Evaluation of a novel antiviral compound effective against multiple pestivirus species.

by

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Abstract

Bovine viral diarrhea virus (BVDV) is a viral pathogen of cattle and other wild and domestic ungulates that is capable of affecting multiple body systems, most notably the gastrointestinal, reproductive, immune and respiratory systems. Aside from causing clinical concern, BVDV is a significant contaminant in laboratory systems and biological products due to its often noncytopathic nature. The virus is also widely used as a surrogate viral model to study Hepatitis C infection in humans. Despite its significance to multiple clinical and research fields, practical methods of BVDV control are limited largely to management protocols and biosecurity safeguards. Specific therapeutics or decontaminants for BVDV are not currently available for use in the farm or the laboratory.

Previous research identified a family of antiviral compounds that were safe and effective when used to treat or prevent BVDV infection in various cell lines. This research continued exploration of the identified compound with the highest therapeutic index (DB772) and its potential to be used as an antiviral preventive or therapeutic *in vivo* as well as it effectiveness against other viruses closely related to BVDV. In the initial study, calves persistently infected with BVDV were treated intravenously with either the antiviral compound or the drug diluent every eight hours over a six day period. In addition to other parameters, viral titers in white blood cells were compared to pre-treatment levels. Treatment with DB772 was associated with a rapid drop in viral titers.

However, with one exception, the titers rebounded to near pre-treatment levels during or immediately following the treatment period.

Further study revealed that virus isolated from the treated calves with rebound titers was resistant to DB772 whereas pre-treatment isolates were susceptible to the compound. Full genome sequencing was performed on the isolates and revealed single or multiple mutations in the region encoding NS5b, which forms the RNA-dependent RNA polymerase. These mutations have not been described in wild-type isolates and consequently the growth curves of wild-type and mutant isolates were studied but significant changes were not detected.

In a second study, calves naïve to BVDV were again treated with either the compound or diluent intravenously before intranasal challenge with BVDV. Treatment with DB772 successfully prevented infection in calves although these same calves were shown to be susceptible to BVDV following treatment after the serum concentration of DB772 was calculated to have fallen below *in vitro* protective levels.

Thirdly, the *in vitro* efficacy of DB772 against BVDV and closely related viruses was examined by infection of cells with BVDV, border disease virus, HoBi virus, pronghorn virus and Bungowannah virus. Infected cells were incubated in in medium containing 0, 0.006, 0.01, 0.02, 0.05, 0.1, 0.2, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5 or 25 μM DB772 and subsequently assayed for the presence of virus by virus isolation and titration or polymerase chain reaction. Cytotoxicity of the compound for different cell types used in the study was evaluated using a commercially available cell counting kit. The compound was shown to effectively inhibit all pestiviruses studied at concentrations $\geq$ 0.20 μM. Cytotoxicity was not evident until DB772 concentrations exceeded 25 μM.

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List of Abbreviations

ACE	antigen capture ELISA
ACM	aromatic cationic molecule
AI	artificial insemination
AUMC	area under the curve and its first moment
BCoV	bovine coronavirus
BDV	border disease virus
BHV-1	bovine herpesvirus 1
BHV-4	bovine herpesvirus 4
BLV	bovine leukemia virus
BoIFN	bovine interferon
BRSV	bovine respiratory syncytial virus
BRV	bovine rotavirus
BVDV	bovine viral diarrhea virus
CBC	complete blood count
$CCID_{50}$	50% cell culture infective dose
Cl	clearance
C_{max}	maximal serum concentration
CSFV	classical swine fever virus
CP	cytopathic
CPE	cytopathogenic effects

DB606	2-(4-[2-imidazolinyl]phenyl)-5-(4-methoxyphenyl)furan
DB772	2-(2-benzimidazolyl)-5-[4-(2-imidazolino)phenyl]furan dihydrochloride
dsRNA	double-stranded ribonucleic acid
E1	primary envelope glycoprotein
E2	secondary envelope glycoprotein
E^{rns}	ribonuclease soluble envelope glycoprotein
FMDV	foot-and-mouth disease virus
GGT	gamma-glutamyltransferase
HCV	hepatitis C virus
HPLC	high performance liquid chromatography
HuIFN	human interferon
IFN	interferon
IHC	immunohistochemistry
IL	interleukin
IRES	internal ribosome entry site
IVF	*in vitro* fertilization
Jiv	J-domain protein interacting with viral protein
MD	mucosal disease
MDBK	Madin-Darby Bovine Kidney (cells)
MEM	minimum essential medium
MOI	multiplicity of infection
mRNA	messenger RNA
MRT	mean residence time

MW	molecular weight
NCP	noncytopathic
NS2-3	the fused second and third nonstructural proteins
NS4A	the amino-terminal portion of the fourth nonstructural protein
NS4B	the carboxy-terminal portion of the fourth nonstructural protein
NS5A	the amino-terminal portion of the fifth nonstructural protein
NS5B	the carboxy-terminal portion of the fifth nonstructural protein
N^{pro}	amino terminal autoprotease
OFTU	ovine fetal turbinate (cells)
ORF	open reading frame
PCR	polymerase chain reaction
PEG 200	polyethylene glycol 200
PI	persistently infected
PI_3	parainfluenza 3 virus
PK	porcine kidney (cells)
ProTI	prolonged testicular infection
PTI	persistent testicular infection
qPCR	quantitative polymerase chain reaction
RdRP	RNA-dependent-RNA-polymerase
RNAi	RNA interference
rtPCR	reverse transcriptase polymerase chain reaction
SBP	serum biochemical profile
SDH	sorbitol dehydrogenase

siRNA	small interfering RNA
SUN	serum urea nitrogen
t½	terminal elimination half-life
UTR	untranslated region
Vd_{ss}	volume of distribution
VSV	vesicular stomatitis virus
YFV	yellow fever virus

Introduction

In 1946, Peter Olafson described "an apparently new transmissible disease of cattle" in the *Cornell Veterinarian* (Olafson et al., 1946). The main clinical symptoms exhibited by affected cattle were severe diarrhea, abortion and respiratory disease and though an infectious etiology was suspected, it was not until the following decade that the virus was first isolated and propagated in the laboratory (Baker et al., 1954;Lee and Gillespie 1957). Due to the observed symptoms in affected cattle, the virus was given the unfortunate name of bovine viral diarrhea virus (BVDV) and remains so-named to this day. Despite its moniker, the effects of BVDV infection are manifested not only in the gastrointestinal tract but also the respiratory, reproductive, cardiovascular, lymphatic, immune, integumentary or central nervous systems (Baker 1995;Walz et al., 2010) of not only cattle, but in several domestic and feral mammalian species of the order Artiodactyla (Passler and Walz 2010). However, reproductive losses associated with BVDV infection are thought to have the largest negative economic impact for producers (Grooms 2004).

Not only significant clinical pathogens, BVDV and other pestiviruses are important laboratory contaminants as well. Despite manufacturer testing, BVDV has been isolated from commercially available lots of fetal bovine serum (Yanagi et al., 1996). *In vitro* produced bovine embryos are potential vectors for pestivirus transmission (Zurovac et al., 1994); (Bielanski and Jordan 1996). Additionally, commercially available bovine,

canine, feline and primate cell lines (Fernelius et al., 1969); (Harasawa and Mizusawa 1995), human viral vaccines (Harasawa and Tomiyama 1994); (Giangaspero et al., 2001) and interferons for human use (Harasawa and Sasaki 1995) have been found to be contaminated by pestiviruses. No specific antiviral therapeutics to control pestivirus infection in the laboratory or on the farm are currently available commercially.

Aromatic cationic molecules (ACMs) possess inhibitory action against several RNA viruses, including human immunodeficiency virus (Kumar et al., 1995), rotavirus (Vonderfecht et al., 1988) and respiratory syncytial virus (Dubovi et al., 1980). One particular compound, 2-(2-benzimidazolyl)-5-[4-(2-imidazolino)phenyl]furan dihydrochloride, (DB772; MW=410.28) has been shown to inhibit BVDV growth in cell culture at micromolar concentrations lacking cytotoxicity (Givens et al., 2003a). The 99% endpoint for prevention of viral replication by DB772 was found to be 6 nM (Givens et al., 2004). Further, DB772 has been shown to eliminate BVDV infection in contaminated bovine fetal fibroblast cells with a single passage in culture media supplemented with 4μM of DB772 (Givens et al., 2004). Thus, DB772 exhibits antiviral activity against BVDV at micromolar concentrations *in vitro* without causing detectable signs of cytotoxicity. The research described in this dissertation evaluates the antiviral effects of DB772 when used as either a preventive or therapeutic strategy to combat BVDV infection in live animals. Additionally, the research seeks to establish the *in vitro* antiviral activity of DB772 against other related pestiviruses.

Literature Review

Bovine Viral Diarrhea Virus

Introduction

On July 10, 1946, at a meeting of the New York State Veterinary Medical Association in Syracuse, New York, the first description of the disease we now know as BVDV was delivered to a professional audience composed of members of the New York State Veterinary College (Olafson et al., 1946). Working independently, Dr. T. Childs of the Canadian Department of Agriculture confirmed the presence of a similar disease in cattle herds in Saskatchewan, Canada but a few months later in an address before the Ottawa Society for Comparative Medicine in which he speculated that the syndrome was not necessarily new but certainly newly recognized (Childs 1946). While a viral etiology of the observed syndromes was suspected, it was not until 1957 that the virus was first isolated and named BVDV (Lee and Gillespie 1957). In the nearly 60 years that have ensued, BVDV has emerged as one of the most studied veterinary viruses due to its importance in clinical veterinary medicine worldwide (Walz et al., 2010), its significance as a contaminant in human and animal biologicals (Bolin et al., 1991;Harasawa and Tomiyama 1994;Harasawa and Sasaki 1995) and its value as a laboratory model for human viral infections, particularly hepatitis C virus (HCV) (Buckwold et al., 2003).

Despite decades of study, the discovery and development of antiviral therapeutics specific to BVDV have proved elusive and the virus continues to pose significant challenges in the field and in the laboratory.

Taxonomy

In 1993, BVDV was elucidated as the causative agent of severe morbidity and mortality in adult cattle and veal calves in Canada (Carman et al., 1998). Subsequent genomic characterization of virus isolates from the outbreaks revealed large differences in sequence homology to known BVDV strains (Pellerin et al., 1994), leading to the division of BVDV into two distinct genotypes, BVDV1 and BVDV2 (Thiel et al., 2005). The two viruses were first separated by noting phylogenetic differences (Ridpath et al., 1994). Although antigenic differences in the two species have also been demonstrated, the two species remain serologically cross-reactive (Ridpath et al., 2000). Sequence differences between BVDV1 and BVDV2 can be found throughout the genome but because the 5′ untranslated region (UTR) is highly conserved among viral strains and thus accommodating to polymerase chain reaction (PCR) amplification, it is the region most commonly used to characterize viral strains (Ridpath and Bolin 1995). Both species can be further divided into subgenotype groupings by phylogenetic analysis of conserved regions of the genome although the clinical significance of such groupings is unknown (Vilcek et al., 2001;Flores et al., 2002).

BVDV1 can be subdivided into at least 11 groups denoted as types 1a, 1b, 1c, 1d, 1e, 1f, 1g, 1h, 1i, 1j and 1k (Vilcek et al., 2001). Subtypes 1a and 1b are the predominant strains in North America (Ridpath and Bolin 1998;Fulton et al., 2005). Classification of

BVDV2 isolates into subgenotypes is less common due to the smaller number of described isolates (Vilcek et al., 2005a). Two subgenotypes of BVDV2 have been described in North America with type 2a more commonly isolated than type 2b (Ridpath 2005).

Bovine viral diarrhea virus is the prototypical virus in the *Pestivirus* genus of the *Flaviviridae* family (Walz et al., 2010). First proposed in 1973 by M.C. Horzinek, the pestivirus genus also included the antigenically-related classical swine fever (CSFV) and border disease viruses (BDV) (Horzinek 1973;Plant et al., 1973). The genus was originally placed in the *Togaviridae* family but later reassigned to the *Flaviviridae* family based on molecular characterization of the member viruses (Horzinek 1973;Collett et al., 1989). Additionally, atypical pestiviruses isolated from a giraffe (Avalos-Ramirez et al., 2001), a pronghorn antelope (Vilcek et al., 2005b), cattle (Schirrmeier et al., 2004), and swine (Kirkland et al., 2007) have been proposed as members of the *Pestivirus* genus and are referred to as giraffe, pronghorn, HoBi and Bungowannah viruses, respectively. References to the chamois pestivirus in the literature describe an atypical pestivirus that was first isolated from Pyrenean chamois in Spain and later determined to be a substrain of BDV (Arnal et al., 2004).

The *Flaviviridae* are enveloped spherical viruses ranging from 40-50 nm in diameter (Heinz et al., 2000). In addition to the pestiviruses, the *Flaviviridae* comprise the hepaciviruses and flaviviruses. Hepatitis C virus is the lone virus assigned to the *Hepacivirus* genus while the flaviviruses include the West Nile virus, dengue virus, tick-borne encephalitis virus, yellow fever virus (YFV), Japanese encephalitis virus and St. Louis encephalitis virus, among others (Lindenbach and Rice 2001). The lipid envelope

of most member viruses is constructed from the cell membrane of infected cells; however, unlike that of related genera, the envelope of pestiviruses is pleomorphic (Heinz et al., 2000). Additionally, pestiviruses encode two proteins unique among the *Flaviviridae*, both of which are thought to be involved in the suppression of host immune defenses (Lindenbach and Rice 2001). Another distinguishing feature of pestiviruses is their relative stability over a wide pH range; dissimilar *Flaviviridae* are inactivated in acidic environments (Ridpath 2008).

Genomic Structure

The BVDV genome consists of a single strand of positive sense RNA. Unlike most eukaryotic messenger RNA, the BVDV genome lacks a 5′methylguanosine cap, instead possessing a 5′UTR of approximately 380 nucleotides (Brock et al., 1992). Likewise, a 229-273 untranslated nucleotide sequence on the 3′ end of the genome (3′UTR) takes the place of a 3′polyadenine tail (Collett et al., 1988;Brock et al., 1992). The genome of approximately 12.5 kb encodes a lone open reading frame (ORF) that is translated into a single polyprotein of approximately 3,898 amino acids (Donis 1995). Cytopathic (CP) strains of the virus commonly contain insertions, duplications and other rearrangements resulting in larger genomic RNAs (Lindenbach and Rice 2001); however, nucleotide deletions have resulted in genomes smaller than the type strain (Tautz et al., 1994;Donis 1995). Both viral and host endoproteinases are involved in co- and post-translational processing of the polypeptide product into at least 11 mature viral proteins (Rumenapf et al., 1993). The individual proteins in order from 5′ to 3′ are the amino-terminal autoprotease (N^{pro}), the nucleocapsid or core protein (C), the ribonuclease

soluble envelope glycoprotein (E^{rns}), the primary envelope glycoprotein (E1), the secondary envelope glycoprotein (E2), protein p7, the fused second and third nonstructural proteins (NS2-3), the amino- and carboxy-terminal portions of the fourth nonstructural protein (NS4A, NS4B) and the amino- and carboxy-terminal portions of the fifth nonstructural protein (NS5A, NS5B) (Collett et al., 1988) (Donis 1995). The BVDV capsid containing the single-stranded RNA genome is enveloped in a host-derived lipid membrane and is comprised of the four virion-encoded structural proteins, core (C), E(rns), E1, and E2 (Murray et al., 2008). The remaining proteins are nonstructural, meaning they are not contained within the virion and must be translated from the genomic RNA after infection of the target cell.

The 5´ untranslated region. The 5´ UTR is a highly conserved region of approximately 380 base pairs of the pestivirus genome. Between BVDV genotypes, sequence homology of the 5´ UTR approaches or exceeds 90% (Pellerin et al., 1994;Ridpath et al., 1994). However, between pestivirus species, a significant degree of heterology is present, a difference that approaches 25% between BVDV1 and BVDV2. This degree of heterology is nearly equal to the differences seen between the 5´ UTR sequences of BVDV1 and HCV. With the advent of molecular diagnostics, the conserved nature of the 5´ UTR has been exploited to enable differentiation and classification not only of different BVDV genotypes but of other diverse pestivirus species as well (Pellerin et al., 1994;Ridpath et al., 1994;Letellier et al., 1999;Giangaspero and Harasawa 2011).

Despite large differences in the primary nucleotide sequences of the 5´ UTR region between pestivirus species, the secondary structure of the RNA appears to be very

similar, suggesting a similar function between viruses. The secondary structure has been described as consisting of four separate domains, I, II, III and IV in order from 5´ to 3´ (Brown et al., 1992;Deng and Brock 1993). The 3´ half of the region forms a complex conserved stem-loop structure that functions as an internal ribosomal entry site (IRES). An IRES is a sequence within the genome that allows initiation of translation of a messenger RNA (mRNA) by mediating binding of ribosomes directly to the RNA template. Thus the 5´ UTR of BVDV and other pestiviruses performs much the same function as the 5′methylguanosine cap found in most mRNAs. In addition to its function during translation, the 5´ UTR may contain virulence markers (Topliff and Kelling 1998). In a study examining the primary and secondary structures of the 5´ UTRs of low and high virulence isolates of BVDV2, two mutations absent in low virulence isolates were found within the IRES of high virulence isolates. The authors speculated these mutations could be used to identify other BVDV2 isolates of high virulence.

The amino-terminal autoprotease. The amino-terminal autoprotease (N^{pro}) is the first protein to be translated from the ORF and consists of 168 amino acids with an observed mass of 20 kD (Donis 1995). The N^{pro} acts as an autoprotease to release itself from the nascent protein product at a cysteine-serine sequence at the 3´ end of the protein chain (Donis 1995;Ridpath 2010b). While unique to the pestiviruses among the *Flaviviridae*, at least two other RNA viruses of veterinary importance have a similar accessory protease that are produced at the onset of translation, namely foot and mouth disease virus and equine arteritis virus (Gorbalenya et al., 1991). While it was initially believed the N^{pro} had no other function than as an autoprotease, further research has

revealed that the protein is also involved in the suppression of host immune defenses. The N^{pro} interferes with production of type I interferon through the activity of interferon regulatory factor 3 (Hilton et al., 2006;Seago et al., 2007).

The nucleocapsid protein. The second translation product of the BVDV ORF is the nucleocapsid, or core, (C) protein comprised of 102 amino acids (Donis 1995). The protein is believed to be located in the cytosol or endoplasmic reticulum of infected cells where it functions in the processing and packaging of genomic RNA into the nascent virion. Three enzymatic cleavages are necessary to finalize processing of the peptide before inclusion into the virion where it lacks significant secondary structure and is "intrinsically disordered" (Murray et al., 2008). The protein is well-conserved across the *Pestivirus* genus; however, important differences exist. In contrast to the high immunogenicity of the capsid protein of HCV in humans, cattle do not appear to produce antibodies after infection with either biotype of BVDV (Donis and Dubovi 1987).

The ribonuclease soluble envelope glycoprotein. The ribonuclease soluble envelope glycoprotein (E^{rns}) envelope glycoprotein is made up of 227 amino acids and has a predicted mass of 26 kD though carbohydrate molecules attached to various glycosylation sites result in an observed migration on sodium dodecyl sulfate polyacrylamide gel electrophoresis of 48 kD (Donis 1995). The amino-terminus, created when the nucleocapsid protein is cleaved from the polypeptide chain, contains a signal sequence for translocation into the endoplasmic reticulum. Though the E^{rns} contains no typical transmembrane anchor, the protein remains loosely membrane-bound. It is now known that the presence of an 11 amino acid sequence in the carboxy-terminus of the protein is necessary but not sufficient for membrane binding (Fetzer et al., 2005).

Additional moieties involved in the membrane binding of the E^{rns} remain to be characterized.

Because the E^{rns} is an envelope protein, it was believed to play a role in virus binding and cell entry as well as immune recognition by the host. Indeed, infected cattle generate high levels of antibody to the E^{rns} protein, which forms the basis of a commercially-available diagnostic test for BVDV (Kuhne et al., 2005) (Kampa et al., 2007). However, these antibodies are somewhat limited in their ability to neutralize the virus (Boulanger et al., 1991). Furthermore, E^{rns} has been shown to be dispensable for virus entry (Ronecker et al., 2008). More recent research has focused on the role of the E^{rns} in evasion of the host's immune system. Like the N^{pro} protein, the E^{rns} protein is unique to pestiviruses among *Flaviviridae* and is also involved in the suppression of host immune defenses; the E^{rns} is believed to prevent the induction of alpha and beta interferon (Iqbal et al., 2004;Matzener et al., 2009).

The primary envelope glycoprotein. Subject to posttranslational modification, the primary envelope glycoprotein (E1) is predicted to be 195 amino acids long (Donis 1995). The protein is anchored in the cell membrane by two hydrophobic domains that also function in the translocation of the secondary envelope glycoprotein (E2). Sera from convalescent cattle do not show high levels of antibody to E1 (Donis and Dubovi 1987) but the protein is essential for cell entry (Rumenapf et al., 1993). Cell entry appears contingent on the formation of heterodimers with E2; heterodimer formation is mediated through the lysine and arginine residues within the two hydrophobic domains of E1 and the arginine residue in the transmembrane domain of E2 (Ronecker et al., 2008).

The secondary envelope glycoprotein. The final structural protein to be translated is the secondary envelope glycoprotein (E2). The E2 protein is of variable size, with a polypeptide skeleton ranging from 375 to 400 amino acids long depending on the carboxy-terminal processing site (Donis 1995). Not only variable in length, three hypervariable regions exist within the amino acid sequence and this hypervariablity is believed to reflect immunologic selective pressure. As the immunodominant protein, E2 is extremely antigenic and exposure to the protein through vaccination or natural infection results in the stimulation of neutralizing antibody production in exposed cattle (Donis 1995;Toth et al., 1999). In a characterization study of 40 monoclonal antibodies specific for BVDV, the investigators concluded that antigenic variation amongst BVDV isolates is essentially confined to the envelope glycoproteins, particularly E2 (Corapi et al., 1990). More recent research has continued to focus on E2 and its role in successful antigenic stimulation following vaccination with killed or modified-live vaccines. Serum antibodies produced in response to vaccination with a killed vaccine are almost entirely directed at the E2 protein; when using modified-live vaccines, serum antibodies are still primarily directed at the E2 protein but also to the fused second and third nonstructural protein (NS2-3) and to a lesser degree, the other envelope glycoproteins (Ridpath 2012). Vaccination with E2 subunit vaccines has also been explored but as yet has not been widely exploited commercially (Ferrer et al., 2007;Donofrio et al., 2007;Pecora et al., 2012).

Protein p7. Separating the genes encoding E2 and NS2-3 is a small polypeptide that was originally described as a hypothetical peptide (p?) (Elbers et al., 1996). Since then, the protein has been termed, somewhat unimaginatively, the protein of unknown

function, or p7 as a result of an analogous protein translated from the HCV genome. The original description of p7 as a hypothetical peptide is due to incomplete cleavage from E2; thus p7 may exist as a distinct peptide or may form the E2-p7 polypeptide, much like the HCV peptide (Lin et al., 1994;Harada et al., 2000). While the role of p7 is still incompletely understood, the "term protein of unknown function" may be somewhat dated as the protein appears to be involved in replication of the BVDV virion (Harada et al., 2000). In HCV, p7 is required for replication, acting in late stage assembly and release of the virion and also potentially acts as a virulence factor that modulates viral fitness (Steinmann et al., 2007). Due to its importance for replication of the HCV virion, p7 has been identified as a potential target for antiviral therapy (Luscombe et al., 2010). Although knowledge of p7 has clearly advanced since its original description as a hypothetical peptide, further research is needed to fully understand its role and function in BVDV replication and infection.

The fused second and third nonstructural proteins. The NS2-3 polypeptide is roughly 1300 amino acids in length and is slightly basic (Donis 1995). Posttranslational processing can result in the cleavage of NS2-3 into the second (NS2) and third (NS3) nonstructural proteins, each with distinct chemical properties. Forming the amino-terminus of NS2-3 is a very hydrophobic region that is homologous to NS2. On the contrary, the carboxy-terminus is hydrophilic and homologous to NS3. Downstream of the amino-terminus lies three distinct regions, namely a zinc finger domain, a protease and a helicase. High levels of neutralizing antibodies to NS2-3 are seen after natural infection with BVDV or following vaccination with a modified-live vaccine (Donis 1995;Ridpath 2012). However, humoral responses to NS2-3 following vaccination with a

killed vaccine are heavily muted. Cross-reaction of NS2-3 antibodies between pestivirus species has been demonstrated with BVDV, CSFV and BDV (Donis 1995).

The NS2 protein is composed of the hydrophobic amino-terminus and the zinc finger domain. The zinc finger is believed to bind RNA and may function to maintain close proximity of the viral replication complex to the cell membrane (De Moerlooze et al., 1990). The size of the NS2 protein is highly variable due to the presence of foreign sequence insertions or additional genetic elements subsequent to BVDV sequence rearrangement (Donis 1995). Unlike, the fused NS2-3 protein, NS2 does not stimulate high levels of antibody production following infection (Donis and Dubovi 1987).

The NS3 protein includes the protease and helicase domains of NS2-3 and is one of the more widely studied proteins of BVDV. The protease acts on the NS2-3 polypeptide itself to cleave the molecule into the individual NS2 and NS3 proteins (Wiskerchen and Collett 1991). The protease is also necessary for all proteolytic processing of the other viral nonstructural proteins. Finally, the domain closest to the carboxy-terminus forms a helicase that is able to catalyze ATP-dependent separation of double-stranded RNA (dsRNA) and is theorized to function during the initiation of translation as well as viral replication (Warrener and Collett 1995). The NS3 protein is a necessary component of the replication complex required for viral RNA replication (Behrens et al., 1998). The important role played by NS3 likely explains why it is one of the most conserved of the viral proteins amongst the pestiviruses. Unlike NS2, NS3 is highly immunogenic (Donis and Dubovi 1987).

It was originally thought that the NS2-3 protein is not cleaved to individual NS2 and NS3 subunits except in CP isolates. Consequently, NS3 has historically been

considered a molecular marker of CP strains. However, the answer to whether NS2-3 is cleaved into NS2 and NS3 subunits in noncytopathic (NCP) infection is not of the categorical type but one of magnitude and timing. Expression of cleaved NS2 and NS3 does occur early in the post-infection period even with NCP BVDV (Lackner et al., 2005;Kameyama et al., 2008). Cleavage rates of NS2-3 are largely similar between CP and NCP isolates until approximately six to nine hours post-infection when the cleavage rates for NCP isolates diminish rapidly while rates for CP isolates show only a moderate decrease (Lackner et al., 2004). Consequently, constitutive expression of NS3 remains a hallmark of CP strains but NCP strains also result in NS3 expression for a short period following infection. Many CP isolates contain insertions, rearrangements or duplications of foreign or autologous genetic material in the NS2-3 gene that results in high levels of NS3 expression (Qi et al., 1992;Donis 1995;Vilcek et al., 2000;Balint et al., 2005b). Constitutive expression of NS3 may result from unchecked cleavage of NS2-3 or de novo expression of the protein.

The amino- and carboxy-terminal portions of the fourth nonstructural protein. Like NS2-3, the fourth nonstructural protein (NS4) is subject to posttranslational proteolytic processing that yields two distinct peptides, NS4A and NS4B. Proteolytic processing is accomplished by the protease found in NS3 (Tautz et al., 2000). Unlike NS2-3, the boundaries of the NS4 polypeptide have been poorly characterized and relatively little information is available about its role in viral replication (Donis 1995;Lindenbach and Rice 2001). The only function of the protein to be elucidated is its role as a cofactor to the serine protease activity of NS3 (Tautz et al., 2000). The region of

importance to maintain cofactor activity is found within the central region of NS4A. Currently, the role of NS4B has not been clearly established.

The amino- and carboxy- terminal portions of the fifth nonstructural protein. The fifth nonstructural protein (NS5) is also processed by the protease of NS3 to yield two distinct peptides, NS5A and NS5B (Tautz et al., 2000). Cleavage is incomplete and expression of the uncleaved NS5 can also be found in infected cells. Among the *Flaviviridae*, cleavage of NS5 into two distinct proteins is limited to the *Pestivirus* and *Hepacivirus* genera (Donis 1995). NS5A has been postulated to play a role in viral replication as it has been shown to interact with host cellular proteins including bovine translation elongation factor (Johnson et al., 2001) and bovine NIK- and IKKbeta-binding protein (Zahoor et al., 2010). The analogous protein in other pestiviruses as well as HCV has been shown to play a crucial role in genome replication (Tellinghuisen et al., 2006;Xiao et al., 2009;Chen et al., 2012). The specific role of NS5A in replication of the BVDV genome will only be identified through continued study.

The NS5B gene is highly conserved among the pestiviruses and its protein product is one of the best characterized NS proteins. As an integral component of the replicase complex, NS5B functions as the RNA-dependent RNA polymerase (RdRP) during genome replication (Steffens et al., 1999). The structure of the RdRP is roughly analogous to the right hand with separate fingers, palm and thumb domains. In addition, the RdRP of BVDV also contains a unique amino-terminal domain of unknown function (Choi et al., 2004). During elongation, the fingers and thumb domains are thought to form a channel to direct the template to the catalytic residues of the palm domain with the thumb domain acting as a ratchet to advance the template and nascent product over the

catalytic residues. The catalytic site is critical for several enzyme activities important to de novo and elongative RNA synthesis including template translocation, protein-protein interactions and formation of the replication complex (Lai et al., 1999;Tonelli et al., 2010a).

The 3´ untranslated region. The 3´ UTR which takes the place of 3´ poly A tail found in most eukaryotic mRNA, is 226 nucleotides in length (Donis 1995). Though it may lack a poly A tail, the closely related HCV does have a homopolymeric tail; it consists of a chain of uracil residues (Brock et al., 1992). The 3´ UTR consists of a highly variable region at the 5´ terminus while the 102 nucleotides at the 3´ terminus of the sequence are more highly conserved (Deng and Brock 1993). Due to the variable nature of the amino-terminus of the 3´ UTR, it has been assumed that the region may play a less critical role in virus replication than the carboxy-terminus. However, the variable region has been shown to form a complex RNA motif that is critical for the coordination of viral translation and replication and is necessary for the termination of translation at the stop-codon of the viral ORF (Isken et al., 2004). The RNA motif also forms a site for interaction with cellular host factors. The conserved region of the 3´ UTR is part of the replication complex. Thus the different roles of the 3´ UTR are manifested through the different domains of the sequence.

Biotypes

Viral isolates from either BVDV genotype can be characterized as either CP or NCP biotypes based on their activity in cultured epithelial cell lines (Gillespie et al.,

1960). Vacuolation and cell death are seen in cultured cell monolayers after CP, but not NCP, infection (Figure 1) (Walz 2009). Biotype is not an accurate predictor of *in vivo*

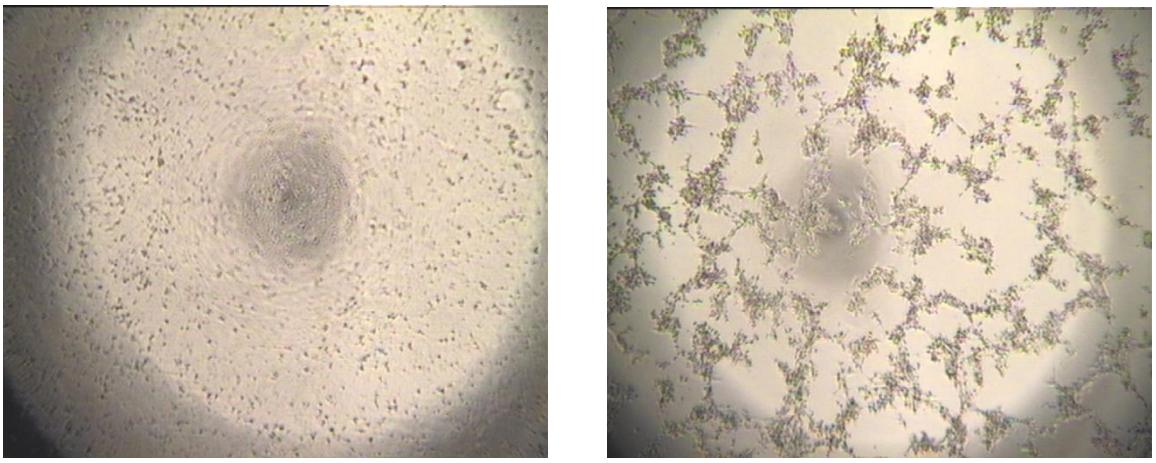


Figure 1. Microscope photographs (100x) of Madin-Darby Bovine Kidney cell cultures 72 hours after infection with a noncytopathic (left) or cytopathic (right) strain of BVDV. (Photographs courtesy of Dr. M. S. Marley).

pathogenicity (Bezek et al., 1994). In nature, NCP strains predominate and are associated with the most clinically virulent form of acute BVDV infection (Pellerin et al., 1994). The CP strains are believed to arise from NCP strains by mutagenesis and the CP strain and its NCP counterpart are known as a viral pair. This occurs most commonly as a result of a recombination event where extra pieces of genetic code are inserted into an NCP genome. However, not all biotype changes are associated with a recombination event as CP viruses without insertions have been identified (Quadros et al., 2006). Consequently, CP strains are more variable in length than NCP strains (Meyers et al., 1992;Tautz et al.,

1994). A third biotype of BVDV has been proposed based on the cytopathogenic traits of certain viral strains in lymphoid cells, which are the principal site of viral replication *in vivo* (Ridpath et al., 2006). Biotype is not an indicator of virulence as animals infected with either CP or NCP strains exhibit a wide range of clinical syndromes.

The cytopathogenic effects (CPE) caused by CP infection are not completely understood and are likely mediated by several distinct pathways. Infection of cell culture monolayers with CP strains of BVDV results in apoptosis of infected cells rather than cell lysis and necrosis (Zhang et al., 1996;Hoff and Donis 1997;Schweizer and Peterhans 1999). Necrosis is characterized by leakage of the plasma membrane and spilling of the intracellular contents; apoptosis is a more tightly regulated process that limits damage to the surrounding tissue (Steller 1995). Infection with the closely-related HCV has also been shown to induce apoptosis rather than cell lysis and necrosis (Mita et al., 1994). Various pathways are involved in apoptosis induction following CP infection. The extrinsic pathway appears to be involved to a small extent (Bielefeldt-Ohmann and Babiuk 1988;Yamane et al., 2005) with the oxidative stress-mediated (Hoff and Donis 1997;Schweizer and Peterhans 1999;Grummer et al., 2002), the intrinsic (Grummer et al., 2002), and the ER stress-mediated apoptosis (Jordan et al., 2002;Lackner et al., 2005;Maeda et al., 2009) pathways the primary mediators of CPE in CP-infected cells.

The main pathways can all be induced by elevated levels of dsRNA, such as those seen with CP infection (Yamane et al., 2006). Until recently, the factors stimulating higher levels of dsRNA in CP-infected cells compared to NCP-infected cells were unknown but it appears that a J-domain protein interacting with viral protein (Jiv) plays a pivotal role in RNA production and virion formation (Lackner et al., 2004). This cellular

chaperone is an essential component in the protein complex responsible for cleavage of NS2-3 to NS2 and NS3 (Rinck et al., 2001). The NS3 protein is a necessary component of the replication complex required for viral RNA replication (Behrens et al., 1998). A model has been proposed whereby each NS2-3 protein binds a single Jiv molecule after which time the Jiv molecule is no longer capable of interacting with other NS2-3 molecules (Lackner et al., 2005). Because intracellular levels of Jiv are normally low and the synthesis rate is much lower than the synthesis rate of viral NS2-3, the production of NS2-3 rapidly depletes cellular Jiv levels, inhibiting further processing of NS2-3 and effectively down-regulating viral replication. Low endogenous intracellular levels of Jiv therefore inhibit unchecked replication of BVDV. Since at least some CP isolates encode Jiv (Muller et al., 2003;Balint et al., 2005a;Kameyama et al., 2006), viral replication continues unimpeded and leads to increased levels of dsRNA within infected cells. Interestingly, insertions encoding Jiv or Jiv-like proteins have also been described in CP strains of other member and atypical pestiviruses (Hughes 2004;Gallei et al., 2008;Decaro et al., 2012). Further work is necessary to characterize additional triggering factors of CPE in CP-infected cells. Comparable *in vitro* data is lacking and is necessary to understanding the pathogenicity of various strains in natural infections (Zhang et al., 1996). Because not all CP isolates are known to encode Jiv, future work must also explore the inducing factors for CPE caused by those strains.

Viral Structure

The BVDV virion is typical of the pestiviruses and measures 40 to 60 nanometers in diameter with genomic RNA and the nucleocapsid protein contained in the central core

of an icosahedral capsid (Givens 2000;Lindenbach and Rice 2001). Two glycoproteins are anchored in the membranous envelope with a third peptide loosely attached by undefined interactions. The virus does not package any proteins necessary for viral replication in the virion (Donis 1995). The virion is comprised of genomic RNA, the lipid bilayer and four structural proteins: the nucleocapsid protein (C) and the envelope proteins E^{rns}, E1 and E2 (Lindenbach and Rice 2001).

Viral Replication

Due to the structure and polarity of its genome, BVDV is classified as a class IV virus in the Baltimore classification scheme (Heinz et al., 2000). As the prototypical virus in the *Pestivirus* genus of the *Flaviviridae* family, replication of BVDV is similar to other flaviviruses and can be thought of as occurring in three phases; attachment and cell entry, gene translation and genome replication (Donis 1995). Attachment of BVDV is thought to be mediated primarily through the interaction of the viral E^{rns} envelope protein with glycosaminoglycans in the host cell membrane. Preventing E^{rns} binding *in vitro* is sufficient to prevent BVDV infection (Iqbal et al., 2000). After attachment, a second viral envelope protein, E2, interacts with a cellular receptor of 50 kD that has not yet been clearly defined. Binding of E2 triggers receptor mediated endocytosis that delivers the genome to the cytosol through an acid-dependent step. Compounds that block cellular endocytosis or endosomal acidification are effective at preventing BVDV infection (Donis 1995).

Release of the viral RNA into the cytosol signals the start of the second phase of BVDV replication. The approximately 380-nucleotide 5′UTR serves as the IRES when

the RNA template comes into contact with ribosomes (Donis 1995). Localization signals for the viral RNA have yet to be identified. Translation and peptide processing result in the expression of 10 to 12 mature proteins (Figure 2). As alluded to earlier, BVDV is an enveloped virus and the envelope proteins are processed in the endoplasmic reticulum. Incompletely processed polypeptides are present in infected cells throughout infection and it is thought these may serve a catalytic or regulatory function in viral replication. Detection of viral polypeptides can be detected as early as 3 hours after BVDV infection and reaches a peak 12-14 hours after infection (Donis 1995).

Protein synthesis precedes genomic replication as the virus does not package any proteins necessary for viral replication in the virion (Donis 1995). The carboxy-terminus of the NS5 protein encodes the replicase but other non-structural proteins are also necessary for efficient genomic replication (Choi et al., 2004). First, a full-length negative sense template is made. This negative sense strand then serves as a template to synthesize progeny genomes (Donis 1995). Regulation of genome synthesis is currently an area of intense study. It is believed that BVDV assembly takes place in either the endoplasmic reticulum or the Golgi apparatus as electron microscopy studies have revealed no clearly visible nucleocapsid structures in the cytosol of infected cells. The viral envelope is attained by budding of the capsid through the lipid envelope of the organelle and virions accumulate in the vesicular lumen before reaching the extracellular compartment by exocytosis. The latent phase of BVDV infection can be as short as 10 hours (Donis 1995).

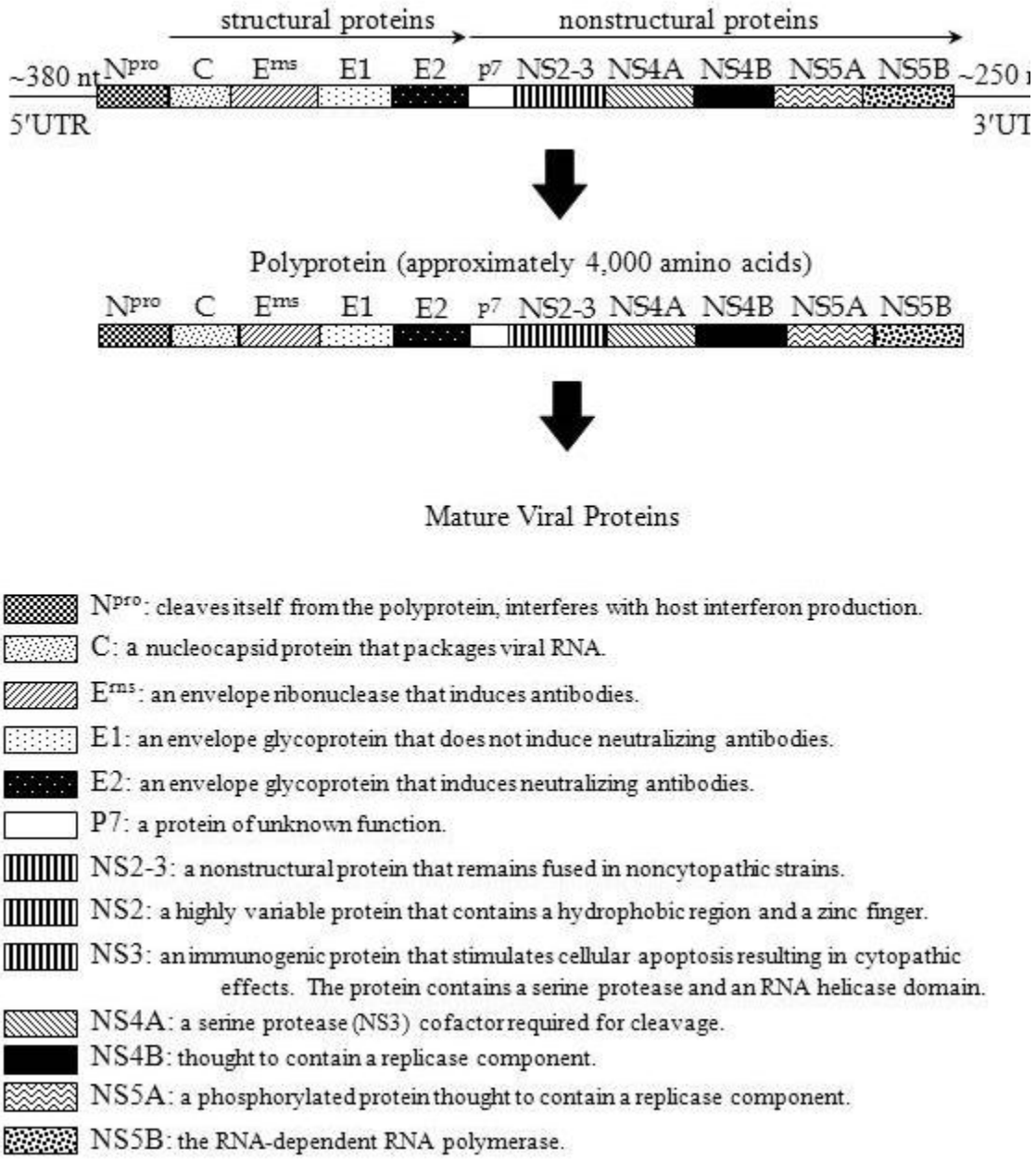


Figure 2. Organization of the bovine viral diarrhea virus genome. The genome encodes a single polypeptide that is cleaved into 10 peptides. The NS2-3 and NS5A-B peptides may be further processed to their individual subunits as shown for a possible total of 12 mature proteins.

Viral Host Range

Based on name alone, one can surmise that cattle serve as the natural host of BVDV. However, the virus does not exhibit strict host specificity and evidence of infection has been documented in greater than 50 families within the order Artiodactyla which comprises the even-toed ungulates (Walz et al., 2010;Passler and Walz 2010). Domesticated or farmed species at risk for BVDV infection other than cattle include bison, water buffalo, goats, sheep, swine and various cervids as well as both New World and Old World camelids. Infection has also been demonstrated in non-domesticated species, which poses a risk to domestic species as the establishment of wildlife reservoirs may be important to the epidemiology of the disease where the wildlife-livestock interface is not well controlled. In North America, the white-tailed deer (*Odocoileus virginianus*) represents a potential likely wildlife reservoir of importance to cattle farmers as the most abundant free-ranging ruminant on the continent (Passler et al., 2010). Persistent infection has been demonstrated in both captive and free-ranging deer in the United States; further study is needed to characterize the importance of cross-species transmission to the epidemiology of BVDV infection in cattle.

Although minor controversy exists, humans are not considered susceptible to BVDV infection. By its very nature as an RNA virus, BVDV is highly mutable. The RdRP lacks an efficient exonuclease; thus misincorporated nucleotides are not readily removed (Donis 1995). The error rate of the BVDV RdRP approaches one error for every 10,000 bases polymerized. With a genome length of approximately 12.5 kb, this translates to an average of 1.25 base substitutions per virus replication cycle.

Consequently, the potential of BVDV to emerge as a human pathogen has been studied. (Giangaspero et al., 1993a;Giangaspero et al., 1997).

Human cells are able to endocytose the BVDV virion after cell surface binding through the action of low density lipoprotein receptors (Agnello et al., 1999). Furthermore, the virus is capable of replicating within human cancer cells (Fernelius et al., 1969) although efficient replication is limited to cells derived from Artiodactyla (Bolin et al., 1994). However, *in vivo* evidence of human infection is largely lacking. Much of the early data cited as evidence of BVDV infection in humans was based on the demonstration of serum neutralizing antibodies to BVDV. Giangaspero and colleagues performed an epidemiological sampling of 1272 individuals in Europe and Zambia and found that 15.3% of the sample population was seropositive to BVDV (Giangaspero et al., 1993a). In Zambian individuals, the percentage of seropositive individuals was highest in individuals showing signs of human immunodeficiency virus infection (Giangaspero et al., 1993b). However, other researchers who demonstrated the presence of very small antibody titers to BVDV in individuals with no previous close contact with likely animal hosts questioned the value of such tests in diagnosing human infection (Wilks et al., 1989). Furthermore, individuals who have spent many years in BVDV research with known exposure to the virus have failed to produce serum neutralizing antibodies in response to exposure (M.D. Givens, personal communication, 2009). Understanding the meaning of seropositivity to BVDV in humans is obviously not fully understood and requires additional study.

Isolation of a pestivirus, purportedly BVDV, was reported to occur from two clinically healthy individuals (Giangaspero et al., 1993a). Pestivirus antigens were

detected in the feces of children under two years of age suffering from episodes of gastroenteritis that could not be attributed to other commonly recognized enteric pathogens (Yolken et al., 1989). More recently, evidence of pestivirus infection was found in one patient suffering from Crohn's disease by PCR (Van Kruiningen et al., 2007). Early hypotheses as to the etiology of white matter damage in the brain of preterm infants involved prenatal infection with a pestivirus; however, such hypotheses have largely been discarded due to a lack of evidence (Dammann et al., 2006). Further study is needed to determine what role, if any, BVDV and other pestiviruses play in human disease processes.

Diagnosis of BVDV in animals or humans could be complicated by the demonstrated presence of contaminated reagents used in various diagnostic tests. By definition, NCP strains of BVDV do not result in visible changes to infected cells; thus, infection may go unnoticed unless rigorous quality control measures are implemented and enforced. Despite manufacturer testing, BVDV has been isolated from commercially available lots of fetal bovine serum (Yanagi et al., 1996). Additionally, commercially available bovine, canine, feline and primate cell lines (Fernelius et al., 1969;Harasawa and Mizusawa 1995), human viral vaccines (Harasawa and Tomiyama 1994;Giangaspero et al., 2001) and interferons for human use (Harasawa and Sasaki 1995) have been found to be contaminated by pestiviruses. Contaminated reagents and biologicals represent a potential source of adulteration that could alter the results of diagnostic tests.

Clinical Disease

Clinical disease caused by BVDV infection can take several forms and is a source of significant economic losses in cattle worldwide. The clinical effects of BVDV are manifested in the respiratory, gastrointestinal, reproductive, cardiovascular, lymphatic, immunologic, integumentary or central nervous systems of affected cattle throughout the world (Baker 1995;Brock 2004). However, reproductive losses associated with BVDV infection are thought to have the largest negative economic impact for producers (Grooms 2004). Economic losses are substantial in both the beef and dairy industries. Clinical manifestations of BVDV infection are often broadly discussed in the categories of (1) acute disease, (2) persistent infection (3) reproductive tract infections and (4) testicular infections (Bielefeldt-Ohmann 1995;Walz et al., 2010).

Acute infection. Acute infection occurs when the virus infects seronegative, immunocompetent cattle and can cause several different clinical syndromes which are influenced by viral strain and several host factors (e.g. age, immune status, gestational status) (Baker 1987;Scruggs et al., 1995). Animals are usually infected through the respiratory or gastrointestinal mucosa; subsequent primary viremia results in widespread dissemination of the virus (Walz 2009). Although typically much shorter, viremia may last up to 15 days (Duffell and Harkness 1985) and is often accompanied by pyrexia and leukopenia (Traven et al., 1991;Polak and Zmudzinski 2000;Stoffregen et al., 2000;Liebler-Tenorio et al., 2002). Leukopenia in affected cattle is characterized by a lymphopenia and potentially neutrophilia (Ellis et al., 1988;Newcomer et al., 2013). Clinical signs due to acute BVDV infection include depression, inappetance, decreased milk production, oculonasal discharge and oral ulcerations (Walz et al., 2010). Despite its

name, acute BVDV infection resulting in diarrhea is poorly characterized and inconsistently seen in acute infections, particularly of adult cattle.

Likewise, the contribution of BVDV infection to respiratory disease is not well understood but is thought to be due to its immunosuppressive qualities that enhance the pathogenesis of other viral or bacterial pathogens (Bielefeldt-Ohmann 1995). Tropism of BVDV is incompletely understood but the virus is most reliably isolated from cells of hematopoietic or lymphoid tissue (e.g. white blood cells, spleen) but can also be found in tissues from multiple organ systems. The lymphotropism exhibited by BVDV affects cells of both the innate and adaptive immune systems (Walz et al., 2010). Consequently, BVDV has been implicated along with other pathogens (e.g., *Pasteurella multocida, Manheimia hemolytica*, parainfluenza-3 virus, bovine respiratory syncytial virus) in the pathogenesis of the bovine respiratory disease complex (Potgieter 1997;Fulton et al., 2000;Ridpath 2010a).

More recently, a severe syndrome caused by acute BVDV infection marked by severe thrombocytopenia and hemorrhage has been described in Canada and the United States (Rebhun et al., 1989;Pellerin et al., 1994). Mortality rates in both mature cattle and heifers were higher than commonly seen in previous outbreaks of BVDV, reaching 9% and 53%, respectively (Carman et al., 1998). The viral isolates responsible for the outbreak were classified as BVDV2 due to differences in sequence homology from previous BVDV isolates (Pellerin et al., 1994). Subsequent experimental reproduction of thrombocytic BVDV infection has been largely limited to NCP BVDV2 strains (Bolin and Ridpath 1992;Walz et al., 2001). BVDV-induced thrombocyopenia is believed to be due to infection of megakaryocytes in the bone marrow (Ellis et al., 1998).

Venereal transmission from bulls suffering from acute BVDV infection has been described (Gard et al., 2007). Virus (5 to 75 $CCID_{50}$/mL) may be detected in the semen of infected bulls from 2 to 20 days after infection (Kirkland et al., 1991). Contaminated samples may have acceptable motility, morphology and concentration of spermatozoa. Virus can rarely be isolated from semen after viremia has resolved but can no longer be cultivated from semen after antibodies are detectable in serum (Paton et al., 1989). Approximately 5% of seronegative heifers were infected when inseminated with contaminated semen collected from an acutely infected bull (Kirkland et al., 1997). Subsequent horizontal transmission to pregnant animals resulted in the creation of PI calves.

Persistent infection. Of utmost importance in the propagation of BVDV infection is the persistently infected (PI) animal. In utero exposure to NCP strains before development of fetal immunocompetence (generally before 125 days of gestation) can result in a calf that is PI with the virus (McClurkin et al., 1984). PI calves are often weak at birth and over 50% will die before one year of age. However, others may not show signs of disease but continuously shed virus and are important in the epidemiologic aspects of BVDV propagation. Superinfection of PI calves with homologous CP strains of BVDV may result in mucosal disease (MD). Such strains may arise by mutation of the NCP strain causing the persistent infection or may come from external sources such as modified-live vaccines or herdmates (Brownlie 1990). The prevalence rate of MD is very low but the mortality is very high. Most calves with MD have widespread ulceration of the upper gastrointestinal tract as well as hemorrhagic lesions in the abomasum and elsewhere (Walz et al., 2010). Histologically, the Peyer's patches are often depleted of

cells or atrophic and the lesions show infiltrates of major histocompatibility complex II antigen positive macrophages and dendritic cells (Bolin 1995). Lesions similar to those seen in MD can be induced experimentally by overexpression of several inflammatory cytokines including TNF_{α}, TGF_{β} and IL-6 but it remains to be determined exactly what role these play in the pathogenesis of MD (Bielefeldt-Ohmann 1995).

As with bulls acutely infected with BVDV, PI bulls also pose a threat of venereal BVDV transmission and thus should be identified and culled. Such bulls may display normal growth and development characteristics (Howard et al., 1990) and can produce semen with normal concentration, motility and morphology of spermatozoa (Kirkland et al., 1994). However, large quantities of infectious BVDV ($10^{7.6}$ $CCID_{50}$/mL) are shed in the semen of PI bulls. Contaminating virus survives the cryopreservation and processing steps and will consistently infect susceptible inseminated females, occasionally resulting in the birth of PI calves (Meyling and Jensen 1988;Kirkland et al., 1994). Contaminated semen collected from PI bulls cannot be cleared of the virus by swim up techniques, glass wool filtration, glass bead filtration or centrifugation through Percoll gradients as the virus is closely associated with the sperm (Bielanski et al., 1992). Consequently, semen collected from PI bulls is very likely to result in viral transmission if used in artificial insemination (AI) programs.

Reproductive tract infections. As with other pestiviruses, BVDV is able to readily cross the placenta of pregnant animals and infect the fetus (Moennig and Liess 1995). If this occurs in the last trimester, the infection is generally neutralized by the fetus's immune response but prior to this, infection may result in persistent infection or other sequelae. This is largely determined by the age of the fetus when infected. A naïve cow

infected during the first month and a half of gestation may suffer early embryonic death, possibly due to endometrial inflammation resulting from the viral infection. Later in gestation, but before the fetus has developed immunocompetence, BVDV infection can result in central nervous system malformations due to the ability of BVDV to cross the blood-brain-barrier. Cerebellar hypoplasia is the most notable sign seen, but other congenital defects include growth retardation, hypomyelinogenesis, hydrancephaly, hydrocephalus, microencephaly, micropthalmia, ocular cataracts, retinal degeneration, optic neuritis, alopecia, hypotrichosis, brachygnathism, thymic aplasia and pulmonary hypoplasia (Grooms 2004). It is unclear if these malformations are a result of direct viral interactions with target cells or due to the nascent immune system responding to the infection (Moennig and Liess 1995).

Testicular infections. In 1998, a new face was added to the multifaceted spectrum of clinical BVDV syndromes when a seropositive, nonviremic bull at an AI center was found to have a unique, localized persistent testicular infection (PTI) (Voges et al., 1998). Since then, only one other report exists of a similarly infected bull, also found at a bull stud (Givens et al., 2012). Experimental studies following the description of the original bull with PTI have been unable to reproduce the exact syndrome although similar results have been achieved, leading to the distinction of PTI and prolonged testicular infection (ProTI) (Givens et al., 2003b).

Bulls with PTI are nonviremic but consistently shed virus in the semen that is detectable using routine virus isolation techniques. The original PTI bull was reported to have consistently shed BVDV in his semen at a concentration ($< 2 \times 10^3$ $CCID_{50}$/mL) intermediate between PI and transiently infected bulls (Kirkland et al., 1991;Voges et al.,

1998). Because virus could not be isolated from blood samples, the bull was admitted to an AI center and cryopreserved semen resulted in the infection and seroconversion of a seronegative heifer (Niskanen et al., 2002). At slaughter, virus was isolated only from the bull's testicles (Voges et al., 1998). The prevalence of bulls with PTI is thought to be very low (Givens and Waldrop 2004).

Prolonged testicular infection differs from PTI in that virus cannot be readily isolated from the semen of infected bulls, even when using the more sensitive roller bottle virus isolation technique (Givens et al., 2003b). Prolonged testicular infection can be reliably induced experimentally by exposing seronegative, peri-pubertal bulls to BVDV (Givens et al., 2009b). Viral RNA could be detected in semen of infected bulls for nearly three years but virus could not be isolated from semen samples although it could be grown from testicular tissue. Despite the presence of viral RNA in semen, transmission of the virus using either fresh or cryopreserved semen remains to be demonstrated (Givens et al., 2009b). Consequently, the risk of viral transmission from ProTI bulls appears to be low.

Diagnosis

A definitive diagnosis of BVDV infection is only made through laboratory testing due to the diverse clinical and subclinical syndromes displayed by infected animals. Several tests are available and selection of the appropriate test will be dictated by several factors, including the management system of the affected farm, financial constraints, and availability of tests at a given laboratory (Walz et al., 2010). Since not all available tests are appropriate for each clinical situation, care should be made when selecting a test in

order to reach a successful solution quickly and efficiently (Saliki and Dubovi 2004). Most BVDV testing focuses on the detection and identification of PI animals in order to remove said animals from the herd to limit virus shedding and spread; not all tests are appropriate for identification of PI animals. Essentially all diagnostic tests for BVDV fall into one of four main categories: virus isolation; antigen detection; molecular techniques and serology.

Virus isolation. Isolating virus from tissues or secretions of infected animals remains the "gold standard" diagnostic test for BVDV (Saliki and Dubovi 2004;Edmondson et al., 2007;Walz et al., 2010). Many cells from several different species will support the growth of BVDV in culture but cell lines used in BVDV isolation assays are generally limited to bovine turbinate, bovine testicle and Madin Darby Bovine Kidney (MDBK) cells (Saliki and Dubovi 2004). By definition, infection with CP strains of BVDV results in characteristic CPE in cultured monolayers; cells display vacuolization and apoptosis within 48 hours of infection. However, most field strains of BVDV are of the NCP biotype and do not result in observable CPE. Consequently, alternative visualization techniques such as immunofluorescence or immunostaining must be employed to detect infected cells.

White blood cells extracted from whole blood samples are the preferred sample when collecting samples from the live animal for detection of BVDV by virus isolation (Saliki and Dubovi 2004). Serum samples are also commonly used although false negative results may be obtained as a result of the presence of serum-neutralizing antibodies in the serum. In one study of 23 separate diagnostic laboratories using seven sample-test combinations, virus isolation performed on serum resulted in the lowest

consistency in detecting positive samples and the lowest level of agreement between laboratories (Edmondson et al., 2007). Nasal swab samples may also be subjected to virus isolation assays as live virus is often shed in the nasal secretions of infected cattle. Tissue from the lymphoid organs such as thymus, spleen, Peyer's patches and mesenteric lymph nodes is the preferred sample to be collected from a dead animal or aborted fetus for submission for virus isolation (Saliki and Dubovi 2004). A single virus isolation test is not capable of differentiating persistent and acute infections. PI animals can be identified by positive virus isolation from serial samples collected no less than 14 days apart (Brock 1995).

Antigen detection. Direct antigen detection is generally less sensitive and less reliable than virus isolation techniques; however, antigen detection assays are generally quicker and more economical to perform than virus isolation assays (Saliki and Dubovi 2004). Consequently, antigen detection methods are most often employed to detect persistent rather than acute infections. Antigen detection methods are limited to antigen capture ELISAs (ACE) and immunohistochemistry (IHC) techniques. Antigen detection tests are generally used as screening tests to detect PI animals although additional testing may be warranted in certain circumstances due to the occurrence of false-positives (Saliki and Dubovi 2004;Fulton et al., 2005). While IHC tests are primarily carried out by trained personnel at diagnostic laboratories, commercially available ACE kits have been used cow-side for the identification of PI animals. Most commercial IHC testing is performed on fresh or formalin-fixed tissue samples commonly taken from the ear and referred to as "ear notches". However, one study found no difference in the test sensitivity when using tail fold biopsies or nasal swab samples as the test sample

(VanderLey et al., 2011). Several studies have demonstrated a high level of sensitivity for the commercial ACE kits when used as a screening test to detect PI animals (Fulton et al., 2005;Kuhne et al., 2005;Edmondson et al., 2007). Commercial ACE kits rely on monoclonal antibodies targeting the E^{rns} glycoprotein of BVDV; consequently, differing strains of BVDV may not be detected by the test (Gripshover et al., 2007).

Molecular techniques. Since the publication of the BVDV genome, detection of viral nucleic acid has been explored as a diagnostic test. Today, amplification of the RNA genome by reverse-transcriptase polymerase chain reaction (rtPCR) and subsequent detection has become a widely used diagnostic method (Driskell and Ridpath 2006). Commercially available kits with simple viral RNA extraction steps have encouraged the acceptance of rtPCR as the primary herd screening assay used by many diagnostic laboratories. The sensitivity and specificity of the rtPCR assay are high and a variety of samples can be used, including serum, whole blood, milk, tissues, nasal swabs, semen and embryos (Radwan et al., 1995;Da et al., 1995;Givens et al., 2001;Edmondson et al., 2007;Fulton et al., 2009). The rtPCR assay should be validated for each sample type as poor RNA extraction will obviously drastically affect the sensitivity of the assay (Saliki and Dubovi 2004). Detection of viral nucleic acid does not necessarily mean that infectious virus is present in the sample; thus clinicians must take care to interpret assay results in conjunction with the clinical scenario.

The high sensitivity of the rtPCR assay has made the testing of pooled milk or serum samples an effective and economical means of screening whole herds or other groups of animals. Weinstock, et al., showed that a single viremic sample in a pool of 100 negative sera could be detected by rtPCR and estimated the sensitivity of the assay to

exceed that of virus isolation by 10^1-10^4-fold (Weinstock et al., 2001). The sensitivity of rtPCR when using milk samples exceeds that of virus isolation nearly 15-fold; one researcher was able to detect BVDV using rtPCR when the sample was diluted 1:640 (Radwan et al., 1995). However, sensitivity of the test decreases as the number of samples included in the pooled aliquot increases leading to controversy as to the optimal number of samples to be included in each test (Ridpath 2011). When using pooled tissue, whole blood or serum samples, it is also important to remember that each animal in the herd must be sampled and that bulk-tank milk samples will not reflect the infection status of any non-lactating animals (e.g., bulls, dry cows, heifers) on the farm. Recently, a diagnostic test using rtPCR has been described for non-lactating animals that does not require individual sampling (Givens et al., 2011). By swabbing consumption surfaces and assaying the swabs by rtPCR, the investigators were able to determine if PI animals were present in a group of cattle.

Serology. Serologic tests measure the level of antibody response to previous exposure or challenge with a particular infectious agent and can be used to demonstrate BVDV infection. However, care must be taken when interpreting the results as most serologic tests are unable to differentiate antibodies produced in response to natural exposure versus those produced after vaccination versus those present as a result of passive transfer such as through maternal colostrum (Walz et al., 2010). However, in the right circumstances, serologic testing may be beneficial to assess vaccine efficacy or compliance with a vaccine protocol or to assess the herd's exposure status to BVDV (Saliki and Dubovi 2004). In vaccinated herds, testing of seronegative sentinel animals may be useful to determine herd exposure. The serum neutralization assay is the most

commonly used serologic test for BVDV infection and can be used to differentiate exposure to BVDV1 and BVDV2. However, comparison of results between different laboratories is complicated as different reference strains are likely used to determine the antibody titer (Walz et al., 2010).

Antiviral agents in cattle

Introduction

Viral diseases, including BVDV, cause significant financial loss in cattle production systems in addition to widespread animal suffering in the face of disease outbreaks. Despite the importance of viral infections, no antiviral agents are currently licensed for use by veterinarians or producers in cattle. Because viral replication often involves use of the host cell machinery, viruses present few specific targets for chemotherapy that will not also affect host cell metabolism (White 1981). Notwithstanding, antiviral research has increased significantly during the past 20 years and while several promising compounds have been identified from *in vitro* testing, further *in vivo* testing is lacking and must be completed for the promise of such compounds to be realized clinically. Viral pathogens of cattle, like BVDV, that serve as a model for human disease have been the focus of the most intense research due to the greater potential market that exists for human medical compounds (White 1981). However, this research has also yielded discoveries applicable to veterinary medicine. Proven antiviral agents would not be useful solely in the treatment of disease but also could be employed in disease prophylaxis, control of disease outbreaks, in embryo transfer and assisted reproduction programs and in the laboratory.

Hepatitis C

Hepatitis C virus is the lone member of the *Hepacivirus* genus, which along with the *Pestivirus* and *Flavivirus* genera compose the *Flaviviridae* family. Infection with HCV in humans is often asymptomatic but chronic infection can lead to liver cirrhosis and subsequent liver failure, hepatocellular carcinoma or dilation of submucosal veins in the stomach and esophagus that are at risk of rupture leading to life-threatening hemorrhage (Sanyal et al., 2006). Chronic HCV infection is estimated by the World Health Organization to affect 170 million people, roughly 3% of the human population, worldwide (Baginski et al., 2000), and is the leading reason for liver transplantation in the United States (Zitzmann et al., 1999).

Despite its importance as a human pathogen, direct HCV research is hampered by the absence of a robust *in vitro* infection model. Efficient HCV propagation in cell culture has proven difficult and has limited opportunities to study HCV replication, pathogenesis and potential treatments (Bartenschlager and Lohmann 2001). In 1999, the study of HCV was greatly aided by the development of a subgenomic HCV replicon system (Lohmann et al., 1999). However, the replicon assay is not without its disadvantages since the system fails to faithfully mimic all steps of the HCV replication cycle. Antiviral drugs that target virus attachment and entry, subsequent uncoating or virion maturation and egress cannot be identified or studied using the replicon system because it fails to yield infectious virions (Buckwold et al., 2003). Another drawback to the replicon system is its relatively high cost compared to the culture of surrogate viruses and the use of cell culture systems.

Yellow fever virus, the prototypical member of the *Flavivirus* genus, has been proposed as a possible surrogate to study and characterize potential antiviral therapies effective against HCV (Neyts et al., 1996). However, the genomic structure of YFV is less similar to HCV than is BVDV and furthermore, the virion does not employ an IRES-mediated mechanism to initiate translation as do both HCV and BVDV (Buckwold et al., 2003). Therefore, YFV is no longer commonly used as an *in vitro* model for HCV research. As the prototypical member of the pestiviruses, BVDV shares many similarities with HCV in regards to virion structure, genome organization, replication cycle as well as clinical disease. Consequently, BVDV is now essentially the universal choice of surrogate viruses used for the study of HCV and to assess the efficacy of novel antiviral compounds (Buckwold et al., 2003;Birk et al., 2008;Finkielsztein et al., 2008).

At approximately 9.6 kb, the enveloped, positive-sense single-stranded RNA genome of HCV is slightly smaller than that of BVDV (Kato 2000). The genomic structure of BVDV is closely related to that of HCV and both genomes encode several proteins with similar functions (Baginski et al., 2000). For example, the protease and helicase functions of the NS3 protein are common to both viruses (Buckwold et al., 2003). Likewise, the RdRP of both viruses is encoded by NS5b and is mechanistically analogous (Choi et al., 2004). Low density lipoprotein receptors in host cells are utilized by both HCV and BVDV for cell entry and the processes of virion maturation, assembly and egress are similar for both viruses (Agnello et al., 1999;Buckwold et al., 2003). These similarities have made BVDV the popular surrogate for HCV research, particularly that focused on antiviral treatment and the elucidation of drug mechanism of action.

Interferons

Interferons (IFNs) are multifunctional proteins produced by several cell types in the body, particularly those of the reticuloendothelial system (Rosenquist 1973). Interferons are produced and released from host cells in response to the presence of pathogens, particularly viruses. Peak IFN levels are generally found 72-96 hours after viral challenge (Cummins et al., 1999a). Interferons exert their effect by binding to specific receptors on target cells, inducing the transcription of target genes (Goodbourn et al., 2000); classification of IFNs is based on the type of cell receptor to which they bind. While IFN-α and IFN-β are produced in direct response to infection, IFN-γ is produced after recognition of infected cells by activated T lymphocytes and natural killer cells (Rosenquist 1973). Termed the first cytokine of nonspecific immunity, IFN-α has been the most frequent target of therapeutic antiviral studies (Tompkins 1999). In addition to the induction of transcription of multiple cytokines including IFN-γ, interleukin (IL) IL-1, IL-2, and IL-10 (Brassard et al., 2002) IFN-α has been shown to affect local (Namangala et al., 2006) and peripheral (Ohmann and Babiuk 1985;Ohtsuka et al., 2006) lymphoid cells in the bovine patient. Therapeutic studies have been made easier by the advent of recombinant DNA technologies that have made large quantities of IFNs commercially available for study and clinical use.

Bovine cells possess receptors for human IFN (HuIFN) (Chambers et al., 1990) and are highly sensitive to HuIFN relative to other domestic species (Gresser et al., 1974;Bridgman et al., 1988). Consequently, both HuIFN and bovine interferon (BoIFN) have been used in numerous studies to examine the therapeutic potential of IFNs against BVDV and other viral pathogens of interest to bovine medicine. Replication of BVDV in

peripheral blood mononuclear leukocytes from PI cattle was inhibited by IFN-α but not by IFN-γ in one study (Sentsui et al., 1998). In addition to BVDV (Sentsui et al., 1998), *in vitro* studies have shown an inhibitory effect of HuIFN against bovine leukemia virus (BLV) (Ike et al., 1989), malignant catarrhal fever virus (Wan et al., 1988), vesicular stomatitis virus (VSV), bovine herpesvirus-1 (BHV-1), parainfluenza 3 virus (PI_3), and bovine respiratory syncytial virus (BRSV) (Fulton et al., 1986). In addition to these viruses, BoIFN has also exhibited antiviral activity against bovine adenovirus 7 and foot-and-mouth disease virus (FMDV) (Fulton et al., 1984;Wan et al., 1988;Sentsui et al., 2001) (Perez-Martin et al., 2012). Viruses vary in their susceptibility to *in vitro* IFN treatment with VSV and both cytopathic and noncytopathic strains of BVDV being highly susceptible, BHV-1 possessing low susceptibility and PI_3 possessing intermediate susceptibility (Gillespie et al., 1985;Peek et al., 2004a).

In vivo studies of IFN treatment of BVDV have not produced the promising results seen in the treatment of other bovine viral diseases. Oral treatment of calves with HuIFN-α prior to BHV-1 challenge lessened clinical symptoms, duration of fever, fatality rate and antibiotic costs while improving weight gain (Cummins et al., 1993;Georgiades 1993;Cummins et al., 1999b). Calves treated intranasally with HuIFN-α (Roney et al., 1985) or BoIFN-α (Babiuk et al., 1985;Babiuk et al., 1987) before infection with BHV-1 had decreased severity of clinical signs due to subsequent challenge with *Pasteurella hemolytica*. In one study, intramuscular injections of BoIFN decreased morbidity and increased survival rates in calves during a parainfluenza outbreak (Kishko and Mukvych 1998). However, another author found no protective effect of intramuscular injections of HuIFN before challenge with PI_3 (Bryson et al., 1989).

Prophylactic treatment of 30 calves with BoIFN significantly decreased the severity of bovine respiratory disease as compared to the placebo group (Akiyama et al., 1993). Likewise, calves treated with a low dose of HuIFN before shipping resulted in greater weight gain if the calves were treated when afebrile. Calves treated after shipment experienced fewer sick days than those from the negative control group (Cummins et al., 1999b). Despite the demonstrated success of IFN in research studies both as a therapeutic or prophylactic agent, the proteins are not commercially licensed or available for use in clinical practice. This may be due in part to the conclusion by several authors that the observed results were not due to a direct antiviral effect of the treatment but rather a general immunomodulatory effect (Roney et al., 1985;Babiuk et al., 1987). Additionally long-term administration of IFNs in cattle has the potential to result in the production of anti-interferon antibodies and subsequent microcytic anemia (Peek et al., 2004b).

However, unlike the benefits described in other viral infections of cattle, the value of IFN administration to treat or prevent BVDV infection in the live animal has been difficult to demonstrate. In contrast to the viruses in the studies listed above which all contain a genome comprised of DNA, BVDV is an RNA virus and thus, highly mutable, which may account for some of the differences seen in research studies involving IFNs. Viral load in PI cattle decreased slightly after subcutaneous treatment with BoIFN-τ when animals were treated with 10^6 U/kg ten times over the course of a two week period (Kohara et al., 2012). A similar decrease was not seen when cattle were treated at a dosage of only 10^5 U/kg or with oral or subcutaneous administration of HuIFN-α (Kohara et al., 2009). However, decreases in viral titer seen with administration of the higher dose of BoIFN-τ were transient and were erased following cessation of treatment. In an

extended study, where five Holstein PI heifers were treated with recombinant HuIFN-α every other day for 84 days, no antiviral activity of the treatment could be demonstrated (Peek et al., 2004b). Furthermore, the treated heifers all developed a microcytic anemia during the treatment period secondary to the production of anti-interferon antibodies. Interestingly, interferon administration is commonly employed to treat HCV infection in humans. Combination therapy with interferons and ribavirin has been shown to be more effective than monotherapy in HCV patients and is the mainstay of current treatment protocols (Seeff and Hoofnagle, 2002). The efficacy of BoIFN as a component of combination therapy has not been studied to date.

Aromatic cationic molecules

Aromatic cationic molecules have demonstrated inhibitory activity against several RNA viruses, including human immunodeficiency virus (Kumar et al., 1995), rotavirus (Vonderfecht et al., 1988) and respiratory syncytial virus (Dubovi et al., 1980). *In vitro* screening of 93 ACMs for cytotoxicity and antiviral activity against the NCP SD-1 strain of BVDV revealed five compounds that inhibited BVDV at nanomolar concentrations and yet did not display evidence of cytotoxicity at concentrations as high as 25 μM (Givens et al., 2003a). The compound exhibiting the widest therapeutic window of the selected compounds, DB772, was used in further characterization studies and is the antiviral compound of interest to this dissertation. The 99% endpoint for prevention of viral replication by DB772 was found to be 6 nM (Givens et al., 2004). Further, DB772 will eliminate BVDV infection in contaminated bovine fetal fibroblast cells with a single passage in culture media supplemented with 4 μM of DB772.

Subsequently, ACMs were studied for their use in bovine embryo production systems. If such systems are contaminated with BVDV, the developing embryo could conceivably act as a vector of transmission, resulting in early embryonic death, abortion, or the birth of persistently infected calves (Givens et al., 2004). Givens et al. used two closely related compounds, 2-[5(6)-{2-imidazolinyl}-2-benzimidazolyl]-5-(4-aminophenyl)furan or 2-(4-[2-imidazolinyl]phenyl)-5-(4-methoxyphenyl)furan (DB606) to demonstrate the ability of ACMs to prevent or eliminate BVDV from fetal fibroblast cell lines and embryo culture media (Givens et al., 2004;Givens et al., 2005). Furthermore, bovine embryos obtained from *in vitro* fertilization (IVF) that were cultured in medium containing 0.4 μM DB606 were successfully implanted into recipients and yielded healthy calves (Givens et al., 2006). Blastocyst development, pregnancy rate, abortion rate, gestation length, gender ratio, birth weights, neonate viability, clinical pathology values of the calves at 3 months of age and 205 d weaning weights did not differ significantly from those of controls using IVF embryos that were cultured in medium not supplemented with DB606. The reproductive capacity of heifers born from IVF embryos cultured in medium supplemented with DB606 was not impaired relative to that of heifers born from natural breedings or from IVF embryos not cultured with ACMs (Givens et al., 2009a). Seven of seven heifers born from DB606 cultured embryos were diagnosed pregnant after being exposed to a fertile bull for a 63 d breeding period. All seven gave birth to clinically normal calves. Thus, the antiviral properties of ACMs can be safely employed in multiple situations to either treat or prevent BVDV infections.

Nuceloside analogues

RNA viruses are highly susceptible to mutagenesis during viral replication. Research has sought to exploit the fallibility of the RdRP by even further increasing the error frequency during viral replication to a level that results in decreased infectivity or viral extinction (Goris et al., 2008). Most mutagenic agents are nucleoside analogues that terminate RNA synthesis when incorporated into the emerging strand or otherwise disrupt translation of the nascent mRNA. Such compounds may also act as antimetabolites on various enzymes necessary for viral replication (Finkielsztein et al., 2010). Enzymatic systems specific to the *Flaviviridae*, particularly the protease of NS3 and the RNA polymerase of NS5b are particularly attractive targets for antiviral therapy (Leyssen et al., 2000;Finkielsztein et al., 2010). Targeted antivirals are expected to exhibit less risk of toxicity due to the absence of an analogous enzyme system in the host that would be negatively affected by treatment with the antiviral compound.

Nucleoside analogues have been shown to inhibit viral replication of rinderpest virus (Ghosh et al., 1996), FMDV (Sierra et al., 2000;Pariente et al., 2005;Goris et al., 2007) and reduce the cytopathogenicity of peste des petits ruminants virus (El-Sabbagh et al., 2007) *in vitro*. The *in vitro* antiviral properties of several nucleosidic compounds against BVDV have been assessed using the virus as a model for HCV infection in humans (Finkielsztein et al., 2010). Recognized mechanisms of action of various nucleosidic compounds effective against BVDV include inhibition of the inosine-monophosphate dehydrogenase enzyme system (Yanagida et al., 2004), antagonism of the RdRP (Manfredini et al., 2004;Angusti et al., 2008) and the ability to act as highly efficient chain terminators (Hollecker et al., 2004). However, with the primary objective of most studies focused on HCV infection, reports of *in vivo* studies are lacking from the

scientific literature. For these compounds to find useful employ in the arsenal of the bovine clinician, further studies to demonstrate efficacy and safety are needed.

Ribavirin, a nucleoside analog discovered in 1972, is currently used extensively to treat various RNA viral infections in humans (Crotty et al., 2002). In 1989, ribavirin was shown to inhibit the proliferation of BRSV *in vitro* (Bartzatt and Anderson 1989). Ribavirin alone (de la Torre et al., 1987;Airaksinen et al., 2003) or in combination with other mutagenic agents (Pariente et al., 2005) has also been shown to cure cultured cells of a persistent infection with FMDV. Similarly, combination therapy involving ribavirin, iminosugars derivatives and interferon is effective at clearing cell lines infected with BVDV (Durantel et al., 2004;Woodhouse et al., 2008). Similar protocols form the mainstay of HCV treatment in human patients. Demonstrable evidence of clinical activity in veterinary patients is lacking. However, pigs fed a compound related to ribavirin for six days beginning one hour before challenge with FMDV were protected from clinical disease (Sakamoto et al., 2006). The lone report describing ribavirin use in live cattle detailed the intravenous treatment two calves experimentally infected with BRSV (Bartzatt and Anderson 1989). The authors reported no adverse side effects associated with the treatment and a significant decrease in nasal discharge following administration of the compound. Although *in vitro* data is promising, ribavirin is not currently licensed for use in cattle and will not likely be suitable for use by veterinary practitioners without significant outlay of resources for further study.

Polyprotein processing molecules

Proteolytic processing plays an important role in the life cycle of many viruses and thus has become a target for potential antiviral therapeutics. For example, the leader (L) protease of FMDV enables cleavage of a host protein complex involved in the initiation of translation, resulting in shut-off of host protein synthesis (Etchison et al., 1982;Devaney et al., 1988). Consequently, inhibition of L protease by a thiol protease inhibitor results in increased competition between host and FMDV mRNA for access to the transcription machinery of the cell, ultimately resulting in decreased viral protein and RNA synthesis (Kleina and Grubman 1992). A ribozyme that targets rex/tax, a regulatory gene of BLV, results in decreased viral expression in cell culture (Cantor et al., 1993). Similarly, BHV-1 proteases are essential for the production of infectious virions; inhibition of their proteolytic activity by aprotinin results in the production of empty non-infectious viral capsids (Krasota et al., 2002). Both proteases have yet to be tested in a clinical setting. As discussed earlier, the NS3 serine protease is essential for proper processing of the polyprotein transcribed from the BVDV genomic template (Wiskerchen and Collett 1991). A boron-modified peptidyl mimetic of the BVDV NS4A/NS4B cleavage site has been shown to decrease efficiency of the NS3 protease by approximately 75% (Bukhtiyarova et al., 2001). However, attempts to inhibit viral replication in BVDV-infected MDBK cells were unrewarding, even using concentrations of the antiviral compound reaching 90 pM leading the authors to speculate cellular-derived limitations (e.g. permeability) neutralized the antiviral activity of the compound. The specificity of the NS3 protease has made the molecule a continued target for antiviral researchers.

Like the ACMs, the antiviral activity of polyprotein processing molecules has proven effective for treating infected bovine embryos and reagents used in embryo transfer systems. Bovine herpesvirus adheres to bovine embryos with an intact zona pellucida and thus infected embryos serve as a theoretical route of transmission of the virus to susceptible recipients (Givens 2000). Treatment of infected bovine embryos with trypsin, a serine protease, is capable of clearing BHV-1 and bovine herpesvirus-4 (BHV-4) from the zona pellucida and rendering the embryos non-infectious for uterine tubal cells (Stringfellow et al., 1990;Edens et al., 2003). A recombinant trypsin-like protease has also been evaluated as an antiviral treatment effective against BHV-1 infected bovine embryos (Marley et al., 2008a). The product was found to highly effective in treating *in vivo*-derived embryos but was not recommended for the treatment of *in vitro*-derived embryos.

Antisense strategies

Gene silencing is a form of epigenetic regulation of gene expression that occurs at either the transcriptional or post-transcriptional level. The phenomenon of gene silencing was originally discovered by researchers working to introduce transgenes into the genome of plants (Waterhouse et al., 2001a). The most avidly pursued strategy currently involves RNA interference (RNAi) which is the process of gene silencing in plants or animals induced by ribonucleoprotein complexes called small interfering RNA (siRNA) (Pratt and MacRae 2009). These complexes consist of 20-30 nucleotides and can be theoretically programmed to target a specific gene within any known nucleic acid sequence. The siRNA sequences are designed antisense to the targeted gene of interest

and thus can effectively block transcription of the given gene. The highly specific nature of siRNA results in minimal disruption to the host's cellular processes. In addition to altering gene expression, RNAi may activate the immune system of the host organism as a defense mechanism against viruses or other invaders (Waterhouse et al., 2001b).

Consequently, the therapeutic potential of siRNA is being actively studied as it applies to the prevention of viral infection and/or replication. Interestingly, results of one study suggest that siRNA may be more effective against the positive-sense strand of viral RNA (Schubert et al., 2007). Consequently, siRNAs developed for prevention of BVDV replication should be designed antisense to the genomic strand of RNA rather than the replicative intermediate strand. However, as reports of siRNA designed against BVDV are lacking, results must currently be extrapolated from work with other single-stranded RNA viruses. Viral replication of two closely related morbilliviruses, rinderpest and peste-de-petites-ruminants virus, was reduced 80% in one study by siRNA targeting specific viral nucleocapsid genes (Servan de Almeida et al., 2007). Similar results were seen with FMDV in cell culture (Chen et al., 2004). In the same study, mice treated with siRNA were significantly less susceptible to FMDV infection than non-treated controls. However, antisense sequences designed to target a regulatory gene of BLV were found to be ineffective, particularly when compared to the activity of a previously described ribozyme protease (Cantor et al., 1996). The role of antisense strategies in the control and prevention of viral pathogens remains unclear and further studies are needed to determine the feasibility of such strategies in a clinical setting.

Vegetal extracts

The antiviral activity of several natural products has been explored in relation to pathogens of cattle. Some studies would suggest that BVDV is more susceptible than other bovine viruses to some plant extracts (Ruffa et al., 2002;Romero et al., 2006). However, further studies are necessary before their clinical efficacy can be ascertained. Extracts of *Petiveria alliacea* and *Phyllantus amarus* have proven to inhibit BVDV replication and associated CP effects in cultured cells (Ruffa et al., 2002;Bhattacharyya et al., 2003). Theaflavins are polyphenols extracted from black tea and have been shown to possess antiviral activity against several human viruses, including influenza (Nakayama et al., 1993), enterovirus and rotavirus (Mukoyama et al., 1991). They have also been shown to have *in vitro* virucidal activity against bovine rotavirus (BRV) and bovine coronavirus (BCoV) (Clark et al., 1998a). In another study of BRV and BCoV, the adsorbent qualities of clay, clay minerals and charcoal were characterized (Clark et al., 1998b). A high affinity binding was found for both viruses but the resulting adsorbent-bound virus complex appeared to actually enhance viral infectivity in some cell lines. Bovine herpesvirus-4 replication in endothelial cells is inhibited by arsenite (Jiang et al., 2004). However, the clinical relevance of this finding is unclear given that arsenite is a documented carcinogen. Genistein, a natural compound found in soybeans, is a potent tyrosine kinase inhibitor and by this mechanism inhibits BHV-1 replication (Akula et al., 2002). Further studies are indicated to determine if feeding genistein-rich feeds before periods of stress has a protective effect against BHV-1 infection. Vegetal extracts will continue to be studied for their antiviral properties. However, the veterinary use of such compounds must most likely piggyback on research and development performed to

establish products of use in the field of human medicine due to the relatively small return-on-investment that could be expected in the veterinary market.

Miscellaneous agents

New compounds are constantly being synthesized and subsequently evaluated for potential antiviral activity (Baginski et al., 2000). Alternatively, known substances with other described uses are assessed for possible use as antiviral therapeutics. Thiosemicarbazone derivatives have *in vitro* antiviral activity against BVDV (Finkielsztein et al., 2008) and BHV-1 (Munro and Sabina 1970;Field and Reading 1987) that is thought to be mediated through specific ion-binding within the host cell. The structure of the molecule may play a role in the spectrum of activity of various derivatives as the antiviral activity of some appears to be specific to the *Pestiviridae* (Finkielsztein et al., 2008). Benzimidazole derivatives (Tonelli et al., 2010b), an aminoglycoside antibiotic (Birk et al., 2008) and iminosugars (Durantel et al., 2001) have also exhibited antiviral activity against BVDV *in vitro* but clinical studies are absent from the published literature. Iminosugars have been shown to inhibit the secretion and infectivity of newly released viral particles of BVDV by inhibiting an endoplasmic reticulum alpha-glucosidase in a dose-dependent manner (Zitzmann et al., 1999;Branza-Nichita et al., 2001;Durantel et al., 2001). Synergistic effects are seen when iminosugars are combined with IFNs alone (Ouzounov et al., 2002) or IFNs and ribavirin (Woodhouse et al., 2008). However, since many compounds possessing *in vitro* antiviral activity are toxic to live animals or embryos, their safety must be assessed in clinical trials before they can be made available for commercial use (Schwers et al., 1980;Marley et al., 2006).

Summary

In summary, despite the importance of viral pathogens in cattle, no antiviral compounds are licensed for use by the bovine practitioner. Several bovine viral pathogens serve as models for pathogens of importance to the field of human medicine, most notably BVDV serving as the de facto surrogate of HCV for research purposes. Because of this, several compounds have been identified that possess *in vitro* antiviral activity but clinical assessments have yet to be completed due to the relative expense and breakdown of the surrogate models in clinical situations. However, research has identified several compounds with demonstrated usefulness in clinical situations, such as the use of ACMs to clear infected cell lines of BVDV or the use of trypsin to rid infected bovine embryos of bovine herpesviruses. Further research must be completed to allow the employ of compounds identified *in vitro* for clinical applications.

Statement of Research Objectives

In order to better characterize the antiviral effects, mechanism of action and scope of efficacy of 2-(2-benzimidazolyl)-5-[4-(2-imidazolino)phenyl]furan dihydrochloride (DB772), this work focuses on the following objectives:

The first objective was to evaluate the antiviral activity of DB772 when administered to persistently infected calves.

The second objective was to initiate an evaluation of the antiviral activity of DB772 when administered to healthy calves subsequently challenged intranasally with BVDV.

The third objective was to determine the scope of *in vitro* antiviral efficacy of DB772 among various pestiviruses, including BVDV2, border disease virus, HoBi virus and Bungowannah virus.

The fourth objective was to characterize mutations seen in the NS5B gene of BVDV isolates from PI calves treated with DB772 that were subsequently found to be resistant to the compound and to assess the hypothesis that the mutations in the NS5B gene observed after treatment with DB772 would hinder replication efficiency.

Antiviral Treatment of Calves Persistently Infected with

Bovine Viral Diarrhea Virus

Abstract

Animals persistently infected (PI) with bovine viral diarrhea virus (BVDV) are a key source of viral propagation within and among herds. Currently, no specific therapy exists to treat PI animals. The purpose of this research was to initiate evaluation of the pharmacokinetic and safety data of a novel antiviral agent in BVDV-free calves and to assess the antiviral efficacy of the same agent in PI calves. One BVDV-free calf was treated with 2-(2-benzimidazolyl)-5-[4-(2-imidazolino)phenyl]furan dihydrochloride (DB772) once at a dose of 1.6 mg/kg intravenously and one BVDV-free calf was treated three times a day for 6 days at 9.5 mg/kg intravenously. Subsequently, four PI calves were treated intravenously with 12 mg/kg DB772 three times a day for six days and two PI control calves were treated with an equivalent volume of diluent only.

Prior to antiviral treatment, the virus isolated from each calf was susceptible to DB772 *in vitro*. The antiviral treatment effectively inhibited virus for 14 days in one calf and at least three days in three calves. Subsequent virus isolated from the three calves was resistant to DB772 *in vitro*. No adverse effects of DB772 administration were detected. Results demonstrate that DB772 administration is safe and exhibits antiviral

properties in PI calves while facilitating the rapid development of viral resistance to this novel therapeutic agent.

B.W. Newcomer, M.S. Marley, P.K. Galik, P.H. Walz, Y. Zhang, K.P. Riddell, C.C. Dykstra, D.W. Boykin, A. Kumar, C. Cruz-Espindola, D.M. Boothe, M.D. Givens. "Antiviral treatment of calves persistently infected with bovine viral diarrhea virus". *Antiviral Chemistry and Chemotherapy*, 2012; 22(4): 171-179.

Introduction

Bovine viral diarrhea virus (BVDV) is the prototypical virus of the *Pestivirus* genus of the *Flaviviridae* family (Walz et al., 2010). Based on genomic and serotypic differences, BVDV is divided into two genotypes, BVDV 1 and BVDV 2 (Heinz et al., 2000). Clinical disease caused by BVDV infection is a source of significant economic losses in cattle worldwide (Houe 1999). The effects of BVDV infection are manifested in the respiratory, gastrointestinal, reproductive, cardiovascular, lymphatic, immune, integumentary or central nervous system of affected cattle (Baker 1995). Of utmost importance in the propagation of BVDV infection is the persistently infected (PI) animal (Campbell 2004). In utero exposure to noncytopathic BVDV before development of fetal immunocompetence can result in a calf that is PI with the virus (McClurkin et al., 1984). Such cattle consistently shed large quantities of virus throughout their life and serve as a constant reservoir of infection to their herdmates (Brock et al., 1991). Previous studies involving the treatment of PI animals with human or bovine interferon (IFN) were largely

unsuccessful and in some cases associated with significant negative side effects (Peek et al., 2004b;Kohara et al., 2009). As no specific therapies are available to cure PI animals, treatment is currently limited to supportive care or humane euthanasia.

Aromatic cationic compounds possess inhibitory action against several RNA viruses, including human immunodeficiency virus (Kumar et al., 1995), rotavirus (Vonderfecht et al., 1988) and respiratory syncytial virus (Dubovi et al., 1980). Givens and others used a similar novel compound, 2-(2-benzimidazolyl)-5-[4-(2-imidazolino) phenyl]furan dihydrochloride, (DB772; MW=410.28) to inhibit BVDV growth in cell culture while demonstrating the compound's lack of cytotoxicity (Givens et al., 2003a). The 99% endpoint for prevention of viral replication by DB772 was found to be 6 nM (Givens et al., 2004). Further, DB772 has been shown to eliminate BVDV infection in contaminated bovine fetal fibroblast cells with a single passage in culture media supplemented with 4µM of DB772 (Givens et al., 2004). Blastocyst development was not hindered by exposure to a closely related compound and heifers resulting from treated blastocysts displayed normal characteristics during puberty, breeding, gestation and lactation (Givens et al., 2005;Givens et al., 2006). The mechanism of action of similar compounds has shown to be through inhibition of the RNA-dependant RNA-polymerase (Giliberti et al., 2010;Tonelli et al., 2010a).

However, the effects of DB772 in animals are not known. Therefore, the purposes of this study were three-fold; 1) to establish pharmacokinetic data of DB772 after intravenous injection in BVDV-free calves; 2) to assess the safety of DB772 administration in BVDV-free calves; and 3) to evaluate the antiviral activity of DB772 when administered to PI calves.

Materials and Methods

2.1. Pharmacokinetic and safety and toxicity study

2.1.1. Sample collection

A 10-day-old miniature calf (Calf 601) and its dam were isolated within a 9.3 square meter pen inside a humidity- and temperature-controlled room. The calf nursed milk from its dam as desired throughout the study. The 17.7 kg calf was administered 1.6 mg/kg DB772 via intravenous catheter in the left jugular vein at Time 0. Blood samples were collected and analyzed for serum concentration of DB772 at 0, 5, 10, 15, 30 and 45 minutes, and 1, 1.5, 2, 3, 4, 6, 8, 10 and 12 hours and then every four hours through 36 hours post-administration. Samples were submitted for analysis to measure serum DB772 concentration, serum biochemical profile (SBP) and complete blood count (CBC) on Days 0, 3, and 5. Clinical scores for appetite, appearance, dehydration status, and fecal appearance were assigned daily.

A second 10-day old miniature calf (Calf 701) and its dam were isolated within a 9.3 square meter pen inside a humidity- and temperature-controlled room. The calf nursed milk from its dam as desired throughout the study. The calf weighed 20.5 kg and was treated intravenously with 9.5 mg/kg of DB772 every eight hours for six consecutive days with the first administration of DB772 defined as Time 0. Serum samples were collected immediately before and one hour after each administration of DB772 for analysis of serum concentration of DB772. Serum was separated from clotted

blood and frozen at -80°C until analysis. Additional samples collected 3, 5, 9, 13, 17, 25, 37 and 63 h after the final administration of DB772 were also analyzed for drug concentration. Clinical scores for appetite, appearance, dehydration status, and fecal appearance were assigned daily. Clinical scores were assigned on a scale from 0.0 (normal) to 1.0.

2.1.2. Sample analysis

Serum samples were thawed at room temperature and vortexed to homogeneity. Samples were prepared for analysis by the combination of 200 µl serum with 500 µl methanol, vigorous vortexing for 15 seconds, and centrifugation at 1900 g for 30 min at room temperature; 30 µl of the supernatant were injected through the high performance liquid chromatography (HPLC) system (Waters Corporation, Milford, MA, USA).

DB772 was analyzed by HPLC based on a combination of previously described methods (Morton et al., 1986;Lansiaux et al., 2002;Zhou et al., 2004). The mobile phase consisted of solvent A (15 mM ammonium formate [Sigma Aldrich, St. Louis, MO, USA], 35 mM formic acid (v/v) [Sigma Aldrich], in distilled water) and solvent B (15 mM ammonium formate, 35 mM formic acid (v/v) in 80:20 acetonitrile [VWR, West Chester, PA, USA] distilled water solution [v/v]). The solvents were mixed using an initial gradient of 14% B that progressed to 34 % over 5 min, followed by a second gradient to 95 % over 0.5 min. After 10 min at this point, a return gradient to 14 % occurred over 5 min before recycling. The flow rate of the system was 0.8 mL/min. DB772 separation was achieved at room temperature using a Zorbax Bonus-RP, 5 µm, 2.1 x 150 mm chromatographic column (Agilent Technologies, Santa Clara, CA, USA),

protected by a Zorbax Bonus RP, 5 µm, 2.1 x 12.2 mm precolumn (Agilent Technologies, Santa Clara, CA, USA). DB772 was detected using a fluorescence detector (Waters Corporation, Milford, MA, USA), with excitation occurring at 366 nm and emission at 500 nm.

Unknown concentrations in animal samples were quantitated by comparing the fluorescent signal to the signal generated from standards prepared by the addition of known amounts of DB772 to plasma and serum. The calibration curve contained known concentrations ranging from 25 to 1000 ng/ml. The linear correlation coefficient of the calibration curve was 0.9995 and the lower limit of detection was 10 ng/ml. The lower and upper limits of quantitation were 25 ng/ml and 1000 ng/ml, respectively. The coefficient of variation for controls was 22.87%, 8.82%, 2.41% and 0.77% for 25 ng/ml, 50 ng/ml, 500 ng/ml, and 1000 ng/ml, respectively.

Samples were submitted to the clinical pathology service at Auburn University Teaching Hospital on Days 0, 3, 5, 7 and 11 for SBP and CBC analysis. Parameters assessed in the SBP included total protein, albumin, globulins, albumin/globulin ratio, sorbitol dehydrogenase (SDH), aspartate aminotransferase, gamma-glutamyltransferase (GGT), total bilirubin, creatine kinase, urea nitrogen, creatinine, calcium, phosphorus, magnesium, glucose, bicarbonate, sodium, potassium, chloride, anion gap, osmolality and iron.

2.1.3. Pharmacokinetic Analysis

Serum DB772 concentration versus time curves were subjected to non-compartment analysis using a commercial computer software program (WinNonlin v.

5.1, Pharsight Crp., Mountain View, CA). Terminal elimination half-life (t½) was calculated and a minimum of three data points were used to calculate k_{el}. Area under the curve and its first moment (AUMC) were calculated to infinity ($AUC_{0-\infty}$) using the log-linear trapezoidal rule. From these values, other kinetic parameters were calculated: clearance (Cl) = dose/AUC, volume of distribution (Vd_{ss}) = dose x $AUMC/AUC^2$, and mean residence time (MRT) = AUMC/AUC. The maximal serum concentration (C_{max}) was also recorded. Concentration at Time 0 (for IV) ($C_{0,}$) was estimated by back extrapolation of the first two time points via log-linear regression to Time 0. For multiple dosing, weekly peak (time 1) and trough (time 2) concentrations were also used to determine elimination half-life based on the relationship of $t_{1/2} = 0.693/k_{el}$ and kel = $\ln(C_1/C_2)/t_2\text{-}t_1$), where C = concentration at time (t).

2.2. In vivo antiviral evaluation of DB772 in PI calves

2.2.1. Animals

Six crossbred beef calves (A to F) born between September and October 2008 at the Upper Coastal Plain Agricultural Research Center were separated from their dams and moved to the research facility at 2 to 5 d of age. All six calves were confirmed to be PI animals as reported earlier (Rodning et al., 2010). Calves B and D were bulls, and the remaining four calves were heifers. Calves A, C and D were PI with BVDV 1 while calves B, E and F were PI with BVDV 2. On Day 0, the calves were 15 to 17d of age and weighed 25.9 to 33.3 kg.

2.2.2. Housing

The calves were housed in individual 1.9 square meter pens arranged two per humidity- and temperature-controlled room. The calves were fed two quarts of a commercial milk replacer twice daily and had free choice access to water throughout the study. Clinical scores for appetite, appearance, dehydration status, and fecal appearance were recorded daily. Clinical scores were assigned on a scale from 0.0 (normal) to 1.0.

2.2.3. Treatment

The compound used in this study was synthesized in the laboratory of David W. Boykin (Georgia State University, Atlanta, GA, USA). A 25 mg/ml solution of DB772 was made by dissolving the compound in polyethylene glycol 200 (PEG 200) (Mallinckrodt Baker, Phillipsburg, NJ, USA). After a 10 d adjustment period to the isolation facilities, jugular catheters were placed bilaterally in all six calves. Four treated calves (C, D, E and F) were administered DB772 intravenously three times a day via the right jugular catheter for six days at a dose (12 mg/kg) calculated to achieve a 4 μM concentration (1.6 μg/mL) in serum. Two control calves (A and B) were intravenously administered an equivalent volume of only diluent three times a day for six days. The day treatment was first initiated was defined as Day 0. Serum samples were collected once daily from each calf immediately prior and one hour following DB772 administration and analyzed for DB772 concentration as described earlier.

2.2.4. Sample collection

Whole blood and serum samples were collected on Day 0, 1, 3, 5, 7, 14, 21, 28 and 35 via the left jugular catheter or by jugular venipuncture if no catheter was patent. Nasal swab samples were collected at the same time points. Serum was removed from clotted blood after centrifugation and processed immediately or refrigerated until processing. Whole blood samples were processed as described earlier (Walz et al., 2008) to obtain the buffy coat with the exception that samples were resuspended in 1 mL of minimum essential medium (MEM). Whole blood, serum, and nasal swabs were refrigerated for <72 h before sample analysis. Whole blood and serum samples from each calf were submitted weekly to the clinical pathology service at Auburn University Teaching Hospital for CBCs and SBP analysis.

2.2.5. Virus detection and quantification

Serum samples from Day 0, 1, 3, 5, 7, 14, 21, 28 and 35 were analyzed for concentration of BVDV by quantitative polymerase chain reaction (qPCR) as described previously (Marley et al., 2008b) with the exception that only BVDV primers were used. Virus isolation was performed on buffy coat and nasal swab samples as described previously (Walz et al., 2008) with the exception that 1 mL aliquots of buffy coat sample were used and the procedure was performed in six-well (9.6 cm^2) plates. Virus titration of nasal swab samples was performed. Multiple, serial ten-fold dilutions of 10 μL sample diluted in 90 μL MEM were performed in triplicate and the statistical method of Reed and Muench (Reed L.J. and Muench H 1938) was used to quantify the concentration of virus.

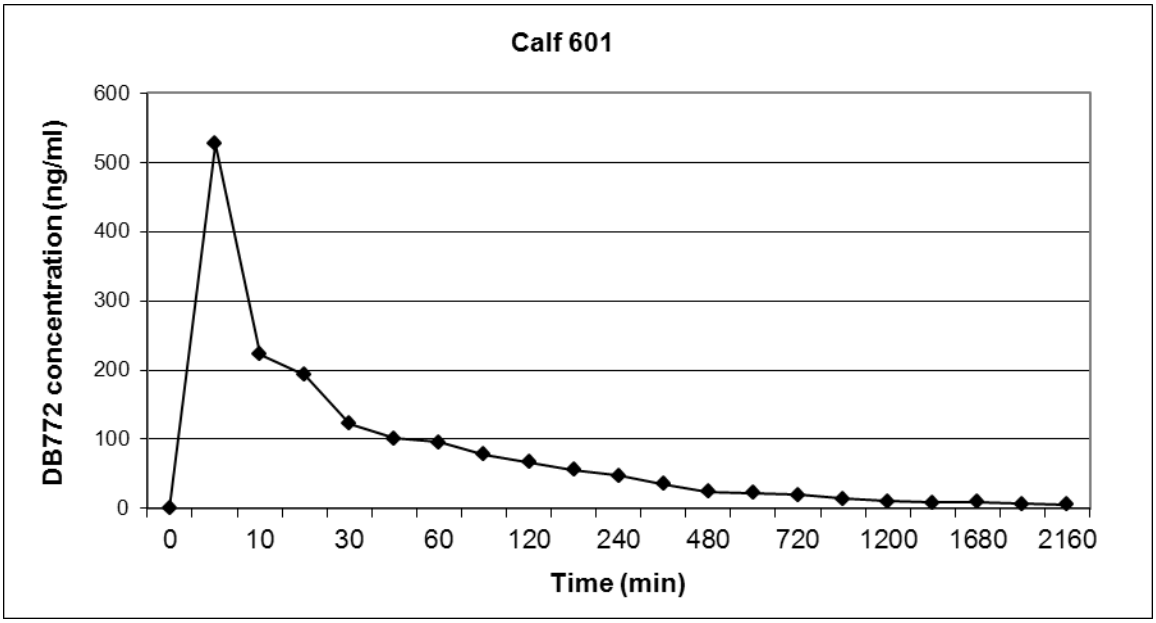
2.2.6. In vitro viral susceptibility to DB772

Cell lysate from buffy coat samples that had been passaged as described in 2.2.5 were stored at -80 °C. Virus from cell lysate samples obtained on Day 0 and on the first day after treatment initiation when virus was isolated from buffy coat samples in sufficient concentrations were tested for susceptibility to DB772. Virus was isolated in sufficient quantity to perform this *in vitro* testing from buffy coat samples of Calves C, D, E and F on Day 14, 5, 7 and 3, respectively. Virus from each cell lysate sample was incubated in the presence and absence of 4 μM of DB772 for 4 d in a 24-well (2 cm^2) plate. Plates were then frozen and thawed and virus titration was performed as described in 2.2.5. Presence of BVDV was confirmed by an immunoperoxidase monolayer assay (Givens et al., 2003b) and quantified by the method of Reed and Muench (Reed L.J. and Muench H 1938).

Results

3.1. Pharmacokinetic data

The C_{max} achieved in serum after a single dose of DB772 (1.6 mg/kg) was 527 ng/mL (1.28μM) obtained 5 min after intravenous administration (Figure 3). The serum half-life of DB772 was determined to be 13 h and the clearance rate was calculated to be 16.7 mL/min/kg. The last time point at which DB772 could be detected in serum was 36



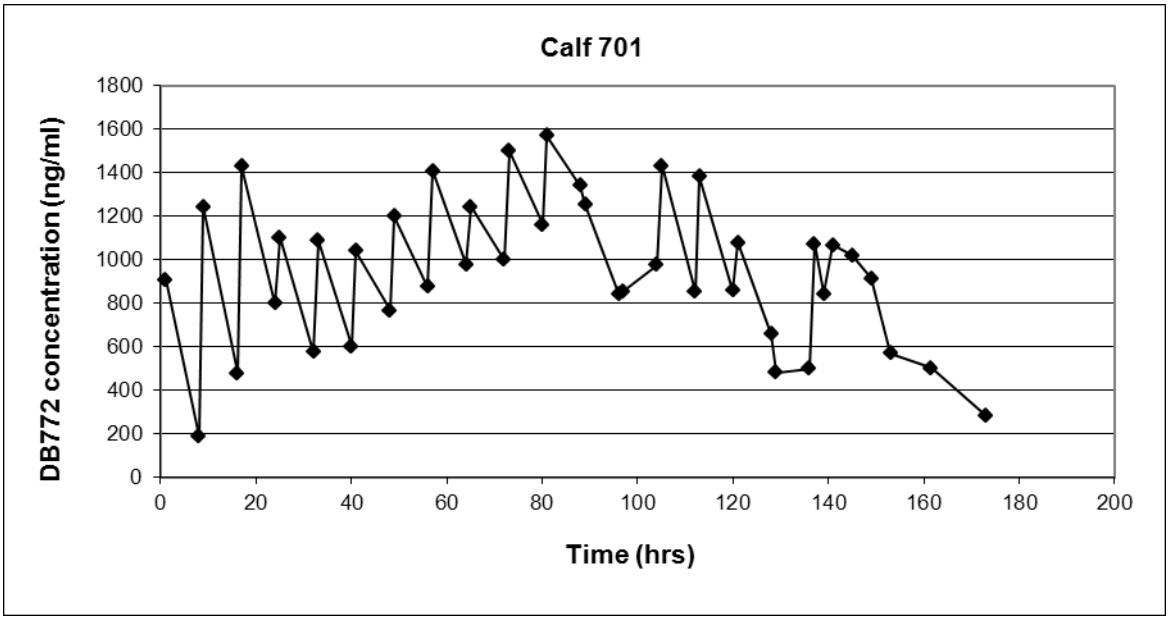


Figure 3. Serum DB772 concentrations after intravenous administration after a single dose of 1.6 mg/kg DB772 (Calf 601) or after treatment with 9.5 mg/kg of DB772 every eight hours for six consecutive days (Calf 701).

hours following administration at a concentration of 5.44 ng/ml. The MRT of DB772 was observed to be 14 h and the Vd_{ss} was calculated to be 24,022 mL/kg.

Intravenous administration of DB772 at a dose of 9.5 mg/kg every eight hours resulted in serum concentrations >575 ng/mL (1.4 μM) after 16 h (Figure 3). This concentration was maintained throughout the remainder of the administration period. The first time point at which the measured serum concentration of DB772 fell below the *in vitro* therapeutic goal was at 129 h, or 9 h after the final administered dose. The C_{max} of 1073 ng/mL (2.6 μM) was obtained 137 h after the first administration of DB772. The serum half-life of DB772 was determined to be 16.2 h and the clearance rate was calculated to be 28.6 mL/min/kg. The Vd_{ss} of DB772 in the calf treated every 8 h for six days was similar to the Vd_{ss} in the calf treated once at 26,613 mL/kg. Meanwhile, the MRT nearly doubled to 26.6 h.

3.2 Safety and toxicity data

Clinical evaluations of the initial miniature calf treated with DB772 indicated no sign of toxicity from treatment. A mild lymphopenia (1,892 cells/μL; reference range 2,500 to7,500 cell/μL) was detected before treatment and attributed to a stress leukogram. On Day 3, the lymphocyte count had increased to 2,263 cells/μL, before decreasing to 1,833 cells/μL on Day 5. Similarly, GGT was elevated on Days 1, 3 and 5 (142, 116, and 85 U/L, respectively; reference range 3.7 to 31 U/L). No changes in clinical scores were seen at any point in the study period.

Similarly, no clinical signs of toxicity or adverse health effects due to DB772 administration were seen when 9.5 mg/kg of DB772 was administered intravenously to a

single calf every eight hours for six consecutive days. A decrease in lymphocytes occurred on Days 7 and 11 post-administration, decreasing from 4,649 cells/μL on Day 0 to 3,165 and 1,833 cells/μL, respectively. The lymphopenia did not result in clinical evidence of infection. No other significant changes in the hematologic or biochemical profiles of the calf were detected during or following DB772 administration.

3.3 In vivo antiviral evaluation of DB772 in PI calves

3.3.1. Physical and hematologic findings

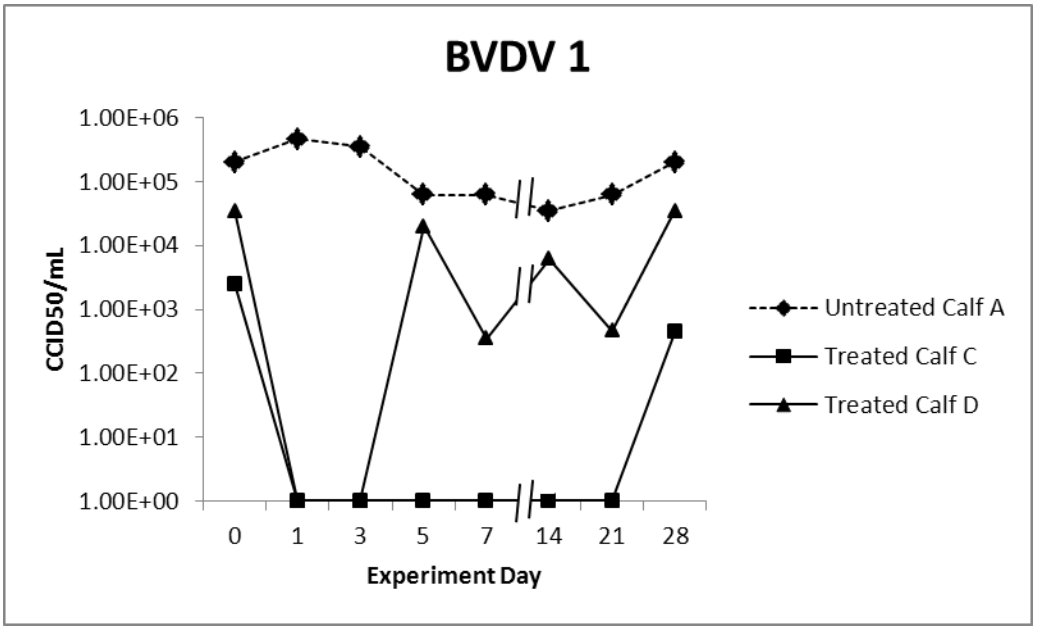
Serum concentrations of DB772 exceeded 4 μM 24 hours following the initial DB772 administration in three of four treated calves (A, B, C) and in all four treated calves by 48 hours following initial treatment. Daily clinical scores did not differ among treatment groups or change during the course of the study. There were no consistent, consequential changes in the CBCs of either treatment group. Two of the treated calves had lymphocytes of 1,500 cells/μL or less on Day 0. However, as with the other calves in this study, lymphocyte counts did not fluctuate during the study period. One of the control calves (A) had a consistently high fibrinogen level (800 to 1300 mg/dL) throughout the study (reference range 200 to 730mg/dL) but no other indicators of inflammation were seen. The concentration of GGT in all six calves (range 20 to 185 U/L) was elevated above the reference range (18.3 U/L) at all time points during the study with the exception of one control calf who had values of 16 U/L and 17 U/L on Days 14 and 21, respectively, and one treated calf who had a value 17 U/L on Day 28. Likewise, SDH was elevated in all 6 calves on Days 28 and 35. Values on Day 28 ranged

from 16.9 to 101.4 U/L (reference range 2.6 to16 U/L). Values on Day 35 increased to 30.9 to 259 U/L.

3.3.2. Virus detection and quantification

Virus was isolated from the buffy coat samples of all six calves on Day -7 and 0. On Day 1, the control calves remained positive on virus isolation while no virus was isolated from the buffy coat samples of any of the four treated calves. On Day 3, the treated calves PI with type 1 BVDV (C and D) remained negative while both treated calves PI with type 2 (E and F) were positive on virus isolation from buffy coat samples and remained so throughout the remainder of the study. One calf PI with type 1 BVDV (C) remained negative on virus isolation from buffy coat samples until Day 14 while virus was first isolated from the other calf PI with type 1 BVDV (D) on Day 5.

On Day 0 the treated calves PI with type 1 BVDV (C and D) were positive for virus isolation in nasal swab samples with a mean of 1.9×10^4 cell culture infectious doses ($CCID_{50}$)/mL (Figure 4). Samples from both calves were negative on Day 1 and 3. Calf C remained negative until Day 21 and calf D remained negative until Day 5. Viral concentration in nasal swab samples of calf C and D returned to pre-treatment concentrations on Day 35 and 28, respectively. On Day 0 the treated calves PI with type 2 BVDV (E and F) were positive for virus isolation in nasal swab samples with a mean of 1.2×10^5 $CCID_{50}$/mL (Figure 4). The amount of virus in the nasal swab samples decreased three log scores to a mean of 4.9×10^2 $CCID_{50}$/mL on Day 5. Viral concentration in nasal swab samples of calf E and F returned to pre-treatment concentrations on Day 14 and 7, respectively. In contrast, the control calf PI with type 1



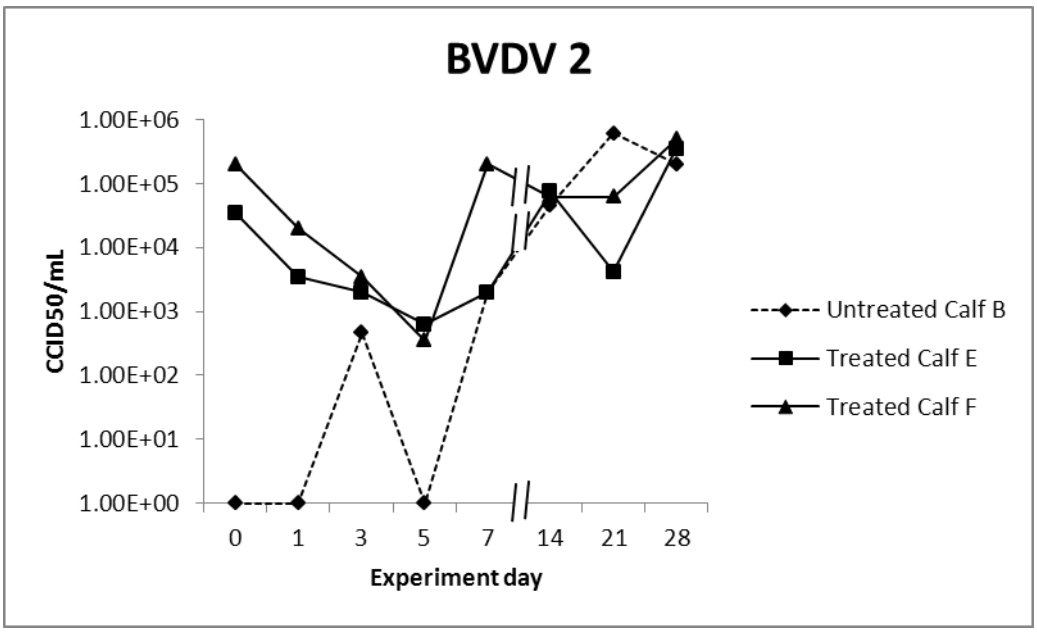


Figure 4. Number of cell culture infectious $doses_{50}$/mL (CCID50) of nasal swab media in calves persistently infected with BVDV 1 or BVDV 2 by experiment day.

BVDV (A) had 2 x 10^5 $CCID_{50}$/mL of virus in nasal swab sample on Day 0 and remained at this concentration throughout the study. On Day 0 and 1 the control calf PI

with type 2 BVDV (B) was only positive for virus in nasal swab samples following passage. However, 4.7 x 10^2 $CCID_{50}$/mL of virus in nasal swab sample was detected on Day 3 and increased to 6.2 x 10^5 $CCID_{50}$/mL on Day 21.

On Day 0, the treated calves PI with type 1 BVDV (C and D) had an average of 65 viral copies of RNA/μL of serum (Figure 5). The amount of viral RNA decreased to a mean of 1.4 viral copies of RNA/μL of serum on Day 14 before subsequently increasing to an average of 740 viral copies of RNA/μL of serum on Day 28. Likewise, the treated calves PI with type 2 BVDV (E and F) decreased from a Day 0 mean of 46 viral copies of RNA/μL of serum to a mean of 8 viral copies of RNA/μL of serum on Day 14 (Figure 5). A subsequent increase to an average of 396 viral copies of RNA/μL of serum occurred on Day 28. In contrast, the number of viral copies of RNA/μL of serum increased steadily in the control calf PI with type 1 BVDV (A) from 356 on Day 0 to 1,360 on Day 14 and 3,100 on Day 28. A similar increase was seen in the control calf PI with type 2 BVDV (B), from 0.6 viral copies of RNA/μL of serum on Day 0, 10 on Day 14 and 2,100 on Day 28.

3.3.3. In vitro viral susceptibility to DB772

Virus was detected from all Day 0 samples at a concentration of 6.2 x 10^4 to 6.2 x 10^6 $CCID_{50}$/mL when cultured in the absence of DB772. Virus was completely inhibited in all Day 0 samples when cultured in the presence of 4 μM concentration of DB772. Likewise, cell lysate samples from the day virus was first detected in buffy coat samples

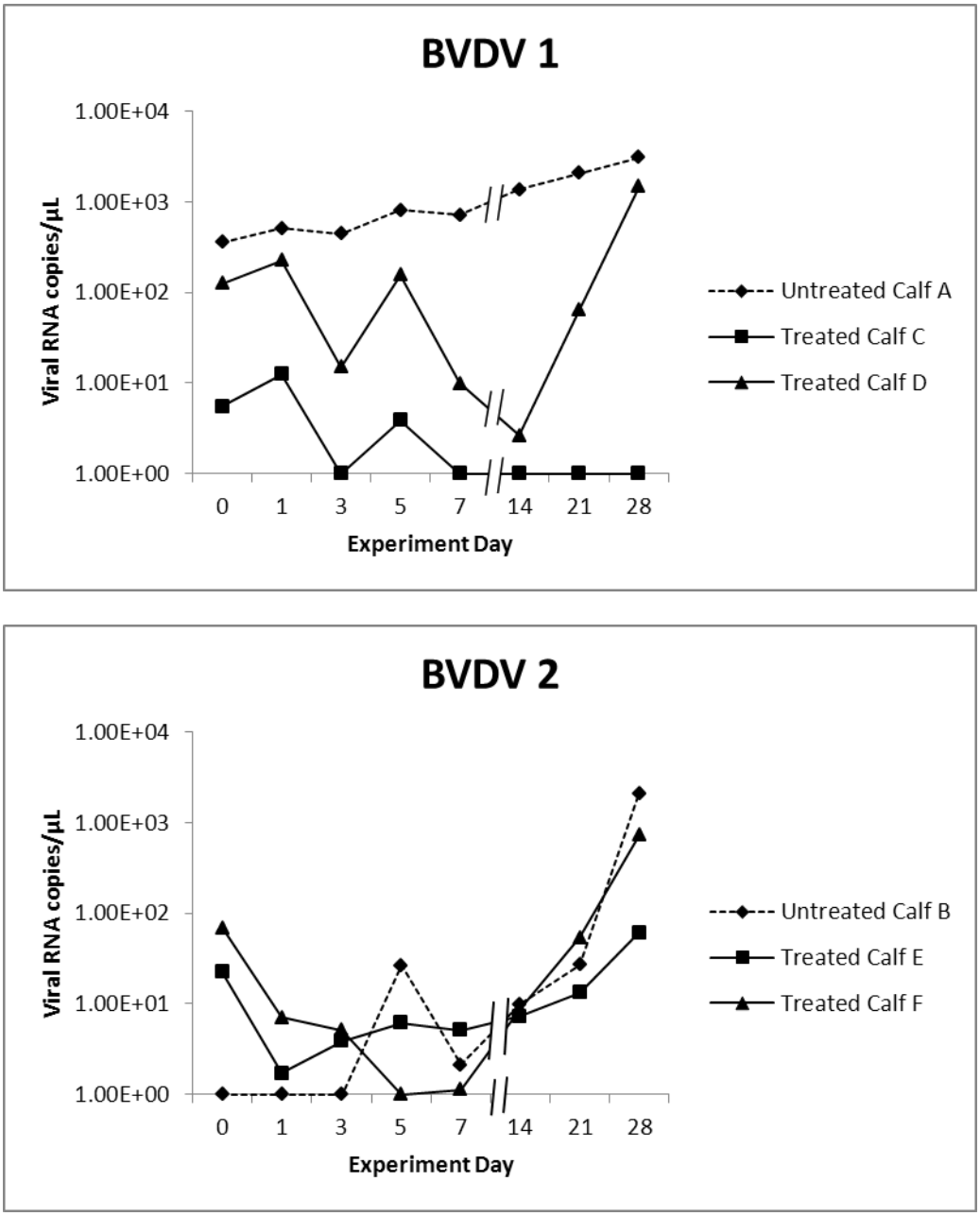


Figure 5. Number of viral RNA copies/µL of serum in calves persistently infected with BVDV 1 or BVDV 2 by experiment day.

yielded a concentration of 3.5 x 10^5 to 2 x 10^7 $CCID_{50}$/mL when cultured in the absence of DB772. However, when cultured in the presence of DB772, growth was inhibited one

log score or less in calves D, E and F. In contrast, virus was completely inhibited in the sample from calf C when cultured in the presence of 4 μM concentration of DB772.

Discussion

Treatment of PI calves with 12 mg/kg of DB772 every 8 h for 6 d resulted in temporary reduction of viral load in all treated calves below the detection threshold of virus isolation after passage of buffy coat samples. In a similar experiment, six PI animals were treated with bovine IFN for two rounds of five daily treatments in a two week period (Kohara et al., 2009). A transient decrease was seen in mean serum BVDV titers during the treatment period. However, in a follow-up study three months later, no antiviral activity of human INF was seen when given either orally or by intramuscular injection. In our study, all four calves treated with DB772 were negative by virus isolation 24 h after the initial treatment while the two control calves remained viremic. However, the two treated calves PI with BVDV type 2 (E and F) were again positive on virus isolation on Day 3 while virus was not isolated from Calves C and D (PI type 1 calves) until Day 14 and 5, respectively. Due to the small subject numbers in this pilot study, it is unclear if this represents a true difference of efficacy of DB772 against the different genotypes.

While the antiviral treatment decreased the viral concentration to below the detection threshold in WBC, we theorize that the virus was able to replicate despite continued treatment with DB772. The exception is Calf C from whom virus was not isolated until after the treatment period was over. This indicates that a longer duration of

treatment most likely would not have changed the outcome for calves D, E and F. It is possible that treatment with higher dosages would reduce the viral load even further than seen with the current dose which may allow additional treatments to be successful.

Results indicate that the treatment of PI calves with DB772 drastically reduced the viral concentration in the calves at initiation of treatment and then subsequently selected for resistant mutants of the virus which gained a competitive edge and replicated in the face of treatment with DB772. This conclusion is supported by isolation of virus from three of the four treated calves (D, E and F) in the face of DB772 administration. When viral isolates obtained during the treatment period from these three calves were tested for DB772 susceptibility *in vitro,* viral replication was similar to replication in media lacking DB772. This demonstrates that strains resistant to the antiviral effects of DB772 were selected for and became the strain persisting within the animal. This research also describes the first documentation of strains of BVDV that are resistant to DB772.

To our knowledge, this is the first *in vivo* study involving an aromatic cationic molecule as an antiviral agent. No evidence of toxicity was seen in any of the calves at any point during the study. Data obtained from subjective clinical scoring as well as objective data from hematologic and biochemical profiles revealed no significant differences among treated animals and their untreated cohorts. No significant changes in hematologic or biochemical profiles of treated animals were detected from pre-treatment time points through the treatment period or up to four weeks post-treatment. All calves in this study were unweaned and consuming a diet consisting of either milk or milk replacer. The effects of DB772 have not yet been studied in weaned or ruminating

animals. Neither a meat nor a milk withdrawal time has been established for DB772 but the pharmacokinetic data from this study indicate that the drug has a relatively short half-life in serum of 13 to 16.2 h when administered intravenously.

Due to the rapid selection for or development of virus resistant to the antiviral effects of DB772, therapeutic potential of the compound for PI animals is likely to require combination therapy. To date, reports evaluating the efficacy of combination therapy for persistent BVDV infections are lacking from the scientific literature. In hepatitis C, a closely related flavivirus that can also cause persistent infection, combination therapy with interferons and ribavirin has been shown to be more effective than monotherapy (Seeff and Hoofnagle 2002). Treatment of PI animals with bovine interferon resulted in a transient decrease in viral titers (Kohara et al., 2009) but its efficacy as a component of combination therapy has yet to be studied.

This is the first reported study of the use of antivirals in calves PI with BVDV type 2. Previous studies involved only calves PI with BVDV type 1. In hepatitis C infections, certain genotypes have proven more amenable to treatment than others (Seeff and Hoofnagle 2002). Interestingly, treatment with DB772 appeared to be more successful in calves PI with BVDV 1 in this pilot study. The calves PI with BVDV 1 remained negative on buffy coat virus isolation longer than their type 2 counterparts, including one calf (C) who remained negative until Day 14, 9 d after treatment had ceased. Additionally, a larger decrease was seen in the number of viral RNA copies/μL of serum in calves PI with type 1 than PI type 2 calves. The decrease in $CCID_{50}$/mL found in nasal swab samples was also greater in PI BVDV 1 calves. The reason for this is currently unknown as the mechanism of DB772 has yet to be elucidated. Given the small

number of animals in this study, it remains to be seen if this response difference by genotype is repeatable on a larger scale.

A single intravenous dose of DB772 at 1.6 mg/kg in this research achieved a maximum serum concentration (1.28 µM) that was 213 times the 99% endpoint previously determined to prevent infection of primary fetal fibroblast cells *in vitro* (0.006 µM); however, the serum concentration that was achieved with this single dose may not have been sufficient to consistently clear BVDV from infected cells *in vitro* as has been demonstrated with 4 µM concentrations of DB772 (Givens et al., 2004). Yet, when administering DB772 at 9.5 mg/kg intravenously every 8 h for 6 d, the serum concentration of DB772 consistently exceeded 1.4 µM after 16 h which corresponded to the second dose. When administered at 12 mg/kg intravenously every 8 h for 6 d, the serum concentration of DB772 in nursing calves consistently exceeded 1.9 µg/mL (3µM) after 24 h and 1.4 µg/mL (3.6µM) after 48 h.

In summary, the dosing regimen used in this study resulted in serum concentrations of DB772 sufficient to inhibit viral replication in calves PI with BVDV types 1 and 2. Furthermore, no adverse effects of DB772 were detected in any treated calf. The rapid development of viral resistance to the compound is a significant concern but does not preclude its use in combination therapy. Thus, DB772 or related compounds continue to represent a potential therapeutic agent for BVDV infections.

Effect of Treatment with a Cationic Antiviral Compound on Acute Infection with Bovine Viral Diarrhea Virus

Abstract

Bovine viral diarrhea virus (BVDV) is a widespread bovine pathogen capable of causing disease affecting multiple body systems. Previous studies have shown 2-(2-benzimidazolyl)-5-[4-(2-imidazolino)phenyl]furan dihydrochloride (DB772) effectively prevents BVDV infection in cell culture. The aim of this project was to assess the efficacy of DB772 for the prevention of acute BVDV infection. Four calves seronegative to BVDV were treated with DB772 and four were treated with diluent only on the same dosing schedule. Each calf was subsequently challenged intranasally with BVDV. Virus was isolated consistently from untreated calves on Days 4 to 8 while treated calves remained negative by virus isolation during this period. Azotemia was exhibited by all treated calves on Day 4 resulting in the euthanasia of one calf on Day 10 and the death of another on Day 13. Virus was isolated from the two remaining treated calves on Day 14 or 21. On Day 21, both remaining treated calves and all four untreated calves had anti-BVDV antibody titers >1:2,048. This pilot study indicates that DB772 temporarily prevented acute disease due to BVDV but carries a significant concern of renal toxicity.

B.W. Newcomer, M.S. Marley, P.K. Galik, Y. Zhang, K.P. Riddell, D.W. Boykin, A. Kumar, L.A. Kuhnt, J.A. Gard, M.D. Givens. “Effect of treatment with a cationic antiviral compound on acute infection with bovine viral diarrhea virus”. *Canadian Journal of Veterinary Research.* (Manuscript in press).

1. Introduction

Bovine viral diarrhea virus (BVDV) is the prototypical virus of the *Pestivirus* genus of the *Flaviviridae* family (Walz et al., 2010). Clinical disease caused by BVDV infection is a source of significant economic losses in cattle worldwide (Houe 1995). Acute disease caused by BVDV can cause several different clinical syndromes which are influenced by viral strain and several host factors (e.g. age, immune status, stage of gestation) (Scruggs et al., 1995). The clinical effects of BVDV are manifested in the respiratory, gastrointestinal, reproductive, cardiovascular, lymphatic, immune, integumentary or central nervous systems of affected cattle (Baker 1995). Animals are usually infected through the respiratory or gastrointestinal mucosa but virus can also be transmitted transplacentally or via artificial insemination with contaminated semen (Walz et al., 2010). Subsequent primary viremia results in widespread dissemination of the virus. Acute BVDV infection marked by severe thrombocytopenia and hemorrhage has also been described (Rebhun et al., 1989). Other than maintaining a strong biosecurity program, including identification and culling of persistently infected animals, current preventatives for BVDV infection are limited to killed or modified live vaccines.

Aromatic cationic compounds possess inhibitory action against several RNA viruses, including human immunodeficiency virus (Kumar et al., 1995), rotavirus (Vonderfecht et al., 1988) and respiratory syncytial virus (Dubovi et al., 1980). Givens and others (Givens et al., 2003a) used a similar novel compound, 2-(2-benzimidazolyl)-5-[4-(2-imidazolino) phenyl]furan dihydrochloride (DB772; MW=410.28), to inhibit BVDV growth in cell culture while demonstrating the compound's lack of cytotoxicity. The 99% endpoint for prevention of BVDV replication by DB772 was found to be 6 nM. Furthermore, DB772 has been shown to eliminate BVDV infection in contaminated bovine fetal fibroblast cells with a single passage in culture media supplemented with 4μM of DB772 (Givens et al., 2004). Blastocyst development was not hindered by exposure to a closely related compound and heifers resulting from treated blastocysts displayed normal characteristics during puberty, breeding, gestation and lactation (Givens et al., 2004;Givens et al., 2005). Administration of the compound to calves persistently infected with BVDV resulted in a decrease in viral concentration with no detectable negative side effects (Newcomer et al., 2012a). However, administration of DB772 to calves as a means to prevent BVDV infection has not been studied. Therefore, the purpose of this pilot study was to initiate an evaluation of the antiviral activity of DB772 when administered to healthy calves subsequently challenged intranasally with BVDV.

Materials and Methods

2.1. Animals and housing

Eight Miniature Brahma-cross calves (four heifers and four bulls) that were between five and six months of age were used in this study. Five days prior to study initiation, the calves were weaned, weighed and allowed to acclimate to the project housing until the study onset. Calves were allowed free choice access to a commercially available calf starter, grass hay and clean water when introduced to the study housing and throughout the study period. Reconstituted oral electrolyte powder was offered daily to calves from Day 9 to Day 18. All calves were determined to be seronegative to BVDV-2 ($\leq$1:4) by virus neutralization prior to study initiation. Likewise, BVDV was not isolated in serum, buffy coat, or nasal swab samples from any calf using the immunoperoxidase monolayer assay before commencement of treatment.

The calves weighed between 46.7 and 95.2 kg (mean: 72.4, standard deviation: 18.2) at the study onset. The calves were stratified by weight into four strata (two calves per strata) and assigned random numbers using Microsoft Excel. In each strata, the calf with the higher random number was assigned to Group A, the calf with the lower number to Group B. Based on a coin toss, Group A (three males, one female) was designated the treatment group and Group B (one male, three females) was designated the control group.

The calves were housed in individual 1.9 square meter crates arranged two per room within a 9.3 square meter pen inside a humidity- and temperature-controlled room. Each room housed one calf from the treatment group and one calf from the untreated group. This study design was intended to minimize potential environmental bias between the treatment and control groups that may have been introduced by housing them in separate rooms. Rooms were cleaned and calves observed at least twice daily. During the

daily cleaning period, calves were released into a communal area within each room to allow for cleaning of the individual crates.

2.2. Clinical monitoring

Physical exams including rectal temperatures were performed on all calves daily throughout the study period and a second daily temperature reading was obtained beginning on Day 6 until Day 12. Calves were considered febrile when rectal temperatures exceeded 102.8°F. Clinical scores for appearance, dehydration status, appetite, and fecal appearance were recorded daily. Appearance scores were based on attitude and behavior with a score of 0 being normal to 1.0 being defined as severe depression, recumbency and refusal to rise or move even with encouragement. Dehydration scores were based on capillary refill time, skin tent and sunken eyes with a score of 0 being normal to 1.0 being defined as a capillary refill time exceeding three seconds, skin tent exceeding 10 seconds accompanied by sunken eyes and cold extremities. Appetite scores were based on severity and duration of anorexia with a score of 0 being normal, a score of 0.2 defined as a decreased appetite with limited interest in eating and a score of 0.4 defined as complete anorexia. Fecal scores were based on the severity of diarrhea and the presence or absence of fecal blood with a score of 0 being normal, a score of 0.2 indicating watery diarrhea and a score of 0.4 denoting blood in the feces. Humane endpoints for euthanasia based on clinical scores or physical exam findings were established prior to study onset and defined as a dehydration score of 1 (sunken eyes, severe loss of skin elasticity, cold extremities) at any time point or when

appearance, appetite, dehydration and fecal clinical scores summed to a total of 1.0 or greater for two consecutive days.

2.3. Treatment

The compound DB772 was synthesized in the laboratory of one of the authors (D.W. Boykin, Georgia State University, Atlanta, GA, USA). A 25 mg/mL solution of DB772 was made by dissolving the compound in polyethylene glycol 200 (PEG 200) (Mallinckrodt Baker, Phillipsburg, NJ, USA). After a 5 d adjustment period to the isolation facilities, jugular catheters were placed bilaterally in all eight calves. The following day treatment with DB772 was initiated in the treatment group calves (A, B, C, D) and administration of diluent in the untreated group calves (E, F, G, H). Treatments in each group were administered intravenously via the right jugular catheter every eight hours for a total of 11 treatments over four days. Day 0 was defined as the day treatment was first initiated with the final treatment administered on Day 3. The antiviral dose (12 mg/kg) was calculated to achieve a 4 μM concentration (1.6 μg/mL) in serum.

2.4. Virus

The virus used in these studies (courtesy of J. Ridpath, USDA National Animal Disease Center, Ames, Iowa, USA) was a noncytopathic BVDV-2 (strain 1373) originally isolated from a 1993 outbreak of clinically severe acute BVDV infection in Ontario, Canada (Carman et al., 1998). After administration of the second treatment on Day 0, each calf was inoculated with 40,000 cell culture infectious doses ($CCID_{50}$) of BVDV-2 (1373) by aerosol inoculation using a DeVilbiss aerosolizer. This selected dose

of virus was one to two logs less than used in previous research (Liebler-Tenorio et al., 2002;Brock et al., 2007) in an effort to slow the rapid course of severe disease which can be seen with this strain (Stoffregen et al., 2000).

2.5. Sample collection

Whole blood and serum samples were collected on Day 0, 2, 4, 6, 8, 14, 21 and 28 via the left jugular catheter or by jugular venipuncture if no catheter was present. Nasal swab samples were collected on Day 0, 4, 8, 14, 21 and 28. Serum was removed from clotted blood after centrifugation and processed immediately or refrigerated until processing. Buffy coat samples were obtained from whole blood samples as described earlier (Walz et al., 2008) with the exception that samples were resuspended in 1 mL of minimum essential medium. Whole blood, serum, and nasal swabs were refrigerated for <72 h before sample analysis. Whole blood and serum samples from each calf were submitted to the clinical pathology service at Auburn University Teaching Hospital for complete blood count (CBC) and serum biochemical profile (SBP) analysis on Day 0, 4, 8, 14, 21 and 28. Analytes assessed by SBP evaluation included total protein, albumin, globulins, serum dehydrogenase, aspartate transaminase, gamma-glutamyl transpeptidase, total bilirubin, creatine kinase, urea nitrogen, creatinine, calcium, phosphorous, glucose, magnesium, bicarbonate, sodium, potassium, chloride and iron.

2.6. Virus and antibody detection

Virus isolation was performed on buffy coat, serum and nasal swab samples by using the immunoperoxidase monolayer assay as described elsewhere (Walz et al., 2008)

with the exception that 1 mL aliquots of buffy coat sample were used and the procedure was performed in six-well (9.6 cm^2) plates. Virus neutralization assays were performed on serum samples collected on Days 0, 7, 14 and 21 as described previously except that serum was not initially diluted (Givens et al., 2003b). The virus neutralization assays were performed using BVDV2 (1373). Plates were incubated for 3 d at 38.5 °C in a humidified atmosphere of 5% CO_2 and air and then underwent the immunoperoxidase monolayer assay procedure for detection of BVDV (Givens et al., 2003b).

Results

3.1 Physical and hematologic findings

At study initiation, all calves were judged to be healthy based on a complete physical exam. Whole blood and serum samples submitted for CBC and SBP analysis on Day 0 revealed no remarkable differences between individual calves or between groups. However, on Day 4, all calves in the treated group had elevated serum creatinine concentrations ranging from 2.6 to 7.6 mg/dL (reference range 1.0 to 2.3 mg/dL) and elevated serum urea nitrogen (SUN) concentrations of 38 to 84 mg/dL (range 6.9 to 16.8 mg/dL). The same analytes were within the reference range for all calves in the untreated group. On Day 8, serum creatinine concentrations in calves A and D had returned to normal but remained high in calves B and C (6.9 and 19.3 mg/dL, respectively). Likewise, SUN concentrations remained elevated in treated calves B, C and D (80.7, 131.9 and 22.7 mg/dL, respectively) and were also slightly outside the reference range in untreated calves E and F (17.2 and 17.7 mg/dL respectively). Azotemia resolved in

calves E and F by Day 14 but SUN remained mildly elevated in calf D (20.7 to 28.7 mg/dL) throughout the study.

Transient inappetence was seen in calf A on Day 3 and mild inappetence and depression in calf C on Day 4. Calves B and C exhibited decreased appetite beginning on Day 3 that progressed to complete anorexia on Day 11 and Day 8, respectively. No evidence of clinical dehydration was noted at any point during the study. Calf C was humanely euthanized on Day 10 due to continued depression and inappetence. Intravenous fluid and electrolyte therapy was instituted for calf B beginning on Day 11. Despite aggressive treatment, the calf died on Day 13.

Tissues from Calves B and C were examined by a board certified veterinary pathologist and revealed multifocal tubulointerstitial nephritis with tubular epithelial degeneration, necrosis and regeneration. The renal tubules contained variable numbers of sloughed cells and inflammatory cells and moderate amounts of eosinophilic fluid. Mild to moderate splenic lymphoid depletion was present in both calves.

On Day 14, two untreated calves (E and H) developed loose stool that progressed to watery diarrhea over a 24 hour period. Calf H exhibited complete anorexia and mild depression that continued for three days. No treatment was necessary beyond the provision of free-choice reconstituted oral electrolyte solution. Fecal scores returned to normal after three (calf E) to seven days (calf H). Diarrhea was not present in the treated calves at any point during the study. Clinical scores for appearance were normal throughout the study period for all untreated calves.

The average lymphocyte count in treated calves on Day 0 was 5,306 x 10^3/µL (reference range 2,500-7,500 x 10^3/µL) with a standard deviation of 1,654 and 5,193 x 10^3/µL (standard deviation: 1,574) in untreated calves (Figure 6). All individual

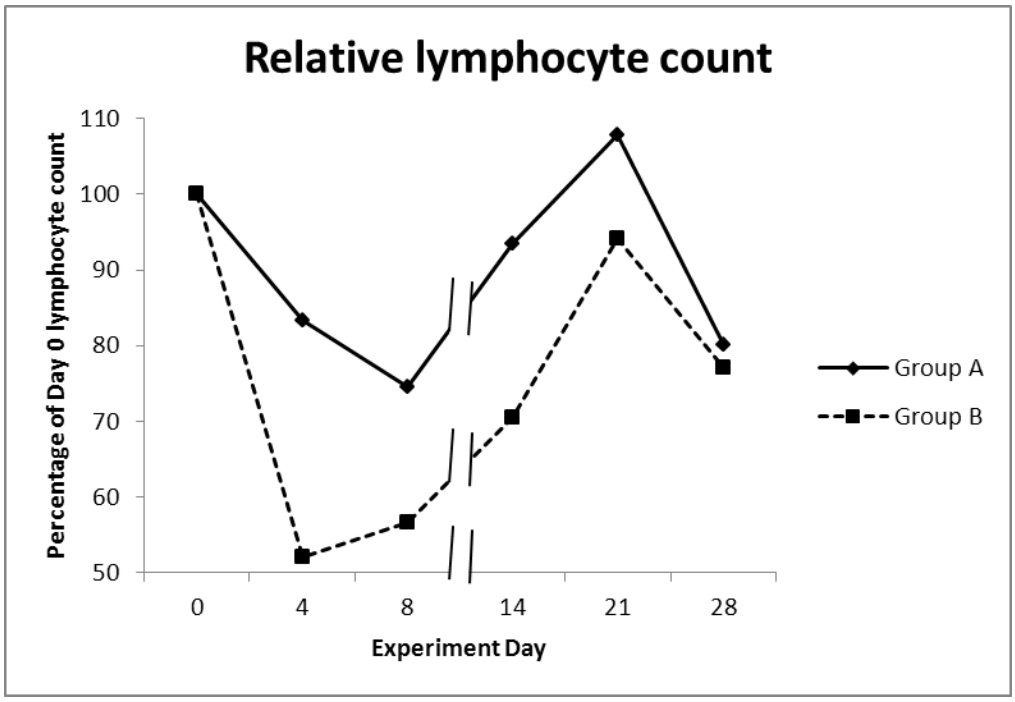


Figure 6. Average lymphocyte counts relative to Day 0 after intranasal challenge with 40,000 cell culture infectious doses of type 2 bovine viral diarrhea virus (BVDV-2) (strain 1373) in calves treated with the antiviral compound DB772 (Group A) and untreated (Group B) calves.

lymphocyte counts were within the reference range on Day 0. The average lymphocyte count in untreated calves decreased 48% to 2,703 x 10^3/µL (SD: 611) from Day 0 to Day 4 before increasing slightly to 2,947 x 10^3/µL (SD: 983) on Day 8. On Day 4, Calf E had

a lymphocyte count of 1,863 x 10^3/µL which remained outside the reference range on Day 8 at 2,260 x 10^3/µL. Calf H had a low lymphocyte count on Day 8 at 2,134 x 10^3/µL and Calf F had a count of 2,373 on Day 14. Lymphocyte counts of Calf G were within the reference range at all time points. The average lymphocyte count in treated calves decreased to 4,700 x 10^3/µL (SD:1,604) on Day 4 and 4,020 x 10^3/µL (SD: 634) on Day 8, a decline of 11% and 24%, respectively with no individual lymphocyte counts falling outside the reference range. The average lymphocyte count returned to within 90% of baseline on Day 14 in the treated group and Day 21 in the untreated group. Differences in average lymphocyte count were not statistically significant ($p= 0.205$) between groups using a multivariate repeated measure ANOVA with differences between treatment groups assessed over time.

On Day 14, two untreated calves (F and H) had platelet counts of less than 100,000/µL (reference range 265,000 to 886,000/µL). Clinically this manifested as ecchymotic hemorrhages on the distal limbs of calf F. The platelet count returned to normal by Day 21 in calf F but remained below the reference range in calf H throughout the study period. Neither thrombocytopenia nor clinical signs of bleeding diatheses were noted in any of the other calves.

Average baseline rectal temperatures obtained after acclimation to study housing and before initiation of treatment were 101.8°F and 102.3°F for treated and untreated calves, respectively (Figure 7). A biphasic fever pattern in untreated calves was evidenced by average rectal temperatures of at least 102.8°F on Days 5 and 6 and again on Days 9 through 14. Average rectal temperatures were near baseline on Days 7 and 8 for the same calves. Three of four untreated calves (E, G and H) exhibited fever of at

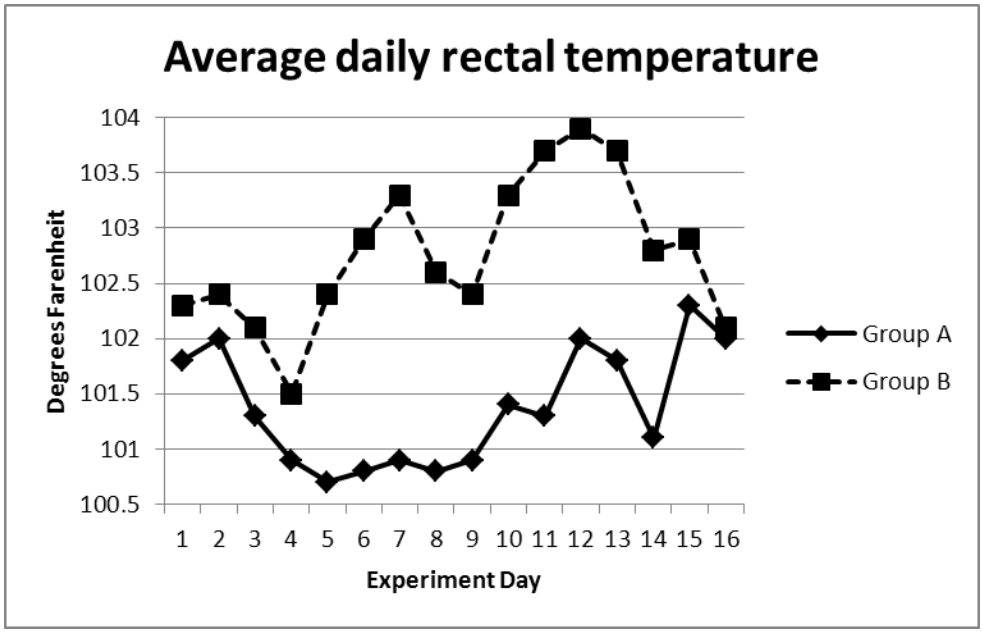


Figure 7. Comparison of the average rectal temperature readings between calves treated with the antiviral compound DB772 (Group A) and untreated (Group B) calves through Day 16 after aerosol challenge with type 2 bovine viral diarrhea virus (BVDV) . Intranasal challenge with 40,000 cell culture infectious doses of BVDV-2 (1373) occurred on Day 0 after the second dose of the antiviral compound was administered.

least 103.6°F on Day 5. Similarly, rectal temperatures of three calves (F, G and H) were consistently 103.2°F to 106.1°F for a four day period beginning on Day 9. The average daily rectal temperature for treated calves never reached 102.8°F. The average rectal temperature exceeded baseline by more than 0.5°F only on Days 16 and 17 at 102.7 °F and 102.4°F, respectively. Individually, one treated calf exhibited fever over 102.8°F. Calf A had a rectal temperature of 103.4°F on Day 16 and 103.2°F on Day 17 before returning to baseline. Differences in average rectal temperatures were not statistically

significant (p=0.446) between groups using a multivariate repeated measure ANOVA with differences between treatment groups assessed over time.

3.2 Virus and antibody detection

Virus was not detected in serum, buffy coat, or nasal swab samples from any calf on Day 0 or Day 2 (Table 1). On Day 4, virus was isolated from all the untreated calves in either buffy coat (Calves F and G), serum (E, F and H), or nasal swab samples (E and F). On Days 6 and 8, all samples collected from the untreated calves were positive on virus isolation. Virus was isolated from the buffy coat of three calves (F, G and H) on Day 14 but by Day 21 there was no virus isolated from samples collected from the untreated calves. No virus was detected in samples from treated calves before Day 14. Virus was isolated from the serum and buffy coat of calf A on Day 14 only. Calf D had positive virus isolation results on buffy coat on Days 21 and 28 and on nasal swab sample on day 28. No virus was detected in samples collected at, or any time prior to, the euthanasia (Day 10) or death (Day 13) of calf C and B, respectively.

All calves were seronegative to BVDV-2 ($\leq$1:4) on Day 0 (Figure 8). By Day 14, all four untreated calves developed antibody titers >1:256 to BVDV-2. On Day 21, the titers in untreated calves rose to at least 1:2048 and remained high through the end of the study. Serum antibodies to BVDV-2 were not detected in the treated calves on Day 14, 21 or prior to euthanasia or death (calves B and C). Calf A had an antibody titer of 1:256 on Day 28 but calf D remained seronegative throughout the study period.

	Treated group (positive animals/total animals)			Untreated group (positive animals/total animals)		
	WBC	Serum	Nasal	WBC	Serum	Nasal
Day 0	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
Day 2	0/4 (0%)	0/4 (0%)	N/A	0/4 (0%)	0/4 (0%)	N/A
Day 4	0/4 (0%)	0/4 (0%)	0/4 (0%)	2/4 (50%)	3/4 (75%)	2/4 (50%)
Day 6	0/4 (0%)	0/4 (0%)	N/A	4/4 (100%)	4/4 (100%)	N/A
Day 8	0/4 (0%)	0/4 (0%)	0/4 (0%)	4/4 (100%)	4/4 (100%)	4/4 (100%)
Day 14	1/2 (50%)	1/2 (50%)	0/2 (0%)	3/4 (75%)	0/4 (0%)	0/4 (0%)
Day 21	1/2 (50%)	0/2 (0%)	0/2 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
Day 28	1/2 (50%)	0/2 (0%)	1/2 (50%)	1/4 (25%)	0/4 (0%)	0/4 (0%)

Table 1. Results of virus isolation of BVDV-2 from buffy coat (WBC), serum or nasal swab media (nasal) samples from calves inoculated with 40,000 cell culture infectious doses ($CCID_{50}$) of BVDV-2 (strain) 1373 by aerosol inoculation on Day 0. DB772 was administered to the treatment group intravenously every 8 hours for a total of 11 treatments beginning nine hours prior to challenge. Untreated calves were administered an equivalent volume of diluent only on the same dosing schedule. One treated calf was euthanized on Day 10 and a second calf died on Day 13; thus, Days 14, 21 and 28 demonstrate 2 total animals in the treated group.

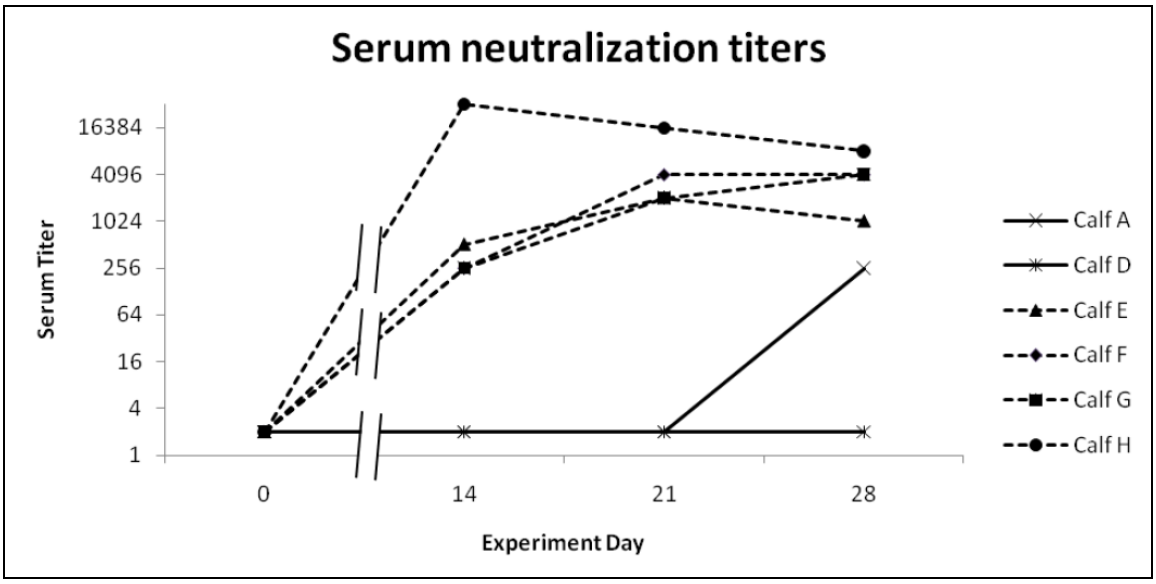


Figure 8. Serum neutralization titers to type 2 bovine viral diarrhea virus (BVDV-2) in miniature calves after aerosol BVDV challenge. Intranasal challenge with 40,000 cell culture infectious doses of BVDV-2 (1373) occurred on Day 0 after the second dose of the antiviral compound was administered. Calves treated with DB772 are denoted by solid lines and untreated calves in are denoted by dashed lines.

Discussion

In this preliminary study, eleven intravenous treatments with DB772 at a dose of 12 mg/kg effectively prevented BVDV-associated viremia until long after cessation of therapy in Miniature Brahma-cross calves following nasal inoculation with 40,000 $CCID_{50}$ of BVDV-2 (1373) and continued exposure to infected, untreated calves. Virus was consistently isolated from each untreated calf on Days 4, 6 and 8 and from three of four calves on Day 14, consistent with similar previous experimental inoculation studies

(Archambault et al., 2000). Virus was not isolated from treated calves until Day 14 (calf A) or Day 21 (calf D) indicating treatment with DB772 delayed infection with, or viral replication of, BVDV-2 (1373). Though two of the treated calves were removed from the study prior to Day 14, no virus was isolated from either calf preceding removal from the study or from post-mortem samples. The results suggest DB772 effectively inhibits BVDV infection or initial replication when present in serum at tested concentrations. However, as serum concentrations decline, the calves were once more susceptible to BVDV infection or continuation of initial replication.

In this study, calves were challenged with BVDV after the second administration of DB772 or diluent. Therefore, the serum concentration of DB772 in treated calves was projected to reach or exceed levels sufficient to inhibit BVDV replication *in vitro* at the time of challenge. Administration of DB772 continued for 72 hours after challenge to maintain adequate serum concentrations of the antiviral compound. In a previous study using similar doses of DB772, the serum half-life of DB772 following intravenous injection was reported to be 16.2 h (Newcomer et al., 2012a). Thus, serum concentrations of DB772 were expected to drop quickly after the final administered dose on Day 3.

In the current study protocol, calves were maintained in the same room as an infected untreated calf through the end of the study on Day 28. Though the calves were housed in individual crates within the room, the potential for nose-to-nose contact existed when each calf was released from its individual crate into the common room twice a day to allow for cleaning of the individual crates. Virus could still be isolated from three of the four untreated calves on Day 14 of the study at which time serum DB772 concentrations of their cohorts were projected to be below *in vitro* protective levels

(Givens et al., 2004). Thus, viral challenge in this study was not limited to a single intranasal inoculation on Day 0 but was on-going due to continued viral shedding by the untreated cohorts. We hypothesize that the treated calves were protected from BVDV infection when first exposed by aerosol inoculation but were left vulnerable to cross-infection from their untreated cohorts in the same room as serum DB772 concentrations waned following the cessation of treatment. A second possibility is that DB772 administration prevented acute viremia and masked clinical signs of disease until therapy ceased and concentrations of the antiviral compound decreased to a degree sufficient to allow progression of the infection.

The first hypothesis is supported by the time of development of serum neutralizing antibodies in the untreated calves compared to their treated cohorts. All untreated calves had serum titers $\geq$1:256 by Day 14 and $\geq$1:2,048 by Day 21 while the two remaining treated calves remained seronegative. Serum neutralizing antibodies were not detected in calf A until Day 28 (1:256) and calf D remained seronegative throughout the study period. Multiple authors have reported that seroconversion to BVDV occurs within 10 to 21 days after initial viral exposure (Traven et al., 1991;Polak and Zmudzinski 2000). Thus, the delay in development of serum neutralizing antibodies in treated calves compared to untreated controls suggests treated calves were not infected by the initial viral challenge but later in the study period after serum concentrations of DB772 had declined below effective levels. Viremia and seroconversion that occurred in treated calves late in the study period suggests treatment with DB772 does not provide long-lasting protection from infection. Though challenged on Day 0, treated calves failed to produce adequate amounts of serum neutralizing antibodies to prevent infection from

subsequent challenge with BVDV. Consequently, animals treated with DB772 remain susceptible to BVDV after serum antiviral concentrations wane.

An unexpected and concerning result of this study was the development of signs of acute renal toxicity associated with administration of DB772 that resulted in evidence of renal toxicity in all four treated calves and the death or euthanasia of two of the four treated calves. This is the first documented report of negative side effects associated with DB772 administration. In previous studies involving healthy calves or calves persistently infected with BVDV, no signs of toxicity were evident clinically or upon evaluation of hematologic parameters (Newcomer et al., 2012a). Consequently, renal disease associated with DB772 treatment was not foreseen but represents a significant concern that warrants further investigation into the safety of the compound. Earlier safety studies should be repeated with more calves using lower dosages of DB772 to accurately characterize the risk of toxicity to the compound.

An important difference in this study with previous *in vivo* studies was the age and husbandry of the calves treated with DB772. Calves treated previously were 10 to 17 d old and had not yet been weaned. Those calves were allowed to nurse freely from their dams or bottle fed twice daily with a commercial milk replacer. In this study, the calves were five to six months old and weaned immediately prior to the housing adjustment period. Though no changes in daily hydration clinical scores or total protein concentrations were appreciated in treated calves, the possibility exists that the calves in the present study were not as well hydrated as previously studied calves, leaving them susceptible to renal insult.

Another difference in this study that may have contributed to the signs of acute renal toxicity was the amount of DB772 administered. In earlier studies, calves were treated with 1.6 mg/kg once, 9.5 mg/kg every 8 h for 6 d or 12 mg/kg every 8 h for 6 d. In this study, calves were administered 12 mg/kg every 8 h for a total of 11 treatments, a dose equivalent to the highest dose reported previously but for a shorter duration. However, because the calves used in this study were heavier than calves in previous studies, the total cumulative dose of DB772 administered to calves was higher by two- to three-fold in some calves in this study. Interestingly, the two calves most severely affected in this study (calves B and C) were the two lightest calves in the treatment group and so received the lowest cumulative doses of DB772.

An important limitation to this study was the small sample size in both the treated and untreated groups. This study was designed as a pilot study to evaluate the antiviral activity of DB772 when administered to healthy calves subsequently challenged intranasally with BVDV. Because of the small sample size, no statistically significant differences were detected in biochemical parameters or rectal temperatures between the treated and untreated groups. To achieve the statistical power necessary to detect expected differences as statistically significant, many more calves would need to be enrolled in this research. For instance, to achieve a statistical power of 0.80 at $\alpha = 0.05$ for detecting a 25% difference in a biochemical parameter which exhibits a standard deviation of 25% of the mean, 17 calves would be needed in each experimental group.

Though clinical signs of BVDV infection were seen in the untreated calves, the severe disease and high mortality rates observed in previous experiments using BVDV-2 (1373) (Liebler-Tenorio et al., 2002;Brock et al., 2007) were not observed in this study.

Previous studies have used both older and younger animals (Stoffregen et al., 2000;Brock et al., 2007). The virus titer of the challenge inoculum in our study was one to two log scores lower than in previously reported studies (Archambault et al., 2000;Liebler-Tenorio et al., 2002;Brock et al., 2007)and may have accounted for the decreased morbidity and mortality seen in this study. In our study, two of four untreated calves developed watery diarrhea and two of four also exhibited thrombocytopenia 14 d after experimental inoculation which manifested as ecchymotic hemorrhages on the distal limb of one calf (calf F). In previous studies using BVDV-2 (1373), disease was characterized by thrombocytopenia and bloody diarrhea which was not evident in this study. The decrease in severity of clinical signs was thought to be as a result from lower inoculum used during challenge.

Elevated rectal temperatures were detected in untreated calves on Days 5 and 6 and again on Days 9 through 14 with the maximum fever being reached on Days 10-12. Biphasic fever has been previously documented with BVDV infection (Traven et al., 1991;Polak and Zmudzinski 2000) with the maximal rectal temperatures in those studies being reached on Day 6 or Days 8 and 9, respectively. Fever was not seen in three of the calves receiving the antiviral compound but calf A spiked a fever on Day 16 and 17. Likewise, untreated calves exhibited severe lymphopenia beginning on Day 4, similar to what has been described in previous studies also using BVDV-2 (1373) (Stoffregen et al., 2000;Liebler-Tenorio et al., 2002). While the average lymphocyte count in untreated calves declined 48% by Day 4, the average lymphocyte count in treated calves only declined 24% by Day 8. These results suggest intravenous treatment with DB772 at 12

mg/kg was sufficient to prevent infection or viral replication after intranasal inoculation of BVDV.

Previous *in vitro* studies indicate the 90% viral inhibitory concentration of DB772 against BVDV-1 is 0.02 µM (Givens et al., 2003a). Similar antiviral efficacy against BVDV-2 is seen with DB772 and closely related compounds (Givens et al., 2005). The dose used in this study was designed to achieve a 4 µM concentration in serum; this dose has been previously demonstrated to eliminate BVDV from cells *in vitro* (Givens et al., 2004). Serum DB772 concentrations consistently exceeded 3µM after 24 h in nursing calves on the same dosing regimen (Newcomer et al., 2012a). While this suggests a lower concentration of DB772 may still be effective at preventing viral replication while minimizing the signs of renal toxicity evident in this study, this remains to be proven in additional studies.

Identifying and eliminating animals persistently infected with BVDV is the cornerstone of BVDV control in North America and much of the world (Walz et al., 2008). However, an antiviral agent such as, or similar to, DB772 that could prevent BVDV infection as well as maintain the seronegative status of animals exposed to the virus would be of great benefit in regions free of BVDV where serology is an important component of disease surveillance (Sandvik 2004). Maintaining seronegativity in treated animals in North America and other regions where vaccination is widespread may be less crucial because serology is less commonly used as a diagnostic test of BVDV exposure. Further studies must examine the effect of DB772 in seropositive animals. Additionally, the availability of an easily administered specific antiviral compound for use during an outbreak that would maintain the integrity of the diagnostic infrastructure would be

invaluable in BVDV-free regions. In this study, DB772 was administered intravenously three times a day which is impractical for commercial or large-scale use. If DB772 or a related compound is to be developed as a prophylactic compound to be used in the face of BVDV outbreaks, a formulation that is easy to administer and demonstrates long-term efficacy must be developed.

In summary, BVDV viremia and clinical signs were effectively prevented in miniature calves during treatment with 12 mg/kg of DB772 intravenously every 8 h. However, DB772 administration in this study was associated with signs of acute renal toxicity that resulted in the death or euthanasia in two of four treated animals. Because the calculated serum concentrations of DB772 greatly exceeded the concentrations shown to inhibit BVDV *in vitro*, a lower dose could potentially be used to achieve the same antiviral effects without the negative side effects. Thus, this research proves the concept that an antiviral agent can be used to prevent acute disease caused by BVDV although further investigation is required to ensure that acute renal toxicity does not result from antiviral treatment.

Efficacy of an Antiviral Compound to Inhibit Replication of Multiple Pestivirus Species

Abstract

Pestiviruses are economically important pathogens of livestock. An aromatic cationic compound (DB772) has previously been shown to inhibit bovine viral diarrhea virus (BVDV) type 1 *in vitro* at concentrations lacking cytotoxic side effects. The aim of this study was to determine the scope of antiviral activity of DB772 among diverse pestiviruses. Isolates of BVDV 2, border disease virus (BDV), HoBi virus, pronghorn virus and Bungowannah virus were tested for *in vitro* susceptibility to DB772 by incubating infected cells in medium containing 0, 0.006, 0.01, 0.02, 0.05, 0.1, 0.2, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5 or 25 μM DB772. The samples were assayed for the presence of virus by virus isolation and titration (BDV and BVDV 2) or PCR (HoBi, pronghorn and Bungowannah viruses). Cytotoxicity of the compound was assayed for each cell type. Complete inhibition of BVDV 2, BDV, and pronghorn virus was detected when DB772 was included in the culture media at concentrations of 0.20 μM and higher. In two of three tests, a concentration of 0.05 μM DB772 was sufficient to completely inhibit HoBi virus replication. Bungowannah virus was completely inhibited at a concentration of 0.01 μM DB772. Thus, DB772 effectively inhibits all pestiviruses

studied at concentrations ≥ 0.20 μM. As cytotoxicity is not evident at these concentrations, this antiviral compound potentially represents an effective preventative or therapeutic for diverse pestiviruses.

B.W. Newcomer, M.S. Marley, J.F. Ridpath. J.D. Neill, D.W. Boykin, A. Kumar, M.D. Givens. "Efficacy of an Antiviral Compound to Inhibit Replication of Multiple Pestivirus Species". *Antiviral Research* 2012; 96(2): 127-129.

The *Pestivirus* genus of viruses is comprised of four virus species: bovine viral diarrhea virus 1 and 2 (BVDV -1 and -2), classical swine fever virus (CSFV) and border disease virus (BDV). Additional isolates from cattle (Schirrmeier et al., 2004), a pronghorn antelope (Vilcek et al., 2005b) and swine (Kirkland et al., 2007) have been proposed as member viruses and are referred to as HoBi, pronghorn and Bungowannah viruses, respectively.

Aromatic cationic compounds possess inhibitory action against RNA viruses, (Dubovi et al., 1980;Vonderfecht et al., 1988;Kumar et al., 1995). One particular compound, 2-(2-benzimidazolyl)-5-[4-(2-imidazolino) phenyl]furan dihydrochloride, (DB772; MW=410.28) has been shown to inhibit BVDV1 growth in cell culture at concentrations lacking cytotoxicity (Givens et al., 2003a) but preliminary studies to assess the antiviral efficacy of DB772 as an antiviral agent for use against Hepatitis C virus did not warrant further investigation (Dan Givens, personal communication, 2008). The *in vitro* efficacy of DB772 has not been examined with pestiviruses other than BVDV1. The sequence homology of current and proposed pestiviruses may vary by as

much as 46% in the well-conserved 5` UTR (Kirkland et al., 2007); thus, the aim of this study was to determine the scope of antiviral efficacy of DB772 among various pestiviruses.

The antiviral efficacy of DB772 was tested with isolates of BVDV2, BDV, HoBi, pronghorn and Bungowannah viruses. A 24-well plate was seeded with 300 µL of reconstituted cells and 1 mL of minimum essential medium (MEM) and incubated for 24 hours at 38.5°C. Madin-Darby bovine kidney (MDBK) cells were used to evaluate BVDV2 and HoBi virus; ovine fetal turbinate (OFTU) cells for BDV and pronghorn virus; and porcine kidney (PK) cells for Bungowannah virus. The media was then replaced with 200 µL MEM and DB772 was added to achieve a final concentration of 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.10, 0.05, 0.02, 0.01, or 0.006 µM DB772 except for the positive and negative control wells containing no DB772. Plates were incubated for 15 min before infection at a multiplicity of infection of 0.5. After one hour, the inoculum was removed and the wells washed twice with 500 µL phosphate buffered solution without calcium or magnesium. One mL of MEM containing the appropriate concentration of DB772 was added back to each well and the plates incubated for four days and then frozen. Each virus was tested in triplicate using separate plates.

Using the lysate obtained after a single freeze-thaw cycle, virus titration of BVDV2 and BDV samples was performed. Multiple, serial ten-fold dilutions of 10 µL lysate diluted in 90 µL MEM were performed in triplicate and assayed by immunoperoxidase staining (Walz et al., 2008)or direct visualization of cytopathic effect, for BVDV2 and BDV, respectively. The statistical method of Reed and Muench (Reed

L.J. and Muench H 1938) was used on the limited number of replicates to estimate the concentration of BVDV2 and BDV.

Samples containing HoBi, pronghorn and Bungowannah viruses were assayed by rtPCR. Samples spiked with 25µM DB772 does not result in detectable inhibition of reverse transcriptase, Taq polymerase or other enzymes involved in the rtPCR reaction (Newcomer, unpublished data). Genomic RNA was isolated using a commercial viral RNA isolation kit (Qiagen, Inc., Valencia, CA). Viral RNA yields from biological samples are typically less than 1 µg per final extracted volume of 60 µL using this kit. For the PCR reactions for pronghorn virus and Bungowannah virus, 10 µL of purified RNA was added to a master mixture consisting of 10 µL 10X PCR +Mg buffer, 8 µL dNTPs, 50 u M-MLV reverse transcriptase, 0.5 µL DNA Taq polymerase, 20 u RNase inhibitor and 1 µL each of forward and reverse primers. For HoBi virus, 20 µL of purified RNA template was used. The rtPCR reactions for HoBi virus and pronghorn virus were run by heating to 56°C for 60 min, then 94°C for 2 min, followed by 38 cycles of 94°C for 30 s, 55°C for 30s and 72°C for 30 sec. After completion of the 38th cycle, the reaction was kept at 72°C for an additional seven minutes and maintained at 4°C until the amplicon was run on 1% agarose/ethidium bromide gels. For Bungowannah virus PCR reactions, the samples were heated to 56°C for 60 min, then 94°C for 4 min, followed by 40 cycles of 94°C for 10 s, 50°C for 15s and 72°C for 45 sec. The amplicon remained at 72°C for 10 min before maintenance at 4°C until run on the gel. All gels were run within 12 h of cycle completion. The forward and reverse primers for HoBi and pronghorn viruses were 5´-ATGCCCATAGTAGGACTAGCA-3´ and 5´-TCAACTCCATGTGCCATGTAC-3´. The primers for Bungowannah virus were

5´-AAGCGGTGAGTACACCGTATTCGT-3´ and

5´- ATGTTTCCTTCCTCACTCCCTCCA-3´.

The cytotoxicity of DB772 in MDBK, OFTU and PK cells was tested using a commercially available cell counting kit (Dojindo Molecular Technologies, Inc., Rockville, MD) after a 96 hour incubation period. 96-well plates containing approximately 5,000 cells per well were incubated for 24 h at 38.5°C. Dilutions of DB772 were added to the appropriate wells to obtain a final concentration of 0, 1.56, 3.12, 6.25, 12.5, 25, 50 and 100 µM DB772 and plates were incubated for 96 h. Ten microliters of assay solution were then added to each well. The plates were incubated for 1 h before the optical density (OD) at 450 nm was measured using a microplate reader. Each concentration of the compound was assayed in six replicates.

Each virus was successfully cultured in the absence of DB772 (Table 2). The mean titer of BVDV2 cultured in the absence of DB772 was 3.9×10^7 $CCID_{50}$/mL. The titer remained relatively constant when cultured in increasing concentrations of DB772 before virus replication was completely inhibited in all replicates at concentrations $\geq$ 0.2 µM DB772. Like BVDV2, BDV replication was completely inhibited at concentrations $\geq$ 0.2 µM DB772. In the absence of DB772, the mean viral titer was 1.79×10^8 $CCID_{50}$/mL which decreased to 2.07×10^3 $CCID_{50}$/mL and 1.17×10^3 $CCID_{50}$/mL at concentrations of 0.05 and 0.1 µM DB772, respectively. Pronghorn virus and two of three replicates of

VIRUS and REPLICATE #	Concentration of DB772 (µM)													
	25	12.5	6.25	3.13	1.56	0.78	0.39	0.2	0.1	0.05	0.02	0.01	0.006	0
BVDV2 #1	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	2E+06	6E+06	4E+06	4E+05	6E+06	6E+07
BVDV2 #2	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	4E+03	2E+04	6E+05	2E+06	4E+06	4E+07
BVDV2 #3	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	6E+03	5E+05	4E+06	4E+06	2E+07
(Mean)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	7E+05	2E+06	2E+06	2E+06	4E+06	3E+07
BDV #1	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	6E+04	6E+07	4E+08	4E+07
BDV #2	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	6E+03	5E+03	6E+03	1E+05	6E+07
BDV #3	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	4E+03	(-)	6E+06	2E+05	2E+07	4E+08
(Mean)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	1E+03	2E+03	2E+06	2E+07	1E+08	2E+08
HoBi #1	(-)	(-)	(-)	(-)	+	+	+	(-)	+	+	+	+	+	+
HoBi #2	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	+	+	+	+
HoBi #3	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	+	+	+
Phorn #1	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	+	+	(-)	+	+	(-)
Phorn #2	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	+	+	+	+	+
Phorn #3	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	+	+	+	+	+	+
Bungo #1	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	+
Bungo #2	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	+
Bungo #3	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	+	+

Table 2. Results of inhibitory testing of several pestiviruses with different concentrations of DB772. Isolates of bovine viral disease virus (BVDV) and border disease virus (BDV) were assayed by virus isolation and titration with the individual and mean estimated viral titers shown ($CCID_{50}$/mL). HoBi, Pronghorn (Phorn) and Bungowannah (Bungo) viruses were assayed by RTPCR with positive results indicated by (+). Negative test results are indicated by (-).

Hobi virus were completely inhibited at a DB772 concentration of 0.2 µM or greater. In the third HoBi virus replicate, faint bands were observed on the gel at concentrations of 0.39, 0.78 and 1.56 µM DB772. A concentration of 0.01 µM DB772 or greater completely inhibited viral replication of Bungowannah virus. Cytotoxicity was first detected in MDBK, OFTU and PK cells at concentrations of 100 µM, 50 µM and 25 µM DB772, respectively.

All pestiviruses tested were completely inhibited by concentrations $\geq$ 0.2 µM DB772 with the exception of one replicate of HoBi virus which was completely inhibited at concentrations of DB772 greater than 1.56 µM, similar to earlier studies using BVDV1 isolates. (Givens et al., 2003a;Givens et al., 2004). Thus, the compound exhibits pan-pestivirus antiviral activity at micromolar concentrations. The mechanisms of action of DB772 have yet to be determined. The lack of detection of genomic RNA in this study is consistent with inhibition of viral replication as has been seen with other anti-BVDV compounds (Givens et al., 2003a).

Development of an antiviral compound such as DB772 effective against multiple pestiviruses holds potential for multiple uses. The availability of an easily administered specific antiviral compound for use during an outbreak that would maintain the integrity of the diagnostic infrastructure would be invaluable in regions free of specific pestiviruses. Likewise, pestivirus contamination of both medical and veterinary biologicals is of significant concern and often remains undetected as many pestiviruses are noncytopathic (Givens et al., 2004). Adventitious viruses or viral particles may contaminate live vaccines or disrupt cell cultures and diagnostic assays (Yanagi et al.,

1996). A compound able to clear infected biologicals at noncytotoxic concentrations would provide an important impediment to viral spread.

The antiviral efficacy of DB772 against CSFV was not evaluated in this study because it is an exotic agent in the US and thus not available for testing, but results suggest the virus would likely be susceptible to the compound *in vitro*. Molecular analyses of complete genome sequences of CSFV have consistently exhibited a high level of sequence identity with other member pestiviruses, particularly isolates of BDV (Becher et al., 1995;Ridpath and Bolin 1997). Thus we expect DB722 to inhibit CSFV replication at or near the 0.2 uM level.

In summary, micromolar concentrations of DB772 are sufficient to completely inhibit viral replication of all pestiviruses against which it was tested. While the effects of DB772 on CSFV infection remain to be demonstrated, we believe the virus will also prove susceptible to *in vitro* antiviral treatment. Cytotoxic effects are not observed until concentrations exceed therapeutic concentrations approximately 100-fold. Thus, DB772 or related compounds continue to represent a potential therapeutic agent for diverse pestiviral infections.

Acknowledgements

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Mutations induced in the NS5B gene of bovine viral diarrhea virus by antiviral treatment convey resistance to the compound.

Abstract

Bovine viral diarrhea virus (BVDV) is a widespread bovine pathogen for which there is no specific therapeutic agent. A previous study using 2-(2-benzimidazolyl)-5-[4-(2-imidazolino)phenyl]furan dihydrochloride (DB772) to treat calves persistently infected with BVDV resulted in a decrease in the viral load of infected calves but treatment resulted in the rapid selection of drug-resistant mutant isolates. In this manuscript we describe three mutations found in the mutant isolates associated with *in vivo* and *in vitro* resistance to DB772. All three mutations are found in the NS5B which functions as the RNA-dependent-RNA-polymerase during viral replication. Growth curves for the mutant isolates were not largely different from those of wild-type isolates when cultured in the absence of DB772. Thus, DB772 appears to act by binding to the specified domain but binding is disrupted or inhibited by the described mutation.

Benjamin W. Newcomer, John D. Neill, M. Shonda Marley, Julia F. Ridpath, M. Daniel Givens. "Mutations induced in the NS5B gene of bovine viral diarrhea virus by antiviral treatment convey resistance to the compound." *Virus Research*. (Manuscript in press).

Introduction

As the prototypical virus of the *Pestivirus* genus of the *Flaviviridae* family, bovine viral diarrhea virus (BVDV) contains a single-stranded, positive sense RNA genome approximately 12.5 kb in size. A lone open reading frame encodes a single polyprotein of roughly 3900 amino acids that yields 11 mature proteins after co- and post-translational processing. The individual proteins in order from 5′ to 3′ are the amino-terminal autoprotease (N^{pro}), the nucleocapsid protein (C), the ribonuclease soluble envelope glycoprotein (E^{rns}), the envelope protein 1 (E1), the envelope protein 2 (E2), a protein of unknown function (p7), the fused second and third nonstructural proteins (NS2-3), the amino- and carboxy-terminal portions of the fourth nonstructural protein (NS4A, NS4B) and the amino- and carboxy- terminal portions of the fifth nonstructural protein (NS5A, NS5B) (Collett et al., 1988). The first four proteins are structural while the remainder are nonstructural (NS) and function in viral assembly, replication and host immune evasion (Meyers and Thiel 1996). The NS5B gene is highly conserved among the pestiviruses and its protein product is one of the best characterized NS proteins. As an integral component of the replicase complex, NS5B functions as the RNA-dependent RNA polymerase (RdRP) during genome replication (Choi et al., 2004).

A large number of ruminant and non-ruminant species across the globe can be infected with BVDV. Infection with BVDV may be manifested in the respiratory, gastrointestinal, reproductive, cardiovascular, lymphatic, immune, integumentary or central nervous systems of affected cattle and is a significant source of economic loss to the dairy and beef industries (Walz et al., 2010). Acute infection with virulent BVDV has resulted in mortality rates in excess of 30% in naïve groups of animals (Pellerin et al., 1994). Additionally, BVDV is unique in its ability to cross the placenta of susceptible animals and cause persistent infection of the growing fetus. Animals persistently infected (PI) with BVDV serve as reservoirs of the virus, continuously shed virus and are important in the epidemiologic aspects of BVDV propagation (McClurkin et al., 1984). Other than maintaining a strong biosecurity program, including the identification and elimination of PI animals, current preventatives for BVDV are limited to killed or modified live vaccines.

Not only a significant clinical pathogen, BVDV is an important laboratory contaminant as well. Isolates of BVDV can be characterized as either cytopathic (CP) or noncytopathic (NCP) biotypes based on their activity in cultured epithelial cell lines (Gillespie et al., 1960). Despite manufacturer testing, NCP BVDV has been isolated from commercially available lots of bovine fetal serum (Yanagi et al., 1996). Additionally, commercially available bovine, canine, feline and primate cell lines (Fernelius et al., 1969), (Harasawa and Mizusawa 1995), human viral vaccines (Giangaspero et al., 2001), (Harasawa and Tomiyama 1994) and interferons for human use (Harasawa and Sasaki 1995) have been found to be contaminated with NCP BVDV. No specific antiviral

therapeutics to control BVDV infection in the laboratory or on the farm are currently available.

Aromatic cationic compounds possess inhibitory action against several RNA viruses, including human immunodeficiency virus (Kumar et al., 1995), rotavirus (Vonderfecht et al., 1988) and respiratory syncytial virus (Dubovi et al., 1980). One particular compound, 2-(2-benzimidazolyl)-5-[4-(2-imidazolino)phenyl]furan dihydrochloride, (DB772; MW=410.28) has been shown to inhibit BVDV growth in cell culture at micromolar concentrations lacking cytotoxicity (Givens et al., 2003a). The 99% endpoint for prevention of viral replication by DB772 was found to be 6 nM (Givens et al., 2004). Further, DB772 has been shown to eliminate BVDV infection in contaminated bovine fetal fibroblast cells with a single passage in culture media supplemented with 4μM of DB772 (Givens et al., 2004). Administration of the compound to calves persistently infected with BVDV resulted in a decrease in viral concentration with no detectable negative side effects (Newcomer et al., 2012a). When administered to calves naïve to BVDV, the compound successfully delayed BVDV infection after experimental viral inoculation (Newcomer et al., 2013).

However, the mechanism of action of DB772 is currently unknown. The primary objective of this research was to characterize mutations seen in the NS5B gene of BVDV isolates from PI calves treated with DB772 that were subsequently found to be resistant to the compound. The secondary objective of this research was to assess the hypothesis that the mutations in the NS5B gene observed after treatment with DB772 would hinder replication efficiency.

Materials and methods

2.1 Virus

The isolates sequenced in this study were obtained from six PI Angus-cross beef calves treated with DB772 intravenously three times a day for six days (A, B, C, D) or diluent only on the same dosing schedule (E, F) as described earlier (Newcomer et al., 2012a). Day 0 isolates were obtained from each calf before treatment initiation and shown to be susceptible to DB772 *in vitro*. Virus isolated from calves B, C and D during the treatment period on Days 5, 3 and 3, respectively, exhibited *in vitro* resistance to the compound. Isolates from calf A and the untreated calves E and F did not demonstrate resistance at any point during the study. The first isolates shown to be resistant to DB772 *in vitro* obtained in sufficient quantity for further study were selected for further characterization. These isolates were obtained during the treatment period (Day 7 for Calf C and Day 3 for Calf D) or following treatment (Day 56 for Calf B). Samples obtained from Calf A on Day 14 were used for further evaluation but remained susceptible to DB772 *in vitro* (Newcomer et al., 2012a). All viruses were purified by selecting clones created by serial limiting dilutions of virus isolates from passaged white blood cell samples in the absence (wild-type isolates) or presence (mutant isolates) of 4 μM DB772. The GenBank accession numbers for the wild-type isolates from Calves B, C and D are HQ174299, HQ174300 and HQ174293, respectively.

2.2 Reverse transcriptase PCR and DNA sequencing

Genomic RNA was isolated from viral samples using a commercially available viral RNA isolation kit (Qiagen, Inc., Valencia, CA). The nucleotide sequence of the open reading frame (ORF) was determined for each wild-type and mutant isolate as previously described (Neill et al., 2011). Amplified reactions were run on 1% agarose/ethidium bromide gels. After excision, a GeneClean spin column kit (MP Biomedicals, Inc., Solon, OH) was used to purify the amplicons. An ABI PRISM 3100 automated sequencer with ABI BigDye Terminator v3.1 chemistry (ABI, Inc., CA) was used to sequence the purified DNA amplicons. CodonCode Aligner software (Codoncode, Inc., Dedham, MA) was used to edit and assemble the sequences.

2.3 Growth kinetics

Assays were performed to compare the growth kinetics of the mutant isolates to the wild-type isolates causing the persistent infection in the calves. Pre-treatment (Day 0) isolates from the buffy coat of each treated calf were compared to mutant isolates obtained during or following treatment that were shown to be resistant to DB772 *in vitro*. A 96 well plate was seeded with 50 μL of Madin-Darby bovine kidney (MDBK) cells (approximately 3 x 10^5 cells) and 100 μL of minimum essential medium (MEM) supplemented with 10% equine serum. After incubation at 38.5°C for 24 hours, the total number of cells in a well was calculated with the use of a gridded microscope ocular. The number of cells in at least five wells was counted and the average was used to calculate the number of cell culture infectious doses (CCID) to achieve a multiplicity of infection (MOI) of 3.0. A final concentration of 4 μM per test well was achieved by adding 10 μL of DB772 (1.6μg/mL); 10 μL of MEM was added to each control well.

After incubation for 15 minutes at 38.5°C, 10 μL of passaged white blood cell clone was added to each well. After further incubation for one hour, the media was removed from each well and the wells were washed twice with 100 μL of PBS without Ca+ or Mg+. One hundred μL of media or 100 μL of media with 4 μM DB772 was then added to the control and test wells, respectively. The plates were then incubated for the indicated time period (1,3,5,7,9,11,13,23,48 or 72 hours) before freezing to stop viral replication. Viral titrations were performed using the immunoperoxidase staining assay described earlier (Givens et al., 2003b). Each isolate was assayed in triplicate.

2.4 Clonal Fitness

Clonal fitness was assessed in the three mutant strains by co-incubating wild-type and mutant isolate clones in 24-well plates at known ratios. One day before *in vitro* infection, a 24-well plate was seeded with 50 μL of MDBK cells (approximately 3×10^5 cells) and 1 mL MEM and incubated at 38.5°C for 24 hours. The cells were counted in at least five wells and the average was used to calculate the number of CCID to achieve an MOI of 1.0 for each isolate. The total infectious dose was comprised of a 90:10 or 10:90 ratio of wild-type and mutant isolates. The plates were incubated at 38.5°C for one hour before the inoculum was removed and the cells were washed twice with 100 μL of D-PBS without Ca+ or Mg+. One mL of MEM was then added to each well and the plate was incubated for four days at 38.5°C at which time the plate was subjected to a freeze-thaw cycle to stop viral replication. The same procedure was repeated for a total of five passages with the exception that subsequent plates were infected using the inoculum taken from the previous passage. After the fifth passage and freeze cycle, each virus

population was assayed by PCR using Pfx50 high fidelity polymerase (Life Technologies, Grand Island, NY). After gel purification, the PCR product was cloned using a proprietary vector system contained in a commercial blunt PCR cloning kit (Agilent Technologies, Palo Alto, CA) according to the manufacturer's instructions and plated on X-gal containing plates. White colonies were selected and grown in 96 well plates. Colonies were purified using a commercial plasmid purification kit (Qiagen, Inc., Valencia, CA) before sequence analysis as described above.

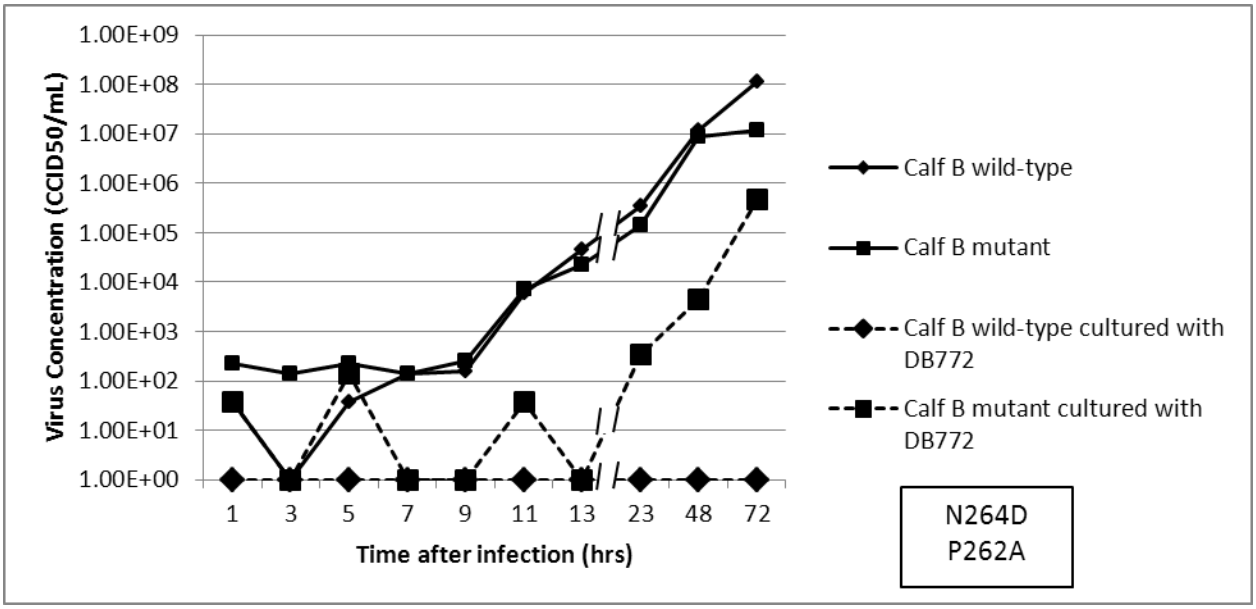
Results

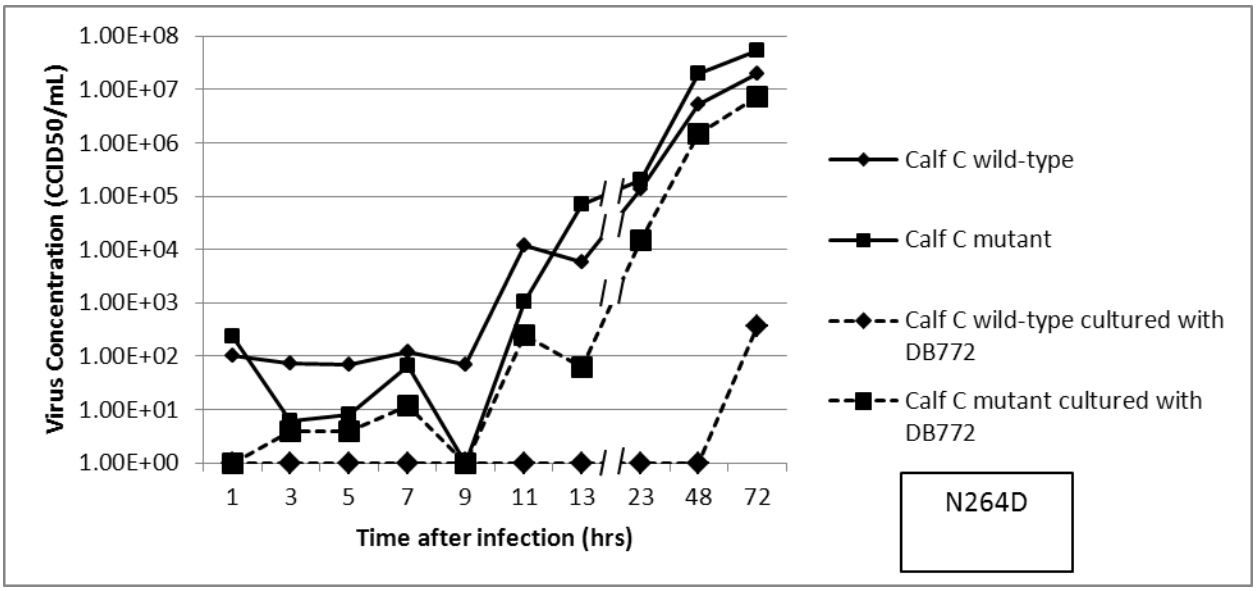
3.1 Genetic sequencing

Genetic sequencing of the open reading frame (ORF) encoded by the genomic RNAs from isolates collected from treated calves revealed three common point mutations in the NS5B that resulted in an amino acid change. One mutation was common to all three resistant isolates (B, C, D) but was absent from the isolate that remained susceptible to DB772 *in vitro* (A) and the isolates from the untreated controls. This common mutation resulted in an asparagine to aspartic acid amino acid change at residue position 264 (N264D). Two of the three isolates displaying the common mutation displayed an additional point mutation in the same region. The isolate from Calf B displayed a proline to alanine change at residue position 262 (P262A) and the isolate from Calf C displayed an isoleucine to methionine change at residue position 261 (I261M). No other amino acid changes were observed in any of the wild-type/mutant isolate pairs.

3.2 Growth kinetics

The multi-step growth curve of each isolate is depicted in Figure 9. After one hour of incubation, the average virus concentrations for each wild-type/mutant pair were similar when incubated in the absence of DB772. Similar titers were seen after nine hours





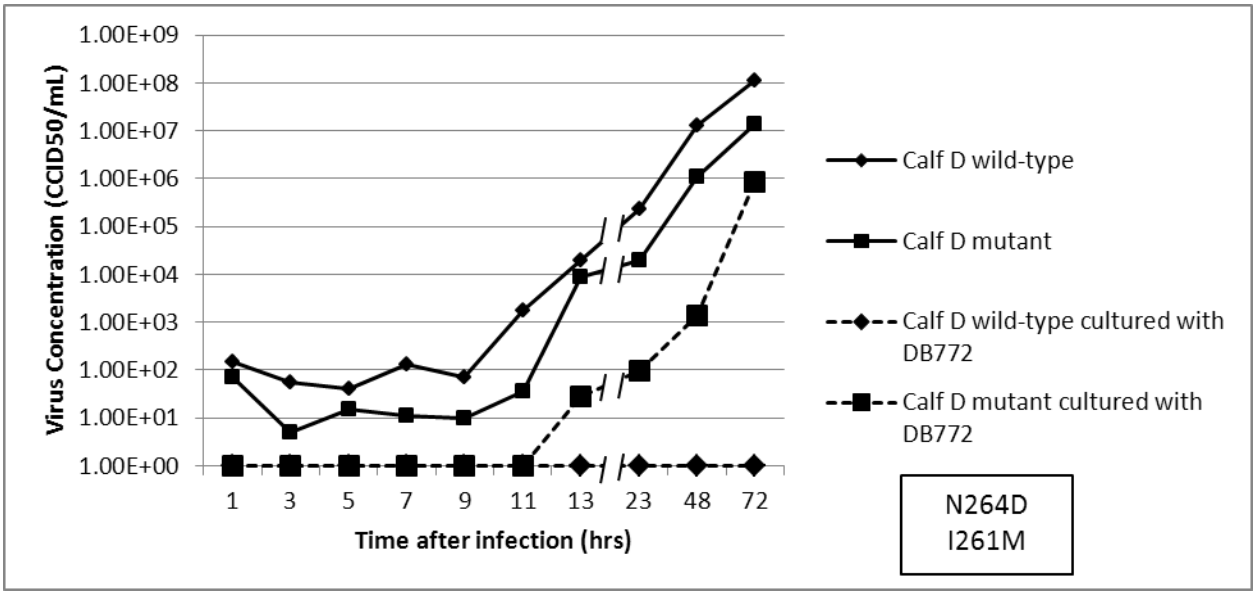


Figure 9. Virus concentration after infection of cells with wild-type (diamond) or mutant (square) BVDV isolates from Calves B, C and D after incubation for various time periods in the absence (solid line) or presence (dashed line) of DB772. The mutations associated with each isolate are shown in the inset.

of incubation for the wild-type isolates although mutant isolates C and D were lower than their wild-type counterparts. Thereafter, the average concentration for each isolate increased at each time point with the exception of wild-type isolates incubated in the presence of the antiviral compound. The average virus concentration of the isolate from Calf D containing the single N264D mutation was consistently one log score lower than the average concentration of the susceptible isolate with the exception of the 13 hr incubation time point when the virus concentration of the wild-type isolate was 20,000 $CCID_{50}$/mL while that of the mutant isolate reached 9,000 $CCID_{50}$/mL. The average virus concentration of the mutant isolate from Calf C containing the N264D and I261M

mutations lagged behind that of its wild-type counterpart until the 13 hour time point, after which time the average concentrations were largely similar. The growth kinetics pattern for the isolates from Calf B were largely similar to those of Calf C with the exception that comparable concentrations between the wild-type and resistant isolates were seen beginning at the seven hour time point and onward.

Virus was not detected consistently when mutant isolates were incubated in the presence of DB772 until after at least nine hours of incubation. The concentration of each mutant isolate was lower when incubated in the presence of DB772 then when cultured in media alone. This difference was most evident for isolates from calves B and D where the titer was at least two log scores lower at each time point with the exception of the isolate from Calf D at the final time point when there was only a 1.3 log score decrease. Mutant isolate C also reached lower titers in the presence of DB772 but to a lesser extent than the other isolates with approximately only one log score difference seen after 13 hours of incubation. With the exception of a single replicate of isolate C at a single time point, wild-type isolates did not grow in the presence of DB772, confirming their susceptibility to the compound.

3.3 Clonal Fitness

Over 85 individual clones were sequenced for each of the co-infected inocula from calves B, C and D at both the 90:10 and 10:90 ratios of wild-type and mutant isolates (Table 3). All sequenced NS5b segments, at both ratios from calves B and D**,** were lacking the mutations associated with DB772 resistance after serial passage. For Calf C, 96 replicates were sequenced from the inoculum that was originally co-infected

Virus	Infectious Portion		Replicates	Sequence Result		% Wild-type	Mutation(s)
	Wild-type	Mutant		Wild-type	Mutant		
B	90	10	86	86	0	100.0	N264D, P262A
C	90	10	96	87	9	90.6	N264D, I262M
D	90	10	100	100	0	100.0	N264D
B	10	90	87	87	0	100.0	N264D, P262A
C	10	90	90	1	89	1.1	N264D, I262M
D	10	90	86	86	0	100.0	N264D

Table 3. Sequencing results following five passages in white blood cells after co-infection with fixed ratios of wild-type and mutant virus isolates.

with 90% wild-type isolate; 87 replicates mirrored the wild-type isolate while 9 contained the two mutations described in the resistant isolate. From the inoculum that was originally co-infected with 10% wild-type isolate, 90 replicates were sequenced with all but one containing the mutations in the NS5B region associated with resistance.

Discussion and Conclusions

We recently reported, for the first time, the use of directed antiviral therapy to markedly reduce viremia in calves persistently infected with BVDV (Newcomer et al., 2012a). In this manuscript we describe three mutations associated with *in vivo* and *in vitro* resistance to the antiviral compound DB772 isolated from treated calves in the previous study. All three mutations are found in the NS5B which functions as the RdRP during viral replication. In addition to the fingers, palm and thumb domains shared by other RdRPs, the RdRP of BVDV also contains a unique N-terminal domain of unknown function (Choi et al., 2004). The mutations identified in the drug-resistant isolates in this study are all located within the region of the fingers domain that associates with the thumb domain. During elongation, the fingers and thumb domains are thought to form a channel to direct the template to the catalytic residues of the palm domain. The domain containing the mutations is believed to critically affect several enzyme activities important to de novo and elongative RNA synthesis including template translocation, protein-protein interactions and formation of the replication complex (Lai et al., 1999;Tonelli et al., 2010). The location of the mutations suggests the mechanism of

action of DB722 may be through binding to this domain but binding is inhibited or disrupted by the mutation.

As *in vivo* BVDV antiviral studies are severely limited, we describe for the first time evidence of mutations to the BVDV genome following *in vivo* antiviral treatment. However, prior *in vitro* work supports the results of this study. The mutation common to all three isolates, N264D, has been described following *in vitro* studies involving thiosemicarbazone (Castro et al., 2011) and derivatives of 2-phenylbenzimidazole (Tonelli et al., 2010). The second mutation seen in isolates from Calf C, I261M, has also been described in *in vitro* studies of arylazoenamine derivatives in conjunction with other mutations in the same domain (Giliberti et al., 2010). To our knowledge, the second mutation seen in Calf B, P262A, has not been previously reported following antiviral treatment. The same pair of mutations we observed in the isolate from Calf C was also described following *in vitro* antiviral treatment when using derivatives of 2-phenylbenzimidazole which have been shown to inhibit the RdRP (Tonelli et al., 2010). For the described *in vitro* studies, the mutations were selected for by using the drugs at 10-32 times the half maximal effective concentration (EC_{50}). In our study, the dose of antiviral compound administered to the persistently infected calves was calculated to achieve a 4 μM concentration in serum, the same concentration shown to eliminate BVDV infection in contaminated bovine fetal fibroblast cells in a single passage (Givens et al., 2004).

This is the first published report of resistance to DB772. All previously tested strains of BVDV and other pestiviruses have been uniformly susceptible to the compound *in vitro* at micromolar concentrations of DB772 (Givens et al., 2003a; Newcomer et al.,

2012b). However, this was the first *in vivo* test of the compound. The calves in the study weighed between 26 and 34 kg and had an average nasal titration of 7.9×10^4 $CCID_{50}$/mL immediately before initiation of treatment (Newcomer et al., 2012a). Serum levels of viremia in persistently infected cattle typically range between 5×10^2 and 5×10^5 $CCID_{50}$/mL (Brock et al., 1998). Therefore, the PI animal represents an extremely robust test for antiviral therapeutics due to the consistently high level of viremia exhibited by such animals.

Because of its important role in genome replication, the NS5B region is well conserved among the pestiviruses. The described mutations are not found in any wild-type strains of BVDV currently in GenBank indicating considerable selective pressure is at work to maintain sequence homology at the defined residue positions. Mutant isolates consistently grew better when cultured in the absence of DB772 compared to when cultured in a concentration of 4 µM DB772. Since the mutations associated with resistance were located in such a critical region of NS5B we were interested to compare the growth kinetics of each mutant strain to its wild-type counterpart. Increases in average viral concentration were not seen in wild-type or mutant isolates until nine hours post infection, consistent with previously established one-step growth curves for BVDV (Gong et al., 1996). Thereafter, the growth curves for wild-type and mutant isolates were largely similar, particularly for isolates B and C. The average virus concentration of isolate D containing only the N264D mutation lagged behind the wild-type isolate by approximately one log score at almost all time points. Virus was not found after incubation of wild-type isolates in the presence of DB772 with the exception of a single time-point (72 hrs) in a single replicate from the Calf C isolate. Because no evidence of

growth was present at any previous time-points in any replicates of any isolate, this is assumed to be a contaminant although sequencing of the isolate is needed to confirm the nature of the isolate. Absence of growth in DB772 by the wild-type isolates is consistent with the suggested mechanism of RdRP interference, especially when coupled with the location of the observed mutations. Molecular modeling could be used to confirm the target site of DB772.

In addition to a comparison of growth curve kinetics, the level of fitness of mutant isolates was evaluated by serially passaging a fixed ratio of mutant and wild-type isolates in MDBK cells. We believe this to represent a more stringent test of viral fitness as the isolates are forced to compete against the wild-type isolates under controlled conditions for multiple passages. After five serial passages, the mutant isolates B and D were unable to outcompete their respective wild-type isolates and in fact, were outcompeted by the wild-type isolates. Even when cells were infected with nine times the number of the respective mutant isolate compared to the wild-type isolate, no isolates containing the original mutations were observed after serial passage. Consequently, even though the growth curves were similar for the mutant and wild-type isolates, the wild-type isolates appear to have a competitive advantage after multiple passages through white blood cells. Further study is needed to fully characterize the effects of the observed mutations.

Recently, another study using three 2-phenylbenzimidazole derivatives selected BVDV resistant mutants containing the same N264D mutation (Tonelli et al., 2010). The authors of that study used molecular modeling to determine the mutation resulted in a decrease in both van der Waals and electrostatic forces in the BVDV RdRP. A third isolate in our study (C), containing the I261M mutation in addition to the N264D

mutation, was not outcompeted by its wild-type counterpart. The growth curve of the isolate from Calf C was most similar to the curve of its corresponding wild-type isolate in comparison to the isolates from Calves B and D. As in our study, previous documentation of the I261M mutation has only been reported in conjunction with other mutations in the same region following antiviral treatment (Giliberti et al., 2010;Tonelli et al., 2010). Thus, the mutation appears to be associated with resistance to several antiviral compounds. The similar location of the mutations described in this study to those observed previously in treated cell cultures suggests a similar mechanism of action of the compounds.

In summary, the mutations in DB772 resistant isolates after *in vivo* treatment of PI calves are similar to mutations described previously following *in vitro* treatment with separate antiviral compounds. The mutations suggest the mechanism of action of the antiviral compound DB772 is through inhibition of the RdRP. Continued investigation of the compound for prophylactic and therapeutic use for diverse pestivirus infections should be directed at developing combination therapies with compounds with separate mechanisms of action to limit the development of mutations associated with compound resistance.

Summary and Conclusions

Over the past three-quarters of a century, BVDV has evolved from an undiscovered entity to a significant clinical pathogen, laboratory contaminant and virus research surrogate. Despite targeted study, specific therapeutics to treat BVDV infection have eluded commercial development and prophylactics are limited to killed or modified-live vaccines. Many compounds showing promise *in vitro* have lacked efficacy when tested in the live animal or have been shown to carry significant negative side effects which outweigh the benefits of the drug. Consequently, BVDV remains a costly viral pathogen for farmers, veterinarians and laboratory managers alike and the hunt for effective compounds to combat BVDV infection continues. The objective of this research was to further characterize an antiviral compound (DB772) that was found to clear BVDV from infected cells or prevent cellular infection at concentrations several-fold smaller than those required to cause evidence of cytotoxicity (Givens et al., 2003a; Givens et al., 2004).

The initial experimental method was to determine if treatment of PI calves with DB772 could clear the BVDV infection. Preliminary results were highly encouraging as virus isolation assays on all treated calves were consistently negative after one day of treatment. However, virus was subsequently isolated from three of the four treated calves during or immediately following the treatment period and virus was isolated from the

fourth treated calf eight days after cessation of treatment indicating that the described dosing regimen was insufficient to clear BVDV from PI calves. Additional studies were designed to characterize the pre- and post-treatment isolates obtained from all four calves. Pre-treatment isolates from all calves were uniformly susceptible to DB772 *in vitro* but isolates obtained from the three calves during or immediately following the treatment period consistently demonstrated *in vitro* resistance to the compound. Isolates from the fourth calf remained susceptible to DB772 *in vitro*, suggesting that treatment for a longer period or at a higher duration may have been successful at clearing the infection.

Regardless, it appears clear that DB772 does exhibit antiviral activity against BVDV *in vivo*; however selection of resistant mutant phenotypes is a significant, although not unexpected, concern. Due to the poor fidelity of the RNA polymerase, BVDV and other RNA viruses have a high rate of mutagenesis and are often talked about as existing as a quasi-species swarm (Brock 2004). Consequently, the use of DB772 as a therapeutic agent may be most advantageous as a component of combination therapy such as that used to treat closely related persistent HCV infections (Seeff and Hoofnagle, 2002). Genomic sequencing of the resistant isolates revealed single or multiple point mutations within NS5b. Further research using cDNA clones and site-directed mutagenesis will provide definitive proof that the associated mutations are sufficient to convey DB772 resistance.

The potential for DB772 to be used prophylactically was also examined by administering the compound to naïve calves. Calves seronegative to BVDV were challenged intranasally with the virus after receiving two doses of DB772; DB772 administration continued for an additional three days after challenge. Cohort calves were

administered diluent only while challenged with the virus on the same experimental schedule. Virus was isolated consistently from untreated calves on Days 4 to 8 while treated calves remained negative by virus isolation during this period. However, the treated calves remained susceptible to subsequent BVDV challenge after serum concentrations of DB772 were calculated to have fallen below the minimum *in vitro* inhibitory concentration. However, this study showed that DB772 holds potential in outbreak situations where the disease has previously been eradicated. Treating animals at the perimeter of the outbreak may halt transmission by establishing a barrier of animals resistant to infection. More concerning was the evidence of renal toxicity associated with DB772 administration. Unlike previous studies, treatment with the compound was associated with azotemia and tubulointerstitial nephritis. Further study must address the risk of renal toxicity before the antiviral benefits of the compound can be realized.

A third focus of this research was a determination of the scope of *in vitro* efficacy of DB772 amongst the pestiviruses. Cells were infected with BVDV2, BDV, HoBi virus, pronghorn virus or Bungowannah virus and incubated with concentrations of DB772 ranging from 0-25 μM. A concentration of $\geq$ 0.2 μM DB772 was sufficient to completely inhibit replication of all pestiviruses tested. Earlier studies investigating DB772 as an antiviral therapeutic for the closely related HCV were not continued due to perceived lack of efficacy and commercial potential. Thus, the antiviral scope of DB772 appears to span all the pestiviruses but has limited activity against viruses of other genera. While the susceptibility of CSFV to DB772 was not specifically tested in this study, results suggest the virus would likely be susceptible to the compound *in vitro*. Molecular analyses of complete genome sequences of CSFV have consistently exhibited a high level of

sequence identity with other member pestiviruses, particularly isolates of BDV (Becher et al., 1995;Ridpath and Bolin 1997) suggesting a similar pattern of susceptibility. Further study is needed to confirm the expected susceptibility of CSFV.

In summary, DB772 is shown to inhibit viral replication in calves PI with BVDV although the rapid development of viral resistance to the compound is a significant concern. The development of resistance after *in vivo* treatment of PI calves is associated with single or multiple point mutations in NS5b which suggests the mechanism of action of the antiviral compound DB772 is through inhibition of the RdRP. Additionally, BVDV viremia and clinical signs were effectively prevented in naïve calves during treatment with DB772. However, DB772 administration was associated with signs of acute renal toxicity that resulted in the death or euthanasia in two of four treated animals. Thus, this research proves the concept that an antiviral agent can be used to prevent acute disease caused by BVDV although further investigation is required to ensure that acute renal toxicity does not result from antiviral treatment. Micromolar concentrations of DB772 are sufficient to completely inhibit viral replication of all pestiviruses against which it was tested. Continued investigation of the compound for prophylactic and therapeutic use for diverse pestivirus infections should be directed at minimizing renal insult and developing combination therapies with compounds with separate mechanisms of action to limit the development of mutations associated with compound resistance.

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