Efficacy of oleandrin and PBI-05204 against bovine viruses of importance to commercial cattle health

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Abstract

Background: Bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), and bovine coronavirus (BCV) threaten the productivity of cattle worldwide. Development of therapeutics that can control the spread of these viruses is an unmet need. The present research was designed to explore the in vitro antiviral activity of the Nerium oleander derived cardiac glycoside oleandrin and a defined N. oleander plant extract (PBI-05204) containing oleandrin.

Methods: Madin Darby Bovine Kidney (MDBK) cells, Bovine Turbinate (BT) cells, and Human Rectal Tumor-18 (HRT-18) cells were used as in vitro culture systems for BVDV, BRSV and BCV, respectively. Cytotoxicity was established using serial dilutions of oleandrin or PBI-05204. Noncytotoxic concentrations of each drug were used either prior to or at 12 h and 24 h following virus exposure to corresponding viruses. Infectious virus titers were determined following each treatment.

Results: Both oleandrin as well as PBI-05204 demonstrated strong antiviral activity against BVDV, BRSV, and BCV, in a dose-dependent manner, when added prior to or following infection of host cells. Determination of viral loads by PCR demonstrated a concentration dependent decline in virus replication. Importantly, the relative ability of virus produced from treated cultures to infect new host cells was reduced by as much as 10,000-fold at noncytotoxic concentrations of oleandrin or PBI-05204.

Conclusions: The research demonstrates the potency of oleandrin and PBI-05204 to inhibit infectivity of three important enveloped bovine viruses in vitro. These data showing non-toxic concentrations of oleandrin inhibiting infectivity of three bovine viruses support further investigation of in vivo antiviral efficacy.

Keywords

PBI-05204, oleandrin, bovine respiratory syncytial virus, bovine coronavirus, bovine viral diarrhea virus, Nerium oleander

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Introduction

According to the United States Department of Agriculture, in 2021 there were a total of 93.6 million head of cows and heifers in the United States, and of these, 31 million and 9.4 million were used for beef and milk production, respectively.1 The health of these commercial animals is of paramount importance as they represent a vital human food supply.2 Unfortunately, cattle are susceptible to numerous types of viral infections and the resulting Diseases are a constant threat to their health and well-being.2 Because these animals are an important component of the food supply, significant losses can lead to widespread food shortages. Importantly, infected cattle also present a major economic burden on ranchers as adequate control and elimination of the diseases caused by these viruses is often
difficult and problematic.\textsuperscript{3,4} Three of the most common viruses affecting cattle health are bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), and bovine coronavirus (BCV).

Bovine Viral Diarrhea caused by BVDV affects herd health and reproduction in many countries around the world leading to massive economic losses.\textsuperscript{5–7} BVDV is a member of the genus \textit{Pestivirus} of the family \textit{Flaviviridae}. The enveloped virus consists of a single, linear, positive-sense, single stranded RNA.\textsuperscript{8} BVD is currently one of the costliest diseases of cattle.\textsuperscript{7} Abortions, infertility, and/or embryonic death associated with BVD significantly reduce reproductive performance and increase premature culling.\textsuperscript{9} Persistent infection that occurs during fetal development results in animals that continually shed BVDV making them a constant threat to the herd.\textsuperscript{8,9} Animals that develop acute diarrhea and fever may die or have long, costly recovery periods with decreased milk production and/or growth. During recovery their immune system is often depressed, making them more susceptible to other diseases.\textsuperscript{10–13}

Bovine respiratory syncytial virus (BRSV) is an enveloped, negative sense, single stranded RNA virus belonging to the \textit{Pneumovirus} genus within the family \textit{Paramyxoviridae}.\textsuperscript{8} BRSV causes respiratory disease in young calves and is a major pathogen associated with BRDC.\textsuperscript{14} The infection with BRSV results in significant economic losses in the cattle industry due to morbidity and mortality, along with treatment and prevention costs that lead to reduced production.\textsuperscript{15,16}

Bovine coronavirus (BCV or BCoV), a member of the species \textit{Betacoronavirus 1} is an enveloped, positive sense, single stranded RNA virus.\textsuperscript{8} It has up to 95% similarity with human coronaviruses and 93% similarity to porcine hemagglutinating encephalomyelitis virus.\textsuperscript{3,14,17,18} BCV infection causes calf enteritis and frequently leads to profuse diarrhea, dehydration, reduced weight gain and anorexia. It can also cause winter dysentery in adult cattle.\textsuperscript{19} Because of its tropism for both the gastrointestinal tract and the respiratory tract, BCV has recently become an important respiratory pathogen associated with bovine respiratory disease complex (BRDC).\textsuperscript{8}

Although vaccines are important tools for the control of many bovine viral pathogens, in some situations they have proven insufficient to control the associated diseases, despite decades of use.\textsuperscript{20} Likewise, no antiviral products are currently licensed for use in cattle within the USA which indicates that an effective prophylactic and/or therapeutic agent against viruses of importance to commercial cattle production is an urgent need. The present studies demonstrate the use of low, nontoxic concentrations of oleandrin or PBI-05204, a defined extract of \textit{N. oleander} that contains this molecule against three separate viruses affecting cattle health.

### Materials and methods

#### Cells, viruses, and antiviral treatments

Bovine Viral Diarrhea virus (BVDV) Type 1 Singer Lot# 040917 was obtained from the National Veterinary Services Laboratory (NVSL Lot# 140BDV0401) and propagated on Bovine Turbinate (BT) cells. Bovine Respiratory Syncytial Virus (BRSV) AS1908 (ATCC VR794) Lot# 181219 was purchased from NVSL (Lot# 110BDV1401) and propagated on BT cells. Bovine Coronavirus (BCV) Nebraska strain Lot# 010321 was obtained from NVSL (Lot# 020BDV0701) and was propagated on Human Rectal Tumor-18 (HRT-18) cells. Prior to the assays, working stock for each virus was titrated on the appropriate cell line used for the assay. These positive-sense RNA viruses have one-step growth curves with detection at 12 hr, are at 70-80% peak production at 24 h and peak prior to 48 h.

MDBK, BT, and HRT cells were used for the prophylactic and therapeutic assays for BVDV, BRSV, and BCV, respectively. The cells were maintained in a 37°C incubator with 5% CO\textsubscript{2} and propagated using Eagle’s Minimum Essential Medium (MEM) (Corning, Manassas, VA) supplemented with 10% Fetal Bovine Serum (PAA GE Healthcare, Westborough, MA), 1× Antibiotic/ Antimycotic solution (Ab/Am) (Cytiva, Logan, UT), and 1× L-glutamine solution (Gibco, Grand Island, NY). Additionally, 1% Lactalbumin hydrolysate (BD, Sparks, MD) and 1× MEM vitamins (Cytiva, Logan, UT) were added to the growth media for BT cells. Maintenance media for all viruses was comprised of MEM containing 2% horse serum (Gibco, Grand Island, NY), 1× Ab/Am, and 1× L-glutamine.

PBI-05204 is a supercritical CO\textsubscript{2} extract of \textit{Nerium oleander} leaves and was provided by Phoenix Biotechnology, Inc. (San Antonio, Texas). Characterization of PBI-05204 was carried out using an AccuTOF-DART mass spectrometer (Jeol UAS, Peabody, MA). Specific content of the extract was previously reported.\textsuperscript{21} The extract contains cardiac glycosides, oleandrin (2.99%) and oleandrigenin (3.31%); triterpenic acids, ursolic and betulinic acids (combined total of 15.29%) and oleanolic acid (0.60%) and odoroside (0.8%); Urs-12-ene-3β, 28-diol/biutin (5.44%); 3β, 3β-hydroxy-12-olean-28-oic acid (14.26%); 28-nours-12-3β-ol (4.94%); and urs-12-EN-3β-ol (4.76%). Other triterpenoids present in the extract of \textit{Nerium oleander} have been reported by others.\textsuperscript{22} A stock solution of PBI-05204 at a concentration of 1 mg/ml in DMSO was diluted based on the oleandrin content as shown in the figures and tables for use in the different assays. A stock solution of oleandrin (PhytoLab, Vestenbergsgreuth, Germany) was dissolved at a concentration of 1 mg/ml in DMSO (Invitrogen, Eugene, OR) and concentrations ranging between 1000 to 5 ng/ml were used in the different assays. The relative cytotoxicity as well as
data pertaining to antiviral efficacy of PBI-05204 are reported as equivalent oleandrin concentrations to permit a direct comparison to pure oleandrin. Actual PBI-05204 concentrations ranged from 294 ng/ml to 58,800 ng/ml which were equivalent to 0.005 μg/ml to 1 μg/ml of oleandrin, respectively.

**LDH toxicity assay**

The optimum cell number to use in the toxicity assay was determined for each of the three different cell types used for the three viruses. Different cell seeding capacities were prepared in 100μl volumes starting at 1000 cell/100μl to 80,000 cell/100μl in a 2-fold serial dilution manner. This serial dilution was done in triplicate for 2 sets of cells. One set was used as a cell control to report the spontaneous LDH release, and the other set was treated with cell lysis buffer to report the maximum LDH release. After performing the test according to the manufacturer’s instructions, the average OD value reading of the triplicate samples for each dilution in each set was recorded and plotted against the number of cells. The best cell seeding capacity/100μl volume was the one that achieved maximum LDH release of 1.2-2 and spontaneous LDH release of less than 0.5 after 30 min of incubation with the kit mix (manufacturer instructions) at both time points.

For testing the toxicity of oleandrin and PBI-05204, tissue culture plates (96-well) were seeded with 2–4 × 10^4 of MDBK, BT or HRT cells in 100μl growth media and cultured in a 37°C/5% CO_2 incubator overnight. The following day, the growth media was removed and replaced with 100μl maintenance media containing either different concentrations of pure oleandrin or PBI-05204 in DMSO along with matching dilutions of DMSO or untreated media to serve as controls. All treatments were added to triplicate wells and cultured in the 37°C/5% CO_2 incubator. At either 24- or 48-h post-treatment, the cell viability was assessed by determining the LDH released into the supernatant using the CyQUANT LDH toxicity assay kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s directions. Absorbance was measured at 490 nm and 680 nm on the Spectramax i3x (Molecular devices, San Jose, CA).

**In vitro assays to determine the antiviral activities of oleandrin and PBI-05204**

Confluent monolayers (approximately 5 × 10^5 cells per well in 12-well plates) of respective target cells for each of the three viruses were infected and treated at different time points (pre-, 12h-, and 24h-post challenge) with serial dilutions of oleandrin (5-1000 ng/ml) or PBI-05204 (5–50 ng/ml of oleandrin), selected based on the toxicity assay, along with corresponding dilutions of DMSO controls in fresh maintenance media.

For the pre-challenge treatment, the cells in triplicate wells were incubated with the drug concentrations for 30 min followed by the addition of virus (5 × 10^3 TCID_{50} virus units in a volume of 500 μl maintenance media) at a multiplicity of infection (MOI) of 0.01. The infection was allowed to proceed for one hour at 37°C/5% CO_2. After infection, cells were washed gently 3 times with phosphate buffered saline (PBS). Finally, 2 ml of maintenance media containing the original concentrations of oleandrin or PBI-05204 in DMSO, or DMSO was added. Samples of cell culture supernatants were collected at 24- and 48-h post infection for determining the amount of virus and its infectivity.

For the post-challenge treatments, the cells in triplicate wells were treated at either 12- or 24-h post-infection with the different concentrations of oleandrin or PBI-05204 in DMSO or matched DMSO-only controls. Samples of cell culture supernatants from the 12-h post-infection treatment were collected at 24- and 48-h post-infection. Similarly, samples of cell culture supernatants from the 24-h post-infection treatment were collected at 48-h post-infection.

One set of culture supernatant samples collected were used immediately for determining the infectivity of the progeny virus by the TCID_{50} assay and the second set of samples were stored at −80°C for determining the amount of virus by the PCR assay.

**TCID_{50} assay**

Cells appropriate for each virus were seeded in 96-well plates to confluence. Samples of culture supernatant samples collected as described above were serially diluted 10-fold in maintenance medium. Diluted samples were plated onto cells in triplicate in a volume of 200 μL/well and incubated at 37°C/5% CO_2 for the appropriate time established for each virus. BVDV samples were incubated for 3 days, BRSV samples were incubated for 9 days, and BCV samples were incubated for 3 days. After incubation, plates were fixed with acetone and stained using an appropriate monoclonal antibody specific for each virus (BVD 157, VMRD, Pullman, WA); (BRSV RSV3216[0016], GeneTex, Irvine, CA); (BCV BC6-4, RTI, Brookings, SD). Virus was visualized with a goat anti-mouse FITC secondary conjugate (Invitrogen, Carlsbad, CA) and plates were read using a fluorescence microscope. Wells positive for virus were recorded and TCID_{50} virus units were calculated using the Spearman-Karber method.

**Determination of the virus load in the culture supernatants**

An aliquot of each sample from all treatments and time points for each virus was used for Viral nucleic acid extraction and real-time PCR (RT-qPCR) at South Dakota State
University. Also, 10-fold serial dilutions of each virus stock were used to create a corresponding standard curve. Viral nucleic acid was extracted from samples using the MagMAX viral RNA/DNA isolation kit (Life Technologies) following the manufacturer’s instructions. Detection of BRSV, BCV, and BVDV RNA was performed using a commercial RT-qPCR kit, PATH-ID MPX One Step (Life Technologies). For BRSV, the primers and probe target the conserved N gene of BRSV. For BCV, the PCR amplifies the spike (S) protein encoded by ORF 4. For BVDV, the 5’ untranslated region of the viral genome is targeted for PCR.

The qPCR was performed using ABI 7500 Realtime PCR system. The CT values were converted to TCID₅₀ equivalent/mL by plotting the average CT values for each sample against the standard curve created for each virus.

Results

Relative in vitro cytotoxicity of oleandrin and PBI-05204

The relative cytotoxicity of PBI-05204 as well as oleandrin was determined separately for all three cell lines used in this study (Table 1). Oleandrin and PBI-05204 were dissolved in DMSO and diluted to achieve equivalent oleandrin concentrations ranging between 5 and 500 ng/ml. The highest DMSO control concentrations were 0.05% for oleandrin and 0.058% for PBI-05204. None of the DMSO only solutions showed cytotoxicity after 48 h of continuous incubation. Oleandrin was nontoxic to MDBK cells up to and including a concentration of 50 ng/ml and less than 10% toxicity between 100-500 ng/ml while PBI-05204 at equivalent oleandrin concentrations was nontoxic up to a concentration of 50 ng/ml. Oleandrin was completely nontoxic at all concentrations tested against HRT and BT cells, while the relative nontoxic oleandrin concentrations within PBI-05204 were between 5 and 50 ng/ml for the cell lines. Based on these results, oleandrin at 5, 10, and 50 ng/ml and PBI-05204 with matching oleandrin concentrations were tested for antiviral activities against BVDV, BRSV, and BCV using the corresponding host cells.

Effect of PBI-05204 and oleandrin on production of virus and its infectivity

The host cells were either pretreated with oleandrin or PBI-05204 for 30 min prior to infection or added at 12 h and 24 h post-infection with each virus. One set of the culture supernatants from each time point were assessed for the total amount of virus produced by the RT-qPCR assay. The CT values from the assay representing virus production were converted to TCID₅₀ equivalent/mL and plotted (Figures 1, 2, 3; panels A and C). For each of the viruses, the supernatants from infected cultures were assessed for the infectivity of the progeny virus by the TCID₅₀ assay using fresh host cells (Figures 1, 2, 3; panels B and D).

BVDV. For BVDV infection of MDBK cells, both pure oleandrin and PBI-05204 (with matching oleandrin content) exhibited dose dependent inhibition of the total amount of virus produced (Figure 1A and 1C) and its infectivity (Figure 1B and 1D) when the treatment was initiated prior to infection, and to a lesser extent at 12 h post-infection. Furthermore, the inhibitory effects of oleandrin as well as PBI-05204 on both the amount of virus produced and its infectivity are more pronounced after 48 h culture relative to 24 h, particularly at the two higher oleandrin concentrations tested even when the treatment started post-infection and the drug was not replenished.

Overall, the inhibition of infectivity of the progeny virus at each of the concentrations tested for both oleandrin and PBI-05204 was more pronounced relative to that of the total amount of virus produced (Table 2). Interestingly, while treatment 24 h post-infection was ineffective in reducing the amount of virus produced (Figure 1A and 1C), the infectivity of the progeny virus was reduced (reaching 50 to 100 fold) at the highest non-toxic concentration tested (Table 2).

Table 1. Cytotoxicity of oleandrin and PBI-05204 on the different target cells used for viral infections.

| Oleandrin concentration (ng/ml) | PBI-05204 | Oleandrin |
|--------------------------------|-----------|-----------|
|                               | MDBK cells* | BT cells | HRT-18 cells | MDBK cells | BT cells | HRT-18 cells |
| 5                              | 0%         | 0%       | 0%         | 0%         | 0%       | 0%         |
| 10                             | 0%         | 0%       | 0%         | 0%         | 0%       | 0%         |
| 50                             | 0%         | 0%       | 0%         | 0%         | 0%       | 0%         |
| 100                            | 13.8%      | 18%      | 14.5%      | 4.8%       | 0%       | 0%         |
| 500                            | 38.3%      | 9%       | 19.2%      | 6.6%       | 0%       | 0%         |

*MDBK: Madin Darby Bovine Kidney cells used as target cells for BVDV infection; BT: Bovine Turbinate cells used as target cells for BRSV infection; HRT-18: Human Rectal Tumor-18 cells used as target cells for BCV infection. The results are reported as % cytotoxicity according to the following formula %cytotoxicity of a treatment = (OD value of the treatment well-OD value of negative control)/(OD value of positive cytotoxicity control-OD value of negative control) x 100.
In the case of BRSV infection of BT cells, both oleandrin and PBI-05204 were marginally effective in reducing the amount of virus produced (Figure 2A and 2C), but strong concentration-dependent reduction in the infectivity of progeny virus was observed, even when the treatment was initiated 12 h and 24 h post-infection (Figure 2B and 2D; Table 3).

Both oleandrin and PBI-05204 reduced infectivity of the progeny virus produced in a concentration-dependent manner and this effect increased with culturing time from 24 h to 48 h (Table 3). It is noteworthy that the magnitude of inhibitory effects of both oleandrin and PBI-50204 against BRSV are relatively lower compared to that seen against BVDV (Table 2), which may be because the replication kinetics of BRSV require longer culture time to increase the viral titers.

Treatment with oleandrin and PBI-05204 reduced the amount of virus produced as well as its infectivity in case of BCV infection of HRT cells (Figure 3A-D). This antiviral effect was more pronounced at 48 h, relative to 24 h of culturing, specifically at the 50 ng/ml concentration, irrespective of when the treatment was initiated. Similar to the observations with infections by BVDV and BRSV, in case of BCV infection also, treatment with oleandrin and PBI-05204 containing matching oleandrin concentrations resulted in the reduction of the infectivity of the progeny virus that is more evident relative to that in the amount of total virus produced (Figure 3A and 3C relative to 3B and 3D, respectively).

Overall, treatment prior to and 12 h post-infection resulted in stronger inhibition of infectivity with both pure oleandrin and PBI-05204 with matching oleandrin concentrations (Table 4).

**Discussion**

Our research has shown that oleandrin, a cardiac glycoside (CG) and PBI-05204, a defined N. oleander plant extract containing this molecule are highly effective as antiviral agents against viruses of importance to human health, little has been reported of the efficacy of CGs against viruses of importance to commercial animal production. Yang et al. have demonstrated the ability of several CGs including oleandrin to potently inhibit porcine
transmissible gastroenteritis while McGill et al.\textsuperscript{33} have shown that digoxin can be given safely to calves to achieve moderation of BRSV. The authors of both studies have suggested that additional research against viruses of importance to commercial animal production should be actively considered.

We reported earlier that oleandrin mediated inhibition of infectivity against HIV,\textsuperscript{30} HTLV-1\textsuperscript{31} and SARS-CoV-2,\textsuperscript{29} three viruses affecting human health and welfare. Beyond the established ability of CGs to inhibit transmembrane cellular ion flux through inhibition of Na-K-ATPase, a wide variety of other mechanisms of action have been reported of relevance to cancer and neurodegenerative disease. With respect to antiviral activity, CGs such as oleandrin and digoxin have multiple mechanisms of action. These include inhibition of an endoplasmic reticulum stress response that facilitates virally infected cells to undergo apoptosis,\textsuperscript{34,35} inhibition of viral protein translation,\textsuperscript{36–38} inhibition of viral gene expression,\textsuperscript{39–41} inhibition of viral pre-RNA splicing,\textsuperscript{42} inhibition of viral entry through ATP-mediated Src signaling\textsuperscript{43} and decreased content of viral envelope leading to greatly reduced progeny virus infectivity.\textsuperscript{30,31}

A particularly intriguing aspect of the antiviral activity of oleandrin observed in the present investigation is the reduction in the infectivity of the progeny virus that is more pronounced than the viral replication, measured in terms of the amount of virus produced, within the treated cultures for each of the three viruses tested. We observed similar outcome for the activity of oleandrin against HIV-1 where it also showed significant reduction in the envelope content of the progeny virus as the likely underlying mechanism for reduced infectivity.\textsuperscript{30} Together, our earlier published studies and the current investigation exemplify the importance of antiviral activity manifested in terms of reduction in infectivity and underscore the potential practical constraints of viral load measurements such as the RT-PCR methodology employed here that detect viral particles irrespective of infective functional significance.

Like most compounds that are used for therapeutic effect, CGs including oleandrin, which is found in the poisonous plant oleander (N. oleander), can be toxic at certain concentrations or doses. In cattle, poisoning from oleander is rare although it has been reported.\textsuperscript{44,45} It is mainly due to contamination of feed products and not through grazing as oleander plants have a bitter taste.\textsuperscript{44} While in vivo pilot studies of PBI-05204 and related products prepared from N. oleander have yet to be carried out, human clinical trials with PBI-05204 have shown that it can be safely administered to patients with cancer without serious adverse consequences.\textsuperscript{46,47} The documented plasma

![Figure 2](image-url)
Figure 3. Effect of time of PBI-05204 or oleandrin treatment on BCV infection. Target HRT cells were treated with various concentrations of PBI-05204 (A and B) or Oleandrin (C and D) either 30 min before (Pre) or 12h-Post and 24h-Post infection with BCV (MOI = 0.01 based on TCID50) followed by determining at the end of 24 h and 48 h infection the amount of produced virus (A and C) or its infectivity (B and D). The amount of BCV, in terms of 50% Tissue Culture Infectious Dose (TCID50) equivalent titers were quantified via real-time RT-qPCR assay of serial stock virus dilutions, and infectious BCV titers were quantified directly by the TCID50 assay on fresh target cells. Bar heights represent the mean and error bars represent the standard deviation.

Table 2. Percent inhibition in the infectivity of BVDV in the cultures treated with oleandrin or PBI-05204.

| Concentration (ng/ml) | Pre-24h | Pre-48h | 12h-24h | 12h-48h | 24h-48h |
|-----------------------|---------|---------|---------|---------|---------|
| PBI-05204             |         |         |         |         |         |
| 5                     | 68.34   | 99.07   | 0       | 97.38   | 61.87   |
| 10                    | 99.37   | 99.94   | 82.77   | 99.87   | 87.22   |
| 50                    | 98.78   | 100     | 98.28   | 99.99   | 99.00   |
| Oleandrin             |         |         |         |         |         |
| 5                     | 21.73   | 90.22   | 41.93   | 0       | 0       |
| 10                    | 92.69   | 99.32   | 46.71   | 97.13   | 92.58   |
| 50                    | 98.44   | 99.99   | 93.18   | 99.97   | 98.21   |

Table 3. Percent inhibition reduction in the infectivity of BRSV in the cultures treated with oleandrin or PBI-05204.

| Concentration (ng/ml) | Pre-24h | Pre-48h | 12h-24h | 12h-48h | 24h-48h |
|-----------------------|---------|---------|---------|---------|---------|
| PBI-05204             |         |         |         |         |         |
| 5                     | 35.17   | 93.67   | 52.76   | 85.10   | 70.43   |
| 10                    | 34.73   | 97.52   | 34.74   | 84.66   | 83.78   |
| 50                    | 58.98   | 96.98   | 83.29   | 97.96   | 89.46   |
| Oleandrin             |         |         |         |         |         |
| 5                     | 41.22   | 83.29   | 39.95   | 63.50   | 0       |
| 10                    | 38.11   | 94.91   | 77.07   | 63.92   | 34.85   |
| 50                    | 72.86   | 97.51   | 85.03   | 98.76   | 95.02   |
concentrations of oleandrin achieved in these clinical trials believed to be important for elimination of malignant cell proliferation are higher than the low concentrations shown in the present study to be effective for virus control.

Our research has shown that PBI-05204 as well as its active principal ingredient, oleandrin, target viruses with envelope structures and provides effective inhibition of progeny virus infectivity. The relevance to enveloped viruses important to commercial cattle production is clear when it is recognized that multiple diseases affecting commercial cattle production are attributed to those pathogens within the following virus families: Herpesviridae, Paroviridae, Rteroviridae, Bunyaviridae, Coronavirus, Flaviviridae, Paramyxoviridae, Rhabdoviridae and Togaviridae, all of which consist of enveloped viruses. Our research has shown highly effective inhibition of viral infectivity against BVDV, BCV and BRSV that belong to three distinctly different virus families, namely, Flaviviridae, Coronaviruses, and Paramyxoviridae, respectively. Additional planned research will explore the antiviral ability of PBI-05204 to reduce infectivity of viruses in additional families of enveloped viruses, including those of importance to commercial porcine production. Successful application of the present research to effective prevention or control of targeted bovine virus mediated disease will require adequate safety studies and suitable formulation development.

Authors’ contributions
KJS, CCLC and AW designed experiments, RB, HA and KA performed the experiments with great help from SVH. HA, AW, KA and RB were major contributors in analyzing the data and interpreting the results. RAN, KJS and CCLC were major contributors in writing the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests
The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Xxxxxxx. RAN serves as the Chief Science Officer and Director of Phoenix Biotechnology, Inc.; JRM is a Director and KJS is a paid consultant (Phoenix Biotechnology, Inc.). All remaining coauthors declare no conflict of interest.

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Table 4. Percent inhibition in the infectivity of BCV in the cultures treated with oleandrin or PBI-05204.

| Concentration (ng/ml) | Pre-24h | Pre-48h | 12h-24h | 12h-48h | 24h-48h |
|-----------------------|---------|---------|---------|---------|---------|
| 5                     | 66.06   | 37.15   | 0       | 53.58   | 49.51   |
| 10                    | 44.01   | 83.14   | 0       | 94.67   | 88.26   |
| 50                    | 86.32   | 99.99   | 47.86   | 99.32   | 99.15   |
| Oleandrin             |         |         |         |         |         |
| 5                     | 91.71   | 98.72   | 31.96   | 34.26   | 0       |
| 10                    | 93.57   | 99.98   | 68.42   | 95.36   | 0       |
| 50                    | 100.00  | 100.00  | 74.38   | 99.97   | 97.58   |

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