starting material. White amorphous solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.55 (dd, 1H, J = 8.0, 1.2 Hz), 8.29–8.22 (m, 3H), 7.92–7.89 (m, 1H), 7.81–7.77 (m, 1H), 7.73 (d, 1H, J = 8.0 Hz), 7.60 (t, 3H, J = 8.0 Hz), 7.56–7.50 (m, 2H), 4.59 (t, 2H, J = 7.3 Hz), 1.93–1.86 (m, 2H), 1.26–1.18 (m, 2H), 0.83 (t, 3H, J = 7.3 Hz). HRMS (FAB) calcd for $C_{21}H_{19}NO$ 302.1545; found: 302.1574 $(M+H)^+$.

4.36. 6-Butylbenzo[*a*]phenanthridin-5(6*H*)-one (42)

The title compound was prepared by the same method as described for the synthesis of **39**. The title compound was fractionated by means of HPLC. Colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.80 (d, 1H, J = 8.5 Hz), 8.68 (d, 1H, J = 8.5 Hz), 8.64 (dd, 1H, J = 8.5, 1.5 Hz), 7.95 (t, 2H, J = 8.5 Hz), 7.79 (td, 1H, J = 8.5, 1.5 Hz), 7.65–7.60 (m, 3H), 7.52 (t, 1H, J = 8.5 Hz), 4.50 (t, 2H, J = 7.5 Hz), 1.88–1.80 (m, 2H), 1.58–1.53 (m, 2H), 1.03 (t, 3H, J = 7.3 Hz). HRMS (FAB) calcd for $C_{21}H_{19}NO$ 302.1545; found: 302.1534 $(M+H)^+$.

4.37. 5-Butylbenzo[*k*]phenanthridin-6(5*H*)-one (43)

The title compound was prepared by the same method as described for the synthesis of **40**. The title compound was fractionated by means of HPLC. White solid. Mp 54.0–60.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.88 (d, 1H, J = 7.3 Hz), 8.66 (d, 1H, J = 8.5 Hz), 8.51 (d, 1H, J = 8.5 Hz), 8.01 (dd, 2H, J = 7.3, 3.0 Hz), 7.95 (d, 1H, J = 8.5 Hz), 7.70–7.63 (m, 3H), 7.59 (t, 1H, J = 7.3 Hz), 7.53 (d, 2H, J = 8.5 Hz), 7.36 (t, 2H, J = 8.5 Hz), 4.45 (t, 2H, J = 7.9 Hz), 1.90–1.83 (m, 3H), 1.57–1.51 (m, 2H), 1.03 (t, 3H, J = 7.5 Hz). HRMS (FAB) calcd for $C_{21}H_{19}NO$ 302.1545; found: 302.1567 $(M+H)^+$.

4.38. 6-Butylbenzo[*i*]phenanthridin-5(6*H*)-one (44)

The title compound was prepared by a method similar to that described for the synthesis of **40**. 1-Naphthoyl chloride was used instead of 2-naphthoyl chloride. White amorpous solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.30 (d, 1H, J = 9.0 Hz), 8.44 (dd, 1H, J = 8.0, 1.2 Hz), 8.39 (d, 1H, J = 9.0 Hz), 8n.17 (d, 1H, J = 9.0 Hz), 7.94 (dd, 1H, J = 8.0, 1.2 Hz), 7.77–7.73 (m, 1H), 7.65–7.60 (m, 2H), 7.49 (d, 1H, J = 8.0 Hz), 7.36 (t, 1H, J = 8.0 Hz), 4.50 (t, 2H, J = 7.6 Hz), 1.90–1.83 (m, 2H), 1.63–1.53 (m, 2H), 1.06 (t, 3H, J = 7.3 Hz). HRMS (FAB) calcd for $C_{21}H_{19}NO$ 302.1545; found: 302.1540 $(M+H)^+$.

4.39. Bioassay

NNC #2 cells carrying full-genomic HCV RNA replicons were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% (v/v) fetal bovine serum (FBS) and 1 mg/mL G418 (Sigma). Cells at 70% confluence were collected after treatment with trypsin and resuspended in the same medium (5×10^4 cells/mL). One hundred microliters of the cell suspension was transferred to each well of a 96-well plate and cultured at 37 °C for 24 h. Then the medium was removed, and 200 µL of DMEM supplemented with 10% FBS containing various concentrations of test compound was added to each well. After incubation for 3 days, the cells were treated with lysis buffer of a TaqMan Gene Expression Cell-to-CT™ Kit. Expression levels of HCV RNA and GAPDH RNA were measured with the kit, according to the manufacturer's instructions. The RNA levels were quantified by real-time RT-PCR using an ABI 7500 Real-Time PCR System (Applied Biosystems). The anti-HCV activity and cytotoxicity of test compounds were expressed as EC_{50} and CC_{50} determined from the decrease of HCV RNA and GAPDH RNA levels, respectively, as described above.

Acknowledgments

The work described in this paper was partially supported by Grants-in Aid for Scientific Research from the Science and Technology Incubation Program in Advanced Regions, Japan Science and Technology Agency (JST), Japan and The Ministry of Education, Culture, Sports, Science and Technology, Japan, and a Grant from the Japan Society for the Promotion of Science.

References and notes

1. Hashimoto, Y. *Arch. Pharm. Life Sci.* **2008**, *341*, 536.
2. Hosoda, S.; Matsuda, D.; Tomoda, H.; Hashimoto, Y. *Mini-Rev. Med. Chem.* **2009**, *9*, 572.
3. Hosoda, S.; Aoyama, H.; Goto, Y.; Salim, M. T. A.; Okamoto, M.; Hashimoto, M.; Baba, M.; Hashimoto, Y. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3157.
4. Nakamura, M.; Hamasaki, T.; Tokitou, M.; Baba, M.; Hashimoto, Y.; Aoyama, H. *Bioorg. Med. Chem.* **2009**, *17*, 4740.
5. Hosoda, S.; Matsuda, D.; Tomoda, H.; Hashimoto, M.; Aoyama, H.; Hashimoto, Y. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 4228.
6. Koonin, E. V.; Wolf, Y. I.; Karev, G. P. *Nature* **2002**, *420*, 218.
7. Grishin, N. V. *J. Struct. Biol.* **2001**, *134*, 167.
8. Koch, M. A.; Wittenberg, L.-O.; Basu, S.; Jeyaraj, D. A.; Gourzoulidou, E.; Reinecke, K.; Odermatt, A.; Waldmann, H. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 16721.
9. Hashimoto, Y. *Curr. Med. Chem.* **1998**, *5*, 163.
10. Hashimoto, Y. *Bioorg. Med. Chem.* **2002**, *10*, 461.
11. Hashimoto, Y. *Mini-Rev. Med. Chem.* **2002**, *2*, 543.
12. Hashimoto, Y.; Tanatani, A.; Nagasawa, K.; Miyachi, H. *Drugs Future* **2004**, *29*, 383.
13. Hashimoto, Y. *Cell Biol. Rev.* **1991**, *25*, 209.
14. Sako, K.; Aoyama, H.; Sato, S.; Hashimoto, Y.; Baba, M. *Bioorg. Med. Chem.* **2008**, *16*, 3780.
15. Aoyama, H.; Sako, K.; Sato, S.; Nakamura, M.; Miyachi, H.; Baba, M.; Hashimoto, Y. *Heterocycles* **2009**, *77*, 779.
16. Liang, T. J.; Rehermann, B.; Seeff, L. B.; Hoofnagle, J. H. *Ann. Intern. Med.* **2000**, *132*, 296.
17. Hayashi, P. H.; Di Bisceglie, A. M. *Med. Clin. North Am.* **2005**, *89*, 371.
18. Memon, M. I.; Memon, M. A. *J. Viral Hepat.* **2002**, *9*, 84.
19. Echevarria-Mayo, J. M. *Enferm. Infecc. Microbiol. Clin.* **2006**, *24*, 45.
20. Bosch, F. X.; Ribes, J.; Cleries, R.; Diaz, M. *Clin. Liver Dis.* **2005**, *9*, 191.
21. McHutchison, J. G.; Gordon, S. C.; Schiff, E. R.; Shiffman, M. L.; Lee, W. M.; Rustgi, V. K.; Goodman, Z. D.; Ling, M. H.; Cort, S.; Albrecht, J. K. *N. Eng. J. Med.* **1998**, *339*, 1485.
22. Tan, S. L.; Pause, A.; Shi, Y.; Sonenberg, N. *Nat. Rev. Drug Disc.* **2002**, *1*, 867.
23. Buckwold, V. E.; Beer, B. E.; Donis, R. O. *Antiviral Res.* **2003**, *60*, 1.
24. Buckwold, V. E.; Wei, J.; Wenzel-Mathers, M.; Russell, J. *Antimicrob. Agents Chemother.* **2003**, *47*, 2293.
25. Yanagida, K.; Baba, C.; Baba, M. *Antiviral Res.* **2004**, *64*, 195.
26. Aoyama, A.; Aoyama, H.; Dodo, K.; Makishima, M.; Hashimoto, Y.; Miyachi, H. *Heterocycles* **2008**, *76*, 137.
27. Miyachi, H.; Aoyama, A.; Hashimoto, Y. *MedChemNews* **2009**, *19*(2), 30.
28. Ishii, N.; Watashi, K.; Hishiki, T.; Goto, K.; Inoue, D.; Hijikata, M.; Wakita, T.; Kato, N.; Shimotohno, K. *J. Virol.* **2006**, *80*, 4510.
29. Li, L.; Liu, J.; Zhu, L.; Cutler, S.; Hasegawa, H.; Shan, B.; Medina, J. C. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1638.

Antiviral Chemistry & Chemotherapy 2010 **20**:193–200 (doi: 10.3851/IMP1528)

Original article

Anti-bovine viral diarrhoea virus activity of novel diphenylmethane derivatives

*Mohammed TA Salim[1], Mika Okamoto[1], Shinnosuke Hosoda[2], Hiroshi Aoyama[2], Yuichi Hashimoto[2] and Masanori Baba[1]**

[1]Division of Antiviral Chemotherapy, Center for Chronic Viral Diseases, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan
[2]Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan

*Corresponding author: e-mail: m-baba@vanilla.ocn.ne.jp

Background: A number of compounds were examined for their inhibitory effects on bovine viral diarrhoea virus (BVDV), a surrogate model of hepatitis C virus, in cell cultures. Among them, some diphenylmethane derivatives were found to be selective inhibitors of BVDV.
Methods: Determination of compounds for their anti-BVDV activity was based on the inhibition of virus-induced cytopathic effect in Madin–Darby bovine kidney cells and reduction of infectious virus particles in culture supernatants. To gain insight into the mechanism of action, the inhibition of viral entry and RNA synthesis in the host cells was also determined by real-time reverse transcription-PCR.
Results: Among the test compounds, four diphenylmethane derivatives significantly inhibited BVDV replication with a 50% effective concentration ranging between 6.3 and 10.8 μM. They were not cytotoxic at concentrations up to 100 μM. The representative compound, SH-595A, reduced the virus titre of culture supernatants in a dose-dependent manner. In addition, the compound appeared to somewhat affect viral entry to the host cells. Although SH-595A was inhibitory to viral RNA synthesis, the inhibition was achieved only at high concentrations and was not comparable to its antiviral activity.
Conclusions: The novel diphenylmethane derivatives are effective against BVDV replication and might have a unique mechanism of action.

Introduction

Human chronic hepatitis is often caused by persistent infection with hepatitis C virus (HCV). This persistent infection commonly leads to liver fibrosis, cirrhosis and hepatocellular carcinoma [1]. Pegylated interferon-α in combination with the nucleoside analogue ribavirin is currently used for the treatment of HCV infection [2]; however, the treatment outcome is dependent on the genotype of HCV. Indeed, the treatment is not effective in approximately 50% of patients who are infected with genotype 1b. Furthermore, this combination therapy is expensive and is often associated with unacceptable side effects in many patients; therefore, novel therapies aiming at complete and permanent eradication of HCV from the patient are still mandatory.

HCV belongs to the family *Flaviviridae*, which include three genera: hepacivirus, flavivirus and pestivirus. Bovine viral diarrhoea virus (BVDV), a member of the pestivirus genus is a causative agent of mucosal disease in cattle. The virus possesses a positive sense RNA genome of approximately 12.6 kb. All members of *Flaviviridae* share similarities in virion structure, genome organization and replication machinery. Because BVDV shares many important properties with HCV, BVDV provides a surrogate model of HCV [3,4], in particular, for evaluation of antiviral compounds [5]. Both HCV and BVDV utilize an internal ribosomal entry site within the 5′-non-translated region (NTR) for translating the viral polyprotein. Because of some similarities in their non-structural proteins, antiviral agents active against BVDV are likely to inhibit HCV replication.

The subgenomic HCV RNA replicon cell system has been recognized as a useful tool for discovery of novel anti-HCV agents [6,7]. However, this system does not produce infectious progeny; therefore, it cannot be used for the identification and characterization of antiviral agents acting on early and late steps in the viral replication cycle, such as viral attachment, entry, uncoating, maturation or release. In addition, antiviral agents that

©2010 International Medical Press 1359-6535 (print) 2040-2066 (online)

Figure 1. Structures of diphenylmethane derivatives

©2010 International Medical Press

reduce the infectivity of progeny cannot be identified in this system [3]. By contrast, a cell culture system of productive HCV infection has recently been established with the replication-competent strain, JFH-1, which makes it possible to identify inhibitors of every step in the viral replication cycle [8–10]. However, this strain was isolated from a patient of fulminant hepatitis C and classified as genotype 2a with several mutations; thus, BVDV is still widely used as a surrogate model for the investigation of anti-HCV agents.

A simple and sensitive colorimetric assay of compounds, which evaluated their anti-BVDV activity, has previously been reported by our group [11]. In this study, we examined novel diphenylmethane derivatives (Figure 1) for their inhibitory effect on BVDV replication and found that some compounds were selective inhibitors of the virus. To gain insight into their mechanism of action, assays for viral entry and RNA inhibition were also conducted.

Methods

Compounds

Twelve diphenylmethane derivatives (Figure 1) and the reference compound, γ-carboline, were selected for antiviral assay. The synthesis of these compounds has been described elsewhere [12,13]. All compounds were dissolved in dimethyl sulfoxide at a concentration of 20 mM and stored at -20°C until use.

Cells and virus

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's medium with high glucose (4.9 mg/ml; Gibco/BRL, Grand Island, NY, USA). The medium was supplemented with 10% heat-inactivated horse serum (Gibco/BRL), 100 units/ml penicillin G and 100 μg/ml streptomycin. The cells were certified as BVDV-contamination-negative. For antiviral assays, medium supplemented with 3% heat-inactivated horse serum and antibiotics was used. The cytopathic BVDV strain Nose was obtained from Kyoto Biken (Kyoto, Japan). BVDV was harvested from culture supernatants of virus-infected cells after incubation for 3 days. Virus stocks were stored at -80°C until use. The infectivity of the stocks was determined in MDBK cells by an end point serial dilution method and expressed as the 50% tissue culture infectious dose per ml ($TCID_{50}$/ml).

Anti-BVDV assays

Determination of compounds for their anti-BVDV activity was based on the inhibition of virus-induced cytopathic effect (CPE) in MDBK cells, as described previously [11]. Briefly, the cells (1×10^5 cells/ml) were infected with BVDV at a multiplicity of infection (MOI) of 0.01 and 100 μl of the cell suspension was brought into each well in a microtitre plate. The cells were incubated in the presence of various concentrations of test compounds, including the reference compound, γ-carboline, for 3 days at 37°C in a humidified CO_2 incubator. After incubation, culture supernatants were collected to determine their lactate dehydrogenase (LDH) levels by an LDH detection kit (Takara Biochemicals, Otsu, Japan) according the manufacturer's instructions. The cytotoxicity of the compounds was evaluated in parallel with their antiviral activity. The mock-infected MDBK cells (1×10^4 cells/well) were incubated in the presence of various concentrations of test compounds for 3 days. The viability was determined by a dye method using water-soluble tetrazolium Tetracolor One® (Seikagaku Corporation, Tokyo, Japan).

Virus yield reduction assays were conducted for the representative compound, SH-595A, and for γ-carboline. Briefly, MDBK cells (1×10^5 cells/ml) were infected with BVDV at a MOI of 0.01 and 500 μl of the infected cell suspension was brought into each well of a 24-well plate in the presence of various concentrations of SH-595A or γ-carboline. The cells were washed with culture medium at 24 h after virus infection and replaced with fresh culture medium containing appropriate concentrations of the test compounds. The plate was incubated for 2 days at 37°C. The culture supernatant of each well was collected and stored at -80°C until virus titration. The virus titre for each sample was determined and expressed as $TCID_{50}$/ml.

ELISAs

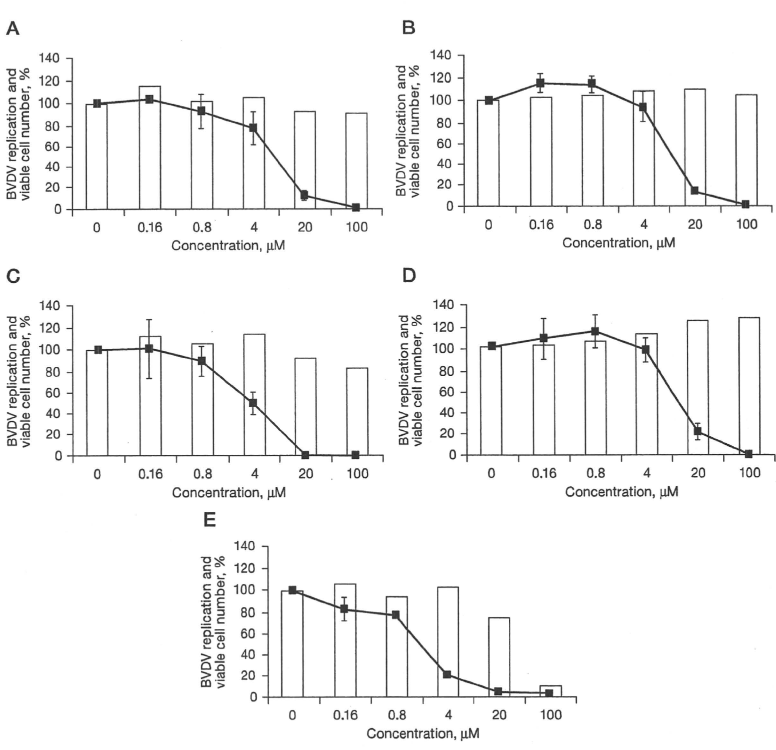
To confirm the anti-BVDV activity of SH-595A, its inhibitory effect on viral antigen production was determined by a BVDV antigen ELISA kit (Bio-X Diagnostics, Jemelle, Belgium). MDBK cells (1×10^4 cells/well) were infected with the virus at a MOI of 0.01 and 100 μl of the cell suspension was brought into each well in a microtitre plate. The cells were incubated in the presence of various concentrations of the compound. After incubation for 3 days at 37°C, the supernatants were collected and their BVDV antigen levels were determined by the ELISA kit according to the manufacturer's instructions.

Real-time reverse transcription-PCR

The anti-BVDV activity of SH-595A and γ-carboline was also determined by the inhibition of viral RNA synthesis in MDBK cells by real-time reverse transcription (RT)-PCR. The cells (2×10^4 cells/well) were infected with the virus at a MOI of 1.0 and cultured in the presence of various concentrations of the test compounds. After incubation for 12 h, the cells

were washed with phosphate-buffered saline (PBS), treated with lysis buffer of TaqMan® Gene Expression Cell-to-CT™ kit (Applied Biosystems, Branchburg, NJ, USA) and subjected to real-time RT-PCR. The BVDV RNA level was determined using the sense primer 5′-TGGTCCGACGCCTTAGTATAAAGG-3′, the antisense primer 5′-GGCTGTATTCGTAAC AGTTGGTTAAA-3′ and the fluorescence probe 5′-ACGAGGGCACGCCCAAAGCA-3′ (Applied Biosystems). The primer pair amplifies the 5′-NTR of BVDV RNA. RT and PCR reagents of the kit were used for real-time RT-PCR according to the manufacturer's instructions. Non-specific inhibition of host cellular messenger RNA (mRNA) synthesis by SH-595A and γ-carboline was determined by amplification of a part of the bovine β-actin mRNA using the sense primer 5′-GCC CTGAGGCTCTCTTCCA-3′, the antisense primer 5′-GCGGATGTCGACGTCACA-3′ and the fluorescence

Figure 2. Inhibitory effect of diphenylmethane derivatives and γ-carboline on BVDV replication in MDBK cells



Madin–Darby bovine kidney (MDBK) cells were infected with bovine viral diarrhoea virus (BVDV) at a multiplicity of infection of 0.01 and incubated in the presence of various concentrations of (A) SH-164, (B) SH-184, (C) SH-583A, (D) SH-595A or (E) γ-carboline for 3 days. Lines indicate the virus-induced cytopathic effect in infected cells, as determined by the lactate dehydrogenase method. Columns indicate the viable cell number of mock-infected cells, determined by the tetrazolium dye method. Experiments were repeated at least twice for each compound and representative results are shown.

©2010 International Medical Press

probe 5′-CATGGAATCCTGCGGCATTCACG-3′ (Applied Biosystems).

Viral entry inhibition assays

MDBK cells (2×10^4 cells/well) were seeded in a microtitre plate and incubated at 37°C for 24 h. The cells were infected with BVDV at a MOI of 2.0. SH-595A (100 μM) and γ-carboline (20 μM) were added at the time of infection and incubated without their removal, added at the time of infection but removed after incubation for 2 h, or added only after incubation for 2 h. The cells were incubated for 12 h after viral infection, washed with PBS and subjected to real-time RT-PCR, as described above.

Results

When 12 diphenylmethane derivatives (Figure 1) were examined for their inhibitory effect on BVDV replication in MDBK cells, 4 compounds displayed dose-dependent inhibition of virus-induced CPE (Figure 2). The 50% effective concentrations (EC_{50}) of SH-164, SH-184, SH-583A and SH-595A were 9.0, 10.8, 6.3 and 10.5 μM, respectively (Table 1). None of the compounds reduced the viability of the mock-infected MDBK cells at a concentration of up to 100 μM. Compounds SH-123, SH-186, SH-199, SH-259, SH-355, SH-394, SH-595B and SH-605 also showed some inhibition of BVDV replication; however, their selectivity indices based on the ratio of 50% cytotoxic concentration (CC_{50}) to EC_{50} were marginal. The EC_{50} and CC_{50} values of the reference compound γ-carboline were 1.9 and 42.6 μM, respectively (Table 1). In the next experiment, SH-595A and γ-carboline were examined for whether they could reduce the amount of infectious virus particles in culture supernatants of the cells. Dose-dependent reduction of the virus titre was observed for both compounds (Figure 3). These results indicate that diphenylmethane derivatives selectively inhibit BVDV replication in cell cultures. The antiviral activity of SH-595A against BVDV was also examined by a sandwich ELISA. It reduced the amount of viral antigen in culture supernatants of the infected cells in a dose-dependent manner (Figure 4). Its EC_{50} was 9.2 μM, which is comparable to the EC_{50} obtained by the CPE inhibition assays (Table 1).

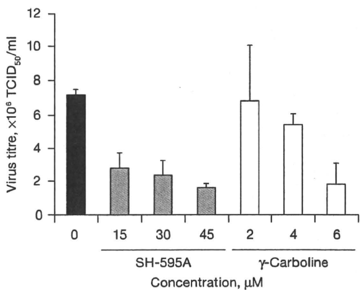
When the inhibitory effect of SH-595A and γ-carboline on BVDV RNA synthesis was examined, SH-595A showed modest inhibition of viral RNA synthesis. Although it achieved approximately 70% inhibition of viral RNA synthesis at a concentration of 100 μM, little inhibition was observed at a concentration of 20 μM (Figure 5A). By contrast, γ-carboline almost completely inhibited viral RNA synthesis at 4 μM without affecting β-actin mRNA synthesis (Figure 5B). Considering the finding that both SH-595A and γ-carboline achieved approximately 80% inhibition of BVDV replication at a concentration of 20 and 4 μM, respectively (Figure 2), the mechanism of BVDV inhibition by SH-595A seems to differ from that by

Table 1. Anti-BVDV activity of diphenylmethane derivatives in MDBK cells

Compound	EC_{50}, μM	CC_{50}, μM	SI
SH-123	21.1 ±7.2	>100	>4.7
SH-164	9.0 ±2.7	>100	>11.1
SH-184	10.8 ±2.0	>100	> 9.3
SH-186	67.1 ±2.6	>100	>1.5
SH-199	7.1 ±3.0	45.1 ±4.0	6.4
SH-259	31.7 ±5.7	43.0 ±6.9	1.4
SH-355	12.5 ±3.5	42.6 ±0.6	3.4
SH-394	16.3 ±7.1	42.0 ±7.5	2.6
SH-583A	6.3 ±1.9	>100	>15.9
SH-595A	10.5 ±2.3	>100	>9.5
SH-595B	9.5 ±2.7	46.8 ±1.1	4.9
SH-605	16.9 ±3.9	>100	>5.9
γ-Carboline	1.9 ±0.4	42.6 ±5.5	22.4

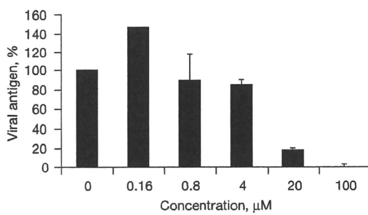
All data represent means ±SD for at least three separate experiments. CC_{50}, 50% cytotoxic concentration based on the reduction of viable cell number; EC_{50}, 50% effective concentration based on the reduction of cell destruction induced by bovine viral diarrhoea virus (BVDV) replication; MDBK, Madin–Darby bovine kidney; SI, selectivity index (a ratio of CC_{50} to EC_{50}).

Figure 3. Inhibitory effect of SH-595A and γ-carboline on viral infectivity in culture supernatants



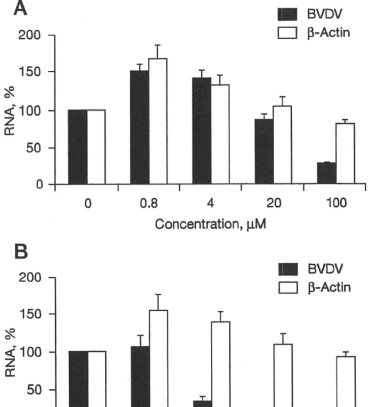
Madin–Darby bovine kidney cells were infected with bovine viral diarrhoea virus at a multiplicity of infection of 0.01 and incubated in the presence of various concentrations of SH-595A or γ-carboline for 24 h. The cells were washed with culture medium and replaced with fresh culture medium containing appropriate concentrations of the test compounds. The plate was further incubated for 2 days. The culture supernatant of each well was collected and, its virus titre was determined and expressed as the 50% tissue culture infectious dose per ml ($TCID_{50}$/ml). All data represent means ±SD for triplicate experiments.

Figure 4. Inhibitory effect of SH-595A on viral antigen production in culture supernatants



Madin–Darby bovine kidney cells were infected with the bovine viral diarrhoea virus (BVDV) at a multiplicity of infection of 0.01 and incubated in the presence of various concentrations of the compound. After incubation for 3 days, the supernatants were collected and their viral antigen levels were determined by a BVDV antigen ELISA kit (Bio-X Diagnostics, Jemelle, Belgium). All data represent means ±SD for triplicate experiments.

Figure 5. Inhibitory effect of SH-595A and γ-carboline on viral RNA synthesis in MDBK cells



Madin–Darby bovine kidney (MDBK) cells were infected with the bovine viral diarrhoea virus (BVDV) at multiplicity of infection of 1.0 and cultured in the presence of various concentrations of (A) SH-595A or (B) γ-carboline. After incubation for 12 h, the cells were washed with phosphate-buffered saline, treated with lysis buffer and subjected to real-time reverse transcription-PCR (see *Methods*). All data represent mean ±SD for triplicate experiments.

γ-carboline. To gain further insight into the mechanism of BVDV inhibition by SH-595A, the effect of delayed compound addition on the viral RNA synthesis was investigated. The concentrations 100 and 20 μM were used for SH-595A and γ-carboline, respectively. When the compounds were added to the cell cultures simultaneously with the virus and were not removed during the whole culture period (condition 1), 87% and almost complete inhibition of viral RNA synthesis was observed for SH-595A and γ-carboline, respectively (Figure 6). When the compounds existed in the cell cultures during only the first 2 h after virus infection (condition 2), SH-595A and γ-carboline generated 47% and 19% inhibition, respectively. When the compounds were added after the virus adsorption period for 2 h (condition 3), SH-595A and γ-carboline achieved 64% and almost complete inhibition of viral RNA synthesis, respectively. Again, these results indicate that the mechanism of BVDV inhibition by SH-595A seems to differ from that by γ-carboline and that it might interfere with the viral adsorption step to some extent in addition to a step after virus adsorption.

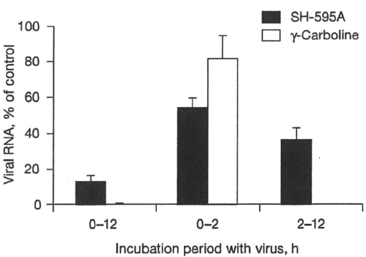
Discussion

BVDV is considered to be a valuable surrogate for identifying and characterizing anti-HCV agents [14]. Our strategy is to discover novel anti-BVDV compounds that can be developed as anti-HCV agents. Recently, we have reported the synthesis of novel diphenylmethane derivatives having anti-BVDV activity in cell cultures [12]. In this study, we attempted to further investigate their antiviral activity and mechanism of action.

Among the active compounds, SH-595A has been considered as a representative compound and was compared with γ-carboline [13]. SH-595A contains a silicon atom in its chemical structure; therefore, the compound is indeed novel and unique (Figure 1). According to the results obtained by real-time RT-PCR, SH-595A was found to be a modest inhibitor of BVDV RNA synthesis. It only marginally inhibited viral RNA synthesis at a concentration of 20 μM (Figure 5A), at which the compound achieved approximately 80% inhibition of viral replication as determined by the CPE inhibition assay (Figure 2D) and the viral antigen ELISA for culture supernatants (Figure 4). By contrast, γ-carboline was a potent inhibitor of viral RNA synthesis and its inhibitory concentration for viral RNA synthesis corresponded well with that for viral replication (Figures 2E and 5B). It is assumed that γ-carboline exerts its anti-BVDV activity through the inhibition of viral RNA polymerase

©2010 International Medical Press

Figure 6. Inhibitory effect of SH-595A and γ-carboline on viral entry to MDBK cells



Madin–Darby bovine kidney (MDBK) cells were seeded in a microtitre plate and incubated for 24 h. The cells were infected with bovine viral diarrhoea virus at multiplicity of infection of 2.0. SH-595A (100 μM) and γ-carboline (20 μM) were added at the time of infection and incubated without their removal (0–12), added at the time of infection but removed after incubation for 2 h (0–2) or added only after incubation for 2 h (2–12). The cells were incubated for 12 h after viral infection, washed with phosphate-buffered saline and subjected to real-time reverse transcription-PCR. All data represent mean ±SD for triplicate experiments.

activity (MTAS *et al.*, unpublished data). These results suggest that SH-595A interacts, in part, with a step after viral RNA synthesis.

Another possible explanation for the discrepancy between viral RNA inhibition by SH-595A and its antiviral activity is the difference of the assay conditions used for the experiments. The CPE inhibition assay reflects the inhibitory effect of test compounds on multi-round viral replication because of the low MOI (0.01) used for the experiment, whereas the viral RNA inhibition assay reflects the inhibition of single-round viral replication by test compounds because of the high MOI (1.0). Indeed, no viral antigen could be detected in the culture supernatants of the infected cells with BVDV at a low MOI (0.01) and exposed to SH-595A (100 μM) after a virus adsorption period for 2 h (MTAS *et al.*, data not shown). If SH-595A could interfere with an early step of the BVDV replication cycle, such as virus adsorption, the activity of the compound should be enhanced in a multi-round viral replication assay. To prove this hypothesis, a viral entry inhibition assay was examined for SH-595A and γ-carboline under a high MOI condition. It has been reported that a single cycle of BVDV replication takes 13 h on average and that gradual increase of intracellular viral RNA is noted at 6–8 h after virus infection [15]; therefore, like γ-carboline, viral RNA polymerase inhibitors are expected to suppress viral RNA synthesis, even when added after a virus adsorption period. However, SH-595A achieved only 65% inhibition, even at a concentration of 100 μM (Figure 6). In addition, if SH-595A could only inhibit the viral adsorption process, the viral RNA level in condition 2 (0–2 h) would be identical to that in condition 1 (0–12 h). By contrast, similar to γ-carboline, if SH-595A could not inhibit the viral adsorption process but could inhibit a process after viral adsorption, the viral RNA level in condition 1 (0–12 h) would be similar to that in condition 3 (2–12 h). These results suggest that SH-595A might have more than one target for inhibition of BVDV. The isolation and characterization of resistant mutants are also important for learning more about the mechanism of action; however, such studies are difficult to conduct because of the low potency of the current compounds. Further experiments are required to identify diphenylmethane derivatives that have higher antiviral activity and to determine their target molecule(s).

Finally, some diphenylmethane derivatives, including SH-595A, were examined for their inhibitory effect on HCV replication in HCV RNA replicon cells and were found to be inactive (MTAS *et al.*, data not shown). Because the replicon cells do not involve early and late steps of the HCV replication cycle [6,7], the diphenylmethane derivatives might be worth testing for their anti-HCV activity in the cell-free viral replication system [8–10].

Acknowledgements

This work was supported in part by the Science and Technology Incubation Program in Advanced Regions, Japan Science and Technology Agency, Japan.

Disclosure statement

All authors are inventors of the patent currently submitted to Japan Patent Office.

References

1. Liang TJ, Rehermann B, Seeff LB, Hoofnagle JH. Pathogenesis, natural history, treatment and prevention of hepatitis C. *Ann Intern Med* 2000; **132**:296–305.
2. Hayashi N, Takehara T. Antiviral therapy for chronic hepatitis C: past, present, and future. *J Gastroenterol* 2006; **41**:17–27.
3. Buckwold VE, Wei J, Wenzel-Mathers M, Russell J. Synergistic *in vitro* interactions between alpha interferon and ribavirin against bovine viral diarrhea virus and yellow fever virus as surrogate models of hepatitis C virus replication. *Antimicrob Agents Chemother* 2003; **47**:2293–2298.
4. Nam JH, Bukh J, Purcell RH, Emerson SU. High-level expression of hepatitis C virus (HCV) structural proteins by a chimeric HCV/BVDV genome propagated as a BVDV pseudotype. *J Virol Methods* 2001; **97**:113–123.
5. Ouzounov S, Mehta A, Dwek RA, Block TM, Jordan R. The combination of interferon α-2b and *n*-butyl deoxynojirimycin has a greater than additive antiviral effect upon production of infectious bovine viral diarrhea virus (BVDV) *in vitro*: implications for hepatitis C virus (HCV) therapy. *Antiviral Res* 2002; **55**:425–435.

6. Lohmann V, Körner F, Koch JO, Herian U, Thielmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNA in a hepatoma cell line. *Science* 1999; **285**:110–113.
7. Zhou S, Liu R, Baroudy BM, Malcom BA, Reyes GR. The effect of ribavirin and IMPDH inhibitors on hepatitis C virus subgenomic replicon RNA. *Virology* 2003; **310**:333–342.
8. Zhong J, Gastaminza P, Cheng G, *et al.* Robust hepatitis C virus infection *in vitro*. *Proc Natl Acad Sci U S A* 2005; **102**:9294–9299.
9. Wakita T, Pietschmann T, Kato T, *et al.* Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005; **11**:791–796.
10. Lindenbach BD, Evans MJ, Syder AJ, *et al.* Complete replication of hepatitis C virus in cell culture. *Science* 2005; **309**:623–626.
11. Baba C, Yanagida K, Kanzaki T, Baba M. Colorimetric lactate dehydrogenase (LDH) assay for evaluation of antiviral activity against bovine viral diarrhea virus (BVDV) *in vitro*. *Antivir Chem Chemother* 2005; **16**:33–39.
12. Hosoda S, Aoyama H, Goto Y, *et al.* Discovery of diphenylmethane analogs as anti-bovine diarrhea viral agents. *Bioorg Med Chem Lett* 2009; **19**:3157–3161.
13. Sako K, Aoyama H, Sato S, Hashimoto Y, Baba M. γ-Carboline derivatives with anti-bovine viral diarrhea virus (BVDV) activity. *Bioorg Med Chem* 2008; **16**:3780–3790.
14. Buckwold VE, Beer BE, Donis RO. Bovine viral diarrhea virus as a surrogate model of hepatitis C virus for the evaluation of antiviral agents. *Antiviral Res* 2003; **60**:1–15.
15. Paeshuyse J, Leyssen P, Mabery E, *et al.* A novel, highly selective inhibitor of pestivirus replication that targets the viral RNA-dependent RNA polymerase. *J Virol* 2006; **80**:149–160.

Received 4 January 2010, accepted 9 February 2010

©2010 International Medical Press

HETEROCYCLES, Vol. 81, No. 6, 2010, pp. 1419 - 1426. © The Japan Institute of Heterocyclic Chemistry
Received, 8th April, 2010, Accepted, 23rd April, 2010, Published online, 26th April, 2010
DOI: 10.3987/COM-10-11961

SYNYHESIS AND ANTI-HEPATITIS C VIRUS ACTIVITY OF MORPHOLINO TRIAZINE DERIVATIVES

Takashi Misawa,[a] Mohammed T. A. Salim,[b] Mika Okamoto,[b] Masanori Baba,*[,b] Hiroshi Aoyama,[a] Yuichi Hashimoto,[a] and Kazuyuki Sugita*[,a]

[a]Institute of Molecular & Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan. [b]Division of Antiviral Chemotherapy, Center for Chronic Viral Diseases, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan. (*Corresponding authors: K. Sugita for chemical part and M. Baba for biological part)

E-mail : sugitakazu@iam.u-tokyo.ac.jp; E-mail : m-baba@vanilla.ocn.ne.jp

Abstract – A series of morpholino triazines was synthesized and evaluated for anti-hepatitis C virus (HCV) activity. Incorporation of OMe, CN and F into the phenyl moiety afforded analogues with moderate potency and good selectivity, as assessed with the subgenomic HCV RNA replicon assay.

Hepatitis C virus (HCV) infection is a principle cause of chronic liver disease[1,2] leading to cirrhosis, hepatocellular carcinoma or liver failure in humans.[3,4] HCV is estimated to infect over 170 million people worldwide.[5] The best current treatment option is a pegylated interferon / ribavirin combination, but this has only limited efficacy against the most prominent HCV genotype (1a/1b), and has significant side effects.[6] Chronic HCV infection has been associated with liver fibrosis, liver cirrhosis, heptocellular carcinoma, and other forms of liver dysfunction. In industrialized nations, HCV infection has become a major reason for orthotopic liver transplantation. Given the widespread impact of this disease, there is a substantial medical need for new anti-HCV agents to complement current therapies.

We have previously found extremely versatile mother nuclei[7–11] so-called multi-templates[12-16] that act as highly efficient scaffolds for the creation of biologically active compounds. This time, we focused on a diphenylmethane template, which has already yielded several potent compounds.[8,9,11] Along this line, we

have already reported some anti-HCV compounds with good activity.[17] The benzyl triazine unit was chosen as an extension of the diphenylmethane template. A series of 2-benzyl-4-(morpholin-4-yl)-1,3,5-triazine derivatives was synthesized, together with bismorpholino-1,3,5-triazine derivatives, and their anti-HCV activity was examined.

The compounds described in this paper were prepared according to usual organic synthetic methods.[18] The general synthesis of morpholino triazine compounds (1) – (18) is outlined in Chart 1. Starting from cyanuric trichloride, the benzyl unit, morpholine ring and phenyl substituent were introduced.

Chart 1. Synthesis of morpholino triazine compounds

R^2 =

5 : X= H, Y= H
6 : X= 2-OH, Y= H
7 : X= 3-OH, Y= H
8 : X= 4-OH, Y= H
9 : X= 2-OMe, Y= H
10 : X= 3-OMe, Y= H
11 : X= 4-OMe, Y= H
12 : X= H, Y= N
13 : X= 2-CN, Y= H
14 : X= 3-CN, Y= H
15 : X= 4-CN, Y= H
16 : X= 2-F, Y= H
17 : X= 3-F, Y= H
18 : X= 4-F, Y= H

1 : X= 2-OH, Y= H
2 : X= 3-OH, Y= H
3 : X= 4-OH, Y= H

Reagents and conditions : (a) BnMgCl, THF, -78°C, 1hr, 82%; (b) morpholine, $(i\text{-Pr})_2NEt$, dioxane, rt, 1h, 89%; (c) morpholine, $(i\text{-Pr})_2NEt$, THF, 0°C, 0.5h, 61%; (d) arylboronic acid, $Pd(PPh_3)_4$, Na_2CO_3, dioxane-H_2O=2:1, 90°C, 2h, 17-98%; (e) morpholine, $NaHCO_3$, DCM, 0-40°C, 3h, quant.; (f) arylboronic acid, $Pd(PPh_3)_4$, Na_2CO_3, dioxane-H_2O=2:1, 90°C, 3-5h, 98%-quant.

The anti-HCV activity of morpholino triazine compounds was determined in the established HCV RNA replicon cells.[19] Briefly, #50-1 cells carrying sub-genomic HCV RNA replicons were cultured in the presence of various concentrations of the test compounds for 3 days. The intracellular HCV RNA and

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA levels were determined by real-time reverse transcription (RT)-PCR. The anti-HCV activity and cytotoxicity of test compounds were expressed as 50% effective concentration (EC_{50}) and 50% cytotoxic concentration (CC_{50}), defined in terms of decrease of HCV RNA and GAPDH RNA levels to 50% of the respective control levels. The results are shown in Figure 1 and Figure 2.

Table 1. Structures of synthesized morpholino triazine compounds

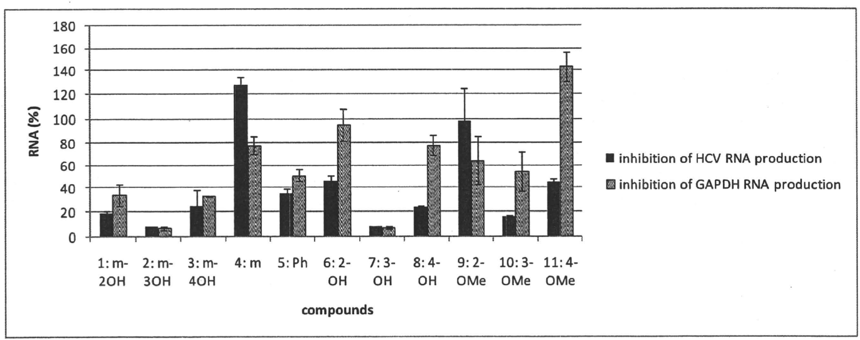
O N N R[1] N N R[2]

R^1 =	R^2 =	compound	R^1 =	R^2 =	compound
O N	OH	1		N	12
	HO	2		CN	13
	HO	3		NC	14
	O N	4		NC	15
		5		F	16
	OH	6		F	17
	HO	7		F	18
	HO	8			
	OMe	9			
	MeO	10			
	MeO	11			

As shown in Figure 1, dimorpholiono-hydroxyphenyl triazine derivatives (1~3) showed relatively strong cytotoxicity. Among these compounds, the 3-hydroxyl analog (2) was the most cytotoxic. Substitution of

the hydroxyphenyl group with a benzyl group, i.e., compound (4), resulted in decreased cytotoxicity, though (4) was completely inactive toward HCV RNA transcription. Then, we replaced one molpholino group with a phenyl group to obtain (5), which showed moderate anti-HCV activity (approximately 60% inhibition) with moderate cytotoxicity (approximately 55% inhibition). This led us to choose a 2-benzyl-4-morpholino-6-phenyl-1,3,5-triazine skeleton as a lead scaffold, and the effects of a substituent introduced into the phenyl moiety were examined. Introduction of a hydroxyl group into position 2 (*ortho*) or 4 (*para*) (6 and 8, respectively) resulted in the appearance of selective anti-HCV activity (Figure 1). In constrast, the 3-hydroxy analog (7) showed strong cytotoxicity. As was the case in the dimorpholino-hydroxypehyl triazine derivatives (1~3), a *meta*-hydroxyl group seems to induced potent cytotoxicity. In the case of methoxybenzyl derivatives (9~11), the 2-methoxy analog (9) was inactive, and the 3- and 4-methoxy derivatives (10 and 11, respectively) showed selective anti-HCV activity. It is noteworthy that compound (11) showed approximately 60% inhibition of HCV RNA transcription at 10 μM, with almost no cytotoxicity.

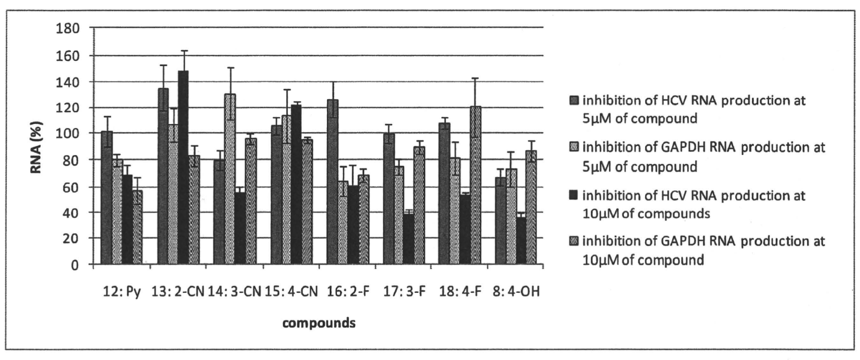
Figure 1. Anti-HCV activity of morpholino triazine compounds at 10 μM
(Percent inhibition of HCV gene and host cell gene (GAPDH) expression at 10 μM)



Based on the results shown in Figure 1, replacement of the phenyl group with a pyridyl group, and introduction of an electron-withdrawing group into the phenyl group were carried out for further structural development (Figure 2). Replacement of the phenyl ring with a 3-pyridine (12; Py) did not improve the anti-HCV activity or selectivity. Among the cyanophenyl derivatives (13~15), only the 3-cyano analog (14) showed selective anti-HCV activity (Figure 2). In the case of fluorophenyl

derivatives, the 3-fluoro and 4-fluoro analogs (**17** and **18**, respectively) showed selective anti-HCV activity at 10 μM.

Figure 2. Anti-HCV activity of morpholino triazine compounds at 5 and 10 μM
(Percent inhibition of HCV gene and host cell gene (GAPDH) expression at 5 and 10 μM)



In summary, compounds which bear a hydroxyl group (**8**; 4-OH), a cyano moiety (**14**; 3-CN) or a fluorine atom (**17**; 3-F), (**18**; 4-F) on the phenyl ring showed moderate anti-HCV activity (approximately 45-65% inhibition of HCV RNA transcription at 10 μM) and good selectivity (almost no cytotoxicity or less than 15% inhibition of GAPDH RNA transcription) in subgenomic HCV RNA replicon assay. Although the values for biological data vary from experiment to experiment, they are intrinsically reproducible. At this stage, we cannot fully characterize the structure-activity relationship. Even though, our substituted triazine derivatives appear to have potential for the development of anti-HCV agents, and further extensive investigation of compounds based on the benzyl triazine nucleus is expected to provide promising drug candidates.

ACKNOWLEDGEMENTS

The work described in this paper was partially supported by Grants-in-Aid for Scientific Research from the Science and Technology Incubation Program in Advanced Regions, Japan Science and Technology Agency (JST), and The Ministry of Education, Culture, Sports, Science and Technology, Japan, and a grant from the Japan Society for the Promotion of Science.

REFERENCES AND NOTES

1. T. J. Liang, B. Rehermann, L. B. Seeff, and J. H. Hoofnagle, *Ann. Intern. Med.*, 2000, **132**, 296.
2. P. H. Hayashi and A. M. Di Bisceglie, *Med. Clin. North Am.*, 2005, **89**, 371.
3. J. M. Echevarria-Mayo, *Enferm. Infecc. Microbiol. Clin.*, 2006, **24**, 45.
4. F. X. Bosch, J. Ribes, R. Cleries, and M. Diaz, *Clin. Liver Dis.*, 2005, **9**, 191.
5. M. I. Memon and M. A. Memon, *J. Viral Hepat.*, 2002, **9**, 84.
6. J. G. McHutchison, S. C. Gordon, E. R. Schiff, M. L. Shiffman, W. M. Lee, V. K. Rustgi, Z. D. Goodman, M. H. Ling, S. Cort, and J. K. Albrecht, *N. Engl. J. Med.*, 1998, **339**, 1485.
7. Y. Hashimoto, *Arch. Pharm. Life Sci.*, 2008, **341**, 536.
8. S. Hosoda, D. Matsuda, H. Tomoda, and Y. Hashimoto, *Mini-Rev. Med. Chem.*, 2009, **9**, 572.
9. S. Hosoda, H. Aoyama, Y. Goto, M. T. A. Salim, M. Okamoto, M. Hashimoto, M. Baba, and Y. Hashimoto, *Bioorg. Med. Chem. Lett.*, 2009, **19**, 3157.
10. M. Nakamura, T. Hamasaki, M. Tokitou, M. Baba, Y. Hashimoto, and H. Aoyama, *Bioorg. Med. Chem.*, 2009, **17**, 4740.
11. S. Hosoda, D. Matsuda, H. Tomoda, M. Hashimoto, H. Aoyama, and Y. Hashimoto, *Bioorg. Med. Chem. Lett.*, 2009, **19**, 4228.
12. Y. Hashimoto, *Curr. Med. Chem.*, 1998, **5**, 163.
13. Y. Hashimoto, *Bioorg. Med. Chem.*, 2002, **10**, 461.
14. Y. Hashimoto, *Mini-Rev. Med. Chem.*, 2002, **2**, 543.
15. Y. Hashimoto, A. Tanatani, K. Nagasawa, and H. Miyachi, *Drugs. Future*, 2004, **29**, 383.
16. Y. Hashimoto, *Cell Biol. Rev.*, 1991, **25**, 209.
17. M. Nakamura, A. Aoyama, M. T. A. Salim, M. Okamoto, M. Baba, H. Miyachi, and Y. Hashimoto, *Bioorg. Med. Chem.*, 2010, doi: 10.1016/j.bmc.2010.02.057.
18. Data for the compounds:

2-(2-Hydroxyphenyl)-4,6-bis(morpholin-4-yl)-1,3,5-triazine (1): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.75–3.80 (8H, m), 3.80–3.88 (8H, m), 6.87–6.92 (1H, m), 6.92–6.96 (1H, m), 7.35–7.41 (1H, m), 8.31–8.34 (1H, m), 13.6 (1H, s). HR-FAB-MS *m/z*: 343.1611 (Calcd for $C_{17}H_{21}N_5O_3$: $(M)^+$, 343.1644).
2-(3-Hydroxyphenyl)-4,6-bis(morpholin-4-yl)-1,3,5-triazine (2): ^{1}H-NMR 500MHz, $CDCl_3$) δ: 3.73–3.80 (8H, m), 3.80–4.10 (8H, m), 6.95–6.98 (1H, m), 7.26–7.29 (1H, m), 7.82–7.84 (1H, m), 7.87–7.91 (1H, m). Anal. Calcd for $C_{17}H_{21}N_5O_3 \cdot 0.5H_2O$: C, 57.94; H, 6.29; N, 19.87. Found: C, 57.53; H, 5.89; N, 19.73.
2-(4-Hydroxyphenyl)-4,6-bis(morpholin-4-yl)-1,3,5-triazine (3): ^{1}H-NMR (500MHz, $CDCl_3$) δ: 3.72–3.79 (8H, m), 3.79–4.02 (8H, m), 5.13 (1H, s), 6.86 (2H, d, *J*=18.3 Hz), 8.30 (2H, d, *J*=18.3 Hz). HR-FAB-MS *m/z*: 343.1692 (Calcd for $C_{17}H_{21}N_5O_3$: $(M)^+$, 343.1644).
2-Benzyl-4,6-dimorpholino-1,3,5-triazine (4): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.68–3.78 (16H, m), 3.80 (2H, s), 7.21 (1H, t, *J*=7.3 Hz), 7.28 (2H, t, *J*=7.3 Hz), 7.36 (2H, d, *J*=7.3 Hz). MS (FAB) *m/z* 342 $(M+H)^+$.

2-Benzyl-4-morpholino-6-phenyl-1,3,5-triazine (5): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.75–3.78 (4H, m), 3.89–4.01 (4H, m), 4.04 (2H, s), 7.23 (1H, t, *J*=7.3 Hz), 7.31 (2H, t, *J*=7.3 Hz), 7.45 (4H, t, *J*=7.3 Hz), 7.51 (1H, t, *J*=7.3 Hz), 8.41 (2H, d, *J*=7.3 Hz). HR-FAB-MS *m/z*: 332.1620 (Calcd for $C_{20}H_{20}N_4O$: (M)$^+$, 332.1637).

2-Benzyl-4-(2-hydroxyphenyl)-6-morpholino-1,3,5-triazine (6): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.72–3.76 (4H, m), 3.88–3.91 (4H, m), 6.98 (1H, d, *J*=7.3 Hz), 7.02 (1H, t, *J*=7.3 Hz), 7.21 (1H, t, *J*=7.3 Hz), 7.30 (2H, t, *J*=7.3 Hz), 7.40 (1H, td, *J*=7.3, 1.8 Hz), 7.48 (2H, d, *J*=7.3 Hz), 7.73 (1H, dd, *J*=7.3, 1.8 Hz). MS (FAB) *m/z* 349 (M+H)$^+$. Anal. Calcd for $C_{20}H_{20}N_4O_2 \cdot 0.2H_2O$: C, 68.24; H, 5.84: N, 15.92. Found: C, 68.12; H, 5.97; N, 16.18.

2-Benzyl-4-(3-hydroxyphenyl)-6-morpholino-1,3,5-triazine (7): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.72–3.78 (4H, m), 3.88–3.98 (4H, m), 4.03 (2H, s), 6.99 (1H, d, *J*=7.3 Hz), 7.23 (1H, t, *J*=7.3 Hz), 7.31 (2H, t, *J*=7.3 Hz), 7.32 (1H, t, *J*=7.3 Hz), 7.42 (2H, d, *J*=7.3 Hz), 7.87–7.88 (1H, m), 7.99 (1H, d, *J*=7.3 Hz). MS (FAB) *m/z* 349 (M+H)$^+$. Anal. Calcd for $C_{20}H_{20}N_4O_2 \cdot 0.1H_2O$: C, 68.59; H, 5.81: N, 16.00. Found: C, 68.52; H, 5.80; N, 16.21.

2-Benzyl-4-(4-hydroxyphenyl)-6-morpholino-1,3,5-triazine (8): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.74–3.76 (4H, m), 3.87–3.99 (4H, m), 4.01 (2H, s), 6.87 (2H, dt, *J*=7.3, 2.0 Hz), 7.22 (1H, t, *J*=7.3 Hz), 7.30 (2H, t, *J*=7.3 Hz), 7.42 (2H, d, *J*=7.3 Hz), 8.33 (2H, dt, *J*=7.3, 2.0 Hz). MS (FAB) *m/z* 349 (M+H)$^+$. Anal. Calcd for $C_{20}H_{20}N_4O_2 \cdot 0.2H_2O$: C, 68.24; H, 5.84: N, 15.92. Found: C, 68.08; H, 5.92; N, 15.94.

2-Benzyl-4-(2-methoxyphenyl)-6-morpholino-1,3,5-triazine (9): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.71–3.75 (4H, m), 3.84 (3H, s), 3.83–3.95 (4H, m), 4.04 (2H, s), 7.00 (1H, d, *J*=7.3 Hz), 7.02 (1H, t, *J*=7.3 Hz), 7.23 (1H, t, *J*=7.3 Hz), 7.31 (2H, t, *J*=7.3 Hz), 7.40 (1H, td, *J*=7.3, 1.2 Hz), 7.44 (2H, d, *J*=7.3 Hz), 7.71 (1H, dd, *J*=7.3, 1.2 Hz). MS (FAB) *m/z* 363 (M+H$^+$)$^+$. Anal. Calcd for $C_{21}H_{22}N_4O_2 \cdot 0.4H_2O$: C, 68.24; H, 6.22: N, 15.16. Found: C, 68.48; H, 6.11; N, 14.98.

2-Benzyl-4-(3-methoxyphenyl)-6-morpholino-1,3,5-triazine (10): ^{1}H-NMR (500 MHz, $CDCl_3$) δ:. MS (FAB) *m/z* 363 (M+H)$^+$. Anal. Calcd for $C_{21}H_{22}N_4O_2$: C, 69.59; H, 6.12: N, 15.46. Found: C, 69.31; H, 6.14; N, 15.54.

2-Benzyl-4-(4-methoxyphenyl)-6-morpholino-1,3,5-triazine (11): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.73–3.81 (4H, m), 3.88 (3H, s), 3.91–3.98 (4H, m), 4.04 (2H, s), 7.05 (1H, dd, *J*=7.3, 2.0 Hz), 7.23 (1H, t, *J*=7.9 Hz), 7.31 (2H, t, *J*=7.3 Hz), 7.36 (1H, t, *J*=7.9 Hz), 7.43 (2H, d, *J*=7.9 Hz), 7 96 (1H, d, *J*=2.0 Hz), 8.38 (1H, td, *J*=7.9, 2.0 Hz). MS (FAB) *m/z* 363 (M+H)$^+$. Anal. Calcd for $C_{21}H_{22}N_4O_2$: C, 69.59; H, 6.12: N, 15.46. Found: C, 69.76; H, 6.09; N, 15.62.

2-Benzyl-4-morpholino-6-(3-pyridinyl)-1,3,5-triazine (12): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.74–3.80 (4H m), 3.90–4.00 (4H, brs), 4.04 (2H, s), 7.24 (1H, t, *J*=7.5 Hz), 7.31 (2H, t, *J*=7.5 Hz), 7.37 (1H, t, *J*=7.5 Hz), 7.43 (2H, d, *J*=7.5 Hz), 7.64 (1H, dt, *J*=7.5, 1.5 Hz), 7.72 (1H, dd, *J*=7.5, 1.5 Hz), 8.58 (1H, d, *J*=1.5 Hz). HR-FAB-MS *m/z*: 333.1613 (Calcd for $C_{19}H_{19}N_5O$: (M)$^+$, 333.1590).

2-Benzyl-4-(2-cyanophenyl)-6-morpholino-1,3,5-triazine (13): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.70–3.76 (4H, m), 3.86–3.94 (4H, m), 4.02 (2H, s), 7.23 (1H, t, *J*=8.0 Hz), 7.31 (2H, t, *J*=7.0 Hz), 7.40 (2H, d, *J*=7.0 Hz), 7.48–7.53 (2H, m), 7.79 (1H, d, *J*=7.0 Hz), 8.09 (1H, t, *J*=7.0 Hz). HR-FAB-MS *m/z*: 357.1635 (Calcd for $C_{21}H_{19}N_5O$: (M)$^+$, 357.1590).

2-Benzyl-4-(3-cyanophenyl)-6-morpholino-1,3,5-triazine (14): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.74–3.80 (4H, m), 3.90–4.00 (4H, m), 4.03 (2H, s), 7.24 (1H, t, *J*=8.0 Hz), 7.56 (1H, t, *J*=8.0 Hz), 7.77 (1H, d, *J*=8.0 Hz), 8.64 (1H, d, *J*=8.0 Hz), 8.72 (1H, s). HR-FAB-MS *m/z*: 357.1629 (Calcd for $C_{21}H_{19}N_5O$: (M)$^+$, 357.1590).

2-Benzyl-4-(4-cyanophenyl)-6-morpholino-1,3,5-triazine (15): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.75–3.78 (4H, m), 3.90–4.00 (4H, m), 4.04 (2H, s), 7.24 (1H, t, *J*=7.5 Hz), 7.32 (2H, t, *J*=7.5 Hz), 7.42 (2H, d, *J*=7.5 Hz), 7.73 (2H, d, *J*=8.5), 8.51 (2H, d, *J*=8.5 Hz). HR-FAB-MS *m/z*: 357.1570 (Calcd for $C_{21}H_{19}N_5O$: (M)$^+$, 357.1590).

2-Benzyl-4-(2-fluorophenyl)-6-morpholino-1,3,5-triazine (16): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.73–3.75 (4H, m), 3.88–3.97 (4H, m), 4.05 (2H, s), 7.14 (1H, dd, *J*=7.5, 2.5 Hz), 7.21–7.24 (2H, m),

7.31 (2H, d, *J*=7.5 Hz), 7.42–7.47 (3H, m), 8.09 (1H, td, *J*=7.5, 2.5 Hz). HR-FAB-MS *m/z*: 350.1527 (Calcd for $C_{20}H_{19}FN_4O$: $(M)^+$, 350.1543).

2-Benzyl-4-(3-fluorophenyl)-6-morpholino-1,3,5-triazine (17): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.73–3.79 (4H, m), 3.90–4.00 (4H, m), 4.03 (2H, s), 7.18–7.25 (3H, m), 7.31 (2H, td, *J*=8.0, 2.0 Hz), 7.39–7.44 (3H, m), 8.01–8.09 (1H, m), 8.21 (1H, dd, *J*=8.0, 2.0 Hz). HR-FAB-MS *m/z*: 350.1521 (Calcd for $C_{20}H_{19}FN_4O$: $(M)^+$, 350.1543).

2-Benzyl-4-(4-fluorophenyl)-6-morpholino-1,3,5-triazine (18): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.73–3.79 (4H, m), 3.86–3.99 (4H, m), 4.06 (2H, s), 7.15 (2H, t, *J*=7.5 Hz), 7.23 (1H, td, *J*=8.0, 2.0 Hz), 7.31 (2H, td, *J*=8.0, 2.0 Hz), 7.42 (2H, d, *J*=7.5 Hz), 8.43 (2H, td, *J*=8.0, 2.0 Hz). HR-FAB-MS *m/z*: 350.1578 (Calcd for $C_{20}H_{19}FN_4O$: $(M)^+$, 350.1543).

2-Benzyl-4,6-dichloro-1,3,5-triazine (20): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 4.18 (2H, s), 7.29 (1H, d, *J*=7.0 Hz), 7.33–7.41 (4H, m). MS (FAB) *m/z* 240 $(M+H)^+$.

2-Benzyl-4-chloro-6-morpholino-1,3,5-triazine (21): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.71–3.73 (4H, m), 3.81–3.87 (4H, m), 3.93 (2H, s), 7.24, (1H, t, *J*=7.3 Hz), 7.31 (2H, t, *J*=7.3 Hz), 7.35 (2H, d, *J*=7.3 Hz). MS (FAB) *m/z* 291 $(M+H)^+$.

2-Chloro-4,6-bis(morpholin-4-yl)-1,3,5-triazine (22): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.65–3.85(16H, m).

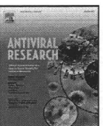
19. N. Ishii, K. Watashi, T. Hishiki, K. Goto, D. Inoue, M. Hijikata, T. Wakita, N. Kato, and K. Shimotohno, *J. Virol.*, 2006, **80**, 4510.

Antiviral Research 88 (2010) 263–268

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral



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

Mohammed T.A. Salim[a], Yukinori Goto[a,1], Takayuki Hamasaki[a], Mika Okamoto[a], Hiroshi Aoyama[b], Yuichi Hashimoto[b], Simone Musiu[c], Jan Paeshuyse[c], Johan Neyts[c], Matheus Froeyen[c], Piet Herdewijn[c], Masanori Baba[a,*]

[a] *Division of Antiviral Chemotherapy, Center for Chronic Viral Diseases, Graduate School of Medical and Dental Sciences, 8-35-1, Sakuragaoka, Kagoshima University, Kagoshima, 890-8544, Japan*
[b] *Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo 113-0032, Japan*
[c] *Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium*

ARTICLE INFO

Article history:
Received 10 June 2010
Received in revised form
10 September 2010
Accepted 17 September 2010

Keywords:
Flavivirus
BVDV
HCV
Carboline
RNA polymerase
Drug-resistance

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.

© 2010 Elsevier B.V. All rights reserved.

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

* Corresponding author. Tel.: +81 99 275 5930; fax: +81 99 275 5932.
E-mail address: m-baba@vanilla.ocn.ne.jp (M. Baba).
[1] Present address: Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Suita 565-0871, Japan.

0166-3542/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2010.09.013

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

AG110

LZ37

BPIP

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

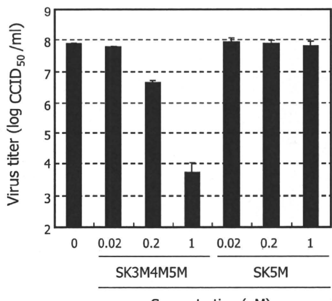


Fig. 2. Inhibitory effect of SM3M4M5M and SK5M on virus production in MDBK cells. The cells were infected with BVDV at a MOI of 1.0 and cultured in the presence or absence of various concentrations of SK3M4M5M or γ-carboline. After incubation for 4 h, the cells were washed with PBS and further incubated 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.