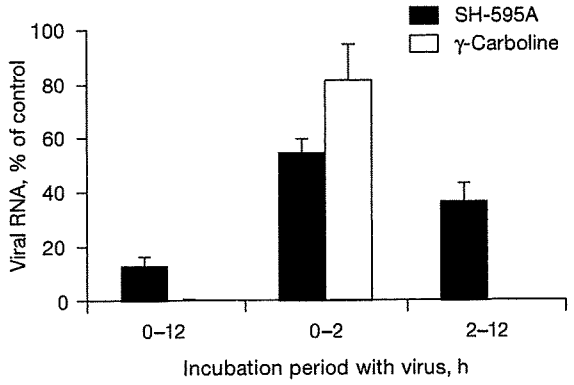
Figure 6. Inhibitory effect of SH-595A and γ-carboline on viral entry to MDBK cells



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 ±so 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activity (MTAS *et al.*, unpublished data).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

Bioorganic & Medicinal Chemistry 18 (2010) 2402–2411

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

Structural development studies of anti-hepatitis C virus agents with a phenanthridinone skeleton

Masahiko Nakamura [a], Atsushi Aoyama [a], Mohammed T. A. Salim [b], Mika Okamoto [b], Masanori Baba [b,*], Hiroyuki Miyachi [c], Yuichi Hashimoto [a], Hiroshi Aoyama [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
[c] Division of Pharmaceutical Sciences, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 1-1-1 Tsushima-Naka, Kita-ku, Okayama 700-8530, Japan

ARTICLE INFO

Article history:
Received 10 February 2010
Revised 24 February 2010
Accepted 25 February 2010
Available online 2 March 2010

Keywords:
Anti-HCV agents
Phenanthridinone
Thalidomide
Am80
Am580

ABSTRACT

A phenanthridinone skeleton was derived from our previous researches on thalidomide and retinoids as a multi-template for generation of anti-viral lead compounds. Structural development studies focusing on anti-hepatitis C virus activity afforded 5-butyl-2-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)phenanthridin-6(5*H*)-one (**10**) and 5-butylbenzo[*b*]phenanthridin-6(5*H*)-one (**39**), which showed EC_{50} values of approximately 3.7 and 3.2 μM, respectively.

1. Introduction

The efficient identification of small-molecular scaffolds for the development of biologically active compounds is very important in chemical genetics and medicinal chemistry. As one approach, we have been utilizing the multi-template hypothesis,[1–5] based on the idea that the number of protein fold structure types that comprise all the domains occurring in natural proteins is quite limited, in spite of the huge number of natural proteins.[6–8] A given fold structure might be characteristic of many natural proteins, and therefore, ignoring physical/chemical interactions, one might expect that a template/scaffold structure which is spatially complementary to one fold structure might serve as a multi-template for structural development of ligands that would interact specifically with many different natural proteins. As candidate multi-template structures, we have focused particularly on thalidomide (**1**) and retinoids, including synthetic retinoids Am80 (**2**) and Am580 (**3**) (Fig. 1).[1,9–13] All of these compounds **1**–**3** elicit a wide range of biological activities, and thalidomide (**1**) is well-established to be multi-target drug.[1,9–13] In fact, we recently applied thalidomide (**1**) and/or Am80 (**2**)/Am580 (**3**) as multi-templates to develop anti-viral agents and/or anti-proliferative agents for virus-infected cells, that is, anti-bovine viral diarrhea virus (anti-BVDV) agents, including SK3M4M5M (**4**) derived from thalidomide (**1**)[14,15] and adult T-cell leukemia (ATL) cell-selective proliferation inhibitors, including TMN($COCH_3$) (**5**) and TMN(OH)($COCH_3$) (**6**) derived from Am80 (**2**)/Am580 (**3**) (Fig. 1).[4] We next aimed to develop anti-hepatitis C virus (HCV) agents.

HCV infection is thought to be a major cause of human hepatitis,[16,17] and it is estimated that at least 170 million people worldwide are chronically infected with this virus.[18] Most infections become persistent and about 60% of cases progress to chronic liver disease, which in turn can lead to development of cirrhosis, hepatocellular carcinoma, and liver failure.[19,20] Currently, no vaccine is available against HCV infection, and the standard treatment for chronic hepatitis C consists of pegylated interferon (IFN)-α in combination with the nucleoside analog ribavirin (1-β-D-ribofuranosyl-1,2,4-triaxole-3-carboxamide). However, the virus cannot be eliminated from approximately half of infected patients treated with these agents.[21] In addition, the side effects of these agents are sometimes serious and unacceptable to patients. Therefore, alternative agents for the treatment and prevention of HCV infection are urgently needed.

As mentioned above, we have been succeeded in the development of potent anti-BVDV agents, including SK3M4M5M (**4**), which

* Corresponding authors. Tel.: +81 3 5841 7848; fax: +81 5841 8495 (H.A.); tel.: +81 99 275 5930; fax: +81 99 275 5932 (M.B.).
E-mail addresses: m-baba@vanilla.ocn.ne.jp (M. Baba), aoyama@iam.u-tokyo.ac.jp (H. Aoyama).

0968-0896/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2010.02.057

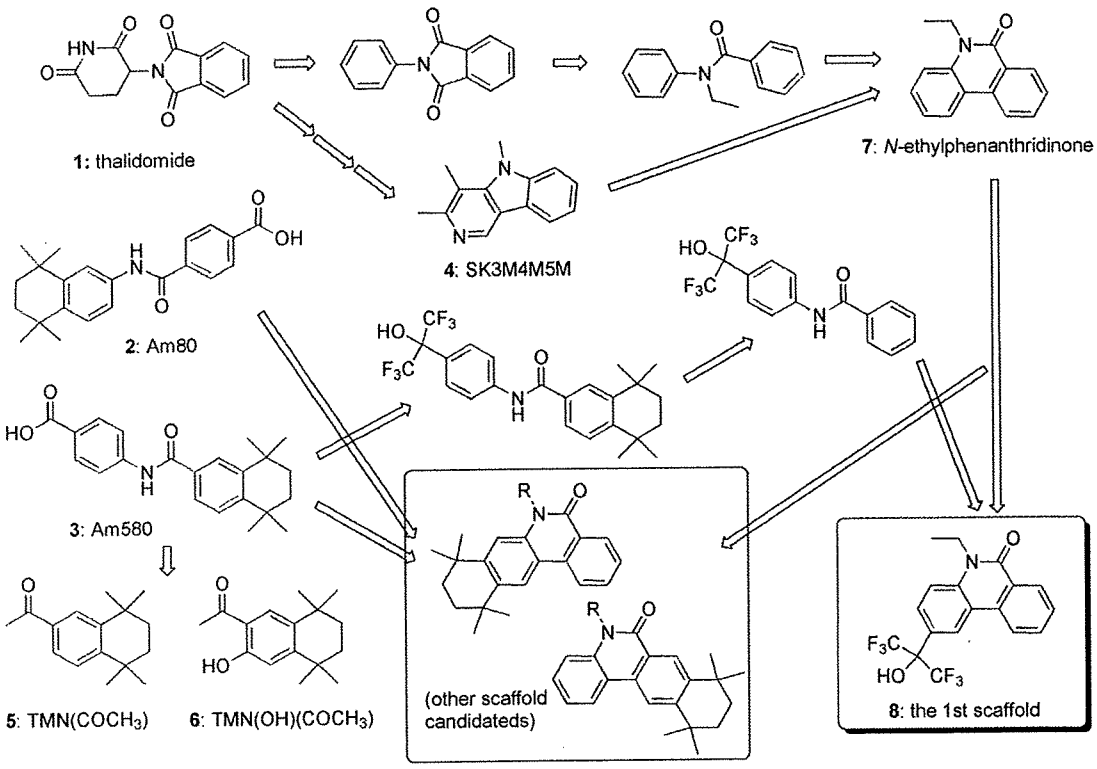


Figure 1. Armchair structural development of thalidomide (**1**) and retinoids (**2**, **3**) to the first scaffold **8**.

have an EC_{50} value of 3.5 nM.[15] Although BVDV belongs to the *Flaviviridae* family, as HCV does,[22] and is thought to be a surrogate model for HCV,[23–25] SK3M4M5M (**4**) showed only a very weak activity against HCV. Therefore, we tried to develop another scaffold for the development of anti-HCV agents, as shown in Figure 1.

2. Results and discussion

2.1. Armchair structural development leading to phenanthridinone derivatives

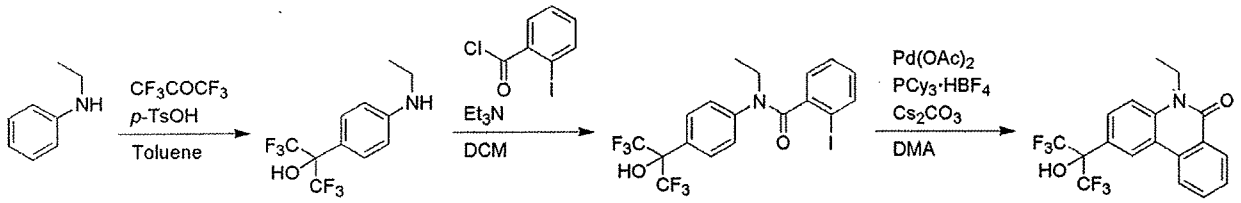
First, we applied armchair structural development to phenylphthalimide, which was itself developed from thalidomide (**1**). The phenylphthalimide skeleton is a superior multi-template, and we have developed various biologically active phenylphthalimide derivatives, including tumor necrosis factor-α production regulators, tubulin polymerization inhibitors, dipeptidylpeptidase type IV inhibitors, liver X receptor antagonists, α-glucosidase inhibitors, and so on.[1,9–12] The armchair ring opening of phenylphthalimide gave *N*-ethylbenzanilide, whose recyclization should afford *N*-ethylphenanthridinone (**7**) (Fig. 1). On the other hand, synthetic retinoids Am80 (**2**) and Am580 (**3**) both possess a benzoic acid moiety. Because our previous studies on anti-BVDV agents suggested that carboxyl acid derivatives are not suitable as lead structures, we considered that a bioisosteric functional group, the 1,1,1,3,3,3-hexafluoropropan-2-ol-substituted phenyl moiety, might be introduced into *N*-ethylphenanthridinone (**7**) to give the first scaffold structure, 5-ethyl-2-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl) phenanthridin-6(5*H*)-one (**8**) (Fig. 1). In addition, both Am80 (**2**) and Am580 (**3**) possess a teterahydrotetramethylnaphthalene moiety, which has been established to be a useful core structure to develop ATL cell-selective proliferation inhibitors, including $TMN(COCH_3)$ (**5**) and $TMN(OH)(COCH_3)$ (**6**).[4] We therefore considered that docking of teterahydrotetramethylnaphthalene moiety and *N*-ethylphenanthridinone (**7**) would afford other scaffold candidate structures, as shown in Figure 1.

Compound **8** was prepared as shown in Scheme 1.[26,27] Briefly, *N*-ethylaniline was treated with hexafluoroacetone to give the 1,1,1,3,3,3-hexafluoro-2-hydroxypropyl derivative, which was coupled with 2-iodobenzoyl chloride. The resulting anilide was cyclized to give compound **8**. The anti-HCV activity of compound **8** was determined in the established HCV RNA replicon cells.[28] Briefly, NNC #2 cells carrying full-genomic HCV RNA replicons were cultured in the presence of various concentrations of the test compounds for 3 days. The cells were examined for HCV RNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA levels 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. As shown in Figure 2, compound **8** showed apparent anti-HCV activity (EC_{50} and CC_{50}: 14.1 and >40 μM, respectively), suggesting that compound **8** could be a lead compound for structural development of anti-HCV agents.

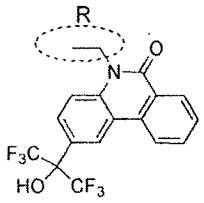
2.2. Effects of N-substituents

Since compound **8** showed anti-HCV activity, we examined the effects of *N*-substituents. Several N-alkylated derivatives of **8** (**9**–**14**) were prepared as shown in Scheme 2, and their anti-HCV activity was measured as described above (Figs. 2 and 3).

As shown in Figures 2 and 3, introduction of three fluorine atoms at the terminal methyl group (**9**) did not improve the activity. However, introduction of a longer-chain alkyl group, *n*-butyl (**10**), *n*-hexyl (**11**), or *n*-nonyl (**12**), resulted in enhancement of anti-HCV activity, though at the same time, the cytotoxicity was increased. The anti-HCV activity of these compounds decreased



Scheme 1.



8: the 1st scaffold

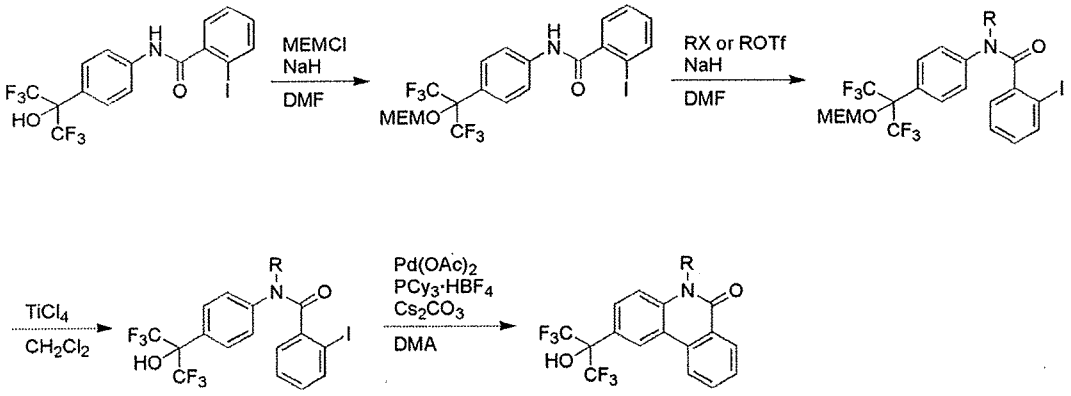
R	EC_{50} (μM)	CC_{50} (μM)
8: CH_3CH_2-	14.1	>40
9: CF_3CH_2-	24.8	30.3
10: $CH_3(CH_2)_3$-	3.7	12.5
11: $CH_3(CH_2)_5$-	4.8	12.2
12: $CH_3(CH_2)_8$-	8.3	10.2
13: $PhCH_2$-	7.7	18.2
14: c-$C_6H_{11}CH_2$-	6.7	14.4

Figure 2. Effects of N-substituents on anti-HCV activity. EC_{50}: 50% effective concentration, based on the decrease of the amount of HCV RNA. CC_{50}: 50% cytotoxic concentration, based on the decrease of the amount of GAPDH RNA.

in the order of: **10** > **11** > **12**. On the other hand, their cytotoxicity tended to decrease in the reverse order, though the differences were small. The benzyl analog (**13**) and the cyclohexylmethyl analog (**14**) showed moderate anti-HCV activity (EC_{50}: 7.7 and 6.7 μM, respectively) with weaker cytotoxicity (CC_{50}: 18.2 and 14.4 μM, respectively). Cytotoxicity was not affected by the length of the introduced alkyl group (CC_{50}: 10.2–12.5 μM). Among this series, compound **10** showed the most potent anti-HCV activity (EC_{50}: 3.7 μM). Therefore, we selected the *N*-butylphenanthridinone skeleton (corresponding to compound **10**) as a scaffold structure for further structural development studies.

2.3. Regioisomers of methyl-substituted *N*-butylphenanthridinone

Based on our previous structure–activity relationship studies of anti-BVDV γ-carboline analogs, which indicated that regio-selective methyl-substitution dramatically influenced the anti-viral activity,[14,15] the effect of methyl-substitution was investigated. For this purpose, we synthesized all the regioisomers of methyl-



Scheme 2.

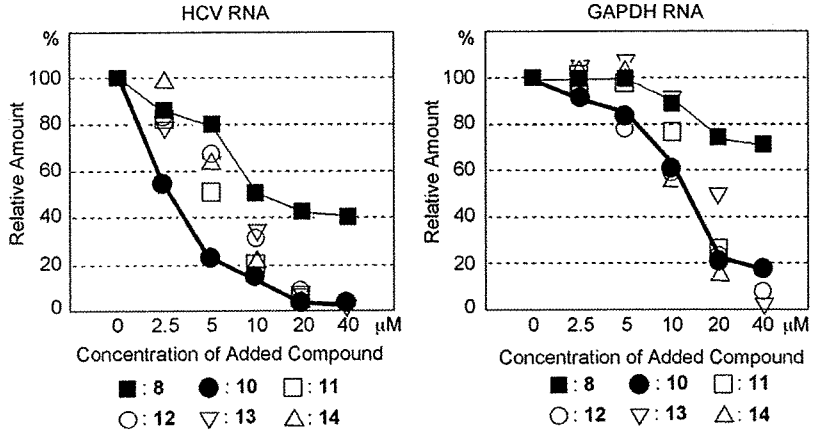


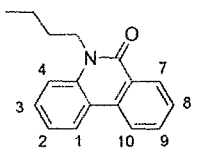
Figure 3. Dose-dependency curves of anti-HCV and cytotoxic activity elicited by compounds **8** and **10–14**.

substituted *N*-butylphenanthridinone (Fig. 4) by the method shown in Scheme 3.

As shown in Figure 4, *N*-butylphenanthridinone itself (an extracted structure from compound **10**) was completely inactive. However, methyl-substitution at position 2, that is, the position at which the 1,1,1,3,3,3-hexafluoropropan-2-ol group was attached in compound **10**, resulted in the appearance of anti-HCV activity (EC_{50}: 42.0 μM). All the other regioisomers, except the 8-methyl analog (**21**), that is, compounds **16**, **18–20**, **22**, and **23**, were inactive, and the anti-HCV activity elicited by **21** was very weak. Therefore, position 2 seems to be the best position at which to introduce a substituent.

2.4. Effect of 2-subsituents on anti-HCV activity

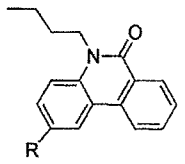
The results described above prompted us to examine the effect of 2-substitutents, and we prepared various derivatives (**24–33**) as shown in Figure 5. Although some derivatives, including compounds **28–33**, showed improved anti-HCV activity compared to the 2-methyl analog **17**, their activity was weaker than that of **10**. As already mentioned, the unsubstituted analog **15** was inactive. Its fluoro analog **24** was also inactive, suggesting that a mono-atomic substituent is inappropriate to improve the activity. Though the 2-methyl analog **17** showed some activity (EC_{50}: 35.8 μM), its trifluoromethyl analog **26** was inactive. On the contrary, its hydroxyl analog **25** showed activity comparable to that of the 2-methyl analog **17**. The 2-ethyl analog **27** also showed activity comparable to that of **17**/**25**.



%-Inhibition of HCV gene and host cell gene (GAPDH) expression at 10 μM

	HCV	GAPDH
15: none	inactive	inactive
16: 1-CH_3	inactive	85.2
17: 2-CH_3	42.0	98.5
18: 3-CH_3	inactive	70.7
19: 4-CH_3	inactive	inactive
20: 7-CH_3	inactive	inactive
21: 8-CH_3	87.0	88.8
22: 9-CH_3	inactive	inactive
23: 10-CH_3	inactive	94.9

Figure 4. Anti-HCV activity of methyl-substituted regioisomers of *N*-butylphenanthridinone, **16–23**.



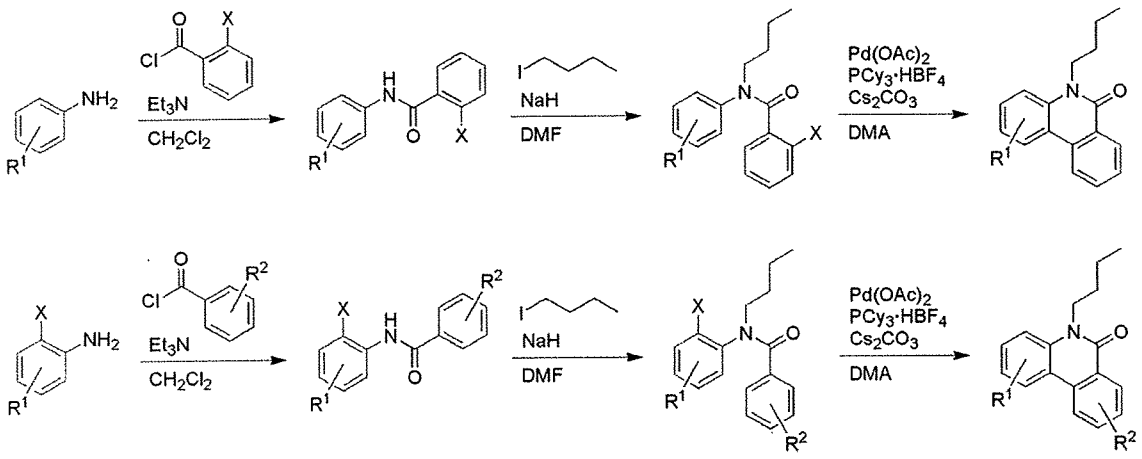
R	EC_{50} (μM)	CC_{50} (μM)
10: $(CF_3)_2(OH)C$-	3.7	12.5
15: H	>58.3	58.3
17: CH_3	35.8	38.4
24: F	>50	>50
25: OH	37.9	43.7
26: CF_3	>50	>50
27: CH_3CH_2	34.7	40.5
28: $(CH_3)_2CH$-	16.2	29.7
29: CH_2OH	21.5	>50
30: $CH_3CH(OH)$-	21.8	51.4
31: $(CH_3)_3CH$	10.6	24.9
32: CH_3CO-	18.7	27.3
33: $Ph(CH_2)_2$-	21.0	24.1

Figure 5. Anti-HCV activity of 2-substituted *N*-butylphenanthridinone, **15**, **17** and **24–33**.

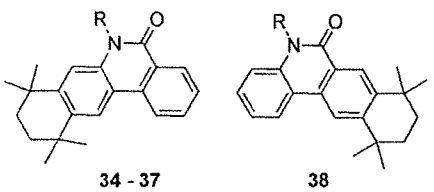
Compounds **28–33** showed moderate anti-HCV activity (EC_{50}: 10.6–21.8 μM). Among the 2-alkylated derivatives, the activity decreased in the order of *t*-butyl (**31**) > *i*-propyl (**28**) > ethyl (**27**) > methyl (**17**), suggesting that the hydrophobicity of the 2-substituent contributes to the activity, at least in part. On the other hand, introduction of a hydroxyl group into the ethyl (**30**) or methyl (**29**) group of **27** and **17**, respectively, seemed to slightly enhance the activity, that is, **27** (EC_{50}: 34.7 μM) versus **30** (EC_{50}: 21.8 μM) and **17** (EC_{50}: 35.8 μM) versus **29** (EC_{50}: 21.5 μM). The 2-acetyl derivative **32** showed slightly more potent activity than **30**.

2.5. Tetrahydrotetramethylnaphthalene-related analogs and benzophenanthridinone analogs

Although the structure–activity relationships of 2-substituted *N*-butylphenanthridinone were clearly interpretable, hydrophobicity around the 2-position also seemed to contribute to the activity. This and the armchair structural development shown in Figure 1 prompted us to prepare compounds **34–38** (Fig. 6). We also prepared benzophenanthridinone derivatives **39–44** (Fig. 7). Among the tetrahydrotetramethylnaphthalene-related analogs **34–38**, only the *N*-butyl and *N*-benzyl derivatives (**35** and **36**, respectively) were moderately active, as shown in Figure 6. The regioisomer of **35**, that is, compound **38**, was inactive.

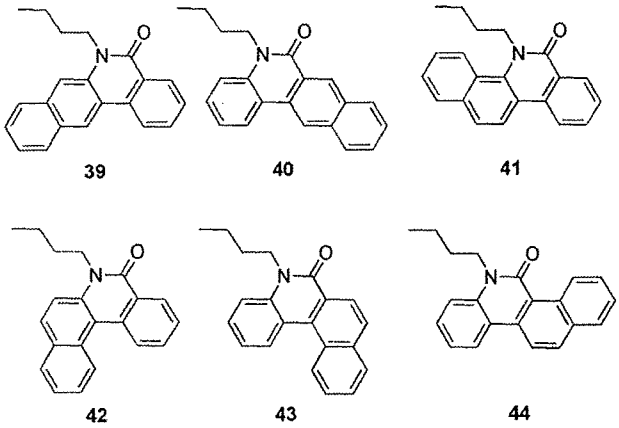


Scheme 3.



R	EC_{50} (μM)	CC_{50} (μM)
34: H	inactive	inactive
35: $CH_3(CH_2)_3$-	18.7	27.3
36: $PhCH_2$-	21.2	31.8
37: c-$C_6H_{11}CH_2$-	inactive	inactive
38: $CH_3(CH_2)_3$-	inactive	inactive

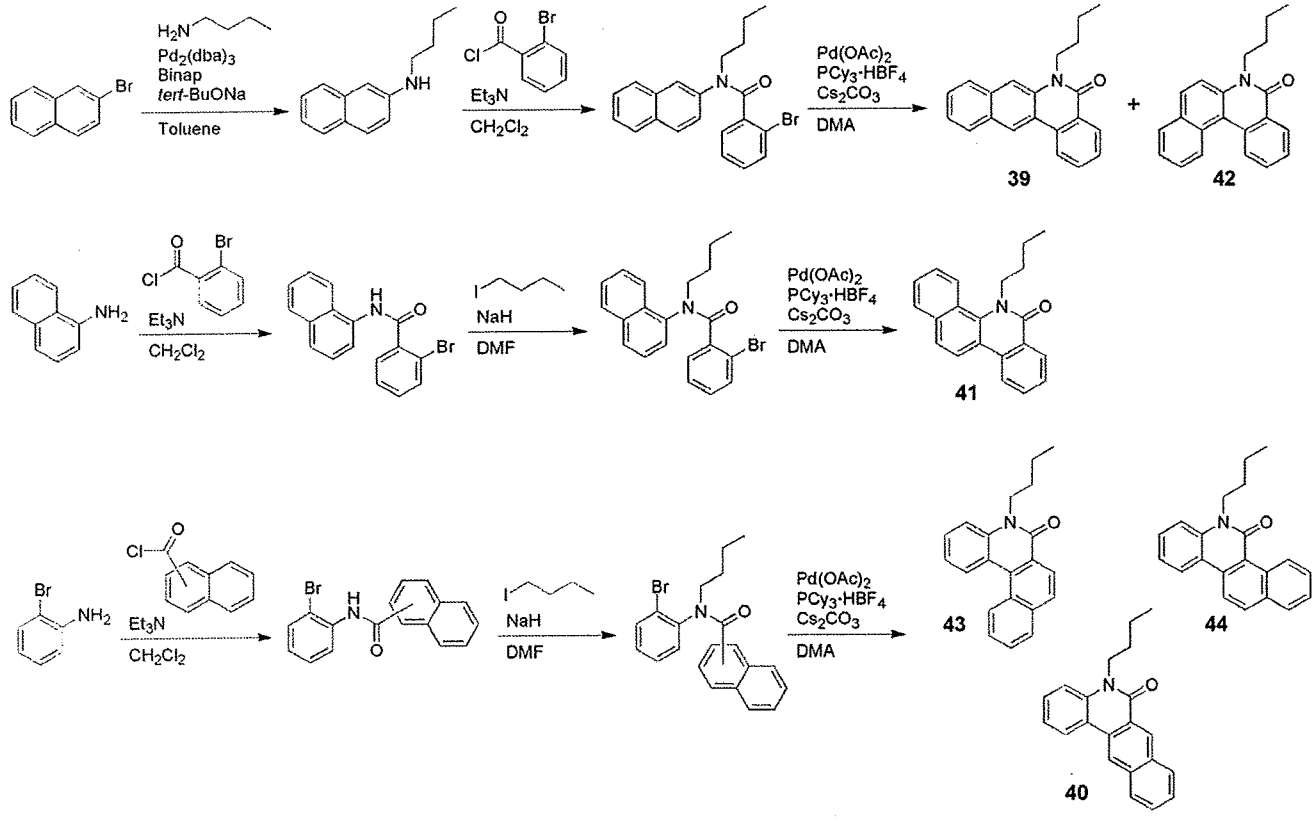
Figure 6. Anti-HCV activity of tetrahydrotetramethylnaphthalene-related analogs, **34–38**.



	EC_{50} (μM)	CC_{50} (μM)
35	18.7	27.3
39	3.2	6.3
40	11.0	>40
41	19.5	24.3
42	29.1	44.5
43	>31.4	31.4
44	29.9	>40

Figure 7. Anti-HCV activity of benzophenathridinone derivatives, **39–44**.

Benzophenanthridinone derivatives **39–44** were prepared as shown in Scheme 4. All the benzophenanthridinone derivatives, except **43**, showed potent or moderate anti-HCV activity. The activity decreased in the order of: benzo[*b*] (**39**) > benzo[*j*] (**40**) > benzo[*c*] (**41**) > benzo[*a*] (**42**) > benzo[*i*] (**44**) >> benzo[*k*] (**43**) as shown in Figures 7 and 8. Although compound **39** showed the most



Scheme 4.

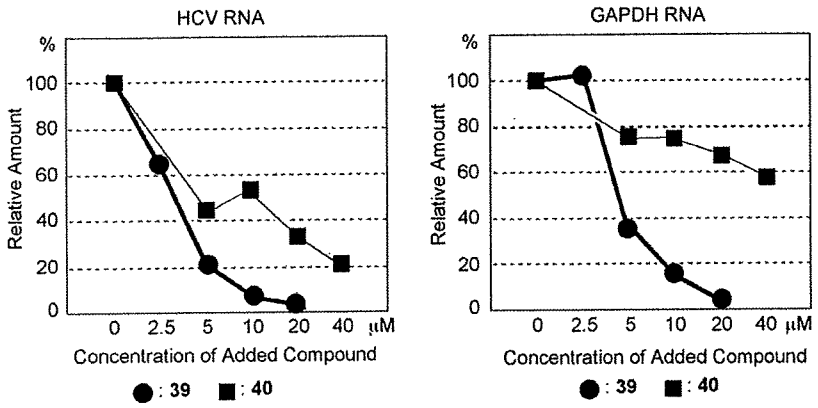


Figure 8. Dose-dependency curves of anti-HCV and cytotoxic activity elicited by compounds **39** and **40**.

potent anti-HCV activity (EC_{50}: 3.2 μM) among the compounds in this paper, its selectivity index (SI = CC_{50}/EC_{50}) was low (SI: 1.97). In terms of SI, compound **40** (EC_{50}: 11.0 μM) seems to be the best (SI: >3.64).

3. Conclusion

Based on a phenanthridinone skeleton derived by armchair structural development of thalidomide and retinoids, we developed candidate anti-HCV agents, **10** (EC_{50}: 3.7 μM,), **39** (EC_{50}: 3.2 μM,), and **40** (EC_{50}: 11 μM, CC_{50}: >40 μM). Further structural development may yield highly potent and selective drug candidates.

4. Experimental

4.1. General comments

Melting points were determined by using a Yanagimoto hot-stage melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a JEOL JNM-GX500 (500 MHz) spectrometer. Chemical shifts are expressed in δ (ppm) values with tetramethylsilane (TMS) as an internal reference. The following abbreviations are used: s = singlet, d = doublet, t = triplet, m = multiplet, dd = double doublet, dt = double triplet, br = broad. Mass spectra (MS) and high-resolution mass spectra (HRMS) were recorded on a JEOL JMS-DX303 spectrometer. Elemental analysis was performed with a Yanagimoto MT-6 elemental analyser.

4.2. 5-Ethyl-2-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)-phenanthridin-6(5*H*)-one (8)

To a solution of 5 mmol of *N*-ethylaniline and 25 mL of toluene were added hexafluoroacetone trihydrate (1.2 equiv) and *p*-TsOH (0.1 equiv). The mixture was stirred for 22 h at 120 °C and the solvent was evaporated in vacuo. The residue was purified by silica gel column chromatography (eluent; *n*-hexane/ethyl acetate) to afford 2-(4-ethylaminophenyl)-1,1,1,3,3,3-hexafluoropropan-2-ol. The obtained compound was dissolved in dichloromethane (0.1 mmol/mL), and then triethylamine (1:40 v/v) and 2-iodobenzoyl chloride (1.2 equiv) were added. The mixture was stirred for 15 h at room temperature and the solvent was evaporated in vacuo. The residue was purified by silica gel column chromatography (eluent; *n*-hexane/ethyl acetate) to afford benzanilide derivatives. To a solution of the obtained benzanilide derivative, cesium carbonate (2 equiv) and *N*,*N*-dimethylacetamide were added palladium (II) acetate (10 mol %) and tricyclohexylphosphine tetrafluoroborate (0.15 equiv), and the mixture was heated to 130 °C. The catalyst was filtered off and washed several times with ethyl acetate. The combined organic layers were washed with water and brine successively, dried over anhydrous magnesium sulfate, and concentrated. The residue was purified by silica gel column chromatography (eluent; *n*-hexane/ethyl acetate) to afford 5-ethyl-2-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)phenanthridin-6(5*H*)-one (**8**) as a white solid. Mp 208.0–209.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.68 (d, J = 1.5 Hz, 1H), 8.56 (dd, J = 7.9, 1.5 Hz, 1H), 8.31 (d, J = 7.9 Hz, 1H), 7.85 (d, J = 9.4 Hz, 1H), 7.80 (td, J = 7.9, 1.3 Hz, 1H), 7.63 (t, J = 7.7 Hz, 1H), 7.50 (d, J = 9.0 Hz, 1H), 4.48 (q, J = 7.3 Hz, 2H), 3.71 (s, 1H), 1.44 (t, J = 7.3 Hz, 3H). HRMS (FAB) calcd for $C_{18}H_{14}F_6NO_2$ 390.0929; found: 390.0918 $(M+H)^+$.

4.3. 2-(1,1,1,3,3,3-Hexafluoro-2-hydroxypropan-2-yl)-5-(2,2,2-trifluoroethyl)phenanthridin-6(5*H*)-one (9)

The title compound was prepared by a method similar to that described for the synthesis of **8**, using aniline as a starting material, with slight modifications. 2-(4-Aminophenyl)-1,1,1,3,3,3-hexafluoropropan-2-ol (prepared from aniline and 1,1,1,3,3,3-hexafluoroacetone)[29] was coupled with 2-iodobenzoyl chloride, followed by protection of the hydroxyl group with 2-methoxylethoxymethyl chloride. It was then N-alkylated by the use of 2,2,2-trifluoroethyl triflate. The 2-methoxyethoxymethyl group of the obtained benzanilide derivative was removed by treatment with titanium tetrachloride, and the deprotected benzanilide derivative was cyclized by the method used for the synthesis of **8**. White solid. Mp 76.1–77.8 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.70 (d, J = 1.1 Hz, 1H), 8.56 (dd, J = 8.1, 1.1 Hz, 1H), 8.33 (d, J = 8.1 Hz, 1H), 7.87 (dd, J = 7.7, 1.1 Hz, 1H), 7.85 (dd, J = 7.7, 1.1 Hz, 1H), 7.66 (td, J = 8.1 Hz, 1H), 7.51 (d, J = 9.0 Hz, 1H), 5.13 (s, 2H), 3.78 (s, 1H). HRMS (FAB) calcd for $C_{18}H_{10}F_9NO_2$ 444.0646; found: 444.0653 $(M+H)^+$.

4.4. 5-Butyl-2-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)-phenanthridin-6(5*H*)-one (10)

The title compound was prepared by a method similar to that described for the synthesis of **9** using 1-iodobutane instead of 2,2,2-trifluoroethyl triflate. White solid. Mp 166.0–166.6 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.67 (s, 1H), 8.55 (d, J = 7.9 Hz, 1H), 8.30 (d, J = 7.9 Hz, 1H), 7.84 (d, J = 7.9 Hz, 1H), 7.79 (dd, J = 7.9, 7.3 Hz, 1H), 7.62 (dd, J = 7.9, 7.3 Hz, 1H), 7.47 (d, J = 7.9 Hz, 1H), 4.39 (t, J = 7.9 Hz, 2H), 3.81 (s, 1H), 1.83–1.76 (m, 2H), 1.56–1.50 (m, 2H),

1.03 (t, J = 7.3 Hz, 3H). HRMS (FAB) calcd for $C_{20}H_{18}F_6NO_2$ 418.1242; found: 418.1262 $(M+H)^+$.

4.5. 2-(1,1,1,3,3,3-Hexafluoro-2-hydroxypropan-2-yl)-5-hexylphenanthridin-6(5*H*)-one (11)

The title compound was prepared by a method similar to that described for the synthesis of compound **9**, using 1-iodohexane instead of 2,2,2-trifluoroethyl triflate. White solid. Mp 132.0–132.2 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.67 (d, J = 1.8 Hz, 1H), 8.54 (d, J = 7.9, 1.8 Hz, 1H), 8.29 (d, J = 7.9 Hz, 1H), 7.84 (d, J = 8.5 Hz, 1H), 7.78 (dd, J = 8.5, 7.3 Hz, 1H), 7.62 (dd, J = 8.5, 7.3 Hz, 1H), 7.46 (d, J = 8.5 Hz, 1H), 4.37 (t, J = 7.9 Hz, 2H), 3.91 (s, 1H), 1.84–1.76 (m, 2H), 1.53–1.47 (m, 2H), 1.42–1.32 (m, 4H), 0.91 (t, J = 7.3 Hz, 3H). HRMS (FAB) calcd for $C_{22}H_{22}F_6NO_2$ 446.1555; found: 446.1516 $(M+H)^+$.

4.6. 2-(1,1,1,3,3,3-Hexafluoro-2-hydroxypropan-2-yl)-5-nonylphenanthridin-6(5*H*)-one (12)

The title compound was prepared by a method similar to that described for the synthesis of compound **9**, using 1-bromononane instead of 2,2,2-trifluoroethyl triflate. White solid. Mp 98.1–99.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.67 (d, J = 1.8 Hz, 1H), 8.54 (d, J = 7.9, 1.8 Hz, 1H), 8.29 (d, J = 7.9 Hz, 1H), 7.84 (d, J = 8.5 Hz, 1H), 7.79 (dd, J = 7.9, 7.3 Hz, 1H), 7.62 (dd, J = 7.9, 7.3 Hz, 1H), 7.46 (d, J = 8.5 Hz, 1H), 4.37 (t, J = 7.9 Hz, 2H), 3.87 (s, 1H), 1.84–1.76 (m, 2H), 1.53–1.46 (m, 2H), 1.43–1.36 (m, 2H), 1.33–1.24 (m, 8H), 0.88 (t, J = 7.3 Hz, 3H). HRMS (FAB) calcd for $C_{25}H_{28}F_6NO_2$ 488.2024; found: 488.1981 $(M+H)^+$.

4.7. 5-Benzyl-2-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)-phenanthridin-6(5*H*)-one (13)

The title compound was prepared by a method similar to that described for the synthesis of compound **9**, using benzyl bromide instead of 2,2,2-trifluoroethyl triflate. White solid. Mp 80.0–80.9 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.67 (d, J = 1.3 Hz, 1H), 8.63 (d, J = 8.1, 1.3 Hz, 1H), 8.34 (d, J = 8.1 Hz, 1H), 7.84 (td, J = 7.2, 1.3 Hz, 1H), 7.68 (q, J = 8.3 Hz, 2H), 7.38 (d, J = 9.4 Hz, 1H), 7.34–7.23 (m, 5H), 5.66 (s, 2H), 3.63 (s, 1H). HRMS (FAB) calcd for $C_{23}H_{15}F_6NO_2$ 452.1085; found: 452.1057 $(M+H)^+$. Anal. Calcd for $C_{23}H_{15}F_6NO_2 \cdot 1/3\ H_2O$: C, 60.40; H, 3.45; N, 3.06. Found: C, 60.46; H, 3.47; N, 3.08.

4.8. 5-(Cyclohexylmethyl)-2-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)phenanthridin-6(5*H*)-one (14)

The title compound was prepared by a method similar to that described for the synthesis of compound **9**, using cyclohexylmethyl bromide instead of 2,2,2-trifluoroethyl triflate. White solid. Mp 200.9–201.7 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.67 (d, J = 1.8 Hz, 1H), 8.55 (d, J = 8.5, 1.8 Hz, 1H), 8.30 (d, J = 8.5 Hz, 1H), 7.83 (d, J = 8.5 Hz, 1H), 7.79 (dd, J = 8.5, 7.3 Hz, 1H), 7.62 (dd, J = 8.5, 7.3 Hz, 1H), 7.46 (d, J = 8.5 Hz, 1H), 4.30 (br s, 2H), 3.75 (s, 1H), 1.98–1.88 (m, 1H), 1.76–1.64 (m, 5H), 1.24–1.17 (m, 5H). HRMS (FAB) calcd for $C_{23}H_{22}F_6NO_2$ 458.1555; found: 458.1569 $(M+H)^+$.

4.9. 5-Butylphenanthridin-6(5*H*)-one (15)

The title compound was prepared by a method similar to that described for the synthesis of **9**, using aniline as a starting material, with slight modifications, that is, 1-iodobutane was used in place of 2,2,2-trifluoroethyl triflate. Colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.54 (dd, 1H, J = 7.9, 1.3 Hz), 8.29 (dd, 1H, J = 8.6, 1.3 Hz), 8.27 (d, 1H, J = 8.6 Hz), 7.74 (ddd, 1H, J = 8.0, 7.4, 1.2 Hz), 7.57 (t, 1H, J = 7.4, Hz), 7.53 (ddd, 1H, J = 8.5, 7.4, 1.2 Hz), 7.40 (d, 1H, J = 8.5 Hz), 7.30 (dd, 1H, J = 8.0, 7.4 Hz), 4.38 (t, 2H, J = 7.9, Hz), 1.78 (td, 2H, J = 7.9, 7.3 Hz), 1.52 (sextet, 2H, J = 7.3 Hz), 1.00 (t, 3H, J = 7.3, Hz). HRMS (FAB) calcd for $C_{17}H_{17}NO$ 252.1388; found: 252.1349 $(M+H)^+$.

4.10. 5-Butyl-1-methylphenanthridin-6(5*H*)-one (16)

The title compound was prepared by a method similar to that described for the synthesis of **15**, using 2-iodo-3-methylaniline as a starting material. Benzoyl chloride was used instead of 2-iodobenzoyl chloride. Pale brown oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.64 (d, 1H, J = 8.0 Hz), 8.44 (d, 1H, J = 8.0 Hz), 7.73 (t, 1H, J = 8.0 Hz), 7.59 (t, 1H, J = 8.0 Hz), 7.42 (t, 1H, J = 8.0 Hz), 7.33 (d, 1H, J = 8.0 Hz), 7.17 (d, 1H, J = 8.0 Hz), 4.40 (t, 2H, J = 7.6 Hz), 2.96 (s, 3H), 184–1.77 (m, 2H), 158–1.48 (m, 2H), 1.02 (t, 3H, J = 7.6 Hz). HRMS (FAB) calcd for $C_{18}H_{19}NO$ 266.1545; found: 266.1568 $(M+H)^+$.

4.11. 5-Butyl-2-methylphenanthridin-6(5*H*)-one (17)

The title compound was prepared by a method similar to that described for the synthesis of **15**, using 4-methylaniline as a starting material. 2-Bromobenzoyl chloride was used instead of 2-iodobenzoyl chloride. Colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.55 (dd, 1H, J = 8.0, 1.2 Hz), 8.28 (d, 1H, J = 8.0 Hz), 8.10 (s, 1H), 7.74 (td, 1H, J = 8.0, 1.2 Hz), 7.57 (m, 1H), 7.37–7.30 (d, 1H, J = 7.6 Hz), 4.38 (t, 2H, J = 7.6 Hz), 2.49 (s, 3H), 1.82–1.75 (m, 3H), 1.56–1.48 (m, 2H), 1.01 (t, 3H, J = 7.3 Hz). HRMS (FAB) calcd for $C_{18}H_{19}NO$ 266.1545; found: 266.1584 $(M+H)^+$.

4.12. 5-Butyl-3-methylphenanthridin-6(5*H*)-one (18)

The title compound was prepared by a method similar to that described for the synthesis of **16**, using 2-bromo-5-methylaniline as a starting material. Pale brown oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.53 (d, 1H, J = 8.0 Hz), 8.24 (d, 1H, J = 8.0 Hz), 8.80 (d, 1H, J = 8.0 Hz), 7.73 (t, 1H, J = 8.0 Hz), 7.55 (t, 1H, J = 8.0 Hz), 7.20 (s, 1H), 7.13 (d, 1H, J = 8.0 Hz), 4.39 (t, 2H, J = 7.5 Hz), 2.52 (s, 3H), 1.83–1.76 (m, 2H), 1.57–1.49 (m, 2H), 1.03 (t, 3H, J = 7.5 Hz). HRMS (FAB) calcd for $C_{18}H_{19}NO$ 266.1545; found: 266.1512 $(M+H)^+$.

4.13. 5-Butyl-4-methylphenanthridin-6(5*H*)-one (19)

The title compound was prepared by a method similar to that described for the synthesis of **17**, using 2-methylaniline as a starting material. Colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.50 (dd, 1H, J = 8.0, 1.2 Hz), 8.22 (d, 1H, J = 8.0 Hz), 8.13 (d, 1H, J = 8.0 Hz), 7.20 (td, 1H, J = 7.6, 1.4 Hz), 7.55 (t, 1H, J = 8.0 Hz), 7.30 (d, 1H, J = 7.6 Hz), 7.21 (t, 1H, J = 7.6 Hz), 4.49 (t, 2H, J = 7.6 Hz), 2.66 (s, 3H), 1.66–1.60 (m, 2H), 1.20–1.19 (m, 2H), 0.86 (t, 3H, J = 7.3 Hz). HRMS (FAB) calcd for $C_{18}H_{19}NO$ 266.1545; found: 266.1557 $(M+H)^+$.

4.14. 5-Butyl-7-methylphenanthridin-6(5*H*)-one (20)

The title compound was prepared by a method similar to that described for the synthesis of **16**, using 2-bromoaniline as a starting material. 2-Methylbenzoyl chloride was used instead of benzoyl chloride. White amorphous solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.27 (d, 1H, J = 7.5 Hz), 8.17 (d, 1H, J = 7.5 Hz), 7.58 (t, 1H, J = 7.5 Hz), 7.51 (t, 1H, J = 7.5 Hz), 7.35 (t, 1H, J = 7.5 Hz), 7.26 (t, 1H, J = 7.5 Hz), 4.31 (t, 2H, J = 8.0 Hz), 2.98 (s, 3H), 1.81–1.74 (m, 2H), 1.58–1.49 (m, 2H), 1.02 (t, 3H, J = 7.3 Hz). HRMS (FAB) calcd for $C_{18}H_{19}NO$ 266.1545; found: 266.1573 $(M+H)^+$.

4.15. 5-Butyl-8-methylphenanthridin-6(5*H*)-one (21)

The title compound was prepared by a method similar to that described for the synthesis of **20**. 3-Methylbenzoyl chloride was used instead of 2-methylbenzoyl chloride. White amorphous solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.36 (s, 1H), 8.28 (d, 1H, J = 8.0 Hz), 8.18 (d, 1H, J = 8.0 Hz), 7.58 (dd, 1H, J = 8.0, 1.8 Hz), 7.52 (t, 1H, J = 8.0 Hz), 7.41 (d, 1H, J = 8.0 Hz), 7.30 (t, 1H, J = 8.0 Hz), 4.40 (t, 2H, J = 8.0 Hz), 2.52 (s, 3H), 1.83–1.76 (m, 2H), 1.56–1.50 (m, 2H), 1.02 (t, 3H, J = 7.3 Hz). HRMS (FAB) calcd for $C_{18}H_{19}NO$ 266.1545; found: 266.1588 $(M+H)^+$.

4.16. 5-Butyl-9-methylphenanthridin-6(5*H*)-one (22)

The title compound was prepared by a method similar to that described for the synthesis of **20**. 4-Methylbenzoyl chloride was used instead of 2-methylbenzoyl chloride. White solid. Mp 70.0–73.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.44 (d, 1H, J = 7.5 Hz), 8.30 (d, 1H, J = 7.5 Hz), 8.07 (s, 1H7.5), 7.53 (td, 1H, J = 7.5 Hz), 7.40 (d, 2H, J = 7.5 Hz), 7.30 (dd, 1H, J = 7.5 Hz), 4.39 (t, 2H, J = 8.0 Hz), 2.57 (s, 3H), 1.81.75 (m, 2H), 1.51.49 (m, 2H), 1.02 (t, 3H, J = 7.3 Hz). HRMS (FAB) calcd for $C_{18}H_{19}NO$ 266.1545; found: 266.1527 $(M+H)^+$.

4.17. 5-Butyl-10-methylphenanthridin-6(5*H*)-one (23)

The title compound was prepared by a method similar to that described for the synthesis of **20**. Colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.53 (d, 1H, J = 8.0 Hz), 8.70 (dd, 1H, J = 8.0, 1.2 Hz), 7.61 (d, 1H, J = 8.0 Hz), 7.56–7.52 (m, 1H), 7.49 (d, 1H, J = 8.0 Hz), 7.46 (dd, 1H, J = 8.0, 1.2 Hz), 7.32–7.27 (m, 1H), 4.40 (t, 2H, J = 7.3 Hz), 2.97 (s, 3H), 1.85–1.78 (m, 2H), 1.50–1.50 (m, 2H), 1.02 (t, 3H, J = 7.3 Hz). HRMS (FAB) calcd for $C_{18}H_{19}NO$ 266.1545; found: 266.1553 $(M+H)^+$.

4.18. 5-Butyl-2-fluorophenanthridin-6(5*H*)-one (24)

The title compound was prepared by a method similar to that described for the synthesis of **17**, using 4-fluoroaniline as a starting material. White solid. FAB-MS m/z 270 $(M+H)^+$. Mp 114.0–117.5 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.56 (dd, 1H, J = 8.0, 1.5 Hz), 8.17 (d, 1H, J = 8.0 Hz), 7.90 (dd, 1H, J = 9.7, 3.0 Hz), 7.77 (t, 1H, J = 8.0 Hz), 7.63 (t, 1H, J = 8.0 Hz), 7.37 (dd, 1H, J = 9.0, 4.5 Hz), 7.29–7.24 (m, 1H), 4.38 (t, 2H, J = 8.0 Hz), 1.82–1.75 (m, 2H), 1.57–1.48 (m, 2H), 1.02 (t, 3H, J = 7.3 Hz). Anal. Calcd for $C_{17}H_{16}NFO$: C, 75.82; H, 5.99; N, 5.20. Found: C, 76.22; H, 6.24; N, 5.24.

4.19. 5-Butyl-2-trifluoromethylphenanthridin-6(5*H*)-one (26)

The title compound was prepared by a method similar to that described for the synthesis of **17**, using 4-trifluoromethylaniline as a starting material. White solid. FAB-MS m/z 320 $(M+H)^+$. Mp 111.0–112.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.56 (d, 1H, J = 8.0 Hz), 8.53 (s, 1H), 8.30 (d, 1H, J = 8.0 Hz), 7.82 (t, 1H, J = 8.0 Hz), 7.77 (dd, 1H, J = 8.0, 1.8 Hz), 7.65 (t, 1H, J = 8.0 Hz), 7.00 (d, 1H, J = 8.0 Hz), 4.41 (t, 2H, J = 7.9 Hz), 1.83–1.75 (m, 2H), 1.58–1.49 (m, 2H), 1.03 (t, 3H, J = 7.3 Hz). Anal. Calcd for $C_{18}H_{16}NF_3O$: C, 67.70; H, 5.05; N, 4.39. Found: C, 67.91; H, 5.33; N, 4.38.

4.20. 5-Butyl-2-ethylphenanthridin-6(5*H*)-one (27)

The title compound was prepared by a method similar to described that for the synthesis of **17**, using 4-ethylaniline as a starting material. Pale brown oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.55 (dd, 1H, J = 8.0, 1.5 Hz), 8.30 (d, 1H, J = 8.0 Hz), 8.12 (d, 1H, J = 1.5 Hz), 7.75 (td, 1H, J = 8.0, 1.5 Hz), 7.58 (t, 1H, J = 8.0 Hz), 7.39 (dd, 1H, J = 8.0, 1.5 Hz), 7.34 (d, 1H, J = 8.0 Hz), 4.39 (t, 2H, J = 8.0 Hz), 2.79 (q, 2H, J = 8.0 Hz), 1.83–1.76 (m, 2H), 1.57–1.49 (m, 2H), 1.34 (t, 3H, J = 7.6 Hz), 1.02 (t, 3H, J = 7.3 Hz). HRMS (FAB) calcd for $C_{19}H_{21}NO$ 280.1701; found: 280.1696 $(M+H)^+$.

4.21. 5-Butyl-2-isopropylphenanthridin-6(5*H*)-one (28)

The title compound was prepared by a method similar to that described for the synthesis of **15**, using 4-isopropylaniline as a starting material. Colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.54 (dd, 1H, J = 8.0, 1.2 Hz), 8.30 (d, 1H, J = 8.0 Hz), 8.12 (d, 1H, J = 1.9 Hz), 7.74 (ddd, 1H, J = 7.9, 7.4, 1.2 Hz), 7.56 (dd, 1H, J = 8.0, 7.4 Hz), 7.41 (dd, 1H, J = 8.6, 1.9 Hz), 7.34 (d, 1H, J = 8.6 Hz), 4.37 (t, 2H, J = 8.0 Hz), 3.05 (septet, 1H, J = 7.4 Hz), 1.77 (quintet, 2H, J = 8.0 Hz), 1.51 (sextet, 2H, J = 7.4 Hz), 1.34 (d, 6H, J = 7.4 Hz), 1.00 (t, 3H, J = 7.4 Hz). HRMS (FAB) calcd for $C_{20}H_{23}NO$ 294.1858; found: 294.1851 $(M+H)^+$.

4.22. 5-Butyl-2-*tert*-butylphenanthridin-6(5*H*)-one (31)

The title compound was prepared by a method similar to that described for the synthesis of **17** using 4-*tert*-burylaniline as a starting material. Pale brown oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.56 (dd, 1H, J = 8.0, 1.5 Hz), 8.32 (d, 1H, J = 8.0 Hz), 8.31 (d, 1H, J = 1.5 Hz), 7.78–7.74 (m, 1H), 7.60 (dd, 1H, J = 8.0, 1.5 Hz), 7.57 (d, 1H, J = 8.0 Hz), 7.37 (d, 1H, J = 8.0 Hz), 4.39 (t, 2H, J = 7.6 Hz), 1.83–1.76 (m, 2H), 1.56–1.50 (m, 2H), 1.44 (s, 9H), 1.02 (t, 3H, J = 7.3 Hz). HRMS (FAB) calcd for $C_{21}H_{25}NO$ 308.2014; found: 308.2008 $(M+H)^+$.

4.23. 5-Butyl-2-hydroxyphenanthridin-6(5*H*)-one (25)

The title compound was prepared by a method similar to that described for the synthesis of **8**, using 4-*tert*-butyldimethylsilyloxyaniline (prepared from *p*-nitrophenol) as a starting material, with slight modifications. 4-*tert*-Butyldimethylsilyloxyaniline was acylated with butyryl chloride in the presence of triethylamine in dichloromethane, and then hydrogenated with lithium aluminum hydride in tetrahydrofuran. Obtained *N*-butylaniline was coupled with 2-iodobenzoyl chloride, and then cyclized in the presence of palladium (II) acetate, tricyclohexylphosphine tetrafluoroborate and potassium carbonate in *N*,*N*-dimethylacetamide. White solid. Mp 187.0–192.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.55 (d, 1H, J = 7.9 Hz), 8.10 (d, 1H, J = 7.9 Hz), 7.78 (d, 1H, J = 2.4 Hz), 7.67 (t, 1H, J = 7.3 Hz), 7.55 (t, 1H, J = 7.3 Hz), 7.28 (d, 1H, J = 9.2 Hz), 7.14 (dd, 1H, J = 9.2, 2.4 Hz), 6.68 (br s, 1H), 4.37 (t, 2H, J = 7.3 Hz), 1.77 (quintet, 2H, J = 7.3 Hz), 1.49 (sextet, 2H, J = 7.3 Hz), 0.97 (t, 3H, J = 7.3 Hz). HRMS (FAB) calcd for $C_{17}H_{17}NO_2$ 268.1338; found: 268.1344 $(M+H)^+$.

4.24. 5-Butyl-2-hydroxymethylphenanthridin-6(5*H*)-one (29)

White solid. FAB-MS m/z 282 $(M+H)^+$. Mp 150.0–153.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 18.54 (d, 1H, J = 8.0 Hz), 8.28–8.26 (m, 2H), 7.75 (t, 1H, J = 8.0 Hz), 7.59 (t, 1H, J = 8.0, 1.8 Hz), 7.52 (dd, 1H, J = 8.0, 1.8 Hz), 7.36 (d, 1H, J = 8.0 Hz), 4.83 (d, 2H, J = 6.0 Hz), 4.37 (t, 2H, J = 7.5 Hz), 1.99–1.94 (m, 1H), 1.81–1.74 (m, 2H), 1.55–1.48 (m, 2H), 1.01 (t, 3H, J = 7.5 Hz). Anal. Calcd for $C_{18}H_{19}NO_2$: C, 76.84; H, 6.81; N, 4.98. Found: C, 76.94; H, 6.71; N, 5.01.

4.25. 5-Butyl-2-(1′-hydroxyethyl)phenanthridin-6(5*H*)-one (30)

The title compound was prepared by a method similar to that described for the synthesis of **25**, using 1-(4-aminophenyl)ethanol as a starting material. Pale yellow solid. Mp 95.0–99.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.51 (dd, 1H, J = 7.9, 1.2 Hz), 8.23 (d, 1H, J = 8.5 Hz), 8.22 (d, 1H, J = 1.8 Hz), 7.72 (td, 1H, J = 7.3, 1.2 Hz),

7.56 (dd, 1H, J = 7.9, 7.3 Hz), 7.49 (dd, 1H, J = 8.5, 1.8 Hz), 7.29 (d, 1H, J = 8.5 Hz), 5.03 (q, 1H, J = 6.7 Hz), 4.33 (t, 2H, J = 7.9 Hz), 1.74 (quintet, 2H, J = 7.9 Hz), 1.57 (d, 3H, J = 6.7 Hz), 1.49 (sextet, 2H, J = 7.3 Hz), 0.99 (t, 3H, J = 7.3 Hz). HRMS (FAB) calcd for $C_{19}H_{21}NO_2$ 296.1651; found: 296.1661 $(M+H)^+$.

4.26. 5-Butyl-2-acetylphenanthridin-6(5*H*)-one (32)

The title compound was prepared by the same method as described for the synthesis of **30**. The title compound was generated by partial oxidization of the alcoholic hydroxy group at the final cyclization step. White solid. Mp 114.0–118.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.92 (d, 1H, J = 1.8 Hz), 8.53 (d, 1H, J = 7.3 Hz), 8.37 (d, 1H, J = 7.9 Hz), 8.10 (dd, 1H, J = 9.2, 1.8 Hz), 7.79 (t, 1H, J = 7.3 Hz), 7.61 (dd, 1H, J = 7.9, 7.3 Hz), 7.44 (d, 1H, J = 9.2 Hz), 4.39 (t, 2H, J = 7.9 Hz), 2.69 (s, 3H), 1.77 (quintet, 2H, J = 7.9 Hz), 1.52 (sextet, 2H, J = 7.3 Hz), 1.01 (t, 3H, J = 7.3 Hz). HRMS (FAB) calcd for $C_{19}H_{19}NO_2$ 294.1494; found: 294.1501 $(M+H)^+$.

4.27. 5-Butyl-2-phenethylphenanthridin-6(5*H*)-one (33)

Compound **29** was oxidized with manganese oxide in dichloromethane to give an aldehyde derivative, which was coupled by Wittig reaction to give a styryl derivative. This product was hydrogenated in the presence of palladium–carbon under a hydrogen atmosphere to give the title compound. Colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.55 (dd, 1H, J = 8.0, 1.2 Hz), 8.22 (d, 1H, J = 8.0 Hz), 8.05 (s, 1H), 7.74 (ddd, 1H, J = 8.0, 6.5, 1.2 Hz), 7.58 (t, 1H, J = 8.0 Hz), 7.37–7.29 (m, 4H), 7.24–7.21 (m, 3H), 4.38 (t, 2H, J = 7.9 Hz), 3.09–2.99 (m, 4H), 1.82–1.76 (m, 2H), 1.55–1.49 (m, 2H), 1.02 (t, 3H, J = 7.3 Hz). HRMS (FAB) calcd for $C_{25}H_{26}NO$ 356.2014; found: 356.1995 $(M+H)^+$.

4.28. 6-Butyl-8,9,10,11-tetrahydro-8,8,11,11-tetramethylbenzo[2,3-*b*]phenanthridin-5(6*H*)-one (35)

The title compound was prepared by a method similar to that described for the synthesis of **17**, using 2-amino-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene as a starting material. Colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.52 (d, 1H, J = 8.0 Hz), 8.26 (d, 1H, J = 8.0 Hz), 8.21 (s, 1H), 7.73 (t, 1H, J = 8.0 Hz), 7.54 (t, 1H, J = 8.0 Hz), 7.31 (s, 1H), 4.39 (t, 2H, J = 7.3 Hz), 1.84–1.75 (m, 6H), 1.59–1.50 (m, 2H), 1.40 (s, 6H), 1.38 (s, 6H), 1.04 (t, 3H, J = 7.3 Hz). HRMS (FAB) calcd for $C_{25}H_{31}NO$ 362.2484; found: 362.2473 $(M+H)^+$.

4.29. 6-Benzyl-8,9,10,11-tetrahydro-8,8,11,11-tetramethylbenzo[2,3-*b*]phenanthridin-5(6*H*)-one (36)

The title compound was prepared by a method similar to that described for the synthesis of **35**. N-Alkylation was performed by using benzyl bromide. White amorphous solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.60 (dd, 2H, J = 8.0, 1.5 Hz), 8.29 (d, 1H, J = 8.0 Hz), 8.18 (s, 1H), 7.79–7.75 (m, 1H), 7.58 (t, 1H, J = 8.0 Hz), 7.37–7.28 (m, 4H), 7.23 (s, 1H), 7.22 (t, 1H, J = 8.0 Hz), 5.65 (s, 2H), 1.69 (d, 4H, J = 1.2 Hz), 1.36 (s, 6H), 1.14 (s, 6H). HRMS (FAB) calcd for $C_{28}H_{29}NO$ 396.2327; found: 396.2296 $(M+H)^+$.

4.30. 6-Cyclohexylmethyl-8,9,10,11-tetrahydro-8,8,11,11-tetramethylbenzo[2,3-*b*]phenanthridin-5(6*H*)-one (37)

The title compound was prepared by a method similar to that described for the synthesis of **35**. N-Alkylation was performed by using cyclohexylmethyl bromide. White solid. Mp 148.0–153.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.52 (dd, 2H, J = 8.0, 1.2 Hz), 8.27 (d, 1H, J = 8.0 Hz), 8.21 (s, 1H), 7.73 (td, 1H, J = 8.0, 1.2 Hz), 7.54 (td, 1H, J = 8.0, 1.2 Hz), 7.29 (s, 1H), 4.30 (br s, 2H), 1.94–1.89 (m, 1H), 1.78 (s, 4H), 1.76 (t, 4H, J = 13.00 Hz), 1.41 (s, 6H), 1.38 (s, 6H), 1.29–1.15 (m, 6H). HRMS (FAB) calcd for $C_{28}H_{35}NO$ 402.2797; found: 402.2791 $(M+H)^+$.

4.31. 5-Butyl-8,9,10,11-tetrahydro-8,8,11,11-tetramethylbenzo[2,3-*j*]phenanthridin-6(5*H*)-one (38)

The title compound was prepared by a method similar to that described for the synthesis of **20**. 5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthoyl chloride (prepared from 5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-carboxylic acid)[4] was used instead of 2-methylbenzoyl chloride. White solid. FAB-MS m/z 362 $(M+H)^+$. Mp 103.0–109.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.51 (s, 1H), 8.29 (d, 1H, J = 7.9 Hz), 8.21 (s, 1H), 7.50 (t, 1H, J = 7.9 Hz), 7.38 (d, 1H, J = 7.9 Hz), 7.29 (t, 1H, J = 7.3 Hz), 4.38 (t, 2H, J = 7.6 Hz), 1.81–1.74 (m, 6H), 1.56–1.50 (m, 2H), 1.42 (s, 6H), 1.40 (s, 6H), 1.02 (t, 3H, J = 7.3 Hz). Anal. Calcd for $C_{25}H_{31}NO{\cdot}1/7\ H_2O$: C, 82.47; H, 8.66; N, 3.85. Found: C, 82.64; H, 8.76; N, 3.71.

4.32. 8,9,10,11-Tetrahydro-8,8,11,11-tetramethylbenzo[2,3-*b*]-phenanthridin-5(6*H*)-one (34)

The title compound was prepared by a method similar to that described for the synthesis of **35**, using 4-methoxybenzyl bromide instead of 1-iodobutane, followed by deprotection of the 4-methoxybenzyl group with trifluoroacetic acid. White solid. Mp 280 °C (decomp). ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.27 (s, 1H), 8.51 (dd, 1H, J = 7.8, 1.3 Hz), 8.28 (t, 1H, J = 7.8 Hz), 8.14 (s, 1H), 7.78 (td, 1H, J = 7.8, 1.3 Hz), 7.57 (t, 1H, J = 7.8 Hz), 7.11 (s, 1H), 1.76 (s, 4H), 1.39 (s, 6H), 1.38 (s, 6H). HRMS (FAB) calcd for $C_{21}H_{23}NO$ 306.1858; found: 306.1817 $(M+H)^+$.

4.33. 6-Butylbenzo[*b*]phenanthridin-5(6*H*)-one (39)

The title compound was prepared by a method similar to that described for the synthesis of **8**, using 2-butylaminonaphthalene as a starting material. 2-Butylaminonaphthalene was prepared from 2-bromonaphthalene by coupling reaction with *n*-butylamine in the presence of tris(dibenzylidenacetone)dipalladium, sodium *tert*-butoxide and n-butylamine. 2-Bromobenzoyl chloride was used instead of 2-iodobenzoyl chloride. White solid. Mp 101.0–104.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.90 (s, 1H), 8.57 (dd, 1H, J = 8.0, 1.8 Hz), 8.46 (d, 1H, J = 8.0 Hz), 7.98 (d, 1H, J = 8.0 Hz), 7.92 (d, 1H, J = 8.0 Hz), 7.82–7.78 (m, 1H), 7.73 (s, 1H), 7.62 (t, 1H, J = 7.3 Hz), 7.55 (t, 1H, J = 7.3 Hz), 7.48 (t, 1H, J = 7.3 Hz), 4.50 (t, 3H, J = 7.9 Hz), 1.92–1.85 (m, 2H), 1.63–1.52 (m, 2H), 1.06 (t, 3H, J = 7.3 Hz). HRMS (FAB) calcd for $C_{21}H_{19}NO$ 302.1545; found: 302.1555 $(M+H)^+$.

4.34. 5-Butylbenzo[*j*]phenanthridin-6(5*H*)-one (40)

The title compound was prepared by a method similar to that described for the synthesis of **20**. 2-Naphthoyl chloride was used instead of 2-methylbenzoyl chloride. White solid. Mp 111.0–115.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.14 (s, 1H), 8.74 (s, 1H), 8.48 (dd, 1H, J = 8.0, 1.5 Hz), 8.09 (d, 1H, J = 8.0 Hz), 8.04 (d, 1H, J = 8.0 Hz), 7.63 (t, 1H, J = 8.0 Hz), 7.57 (d, 1H, J = 8.0 Hz), 7.54 (dd, 1H, J = 8.0, 1.5 Hz), 7.42 (d, 1H, J = 8.0 Hz), 7.35 (t, 1H, J = 8.0 Hz), 4.43 (t, 2H, J = 7.6 Hz), 1.87–1.80 (m, 2H), 1.58–1.52 (m, 2H). HRMS (FAB) calcd for $C_{21}H_{19}NO$ 302.1545; found: 302.1591 $(M+H)^+$.

4.35. 5-Butylbenzo[*c*]phenanthridin-6(5*H*)-one (41)

The title compound was prepared by a method similar to that described for the synthesis of **17**, using 1-aminonaphthlene as a

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 2009 20:47-54 (doi: 10.3851/IMP1372)

Original article

Anti-bovine viral diarrhoea virus and hepatitis C virus activity of the cyclooxygenase inhibitor SC-560

*Mika Okamoto[1], Masashi Sakai[1], Yukinori Goto[1], Mohammed TA Salim[1], Chiaki Baba[1], Kaku Goto[2], Koichi Watashi[2], Kunitada Shimotohno[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]Laboratory of Human Tumor Viruses, Department of Viral Oncology, Institute for Virus Research, Kyoto University, Kyoto, Japan

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

Background: A number of compounds were examined for their inhibitory effect on bovine viral diarrhoea virus (BVDV) replication in cell cultures and found that some cyclooxygenase (COX) inhibitors had antiviral activity against the virus.
Methods: Determination of compounds for their anti-BVDV activity was on the basis of the inhibition of virus-induced cytopathogenicity in Mardin–Darby bovine kidney (MDBK) cells. Anti-hepatitis C virus (HCV) activity was assessed by the inhibition of viral RNA synthesis in the subgenomic HCV RNA replicon cells.
Results: Among the test compounds, 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1*H*-pyrazole (SC-560) was the most active against BVDV, and its 50% effective and cytotoxic concentrations were 10.9 ±2.8 and 93.9 ±24.5 μM in virus and mock-infected MDBK cells, respectively. The compound also suppressed BVDV RNA synthesis in a dose-dependent fashion. Studies on the mechanism of action revealed that SC-560 did not interfere with viral entry to the host cells. Furthermore, it was assumed that the antiviral activity of SC-560 was not associated with its inhibitory effect on COX. The combination of SC-560 and interferon-α was additive to synergistic in inhibiting BVDV replication. More importantly, the compound proved to be a selective inhibitor of HCV replication.
Conclusions: SC-560 and its derivative might have potential as novel antiviral agents against HCV.

Introduction

Hepatitis C virus (HCV), a member of the *hepacivirus* genus from the family *Flaviviridae*, is a major aetiological agent of human liver diseases. The World Health Organization estimates that at least 170 million people in the world are chronically infected with HCV. Although HCV infection is often asymptomatic, it frequently causes chronic hepatitis, which progresses to end-stage liver diseases, such as liver cirrhosis and hepatocellular carcinoma [1]. No vaccine is currently available, and treatment with the nucleoside analogue ribavirin and pegylated interferon (PEG-IFN)-α has efficacy in a subset of patients. However, there is genetic heterogeneity in the HCV genome [2] and HCV genotype 1b cannot be eliminated from approximately half of the patients, even when treated with PEG-IFN-α and ribavirin [3]. In addition, side effects of these agents are sometimes serious and cannot be tolerated in some patients; therefore, alternative agents are highly desired for the treatment of HCV infection.

Because a lot of effort has been made to identify novel anti-HCV agents, several compounds have been developed and are currently under clinical trials [4]; however, progress is not rapid enough because of the inability of HCV replication in cell culture systems. The recent development of the subgenomic HCV RNA replicon system greatly accelerated the speed of anti-HCV drug discovery [5]; nevertheless, the system does not faithfully reproduce all steps in the HCV replication cycle. In addition, relatively high costs are required to use this technology for the screening and development of antiviral agents. Although a cell culture system of productive HCV infection with the replication-competent strain JFH-1 has been established in 2005, which makes it possible to identify inhibitors of every step in the virus replication cycle [6–8], this strain was isolated from a patient of fulminant hepatitis C and classified as genotype 2a with several mutations. Consequently, surrogate viruses are still widely used for the investigation of anti-HCV agents.

Bovine viral diarrhoea virus (BVDV) is considered as a surrogate model of HCV because HCV and BVDV share a significant degree of homology and common replication machinery [9]. BVDV, a member of the *pestivirus* genus of the family *Flaviviridae*, causes mucosal diseases in cattle. BVDV is easy to grow in cell culture. The genome consists of a positive-strand RNA approximately 12.5 kb in length that encodes a single open reading frame flanked by 5′ and 3′ untranslated regions. Similarly to the HCV genome, the 5′ terminus of the BVDV genome is not capped; instead, the initiation of translation is mediated by an internal ribosomal entry site [10]. Based on the effect on host cells, two biotypes of BVDV (cytopathogenic and noncytopathogenic) have been recognized [11]. Common strains of BVDV, such as NADL, are cytopathogenic in cell culture, allowing the evaluation of compounds for their antiviral activity. Thus, BVDV has been most widely utilized *in vitro* as an HCV surrogate model for identification and characterization of antiviral agents against HCV [9].

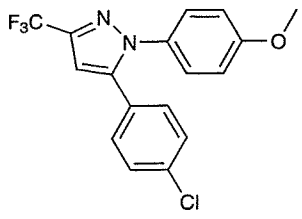
We previously reported a simple and sensitive colorimetric assay of compounds for evaluation of their anti-BVDV activity *in vitro* [12]. Using this assay system, we have now found that some cyclooxygenase (COX) inhibitors are selective inhibitors of BVDV replication in cell culture. Among the compounds, 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1*H*-pyrazole SC-560; Figure 1) was found to be the most potent inhibitor. Studies on its mechanism of action revealed that the compound did not interfere with viral entry to the host cells, but did suppress the viral RNA synthesis. Furthermore, HCV replication was also selectively inhibited by SC-560.

Methods

Compounds

A total of 16 COX inhibitors, that is, aspirin, CAY10404, diclofenac, etodolac, ibuprofen, indomethacin, mefenamic acid, nabumetone, nimesulide, NS-398, phenylbutazone, piroxicam, resveratrol, SC-560, SC-58125 and vareoyl salicylate, and two reference compounds, cyclosporine A and ribavrin, were used for antiviral assays. Among the compounds, aspirin, CAY10404, NS-398, resveratrol, SC-560, SC-58125 and valeroyl salicylate were obtained from Cayman Chemical (Ann Arbor, MI, USA), and the other COX inhibitors and cyclosporine A were purchased from Sigma (Saint Louis, MO, USA). Ribavirin was synthesized by Asahi Kasei Pharma (Tokyo, Japan). All compounds were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 20 mM to avoid any antiviral and cytotoxic effects of DMSO. The stock solution was stored at -20°C until use. Human IFN-α2b was purchased from PBL Biochemical Laboratories (New Brunswick, NJ, USA) and stored at -80°C.

Figure 1. Structure of SC-560



Cells and virus

Madin–Darby bovine kidney (MDBK) cells were purchased from Japan Health Sciences Foundation (Health Science Research Resources Bank, Osaka, Japan). MDBK cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) with high glucose (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. The cytopathogenic BVDV strain Nose was obtained from Kyoto Biken (Kyoto, Japan). BVDV was harvested from culture supernatants of virus-infected cells after a 3-day incubation. Virus stocks were stored at -80°C until use. The infectivity of the stocks was determined in MDBK cells and expressed as the 50% tissue culture infectious dose. The subgenomic HCV RNA replicon cells MH-14 were as described previously [13,14]. The replicon cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Gibco/BRL), 500 μg/ml G418 (Sigma) and the antibiotics listed earlier.

Anti-BVDV assays

Determination of compounds for their anti-BVDV activity was on the basis of the inhibition of virus-induced cytopathogenicity in MDBK cells, as described previously [12]. Briefly, the cells (2×10^5 cells/ml) were infected with BVDV at a multiplicity of infection (MOI) of 0.01 and 100 μl of the cell suspension was added into each well of a microtitre plate. The cells were incubated in the presence of various concentrations of test compounds (each compound alone or in combination with IFN-α) for 3 days at 37°C. After incubation, culture supernatants were collected to determine their lactate dehydrogenase (LDH) levels by an LDH detection kit according to the manufacturer's instructions (Takara Biochemicals, Otsu, Japan). The cytotoxicity of the test compounds was evaluated in parallel with their antiviral activity. The

mock-infected MDBK cells (2×10^4 cells/well) were incubated in the presence of various concentrations of test compounds for 3 days. The cell viability was determined by a dye method using the water-soluble tetrazolium Tetracolor One® (Seikagaku Corporation, Tokyo, Japan).

The anti-BVDV activity of SC-560 was also determined by the inhibition of viral RNA synthesis in MDBK cells by real-time reverse transcription (RT)-PCR. The cells were infected with BVDV at a MOI of 1.0 and cultured in the presence of various concentrations of the test compound. After a 6 h incubation, the cells were extensively washed with phosphate-buffered saline (PBS), trypsinized and then washed again with PBS. Total RNA was extracted from the cells with a RNeasy Mini Kit® (Qiagen, Valencia, CA, 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′-GGCTGTATTCGTAACAGT TGGTTAAA-3′ and the fluorescence probe 5′-ACGA GGGCACGCCCAAAGCA-3′ (Applied Biosystems, Branchburg, NJ, USA). The primer pair amplifies the 5′ unsatulated region of BVDV RNA. The TaqMan® PCR reagent kit and TaqMan® Multiscribe™ RT reagent kit (Applied Biosystems) were used according to the manufacturer's instructions. Non-specific inhibition of host cellular messenger RNA synthesis by SC-560 was determined by amplification of a part of the bovine β-actin RNA using the sense primer 5′-GCCCTGAGGCTCTCTTCCA-3′, the antisense primer 5′-GCGGATGTCGACGTCACA-3′ and the fluorescence probe 5′-CATGGAATCCTGCG GCATTCACG-3′ (Applied Biosystems).

Anti-HCV assays

The anti-HCV activity of SC-560 was determined by the inhibition of viral RNA synthesis in subgenomic HCV RNA replicon cells by real-time RT-PCR as described previously [13,14]. Briefly, MH-14 cells (7×10^3 cells/12-well plate) were seeded and cultured in the presence of various concentrations of the test compound. Every 3 days, the culture medium was replaced by fresh culture medium containing an appropriate concentration of the compound. On day 7, total RNA was extracted from the cells and subjected to real-time RT-PCR. The 5′ untranslated region of HCV RNA was quantified using the sense primer 5′-CGGGAGAGCCATAGTGG-3′, the antisense primer 5′-AGTACCACAAGGCC TTTCG-3′ and the fluorescence probe 5′-CTGCG GAACCGGTGAGTACAC-3′ (Applied Biosystems). As an internal control, ribosomal RNA was also quantified using TaqMan® ribosomal RNA control reagents (Applied Biosystems).

Synergy calculation and statistical analyses

The multidrug effect was evaluated by the median-effect principle and the isobologram method as previously described [15,16]. The combination index (CI) was obtained by this method. CIs of <1, 1 and >1 indicate synergism, additive effect and antagonism, respectively. The statistical significance was determined by the Student's *t*-test. *P*-values <0.05 were considered to be significant.

Results

When 16 COX inhibitors were examined for their inhibitory effect on BVDV replication in MDBK cells, 8 compounds (diclofenac, indomethacin, mefenamic acid, nabumetone, NS-398, resveratrol, SC-560 and SC-58125) displayed selective inhibition of virus-induced cytopathogenicity (Table 1). With the exception of CAY10404, resveratrol and SC-560, these compounds did not reduce the viability of mock-infected MDBK cells at concentrations up to 100 μM. Among the active compounds, SC-560 (Figure 1), proved to be the most potent inhibitor of the virus. Its mean ±SD 50% effective concentration (EC_{50}) and 50% cytotoxic concentration (CC_{50}) were 10.9 ±2.8 μM and 93.9 ±24.5 μM, respectively, indicating that SC-560 is a selective inhibitor of BVDV. Cyclosporine A and ribavirin, both of which are known to inhibit HCV replication in cell cultures [13,14,17,18], also inhibited BVDV replication in this study. The mean ±SD EC_{50} values of cyclosporine A and ribavirin were 2.8 ±0.2 μM and 3.9 ±0.4 μM, respectively (Table 1); however, these compounds were more cytotoxic to the host cells than SC-560.

SC-560 was found to almost completely inhibit BVDV replication at a concentration of 40 μM, and it did not show apparent cytotoxicity at this concentration (Figure 2A). The selective inhibition of BVDV replication by SC-560 was confirmed by dose-dependent inhibition of viral RNA synthesis in the infected cells, as determined by real-time RT-PCR (Figure 2B). In this experiment, almost complete inhibition of viral RNA synthesis was achieved by the compound without affecting the steady state level of β-actin RNA in MDBK cells at a concentration of 50 μM.

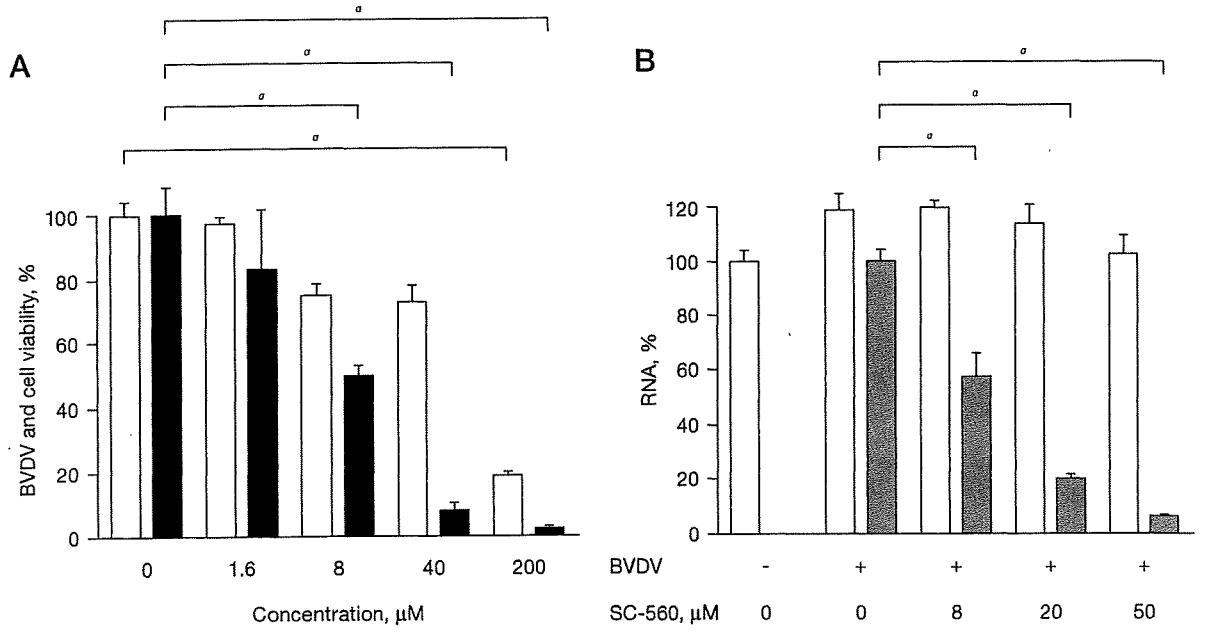
To gain insight into the mechanism of BVDV inhibition by SC-560, an experiment of delayed compound addition was conducted. When SC-560 (50 μM) was added to the cells simultaneously with the virus, almost complete inhibition of viral RNA synthesis was achieved (Figure 3A and 2B). A similar degree of the inhibition was observed, even when the compound was added at 2 h after virus infection (Figure 3B), indicating that SC-560 does not exert its anti-BVDV activity through the inhibition of viral entry to the host cells. In addition, like ribavirin,

Table 1. Anti-BVDV activity of COX inhibitors in MDBK cells

Compound	EC_{50}, μM	CC_{50}, μM	IC_{50}, μM		Reference
			COX1	COX2	
Aspirin	>100	>100	1.2	15.8	[27]
CAY10404	46.3 ±8.0	49.1 ±15.0	>500	<0.001	[20]
Diclofenac	50.0 ±3.9	>100	0.076	0.026	[28]
Etodolac	>100	>100	>100	53	[28]
Ibuprofen	>100	>100	0.34	3.8	[27]
Indomethacin	37.8 ±18.0	>100	0.05	0.15	[27]
Mefenamic acid	62.6 ±7.4	>100	0.01	2.1	[29]
Nabumetone	40.2 ±11.5	>100	149	230	[28]
Nimesulide	93.2 ±17.6	>100	12.5	0.4	[27]
NS-398	71.2 ±20.0	>100	28.9	0.04	[27]
Phenylbutazone	>100	>100	-	-	-
Piroxicam	>100	>100	47	25	[28]
Resveratrol	35.0 ±13.5	78.9 ±11.6	0.83	0.99	[30]
SC-560	10.9 ±2.8	93.9 ±24.5	0.009	6.3	[19]
SC-58125	56.1 ±22.4	>100	13.3	0.07	[27]
Vareoyl salicylate	>100	>100	-	-	-
Cyclosporine A	2.8 ±0.2	16.1 ±2.0	-	-	-
Ribavirin	3.9 ±0.4	15.1 ±2.5	-	-	-

All 50% effective concentration (EC_{50}; on the basis of the reduction of cell destruction induced by bovine viral diarrhoea virus (BVDV) replication) and cytotoxic concentration (CC_{50}; on the basis of the reduction of viable cell number) data represent means ±SD for at least three separate experiments. IC_{50}, 50% inhibitory concentration for human cyclooxygenase (COX) activity. COX-1, cyclooxygenase type-1; COX-2, cyclooxygenase type-2; MDBK, Madin-Darby bovine kidney.

Figure 2. Inhibitory effect of SC-560 on BVDV replication in MDBK cells

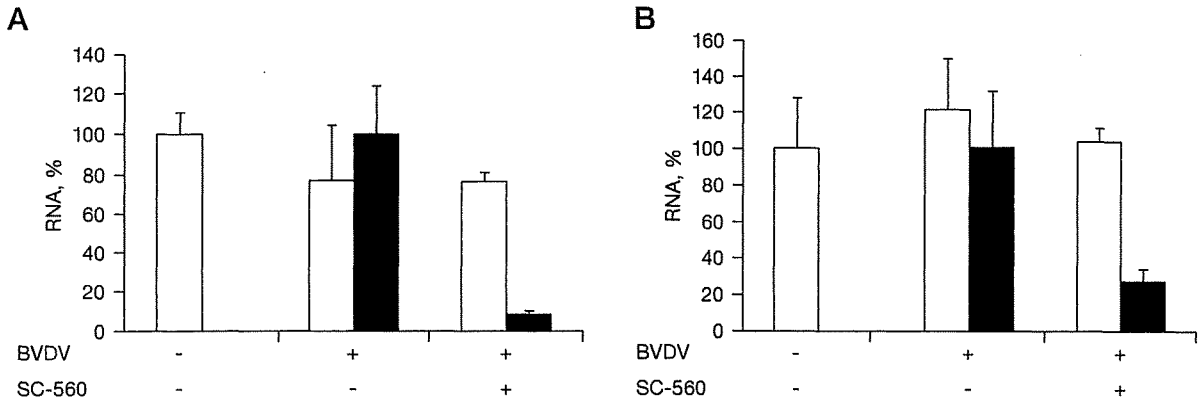


(A) The Madin-Darby bovine kidney (MDBK) cells were infected with bovine viral diarrhoea virus (BVDV) at a multiplicity of infection (MOI) of 0.01 and incubated in the presence of various concentrations of SC-560 for 3 days. White columns indicate the viable cell number in mock-infected cells, as determined by the tetrazolium dye method, and black columns indicate the virus-induced cytopathogenicity in infected cells, as determined by the lactate dehydrogenase method. (B) The cells were infected at a MOI of 1.0 and cultured in the presence of various concentrations of SC-560. After a 6 h incubation, total RNA was extracted from the cells and subjected to real-time reverse transcription-PCR. White and grey columns indicate the amounts of β-actin RNA and BVDV RNA, respectively. All data represent mean ±SD for three separate experiments. Statistical analyses were conducted by Student's *t*-test. $^{a}P<0.05$.

SC-560 could inhibit the negative-strand viral RNA synthesis (Figure 4).

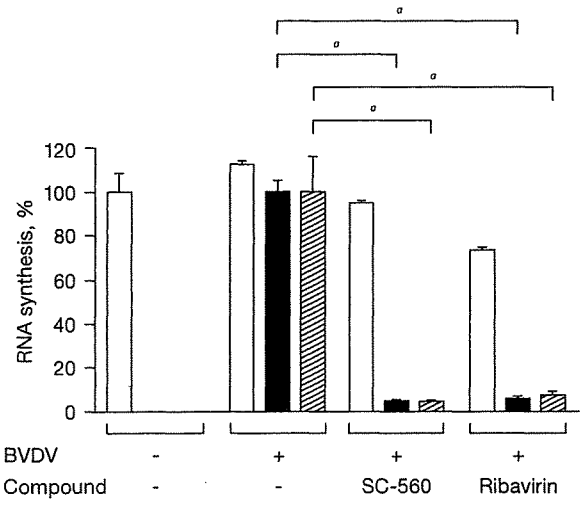
Combination antiviral activity of SC-560 and IFN-α was examined by a cytopathogenicity inhibition assay. Prior to the combination experiment, the optimal concentration ratio of the two compounds was determined (data not shown). Although SC-560 and IFN-α achieved 27.3% and 43.3% inhibition at a concentration of 6.25 μM and 0.625 IU/ml, respectively, their combination resulted in 63.6% inhibition (Figure 5). Furthermore, >90% inhibition of BVDV replication was recorded using the combination of

Figure 3. Inhibitory effect of SC-560 on BVDV replication after viral entry



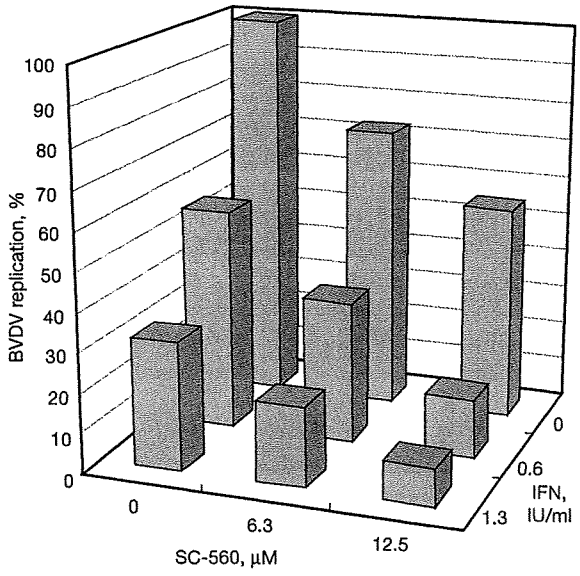
The Madin-Darby bovine kidney cells were infected with bovine viral diarrhoea virus (BVDV) at a multiplicity of infection of 1.0. SC-560 (50 μM) was added to the cells (A) simultaneously with the virus or (B) at 2 h after virus infection. At 6 h after virus infection, total RNA was extracted from the cells and subjected to real-time reverse transcription-PCR. White and black columns indicate the amounts of β-actin RNA and BVDV RNA, respectively. All data represent mean ±so for three separate experiments. Statistical analyses were conducted by Student's *t*-test.

Figure 4. Inhibitory effect of SC-560 on the BVDV negative-strand RNA synthesis



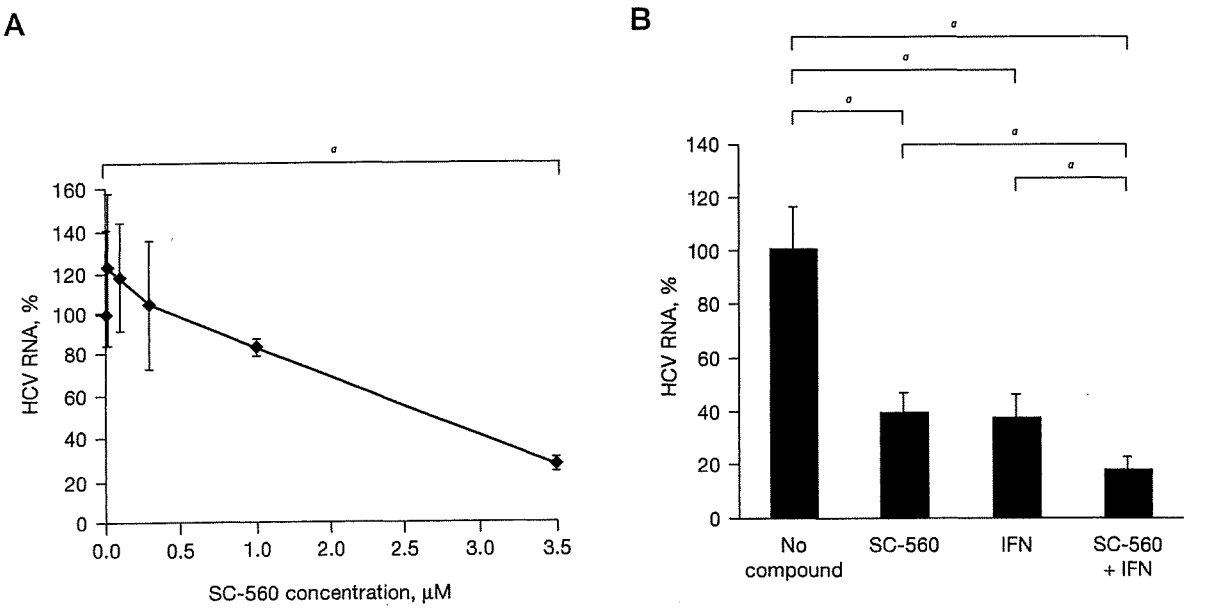
The Madin-Darby bovine kidney cells were infected with bovine viral diarrhoea virus (BVDV) at a multiplicity of infection of 1.0 and incubated in the presence of either SC-560 (50 μM) or ribavirin (20 μM). After a 6 h incubation, total RNA was extracted from the cells and subjected to real-time reverse transcription-PCR. White, black and hatched columns indicate the amounts of β-actin RNA, positive- and negative-strand BVDV RNA, respectively. All data represent mean ±so for three separate experiments. Statistical analyses were conducted by Student's *t*-test. $^{a}P<0.05$.

Figure 5. Inhibitory effect of SC-560 in combination with IFN-α on BVDV replication in MDBK cells



The 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 SC-560 and interferon (IFN)-α for 3 days. Each column indicates the virus-induced cytopathogenicity in virus-infected cells as determined by the lactate dehydrogenase method. Experiments were carried out in triplicate and mean values are shown.

Figure 6. Inhibitory effect of SC-560 on HCV replication in subgenomic HCV RNA replicon cells



MH-14 cells were incubated in the presence of **(A)** SC-560 alone or **(B)** in combination with interferon (IFN)-α. The concentrations of SC-560 and IFN-α used in the combination experiments were 30 μM and 10 IU/ml, respectively. Every 3 days, the culture medium was replaced by fresh culture medium containing an appropriate concentration of the compound. On day 7, total RNA was extracted from the cells and subjected to real-time reverse transcription-PCR. Experiments were carried out in triplicate and a representative result is shown. $^{a}P<0.05$. HCV, hepatitis C virus.

12.5 μM SC-560 and 1.25 IU/ml IFN-α. This combination did not generate any cytotoxicity to the host cells (data not shown). The CIs were 0.99, 0.73 and 0.46 at a level of 50%, 70% and 90% inhibition of BVDV replication, respectively, indicating synergism at higher inhibition levels.

It seems important to know whether SC-560 is inhibitory to HCV replication. When the compound was examined for its inhibitory effect on HCV replication in MH-14 cells, dose-dependent suppression of HCV RNA was observed (Figure 6A). In particular, SC-560 significantly reduced the amount of intracellular HCV RNA at a concentration of 30 μM without apparent cytotoxicity to the replicon cells, indicating that the compound is a selective inhibitor of HCV replication. Furthermore, approximately 80% inhibition of HCV replication was achieved by 30 μM SC-560 in combination with 10 IU/ml IFN-α (Figure 6B).

Discussion

SC-560 is a member of the diaryl heterocycle class of COX inhibitors, which include the selective COX type-2 (COX-2) inhibitors, celecoxib and rofecoxib. Unlike these inhibitors, SC-560 is a highly selective inhibitor of COX type-1 (COX-1). Using human recombinant enzymes, the 50% inhibitory concentrations (IC_{50}) values of SC-560 for COX-1 and COX-2 were 0.009 μM and 6.3 μM, respectively [19]. We did not examine celecoxib and rofecoxib for their antiviral activity against BVDV and HCV; however, it is assumed that the antiviral activity of SC-560 is not associated with its inhibitory effect on COX-1 or COX-2 as there is no correlation between COX inhibition and antiviral activity (Table 1). For instance, CAY10404 is one of the most potent and selective COX-2 inhibitors reported to date. Its IC_{50} values for COX-1 and COX-2 were >500 μM and <1 nM, respectively [20], yet this compound did not show any selective inhibition of BVDV replication (Table 1). Furthermore, the COX-1-specific inhibitors ibuprofen and mefenamic acid were not active against BVDV.

It was recently reported that a triaryl pyrazoline compound, which has a similar chemical structure to SC-560, inhibited West Nile virus replication with an EC_{50} of 28 μM [21]. This compound did not inhibit viral entry or virion assembly but it did specifically suppress viral RNA synthesis. Although the compound was not tested for BVDV and HCV, it was active against other flaviviruses, such as dengue, yellow fever and Saint Louis encephalitis viruses. In our study, SC-560 did not inhibit viral entry but did inhibit BVDV and HCV RNA synthesis. As its anti-HCV activity was determined in HCV RNA replicon cells, the inhibition of virion assembly could

also be excluded as the target of SC-560. Paeshuyse *et al.* [22] reported that two structurally unrelated compounds, BPIP (5-[(4-bromophenyl)methyl]-2-phenyl-5*H*-imidazo[4,5-c]pyridine) and VP32947, were highly potent and selective inhibitors of BVDV. Both compounds inhibited BVDV replication and RNA synthesis at nM concentrations; however, in contrast to SC-560, BPIP and VP32947 did not show any selective inhibition of HCV replication. Studies on the mechanism of action revealed that both BPIP and VP32947 targeted the same region of the BVDV RNA-dependent RNA polymerase.

The nonstructural protein 5B (NS5B) is the virus-encoded RNA-dependent RNA polymerase and is a major target for inhibition of anti-HCV agents [23,24]. Buckwold *et al.* [25] compared the complete amino acid sequences of NS5B between HCV and BVDV and found only 17.2% identity. Therefore, it is unsurprising that inhibitors such as BPIP and VP32947 did not show any activity against HCV replication. By contrast, ribavirin, an inosine monophosphate dehydrogenase inhibitor, has been shown to have antiviral activity against several RNA viruses [26]. It is hypothesized that ribavirin inhibits HCV replication by more than one mechanism, such as viral RNA transcription, elongation and cap formation and thus it might have broad-spectrum antiviral activity. Similar to ribavirin, SC-560 was found to be active against both BVDV and HCV in this study; however, because it is unlikely that SC-560 and ribavirin share the same target molecule for inhibition of these viruses, the target of SC-560 should be elucidated in future studies.

Another important issue to be elucidated is whether anti-COX activity of SC-560 can be dissociated from its antiviral activity by structural modification. A compound that inhibits COX-1 might generate serious side effects, even if it would have potent antiviral activity. Therefore, reducing COX activity as well as increasing antiviral potency seems indispensable for further development of this class of compounds as anti-HCV agents. Combination chemotherapy might be able to circumvent this problem by reducing a required dose of each compound.

In conclusion, the identification of SC-560 described herein represents the first step toward the future development of effective anti-HCV agents. Further studies are in progress to identify more potent and selective inhibitors of HCV replication.

Acknowledgements

We would like to thank Professors Tamotsu Kanzaki and Takuro Kanekura (Department of Dermatology, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan) for their valuable advice.

Disclosure statement

The authors declare no competing interests.

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. Ohno O, Mizokami M, Wu RR, *et al.* New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. *J Clin Microbiol* 1997; 35:201–207.
3. Hayashi N, Takehara T. Antiviral therapy for chronic hepatitis C: past, present, and future. *J Gastroenterol* 2006; 41:17–27.
4. Manns MP, Foster GR, Rockstroh JK, Zeuzem S, Zoulom F, Houghton M. The way forward in HCV treatment – finding the right path. *Nat Rev Drug Discov* 2007; 6:991–1000.
5. 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.
6. 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.
7. 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.
8. Lindenbach BD, Evans MJ, Syder AJ, *et al.* Complete replication of hepatitis C virus in cell culture. *Science* 2005; 309:623–626.
9. Buckwold VE, Beer BE, Conis RO. Bovine viral diarrhea virus as a surrogate model of hepatitis C virus for evaluation of antiviral agents. *Antiviral Res* 2003; 60:1–15.
10. Poole TL, Wang C, Popp RA, Potgieter LN, Siddiqui A, Collett MS. Pestivirus translation initiation occurs by internal ribosome entry. *Virology* 1995; 206:750–754.
11. Mendez E, Ruggli N, Collett MS, Rice CM. Infectious bovine viral diarrhea virus (strain NADL) RNA from stable cDNA clones: a cellular insert determines NS3 production and viral cytopathogenicity. *J Virol* 1998; 72:4737–4745.
12. Baba C, Yanagida K, Kanzaki T, Baba M. Colorimetric lactate dehydrogenase (LDH) assay for evaluation of antiviral activity against bovine viral diarrhoea virus (BVDV) *in vitro*. *Antivir Chem Chemother* 2005; 16:33–39.
13. Watashi K, Hijikata M, Hosaka M, Yamaji M, Shimotohno K. Cyclosporine A suppresses replication of hepatitis C virus genome in cultured hepatocytes. *Hepatology* 2003; 38:1282–1288.
14. Ishii N, Watashi K, Hishiki T, *et al.* Diverse effects of cyclosporine on hepatitis C virus strain replication. *J Virol* 2006; 80:4510–4520.
15. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 1984; 22:27–55.
16. Belen'kii MS, Schinazi RF. Multiple drug effect analysis with confidence interval. *Antiviral Res* 1994; 25:1–11.
17. Zhou S, Liu R, Baroudy BM, Malcolm BA, Reyes GR. The effect of ribavirin and IMPDH inhibitors on hepatitis C virus subgenomic replicon RNA. *Virology* 2003; 310:333–342.
18. Tanabe Y, Sakamoto N, Enomoto N, *et al.* Synergistic inhibition of intracellular hepatitis C virus replication by combination of ribavirin and interferon-α. *J Infect Dis* 2004; 189:1129–1139.

19. Smith CJ, Zhang Y, Koboldt CM, *et al.* Pharmacological analysis of cyclooxygenase-1 in inflammation. *Proc Natl Acad Sci U S A* 1998; 95:13313–13318.
20. Habeeb AG, Praveen Rao PN, Knaus EE. Design and synthesis of diarylisoxazoles: novel inhibitors of cyclooxygenase-2 (COX-2) with analgesic-antiinflammatory activity. *Drug Dev Res* 2000; 51:273–286.
21. Puig-Basagoiti F, Tilgner M, Forshey BM, *et al.* Triaryl pyrazoline compound inhibits flavivirus RNA replication. *Antimicrob Agents Chemother* 2006; 50:1320–1329.
22. 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.
23. Wu JZ, Yao N, Walker M, Hong Z. Recent advances in discovery and development of promising therapeutics against hepatitis C virus NS5B RNA-dependent RNA polymerase. *Mini Rev Med Chem* 2005; 5:1103–1112.
24. Beaulieu PL. Non-nucleoside inhibitors of the HCV NS5B polymerase: progress in the discovery and development of novel agents for the treatment of HCV infections. *Curr Opin Investig Drugs* 2007; 8:614–634.
25. 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.
26. Snell NJ. Ribavirin – current status of a broad spectrum antiviral agent. *Expert Opin Pharmacother* 2001; 2:1317–1324.
27. Miralpeix M, Camacho M, López-Belmonte J, *et al.* Selective induction of cyclo-oxygenase-2 activity in the permanent human endothelial cell line HUV-EC-C: biochemical and pharmacological characterization. *Br J Pharmacol* 1997; 121:171–180.
28. Kato M, Nishida S, Kitasato H, Sakata N, Kawai S. Cyclooxygenase-1 and cyclooxygenase-2 selectivity of non-steroidal anti-inflammatory drugs: investigation using human peripheral monocytes. *J Pharm Pharmacol* 2001; 53:1679–1685.
29. Lora M, Denault JB, Leduc R, de Brum-Fernandes AJ. Systematic pharmacological approach to the characterization of NSAIDs. *Prostaglandins Leukot Essent Fatty Acids* 1998; 59:55–62.
30. Kang SS, Cuendet M, Endringer DC, Croy VL, Pezzuto JM, Lipton MA. Synthesis and biological evaluation of a library of resveratrol analogues as inhibitors of COX-1, COX-2 and NF-κB. *Bioorg Med Chem* 2009; 17:1044–1054.

Received 8 April 2009, accepted 21 May 2009