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Review

Bovine viral diarrhea virus as a surrogate model of hepatitis C virus for the evaluation of antiviral agents

Victor E. Buckwold [a,*], Brigitte E. Beer [a], Ruben O. Donis [b]

[a] *Infectious Disease Research Department, Southern Research Institute, 431 Aviation Way, Frederick, MD 21701, USA*
[b] *Department of Veterinary and Biomedical Sciences, University of Nebraska-Lincoln, Lincoln, NE 68583, USA*

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Abstract

The identification and development of new antiviral agents that can be used to combat hepatitis C virus (HCV) infection has been complicated by both technical and logistic issues. There are few, if any, robust methods by which HCV virions can be grown in vitro. The development of HCV RNA replicons has been a great breakthrough that has allowed for the undertaking of significant screening efforts to identify inhibitors of HCV intracellular replication. However, since replicons do not undergo a complete replication cycle, drug screening programs and mechanism of action studies based solely on these assays will not identify compounds targeting either early (virion attachment, entry, uncoating) or late (virion assembly, egress) stages of the viral replication cycle. Drugs that negatively affect the infectivity of new virions will also not be identified using HCV RNA replicons. Bovine viral diarrhea virus (BVDV) shares a similar structural organization with HCV, and both viruses generally cause chronic long-term infections in their respective hosts. The BVDV surrogate model is attractive, since it is a virus-based system. It is easy to culture the virus in vitro, molecular clones are available for genetic studies, and the virus undergoes a complete replication cycle. Like HCV, BVDV utilizes the LDL receptor to enter cells, uses a functionally similar internal ribosome entry site (IRES) for translation, uses an NS4A cofactor with its homologous NS3 protease, has a similar NS3 helicase/NTPase, a mechanistically similar NS5B RNA-dependent RNA polymerase, and a seemingly equivalent mechanism of virion maturation, assembly and egress. While the concordance between drugs active in either BVDV or HCV is largely unknown at this time, BVDV remains a popular model system with which drugs can be evaluated for potential antiviral activity against HCV and in studies of drug mechanism of action.

Keywords: Hepatitis C; Bovine viral diarrhea virus; Antiviral

1. Introduction

The hepatitis C virus (HCV) is an enveloped virus with a positive-sense RNA genome of approximately 9.6 kb (Choo et al., 1991; Kato, 2000; Rosenberg, 2001). HCV is the major etiologic agent of non-A, non-B hepatitis, and over 170 million people are infected with HCV worldwide (WHO, 2002). These infections are often asymptomatic; however, HCV infection frequently causes chronic hepatitis, which can progress to liver cirrhosis, end-stage liver disease, and hepatocellular carcinoma (Liang et al., 2000). Approximately 3.9 million Americans are infected with HCV, making HCV the most common blood-borne infection in the United States (Kim et al., 2002b). Infection with HCV is also the leading cause for liver transplantation in this country. No vaccine is available for HCV, and the current treatment for HCV infection, ribavirin and interferon-α (IFN-α), is only effective in a subset of patients. Both of these antiviral agents are broadly acting antiviral compounds that are non-specific for HCV. Also, this therapy is not tolerated well by patients. Therefore, new antiviral agents to treat HCV infection are desperately needed.

Technically, we have been retarded in our abilities to identify new antiviral drugs for HCV due to our inability to propagate the virus reproducibly in vitro (reviewed in Bartenschlager and Lohmann, 2001). The invention of a self-replicating subgenomic HCV RNA replicon (Lohmann et al., 1999a) was a great breakthrough in HCV research. These bicistronic replicons are based on constructs containing the authentic 5′ and 3′ non-translated regions (NTR) of the virus genome, a neomycin phosphotransferase (Neo) gene to allow exclusive survival of cells that continue to produce the transgene in the presence of G418, and the internal

* Corresponding author. Tel.: +1-301-694-3232; fax: +1-301-694-7223.
E-mail address: Buckwold@sri.org (V.E. Buckwold).

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ribosome entry site (IRES) element of EMCV mediating the translation of the downstream HCV non-structural (NS) proteins involved in polyprotein processing and RNA replication (reviewed in Bartenschlager and Lohmann, 2001). HCV RNA replicons are robust systems that have shown utility in the evaluation of antiviral agents affecting HCV translation, RNA replication, and polyprotein proteolytic processing (Frese et al., 2001; Guo et al., 2001; Cheney et al., 2002; Lanford et al., 2003; Zhou et al., 2003). However, HCV RNA replicons do not produce infectious virions. Therefore, they cannot be used for the identification and characterization of antiviral agents acting at early steps of the viral replication cycle such as virus attachment to and entry into cells, or the subsequent uncoating process following entry; nor do they allow for the identification of compounds affecting late stages of the replication cycle, such as virion maturation and egress from cells. Similarly, antiviral agents that act through their ability to reduce the infectivity of newly formed virions cannot be identified or characterized using HCV RNA replicons. Since HCV RNA replicons do not faithfully reproduce all steps of the HCV replication cycle, and due to the relatively high costs associated with the use of this technology for the development of antiviral agents, surrogate viruses are still widely utilized for the identification and characterization of anti-HCV antiviral agents.

Other viruses have only been used rarely as surrogate viruses in the study of HCV. The yellow fever virus (YFV) is the type member of the Flavivirus genus of the Flaviviridae family of viruses. This virus has been employed as a surrogate model of HCV replication for the evaluation of antiviral agents (Neyts et al., 1996). However, since its genomic structure is further removed phylogenetically from that of HCV than is bovine viral diarrhea virus (BVDV) and other members of the Flaviviridae, and because it uses a cap-dependent rather than an IRES-mediated mechanism of translation initiation, it is seldom employed as an in vitro surrogate model of HCV. Rather, the YFV is used in the evaluation of antiviral compounds in a range of action studies. These studies are typically performed to see if compounds act specifically against HCV or BVDV, or non-specifically against multiple viruses in order to ascertain whether drugs have a broad spectrum of antiviral activity against all Flaviviridae viruses. This is the case with both of the broadly acting antiviral compounds IFN-α and ribavirin, and the use of YFV has been informative in confirmatory studies on the mechanism of action of these compounds (Buckwold et al., 2003).

The GB virus B (GBV-B) is also an interesting candidate for use as a surrogate model of HCV replication for the evaluation of antiviral agents (Beames et al., 2001). This hepatotrophic virus can undergo a complete replication cycle in tissue culture, making it possible to identify compounds affecting all stages of the viral life cycle. GBV-B has both a relatively high degree of sequence identity with HCV (27–33% amino acid identity with HCV across the polyprotein (Muerhoff et al., 1995)) compared to other members of the Flaviviridae family and a genomic RNA structure very similar to that of HCV. Unfortunately, since the virus can only be grown in the primary hepatocytes of certain non-human primates, this model has not been widely utilized. Clearly it is not possible today to use GBV-B in high-throughput screening (HTS) efforts for drug discovery.

Due to these limitations inherent in the field of HCV drug discovery and development, there has been great interest in the use of BVDV as a surrogate model system. BVDV is the type member of the Pestivirus genus of the Flaviviridae family of viruses (Colett et al., 1988). Other members of this group include classical swine fever virus (formerly known as hog colera virus) and border disease virus of sheep. BVDV is easy to grow in tissue culture, and molecular clones are available for genetic studies (Meyers et al., 1996; Vassilev et al., 1997). Common strains of BVDV such as NADL, which is available from the American Type Culture Collection (ATCC), are cytopathic in tissue culture, allowing for facile assessment of antiviral activity using a cytoprotection assay. The validation of the utility of the BVDV surrogate virus model is incomplete, since most of the few drugs that are known to be active against HCV have not been tested against BVDV; however, its replication in cell culture is inhibited by the drugs currently used to treat HCV infections. Ribavirin and IFN-α are effective antiviral agents against BVDV (Markland et al., 2000; Ouzounov et al., 2002; Buckwold et al., 2003; Stuyver et al., 2003), HCV RNA replicons (Frese et al., 2001; Guo et al., 2001; Cheney et al., 2002; Lanford et al., 2003; Zhou et al., 2003), and related in vitro HCV infection and expression systems (Castet et al., 2002; Contreras et al., 2002). BVDV has been the most widely utilized in vitro HCV surrogate model system for the identification and characterization of antiviral agents for use against HCV. The strengths and weaknesses of the use of the BVDV model system for this indication, as well as examples of its use in this regard, are reviewed below.

2. Similarities and differences between BVDV and HCV

2.1. Overall functional and genetic similarity between HCV and BVDV

The Flaviviridae family of viruses is currently comprised of three genera of viruses: the Hepaciviruses, the Pestiviruses, and the Flaviviruses (Westaway et al., 1985; Robertson et al., 1998; Lindenbach and Rice, 2001). The GB viruses A and B (GBV-A, GBV-B) are awaiting formal classification within the family and may constitute one or more separate genera in the family (Muerhoff et al., 1995; Robertson et al., 1998).

All members of the Flaviviridae family of viruses share similarities in virion structure, genome organization, and presumably in their replication cycle (reviewed in Lindenbach and Rice, 2001). Fig. 1 shows a generalized Flaviviridae replication cycle. Flaviviridae virions are

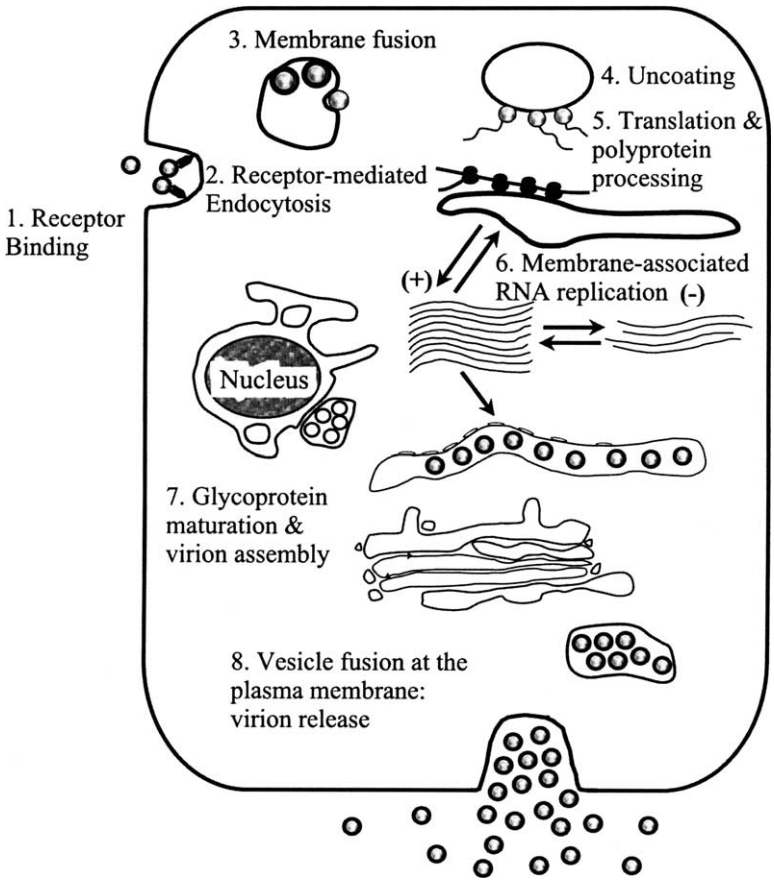

Fig. 1. Generalized Flaviviridae replication cycle. In step 1, Flaviviridae virions bind to specific cellular receptors through their envelope proteins. In step 2, virions are internalized through receptor-mediated endocytosis. A fusion of host and cellular membranes results in step 3, leading to the release of the nucleocapsid into the cytoplasm. Further uncoating of the nucleocapsid (step 4) allows the positive-sense genomic RNA to be translated into a single large polyprotein, which is cleaved into individual viral proteins by a combination of viral and host proteases (step 5). Membrane-associated replication complexes containing the viral RNA-dependent RNA polymerase (step 6) copy the genomic, positive-sense RNA into a negative-sense RNA that serves as the template for the synthesis of progeny positive-sense RNA molecules. The process of glycoprotein maturation and virion assembly occurs in membrane-associated vesicles (step 7). Virions are then secreted from cells through the secretory pathway to the plasma membrane. After Lindenbach and Rice (2001).

enveloped particles composed of a host-cell-derived lipid bilayer containing two or more envelope (E) proteins surrounding a nucleocapsid shell composed of core (C) protein that is associated with the single-stranded positive-sense RNA genome. The initial attachment to and uptake of these viruses into cells is thought to occur through an interaction between specific cellular receptors and the envelope proteins. A fusion of host and cellular membranes results in the release of the nucleocapsid into the cytoplasm. Further uncoating of the nucleocapsid allows the positive-sense RNA to be translated into a single large polyprotein, which is cleaved into individual viral proteins by a combination of viral and host proteases. Inhibition of host macromolecular synthesis is not known to occur until very late stages of infection with cytopathic BVDV variants (Donis and Dubovi, 1987a). Membrane-associated replication complexes containing the viral RNA-dependent RNA polymerase copy the genomic, positive-sense RNA into anti-sense RNA, which serves as the template for the synthesis of progeny positive-sense RNA molecules. The process of glycoprotein maturation and virion assembly is thought to occur along the secretory pathway in the endoplasmic reticulum (ER), Golgi, and intermediate compartments (Donis and Dubovi, 1987c; Grummer et al., 2001). Virions are then secreted from cells by fusion of exocytic vesicles with the plasma membrane.

The structural organization of the genomes of HCV and BVDV is shown in Fig. 2. The overall structure is similar, yet major differences in their genomic organization are also immediately apparent. Both of these Flaviviridae viruses have a single-stranded positive-sense genomic RNA that codes for a single large polyprotein that is processed into multiple proteins both co- and post-translationally by means of both viral and host proteases. The sizes of the genomes are disparate, with HCV at approximately 9.6 kb (Choo et al., 1991) and BVDV at about 12.6 kb in length (Colett et al., 1988). Like other RNA viruses, the high mutation rate resulting from the lack of proofreading function in the viral

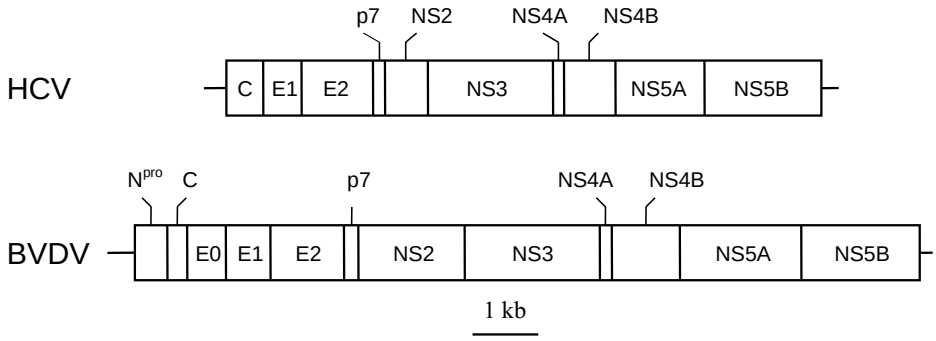

Fig. 2. Structural organization of HCV and BVDV genomes. The organization of the genomic RNAs of HCV and BVDV are displayed diagrammatically. This drawing is based on the cDNA sequences of HCV-1 (Genbank Accession number M62321) and of BVDV NADL strain (AJ133738). Coding sequences in the viral polyprotein are boxed with the names of the proteins indicated. Non-coding regions are shown as straight lines. The scale of the drawing is indicated by the line below the two genomes that shows the size of 1-kb RNA. See text for details.

RNA-dependent RNA polymerase is believed to result in a complex interrelated population of viral quasispecies during infections (Donis et al., 1991). If the complete amino acid sequence of the polyproteins is compared between various HCV genotypes and BVDV, little overall amino acid sequence identity (21%) is found (Miller and Purcell, 1990; Muerhoff et al., 1995). However, this level of identity should be viewed in light of the 27–33% amino acid identity across the polyprotein observed when GBV-B, the virus phylogenetically most similar to HCV, was analyzed (Muerhoff et al., 1995). Nonetheless, from the similarity of the hydropathic profiles of the BVDV and HCV polyproteins (Muerhoff et al., 1995), it appears that both viruses code for essentially functionally equivalent gene products. Likewise, the similar RNA structures of the 5′ NTRs point toward homologous *cis*-acting control elements.

Both genomes are composed of the 5′ NTR that contains an IRES, followed by the viral polyproteins. The N-terminus of the Pestivirus polyprotein consists of an autoprotease (NPro, aka p20) that functions to release the core protein by autoproteolysis (Wiskerchen et al., 1991). This protein is a *cis*-acting papain-like protease that cleaves its own C-terminal end at a Cys/Ser recognition sequence. No other function other than to generate the N-terminus of the core protein (C) has been identified for NPro. Genetic engineering has been used to demonstrate that NPro is not essential for Pestivirus replication (Tratschin et al., 1998; Lai et al., 2000). In contrast, the HCV polyprotein N-terminus encodes a structural protein, termed core (C), that functions as the viral capsid protein. This protein is common to both HCV and BVDV. Following the core protein is the E0 (aka Erns for RNase, soluble) glycoprotein, which is only present in the Pestivirus genus. The E0 is an additional envelope protein that has an associated RNase activity (Schneider et al., 1993). This protein is not an integral membrane protein, and it seems to be either non-covalently attached to virions or simply secreted from infected cells (Silva-Krott et al., 1994). Interestingly, soluble recombinant E0 can inhibit the ability of BVDV to infect cells (Iqbal et al., 2000). The remainder of the viral proteins are common to both HCV and BVDV. The envelope proteins 1 and 2 (E1, E2) are proteolytically processed using host cell ER-associated enzymes. Next are the non-structural proteins derived from the p7-NS5B region, which are involved in polyprotein processing, RNA replication, and virion assembly, followed by the 3′ NTR, which is also common to both viruses. The p7 of both viruses appears to function to allow the traffic of ions across the membrane. Blockage of this ion channel suppresses the replication of BVDV (Griffin et al., 2003; Pavlovic et al., 2003). The NS2–NS3 has a *cis*-acting protease activity that cleaves the NS2/NS3 boundary in HCV, but only in cytopathic isolates of BVDV (Donis and Dubovi, 1987b; Tautz et al., 1997) (discussed below). The NS3 protease cleaves the remaining sites in the polyprotein of all isolates (NS3/NS4A, NS4A/NS4B, NS4B/NS5A, NS5A/NS5B). In addition, the NS3 protein has a helicase and NTPase activity. The NS4A acts as a cofactor for the NS3 protease. The functions of NS4B and NS5A are uncertain, but mutations in the NS5A region are thought to be associated with resistance to IFN in some HCV genotypes (Enomoto et al., 1995). The HCV NS5A protein appears to modulate intracellular signal transduction pathways and may influence the replication, persistence, and pathogenesis of HCV (Khabar and Polyak, 2002). The role of NS5A in the biology of BVDV has not yet been explored. The NS5B protein has RNA-dependent RNA polymerase activity. The polyprotein coding region of the genome is followed by the 3′ NTR sequences, which stabilize the RNA by preventing its degradation and function as the promoter for minus strand RNA synthesis.

2.2. Biology of persistent infections in their natural hosts

Persistent infection is the hallmark of the interaction of Pestiviruses and Hepaciviruses with their hosts and is characterized by chronic viremia in the absence of clinical signs of disease for many years. The frequency of chronic carriers of HCV in the human population or of BVDV in cattle

populations are very similar (between 0.5 and 3%) (Bolin et al., 1985; Kim et al., 2002b); however, the mechanism of establishment of persistent infection is different.

Pestiviruses can only establish persistence if they infect the developing fetus (McClurkin et al., 1984). It appears that evasion of the host innate and adaptive immune responses are required for the establishment of Pestivirus infections. Naïve pregnant animals usually acquire non-cytopathic BVDV by oronasal exposure that results in a transient viremia. The Pestiviruses are placental trophoblast tropic, which facilitates the transplacental infection of virtually every fetus carried by a viremic mother. Unless the fetal infection results in an abortion, a persistent infection develops in most fetuses infected with a non-cytopathic BVDV during the first trimester of pregnancy (Swasdipan et al., 2002). The success of fetal infection is associated with the ability of the non-cytopathic virus to suppress type I interferon production in the fetus (Charleston et al., 2001). Fetal infection with non-cytopathic BVDV during the first trimester results in the birth of calves that are immunotolerant to BVDV. These newborns develop into apparently healthy animals, which remain viremic for life (McClurkin et al., 1984). Viral loads in the peripheral blood range from 10^3 to 10^5 infectious units/ml. The source of the circulating virus appears to be primarily monocytes and lymphocytes (Bielefeldt, 1987, 1988; Bielefeldt et al., 1987). Ephithelial tissues are another primary target of BVDV replication (Odeon et al., 1999).

In contrast, fetal infections with cytopathic BVDV strains are rare, and persistence is not established (Brownlie et al., 1989; Harding et al., 2002). The appearance of cytopathic lethal variants of BVDV in persistently infected animals is common and results in a fatal syndrome known as mucosal disease. This disease is caused by the unchecked replication of these cytopathic BVDV variants due to immunologic tolerance to the viral antigens. Cytopathic variants are characterized by the production of NS3 as free protein (non-cytopathic BVDV produces only NS2–NS3). In the majority of cases, cytopathic variants arise by either mutations in NS2 that allow cleavage at its C-terminus with concomitant release of NS3 or by a gene duplication event that leads to the ectopic production of NS3 (reviewed in Meyers and Thiel, 1996). While non-cytopathic BVDV isolates suppress type I interferon mRNA production, cytopathic BVDV variants are powerful interferon inducers (Adler et al., 1994; Baigent et al., 2002).

Hepaciviruses generally establish persistence following parenteral inoculation of a mature individual. HCV is principally a hepatotropic virus; however, the virus may be able to replicate in peripheral blood mononuclear cells and, to a more limited degree, in other tissues (Gowans, 2000). A minority of infected individuals spontaneously resolve the acute infection (∼15%), while most cases (∼85%) lead to chronic persistent HCV infections. The outcome of HCV infection is determined by interactions between the virus and the host immune system. The persistence of infection in most HCV-infected individuals, despite the presence of anti-HCV antibodies, suggests that such antibodies fail to induce viral clearance. Chronic hepatitis C is generally asymptomatic, but is usually associated with persistent or fluctuating elevations in blood liver enzyme levels (Hoofnagle, 2002). Approximately 20% of chronically infected individuals will develop liver cirrhosis, which can lead to end-stage liver disease and hepatocellular carcinoma. Extra-hepatic manifestations include cryoglobulinemia, which seems to coincide with the development of liver cirrhosis (Kayali et al., 2002).

Although the mechanisms of establishment and maintenance of Pestivirus and Hepacivirus persistence in their hosts are different, the outcome is similar in virologic terms. Both viruses tend to establish chronic long-term infections that either tolerize the host or are insensitive to viral-specific host humoral and cellular immune responses, and the interplay between virologic and host immunologic responses to infection seems to be related to the manifestation of the disease process associated with each virus.

3. Overview of major viral targets for antivirals

3.1. Attachment and entry through homologous cellular receptors

Virions must specifically attach to and enter into cells in order to initiate an infection cycle. Two candidate BVDV receptors have been identified. In one study, a pair of cell surface proteins of 56 and 28 kDa existing as a multimer of about 200 kDa were identified using monoclonal antibodies that inhibited BVDV infections (Schelp et al., 1995, 2000). It appears that this protein complex binds F-actin to aid the endocytosis of BVDV virions (Schelp et al., 2000). In another study, a 50-kDa cellular protein was identified that was able to inhibit BVDV infection (Minocha et al., 1997). It is not known if any of these proteins are responsible for the resistance of a mutant MDBK cell line to Pestivirus infection (Flores and Donis, 1995).

Four putative cellular receptors have been identified that may be involved in the initiation of HCV attachment, penetration, and entry into cells: CD81, SR-BI, L-SIGN, and the low-density lipoprotein (LDL) receptor. The CD81 molecule was the first candidate HCV receptor that was found. It was identified by cloning efforts as a putative HCV receptor by virtue of its binding to HCV E2 (Pileri et al., 1998). This tetraspanin family member is expressed in a variety of cell types, and there is growing evidence that this interaction is not critical for HCV attachment and entry into cells (Petracca et al., 2000; Wünschmann et al., 2000; Triyatni et al., 2002; Zhou et al., 2002; Sasaki et al., 2003), including the fact that transgenic mice expressing the CD81 are not susceptible to HCV infection (Masciopinto et al., 2002). The CD81 receptor may play a role as a coreceptor by aiding the attachment to and entry of HCV into cells, but it is clearly not sufficient to perform this role alone. The role of a CD81-like molecule

in the attachment to and entry of BVDV into cells has not been explored.

A second candidate receptor for HCV that was also identified by its affinity for E2 is the human scavenger receptor class B type I (SR-BI) (Scarselli et al., 2002). The human HepG2 hepatoma cell line was found to bind to E2, yet it did not express the CD81 receptor. An 82-kDa glycosylated protein was identified as the most likely candidate responsible for E2 binding by reversible cross-linking experiments and using specific antibodies. CHO cells stably transfected with the human SR-BI bound E2 while stable cells expressing a different human scavenger receptor did not. Infection of these cell lines with HCV (or BVDV) has not been described.

Very recently, a new candidate HCV receptor has been proposed: the liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin (L-SIGN). This liver-enriched molecule is a calcium-dependent lectin expressed on liver and lymph node endothelial cells. L-SIGN and the dendritic cell-specific homologue DC-SIGN specifically bind HCV virions by engaging E2 (Gardner et al., 2003). Its role in HCV and BVDV infection is unknown.

The fourth candidate HCV receptor that has been identified is the LDL receptor (Agnello et al., 1999; Monazahian et al., 1999). A variety of experimental evidence indicates that this interaction is biologically significant. The most salient of these findings include the facts that the LDL receptor has a liver-enriched expression pattern, that the most highly infectious fraction of HCV is found bound to lipoproteins, and that the binding to and entry of HCV into cultured cells can be upregulated by agents that increase the expression of LDL receptor and downregulated by agents interfering with the HCV–LDL receptor interaction (see Agnello et al., 1999; Monazahian et al., 1999; Wünschmann et al., 2000; Favre et al., 2001; Andre et al., 2002; Triyatni et al., 2002; and references therein). The emerging view is that the LDL receptor may serve as an HCV receptor, although a role for the participation of other receptors and coreceptors cannot be excluded at this time. But the LDL receptor has not yet been shown to be sufficient for HCV entry into cells. The entry of BVDV into cultured cells is also known to be mediated by the LDL receptor (Flores and Donis, 1995; Agnello et al., 1999). Thus, antiviral agents that block the attachment of and entry of HCV virions into cells through the LDL receptor may be identified using BVDV as a surrogate model of HCV replication.

Due in part to the uncertain nature of the true HCV receptor, antiviral agents targeting this stage of the viral life cycle have not yet been identified.

3.2. Polyprotein translation via functionally equivalent IRES elements

Normal translation initiation in eukaryotes occurs by recognition of the 5′ terminus m^7G cap of mRNA by the eukaryotic initiation factor (eIF)4F complex, followed by the addition of other initiation factors and the 40S ribosomal subunit (as a 43S preinitiation complex), which scans the mRNA until the first initiation codon is reached. Factor displacements and the joining of the 60S ribosomal subunit result in the formation of the active 80S ribosome. However, the genomic RNAs of HCV and BVDV are not capped. These viruses utilize similar IRES elements in their 5′ NTR that initiate translation via a single common mechanism that differs from the standard cap-dependent translation mechanism (reviewed in Pestova et al., 2001). These “HVC-like IRES elements” are both structurally and mechanistically distinct from the IRES translation initiation mechanism used by encephalomyocarditis virus or polioviruses (Lopez de Quinto et al., 2001; Beales et al., 2003). The HCV-like IRES element binds directly to the 40S ribosomal subunit and eIF3, allowing 43S complexes to bind directly to the initiation codon to initiate translation (Pestova et al., 1998; Kieft et al., 2001). A variety of protein–IRES RNA interactions are known to affect the HCV IRES translational efficiency, including host proteins such as polypyrimidine tract binding protein (Ali and Siddiqui, 1995), La antigen (Ali and Siddiqui, 1997), heterogeneous ribonucleoprotein L (Hahm et al., 1998), an unidentified 25-kDa cellular protein (Fukushi et al., 1997), and also HCV core protein (Shimoike et al., 1999). The interactions affecting the BVDV IRES have not been examined as thoroughly as that of the HCV IRES. The polypyrimidine binding protein also binds to the BVDV IRES, but it does not seem to affect its translational activity (Sanderbrand et al., 2000).

The 5’ NTR is the most conserved portion of both the HCV genome (341 nt 5′ NTR; Hellen and Pestova, 1999) and the BVDV genome (385 nt 5′ NTR; Donis, 1995) and contains sequences involved in the control of RNA replication and translation. The 3′ end of the IRES element is not well defined, and a small segment of the core coding sequence appears to contribute to this element’s efficiency (Bartenschlager and Lohmann, 2001).

Among other viral IRES elements, the BVDV IRES is quite similar to that of HCV. In the IRES, there is 47% sequence homology between the two viruses (Han et al., 1991); however, the secondary structure of the IRES elements appears to be functionally equivalent. The finding that an HCV IRES could substitute for the BVDV IRES in chimeric virus constructs supports this contention (Frolov et al., 1998). It is believed that the structural elements of the HCV-like IRESes of BVDV and HCV act as a scaffold that orients binding sites such that their interactions with initiation factors and ribosomes leads to the assembly of functional initiation complexes. The secondary structures of the HCV and BVDV IRES elements are shown in Fig. 3.

An examination of the secondary structure of the 5′ NTRs shows that the 3′ half of the BVDV NTRs shows conservation with that of HCV, while the 5′ end of the NTR is not conserved. It is believed that the very 5′ ends of the genome are probably involved in RNA replication rather than in translational control (Chon et al., 1998; Frolov et al., 1998; Becher

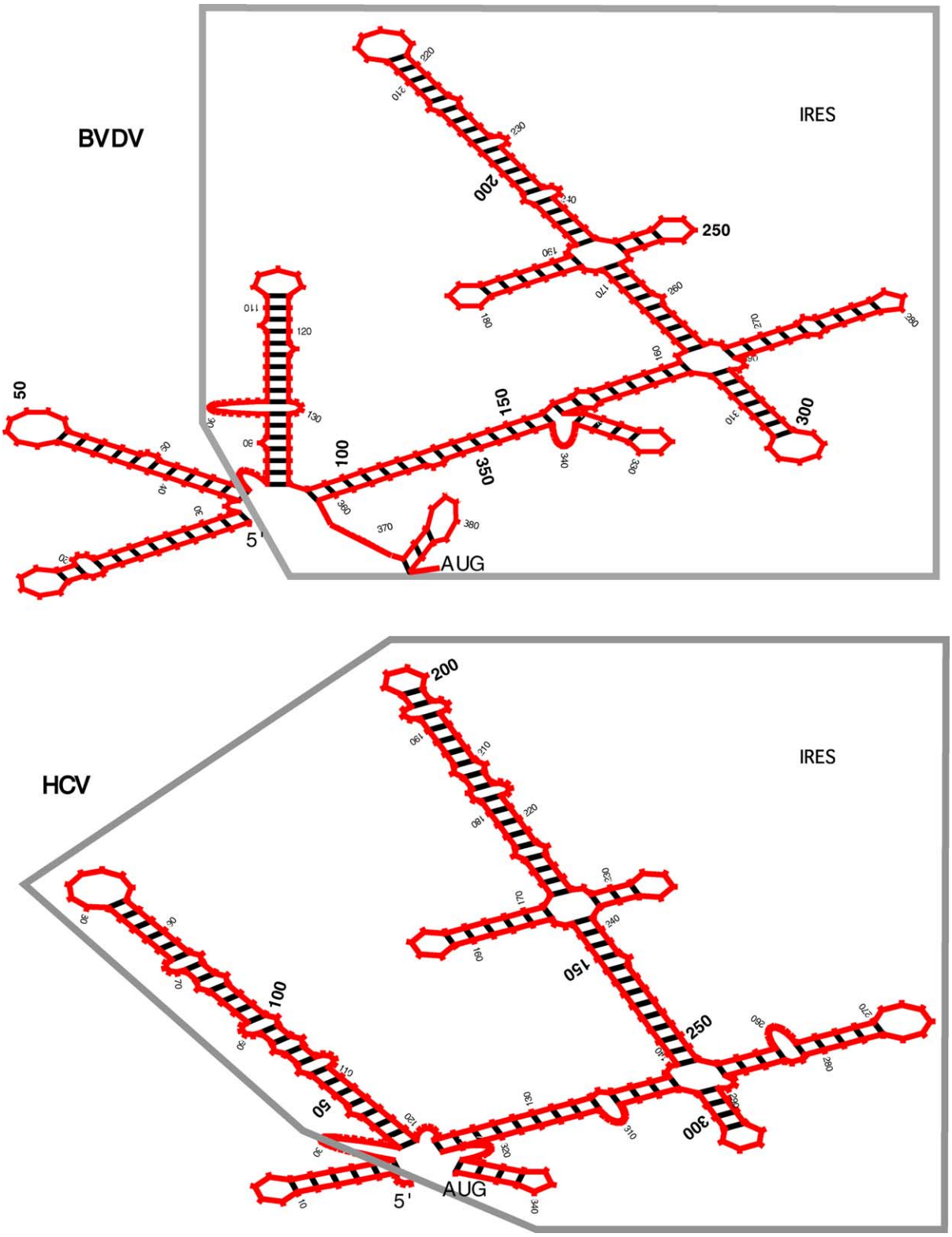

Fig. 3. Comparison of the proposed secondary structures of the HCV and BVDV IRES elements. The folding of the 5′ NTR RNA was predicted by thermodynamic energy rules implemented in the MFOLD software and coincides with the available experimental structure determinations. The similarities in the secondary structures of the complete 5′ NTRs of HCV (Genbank Accession number AF011751) and BVDV (AJ133738) are evident. The IRES elements are boxed. The start codon (AUG) is also indicated.

et al., 2000; Yu et al., 2000; Friebe et al., 2001; Friebe and Bartenschlager, 2002; Kim et al., 2002a). The reverse compliment of the 5′ NTR is the negative-sense 3′ NTR, which serves as the promoter for initiation of positive-sense RNA synthesis.

Since the specific macromolecular interactions involved in the IRES-mediated cap-independent initiation of translation are so different from the manner by which host mRNAs are translated, the HCV IRES is thought to be a good target for HCV antiviral therapy (Jubin, 2001; Gallego and Varani, 2002) using therapeutic antisense molecules (Seki and Honda, 1995; Hanecaki et al., 1996), ribozymes (Krüger et al., 2001), small interfering RNAs (Seo et al., 2003), other small RNAs (Das et al., 1998), and other compounds. Clearly one needs to directly compare the nucleotide sequences of BVDV and HCV when sequence-specific antiviral strategies are to be evaluated using BVDV. We anticipate that antiviral agents targeting other attributes of the HCV IRES that are not dependent on primary sequence may also inhibit the BVDV IRES. Some potentially interesting targets include the IRES secondary and tertiary structure, and the interaction of the IRES with the viral and host proteins that affect its translational efficiency. One example of this type of antiviral agent is the ribozyme directed against the HCV 3′ NTR that actually seemed to inhibit HCV IRES activity. This ribozyme turns out to have targeted the 20S proteosome α-subunit PMA7 RNA (Krüger et al., 2001). It would be interesting to examine the effects of this compound on BVDV IRES activity. Thus, BVDV may serve as an appropriate surrogate model system for the evaluation of antiviral compounds targeting the HCV IRES under some circumstances. The use of replication-competent BVDV chimeras containing the HCV IRES in place of the BVDV IRES (Frolov et al., 1998) will certainly facilitate the use of BVDV to identify inhibitors of the HCV IRES.

3.3. Proteolytic processing using homologous viral NS3 proteins

The HCV and BVDV NS3 proteins are multifunctional proteins. The N-terminal third of NS3 contains a chymotrypsin-like serine protease domain that functions to cleave the viral non-structural proteins NS3-NS5B, while the C-terminal portion codes for RNA helicase and NTPase activities (reviewed in Lindenbach and Rice, 2001). The NS3 protease and the helicase/NTPase activities of HCV and BVDV are each essential for viral replication (Gu et al., 2000; Kolykhalov et al., 2000), and both are considered major targets for HCV antiviral drug discovery efforts (Borowski et al., 2002; Perni and Kwong, 2002). Cleavage of the NS3/NS4A site by the NS3 protease occurs in *cis*, while the remaining sites are cleaved in *trans*. The HCV NS3 protease cleaves a highly conserved sequence of (Asp/Glu)XXXX(Cys/Thr) ↓ (Ser/Ala) while the BVDV NS3 protease targets a sequence of Leu ↓ (Ser/Ala/Asn) and less defined flanking amino acids. Interestingly, both utilize their NS4A as a protein cofactor for NS3 protease activity.

The NS3 proteins of BVDV and HCV are similar in size (683 vs. 631 amino acids, respectively), and both function analogously in terms of their activities in the viral life cycle (Xu et al., 1997). We compared the complete NS3 protein sequences of HCV-1 (Genbank Accession number AF009606) and BVDV NADL (AJ133738) using the program BIOEDIT (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and found 21.6% amino acid identity. The extent of the similarities between the two proteins is more apparent when the conserved NS3 sequence motifs are compared in Table 1. The protease domain motifs show 0–44.4% (mean = 23.7%) amino acid identity with 0–18.2% (mean = 11.3%) conserved amino acid changes at the remaining sites. The helicase/NTPase domain displays 40.0–62.5% (mean = 47.9%) amino acid identity with 0–31.3% (mean 12.7%) conservative amino acid changes at the remaining sites, which indicates that the NS3 NTPase/helicase domain of BVDV is more similar to HCV than is the protease domain.

Due to the relatively low levels of sequence identity between HCV and BVDV NS3 proteases and the differences in cleavage specificity, BVDV may not be an appropriate surrogate model for this target. But due to the high levels of identity between the conserved motifs of the NS3 helicase/NTPase of BVDV and HCV, this BVDV target may serve as an appropriate surrogate for the HCV protein. Certainly it will be important to validate the use of the BVDV model for these indications using the HCV NS3 protease and helicase/NTPase inhibitors that are now being identified before employing it alone to identify inhibitors of these drug targets. The use of the chimeric BVDV containing an HCV NS4A-tethered NS3 protease domain (Lai et al., 2000) might expedite this work.

3.4. RNA replication using homologous NS5B enzymes

The NS5B protein is the virus-encoded RNA-dependent RNA polymerase and is a major target for the development of anti-HCV antiviral agents (Zhong et al., 1998; Kao et al., 1999; Lai et al., 1999; Lohmann et al., 1999b). This activity is essential for the replication of HCV (Lohmann et al., 1999b; Kolykhalov et al., 2000) and BVDV (Grassmann et al., 2001). The HCV NS5B is 591 amino acids in length while the BVDV NS5B codes for a 719-amino-acid-long protein. The size difference is due to the presence of an additional N-terminal domain in the Pestivirus enzyme (Lai et al., 1999). Both the HCV and BVDV NS5B proteins have a highly hydrophobic amino acid domain at the C-terminus (Lai et al., 1999). We compared the complete NS5B protein sequences of HCV-1 (AF009606) and BVDV NADL (AJ133738) using the program BIOEDIT (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and found 17.2% amino acid identity. The extent of the similarities between the two proteins is more apparent when the conserved NS5B sequence motifs are compared, with 13.6–40.0%

Table 1
Comparison of HCV and BVDV NS3 proteins in conserved motifs

NS3 domain	Motif	Comments	Alignment		Percent identity	Conserved changes (%)
Protease	Box 1[a]	Contains the H of the catalytic HDC/S triad	HCV-1 BVDV NADL	NGVCWTVYHGAGTRTIASPK QGGISSVDHVTAGKDLLVCD	15.0	15.0
	Box 2[a]	Contains the D of the catalytic HDC/S triad	HCV-1 BVDV NADL	DQDLVGWPAPQ SNNRLTDETEY	0	18.2
	Box 3[a]	Contains the S of the catalytic HDC/S triad Involved in substrate binding	HCV-1 BVDV NADL	P-----ISYLKGSSGGPLLCP-A TPAFFDLKNLKGWSGLPIFEASS	35.3	11.8
	Box 4[a]	Involved in substrate binding	HCV-1 BVDV NADL	HAVGLFRAAVC RVVG--RVKVG	44.0	0
Helicase/NTPase	I[b,c]	Binding of β and γ phosphate of the NTP	HCV-1 BVDV NADL	LHAPTGSGKSTKVPAA ITLATGAGKTTELPKA	40.0	31.3
	Ia[b]	No function assigned	HCV-1 BVDV NADL	KVLVLNPSVAATLGF RVLVLIPLRAAAESV	40.0	6.7
	II[b,d]	Chelation of Mg^{2+} of Mg-NTP complex	HCV-1 BVDV NADL	ITICDECH YTFLDEYH	50.0	0
	III[b]	No function assigned	HCV-1 BVDV NADL	VLATATPP VAMTATPA	62.5	0
	IV[b]	No function assigned	HCV-1 BVDV NADL	GKAIPL GLKIPV	50.0	16.7
	V[b]	No function assigned	HCV-1 BVDV NADL	TDALMTGFTG-DFDSVI TNAIESGVTLPDLDTVI	43.8	25.0
	VI[b]	Binds nucleic acids	HCV-1 BVDV NADL	DAVSRTQRRGR TVGEQAQRRGR	50.0	9.1

The complete amino acid sequences of the NS3 proteins of HCV-1 (Genbank Accession number M62331) and BVDV NADL (AJ133738) were aligned using the program BIOEDIT (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Blocks of amino acids corresponding to highly conserved sequence motifs[a,b] from that alignment are shown. Asterisks indicate the site of the conserved HDC/S catalytic triad[a] residues. Identical amino acids are shown in black blocks while conservative changes are indicated in shaded blocks.

[a] Bazan and Fletterick (1990).
[b] Kadaré and Haenni (1997).
[c] A-site of Walker et al. (1982).
[d] B-site of Walker et al. (1982).

(mean = 30.4%) amino acid identity and 0–30.4% (mean = 12.8%) conserved amino acid substitutions at the other sites (Table 2).

It has been found that both of these NS5B proteins exhibit similar reactivity profiles in vitro in terms of their RNA polymerase activities (Lai et al., 1999; Steffens et al., 1999). Also, a mutational and deletional analysis of the BVDV NS5B found generally consistent effects on de novo and elongative RNA synthesis compared to analogous mutations and deletions in the HCV NS5B polymerase (Lai et al., 1999).

Ribavirin has a complex and multifaceted mechanism of action that may include both direct and indirect, immune-mediated activities (reviewed in Graci and Cameron, 2002; Lau et al., 2002). When ribavirin is used alone in the treatment of HCV-infected patients, it does not reduce viral loads or lead to sustained virologic responses (Di Bisceglie et al., 1995; Dushieko et al., 1996; Bodenheimer et al., 1997). This drug is active against BVDV in vitro (Markland et al., 2000; Buckwold et al., 2003; Stuyver et al., 2003), and weakly active against HCV RNA replicons (Lanford et al., 2003; Zhou et al., 2003) and related in vitro HCV expression systems (Contreras et al., 2002). However, since the combination therapy of ribavirin and IFN-α leads to enhanced virologic responses in HCV-infected patients relative to the use of either drug alone (Gutfreund and Bain, 2000), and since this drug combination produced a synergy of antiviral activity in vitro against both BVDV and yellow fever virus (Buckwold et al., 2003) and against HCV RNA replicons (Tanabe et al.,

Table 2
Comparison of HCV and BVDV NS5B proteins in conserved sequence motifs

Motif	Comments	Alignment		Percent identity	Conserved changes (%)
Nc[a]	Possible role in RNA template/ primer and/or nucleotide interactions	HCV-1 BVDV NADL	* * * RKPARLIVFPDLGVR EKRPRVIQYPEAKTR	33.3	13.3
A[b]	Magnesium coordination and possibly sugar selection	HCV-1 BVDV NADL	* * KTPMGFSYDTRCFDSTVTESDIR NEPVAVSFDTKAWDTQVTSKDLQ	34.8	30.4
B[b]	Possibly sugar selection and discrimination	HCV-1 BVDV NADL	* * ** ** RRCRASGVLTTSCGN-TLTCYIKARAAC NGQRGSGQPDTSAGNSMLNVLTMMYAFC	37.0	0
C[b]	Magnesium coordination and the invariant “GDD” motif	HCV-1 BVDV NADL	****** GL------QDCTMLVCGDDLVVICES GVPYKSFNRVARIHVCGDDGFLITEK	40.0	15.0
D[b]	Palm core structural element	HCV-1 BVDV NADL	* S-AGVQEDAASLRAFTEAMTRYS KGLGLKFANKGMQILHEAGKPQK	13.6	18.2
E[b]	Hydrophobic interaction with thumb	HCV-1 BVDV	LELITSCSSNVSVAHDG FEDIEFCSHTPVPVRWS	23.5	0

The complete amino acid sequences of the NS5B proteins of HCV-1 (Genbank Accession number M62331) and BVDV NADL (AJ133738) were aligned using the program BIOEDIT (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Blocks of amino acids corresponding to highly conserved sequence motifs[a,b] from that alignment are shown. Identical amino acids are shown in black blocks while conservative changes are indicated in shaded blocks. Asterisks indicate the most highly conserved amino acids[a,b].

[a] Lai et al. (1999).

[b] O’Reilly and Kao (1998).

2002; Yamaga et al., 2002), there is great interest in understanding the mechanism of action of ribavirin. The use of drug–drug combination analysis as above will likely play an important role in the identification and characterization of favorable combinations of new drugs that can be used to combat HCV infection.

Ribavirin acts in part as an antiviral agent through its direct actions as an inhibitor of the HCV RNA-dependent RNA polymerase NS5B (Maag et al., 2001), as an IMP dehydrogenase inhibitor (Wills et al., 1978; Zhou et al., 2003), or by acting as an RNA mutagen following its incorporation into viral RNA by NS5B (Crotty et al., 2000, 2001; Lanford et al., 2001; Contreras et al., 2002; Lanford et al., 2003; Zhou et al., 2003). Since ribavirin also has antiviral activity against BVDV (Markland et al., 2000; Buckwold et al., 2003; Stuyver et al., 2003), one would anticipate that the same NS5B-dependent mechanism is operative here as is with other viruses.

Three additional published studies illustrate how unique HCV NS5B inhibitors might have already been discovered using BVDV. Baginski et al. (2000) identified compound VP32947 (3-[((2-dipropylamino)ethyl)thio]-5H-1,2,4-triazino[5,6-b]indole) as an inhibitor of cytopathic BVDV replication in a cell-based assay. The inhibitor acted at a late stage in the viral life cycle and had a dramatic inhibitory effect on BVDV RNA synthesis. VP32947-resistant BVDV was isolated, and the mutation of NS5B amino acid 224 from phenylalanine to serine (F224S) was found to be responsible for the drug resistance. In an in vitro assay using purified wild-type and F224S mutated BVDV NS5B proteins, they showed that VP32947 inhibited the RNA polymerase activity of wild-type but not F224S NS5B proteins. The compound does not inhibit HCV RNA replicons.

Similarly, Stuyver et al. (2003) screened a nucleoside library with cytopathic BVDV and identified β-D-N^4-hydroxycytidine (NHC) as an inhibitor of BVDV replication. This compound also showed antiviral activity using HCV RNA replicons. In an HCV NS5B polymerase assay, the NHC-triphosphate did not inhibit the reaction, but it did act as an alternate substrate and its incorporation into RNA led to an increased molecular weight of the reaction product. Drug-resistant HCV RNA replicon cell lines could not be identified. The authors speculated that incorporation of NHC into genomic RNA by NS5B led to an alteration in the HCV IRES secondary or tertiary structure, representing one possible mode of action of the compound.

Recently, Sun et al. (2003) identified the cyclic urea derivative compound 1453 as an inhibitor of non-cytopathic BVDV replication in cell culture. The compound was shown to act after viral entry into cells and prior to the release of progeny virus. Compound 1453 reduced viral, but not cellular RNA synthesis. When 1453-resistant BVDVs were selected and isolated, a single specific mutation of NS5B glutamic acid to glycine E291G (E3560G in the polyprotein) was identified. Reverse genetics was used to demonstrate that this mutation was responsible for drug resistance.

Surprisingly, compound 1453 did not inhibit the NS5B polymerase activity in vitro. However, when a membrane assay of the NS5B polymerase activity as replicase complex was utilized, specific inhibition of wild-type, but not mutant, BVDV NS5B was observed. Specifically, compound 1453 is believed to inhibit the elongation of HCV RNA from the NS5B replicase complex. The inhibitory activity of compound 1453 against HCV RNA replicons or HCV NS5B protein was not reported. Indeed, the concordance between the activities of most in vitro inhibitors of the HCV and BVDV polymerases is not known at this time. This is an important area for future studies given the importance of HCV NS5B as a target for antiviral drug discovery efforts.

It is worth pointing out the importance of the BVDV and HCV RNA replicon drug resistance studies mentioned above. By analogy with other viruses such as human immunodeficiency virus, we anticipate that many of the new anti-HCV antiviral agents that are being developed, which target the viral RNA-dependent RNA polymerase NS5B, may lead to the generation of drug-resistant viral strains. A recognition and understanding of this phenomenon will facilitate our abilities to best utilize these new drugs clinically.

3.5. Virion maturation, assembly, and egress

Viral envelope proteins mature as they traverse the ER in a complicated process mediated by the action of host cell enzymes, which add and trim the sugar moieties that are found on the mature envelope glycoproteins. These post-translational modifications are essential for the proper folding and transport of viral glycoproteins and the subsequent secretion of mature virions (Lu et al., 1997; Block et al., 1998). The ER α-glucosidase enzymes mediate the stepwise removal of terminal glucose residues from *N*-glycan chains that are attached to nascent glycoproteins to allow glycoproteins to interact with the ER chaparone proteins calnexin and calreticulin, which bind exclusively to monoglucosylated glycoproteins. Glucosidase inhibitors are known that specifically target proteins dependent on the calnexin interaction (Nehls et al., 2000). These inhibitors and, in particular, the iminosugar *N*-nonyl-deoxynojirimycin (DNJ) and more recently deoxygalactonojirimycins (DGJ, long-alkyl-chain galactose-based iminosugars) were found to inhibit the formation and secretion of BVDV virions in cell culture due to misfolding of host and viral glycoproteins (Zitzmann et al., 1999; Durantel et al., 2001). The infectivity of newly released virions was also curtailed by these agents. As with many such studies, the effects of these anti-BVDV antiviral agents on HCV RNA replicon replication were not described.

4. Conclusions

In spite of the relatively low levels of sequence identity between BVDV and HCV, the BVDV surrogate model of HCV replication continues to be widely utilized in drug discovery programs and in mechanism of action studies. This is due to the similarities between these two viruses in terms of their replication cycles and biology, in terms of their genetic organization, and due to the functionally homologous nature of many of their gene products that are considered to be major targets for development of anti-HCV antiviral agents. It will be important to validate this surrogate model system with the new anti-HCV drugs that are now being identified through other means. Similarly, it is of great interest to determine the concordance between inhibitors of BVDV replication and those of HCV. In all likelihood, the use of BVDV and the myriad of BVDV–HCV chimeras that are emerging may lead to the identification and characterization of unique anti-HCV antiviral agents targeting a variety of stages in the viral life cycle.

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