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Highly potent and selective inhibition of bovine viral diarrhea virus replication by γ-carboline derivatives

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ABSTRACT

Several novel γ-carboline derivatives were identified as selective inhibitors of bovine viral diarrhea virus (BVDV) replication in cell cultures. Among them, 3,4,5-trimethyl-γ-carboline (SK3M4M5M) was the most active against BVDV (Nose strain) in MDBK cells, with a 50% effective concentration of 0.017 ± 0.005 μM and a selectivity index of 435. The compound inhibited viral RNA synthesis in a dose-dependent fashion. In a time of drug-addition experiment during a single viral replication cycle, SK3M4M5M lost its antiviral activity when first added at 8 h or later after infection, which coincides with the onset of viral RNA synthesis. When selected γ-carboline derivatives, including SK3M4M5M, were examined for their inhibitory effect on the mutant strains resistant to some classes of nonnucleoside BVDV RNA-dependent RNA polymerase inhibitors, all of which target the top of the finger domain of the polymerase, the strains displayed cross-resistance to the γ-carboline derivatives. These results indicate that the γ-carboline derivatives may possibly target a hot spot of the RNA-dependent RNA polymerase. Although SK3M4M5M was highly active against BVDV, the compound proved inactive against hepatitis C virus (HCV) in HCV RNA replicon cells.

1. Introduction

Bovine viral diarrhea virus (BVDV), a member of the *pestivirus* genus from the family *Flaviviridae*, causes serious health problems in cattle, which results in great economical loss (Houe, 2003). BVDV infection induces a variety of clinical symptoms, such as respiratory problems, chronic wasting disease, immune system dysfunction, and predisposition to secondary microbial infections. In addition, BVDV infects the fetus by crossing the placenta. Depending on the time of gestation, the infection can result in the birth of a persistently infected calf (Kobrak and Weber, 1997). Two biotypes, namely, non-cytopathic and cytopathic strains can be distinguished. Only non-cytopathic strains have been isolated from persistently infected animals, which are immunotolerant to the virus and are shedding infectious virions in all secretions. The superinfection of a persistently infected animal with a cytopathic strain causes a fatal mucosal disease.

Vaccines are available in some countries in an attempt to control the diseases caused by BVDV (van Oirschot et al., 1999). However, the existence of considerable genetic and antigenic diversity of BVDV is a major concern for the development and efficacy of current vaccines (Kalaycioglu, 2007). An alternative approach could be the use of antiviral agents. Several compounds have recently been identified as selective inhibitors of BVDV replication in cell cultures. These include VP32947 (Baginski et al., 2000), mizoribine (Yanagida et al., 2004), BPIP (Paeshuyse et al., 2006), Acridones (Tabarrini et al., 2006), AG110 (Paeshuyse et al., 2007), SC-560 (Okamoto et al., 2009), iminosugar derivatives (Chang et al., 2009), LZ37 (Paeshuyse et al., 2009), and BIT225 (Luscombe et al., 2010).

We have previously established a simple and sensitive colorimetric assay of compounds for evaluating their antiviral activity against BVDV in cell cultures (Baba et al., 2005). The assay is based on spectrophotometrical assessment of the viability of the cells infected with a cytopathic strain of BVDV by measuring extracellular leakage of lactic dehydrogenase (LDH). Using this system, we screened a number of small molecules for their inhibitory effect on BVDV replication in cell cultures. Among them, some γ-carboline derivatives were identified as highly potent and selective inhibitors of BVDV. In particular, dimethyl- and trimethyl-γ-carbolines could

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exert their anti-BVDV activity in the nanomolar range (Sako et al., 2008; Aoyama et al., 2009). In this study, we evaluated selected γ-carbolines for their inhibitory effect on the replication of mutant strains resistant to other anti-BVDV agents.

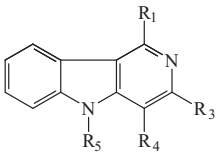
2. Materials and methods

2.1. Compounds

Seven γ-carboline derivatives were used in this study. Their chemical structures are shown in Fig. 1. The synthesis of these compounds has been described previously (Sako et al., 2008; Aoyama et al., 2009). The nonnucleoside BVDV NS5B inhibitors AG110 (Paeshuyse et al., 2007), LZ37 (Paeshuyse et al., 2009), and BPIP (Paeshuyse et al., 2006) were also used in this study (Fig. 1).

2.2. Cells and viruses

Madin-Darby bovine kidney (MDBK) cells were purchased from Japan Health Sciences Foundation (Health Science Research Resources Bank, Osaka, Japan). The cells were grown and maintained in Dulbecco's modified Eagle medium with high glucose (Gibco/BRL, Grand Island, NY). The medium was supplemented with 10% heat-inactivated horse serum (Gibco/BRL), 100 unit/ml penicillin G, and 100 μg/ml streptomycin. The cells were certified as BVDV-contamination negative. For antiviral assays, the medium supplemented with 3% heat-inactivated horse serum and antibiotics was used. The cytopathic BVDV strain Nose was obtained from Kyoto Biken (Kyoto, Japan). The virus was obtained from culture supernatants of infected cells after incubation for 3 days. Virus stocks were stored at −80 °C until use. Their infectivity was determined in MDBK cells by an end point serial dilution method and expressed as 50% cell culture infectious dose per ml ($CCID_{50}$/ml). In addition, three drug-resistant mutant strains and the corresponding wild-type strain NADL were employed (Paeshuyse et al., 2006, 2007, 2009). The strains carry mutations F224S, F224Y, and F291G, and are resistant to BPIP, LZ37, and AG110, respectively. In experiments using these strains, MDBK cells were grown in Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum (Integro, Zaandam, The Netherlands), which proved to be BVDV contamination-free by reverse transcription polymerase chain reaction (RT-PCR).



γ-Carboline derivatives

Compound	R_1	R_3	R_4	R_5
γ-Carboline	H	H	H	H
SK5M	H	H	H	CH_3
SK3M4M	H	CH_3	CH_3	H
SK3M5M	H	CH_3	H	CH_3
SK4M5M	H	H	CH_3	CH_3
SK1M4M5M	CH_3	H	CH_3	CH_3
SK3M4M5M	H	CH_3	CH_3	CH_3

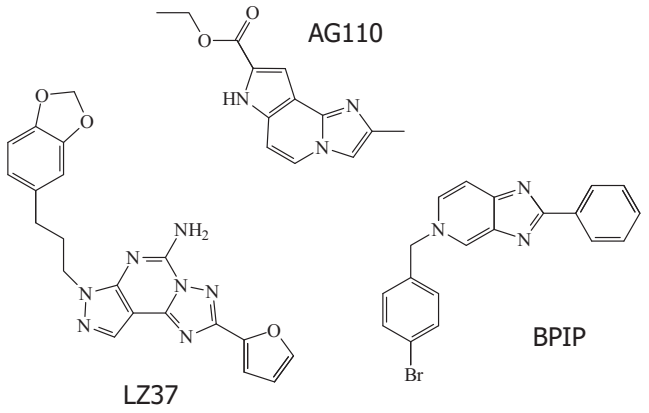


Fig. 1. Chemical structures of γ-carboline derivatives and BVDV RNA-dependent RNA polymerase inhibitors.

2.3. Anti-BVDV assays

Determination of γ-carboline derivatives for their anti-BVDV activity was based on the inhibition of virus-induced cytopathicity in MDBK cells, as previously described (Baba et al., 2005). Briefly, the cells (1×10^5 cells/ml) were infected with BVDV (Nose strain) at a multiplicity of infection (MOI) of 0.01, and 100 μl of the cell suspension was brought into each well in a microtiter plate. The cells were incubated in the presence of various concentrations of the test compounds for 3 days at 37 °C. After incubation, culture supernatants were collected to determine their LDH levels by an LDH detection kit (Takara Biochemicals, Otsu, Japan), according to the manufacturer's instructions. The cytotoxicity of compounds was evaluated in parallel with their antiviral activity. The mock-infected MDBK cells (1×10^4 cell/well) were incubated in the presence of various concentrations of test compounds for 3 days. The viability was determined by a dye method using the water soluble tetrazolium Tetracolor One® (Seikagaku Corporation, Tokyo, Japan).

Antiviral assays using the drug-resistant strains were carried out according to the procedures, as previously described (Paeshuyse et al., 2009). MDBK cells (5×10^3 cells/100 μl) were seeded in a microtiter plate. After incubation for 24 h at 37 °C, culture medium was removed. Serial dilutions of the test compounds were added into each well, and then the cells were infected with the virus at a MOI of 2.0. After 3 days, culture medium was removed, and the virus-induced cytopathicity was quantified by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetra-zolium/phenazinemethosulfate (MTS/PMS) method. The cytotoxicity of compounds was also evaluated by the MTS/PMS method in the mock-infected MDBK cells.

2.4. Virus yield reduction assay

MDBK cells (1×10^5/ml) were infected with BVDV (Nose strain) at a MOI of 1.0 and seeded into a 48-well plate in the presence or absence of various concentrations of SK3M4M5M or SK5M. After incubation for 4 h at 37 °C, the cells were washed twice with phosphate-buffered saline (PBS) and incubated with fresh culture medium containing appropriate concentrations of the test compounds. The cells were further incubated for 3 days. Then, the culture supernatants were collected and examined for their virus titer by the end-point dilution method. The virus titer was expressed as $CCID_{50}$/ml.

2.5. RT-PCR

Inhibitory effect of 3,4,5-trimethyl-γ-carboline (SK3M4M5 M) on viral RNA synthesis was determined by real-time RT-PCR. MDBK cells (2×10^4 cells/well) were infected with BVDV (Nose strain) at a MOI of 2.0 and cultured in the presence of various concentrations of the compound. After incubation for 12 h, the cells were washed with PBS, treated with lysis buffer of TaqMan® Gene Expression Cell-to-CT™ kit (Applied Biosystems, Branchburg, NJ), and subjected to real-time RT-PCR. The BVDV RNA level was determined using the sense primer 5′-TGGTCCGACGCCTTAGTATAAAGG-3′, the antisense primer 5′-GGCTGTATTCGTAACAGTTGGTTAAA-3′, and the fluorescence probe 5′-ACGAGGGCACGCCCAAAGCA-3′ (Applied Biosystems). The primer pair amplifies the 5′-untranslated region

of BVDV RNA. RT and PCR reagents of the kit were used for real-time RT-PCR, according to the manufacturer's instructions. Nonspecific inhibition of host cellular mRNA synthesis by the compound was determined by amplification of part of the bovine β-actin RNA using the sense primer (Applied Biosystems).

2.6. Time of drug-addition experiment

MDBK cells (2×10^4 cells/well) were seeded in a 96-well plate and incubated at 37 °C for 24 h. Then, the cells were infected with BVDV (Nose strain) at a MOI of 2.0. After incubation for 1 h, the inoculum was removed, and the cells were washed three times with PBS. SK3M4M5M (1 μM) or BPIP (2 μM) was added at the time of infection and incubated without their removal or added at different time points after infection. The cells were further incubated until 24 h after virus infection. The cells thoroughly washed with PBS and subjected to real-time RT-PCR, as described above.

2.7. Molecular modeling

The published X-ray structure of the BVDV RNA-dependent RNA polymerase [PDB entry 1S48 (Choi et al., 2004)] was used in all docking experiments. Selenium atoms in the selenomethionine residues were modified back to sulphur atoms to get methionine residues. The inhibitor γ-carboline was drawn using Prodrg (Schuettelkopf and van Aalten, 2010). Polar hydrogen atoms and Gasteiger charges were added to the enzyme and inhibitor structures using autodock tools (Mohamadi et al., 1990). γ-Carboline was docked in the cavity in which F224 is located by means of a Lamarckian genetic algorithm and empirical binding free energy (Morris et al., 1998). Interactions (hydrogen bonds and hydrophobic interactions) were calculated using Ligplot (Wallace et al., 1995).

2.8. Anti-HCV assay

The anti-HCV activity of γ-carboline derivatives was determined by the inhibition of viral RNA synthesis in full-genomic HCV RNA replicon cells by real-time RT-PCR, as previously described (Ishii et al., 2006; Watashi et al., 2003). The replicon cells NNC#2 were kindly provided by Dr. Hijikata (Kyoto University, Kyoto, Japan).

3. Results

3.1. Anti-BVDV activity

We demonstrated earlier that the introduction of a methyl group into γ-carboline enhanced its anti-BVDV activity (Sako et al., 2008). This enhancement was found to be stronger, when more methyl groups were introduced into the molecule (Aoyama et al., 2009). In fact, when the selected methyl-γ-carbolines were examined for their inhibitory effect on BVDV (Nose strain) replication in MDBK cells, the highest activity was achieved by SK3M4M5M followed by 4′,5′-dimethyl-γ-carboline (SK4M5M) (Table 1). The 50% effective concentrations (EC_{50}) of SK3M4M5M and SK4M5M were 0.017 ± 0.005 and 0.057 ± 0.005 μM, respectively. Thus, SK3M4M5M and SK4M5M were approximately 147 and 44-fold more potent than their parental compound γ-carboline (EC_{50}: 2.5 ± 0.3 μM) in inhibiting BVDV replication. Although their cytotoxicity was also higher than γ-carboline, the selectivity indices (SI), based on the ratio of 50% cytotoxic concentration (CC_{50}) to EC_{50}, of SK3M4M5M and SK4M5M were 436 and 174, respectively (Table 1), which were approximately 30 and 12-fold greater than the SI of γ-carboline (14.7). The much higher activity of SK3M4M5M, as compared to SK5M, was also confirmed by a virus yield reduction assay. On average, 1.2 and 4.2 log reduction of virus titer in the culture supernatants was recorded in the presence of SK3M4M5M at 0.2 and 1 μM, respectively (Fig. 2). By contrast, little reduction of virus titer was achieved by SK5M even at a concentration of 1 μM.

Table 1
Antiviral activity of γ-carboline derivatives against BVDV (Nose strain) in MDBK cells.

Compound	EC_{50} (μM)	CC_{50} (μM)	SI
γ-Carboline	2.5 ± 0.3	36.7 ± 3.8	15
SK5M	0.36 ± 0.03	22.6 ± 2.7	63
SK3M4M	0.27 ± 0.11	14.5 ± 0.7	54
SK3M5M	0.26 ± 0.04	17.7 ± 1.9	68
SK4M5M	0.057 ± 0.005	9.9 ± 0.7	174
SK1M4M5M	0.14 ± 0.03	1.9 ± 0.1	14
SK3M4M5M	0.017 ± 0.005	7.4 ± 0.9	435

EC_{50}: 50% effective concentration, based on the inhibition of virus-induced cytopathicity; CC_{50}: 50% cytotoxic concentration, based on the reduction of viable cell number; SI: selectivity index, a ratio of CC_{50} to EC_{50}. All data represent means ± SD for three independent experiments.

3.2. Inhibition of viral RNA synthesis and time-of-drug-addition studies

To determine whether SK3M4M5M inhibits BVDV RNA synthesis without affecting host cellular mRNA synthesis, the intracellular RNA levels of BVDV and β-actin were quantified by real-time RT-PCR. As shown in Fig. 3, SK3M4M5M completely inhibited BVDV RNA synthesis at a concentration of 0.1 μM, whereas no inhibition of β-actin RNA synthesis was observed up to 0.1 μM. In addition, viral RNA synthesis was also strongly inhibited by SK3M4M5M at a concentration of 0.1 μM in the cells infected with non-cytopathic strains of BVDV. Its EC_{50} values were 0.034 ± 0.018 and 0.020 ± 0.005 μM for Pe515 and Os Loss strains, respectively (data not shown).

To gain further insight into the mechanism of action, a time-of-drug-addition experiment was conducted. In this experiment, BVDV RNA levels were determined at different time points of drug-addition to the infected cells. SK3M4M5M was used at a concentration of 1.0 μM, which was 10-fold higher than the concentration that completely inhibited BVDV RNA synthesis (Fig. 3). The compound could retain its full activity against BVDV, when

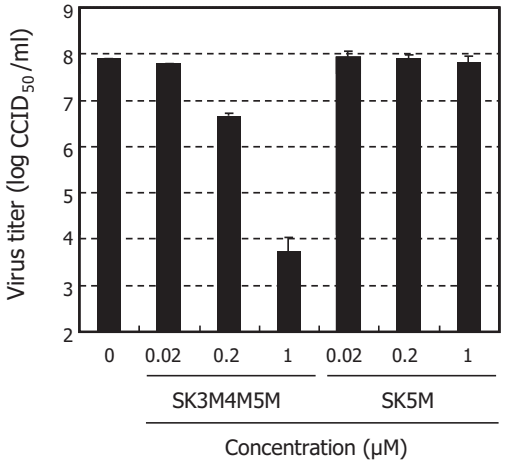


Fig. 2. Inhibitory effect of SM3M4M5M and SK5M on virus production in MDBK cells. The cells were infected with BVDV at a MOI of 1.0 and cultured in the presence or absence of various concentrations of SK3M4M5M or γ-carboline. After incubation for 4 h, the cells were washed with PBS and further incubated with fresh culture medium containing appropriate concentrations of the test compounds. After 3 days, the culture supernatants were collected and examined for their virus titer. The virus titer was expressed as log CCID50/ml. Experiments were repeated three times, and means ± SD are shown.

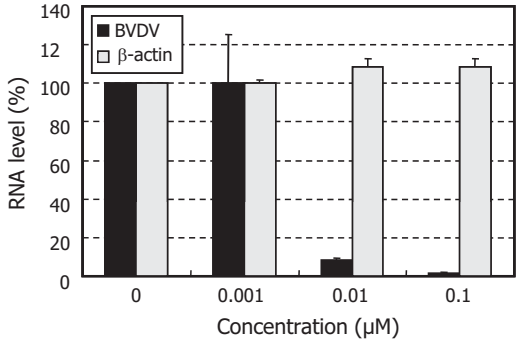


Fig. 3. Effect of SK3M4M5M on BVDV RNA synthesis in MDBK cells. The cells were infected with BVDV at a MOI of 2.0 and cultured in the presence of various concentrations of the compound. After incubation for 12 h, the cells were washed with PBS, treated with lysis buffer and subjected to real-time RT-PCR (see Section 2). Black and gray columns indicate the amounts of BVDV RNA and β-actin RNA, respectively. Data represent means ± SD for triplicate experiments. Experiments were repeated three times, and a representative result is shown.

added during the first 8 h post-infection (Fig. 4A). When the compound was added at a time point later than 8 h after infection, the antiviral activity was lost, and the intracellular viral RNA gradually increased with increasing the time of delayed addition. These results suggest that SK3M4M5M inhibits an event occurring around 8 h after virus infection. Furthermore, an almost identical result was obtained in the experiment with the BVDV RNA polymerase inhibitor BPIP (Fig. 4B). It has been reported that a single cycle of BVDV replication takes 13 h on average and that viral RNA synthesis starts at about 6–8 h after infection (Paeshuyse et al., 2006). Thus, SK3M4M5M appears to interact with a stage in the viral replication cycle that coincides with the onset of viral RNA synthesis.

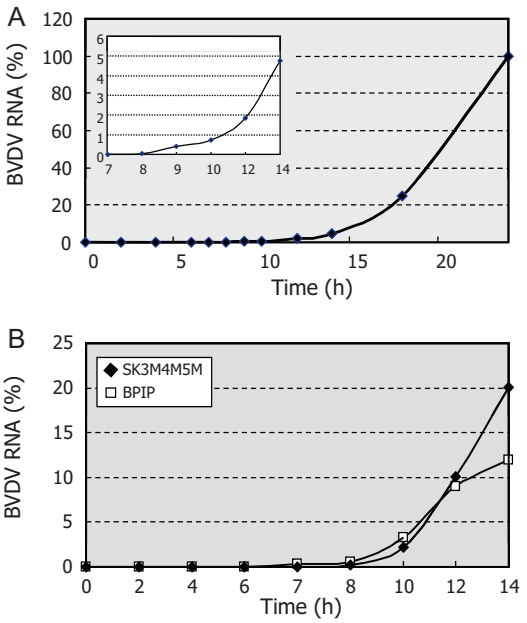


Fig. 4. Effect of time of drug-addition on the antiviral activity of SK3M4M5M. MDBK cells infected with BVDV at a MOI of 2.0. After incubation for 1 h, the inoculum was removed. SK3M4M5M (1 μM) or BPIP (2 μM) was added at the time of infection and incubated without removal of the compound (0 h), or the compound was added at different time points after infection. (A) The cells were further incubated with SK3M4M5M until 24 h after virus infection and subjected to real-time RT-PCR. (B) The cells were further incubated with SK3M4M5 M (filled diamond) or BPIP (open square) up to 24 h after virus infection. Values are expressed as percentage viral RNA of the infected cells untreated with the compound. All data represent means for triplicate experiments. Experiments were repeated twice, and a representative result is shown.

3.3. Activity against NS5B inhibitor-resistant mutants

The antiviral activity of γ-carboline, SK4M5M, SK1M4M5M, and SK3M4M5M was evaluated against three BVDV drug-resistant strains that carry the respective mutations F224S, F224Y, and F291G, which display resistance to BPIP, LZ37, and AG110, respectively. All three molecules have been shown to target the top of the finger domain of the pestivirus RNA polymerase. SK4M5M, and SK1M4M5M, as well as γ-carboline, proved inactive against these mutant strains (Table 2). Interestingly, although SK3M4M5M exhibited reduced activity against the mutant strains, the compound retained overall substantial antiviral activity. Furthermore, the activity of SK3M4M5M against the wild-type of NADL strain was less pronounced than against the Nose strain; however, the anti-BVDV activity was comparable to that of BPIP, which has been reported to be one of the most potent anti-BVDV agents reported so far (Paeshuyse et al., 2006).

3.4. Docking of γ-carboline in the BVDV RNA polymerase crystal structure

The amino acid F224 is located near the tip of the finger domain of the BVDV polymerase. Docking of γ-carboline in this cavity revealed possible interactions between the polymerase and γ-carboline. The following possible interactions were calculated: (i) hydrophobic contacts of γ-carboline with T259, I261 and G223 (Fig. 5A blue shade and Fig. 5B), (ii) a hydrogen bond between the N3 of γ-carboline and the side chain of A392 (Fig. 5A and B), and (iii) aromatic ring stacking interactions between F224 and γ-carboline (Fig. 5A).

3.5. Anti-HCV activity

The γ-carboline derivatives were also examined for their inhibitory effect on HCV replication in replicon cells. However, none of the compounds displayed selective inhibition of HCV replication (data not shown).

4. Discussion

We recently reported two different series of compounds that had selective anti-BVDV activity in cell cultures. One is γ-carboline derivatives, and the other is diphenylmethane derivatives (Sako et al., 2008; Aoyama et al., 2009; Salim et al., 2010). Among them, dimethyl- and trimethyl-γ-carbolines proved to be highly potent and selective inhibitors of BVDV replication (Table 1) (Aoyama et al., 2009). The most potent analogue in this series SK3M4M5M was able to completely inhibit viral RNA synthesis in a single replication cycle assay at a concentration of 100 nM. From a time-of-drug-addition experiment, it appeared that the compound interfered with a step that coincides with the onset of viral RNA synthesis in the replication cycle (Paeshuyse et al., 2009).

Cross-resistance between, two different classes of molecules provides an indirect proof that both have the same molecular target in the viral replication cycle. Therefore, γ-carboline derivatives were examined for their inhibitory effect on the replication of three strains of BVDV that were previously shown to confer resistance to various classes of nonnucleoside NS5B inhibitors (Paeshuyse et al., 2006, 2007, 2009). The antiviral activity of γ-carboline derivatives against the wild-type of NADL strain was several-fold lower than that against the wild-type of Nose strain (Tables 1 and 2). Furthermore, such difference was also observed for their CC_{50} values. This may be attributed to the difference of viral strains and assay conditions used for the experiments, including cytotoxicity evaluation. All of the resistant mutants were less susceptible to the γ-carboline derivatives than the wild-type virus, suggesting that

Table 2
Antiviral activity of γ-carboline derivatives against drug-resistant BVDV in MDBK cells.

Compound	EC_{50} (μM)				CC_{50} (μM)
	WT (NADL strain)	F224S	F224Y	E291G	
γ-Carboline	28.2 ± 9.0	>88.1	>88.1	>88.1	88.1 ± 5.0
SK4M5M	0.38 ± 0.31	>16.7	3.1 ± 0.8	>16.7	16.7 ± 0.7
SK1M4M5M	0.98 ± 0.09	>3.7	>3.7	>3.7	3.7 ± 0.5
SK3M4M5M	0.12 ± 0.06	0.59 ± 0.34	0.58 ± 0.28	0.75 ± 0.34	16.2 ± 1.8
AG110	2.6 ± 1.4	37.8 ± 3.9	30.7 ± 9.0	81.4 ± 6.6	>100
LZ37	12.1 ± 2.6	>92.6	54.7 ± 11.2	6.2 ± 2.0	92.6 ± 5.4
BPIP	0.22 ± 0.02	>76.5	47.8 ± 40.6	1.1 ± 0.1	76.5 ± 2.2

EC_{50}: 50% effective concentration, based on the inhibition of virus-induced cytopathicity; CC_{50}: 50% cytotoxic concentration, based on the reduction of viable cell number. All data represent means ± SD for three independent experiments.

they share the same target with the nonnucleoside NS5B inhibitors AG110, LZ37, and BPIP. The latter compounds were shown to select for drug resistance mutations in the top of the finger domain of the viral polymerase. Interestingly, γ-carboline, SK4M5M, and SK1M4M5M almost completely lost their activity against the resistant mutants. In contrast, SK3M4M5M retained most of its antiviral activity (EC_{50} < 1.0 μM). The methyl group at the 3-position of γ-carboline may possibly play an important role in retaining the antiviral activity against the resistant mutants as well as enhancing the activity against the wild-type strain.

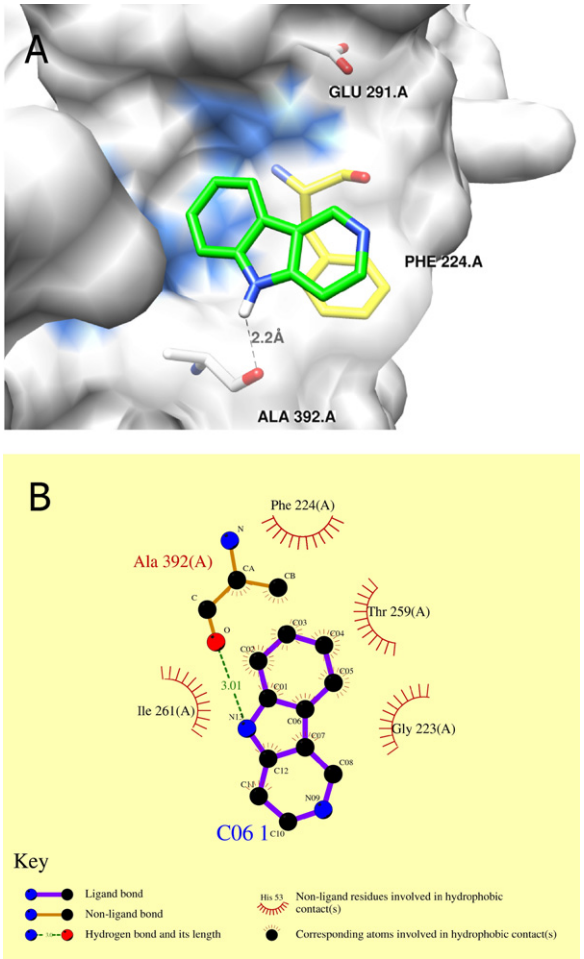


Fig. 5. Modeling of γ-carboline near the position of F224 in BVDV RNA-dependent RNA polymerase. (A) Overview of the entire structure of the RNA polymerase with γ-carboline docked in the vicinity of F224. Picture generated using UCSF Chimera (Pettersen et al., 2004). (B) Ligplot depicting a simplified representation of interactions between γ-carboline and the RNA polymerase. Carbon atoms are black, nitrogen atoms are blue, and oxygen atoms are red. A putative hydrogen bond is drawn as a dotted black line in panel A and a dotted green line in panel B. Spiked red line indicates the hydrophobic interactions.

We hypothesize that the apparent lack of cross-resistance of SK3M4M5M with the drug resistant strains can be explained by a different binding mode of each analogue, as compared to that of γ-carboline; likely due to the different degree of methylation and the position of the methyl groups. For SK3M4M5M, the methyl group in position 3 is optimal to interact with the wild-type RNA polymerase. However, this methyl group will shift the interaction of the molecule, as compared to γ-carboline. Most probably, this shift will result in the loss of the aromatic ring stacking interaction between F224 or Y224 and γ-carboline. Hence, the effect on compound activity may be reduced, when these residues are mutated to non-aromatic residues in the drug-resistant strains.

BVDV is considered to be a valuable surrogate virus for identifying and characterizing anti-HCV compounds. BVDV shares many characteristic similarities with HCV in virion structure, genome organization, and replication machinery (Buckwold et al., 2003; Nam et al., 2001). However, it is not surprising that SK3M4M5M did not have selective anti-HCV activity in HCV RNA replicon cells. A possible explanation is the difference between BVDV NS5B and HCV NS5B molecules. It was recently reported that BPIP, which is inactive against HCV, became active, when one or two fluorine molecules were introduced into certain positions of this compound (Vliegen et al., 2009). Currently, this approach has not been successful for γ-carboline derivatives (data not shown).

In conclusion, our results clearly demonstrate that novel γ-carboline derivatives, especially, 3,4,5-trimethyl-γ-carboline (SK3M4M5M) is a highly potent and selective inhibitor of BVDV replication in cell cultures. Although further studies, such as selection of drug-resistant mutants and inhibition of in vitro transcription, are required, the compound may target NS5B RNA polymerase. It retains sufficient activity against the mutant viruses that are resistant to already known nonnucleoside NS5B inhibitors. Thus, this class of compounds should be further pursued for their use in the field of veterinary medicine.

Acknowledgments

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