Mechanism of action of a pestivirus antiviral compound

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We report here the discovery of a small molecule inhibitor of pestivirus replication. The compound, designated VP32947, inhibits the replication of bovine viral diarrhea virus (BVDV) in cell culture at a 50% inhibitory concentration of approximately 20 nM. VP32947 inhibits both cytopathic and noncytopathic pestiviruses, including isolates of BVDV-1, BVDV-2, border disease virus, and classical swine fever virus. However, the compound shows no activity against viruses from unrelated virus groups. Time of drug addition studies indicated that VP32947 acts after virus adsorption and penetration and before virus assembly and release. Analysis of viral macromolecular synthesis showed VP32947 had no effect on viral protein synthesis or polyprotein processing but did inhibit viral RNA synthesis. To identify the molecular target of VP32947, we isolated drug-resistant (DR) variants of BVDV-1 in cell culture. Sequence analysis of the complete genomic RNA of two DR variants revealed a single common amino acid change located within the coding region of the NS5B protein, the viral RNA-dependent RNA polymerase. When this single amino acid change was introduced into an infectious clone of drug-sensitive wild-type (WT) BVDV-1, replication of the resulting virus was resistant to VP32947. The RNA-dependent RNA polymerase activity of the NS5B proteins derived from WT and DR viruses expressed and purified from recombinant baculovirus-infected insect cells confirmed the drug sensitivity of the WT enzyme and the drug resistance of the DR enzyme. This work formally validates NS5B as a target for antiviral drug discovery and development. The utility of VP32947 and similar compounds for the control of pestivirus diseases, and for hepatitis C virus drug discovery efforts, is discussed.

Pestivirus infections of domesticated livestock (cattle, pigs, and sheep) cause significant economic losses worldwide, as exemplified by recent disease outbreaks in the U.S., Canada, The Netherlands, and Germany (1–7). Bovine viral diarrhea virus (BVDV), the prototypic representative of the pestivirus genus, family Flaviviridae, is ubiquitous and causes a range of clinical manifestations, including abortion, teratogenesis, respiratory problems, chronic wasting disease, immune system dysfunction, and predisposition to secondary viral and bacterial infections (8). Certain BVDV strains can cause acute fatal disease with mortality rates of 17–32% (1, 9, 10). BVDV is also able to establish persistent infections in fetuses (11, 12). When born, these persistently infected animals remain viremic throughout life and serve as continuous sources for virus spread in herds. Persistently infected animals may also succumb to fatal mucosal disease on superinfection with closely related BVDV strains. Vaccines are used in some countries in an attempt to control pestivirus disease with varying degrees of success (8, 13). In other countries, animal culling, quarantine, and slaughter are used to contain pestivirus disease outbreaks (2). Currently, there are no antiviral pharmaceutical options for controlling pestivirus diseases.

Hepatitis C virus (HCV), a member of the hepacivirus genus, family Flaviviridae, is a major cause of human hepatitis throughout the world. The World Health Organization estimates that 170 million people are chronically infected with HCV. Improved therapies are urgently needed to manage the disease burden caused by HCV. Unfortunately, study of HCV has been hampered by the inability to propagate the virus efficiently in cell culture. However, HCV shares many molecular and virological similarities with pestiviruses. Both HCV and BVDV have single strand RNA genomes (approximately 9,600 and 12,600 nucleotides in length, respectively) that encode about nine functionally analogous gene products (14). Thus, comparative studies with the pestiviruses have greatly facilitated our understanding of HCV. Pestiviruses, in fact, have been adopted by many as a model and surrogate for HCV.

In view of the economic impact of pestivirus diseases and the important relationship between pestiviruses and HCV, we sought to identify inhibitors of pestivirus replication. Here, we report the discovery of a potent and selective small molecule inhibitor of pestivirus replication and the characterization of its mechanism of action. The utility of this and similar compounds for the control of pestivirus diseases, and for HCV drug discovery efforts, is discussed.

Materials and Methods

Cells and Viruses. Madin-Darby bovine kidney (MDBK) cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% horse serum (DMEM-HS) at 37°C in a humidified, 5% CO_2 atmosphere. BVDV-1 (strain NADL) has been described (15).

Inhibitor Compound. The preparation of VP32947 (3-[((2-dipropylamino)ethyl)thio]-5H-1,2,4-triazino[5,6-b]indole) is described elsewhere (T. J. Nitz, D. Cebzanov, M. McWherter, S. Caggiano, C. Blackledge, J. Romano, P. Chang, T. Bailey, M.S., and D.C.P., unpublished work). Stock solutions of VP32947 were made in dimethyl sulfoxide (DMSO).

Virus Cytopathic Effect Assay. Ninety-six-well cell culture plates were seeded 24 h before use with 2×10^4 MDBK cells per well in DMEM-HS. Virus inocula were adjusted to result in a greater than 85% destruction of the cell monolayer after 3 days. For drug sensitivity testing, virus inoculum in 150 μl was adsorbed to cells for 1 h. Fifty microliters of each drug dilution adjusted to a final 0.5% DMSO in DMEM-HS was then added. Each dilution was tested in quadruplicate. Uninfected cells and cells receiving virus without compound were included on each assay plate. After 3 days, plates were fixed with 5% glutaraldehyde and were stained with 0.1% crystal violet. After rinsing and drying, the optical density of the wells was read at 570 nm (OD_{570}) in a microplate reader. The 50% inhibitory concentration (IC_{50}) value was

Abbreviations: BVDV, bovine viral diarrhea virus; HCV, hepatitis C virus; DR, drug-resistant; WT, wild-type; RdRp, RNA-dependent RNA polymerase; MDBK cell, Madin-Darby bovine kidney cell; HS, horse serum.

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defined as the concentration of compound that protected 50% of the cell monolayer from virus-induced cytopathic effect.

Time of Drug Addition Assay. MDBK cells seeded onto 24-well plates (4×10^5 cells/well) were infected 24 h later with BVDV at a multiplicity of infection of approximately 1 plaque-forming unit per cell. After a 1-h adsorption period, the inoculum was removed, and cell monolayers were washed twice with PBS and then overlaid with DMEM-HS. VP32947 was added at the indicated times to a final concentration of 167 nM. For the "minus 1 h" time point, VP32947 was included during the 1-h adsorption period. All plates were frozen at 14 h postinfection, and the extent of virus replication was quantified by plaque assay.

Plaque Assay. MDBK cell monolayers in 6-well plates were infected with dilutions of test samples in M199 medium (Sigma) supplemented with 5% HS and antibiotics. Inoculum was removed after 1 h, and cells were overlaid with Earles MEM supplemented with 2.2 g/liter sodium bicarbonate, 12.5 mM Hepes, antibiotics, 5% HS, and 1% Seaplaque agarose (FMC). After 3 days, plaques were either picked for virus plaque purification or were quantified by manual counting after the cells were fixed and stained.

Viral RNA Synthesis. MDBK cells in 96-well plates were infected with virus at a multiplicity of infection of 0.1. After 22 h, cell monolayers were washed with DMEM-HS supplemented with 5 μg/ml actinomycin D and then were overlaid with fresh DMEM-HS containing 5 μg/ml actinomycin D and 25 μCi/ml ^{3}H-uridine supplemented with either 167 nM VP32947 in a final DMSO concentration of 0.5% or DMSO alone. At various times thereafter, the medium was removed, and the cells were washed once with cold PBS and then were lysed on ice for 30 min with 25 μl/well of lysis buffer (20 mM EDTA, pH 8.0/0.3% deoxycholate in water). Nucleic acids were precipitated by addition of 200 μl/well of ice-cold 10% trichloroacetic acid containing 1% sodium pyrophosphate and were collected onto wetted 96-well glass fiber filters (Millipore). Radioactivity was quantified in a 96-well plate liquid scintillation counter (Wallac, Gaithersburg, MD). All data represent the average of six replicate determinations.

Selection of Drug-Resistant Viruses. Wild-type (WT) BVDV stock was generated from triple-plaque purified virus derived from an infectious cDNA clone (16). Virus inoculum was adsorbed to MDBK cells in 6-well culture plates for 1 h and was removed, and the cultures were overlaid with M199 medium supplemented with 2% HS and either 167 nM or 500 nM VP32947. After 3 days, cultures were frozen and thawed. The lysates were used to infect fresh cell monolayers in culture medium containing the respective level of drug. These cultures were frozen and thawed after 3 days, and putative drug resistant viruses were isolated by three cycles of plaque purification in the presence of VP32947.

Sequencing. RNA was extracted from virus-infected MDBK cells with TRIzol Reagent (Life Technologies) according to the manufacturer's protocol. After reverse transcription using Superscript II (Life Technologies), DNA was amplified by PCR using KlenTaq Long-Accurate polymerase and was sequenced by using the ABI PRISM Dye Terminator Cycle Sequencing protocol and AmpliTaq DNA polymerase (Perkin–Elmer). BVDV-specific primers for sequencing reactions were spaced approximately 200 nucleotides apart in both polarities along the entire viral genome. Typical sequencing runs yielded approximately 450 nucleotides of sequence.

Infectious Clone Construction. Plasmid pACNR/NADL, containing a full-length BVDV-1 (NADL) functional clone, has been described (16). Introduction of the F224S change (TTC to TCC) in the NS5B gene used standard site-directed mutagenesis (17) and molecular cloning techniques (16) and resulted in plasmid pACNR/NADL/DR. All plasmid constructions were verified by restriction endonuclease digestion and DNA sequence analyses.

RNA Transcription and Transfection. Procedures used for standard *in vitro* plasmid transcription using the T7-MEGAscript kit (Ambion, Austin, TX) and for transfection of MDBK cells by electroporation have been described (16). Plasmid pACNR/NADL/DR was transcribed, and the resultant RNA was transfected into MDBK cells. After 3 days, the culture was harvested by two freeze-thaw cycles, and the F224S drug-resistant (DR) virus was three-times plaque-purified.

Recombinant Baculovirus Protein Expression. Recombinant baculoviruses containing the WT and DR NS5B genes were constructed by using the pFastBac system (GIBCO/BRL). The amino acid change in the DR viruses (F224S) was introduced into the WT gene by site-specific mutagenesis (17). The WT and DR NS5B coding sequences were amplified by PCR from plasmids using primers that introduced a methionine codon immediately before the first amino acid of the mature protein and the naturally occurring termination codon after the last amino acid and were cloned into the pFastBac plasmid. The protein coding regions and cloning junctions of all recombinant baculovirus transfer plasmids were verified by DNA sequencing. NS5B protein expression in recombinant baculovirus-infected cells was confirmed by Western immunoblot analysis with antiserum specific to NS5B protein (αB3B; ref. 18).

Protein Purification. All operations were carried out at 4°C. Frozen recombinant baculovirus-infected Sf9 cell pellets were resuspended in lysis buffer (50% glycerol/20 mM Tris·HCl, pH 7.5/10 mM DTT/0.5 M NaCl/1 mM EDTA/2% Triton X-100) supplemented with a Complete Protease Inhibitor tablet (Boehringer Mannheim). $MgCl_2$ was added to a final concentration of 10 mM, followed by 10 units of DNase I (RQ-1, Promega). After 30 min on ice, lysates were clarified by centrifugation at 100,000 $\times$ *g* for 35 min, were diluted to 0.3 M NaCl with elution buffer (20% glycerol/20 mM Tris·HCl, pH 7.5/10 mM DTT/1 mM EDTA/0.5% Triton X-100), and were applied to a DEAE Sepharose column equilibrated in elution buffer plus 0.3 M NaCl. Flow-through material was collected, was diluted to 0.2 M NaCl with elution buffer, and was loaded onto a heparin Sepharose column equilibrated in elution buffer plus 0.2 M NaCl. Bound proteins were eluted with a linear NaCl gradient. NS5B-containing fractions were pooled and loaded onto a Cibacron Blue column equilibrated in elution buffer containing 0.4 M NaCl. Bound proteins were again eluted with a linear NaCl gradient. NS5B-containing fractions were pooled, were dialyzed against storage buffer (50% glycerol/10 mM Tris·HCl, pH 7.2/50 mM NaCl/1 mM EDTA/0.01% Triton X-100), and were stored at −20°C.

RNA-Dependent RNA Polymerase Assay. RNA-dependent RNA polymerase (RdRp) reaction mixtures (40 μl) contained 16 mM Hepes (pH 7.5), 4 mM $MgCl_2$, 1 mM DTT, 5 μCi [α-^{32}P]GTP, 0.5 μM GTP, 0.5 mM each of ATP, CTP, and UTP, 20 units of RNasin, and 100 ng of NS5B protein. Enzyme IC_{50} values were determined in reaction mixtures containing 1% DMSO and various concentrations of VP32947. Reactions were initiated by the addition of pOF1213 RNA (150 ng). pOF1213 RNA was transcribed with SP6 RNA polymerase from plasmid pOF1213 (provided by James Flanegan, University of Florida) that had been linearized previously with *Eco*RI. Unincorporated nucleoside triphosphates were removed by Sephadex G50 gel filtration, and the transcript RNA was precipitated with ethanol and stored at −80°C until use. pOF1213 RNA transcripts contain 56 nucleotides of vector sequence, the

poliovirus 3′ terminal sequence (nucleotides 7,206–7,440), a 3′ poly $(A)_{80}$ tract followed by a 3′ terminal –GAAUU. RdRp reactions were incubated for 60 min at 30°C. The reaction product was quantified by trichloroacetic acid precipitation onto nitrocellulose filters followed by liquid scintillation counting. Steady-state kinetic parameters k_{cat} and K_m for RNA substrate were determined in the above reaction mixture by varying the RNA substrate concentration from 0.5 to 38 μM. Values for each nucleoside triphosphate were determined while the other three were maintained at 0.5 mM. Data were fit to the Michaelis-Menten equation by using the GraphPad (San Diego) PRISM program and analyzed by best fit analysis (19).

Results

Identification of a Small Molecule Inhibitor of BVDV Replication. During the course of screening small molecule chemical libraries for inhibition of BVDV replication, we discovered a compound that exhibited potent inhibition of the cytopathic effects of BVDV infection on MDBK cells. This compound, designated VP32947, showed a dose-dependent inhibition of BVDV-1 (NADL) replication with an IC_{50} value of approximately 20 nM. Details of the chemistry of VP32947, a triazinoindole, and structure-activity relationships of the chemical series are described elsewhere (T. J. Nitz, D. Cebzanov, M. McWherter, S. Caggiano, C. Blackledge, J. Romano, P. Chang, T. Bailey, M.S., and D.C.P., unpublished work).

VP32947 showed antiviral activity against virus isolates from all four pestivirus species, including cytopathic and noncytopathic isolates of BVDV-1 (NADL, NY1) and BVDV-2 (125C, 125NC; S. Bolin, personal communication), border disease virus (strain 31), and classical swine fever virus (strain C; C. Bruschke, personal communication). However, the replication of unrelated viruses, such as dengue virus (type 2), yellow fever virus (17D), influenza A virus (WSN), respiratory syncytial virus (A2), coxsackievirus (B3), and herpes simplex virus (type 2), was unaffected by VP32947 at the highest testable drug concentration (2 μM). Under the conditions of these antiviral assays, VP32947 did not exhibit cellular cytotoxicity. When evaluated in a proliferating cell cytotoxicity assay (MTT assay; ref. 20) on MDBK cells, the 75% cytotoxic concentration (CC_{75}) for VP32947 was approximately 2 μM, indicating that the compound has a selectivity index of 100.

We sought to determine the mechanism of action of VP32947 by conducting initially several exploratory experiments. First, we performed a time of drug addition experiment to see at what point during a virus single cycle growth curve the drug was required to exhibit its inhibitory effects. We had previously established that a single cycle of virus growth in our system was 14 h. At various times after infection of MDBK cells with BVDV, VP32947 was added to the culture medium. Then, at 14 h postinfection, cultures were harvested, and the amount of virus produced was quantified by plaque assay. As shown in Fig. 1, VP32947 added to the culture up to 8 h postinfection inhibited greater than 95% of the virus yield whereas addition of the drug after 8 h postinfection allowed some level of virus replication. These results suggest that the drug acts on a process after virus attachment and penetration but before virus assembly and release.

We therefore explored the effect of VP32947 on viral macromolecular synthesis. To examine protein synthesis and polyprotein processing, virus-infected cells were treated with or without VP32947 in the culture medium for 1 h before the addition of [^{35}S]methionine for 2 h. After immunoprecipitation and protein gel analysis, we saw no differences in the radiolabeled viral polypeptide profiles between the drug- and mock-treated samples, suggesting that the drug did not act at the level of viral protein expression.

We next investigated the effect of VP32947 on viral RNA synthesis. Virus-infected cells were treated with actinomycin D (to inhibit cellular RNA synthesis) with or without VP32947 in the culture medium for 1 h, at which time ^{3}H-uridine was added to the medium. Under these labeling conditions, only viral RNA is radiolabeled (21). At various times after the addition of ^{3}H-uridine, the amount of radioactivity incorporated into viral RNA was quantified. Although the drug-free culture showed the time-dependent incorporation of ^{3}H-uridine into viral RNA, there was a clear inhibition of incorporation of tritium into viral RNA in the drug-treated culture (Fig. 2). When this experiment was performed in the absence of actinomycin D, wherein the vast majority of radioactivity is incorporated into ribosomal RNA, no change in ^{3}H-uridine incorporation was observed in the presence of drug, indicating that VP32947 had no obvious effect on uridine uptake by the cells or on cellular RNA synthesis. These results suggest that VP32947 exerted its antiviral effect at the level of viral RNA synthesis.

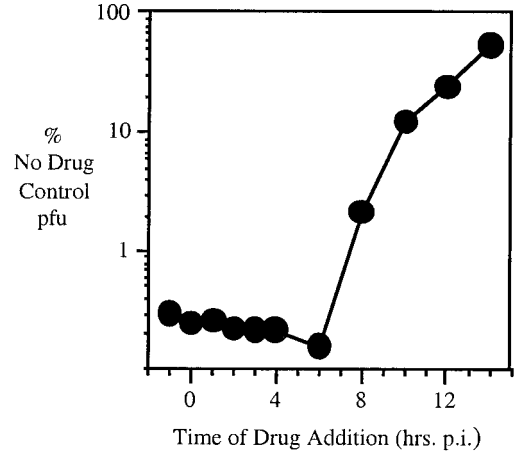

Fig. 1. Effect of time of VP32947 addition on inhibition of BVDV growth. VP32947 was added to BVDV-infected MDBK cell cultures to a final concentration of 167 nM at the indicated times postinfection. For the ''minus 1 h'' time point, VP32947 was included during the 1-h virus adsorption period. All cultures were harvested at 14 h postinfection, and virus yield was quantified by plaque assay. The data are presented as the percent of virus produced in the absence of drug.

Isolation of VP32947-Resistant Mutants. To investigate further the antiviral mechanism of VP32947, we isolated viruses that were

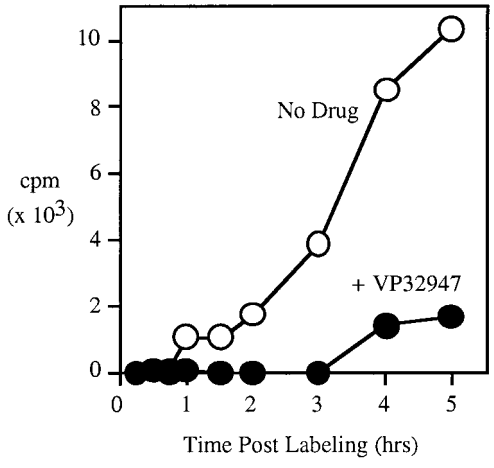

Fig. 2. Effect of VP32947 on viral RNA synthesis in WT virus-infected cells. Incorporation of ^{3}H-uridine into viral RNA in the absence (○) or presence (●) of 167 nM VP32947 was determined as described in *Materials and Methods*.

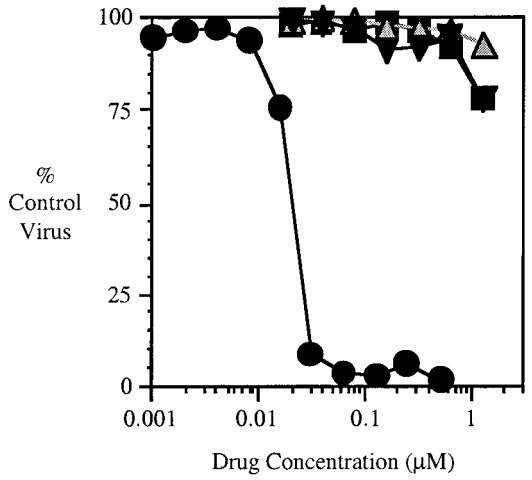

Fig. 3. Effect of VP32947 on DR virus growth. The growth of WT (●), DR clone 2 (▼), DR clone 3 (■), and F224S DR (△) viruses in the presence of increasing concentrations of VP32947 was assessed in a virus cytopathic effect assay. Data are plotted as the percent of control virus cytopathic effect.

resistant to inhibition by the drug and then characterized these drug-resistant (DR) virus variants virologically and at the molecular level. The parental virus used for these studies was BVDV-1 (NADL) derived from an infectious clone (16), hereafter referred to as wild-type (WT) virus. The ability of virus in a BVDV population to survive VP32947 treatment was determined by infecting cells with 10^7 plaque-forming units of virus in the presence of varying concentrations of VP32947 and quantifying surviving virus by plaque assay. Virus survival was inhibited by VP32947 in a concentration-dependent manner up to approximately 167 nM. At this concentration, BVDV plaque formation was reduced by over 4 orders of magnitude but was not further reduced with increasing drug concentration. From this, we estimate the frequency of resistance to VP32947 in the WT virus population was approximately 4.5×10^{-5}.

VP32947-resistant BVDV variants were enriched by two passages in the presence of either 167 nM or 500 nM VP32947 and were isolated by three cycles of plaque purification. Single cycle growth curves of the resultant DR virus variants were similar to that of WT virus, with virus replication rates and yields similar in both the presence and absence of drug. The effect of varying concentrations of VP32947 on the growth of two DR isolates compared with WT virus is shown in Fig. 3. Although plaque formation by the WT virus was inhibited by 50% at a drug concentration of 20 nM, the two DR variants (DR2 isolated at 167 nM drug and DR3 isolated at 500 nM drug) were unaffected by VP32947 concentrations of up to 1,000 nM.

Table 1. Nucleotide and deduced amino acid differences between WT virus and two DR viruses

	Nucleotide position	Base change from WT	Amino acid change from WT	Protein in which change is located
DR 2	5,249	T to A	Phe to Ile	NS2 (NADL insert)
	8,513	G to A	Ala to Thr	NS4B
	10,863	T to C	Phe to Ser	NS5B
DR 3	1,024	G to A	No change	—
	6,981	A to T	Glu to Asp	NS3
	9,424	G to A	No change	—
	10,863	T to C	Phe to Ser	NS5B

Molecular Characterization of DR Viruses. To identify the molecular change(s) responsible for the DR phenotype, we determined the nucleotide sequence of the complete RNA genome of two DR virus isolates and then compared them to the parental WT virus RNA sequence. We note 10 nucleotide differences between the originally reported BVDV-1 (NADL) sequence (ref. 15; GenBank accession no. M31182) and the sequence of our WT virus recovered from the infectious clone. These changes (M31182/WT of present study) and their nucleotide positions are as follows: C/T at 1,315, C/T at 1,729, G/A at 1,989, A/G at 2,653, T/C at 3,877, C/T at 4,341, A/G at 7,261, A/G at 10,546, A/G at 10,838, and G/A at 12,056.

Comparison of the sequence of the two DR virus RNAs with that of the WT virus RNA revealed several changes in the DR sequences (Table 1). DR2 had three and DR3 had four nucleotide changes, all in protein coding regions, and resulted in three and two amino acid changes, respectively, relative to the WT sequence. Both DR clones had a single change in common: the T-to-C transition at nucleotide position 10,863. This change caused an amino acid change of phenylalanine (F) to serine (S) at amino acid residue 224 in the mature NS5B protein sequence. Thus, the F224S change was the candidate mutation implicated in the DR phenotype.

Infectious Clone with the Candidate F224S Drug-Resistance Mutation. To confirm that the F224S change in NS5B was responsible for the DR phenotype, we introduced this single amino acid change into the parental virus infectious clone pACNR/NADL. RNA transcripts derived from plasmid pACNR/NADL/DR were transfected into MDBK cells, and the resultant F224S virus was isolated. As with the original DR variants (DR2 and DR3), the F224S virus caused typical BVDV-induced cytopathic effect on infected MDBK cells and exhibited a single cycle growth curve similar to that of the WT virus. Also like the original DR variants, the F224S virus was completely resistant to the inhibitory effects of VP32947 (Fig. 3). These data confirm that the single F224S amino acid change was sufficient for the DR virus phenotype.

Viral RNA Synthesis in F224S Virus-Infected Cells. Because VP32947 inhibited viral RNA synthesis in WT virus-infected cells (Fig. 2), we investigated whether the synthesis of viral RNA in cells infected with the F224S virus was affected by VP32947. As shown in Fig. 4*A*, VP32947 had no significant effect on the rate or extent of viral RNA synthesis in F224S virus-infected cells. This result was confirmed by analysis of radiolabeled RNA by gel electrophoresis. RNA from MDBK cells infected with either the WT or F224S virus, or mock-infected control cells treated with actinomycin D with or without VP32947 and incubated with ^{3}H-uridine, was extracted and run on a urea-agarose gel. Fig. 4*B* shows that, in the presence of VP32947, viral RNA synthesis by WT virus was completely inhibited (tracks 1 and 2) whereas the level of radiolabeled viral RNA in F224S virus-infected cells was unaffected by the drug (tracks 3 and 4). No radiolabeled viral RNA was apparent in the mock-infected control cells (tracks 5 and 6).

RNA-Directed RNA Polymerase Activity Derived from WT and F224S Viruses. Because the F224S mutation was in the NS5B protein, the viral RdRp, and VP32947 had a clear effect on viral RNA synthesis in virus-infected cells, we examined the RdRp activity of purified NS5B proteins derived from the WT and F224S viruses. We cloned the respective NS5B genes into baculovirus expression vectors and used these to generate recombinant baculoviruses. Infection of Sf9 cells with both WT and F224S NS5B baculoviruses resulted in production of the expected polypeptides. These proteins were purified from infected cell lysates by a series of column chromatography steps that resulted in highly enriched NS5B protein preparations (Fig. 5).

Using the purified WT and F224S NS5B proteins, we evalu-

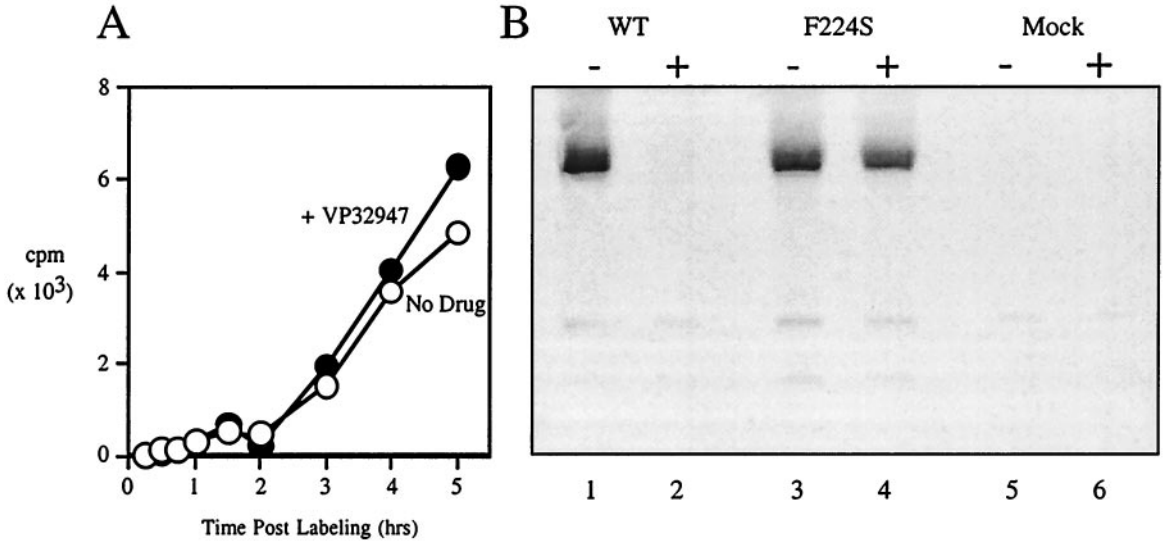

Fig. 4. Effect of VP32947 on viral RNA synthesis in F224S DR virus-infected cells. (*A*) Viral RNA synthesis was determined in cell cultures in the absence (○) or presence (●) of 167 nM VP32947 as described in *Materials and Methods* section. (*B*) MDBK cells were mock-infected or were infected with either WT or F224S DR virus. After 6 h, actinomycin D was added to 5 μg/ml without (−) or with (+) 167 nM VP32947. After an additional 2 h, ^{3}H-uridine (Amersham) was added to the cultures. Cells were harvested 3 h later, and total RNA was extracted and subjected to urea-agarose gel electrophoresis followed by fluorography (21).

ated their ability to synthesize RNA in the presence of VP32947. The WT enzyme was inhibited in a dose-dependent manner by the drug with an IC_{50} value of approximately 700 nM (Fig. 6). In contrast, the RdRp activity of F224S NS5B protein was unaffected by VP32947 up to the highest concentration tested (30 μM).

The mechanism by which VP32947 inhibited NS5B RdRp activity was further investigated by determining the kinetics of inhibition as a function of the concentration of various substrates of the reaction. These studies were carried out individually for each of the four nucleoside triphosphates and the RNA substrate. In all cases, the inhibition appeared noncompetitive. Steady-state kinetic parameters of the WT and F224S enzymes were compared. Substrate K_m values were comparable in all cases. However, the specific activity of the F224S enzyme was about 7-fold greater than that of the WT enzyme (112 vs. 16 pmol/μg/h). This difference was attributable to an increased turnover rate by the F244S enzyme relative to WT (k_{cat} = 0.15 vs. 0.022 min^{-1}).

Discussion

We describe here a small molecule compound, VP32947, that potently inhibits the replication of pestiviruses in cell culture. The compound appears to be pestivirus-specific in that it inhibits the replication of virus isolates from each of the four pestivirus species, yet is inactive against viruses from other groups. The mechanism by which the compound inhibits virus replication involves inhibition of viral RNA synthesis and, more specifically, the NS5B protein. Using drug-resistant virus variants, we further showed that the site of action of the compound on the NS5B

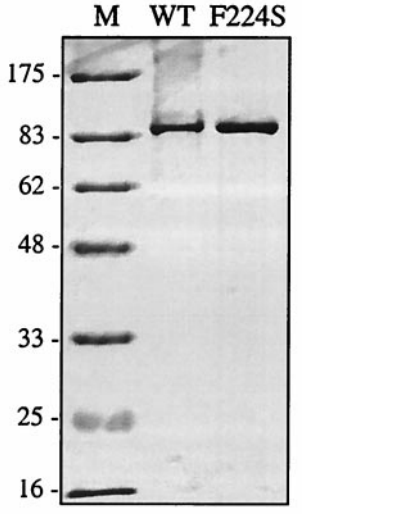

Fig. 5. Purified NS5B proteins from WT and F224S DR viruses. NS5B proteins were purified from Sf9 cells infected with recombinant baculoviruses expressing either the WT or F224S DR NS5B proteins as described in *Materials and Methods*. Aliquots of the final dialyzed preparations were subjected to SDS/10% PAGE, and the resolved proteins were visualized by silver staining. M, molecular mass standards (numbers at left in kilodaltons).

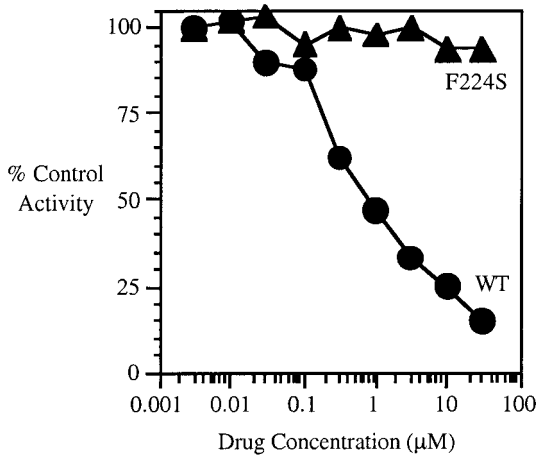

Fig. 6. Inhibition of NS5B polymerase activity by VP32947. The RdRp activity of purified baculovirus-expressed WT (●) and F224S DR (▲) NS5B proteins was measured in the presence of increasing concentrations of VP32947 as described in *Materials and Methods*.

protein involves a phenylalanine at residue 224 in the polypeptide. The NS5B protein is the viral RNA polymerase (22) and thus plays a central role in the virus replication cycle.

Given its antiviral potency and selectivity, VP32947 and related compounds represent potentially useful antiviral compounds for treating or controlling pestivirus infections. A safe and effective pestivirus antiviral drug could be quite valuable if used to control virus transmission and shedding in livestock, such as in cases of congenital infections or insemination procedures, or in the management of persistently infected animals. Additionally, in the face of an acute pestivirus disease outbreak, particularly one caused by virulent virus strains, the availability and use of a suitable antiviral drug may be able to rapidly contain an outbreak and minimize losses. Further utility of a pestivirus antiviral may be found in certain cell culture applications, where BVDV is often a contaminant. Antiviral use during production of biological products, such as vaccines or other products derived from or exposed to bovine or porcine materials, or in *in vitro* fertilization procedures, may be beneficial.

Although we clearly showed that VP32947 acts on the NS5B protein, we found the potency of the compound against BVDV replication in cells (IC_{50} = 20 nM) was significantly greater than its activity against the purified NS5B protein in an RNA polymerase assay (IC_{50} = 700 nM). There are several possible explanations for this difference. One possibility suggests that in cell culture the drug may be actively transported or accumulated inside cells, resulting in higher intracellular drug concentrations.

Alternatively, the difference between the virological and enzymological potencies of VP32947 may reflect fundamental differences between the assays and, possibly, more detailed aspects of the compound's mechanism of action. It is likely that features of NS5B polymerase initiation and elongation on the 12,600-nucleotide viral genome in virus-infected cells and on the 375-nucleotide synthetic RNA molecule used in our *in vitro* assay are not the same. Thus, subtle effects by VP32947 on, for example, the binding affinity of enzyme with RNA template, the efficiency of enzyme initiation or enzyme processivity along these two very different RNA templates may account for the potency differences. The increased turnover rate observed for the F224S enzyme in the *in vitro* polymerase assay may be reflective of such differences. These possibilities may also find support based on the recently reported crystal structures of the HCV NS5B protein (23–25). Although the overall sequence identity between the BVDV and HCV NS5B proteins is low, an alignment of similar sequences by Ago *et al.* (23) suggests that the F224 residue in BVDV NS5B resides in a turn region between two helix domains in the finger portion of the enzyme. This region of the finger domain, which overhangs the palm domain of the enzyme, might be involved in RNA substrate recognition (23). Thus, if the BVDV NS5B is structurally homologous to HCV NS5B, VP32947 may act to affect NS5B interaction with the RNA substrate.

Finally, it is important to remember that NS5B functions in virus-infected cells in the context of membrane-bound replication complexes that consist of several virus-encoded proteins, host proteins and various forms of viral RNA. The NS5B protein may have a role in interactions with these other components or in the assembly or stability of the replication complex. If one or more of these putative functions for NS5B in virus-infected cells is sensitive to VP32947, viral RNA synthesis may be disrupted in a manner distinct from the drug's affect on NS5B polymerase activity by the isolated protein measured in the *in vitro* assay.

Clearly, there is much more to understand about RdRp enzymes in isolation and their functional role in replication complexes in virus-infected cells. Further investigations of VP32947 and similar compounds in the context of viral replication complexes and with viral replicons will hopefully provide more detailed information on the mechanism of inhibition by these compounds in particular and on the process of RNA replication by pestiviruses and related viruses in general. Given the similarities among the NS5B and NS5 RdRp proteins of pestiviruses, hepaciviruses, and flaviviruses (ref. 26; data not shown), knowledge gained in any one of these virus systems is likely to facilitate progress in the others. To this point, the work reported here formally validates NS5B as a target for antiviral drug discovery and development. Given the seriousness of the hepatitis C epidemic and the urgent need for new and effective therapies, it is hoped that compounds such as the one described here may provide insight toward identification of HCV antiviral drugs. Indeed, several groups are engaged in discovery and development efforts in search of HCV NS5B inhibitors. The advancement of these compounds into clinical evaluations is eagerly anticipated.

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1. Sockett, D., Bolin, D., Ridpath, J. & Bolin, S. (1996) in *Bovine Viral Diarrhea Virus: A 50 Year Review.* (Cornell University College of Veterinary Medicine, Ithaca, NY), p. 207.
2. Bitsch, V. & Ronsholt, L. (1995) *Vet. Clin. North Am. Food Anim. Pract.* **11,** 627–640.
3. Carman, S., van Dreumel, T., Ridpath, J., Hazlett, M., Alves, D., Dubovi, E., Tremblay, R., Bolin, S., Godkin, A. & Anderson, N. (1998) *J. Vet. Diagn. Invest.* **10,** 27–35.
4. Herklots, H. & Nasto, B. (1998) *Am. Soc. Microbiol. News* **64,** 10.
5. Houe, H. (1999) *Vet. Microbiol.* **64,** 89–107.
6. Moerman, A., Straver, P. J., de Jong, M. C., Quak, J., Baanvinger, T. & van Oirschot, J. T. (1994) *Vet. Q.* **16,** 115–119.
7. Taylor, L. F., Janzen, E. D. & Van Donkersgoed, J. (1997) *Can. Vet. J.* **38,** 23–28.
8. Thiel, H.-J., Plagemann, P. G. W. & Moennig, V. (1996) in *Virology*, eds. Fields, B. N., Knipe, D. M. & Howley, P. M. (Lippincott, Philadelphia), pp. 1059–1073.
9. Corapi, W. V., French, T. W. & Dubovi, E. J. (1989) *J. Virol.* **63,** 3934–3943.
10. Pellerin, C., Van den Hurk, J., Lecomte, J. & Tijssen, P. (1994) *Virology* **203,** 260–268.
11. Bolin, S. R. (1995) *Vet. Clin. North Am. Food Anim. Pract.* **11,** 489–500.
12. Brownlie, J. & Clarke, M. C. (1993) *Intervirology* **35,** 51–59.
13. Van Oirschot, J. T., Bruschke, C. J. M. & Van Rijn, P. A. (1999) *Vet. Microbiol.* **64,** 169–183.
14. Rice, C. M. (1996) in *Virology*, eds. Fields, B. N., Knipe, D. M. & Howley, P. M. (Lippincott, Philadelphia), pp. 931–959.
15. Collett, M. S., Larson, R., Gold, C., Strick, D., Anderson, D. K. & Purchio, A. F. (1988) *Virology* **165,** 191–199.
16. Mendez, E. M., Ruggli, N., Collett, M. S. & Rice, C. R. (1998) *J. Virol.* **72,** 4737–4745.
17. Picard, V., Ersdal-Badju, E., Lu, A. & Bock, S. C. (1994) *Nucleic Acids Res.* **22,** 2587–2591.
18. Collett, M. S., Larson, R., Belzer, S. K. & Retzel, E. (1988)*Virology* **165,** 200–208.
19. Cleland, W. W. (1979) *Methods Enzymol.* **63,** 103–138.
20. Mosmann, T. (1983) *J. Immunol. Methods* **65,** 55–63.
21. Purchio, A. F., Larson, R. & Collett M. S. (1983) *J. Virol.* **47,** 320–324.
22. Zhong, W., Gutshall, L. L. & Del Vecchio, A. M. (1998) *J. Virol.* **72,** 9365–9369.
23. Ago, H., Adachi, T., Yoshida, A., Yamamoto, M., Habuka, N., Yatsunami, K. & Miyano M. (1999) *Structure (London)* **7,** 1417–1426.
24. Bressanelli S., Tomei, L., Roussel, A., Incitti, I., Vitale, R. L., Mathieu, M., De Francesco, R. & Rey, F. A. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 13034–13039.
25. Lesburg, C. A., Cable, M. B., Farrari, E., Hong, Z., Mannarino, A. F. & Weber, P. C. (1999) *Nat. Struct. Biol.* **6,** 937–943.
26. Steffens, S., Thiel, H.-J. & Behrens, S.-E. (1999) *J. Gen. Virol.* **80,** 2583–2590.