fenbendazole; Akt

INTRODUCTION

Bovine herpesvirus 1 (BoHV-1), a member of the *alpha herpesvirus* subfamily, is an important pathogen causing a range of diseases in cattle, including pneumonia, conjunctivitis, and abortions [1]. Generally, the erosion of mucus and immune suppression due to virus infections may lead to secondary infections by diverse pathogens, resulting in life-threatening pneumonia known as bovine respiratory disease complex (BRDC) [2-4]. BRDC is one of the most important diseases in cattle of all ages and breeds [5,6], and exerts
considerable economic loss on the cattle industry worldwide. BRDC was reported to cost the US cattle industry approximately 3 billion dollars annually [7].

Fenbendazole ([5-(phenylthio)-1H-benzimidazol-2-yl] carboxamid acid methyl ester) is used widely for the treatment of pinworms, helminths, and a variety of other parasitic infections in laboratory animals, livestock, companion animals, and humans [8,9]. Recent studies extended its spectrum of pharmacological effects, and gradually revealed previously unrecognized functions. For example, fenbendazole was recently reported to have anticancer effects by affecting the function of proteasome and microtubules, as well as glucose uptake [9-11]. Fenbendazole treatment could improve the functional recovery of a traumatic spinal cord injury by regulating the function of lymphocytes and the inflammatory response [12]. Fenbendazole can regulate the function of the mitochondria and the cellular immune responses of peripheral blood lymphocytes by reducing the production of inflammatory cytokines, such as IFN-γ, TNF-α, and IL-1β [13,14]. Fenbendazole disrupts the gut microbiota of Amur tiger [15], indicating that it may affect the growth of bacteria. On the other hand, it is unclear if it possesses antiviral effects. Importantly, a fenbendazole treatment has several effects on cellular signaling transduction, such as the Akt and MAPK pathways in porcine trophectoderm [14]. Because both Akt and MAPK pathways are involved in BoHV-1 replication [16-18], it was hypothesized that fenbendazole might inhibit BoHV-1 replication.

This study examined whether fenbendazole affects BoHV-1 productive infections in cell cultures. A fenbendazole treatment reduced virus production by affecting the immediate early (IE) gene transcription and the synthesis of viron-associated proteins differentially. This is the first report showing that fenbendazole has antiviral effects.

**MATERIALS AND METHODS**

**Viruses and cells**

MDBK cells were maintained in DMEM (Gibco BRL) supplemented with 10% horse serum (Solarbio, cat# S9050). BoHV-1, NJ-16-1 isolated from bovine semen samples [19], was propagated in MDBK cells. Aliquots of virus stocks with a titer of 6.5 × 10⁶ pfu/mL were stored at −70°C until use.

**Chemicals and antibodies**

In this study, the following antibodies and chemicals were used, including fenbendazole (MedChemExpress, cat# HY-B0413). The antibodies against phospho-Akt(p-Akt) (S473) (Cell Signaling Technology, cat# 9271), Akt (Cell Signaling Technology, cat # 9272), PLC-γ1(Cell Signaling Technology, cat# 2822S), p-PLC-γ1(Ser1248) (Cell Signaling Technology, cat# 8713), GAPDH (Cell Signaling Technology, cat# 2118), and β-Actin (Cell Signaling Technology, cat# 4970) were used. Goat anti-BoHV-1 serum was purchased from VMDR Inc, Gandhinagar, India [20PAB-IBR]. HRP-(horseradish peroxidase-) conjugated goat anti-mouse IgG (Cell Signaling Technology, cat# 7076), HRP-goat anti-rabbit IgG (Cell Signaling Technology, cat# 7074), and HRP-donkey anti-goat IgG H&L (Abcam, ca# ab97110).

BoHV-1 VP16 antibody (1:2,000) was kindly provided by Prof. Vikram Misra at the University of Saskatchewan [20].
Antiviral effects assay
MDBK cells of confluent in 24-well plates were pretreated for one hour with either DMSO or fenbendazole (20 and 80 nM), respectively. The cells were then infected with BoHV-1 (MOI = 1) along with a chemical treatment. Two hours (h) after infection, the cells were washed three times with PBS (PH, 7.4) and replaced with fresh DMEM medium containing either DMSO or fenbendazole. The cell cultures were collected after infection for 24 h. After frozen-thawing twice, the cell cultures were clarified by centrifugation at 13,000 rpm for 5 min at 4°C. The virus titers in the supernatant were determined in the MDBK cells, with results expressed as TCID₅₀/mL calculated using the Reed-Muench calculation method.

Quantification of viral IE mRNA by qRT-PCR
The confluent MDBK cells in 60 mm dishes, which were pretreated with either DMSO or fenbendazole at a concentration of 20 nM, were infected with BoHV-1 (MOI = 1). After infection for 2 h, the cells were washed three times and the medium was replaced with fresh medium. Throughout infection, the cells were treated with either DMSO or fenbendazole. At 2 and 4 h post-infection (hpi), the cells were washed three times with PBS, and the total RNA was purified with TRIzol LS Reagent (Ambion, Cat#: 10296010) according to the manufacturer’s instruction. Freshly prepared RNA (1 µg) was used as a template for the synthesis of the first-strand cDNA with commercial random hexamer primers for viral mRNA detection using a Thermoscript RT-PCR system Kit (Invitrogen, catalogue #11146-024). The cDNA products were used as templates for qPCR, to measure the mRNA levels of viral IE genes, including BoHV-1 infected cell protein 0 (bICP0), bICP4, and bICP22 as well as cellular gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with the specific primers as described previously in the reference [21]. GAPDH mRNA was used as an internal control to normalize gene expression. The data was analyzed using the equation 2⁻^ΔΔCT method.

Western blotting analysis
MDBK cells in 60 mm dishes pretreated with either DMSO control or fenbendazole for one hour were mock-infected or infected with BoHV-1 (MOI = 1) for 12 and 24 h, respectively. Throughout the infection, the cells were treated with DMSO and fenbendazole at the indicated concentrations, respectively. The cells were collected, and cell lysates were prepared using RIPA buffer (1 × PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail. The clarified cell lysates through centrifugation at 13,000 rpm for 10 min were mixed with 4 X SDS-PAGE loading buffer, boiled for 10 min in a water bath, and subjected to Western blotting analysis using the designated antibodies. Either β-Actin or GAPDH was probed as the protein loading control.

RESULTS
Fenbendazole reduced BoHV-1 productive infection in cell cultures
The cytotoxicity of fenbendazole was initially examined on MDBK cells to determine if it had inhibitory effects on BoHV-1 productive infections. Based on a preliminary study, fenbendazole at a concentration of 80 nM for 24 h did not show apparent cytotoxicity to MDBK cells according to a Trypan-blue exclusion test, as described elsewhere [22] (Fig. 1A). Subsequently, the antiviral effects of fenbendazole at various concentrations (80 and 20 nM) were evaluated. The virus-infected cells were treated with fenbendazole throughout the infection, along with pretreatment for 1 h, as shown in the diagram (Fig. 1B, upper panel). Compared to the DMSO control, the presence of fenbendazole at a concentration of 20 and
80 nM reduced virus production by approximately 1.5- and 2.1-log, respectively (Fig. 1B, lower panel). This suggests that fenbendazole could inhibit BoHV-1 replication in cell cultures in a dose-dependent manner. Because fenbendazole at lower concentrations of 20 nM also showed inhibitory effects, the concentration of 20 nM was applied in the following studies.

A time-of-addition assay was performed to point which step(s) of the BoHV-1 replication cycles were affected by fenbendazole. Fenbendazole at a concentration of 20 nM was added to the virus-infected cell cultures at 0, 1, and 4 hpi, respectively, as shown in the diagram (Fig. 1C, upper panel). Compared to the DMSO control, the fenbendazole treatment starting from 0 hpi reduced the virus titer by approximately 1.2-log (Fig. 1C, lower panel). When fenbendazole was applied from 1 and 4 hpi, the virus yields were reduced consistently by ~0.7 log relative to the DMSO control (Fig. 1C, lower panel). These results suggest that both the early and late stages in the virus replication cycles were potentially affected by fenbendazole.

When fenbendazole was present in the virus-infected cells for one hour starting from 0 hpi, as shown in the diagram (Fig. 1D, upper panel), the virus titer was reduced by ~0.8 log compared to that of the DMSO control (Fig. 1D, lower panel). This further confirmed that fenbendazole affects virus replication at the early stages of infection.

**Fenbendazole affected BoHV-1 IE transcription**

The transcription of BoHV-1 IE genes starts at the early stages of infection, which is essential for a virus-productive infection. Here, the effects of fenbendazole on IE gene transcription were detected. The virus-infected cells were infected for 2 and 4 h together with the fenbendazole treatment at 20 nM. The levels of bICP0, bICP4, and bICP22 mRNA were then detected by qRT-PCR. As a result, in the presence of fenbendazole, the mRNA levels of all the IE genes detected were consistently lower at 2 hpi, which were decreased by ~50% relative to the DMSO control (Fig. 2). At 4 hpi, compared to the mock-treated control, the bICP0 mRNA...
levels were not affected by fenbendazole (Fig. 2A). The \( bICP4 \) mRNA levels significantly decreased to ~26% (Fig. 2B), while the \( bICP22 \) mRNA levels were increased approximately 6-fold (Fig. 2C). These results suggest that fenbendazole affects the transcription of these IE genes in a distinct manner, which corroborates the findings that fenbendazole can inhibit BoHV-1 infections at the early stages in the time-of-addition assay (Fig. 1C and D).

**Fenbendazole affected the expression of viron-associated proteins**

Because fenbendazole differentially affected viral IE transcription (Fig. 2), this study examined whether the expression of viron-associated proteins in the cell culture was affected. MDBK cells were infected with BoHV-1 for 12 and 24 h and treated with either DMSO or fenbendazole (20 nM) during the infection. The virion-associated proteins were detected by Western blot analysis using an antibody against BoHV-1 virion, as described elsewhere [21]. As a result, four molecules arbitrarily denoted by \( \alpha \), \( \beta \), \( \gamma \), and \( \varepsilon \), with distinct molecular weights ranging from 40 to 130 kDa could be detected clearly from the virus-infected cells at 24 hpi (Fig. 3A, right panel). The \( \alpha \) and \( \beta \) proteins were readily detected at 12 hpi (Fig. 3A, left panel). GAPDH protein was probed in the same membrane to indicate the protein loading control accurately (Fig. 3A). Quantitative analysis indicated that \( \alpha \) protein expression was not affected by fenbendazole at 12 hpi (Fig. 3B), but it was reduced by approximately 50% relative to the mock-treated control after infection for 24 h (Fig. 3C). The \( \beta \) protein levels were reduced to approximately 40% by fenbendazole relative to that in the mock-treated control at 12 hpi (Fig. 3B), but no inhibitory effect was observed at 24 hpi (Fig. 3C). At 24 h, following the fenbendazole treatment, the \( \varepsilon \) protein levels were ~63.1% compared to that in the mock-treated control, while \( \gamma \) protein expression was unaffected. Using a virus tegument protein VP16 specific antibody, VP16 protein expression was reduced significantly by fenbendazole at 12 hpi, which was reduced to approximately 45.5% compared to that of the control. After infection for 24 h, VP16 expression was unaffected by fenbendazole (Fig. 3D-E). Overall, the expression of virion-associated proteins, such as \( \alpha \), \( \beta \), \( \varepsilon \), and VP16 but not \( \gamma \) was affected differentially by fenbendazole. This shows that it has inhibitory effects on virus productive infection at the late stages in a time-of-addition assay (Fig. 1C).

**Fenbendazole had no effects on the PLC-\( \gamma \)/Akt cascade stimulated by BoHV-1 infection**

Fenbendazole broadly affects the cellular signaling transduction, such as Akt and MAPK pathways in porcine trophectoderm [14]. A BoHV-1 infection stimulates the PLC-\( \gamma \)/Akt cascade to facilitate virus-productive infection in cell cultures [17,23]. Here, we explored the
Fenbendazole has antiviral effects on BoHV-1 infection

The phosphorylation of PLC-γ1 at site S1248 and Akt at site S473 correlated well with the activation of the individual protein, respectively. Surprisingly, the inhibitory effects on the phosphorylation of either PLC-γ1 (Fig. 4A) or Akt (Fig. 4B) stimulated by a BoHV-1 infection were not observed after the fenbendazole treatment. In addition, in the context without infection, fenbendazole had no effects on the activation of either PLC-γ1 or Akt in MDBK cells (Fig. 4). As expected, the vehicle control, DMSO, had no effects on the phosphorylation of both PLC-γ1 and Akt in the context with or without a BoHV-1 infection (Fig. 4C), excluding the possible interruption by the solvent DMSO. These results suggest

effects of fenbendazole on the LC-γ1/Akt cascade in MDBK cells in a context with or without BoHV-1 infection. The phosphorylation of PLC-γ1 at site S1248 and Akt at site S473 correlated well with the activation of the individual protein, respectively. Surprisingly, the inhibitory effects on the phosphorylation of either PLC-γ1 (Fig. 4A) or Akt (Fig. 4B) stimulated by a BoHV-1 infection were not observed after the fenbendazole treatment. In addition, in the context without infection, fenbendazole had no effects on the activation of either PLC-γ1 or Akt in MDBK cells (Fig. 4). As expected, the vehicle control, DMSO, had no effects on the phosphorylation of both PLC-γ1 and Akt in the context with or without a BoHV-1 infection (Fig. 4C), excluding the possible interruption by the solvent DMSO. These results suggest

Fig. 3. Effects of Fenbendazole on the expression of virion-associated proteins. (A, D) MDBK cells were infected with BoHV-1 at an MOI of 1 for 12 and 24 h, in the presence of fenbendazole or DMSO control throughout infection plus a pretreatment for 1 h. The cell lysates were then prepared for Western blots to detect virion-associated proteins using a commercial anti-BoHV-1 serum (VMDR Inc, cat#: P110325-001) (A) or VP16 specific antibody (D). Given bands were denoted with α, β, γ, and ε. (B, C, and E) The relative band intensity was analyzed with image J, and each analysis was compared with that of uninfected control, which was arbitrarily set to one. The data shown are the means ± SD of three independent experiments. Statistical analyses were performed using Student’s t-test (*P < 0.05).

ns, not significant; hpi, hours post-infection.

Fig. 4. Fenbendazole did not affect the PLC-γ1/Akt cascade stimulated by the BoHV-1 infection. (A, B) MDBK cells were infected with BoHV-1 (MOI = 1) for 24 h. Throughout the infection, the cells were treated with fenbendazole at various concentrations plus a pretreatment for 1 h. The cell lysates were prepared and subjected to Western blots to detect the variation of PLC-γ1(S1248) and PLC-γ1 (A), p-Akt(S473) and Akt (B), respectively. (C) MDBK cells pretreated with or without the vehicle control DMSO for 1 h were infected with BoHV-1 (MOI = 1) together with or without DMSO for 24 h. The cell lysates were collected, and protein levels of both PLC-γ1(S1248) and p-Akt(S473) were detected by using Western blots. β-Actin was detected and used as a protein loading control. Data shown are representative results of two independent experiments.
that the inhibitory effects of fenbendazole on BoHV-1 infection do not rely on blocking the PLC-γ1/Akt cascade essential for the virus infection.

DISCUSSION

Fenbendazole is an anti-parasite medicine approved by FDA that can be administered to sheep, cattle, horses, fish, dogs, cats, rabbits, and seals. The drug can affect many gastrointestinal parasites, such as giardia, roundworms, hookworms, whipworms, and pinworms. Recently, several studies have indicated that fenbendazole is a promising candidate for the treatment of multiple cancers [9,10,24], providing insight on the pharmacological effects of fenbendazole. This study showed that fenbendazole has antiviral effects that can inhibit BoHV-1 productive infection in cell cultures, which would extend its pharmacological effects further.

Fenbendazole could physically associate with the cytoskeletal protein tubulin and disrupt the microtubule structure, a potential mechanism to kill parasites and tumors [9,10,25]. Previous studies reported that microtubules play critical roles in the replication cycles of multiple viruses, including human immunodeficiency virus type 1 (HIV-1), vaccinia virus, rabies virus, and herpes simplex virus I(HSV-1) [26-32]. HSV-1 virions consist of four structural components: DNA, capsid, tegument, and envelope [33]. Upon HSV-1 infection, the cellular microtubules are reorganized and partially mediated by the viral regulatory protein ICP0 [30,34,35]. The incoming cytosolic capsids and tegument protein VP22 are transported along the microtubules to the nucleus [30,35-37]. Both BoHV-1 and HSV-1 belong to the alpha herpesvirus subfamily, and they generally share many biological features. The requirement of microtubules might also be applied to BoHV-1 productive infections. Thus, fenbendazole may inhibit BoHV-1 replication because of its strong capacity to disrupt microtubules.

During productive infection, the BoHV-1 genes are transcribed in an ordered cascade of immediate-early (IE), early (E), and late (L) genes [38], regulated by the viral and cellular transcriptional machinery. This study found that fenbendazole treatment had differential effects on the transcription of IE genes, including bICP0, bICP4, and bICP22 (Fig. 2), as well as the protein expression of various viron-associated proteins (Fig. 3). These results are consistent with the findings that fenbendazole could broadly affect the early and late stages of a virus infection in a time-of-addition assay (Fig. 1D-G). The differential effects on viral gene transcription and protein expression are also in line with its antiviral activities.

Interestingly, this study found that fenbendazole decreased bICP22 transcription at 2 hpi, but it was increased at 4 hpi. A similar phenomenon in other chemicals has been reported, e.g., HMGA1-specific inhibitor netropsin could inhibit BoHV-1 replication. The treatment with netropsin leads to the decreased transcription of bICP0 and bICP22, but increased transcription of bICP4 at 16 hpi [21]. Though cycloheximide could inhibit HSV-1 replication HSV-1 IE gene ICP0 expression was enhanced by cycloheximide treatment [39]. Therefore, an antiviral compound, such as fenbendazole, may also have the capacity to upregulate the transcription of the given viral genes.

The Akt signaling pathway is activated by a BoHV-1 infection, which in turn plays critical roles in the virus productive infection in MDBK cells [16-18]. Fenbendazole was reported to affect Akt cellular signaling transduction in the porcine trophoderm [14]. The present data indicated that Akt phosphorylation was not affected by fenbendazole with or without a
virus infection (Fig. 4). Of note, the bovine kidney cell line, MDBK, has different origins and biological features from the porcine trophectoderm, which may account for this discrepancy. Nevertheless, the data suggest that the antiviral activities of fenbendazole in MDBK cells were not depending on affecting Akt signaling.

In summary, the widely used dewormer, fenbendazole, can inhibit BoHV-1 replication by differentially affecting viral IE transcription and viron-associated protein synthesis. This is a novel finding on the pharmacological effects of fenbendazole.

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