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Short Communication

Efficacy of an antiviral compound to inhibit replication of multiple pestivirus species

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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.

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., 2005) 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, (Kumar et al., 1995; Vonderfecht et al., 1988; 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 BVDV1 growth in cell culture at concentrations lacking cytotoxicity (Givens et al., 2003) 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 h 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 1 h, the inoculum was removed and the wells washed twice with 500 μL phosphate buffered solution without calcium or magnesium. One milliliter of MEM containing the appropriate concentration of DB772 was added back to each well and the plates incubated for

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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 (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 rt-PCR. Samples spiked with 25 μM DB772 does not result in detectable inhibition of reverse transcriptase, Taq polymerase or other enzymes involved in the rt-PCR 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 μL M-MLV reverse transcriptase, 0.5 μL DNA Taq polymerase, 20 μL RNase inhibitor and 1 μL each of forward and reverse primers. For HoBi virus, 20 μL of purified RNA template was used. The rt-PCR 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 30 s and 72 °C for 30 s. 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 15 s and 72 °C for 45 s. 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′-ATGCCCATAG TAGGACTAGCA-3′ and 5′-TCAACTCCATGTGCCATGTAC-3′. The primers for Bungowannah virus were 5′-AAGCGGTGAGTACACCG TATTCGT-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 h incubation period. 96-Well plates containing approximately 5000 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 1). 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 >0.2 μM DB772. Like BVDV2, BDV replication was completely inhibited at concentrations >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 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 >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., 2003, 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., 2003).

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

Table 1
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 RT-PCR with positive results indicated by (+). Negative test results are indicated by (−).

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	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	+	+

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 μM 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.

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