Available online at www.sciencedirect.com
SCIENCE DIRECT®
Antiviral Research 64 (2004) 113–118
Antiviral Research
www.elsevier.com/locate/antiviral

Prevention and elimination of bovine viral diarrhea virus infections in fetal fibroblast cells

M. Daniel Givens[a,*], David A. Stringfellow[a], Christine C. Dykstra[a], Kay P. Riddell[a], Patricia K. Galik[a], Eddie Sullivan[b], James Robl[b], Poothapillai Kasinathan[b], Arvind Kumar[c], David W. Boykin[c]

[a] *127 Sugg Laboratory, College of Veterinary Medicine, Auburn University, AL 36849-5516, USA*
[b] *Hematech, LLC, Westport, CT 06880, USA*
[c] *Department of Chemistry, Georgia State University, Atlanta, GA 30303, USA*

Received 9 March 2004; accepted 26 July 2004

Abstract

Noncytopathic infections with bovine viral diarrhea virus (BVDV) can compromise research and commercial use of cultured cells. The purpose of this research was to evaluate the ability of aromatic cationic compounds to prevent or treat BVDV infections in fetal fibroblast cell lines that are used in somatic cell nuclear transfer. To evaluate preventative use of compounds, 10 cell lines were inoculated with BVDV in the absence or presence of 2-(4-[2-imidazolinyl]phenyl)-5-(4-methoxyphenyl)furan (DB606), 2-(2-benzimidazolyl)-5-[4-(2-imidazolino)phenyl]furan dihydrochloride (DB772), or 2-(1-methyl-2-benzimidazolyl)-5-[4′-(2-imidazolino)-2′-methylphenyl]furan dihydrochloride (DB824). The 99% endpoints for prevention of viral replication by these treatments were 81, 6, and 14 nM. To evaluate therapeutic use of compounds, two fetal fibroblast cell lines infected with a genotype 1a strain of BVDV were cultured through four passages in the absence or presence of either 0.04 or 4 μM concentrations of DB772 or DB824. The presence and concentration of BVDV in media and cell lysates were evaluated using reverse transcription nested polymerase chain reaction and virus isolation from titrated sample. A single passage in 4 μM of either compound was sufficient to eliminate BVDV from cells without causing cytotoxicity. Our results demonstrate that in vitro infections with BVDV can be effectively prevented or eliminated by addition of aromatic cations.

Keywords: Bovine viral diarrhea virus; Somatic cell nuclear transfer; Antiviral; Fetal fibroblast

1. Introduction

Bovine viral diarrhea virus (BVDV), the prototype of the *Pestivirus* genus of the *Flaviviridae* family, causes clinically and economically significant disease in cattle throughout the world (Brownlie, 1990; Houe, 1999). The RNA-dependent RNA polymerase of BVDV permits a high frequency of base substitutions that contributes to tremendous pathogenic and antigenic diversity (Donis, 1995). Some isolates cause subclinical disease while others cause acute infections of immunocompetent cattle that result in mortality rates of 17–32% (Carman et al., 1998; Ellis et al., 1998; Pellerin et al., 1994).

In addition to causing disease on the farm, BVDV can be a problematic contaminant in the laboratory (Yanagi et al., 1996). According to their effect in cell culture, BVDV strains are recognized as either cytopathic or noncytopathic (Harding et al., 2002). As the overwhelming majority of BVDV isolates are noncytopathic, infections can easily go unnoticed in the laboratory (Bezek et al., 1994). Noncytopathic BVDV isolates frequently contaminate commercially available lots of fetal bovine serum despite testing by the manufacturer (Bolin et al., 1991; Yanagi et al., 1996). Consequently, BVDV has been detected in commercially available cell lines from cattle, sheep, goats, dogs, rabbits, cats and

* Corresponding author. Tel.: +1 334 844 4952; fax: +1 334 844 4955.
E-mail address: givenmd@vetmed.auburn.edu (M.D. Givens).

0166-3542/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2004.07.004

primates, human viral vaccines for measles–mumps–rubella, and interferons for human use (Bolin et al., 1994; Fernelius et al., 1969; Giangaspero et al., 2001; Harasawa and Mizusawa, 1995; Harasawa and Sasaki, 1995; Harasawa and Tomiyama, 1994).

Introduction of BVDV into cells cultured for the purpose of producing embryos by somatic cell nuclear transfer is a concern (Stringfellow et al., 2004). Infection of these cells with the virus might reduce the quantity or quality of embryos produced, result in transmission of disease to embryo recipients and the conceptus, or affect the results of research (Shin et al., 2000). No antiviral pharmaceuticals are currently available for controlling BVDV in the laboratory or on the farm.

Aromatic cationic molecules have previously inhibited in vitro replication of BVDV at nontoxic concentrations in established cell lines (Givens et al., 2003). The purpose of this research was to evaluate the ability of compounds, prepared as part of a series of compounds to test antiviral structure activity relationships, to prevent or treat noncytopathic BVDV infections of primary cultures of fetal fibroblast cells such as those that are used in somatic cell nuclear transfer.

2. Materials and methods

2.1. Test compounds

The compounds used in this study were synthesized in the laboratory of one of the authors (D.W.B.). Stock solutions of 10 mM were made in sterile distilled water or dimethyl sulfoxide and stored at −20 °C until use. The synthesis and physical properties of 2-(4-[2-imidazolinyl]phenyl)-5-(4-methoxyphenyl)furan (DB606) and 2-(2-benzimidazolyl)-5-[4-(2-imidazolino)phenyl]furan dihydrochloride (DB772) have been previously described (Lansiaux et al., 2002). The NMR data were obtained using a Varian Unity Plus 300 spectrometer.

2.1.1. 2-(1-Methyl-2-benzimidazolyl)-5-[4′-(2-imidazolino)-2′-methylphenyl]furan dihydrochloride (DB824)

A solution of $NaNO_2$ (3.9 g, 0.056 mol) in 10 mL water was added to a suspension of 4-amino-3-methyl-benzonitrile (5 g, 0.038 mol) in 35 mL water and 5 mL concentrated HCl at 0 °C and allowed to stir at 0 °C for 30 min. The mixture was added slowly with stirring to a solution of distilled 2-furfuraldehyde (3.9 g, 0.042 mol) and $CuCl_2{\cdot}2H_2O$ (10 mol%) in 20 mL acetone and 30 mL water over 30 min; the mixture was allowed to stir at room temperature for 12 h. The resultant brown solid was filtered and washed with water till free from blue color. The solid was dissolved in hot ethanol, treated with charcoal and filtered, triturated with ether to yield 4.3 g (54%) white crystalline solid, m.p. 200–201 °C; ^{1}H NMR(DMSO-d_6): 9.68(s, 1H), 7.94 (d, 1H, J = 8.1 Hz), 7.85 (d, 1H, J = 1.2 Hz), 7.78 (dd, 1H, J = 1.2 Hz, J = 7.1 Hz), 7.68 (d, 1H, J = 3.9 Hz), 7.26 (d, 1H, J = 3.9 Hz), 2.56 (s, 3H); ^{13}C NMR(DMSO-d_6): 178.4, 155.7, 152.0, 136.7, 134.8, 132.2, 129.9, 128.2, 124.1, 118.3, 113.6, 111.3, 20.8; MS: *m*/*e* 211(M^+).

A mixture of the above aldehyde (2.11 g, 0.01 mol), *N*-methyl-1,2-phenylenediamine (1.20 g, 0.01 mol) and 1,4-benzoquinone (1.08 g, 0.01 mol) in 50 mL dry ethanol was heated at reflux (under nitrogen) for 8 h. The reaction mixture was cooled, ether was added, and the solid was filtered. The solid was stirred with a 1:3 mixture (EtOH: ether) for 20 min and the yellow brown solid was filtered, washed with ether and dried under vacuum at 70 °C for 12 h to yield 2.2 g (70%), m.p. 232–233 °C dec., ^{1}H NMR(DMSO-d_6): 7.98 (d, 1H, J = 8 Hz), 7.79 (s, 1H), 7.75 (dd, 1H, J = 1.2 and 8 Hz), 7.67 (brd, 1H, J = 7.2 Hz), 7.61 (brd, 1H, J = 8 Hz), 7.40 (d, 1H, J = 3.6 Hz), 7.30 (ddd, 1H, J = 1.2, 7.2, and 8 Hz), 7.25 (ddd, 1H, J = 1.2, 7.2, and 8 Hz), 7.20 (d, 1H, J = 3.6 Hz), 4.10 (s, 3H), 2.60 (s, 3H). ^{13}C NMR (DMSO-d_6): 151.9, 145.3, 143.2, 142.4, 136.0, 135.3, 134.6, 132.4, 129.6, 127.0, 122.6, 122.1, 118.8, 118.3, 114.3, 113.5, 110.1, 109.9, 31.3, 21.0. MS: *m*/*e* 313 (M^+); analysis calculated for $C_{20}H_{15}N_3O{\cdot}0.25H_2O$: C, 75.49; H, 4.99; N, 13.25; found: C, 75.58; H, 4.95; N, 13.14.

The above nitrile (1.56 g; 0.005 mol) in 75 mL of ethanol was saturated with HCl gas at 0 °C and stirred at room temperature until thin layer chromatography showed the disappearance of the nitrile (ca. 2 d). The mixture was treated with ether and the resulting yellow imidate ester hydrochloride was filtered, washed with ether and dried under vacuum at 30 °C for 5 h to yield 1.9 g (88%). Ethylene diamine (0.18 g, 0.003 mol) was added to the suspension of 0.86 g (0002 mol) imidate ester hydrochloride and heated at reflux for 24 h. After removing the solvent, the solid was treated with ether: ethanol (6:1) and filtered. The yellow solid was dissolved in water and basified with 10% aqueous NaOH, and the precipitated free base was filtered, washed with water and dried at 35 °C. The free base was suspended in dry ethanol, treated with HCl gas and stirred at 50 °C to yield the yellow hydrochloride salt. After filtration the solid was washed with dry ether and dried under vacuum to yield 0.72 g (77%), m.p. > 320 °C dec.; ^{1}H NMR (DMSO-d_6): 10.83 (s, 2H), 8.20 (d, 1H, J = 8.4 Hz), 8.11 (brs, 1H), 8.05 (d, 1H, J = 8.4 Hz), 7.81-7.55 (m, 2H), 7.71(d, 1H, J = 3.9 Hz), 4.18 (s, 3H), 4.03 (s, 4H), 2.68 (s, 3H); ^{13}C NMR (DMSO-d_6/D_2O): 165.2, 154.3, 143.0, 142.7, 139.0, 136.5, 135.7, 135.1, 131.7, 127.8, 126.7, 125.3, 125.2, 121.4, 118.1, 117.9, 115.2, 115.2, 118.8, 45.2, 32.8, 22.5; FABMS: *m*/*e* 357 (M^+, +1); analysis calculated for $C_{22}H_{20}N_4O{\cdot}2HCl{\cdot}2.25H_2O$: C, 56.23; H, 5.68; N, 11.92; found: C, 56.12; H, 5.51; N, 11.85.

2.2. Cell culture

2.2.1. Cells

Ten separate primary fetal fibroblast cell lines (5826, 5832, 6008, 6032, 6228, 6426, 6878, 6879, 6886, and 7672) derived from a bovine fetus at ≤70 days of gestation were used in this research. These cell lines had been established to

provide donor nuclei for production of embryos by somatic cell nuclear transfer and were previously determined to be free of BVDV by virus isolation and reverse transcription-nested polymerase chain reaction (RT-nPCR) (Givens et al., 2001). The two additional primary cell lines (7684 and 10-1-C) used for testing of compounds to treat infection were previously determined to be infected with BVDV using the same assays.

2.2.2. Media

Fetal fibroblast cells were cultured in α MEM (Biowhittaker, Walkersville, Maryland, USA) supplemented with L-alynyl-L-glutamine (2 mM), penicillin (10,000 U/mL), streptomycin (10 mg/mL), 2-mercaptoethanol (0.154 mM) and 15% (vol/vol) equine serum (complete medium). Madin Darby bovine kidney (MDBK) cells were cultured in MEM supplemented with 10% (vol/vol) equine serum (MEM-eq) (Givens et al., 2003).

2.3. Virus

A genotype 1a, noncytopathic strain of BVDV (SD-1) was used for testing of compounds to prevent infection (Deng and Brock, 1992). The BVDV isolate identified in the contaminated fetal fibroblasts was a noncytopathic, genotype 1a strain as determined by growth in cell culture and sequencing of 257 nucleotides within the 5′ nontranslated region of the viral genome.

2.4. Testing of compounds to prevent infection

Pestivirus free fetal fibroblast cells (1×10^4 to 5×10^4 per 2.0 cm^2 well) were incubated in 200 μL of complete medium containing two-fold dilutions of DB606 (0.4–0.006 μM), DB772 (0.08–0.0013 μM), DB824 (0.08–0.0013 μM), or no compound (negative control). Cells were incubated with test compounds for 15 min before inoculation with BVDV (strain SD-1) at a multiplicity of infection of 0.5. Cells were cultured with this inoculum for 1 h at 38.5 °C in 5% CO_2 and humidified air. After an hour, the inoculum was removed and cells were washed with 1 mL of Dulbecco's phosphate buffered saline (D-PBS). Immediately after washing, 1 mL of complete media containing test compound (or negative control) was placed on each of the inoculated monolayers. After 72 h of incubation, cell monolayers were visually observed for cytotoxic effects at 400× magnification. Culture media was then removed from the cell monolayers and stored at −80 °C for later viral assays. One milliliter of fresh medium containing no test compound was added to cell monolayers prior to freezing and thawing to lyse cells for the purpose of releasing any intracellular BVDV.

2.5. Testing of compounds to treat infection

Two aromatic cations, DB772 and DB824, were tested at two concentrations (0.04 and 4.0 μM) on each infected cell line to try to eliminate BVDV contamination. Fetal fibroblast cells that were infected with BVDV were grown to confluence in complete medium. Cell monolayers were washed with D-PBS, trypsinized, suspended in complete media, divided into equal aliquots, centrifuged at 400 × *g* for 5 min and resuspended in complete medium supplemented with DB772, DB824, or no compound (negative control). After resuspension in 3 mL of appropriate medium, cells were placed in 12.5 cm^2 flasks. Medium was removed and replaced with equivalent medium on the day after passage. When cells became confluent (1–6 days after subculture), medium was removed and frozen at −80 °C to be assayed for BVDV by virus isolation and RT-nPCR. Cell monolayers were washed, trypsinized, suspended in equivalent media, divided into equal aliquots, centrifuged and resuspended in equivalent media or media lacking antiviral compound. Cells in media lacking antiviral compound were frozen at −80 °C to be assayed for BVDV by virus isolation and RT-nPCR. Cells in corresponding media were placed in a 12.5 cm^2 flask, which resulted in a 1:2 subculture. Cells were maintained as described in each treatment through four sequential subcultures. During each subculture, cell monolayers were observed with an inverted microscope at 400× magnification.

2.6. Virus detection

Bovine viral diarrhea virus was detected and quantified from cell culture medium and cell lysates using the immunoperoxidase monolayer assay and serial 10-fold dilutions (Afshar et al., 1991). Medium and cell lysates in which BVDV was not detected initially were further evaluated by passaging 200 μL of sample in 2.0 cm^2 wells containing MDBK cells in order to amplify low quantities of residual virus prior to immunoperoxidase monolayer assay (Givens et al., 2003). All medium and cell lysates from testing of compounds to treat ongoing infections were assayed for BVDV using RT-nPCR as previously described (Givens et al., 2000).

2.7. Toxicity evaluation

Viability of MDBK cells treated with DB824 was quantitated using the tetrazolium-based compound XTT (2,3-bis[2-methoxy-4-nitro-5-sulfo-phenyl]-2*H*-tetrazolium-5-carboxanilide) (Weislow et al., 1989) in two-fold dilutions of compound from 100 to 1.56 μM.

2.8. Statistical calculations

The $CCID_{50}$ of BVDV in stock virus, cell culture medium and cell lysates was determined by the statistical method of Reed and Muench (Reed and Muench, 1938). Bovine viral diarrhea virus in cell culture medium and cell lysates was evaluated by comparison to equivalent samples from temporal control cultures in which no compound was added before or

after inoculation (percentage of control = [$CCID_{50}$ of BVDV in compound sample/$CCID_{50}$ of BVDV in control sample lacking compound] × 100). The viral inhibitory concentrations (99%; IC_{99}) of compounds were calculated with JMP software by least-squares regression techniques using the logarithm of the mean percentage of control virus of cell culture medium and cell lysates of each sample (Sall and Lehman, 1996).

The toxicity of DB824 at each concentration was calculated as previously described (Givens et al., 2003). The 50 and 10% cytotoxic concentration endpoints (CC_{50}; CC_{10}) of DB824 were calculated with JMP software by least-squares regression techniques using the square of percent of control of each sample (Sall and Lehman, 1996).

3. Results

3.1. Testing of compounds to prevent infection

No cytotoxicity was observed with any of the concentrations of compounds used. Furthermore, no BVDV was detected by virus isolation in negative control cell cultures. From fetal fibroblast cells inoculated with BVDV in the absence of test compounds, a mean of 4×10^6 (range of 2×10^5 to 2×10^7) $CCID_{50}$ of BVDV /mL was detected in media and 8×10^5 (range of 6×10^4 to 4×10^6) $CCID_{50}$ of BVDV /mL was detected in cell lysates. Virus could not be detected in medium or cell lysates of any cultures supplemented with 0.08 μM of DB772. The 99% endpoints for inhibition of viral replication in fetal fibroblast cell lines are provided in Table 1. Of the 10 cell lines, one cell line (6879) appeared to be afforded less protection from infection by any of the aromatic cationic compounds. For this cell line, each concentration of each compound had been tested in triplicate to validate results.

Table 1
The 99% endpoints (μM) for inhibiting replication of bovine viral diarrhea virus by aromatic cationic compounds (DB606, DB772, and DB824) in 10 different primary fetal fibroblast cell lines

Cell line	DB606	DB772	DB824
5826	0.080	0.005	0.009
5832	0.050	0.012	0.019
6008	0.065	0.003	0.005
6032	0.150	0.006	0.011
6228	0.040	0.003	0.014
6426	0.057	0.002	0.005
6878	0.070	0.008	0.008
6879[a]	0.140	0.014	0.043
6886	0.110	0.009	0.021
7672	0.050	0.003	0.007
Average	0.081	0.006	0.014
±Standard deviation	0.039	0.004	0.012
95% Confidence interval	0.054, 0.108	0.003, 0.009	0.005, 0.023

[a] This cell line was tested in triplicate using seven dilutions of each compound. Other cell lines were tested in a single experiment using seven dilutions of each compound.

3.2. Testing of compounds to treat infection

No cytotoxicity was observed with any of the concentrations of compounds used, and control cell cultures remained infected with BVDV throughout the four passages. Treatment with aromatic compounds reduced or eliminated BVDV in both medium and cell lysate samples as illustrated in Fig. 1.

3.3. Toxicity evaluation

The CC_{50} and CC_{10} of DB824 were 143 and 40 μM, respectively.

4. Discussion

Bovine viral diarrhea virus continues to be a problematic pathogen of cattle both in the field and in the laboratory. The noncytopathic biotype of this virus has resulted in persistent undetected contamination of cultured cells used for both production of vaccines and for production of embryos by somatic cell nuclear transfer (Falcone et al., 1999, 2003; Shin et al., 2000). No antiviral pharmaceuticals are currently available for controlling BVDV, which has been suggested as an attractive surrogate for studying the viral replication or treatment of the uncultivatable human pathogen, hepatitis C virus (Buckwold et al., 2003). The purpose of this research was to evaluate the ability of aromatic cationic compounds to prevent and treat noncytopathic BVDV infections in fetal fibroblast cells.

The 99% endpoints for use of aromatic cationic compounds to inhibit replication of BVDV in the 10 primary fetal fibroblast cell lines correlates with previous values determined by using a continuous cell line (MDBK) (Givens et al., 2003). While a slightly higher concentration of aromatic cationic compounds was necessary to prevent infection of cell line 6879, BVDV could not be detected by virus isolation in the medium or cell lysates of any cell line, including 6879 after culture in 0.08 μM of DB772. Of the compounds evaluated, DB772 exhibited the greatest efficacy for preventing BVDV infections at the lowest concentrations of compound.

The results of our research demonstrate for the first time that aromatic cationic compounds at nontoxic concentrations can eliminate noncytopathic BVDV from previously infected cell cultures. It has been reported that noncytopathic BVDV could not be eliminated from MDBK cells by monotherapy with either novel inhibitors of α-glucosidases or currently available chemotherapies for hepatitis C virus (Durantel et al., 2004). In that research, only the combination of interferon-α and ribavirin eliminated noncytopathic BVDV from infected cells in vitro (Durantel et al., 2004). In our research, both infectious virus and viral RNA in media and cell lysates were eliminated after the first passage of both infected cell lines when the medium contained either DB772 or DB824 at 4.0 μM.

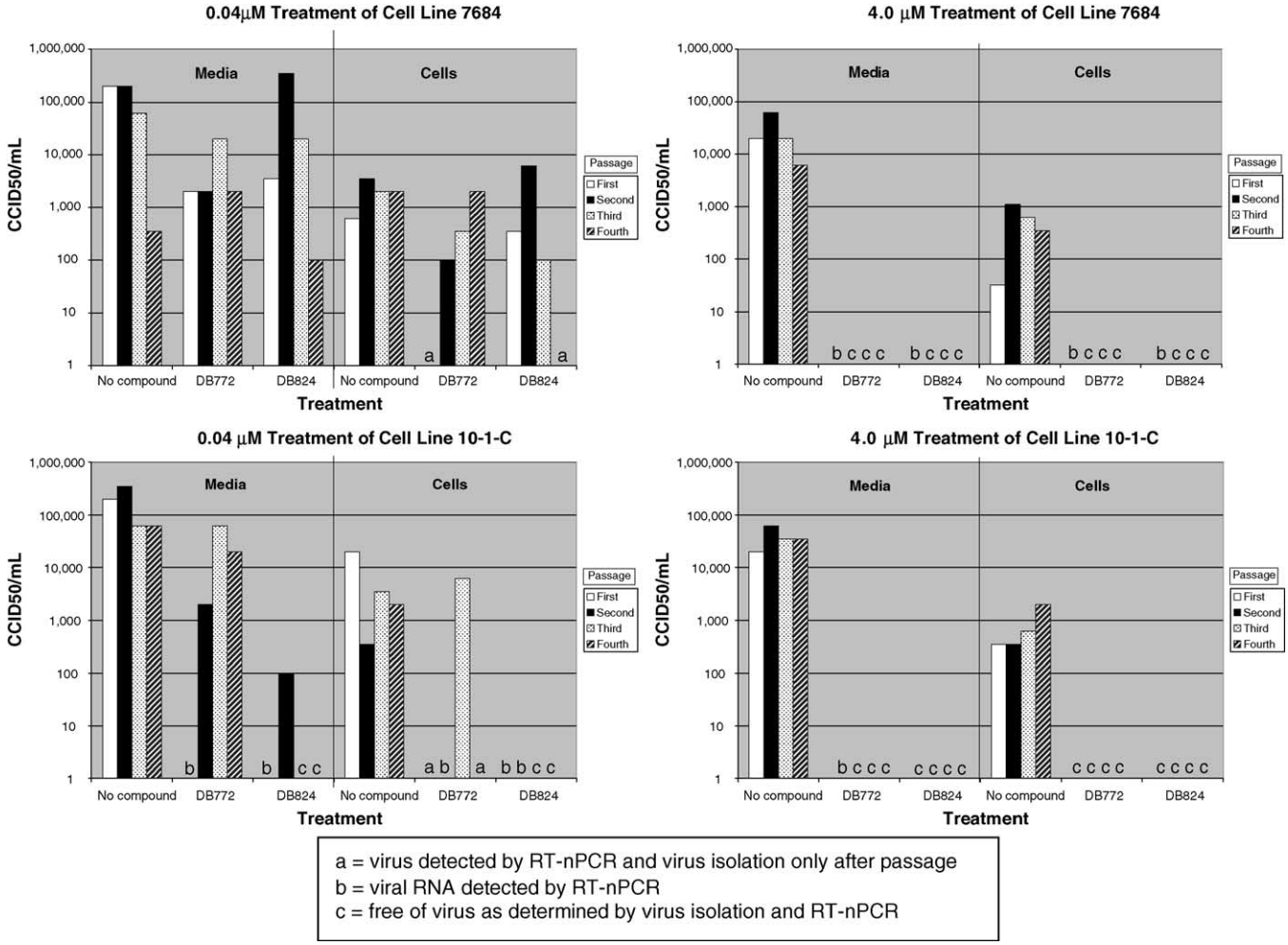

Fig. 1. Detection and quantitation of bovine viral diarrhea virus in infected fetal fibroblast cell lines (7684 and 10-1-C) treated with aromatic cationic compounds (DB772 and DB824) or maintained as negative controls in a single experiment. ($CCID_{50}$/mL = cell culture infective doses, 50% endpoint/mL; RT-nPCR = reverse transcription-nested PCR).

The cytotoxic concentrations of DB824 determined using the tetrazolium-based compound were similar to values previously determined for DB606 and DB772 (Givens et al., 2003). Cytotoxic concentrations were much higher than the IC_{99} necessary to prevent infection of susceptible cells or the 4 μM concentration of DB772 and DB824 used to eliminate ongoing BVDV infections. While DB824 produced a lower CC_{10} and higher IC_{99} than DB772, DB824 exhibited an acceptable therapeutic index and might serve as an alternative if DB772 were subsequently shown to negatively affect the cloning efficiency of exposed cells. These agents exhibit a wide therapeutic window in vitro for prevention or treatment of noncytopathic BVDV infections.

Our results indicate that supplementation of cultures of fetal fibroblast cells with an aromatic cationic compound can prevent or eliminate noncytopathic BVDV infections. If it can be shown that treated bovine fetal fibroblast cells are still competent for production of normal bovine embryos via nuclear transfer, then DB772 could be used as a precautionary measure to ensure that karyoplasts do not transmit BVDV to cloned embryos. The safeguard provided by the addition of an aromatic cationic antiviral compound could ultimately provide protection for bovine embryo recipients and offspring from disease due to BVDV.

Our results also suggest that use of selective aromatic cationic compounds in cell cultures for bovine vaccine production might provide a safeguard against inadvertent introduction and subsequent dissemination of BVDV via vaccination. Compounds might be useful to prevent cultures used for vaccine production from amplifying pathogenic, noncytopathic BVDV contaminants as has been previously reported (Falcone et al., 1999, 2003). Of course, issues such as irritation of tissue after administration and the effects on immunogenicity of bovine pathogens other than BVDV would require investigation before aromatic cationic antivirals could be recommended for use in cell cultures used for production of commercially available bovine vaccines.

In summary, we have discovered that aromatic cationic molecules can be used at nontoxic concentrations to prevent or eliminate noncytopathic BVDV infections in cultured

primary cells. Thus, these compounds might serve as useful instruments of quality assurance in a variety of endeavors that require culture of cells, including advanced embryo technologies and vaccine production.

References

Afshar, A., Dulac, G.C., Dubuc, C., Howard, T.H., 1991. Comparative evaluation of the fluorescent antibody test and microtiter immunoperoxidase assay for detection of bovine viral diarrhea virus from bull semen. Can. J. Vet. Res. 55, 91–93.

Bezek, D.M., Grohn, Y.T., Dubovi, E.J., 1994. Effect of acute infection with noncytopathic or cytopathic bovine viral diarrhea virus isolates on bovine platelets. Am. J. Vet. Res. 55, 1115–1119.

Bolin, S.R., Matthews, P.J., Ridpath, J.F., 1991. Methods for detection and frequency of contamination of fetal calf serum with bovine viral diarrhea virus and antibodies against bovine viral diarrhea virus. J. Vet. Diagn. Invest. 3, 199–203.

Bolin, S.R., Ridpath, J.F., Black, J., Macy, M., Roblin, R., 1994. Survey of cell lines in the American type culture collection for bovine viral diarrhea virus. J. Virol. Methods 48, 211–221.

Brownlie, J., 1990. The pathogenesis of bovine virus diarrhoea virus infections. Rev. Sci. Technol. 9, 43–59.

Buckwold, V.E., Beer, B.E., Donis, R.O., 2003. Bovine viral diarrhea virus as a surrogate model of hepatitis C virus for the evaluation of antiviral agents. Antiviral Res. 60, 1–15.

Carman, S., van Dreumel, T., Ridpath, J., Hazlett, M., Alves, D., Dubovi, E., Tremblay, R., Bolin, S., Godkin, A., Anderson, N., 1998. Severe acute bovine viral diarrhea in Ontario, 1993–1995. J. Vet. Diagn. Invest. 10, 27–35.

Deng, R., Brock, K.V., 1992. Molecular cloning and nucleotide sequence of a pestivirus genome, noncytopathic bovine viral diarrhea virus strain SD-1. Virology 191, 867–879.

Donis, R.O., 1995. Molecular biology of bovine viral diarrhea virus and its interaction with the host. Vet. Clin. North Am. Food Anim. 11, 393–423.

Durantel, D., Carrouee-Durantel, S., Branza-Nichita, N., Dwek, R.A., Zitzmann, N., 2004. Effects of interferon, ribavirin, and iminosugar derivatives on cells persistently infected with noncytopathic bovine viral diarrhea virus. Antimicrob. Agents Chemother. 48, 497–504.

Ellis, J.A., West, K.H., Cortese, V.S., Myers, S.L., Carman, S., Martin, K.M., Haines, D.M., 1998. Lesions and distribution of viral antigen following an experimental infection of young seronegative calves with virulent bovine virus diarrhea virus-type II. Can. J. Vet. Res. 62, 161–169.

Falcone, E., Cordioli, P., Tarantino, M., Muscillo, M., Sala, G., La Rosa, G., Archetti, I.L., Marianelli, C., Lombardi, G., Tollis, M., 2003. Experimental infection of calves with bovine viral diarrhoea virus type-2 (BVDV-2) isolated from a contaminated vaccine. Vet. Res. Commun. 27, 577–589.

Falcone, E., Tollis, M., Conti, G., 1999. Bovine viral diarrhea disease associated with a contaminated vaccine. Vaccine 18, 387–388.

Fernelius, A.L., Lambert, G., Hemness, G.J., 1969. Bovine viral diarrhea virus-host cell interactions: adaptation and growth of virus in cell lines. Am. J. Vet. Res. 30, 1561–1572.

Giangaspero, M., Vacirca, G., Harasawa, R., Buttner, M., Panuccio, A., De Giuli, M.C., Zanetti, A., Belloli, A., Verhulst, A., 2001. Genotypes of pestivirus RNA detected in live virus vaccines for human use. J. Vet. Med. Sci. 63, 723–733.

Givens, M.D., Dykstra, C.C., Brock, K.V., Stringfellow, D.A., Kumar, A., Stephens, C.E., Goker, H., Boykin, D.W., 2003. Detection of inhibition of bovine viral diarrhea virus by aromatic cationic molecules. Antimicrob. Agents Chemother. 47, 2223–2230.

Givens, M.D., Galik, P.K., Riddell, K.P., Brock, K.V., Stringfellow, D.A., 2000. Replication and persistence of different strains of bovine viral diarrhea virus in an in vitro embryo production system. Theriogenology 54, 1093–1107.

Givens, M.D., Galik, P.K., Riddell, K.P., Stringfellow, D.A., Brock, K.V., Bishop, M.D., Eilertsen, K.J., Loskutoff, N.M., 2001. Validation of a reverse transcription nested polymerase chain reaction (RT-nPCR) to detect bovine viral diarrhea virus (BVDV) associated with in vitro-derived bovine embryos and co-cultured cells. Theriogenology 56, 787–799.

Harasawa, R., Mizusawa, H., 1995. Demonstration and genotyping of pestivirus RNA from mammalian cell lines. Microbiol. Immunol. 39, 979–985.

Harasawa, R., Sasaki, T., 1995. Sequence analysis of the 5′ untranslated region of pestivirus RNA demonstrated in interferons for human use. Biologicals 23, 263–269.

Harasawa, R., Tomiyama, T., 1994. Evidence of pestivirus RNA in human virus vaccines. J. Clin. Microbiol. 32, 1604–1605.

Harding, M.J., Cao, X., Shams, H., Johnson, A.F., Vassilev, V.B., Gil, L.H., Wheeler, D.W., Haines, D., Sibert, G.J., Nelson, L.D., Campos, M., Donis, R.O., 2002. Role of bovine viral diarrhea virus biotype in the establishment of fetal infections. Am. J. Vet. Res. 63, 1455–1463.

Houe, H., 1999. Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. Vet. Microbiol. 64, 135–144.

Lansiaux, A., Dassonneville, L., Facompre, M., Kumar, A., Stephens, C.E., Bajic, M., Tanious, F., Wilson, W.D., Boykin, D.W., Bailly, C., 2002. Distribution of furamidine analogues in tumor cells: influence of the number of positive charges. J. Med. Chem. 45, 1994–2002.

Pellerin, C., van den Hurk, J., Lecomte, J., Tijssen, P., 1994. Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virology 203, 260–268.

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent end points. Am. J. Hygiene 27, 493–497.

Sall, J., Lehman, A., 1996. Fitting curves through points: regression. In: JMP Start Statistics. Duxbury Press, Belmont, CA.

Shin, T., Sneed, L., Hill, J.R., Westhusin, M.E., 2000. High incidence of developmental failure in bovine fetuses derived by cloning bovine viral diarrhea virus-infected cells. Theriogenology 53, 243. Abstract.

Stringfellow, D.A., Givens, M.D., Waldrop, J.G., 2004. Biosecurity issues associated with current and emerging embryo technologies. Reprod. Fertil. Dev. 16, 93–102.

Weislow, O.S., Kiser, R., Fine, D.L., Bader, J., Shoemaker, R.H., Boyd, M.R., 1989. New soluble-formazan assay for HIV-1 cytopathic effects: application to high-flux screening of synthetic and natural products for AIDS-antiviral activity. J. Natl. Cancer Inst. 81, 577–586.

Yanagi, M., Bukh, J., Emerson, S.U., Purcell, R.H., 1996. Contamination of commercially available fetal bovine sera with bovine viral diarrhea virus genomes: implications for the study of hepatitis C virus in cell cultures. J. Infect. Dis. 174, 1324–1327.