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Antiviral 2,5-disubstituted imidazo[4,5-*c*]pyridines: From anti-pestivirus to anti-hepatitis C virus activity

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Abstract—A novel class of inhibitors of the hepatitis C virus [substituted 2-(2-fluorophenyl)-5*H*-imidazo[4,5-*c*]pyridines] is described. Introduction of a fluorine in position 2 of the 2-phenyl substituent of the lead anti-pestivirus compound **1** (5-[(4-bromophenyl)methyl]-2-phenyl-5*H*-imidazo[4,5-*c*]pyridine) resulted in an analogue with selective activity against HCV in the subgenomic replicon system.

Hepatitis C virus (HCV) is an enveloped single stranded (+) RNA virus that belongs to the separate genus Hepacivirus of the family Flaviviridae.[1] HCV causes acute and chronic liver disease, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma.[2] Worldwide more than 170 million people are chronically infected with HCV[3] and are thus at increased risk of developing serious life-threatening liver disease. Current standard therapy for chronic hepatitis C consists of pegylated IFN-α 2a in combination with ribavirin.[4] The bovine viral diarrhea virus (BVDV) is the prototype of the Pestivirus genus of the Flaviviridae family. BVDV is a major pathogen of cattle and causes a range of clinical manifestations varying from subclinical symptoms to death.[5] For the United States alone this translates roughly into a loss of $10 to 40 million per million calvings.[6]

We have recently reported on 5-benzyl-2-phenyl-5*H*-imidazo[4,5-*c*]pyridines as a new class of anti-pestiviral compounds.[7,8] Modification of the substitution pattern on the 5-benzyl substituent resulted in an analogue (**1**) with high activity and selectivity against BVDV, but this analogue was inactive against HCV.

To further understand the structure–activity relationships within this class of compounds and to further optimize the antiviral activities we have synthesized additional analogues with modifications in position 2 of the imidazo[4,5-*c*]pyridine ringsystem, keeping the 5-(4-bromobenzyl) substituent as a constant. In a first effort, we have introduced substituents into the 2-phenyl (compounds **2**–**17**, Table 1).[9] With respect to anti-BVDV activity, most substituents were tolerated, resulting in analogues with similar or slightly reduced activity, with the exception of the 3,5-dibromo analogue **17**, which turned out to be totally inactive against BVDV. Surprisingly, the 2-fluoro analogue **8** exhibited potent and selective activity against HCV in the replicon system.[12]

In a second set of analogues (Table 2) the 2-phenyl ring was replaced by ethyl (compound **18**), naphthyl or a (substituted) heterocycle (compounds **19**–**25**), or a linker was introduced between the central imidazo[4,5-*c*]pyridine and the 2-phenyl substituent (compounds **26**–**32**). The 2-ethyl analogue **18** showed reduced anti-BVDV activity and was inactive against HCV. The 2-naphthyl analogue **20** was more active than its 1-naphthyl isomer **19** against BVDV, but was also somewhat more cytotoxic. All heterocyclic analogues exhibited anti-BVDV activity, whereas only the 2-pyridyl analogue **21** exhibited anti-HCV activity, being more cytostatic than the 2-(2-fluorophenyl) analogue **8**. Introduction of a linker resulted in compounds that were inactive against BVDV with the exception of the 2-(*E*)-styryl analogue **32**. None

Keywords: Imidazo[4,5-*c*]pyridines; BVDV inhibitor; HCV inhibitor; Antiviral.
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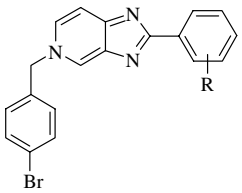
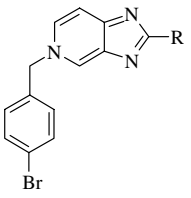
Table 1. Structure, anti-BVDV activity, anti-HCV activity, and cytotoxic/cytostatic activity of compounds **1**–**17**



Compound	R	BVDV			HCV		
		EC_{50}[a] (μM)	CC_{50}[a] (μM)	SI[b]	EC_{50}[a] (μM)	CC_{50}[a] (μM)	SI[b]
1	H	0.070 ± 0.02[c]	83 ± 20	1186	>38	38	n.a.
2	2-CH_3	0.061 ± 0.009	>33	>541	24	21	n.a.
3	3-CH_3	0.06 ± 0.04	23	372	>11	11	n.a.
4	4-CH_3	0.4 ± 0.2	21	53	8.2	13	2
5	2-Cl	0.33 ± 0.05	>33	>100	25	43	2
6	3-Cl	0.15 ± 0.08	>33	>220	83	>125	>2
7	4-Cl	0.7 ± 0.1	>33	>47	13	17	n.a.
8	2-F	0.12 ± 0.01	>33	>275	1.0 ± 0.6	52 ± 16	52
9	3-F	0.04 ± 0.01	>33	>825	>44	44	n.a.
10	4-F	0.17 ± 0.06	>33	>194	31	37	n.a.
11	2-Br	0.5 ± 0.2	>33	>66	21	36	2
12	3-Br	0.11 ± 0.02	>33	>300	>113	>113	n.a.
13	3-OCH_3	0.05 ± 0.02	>33	>660	18	28	2
14	3-$N(CH_3)_2$	0.14 ± 0.01	24	171	>79	79	n.a.
15	3-I	0.4 ± 0.1	>33	>83	5.7	12	2
16	3-CF_3	0.5 ± 0.1	>33	>72	23	>116	>5
17	3,5-Br_2	>33	>33	n.a.	>96	>96	n.a.

The EC_{50} (50% effective concentration) is the concentration of compound that offered 50% protection of the cells against virus-induced cytopathic effect or the 50% reduction of luciferase signal in case of HCV.
The CC_{50} (50% cytostatic concentration) is the concentration that inhibited the proliferation of exponentially growing cells by 50%. n.a., not active.
[a] Data (for active compounds) are mean values ± SD for 4–6 independent experiments.
[b] In vitro selectivity index (CC_{50}/EC_{50}).
[c] The here reported value does not significantly differ from the reported value.[8]

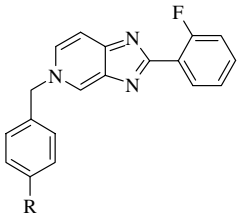
Table 2. Structure, anti-BVDV activity, anti-HCV activity, and cytotoxic/cytostatic activity of compounds **18**–**32**



Compound	R	BVDV			HCV		
		EC_{50}[a] (μM)	CC_{50}[a] (μM)	SI[b]	EC_{50}[a] (μM)	CC_{50}[a] (μM)	SI[b]
18	Ethyl	0.38 ± 0.09	>33	>87	>92	92	n.a.
19	1-Naphthyl	0.3 ± 0.2	23	77	9.7	12	n.a.
20	2-Naphthyl	0.030 ± 0.009	7	259	5. 7	9.7	2
21	2-Pyridyl	0.10 ± 0.02	10	96	0.35 ± 0.3	5.2 ± 1.9	15
22	3-Pyridyl	0.16 ± 0.01	>33	>206	109	126	n.a.
23	4-Pyridyl	0.47 ± 0.07	>33	>70	14	38	3
24	2-Thienyl	0.04 ± 0.02	>33	>825	>35	35	n.a.
25	5-Bromo-2-thienyl	0.6 ± 0.4	>33	>55	>18	18	n.a.
26	Benzyl	>33	>33	n.a.	79	>132	>2
27	2-Phenylethyl	>16	16	n.a.	2.5	7.6	3
28	3-Phenyl-1-propyl	3 ± 1	22	8	7.4	12	2
29	4-Phenyl-1-butyl	>5	5	n.a.	>2.2	2.2	n.a.
30	Phenoxymethyl	>8	>33	4	25	35	1.5
31	Phenylthiomethyl	>25	25	n.a.	4.9	9.7	2
32	(E)-styryl	0.16 ± 0.08	14	88	1.7	2.5	>2

The EC_{50} (50% effective concentration) is the concentration of compound that offered 50% protection of the cells against virus-induced cytopathic effect or the 50% reduction of luciferase signal in case of HCV.
The CC_{50} (50% cytostatic concentration) is the concentration that inhibited the proliferation of exponentially growing cells by 50%. n.a., not active.
[a] Data (for active compounds) are mean values ± SD for 4–6 independent experiments.
[b] In vitro selectivity index (CC_{50}/EC_{50}).

Table 3. Structure, anti-BVDV activity, anti-HCV activity, and cytotoxic/cytostatic activity of compounds **33** and **34**



Compound	R	BVDV			HCV		
		EC_{50}[a] (μM)	CC_{50}[a] (μM)	SI[b]	EC_{50}[a] (μM)	CC_{50}[a] (μM)	SI[b]
33	Cl	0.11 ± 0.05	>33	>300	44	68	1.5
34	I	0.07 ± 0.02	>33	>478	0.79	58	74

The EC_{50} (50% effective concentration) is the concentration of compound that offered 50% protection of the cells against virus-induced cytopathic effect or the 50% reduction of luciferase signal in case of HCV.

The CC_{50} (50% cytostatic concentration) is the concentration that inhibited the proliferation of exponentially growing cells by 50%.

[a] Data (for active compounds) are mean values ± SD for 4–6 independent experiments.

[b] In vitro selectivity index (CC_{50}/EC_{50}).

of these analogues exhibited significant anti-HCV activity.

In an attempt to further prove the importance of the 2-fluoro substituent in compound **8** on HCV activity, two 5-(4-halobenzyl) analogues (**33** and **34**) were prepared (Table 3). Both of these proved active against BVDV, but only the iodo analogue **34** was active against HCV. The size of the substituent at position 4 of the 5-benzyl may thus be a critical component of the anti-HCV activity of this class of compounds.

In summary, introduction of a fluorine in position 2 of the 2-phenyl substituent of the lead compound **1** resulted in analogues with activity against hepatitis C virus. Interestingly, the structure–activity relationships for this class of compounds against BVDV and HCV are rather different. We show here, for the first time, that the appropriate modification of a scaffold of non-nucleoside inhibitors of pestiviruses may result in compounds with activity against the related HCV. We have thus identified, to the best of our knowledge for the first time, non-nucleoside compounds that exhibit potent activity against both pestiviruses and HCV. We recently revealed that imidazo[4,5-*c*]pyridines inhibit pestivirus replication by targeting the viral polymerase in the close neighborhood of residue F224.[8] Since (i) the loop domain of the BVDV RdRp that carries the F224 residue is absent in the HCV RdRp and (ii) the conformation of the finger domain of the RdRp polymerase is different from that of the BVDV polymerase,[13] it is conceivable that the precise molecular mechanism by which these compounds inhibit the replication of BVDV and HCV, respectively, is different. This will be subject of further investigation.

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9. All new compounds were fully characterized by melting point, NMR, MS, and HRMS spectra. The syntheses of the intermediate 2-substituted imidazo[4,5-*c*]pyridines were performed in analogy to published procedures.[7,10,11] The intermediates for compounds **2**–**12**, **14**–**24**, **26**, and **33**–**34** were prepared by reaction of 3,4-diaminopyridine with the corresponding carboxylic acid in polyphosphoric acid (4 h at 190 °C, with an exception in the case of propionic acid, where the temperature of the reaction mixture was kept at 150 °C in the first hour, before heating to 190 °C for 3 h). The cyclizations with 3-methoxybenzoic acid and 5-bromothiophene-2-carboxylic acid were performed in refluxing $POCl_3$. The intermediates for analogues **27**–**32** were obtained by reacting 3,4-diaminopyridine with 3 equiv of the corresponding carboxylic acids at 160 °C for 20 h (neat), followed by washing with diisopropyl ether to remove the excess acid and recrystallization from an appropriate solvent mixture (e.g., diisopropyl ether/ethyl acetate). The final benzyla-

tions were performed as described before.[7] Compound **8**: recrystallized from a mixture of diisopropyl ether and ethyl acetate; off-white powder; mp 156 °C; yield: 53%; ^{1}H NMR (200 MHz, DMSO-d_6) δ 9.18 (d, 1H, H4, J = 1.6 Hz), 8.35–8.26 (m, 1H, arom. H), 8.20 (dd, 1H, H6, J = 6.8, 1.6 Hz), 7.78 (d, 1H, H7, J = 6.8 Hz), 7.64–7.58 (**AA'BB'**, 2H, arom. H), 7.52–7.24 (m, 5H, arom. H), 5.66 (s, 2H, CH_2); HRMS (ESI+): *m/z* calcd for $C_{19}H_{14}BrFN_3$ $(M+H)^+$ 382.0355; found 382.0358.

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12. Anti-HCV assay in Huh 5-2 cells. Huh 5-2 cells were seeded at a density of 5×10^3 per well in a 96-well white view plate (Packard, Canberra, Canada) in complete DMEM supplemented with 250 μg/ml G418. Following incubation for 24 h at 37 °C (5% CO_2) medium was removed and threefold serial dilutions in complete DMEM (without G418) of the test compounds were added in a total volume of 100 μl. After 4 days of incubation at 37 °C, cell culture medium was removed and luciferase activity was determined using the Steady-Glo luciferase assay system (Promega, Leiden, The Netherlands); the luciferase signal was measured using a Luminoskan Ascent (Thermo, Vantaa, Finland). The 50% effective concentration (EC_{50}) was defined as the concentration of compound that reduced the luciferase signal by 50%. Cytostatic assay. MDBK or Huh 5-2 cells were seeded at a density of 5×10^3 cells per well of a 96-well plate in MEM-FCS; 24 h later, serial dilutions of the test compounds were added. Cells were allowed to proliferate for 3 days at 37 °C, after which the cell number was determined by means of the MTS/PMS method (Promega). The percentage cell growth was calculated as follows: $(OD_{treated})/(OD_{control})$; in which $(OD_{treated})$ = the $OD_{490\ nm}$ of cells treated with a certain dilution of compound, $(OD_{control})$ = the $OD_{490\ nm}$ of cells left untreated. The 50% cytostatic concentration (CC_{50}) was defined as the concentration that inhibited the proliferation of exponentially growing cells by 50% and was calculated using logarithmic interpolation.
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