Synthesis and Anti-BVDV Activity of Acridones As New Potential Antiviral Agents[1]

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In this study we report the design, synthesis, and activity against bovine viral diarrhea virus (BVDV) of a *novel* series of acridone derivatives. BVDV is responsible for major losses in cattle. The virus is also considered to be a valuable surrogate for the hepatitis C virus (HCV) in antiviral drug studies. Some of the synthesized acridones elicited selective anti-BVDV activity with EC_{50} values ranging from 0.4 to 4 μg/mL and were not cytotoxic at concentrations that were 25- to 200-fold higher (CC_{50} >100 μg/mL). It was proven that the most potent acridone derivative **10** was able to not only protect cells from virus-induced cytopathic effect but also reduce the production of infectious virus and extracellular viral RNA. Furthermore, compound **10**, as well as a number of other analogues, inhibited HCV replication to some extent. However, there was no direct correlation between anti-BVDV and anti-HCV activity. Thus, the acridone scaffold, when appropriately functionalized, can yield compounds with selective activity against pestiviruses and related viruses such as the HCV.

Introduction

Bovine viral diarrhea virus (BVDV) is the prototype of the *Pestivirus* genus of the *Flaviviridae* family. Besides BVDV, the genus *Pestivirus* contains other animal pathogens such as the classical swine fever virus (CSFV) and the border disease virus (BDV). BVDV causes a range of clinical manifestations including early embryonic death, abortion, teratogenesis, respiratory problems, chronic wasting syndrome, and immune system disorder which resulted in a mortality rate of 17−32%[2−4] in cattle throughout the world; this translates roughly into a loss of $10 to 40 million per million calvings.[5] Moreover, BVDV is a problematic contaminant in the laboratory. Noncytopathic BVDV infection can remain unnoticed and infects laboratory cell lines through its presence in contaminated bovine serum used in cell culture.[6] Hence, contamination of interferons[7] and vaccines[8] for medicinal use have been reported. Regardless of the availability of vaccines[9] against BVDV and the implementation of elaborate eradication or control programs, BVDV remains an agronomical burden. An alternative approach to combat BVDV infections could be the use of antiviral agents that specifically inhibit the replication of the virus. Although not suited to treat large herds, it could be important to have selective anti-pestivirus compounds on hand that could be used prophylactically in case of an outbreak of CSFV to provide protection during the time that is needed until a vaccine became effective (usually 10−14 days later). During this interim period, the pigs are still vulnerable to infection by CSFV. Antiviral drugs do not rely on the immune system to halt viral replication, offering an alternative to large-scale culling of healthy animals. Other possible uses for anti-pestivirus drugs could be to (i) treat valuable animals in zoo collections, (ii) treat expensive animals in breeding programs (in vitro embryo production),[10] or (iii) rid established cell lines from contaminating pestiviruses.[11,12]

Recently, a number of selective anti-BVDV compounds have been reported. These include the following: polymerase inhibitors, i.e., 3-[((2-dipropylamino)ethyl)thio]-5*H*-1,2,4-triazino[5,6-*b*]indole (VP32947),[13] a thiazole urea derivative,[14] and a cyclic urea derivative,[15] and inhibitors of the NS3/NS4A protease, for example, a boron-modified peptidyl mimetic.[16] Aromatic cationic molecules have also been reported to inhibit BVDV replication, although the mechanism of action remains to be elucidated.[17] Other BVDV inhibitors target cellular enzymes such as the α-glucosidase[18−20] and inosine monophosphate dehydrogenase (IMPDH).[21] Recently, a new highly selective BVDV polymerase inhibitor of pestivirus replication, 5-[(4-bromophenyl)methyl]-2-phenyl-5*H*-imidazo[4,5-*c*]pyridine (BPIP), was reported.[22]

Like BVDV, the hepatitis C virus (HCV) also belongs to the *Flaviviridae* family. It is the most common cause of chronic hepatitis throughout the world and is the major risk factor involved in the development of liver cirrhosis and hepatocellular carcinoma.[23,24] The currently available therapy, i.e., the combination of pegylated interferon-α with the broad spectrum antiviral agent ribavirin, has a limited efficacy (sustained response rate of only about 60%) and there are important side effects associated with therapy. Highly effective and selective inhibitors of HCV replication are urgently needed.[25]

BVDV is considered to be a valuable surrogate virus model for identifying and characterizing antiviral agents for use against HCV.[26] In some aspects of viral replication, BVDV is more advantageous than the currently used HCV replicon systems.[27,28] The latter do not undergo a complete replication cycle; hence, early stages (attachment, entry, uncoating) or late stages (virion assembly and release) of the viral replication cycle cannot be studied in the HCV replicon system. However, very recently, robust HCV cell culture systems have been reported.[29−31] Therefore, insight into the mechanism of antiviral activity of

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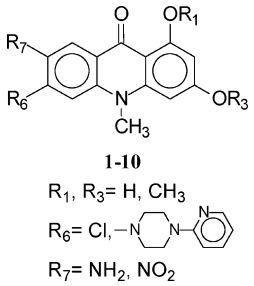


Figure 1. Structural formula of acridones.

anti-pestivirus compounds may provide valuable information for the design of novel antiviral strategies against HCV.[27]

Several types of naturally occurring and synthetic acridones had various bioactivities, including antiviral activity against human immunodeficiency virus (HIV),[32,33] herpes simplex viruses (HSV),[34−40] coxsackie virus type B1, anti-Western equine encephalitis virus,[40] adenovirus type 6,[41] human cytomegalovirus (HCMV),[34,38] and Epstein−Barr virus (EBV).[42]

To date, acridone derivatives have not been reported to exhibit activity against BVDV and/or HCV, even though two recent patents[43,44] report on the activity of various acridones and heterocycle acridones against IMPDH, a potential target for HCV therapy.

We explore here the potential of this class of molecules as inhibitors of the pestiviruses replication by synthesizing a small series of acridone derivatives exploiting as starting molecule the acridone **1** on hand in our laboratory.[45] Structurally, the acridones reported in this study (**1**−**10**, Figure 1) are characterized by a hydroxyl or methoxy group at the C-1 and/or C-3 position and a methyl group at the N-10 position. The functionalization of the C-7 position with an amino group or its nitro precursor, and the C-6 position with a 4-(2-pyridinyl)-1-piperazinyl side chain, was also based on the potent anti-HIV 6-aminoquinolones recently reported by us.[46,47]

Chemistry

The acridones reported in this study were accessed through elaboration of the nitroacridone **1**,[45] prepared according to standard procedure (Scheme 1). Derivative **1** was reduced with $SnCl_2 \cdot 2H_2O$, under acidic conditions, to give the expected 7-amino derivative **2** together with traces of the 1-hydroxy-7-nitro derivative **3**. The successive de-*O*-methylation of amino derivative **2** employing 48% HBr at reflux permitted both 1-hydroxy-3-methoxy acridone **4** and 1,3-dihydroxy acridone **5** to be obtained. It is important to strictly monitor the reaction progression by TLC to avoid the formation of the sole dihydroxy derivative **5**.

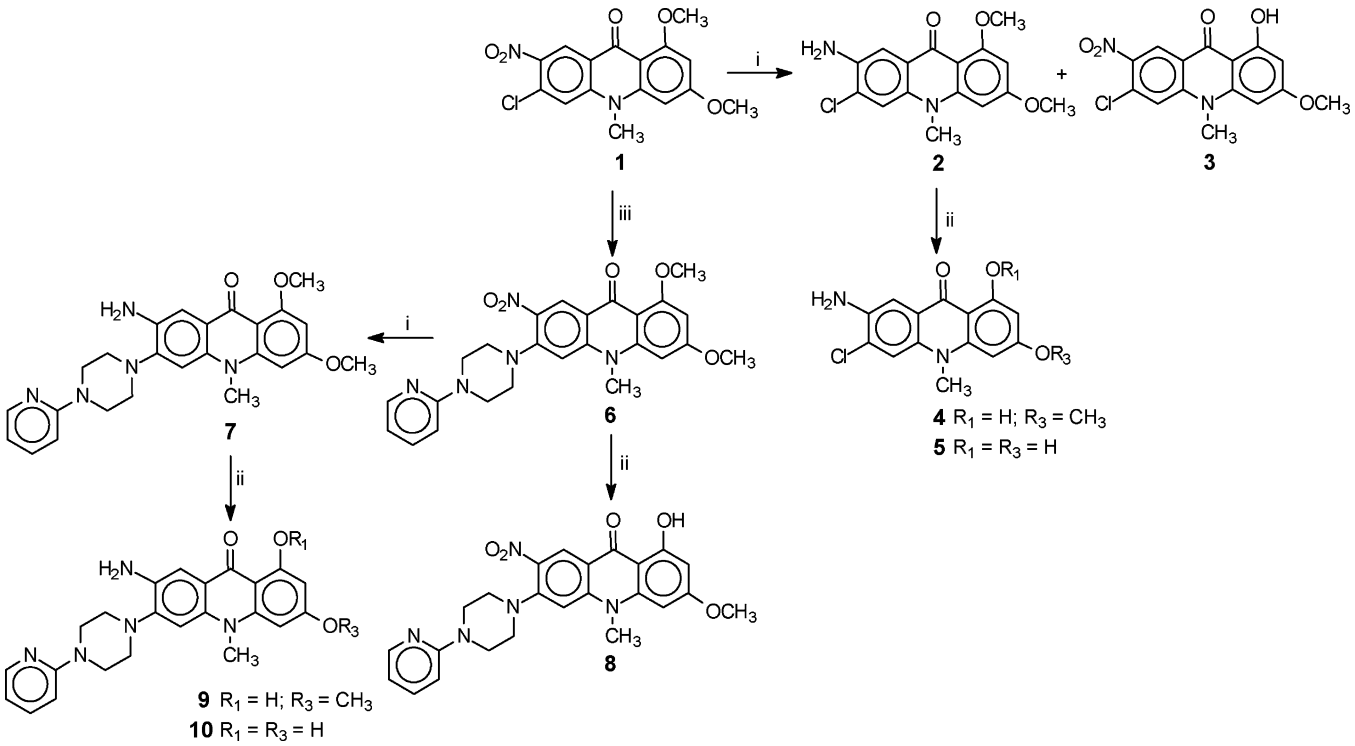
The nucleophilic reaction of acridone **1** with 1-(2-pyridinyl)-piperazine afforded the corresponding 6-[4-(2-pyridinyl)-1-piperazinyl] derivative **6** which was then elaborated to the targets 7-amino-1,3-dimethoxy acridone **7**, 7-amino-1-hydroxy-3-methoxy acridone **9**, and 7-amino-1,3-dihydroxy acridone **10** by using the above illustrated procedure for the corresponding 6-chloro acridone derivatives. In this case, the 7-nitro-1-hydroxy derivative **8** was not obtained as a side product in the reduction step, so it was synthesized by treating **6** with 48% HBr.

Results and Discussion

This novel series of acridones **1**−**10** was evaluated for their anti-BVDV activity (NADL wild-type strain) in MDBK cells. The cytotoxic activity of the compounds was determined in parallel in the same cell lines. VP32947,[13] a potent and selective anti-pestivirus compound, was included for comparison (Table 1).

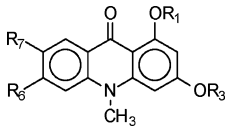
Several compounds inhibited BVDV replication and, with the exception of **5**, **4**, and, to some extent, **3**, they did not inhibit the metabolism of uninfected cells at concentrations up to 100 μg/mL. Derivative **10** elicited the most interesting activity, having EC_{50} values of 0.5 ± 0.2 μg/mL coupled with $CC_{50} > 100$ μg/mL, resulting in a selectivity index (CC_{50}/EC_{50}) of >200. Acridones **4**, **5**, **6**, and **9** also proved to be antivirally active.

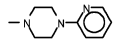
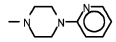
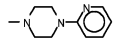
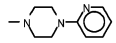
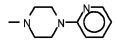
Scheme 1[a]



[a] Reagents: (i) $SnCl_2 \cdot 2H_2O$, 8 N HCl, reflux; (ii) 48% HBr, reflux; (iii) 1-(2-pyridinyl)piperazine, dry DMF 90 °C.

Table 1. Antiviral Activity and Cytotoxicity of Acridone Derivatives against BVDV (Strain NADL) in MDBK Cells



Compd	R_1	R_3	R_6	R_7	EC_{50}[a] (μg/mL)	CC_{50}[b] (μg/mL)	SI[c]
1[45]	CH_3	CH_3	Cl	NO_2	> 100	> 100	1
2	CH_3	CH_3	Cl	NH_2	> 100	> 100	1
3	H	CH_3	Cl	NO_2	> 83	83	1
4	H	CH_3	Cl	NH_2	0.7 ± 0.3	> 33	> 47
5	H	H	Cl	NH_2	0.4 ± 0.2	5.0 ± 0.7	13
6	CH_3	CH_3		NO_2	18 ± 2	> 100	> 6
7	CH_3	CH_3		NH_2	> 100	> 100	1
8	H	CH_3		NO_2	> 100	> 100	1
9	H	CH_3		NH_2	4 ± 2	> 100	> 25
10	H	H		NH_2	0.5 ± 0.2	> 100	> 200
VP32947[13]					0.009 ± 0.006	> 1	> 111
2'-*C*-methyl-adenosine[50,51]					0.22 ± 0.09	> 14	> 63

[a,b] EC_{50} and CC_{50} were obtained as described in the Experimental Section. All the data represent mean values for at least three separate experiments ± standard deviation. [c] SI is the selectivity index which equals CC_{50}/EC_{50}.

The evaluation of this small series of acridones revealed some interesting trends that can be delineated. The presence of an NH_2 group at the C-6 position generally grants higher anti-BVDV activity than the NO_2 group. When comparing the 7-amino-6-chloro series (**2**, **4**, and **5**) with the corresponding 7-amino-6-[4-(2-pyridinyl)-1-piperazinyl] series (**7**, **9**, and **10**), it becomes evident that the anti-BVDV activity increases along the series from the inactive 1,3-dimethoxy derivatives **2** and **7** to the potent 1,3-dihydroxy derivatives **5** and **10.** Thus, the presence of an hydroxyl group at the C-1 position seems to be important for antiviral activity, in agreement with what has been previously reported for anti-HSV acridones.[37] Moreover, contrary to almost all the biologically active acridones,[39,48,49] the C-1 hydroxyl group does not confer cytotoxicity to our molecules.

The effect of the compounds on the replication of other viruses was also assessed, i.e., HSV-1 (KOS strain), HSV-2 (G strain), vaccinia virus, and vesicular stomatitis virus in E_6SM cell cultures; HCMV, (AD-169 strain and Davis strain) in human embryonic lung (HEL) cells and respiratory syncytial virus (RSV) in HeLa cell cultures; coxsackie virus B4, parainfluenza-3 virus, Reovirus-1, Sindbis virus, Punta Toro virus, and Yellow Fever Virus (YFV) (YFV-17D vaccine strain) in Vero cell cultures; HIV-1 (IIIB) and HIV-2 (ROD) in MT-4 cells. With the exception of some minor activity against the flavivirus YFV-17D (that also belongs to the *Flaviviridae* family), none of the compounds were active against any of the other viruses tested.

The anti-BVDV activity of the most active derivative **10** was further confirmed by two additional assays*:* i.e., (i) a RT-qPCR assay to measure viral RNA production and (ii) titration for infectious virus production (Figure 2). The EC_{50} values for the inhibition of viral RNA production in culture supernatant and inhibition of infectious virus yield were 2.3 ± 0.4 and 2.01 ± 0.07 μg/mL, respectively. The overall patterns of the inhibition of viral RNA synthesis and infectious virus yield were very similar. The activity of **10** was also evaluated against a VP32947-resistant strain of BVDV (containing the F224S mutation) in comparison with the nucleoside analogue 2′-*C*-methyl-adenosine[50,51] (Table 2). Not unexpectedly, acridone **10** proved almost equally effective against the wild-type virus and

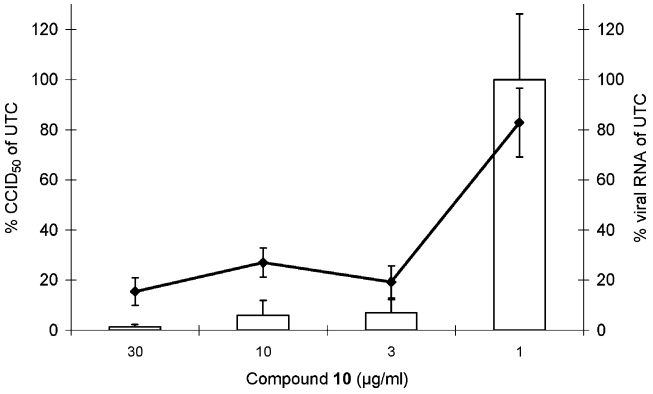


Figure 2. Yields of extracellular viral RNA (diamonds) and infectious virus (open bars) in BVDV NADL infected MDBK cells. Viral RNA levels were monitored by RT-qPCR and infectious virus by a virus yield assay. All data represent mean values for three independent experiments ± standard deviation.

Table 2. Antiviral Activity and Cytotoxicity of Acridone **10** against VP32947-Resistant BVDV in MDBK Cells

compd	EC_{50}[a] (μg/ mL)	CC_{50}[b] (μg/mL)	SI[c]
10	2.24 ± 0.09	>100	>45
VP32947[13]	>1	>1	1
2-*C*-methyl-A denosine[50,51]	0.37 ± 0.06	>14	>38

[a,b] EC_{50} and CC_{50} were obtained as described in the Experimental Section. All the data represent mean values for at least three separate experiments ± standard deviation. [c] SI is the selectivity index which equals CC_{50}/EC_{50}.

this drug-resistant variant. Because the viral RNA-dependent RNA polymerase (RdRp) could be the potential target of this class of molecules, we tested whether compound **10** had any inhibitory activity on the highly purified BVDV RdRp. Unfortunately, very little inhibition of viral polymerase activity was observed. However, this does not exclude that the viral polymerase is the molecular target of the compound. Indeed, we recently reported that the novel imidazopyridine BPIP[22] targets the viral polymerase (as is evident from the fact that a single-point mutation in the viral polymerase gene is responsible for the resistant phenotype) but the compound proved inactive against the purified enzyme. Similarly, the molecular target for the anti-pestivirus activity of VP32947[13] is the viral polymerase, and also for this compound there was no correlation between the potent in vitro antiviral activity and the rather weak effect on the purified polymerase. The lack of activity of these two compounds against the RdRp may perhaps be explained by the fact that viral or cellular factors are involved in the inhibitory effect of these antiviral drugs along with the polymerase. In such case, this would be in-line with what has been previously reported for other antiviral acridones, for which more than one biochemical target, viral, and/or cellular has been identified.[49] Determination of the genotype of virus resistance to compound **10** should reveal which viral protein is the actual antiviral target.

To exclude that an intracellular metabolite, rather than compound **10** itself, was the antiviral active entity, extracts of cells that had been incubated with the compound for 24 h were analyzed. In the infected cultures, compound **10**, when added for the first 24 h after infection, already showed a marked reduction in intracellular viral RNA, indicating that the active component should be present in sufficient quantities in the cell following a 24 h incubation period. Only the intact compound **10** was identified as the molecule that exerts the direct antiviral activity.

Finally, all acridones were also evaluated in the HCV genotype 1 subgenomic replicon system. Preliminary results revealed an interesting antiviral activity for some of the derivatives. However, no strict correlation was observed between the anti-BVDV and anti-HCV activity. In particular, compound **10**, the most potent anti-BVDV acridone, exhibited anti-HCV activity ($EC_{50} = 1.7 \pm 0.2$ μg/mL) that was comparable to that for BVDV. The compound however proved to be somewhat more cytotoxic against the human hepatoma Huh 5-2 cells ($CC_{50} = 21 \pm 3$ μg/mL) than toxic on the bovine kidney cells. An in-depth study on the HCV replication inhibitory activity of these acridones is currently in progress and will be reported in a successive paper.

Experimental Section

All reactions were routinely checked by thin-layer chromatography (TLC) on silica gel $60F_{254}$ (Merck) and visualized by using UV. Flash column chromatography was performed on Merck silica gel 60 (mesh 230−400). Melting points were determined in capillary tubes (Büchi Electrothermal Mod. 9100) and are uncorrected. Elemental analyses were performed on a Carlo Erba elemental analyzer, Model 1106, and the data for C, H, and N are within ±0.4% of the theoretical values. ^{1}H NMR spectra were recorded at 200 MHz (Bruker Avance DPX-200), with DMSO-d_6 as solvent, unless otherwise indicated, and with Me_4Si as internal standard. Chemical shifts are given in ppm (δ). The spectral data are consistent with the assigned structures. Reagents and solvents were purchased from common commercial suppliers and were used as-received. Organic solutions were dried over anhydrous Na_2SO_4 and concentrated with a Büchi rotary evaporator at low pressure. Yields were of purified product and were not optimized. All starting materials were commercially available, unless otherwise indicated.

7-Amino-6-chloro-1,3-dimethoxy-10-methyl-9(10*H*)-acridinone (2) and 6-Chloro-1-hydroxy-3-methoxy-10-methyl-7-nitro-9(10*H*)-acridinone (3). A solution of $SnCl_2 \cdot 2H_2O$ (0.97 g, 4.30 mmol) in 8 N HCl (4 mL) was added at room temperature with stirring to a solution of nitro derivative **1**[45] (0.50 g, 1.43 mmol) in 8 N HCl (5 mL). The mixture was heated under reflux for 1 h. After cooling, the precipitate was filtered, dissolved in water, and made basic with 10% NaOH solution to obtain a solid which was collected, washed several times with water, dried, and purified by flash chromatography eluting with CH_2Cl_2/MeOH (99:1) to give compound **3** (0.024 g, 5%) followed by compound **2** (0.18 g, 40% yield).

Compound **2**: mp 318−322 °C; ^{1}H NMR δ 3.70, 3.75, and 3.90 (s, each 3H, CH_3), 5.20 (bs, 2H, NH_2), 6.25 and 6.50 (d, $J = 2.0$ Hz, each 1H, H-2 and H-4), 7.70 (s, 1H, H-5), 7.80 (s, 1H, H-8). Anal. ($C_{16}H_{15}ClN_2O_3$), C, H, N.

Compound **3**: mp >350 °C; ^{1}H NMR δ 3.80 and 3.85 (s, each 3H, CH_3), 6.45 and 6.70 (d, $J = 2.0$ Hz, each 1H, H-2 and H-4), 8.10 (s, 1H, H-5), 8.90 (s, 1H, H-8), 14.10 (s, 1H, OH). Anal. ($C_{15}H_{11}ClN_2O_5$) C, H, N.

7-Amino-6-chloro-1-hydroxy-3-methoxy-10-methyl-9(10*H*)-acridinone (4) and 7-Amino-6-chloro-1,3-dihydroxy-10-methyl-9(10*H*)acridinone (5). A mixture of **2** (0.30 g, 0.94 mmol) in 48% HBr (4 mL) was heated at 90 °C for about 3 h until the starting material disappeared and two products formed. After cooling, the bromohydrate precipitate was filtered, solubilized in water, and neutralized with saturated Na_2CO_3 solution. The solid obtained was collected by filtration, dried, and purified by flash chromatography eluting with CH_2Cl_2/MeOH (98:2) to give compound **4** (0.03 g, 10%) followed by compound **5** (0.14 g, 52%).

Compound **4**: mp 279−280 °C, ^{1}H NMR δ 3.75 and 4.00 (s, each 3H, CH_3), 5.60 (bs, 2H, NH_2), 6.20 and 6.60 (d, $J = 2.0$ Hz, each 1H, H-2 and H-4), 7.65 (s, 1H, H-5), 7.90 (s, 1H, H-8), 15.00 (s, 1H, OH). Anal. ($C_{15}H_{13}ClN_2O_3$) C, H, N.

Compound **5**: mp >350 °C; ^{1}H NMR δ 3.70 (s, 3H, CH_3), 5.55 (bs, 2H, NH_2), 6.00 and 6.25 (d, $J = 2.0$ Hz, each 1H, H-2 and H-4), 7.85 (s, 1H, H-5), 7.90 (s, 1H, H-8), 10.50 (bs, 1H, OH), 14.80 (s, 1H, OH). Anal. ($C_{14}H_{11}ClN_2O_3$) C, H, N.

1,3-Dimethoxy-10-methyl-7-nitro-6-[4-(2-pyridinyl)-1-piperazinyl]-9(10*H*)-acridinone (6). A mixture of **1**[45] (0.30 g, 0.86 mmol) and 1-(2-pyridinyl)piperazine (0.42 g, 2.58 mmol) in dry DMF (20 mL) was heated at 90 °C for 7 h. The solution was evaporated to dryness, and EtOH was added to the residue. The product was filtered, washed with EtOH, dried, and crystallized by EtOH/DMF to give 0.33 g (81%) of **6**: mp 266−268 °C; ^{1}H NMR ($CDCl_3$) δ 3.20−3.30 and 3.50−3.70 (m, each 4H, piperazine CH_2), 3.75, 3.85, and 4.00 (s, each 3H, CH_3), 6.20 and 6.30 (d, $J = 2.0$ Hz, each 1H, H-2 and H-4), 6.50−6.70 (m, 3H, pyridine CH and H-5), 7.40−7.50 (m, 1H, pyridine CH), 8.10−8.20 (m, 1H, pyridine CH), 8.80 (s, 1H, H-8). Anal. ($C_{25}H_{25}N_5O_5$) C, H, N.

7-Amino-1,3-dimethoxy-10-methyl-6-[4-(2-pyridinyl)-1-piperazinyl]-9(10*H*) -acridinone (7). The title compound was prepared using the procedure as described for **2** starting from 7-nitroderivative **6**. It was obtained in 57% yield: mp 295−298 °C; ^{1}H NMR δ 3.05−3.20 and 3.60−3.75 (m, each 4H, piperazine CH_2), 3.75, 3.80, and 3.90 (s, each 3H, CH_3), 4.80 (bs, 2H, NH_2), 6.25 and 6.50 (d, $J = 2.0$ Hz, each 1H, H-2 and H-4), 6.70 (dd, $J = 4.9$ and 6.6 Hz, 1H, pyridine CH), 6.80 (d, $J = 8.8$ Hz, 1H, pyridine CH), 7.10 (s, 1H, H-5), 7.50 (s, 1H, H-8), 7.55−7.65 and 8.10−8.20 (m, each 1H, pyridine CH). Anal. ($C_{25}H_{27}N_5O_3$) C, H, N.

1-Hydroxy-3-methoxy-10-methyl-7-nitro-6-[4-(2-pyridinyl)-1-piperazinyl]-9(10*H*)acridinone (8). The title compound was prepared using the procedure as described for **4** and **5** starting from 1,3-dimethoxy-7-nitro derivative **6**. It was obtained in 13% yield: mp 261−263 °C; ^{1}H NMR δ 3.10−3.30 (m, 4H, piperazine CH_2), 3.60−3.80 (m, 7H, piperazine CH_2 and CH_3), 3.90 (s, 3H, CH_3), 6.25 and 6.50 (d, $J = 1.7$ Hz, each 1H, H-2, and H-4), 6.70 (dd, $J = 4.9$ and 6.6 Hz, 1H, pyridine CH), 6.80−7.00 (m, 2H, pyridine CH and H-5), 7.50−7.60 (m, 1H, pyridine CH), 8.15 (dd, $J = 1.6$ and 4.9 Hz, 1H, pyridine CH) 8.60 (s, 1H, H-8), 14.40 (s, 1H, OH). Anal. ($C_{24}H_{23}N_5O_5$) C, H, N.

7-Amino-1-hydroxy-3-methoxy-10-methyl-6-[4-(2-pyridinyl)-1-piperazinyl]-9(10*H*)acridinone (9) and 7-Amino-1,3-dihydroxy-10-methyl-6-[4-(2-pyridinyl)-1-piperazinyl]-9(10*H*)acridinone (10). The title compounds were prepared using the procedure as described for **4** and **5** starting from 1,3-dimethoxy-7-amino derivative **7**.

Compound **9**: 25% yield, mp 305−307 °C; ^{1}H NMR δ 3.10−3.25 (m, 4H, piperazine CH_2), 3.60−3.75 (m, 7H, piperazine CH_2 and CH_3), 3.80 (s, 3H, CH_3), 4.75 (bs, 2H, NH_2), 6.15 and 6.40 (d, J = 1.5 Hz, each 1H, H-2 and H-4), 6.70 (dd, J = 5.3 and 6.9 Hz, 1H, pyridine CH), 6.90 (d, J = 8.8 Hz, 1H, pyridine CH), 7.00 (s, 1H, H-5), 7.40−7.60 (m, 2H, pyridine CH and H-8), 8.10−8.20 (m, 1H, pyridine CH), 10.25 (s, 1H, OH). Anal. ($C_{24}H_{25}N_5O_3$) C, H, N.

Compound **10**: 35% yield, mp 331−336 °C; ^{1}H NMR δ 3.10−3.25 and 3.40−3.50 (m, each 4H, piperazine CH_2), 3.80 (s, 3H, CH_3), 5.00 (bs, 2H, NH_2), 6.00 and 6.30 (d, J = 1.5 Hz, each 1H, H-2 and H-4), 6.65−6.75 (m, 1H, pyridine CH), 6.90 (d, J = 8.8 Hz, pyridine CH), 7.15 (s, 1H, H-5), 7.50−7.80 (m, 2H, pyridine CH and H-8), 8.15−8.25 (m, 1H, pyridine CH), 10.35 (bs, 1H, OH), 15.25 (s, 1H, OH). Anal. ($C_{23}H_{23}N_5O_3$) C, H, N.

Cells and Viruses. Madin-Darby bovine kidney (MDBK) cells were grown in a Minimal Essential Medium (Gibco/BRL) supplemented with 5% heat-inactivated fetal bovine serum (FCS) (Integro, Zaandam, The Netherlands). FCS was shown to be free of BVDV-1 and BVDV-2 by an in-house PCR assay.[52] First-passage BVDV NADL stock was generated from pACNRyNADL as previously described.[53] The BVDV NADL F224S mutant strain was selected as described previously.[22] Huh 7 cells containing subgenomic HCV replicons I_{389}luc-ubi-neo/NS3-3′/5.1 (Huh 5-2) were kindly provided by V. Lohmann and R. Bartenschlager.

Antiviral BVDV Assay. MDBK cells (20×10^3/well) were seeded in 96-well cell culture plates in MEM-FCS so that cells reached confluency 24 h later. The medium was then removed and serial 3-fold dilutions of the test compounds were added in a total volume of 100 μL, after which the virus inoculum (MOI = 0.5) was added to each well. This inoculum resulted in a greater than 90% destruction of the cell monolayer 4−5 days after incubation at 37 °C. Uninfected cells and cells receiving virus without compound were included in each assay plate. After 5 days, medium was removed and fixed with 70% ethanol, stained with Giemsa solution (50-fold dilution; 2 h incubation period), washed, and air-dried. The antiviral activity of the compounds was calculated as described previously.[54,55] In brief, the cytopathic effect of the virus on the cells was examined microscopically. Each well was scored on a scale from 0 to 10 in which 0 represents the lack of CPE in the uninfected untreated cell cultures and 10 equals the level of CPE in infected cells without compound. The antiviral activity of the compound is expressed as the concentration required to inhibit virus-induced cytopathic effect (CPE) by 50% (EC_{50}) and was calculated using logarithmic interpolation.

Anti-HCV Assay. Huh 5-2 cells were seeded at a density of 5×10^3 per well in a tissue culture treated white 96-well view plate (Packard, Canberra, Canada) in complete DMEM supplemented with 250 μg/mL G418. Following incubation for 24 h at 37 °C (5% CO_2) the medium was removed and 3-fold serial dilutions in complete DMEM (without G418) of the test compounds were added in a total volume of 100 μL. After 4 days of incubation at 37 °C, the cell culture medium was removed and luciferase activity was determined using the Steady-Glo luciferase assay system (Promega, Leiden, The Netherlands); the luciferase signal was measured using a Luminoskan Ascent (Thermo, Vantaa, Finland). The 50% effective concentration (EC_{50}) was defined as the concentration of compound that reduced the luciferase signal by 50%.

Cytotoxicity Assay. MDBK cells were seeded at a density of 10^4 cells per well of a 96-well plate in MEM-FCS or Huh 5-2 cells were seeded at a density of 5×10^3 cells per well of a 96-well plate in complete DMEM. The next day, serial dilutions of the test compounds were added. Cells were allowed to proliferate for 3 days at 37 °C after which the cell number was determined by means of the MTS/PMS method (Promega, Leiden, The Netherlands). The 50% toxic concentration (CC_{50}) was defined as the concentration that inhibits the growth of cells by 50%.

RNA Isolation. Viral RNA was isolated from a cell culture supernatant using the QIAamp viral RNA minikit (Qiagen, Venlo, The Netherlands) and total cellular RNA was isolated using the RNeasy minikit (Qiagen).

RT-qPCR. A 50 μL RT-qPCR reaction contained TaqMan EZ buffer (50 mmol/L Bicine, 115 mmol/L potassium acetate, 0.01 mmol/L EDTA, 60 nmol/L 6-carboxy-X-rhodamine, and 8% glycerol, pH 8.2; Applied Biosystems Nieuwerkerk, The Netherlands), 200 μmol/L deoxyadenosine triphosphate, 200 μmol/L deoxyguanosine triphosphate, 200 μmol/L deoxycytidine triphosphate, 500 μmol/L deoxyuridine triphosphate, 300 nmol/L forward primer [5′-TGA GCT GTC TGA AAT GGT CGA TT], 300 nmol/L reverse primer [AGA AAT ACT GGG TCA TCT GAT GCA A], 300 nmol/L TaqMan probe [6-FAM-CGA AGC AGG TTA CCA AGG AGG CTG TTA GGA-TAMRA], 3 mmol/L manganese acetate, 0.5 U AmpErase uracil-*N*-glycosylase (UNG), 7.5 U rTth DNA polymerase, and template BVDV RNA. Following an initial activation by uracil-*N*-glycosylase (UNG) at 50 °C for 2 min, the RT step was performed at 60 °C for 30 min, followed by the inactivation of UNG at 95 °C for 5 min. Subsequent PCR amplification consisted of 40 cycles of denaturation at 94 °C for 20 s and annealing and extension at 62 °C for 1 min in an ABI 7000 sequence detector. All samples were analyzed in three replicate reactions.

Virus Yield Assay. MDBK cells were seeded at a density of 5×10^3 cells per well of a 96-well plate (confluency 10−15%) in MEM-FCS; 24 h later they were infected with 10-fold serial dilutions of culture supernatant. After 4 days, the medium was removed and cultures were fixed with 70% ethanol, stained with Giemsa solution, washed, and air-dried. Virus-induced CPE was recorded microscopically and the viral titer was quantified according to the method of Reed and Muench.[56] Viral titers were expressed as cell culture. Infectious dose 50% ($CCID_{50}$/mL).

RNA-Dependent RNA Polymerase Assay. BVDV (NADL) RNA-dependent RNA polymerase (RdRp) was expressed and purified as described before.[57] The purified BVDV polymerase (100 nM) was mixed with 100 μM GTP (containing 8.3 μM [^{3}H]GTP, Amersham) and increasing concentrations of inhibitor (1, 10, 100, and 500 μM) in 50 mM Hepes (pH 8.0), 10 mM KCl, 10 mM DTT, 1 mM $MgCl_2$, 2 mM $MnCl_2$, and 0.5% igepal (Sigma). Enzyme mix and inhibitors were preincubated (30 min) in order to favor enzyme−inhibitor interaction before RNA binding, in case of competition for the RNA binding site. Reactions were started by the addition of 100 nM of poly(C) (about 500 nt in size) template. Reactions were incubated at 30 °C and stopped by addition of 50 mM EDTA after 1, 5, or 15 min. Samples were transferred onto DE-81 filters, washed with 0.3 M ammonium formate, and dried. Radioactivity bound to the filter was determined by liquid scintillation counting. The assay was also carried out as described above except that the poly(C) template was replaced by either oligo-(dT)/Poly(A), oligo(G)/Poly(C), or an artificial hairpin (this is an RNA transcript that encompasses nt 1768−2016 of the sequence with accession number AF305422).

Intracellular Metabolism of Compound 10. MDBK cell cultures were incubated for 24 h with 30 μg/mL of compound **10**. Then cultures were washed 3 times with PBS, cells were trypsinized and resuspended in PBS, and equal volumes of cell suspension and ethyl acetate were combined. Samples were vortexed three times for 30 s and the aqueous phase was snap-frozen in an ethanol dry ice mixture. The organic phase was transferred to a new centrifuge tube and the ethyl acetate was evaporated in a vacuum centrifuge at 40 °C for 1 h. Prior to detection, samples were dissolved in 50% methanol. Separation and analysis was carried out using a capillary liquid chromatograph (CapLC, Waters, Milford, MA) connected to a Q-Tof-2 mass spectrometer (Micromass, Manchester, UK). Samples were separated on a reverse phase column (XTerra column 0.32 × 50 mm, Waters) with a gradient of 0.1% formic acid and acetonitrile at a flow rate of 5 μL/min. This setup has a dynamic

range of over 3 orders of magnitude, allowing the detection of the presence of less than 0.1% impurities and metabolites.

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Supporting Information Available: A table containing elemental analysis data of compounds **2**−**10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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