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Discovery and characterization of a novel 7-aminopyrazolo[1,5-*a*]pyrimidine analog as a potent hepatitis C virus inhibitor

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ABSTRACT

We describe a novel 7-aminopyrazolo[1,5-*a*]pyrimidine (7-APP) derivative as a potent hepatitis C virus (HCV) inhibitor. A series of 7-APPs was synthesized and evaluated for inhibitory activity against HCV in different cell culture systems. The synthesis and preliminary structure-activity relationship study of 7-APP are reported.

Approximately 200 million people worldwide are chronically infected with the hepatitis C virus (HCV). This pathogen is the major cause of acute hepatitis and chronic liver disease including cirrhosis and liver cancer, and thereby HCV is the leading indication for liver transplantation.[1] HCV is an enveloped positive-single stranded RNA virus that belongs to the family of *Flaviviridae*.[2] The HCV genome encodes a polyprotein with structural proteins (C, E1, E2, and p7) and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). Despite the recent approval of two direct acting antivirals (DAAs), telaprevir and boceprevir inhibiting the NS3/4A protease, standard of care for chronic hepatitis C is still a combination therapy of pegylated interferon-alpha (PEG-IFN-α) and ribavirin, which has limitations in HCV genotype 1 patients and causes severe side effects.[3–6] To date, a number of anti-HCV agents are being developed, mainly targeting the non-structural proteins, which still could fail in clinical trials due to severe side effects as described for the recently approved DAAs or because of rapid emergence of drug resistant mutants.[7,8] This unpredictability and unmet medical needs encouraged us to engage in a HCV drug discovery campaign to identify and develop new, safer and potent drugs against HCV with a high genetic barrier to resistance. By using the recently developed infectious cell culture system for HCV (HCVcc), we devised strategies for a phenotypic high throughput screening assay and screened the entire viral life cycle for novel HCV inhibitors. To do so, the green fluorescent protein (GFP) was engineered into the viral NS5A domain III coding region of full length cell culture adapted JFH-1.[9] Insertion of the GFP coding sequence in HCV NS5A allowed us to monitor non-invasively the dynamics of HCV RNA replication, because GFP-fluorescence intensity was proportional to viral RNA replication. The antiviral activity of compounds was determined by dose–response curve analysis. Briefly, serially diluted compounds were incubated with naïve Huh-7 target cells which were plated in 384-well microplates, and subsequently inoculated with HCVcc at a multiplicity of infection (MOI) 1. At 72 h post-infection live cells were analyzed by automated confocal microscopy (Opera®, PerkinElmer) and the half maximal effective concentration against viral replication (EC_{50}) was determined.

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† These authors contributed equally to this study.

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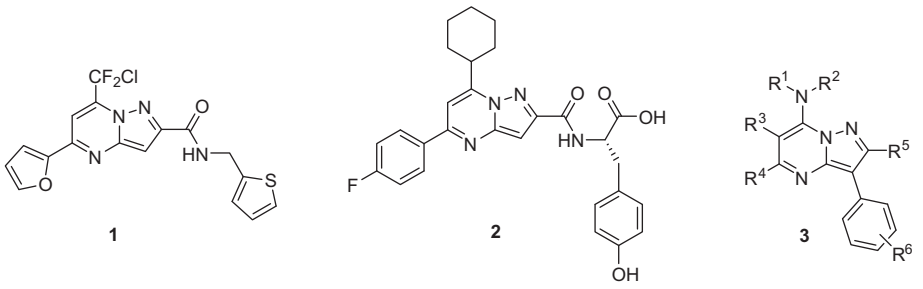
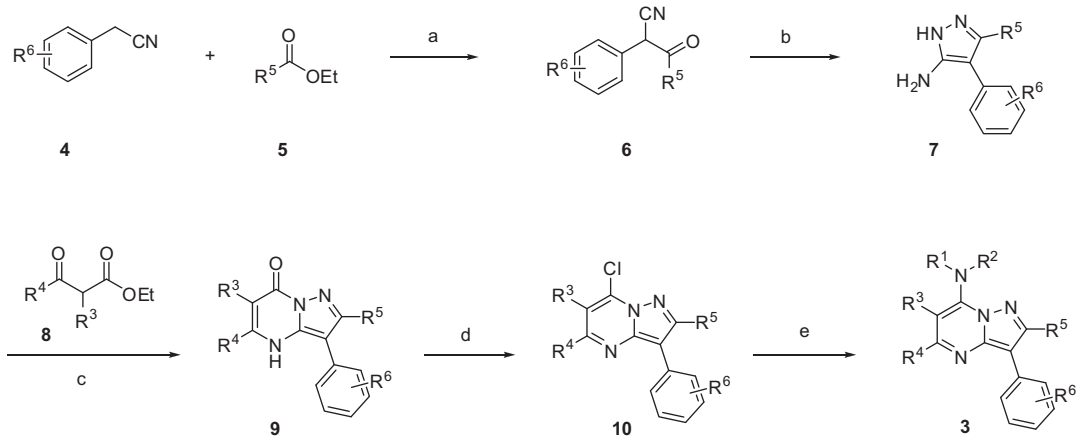
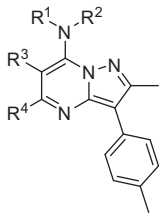


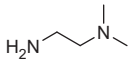
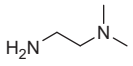
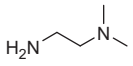
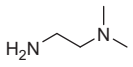
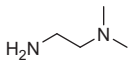
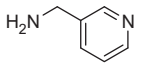
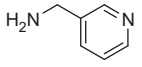
Figure 1. Published HCV NS4B inhibitor (**1**), RdRp inhibitor (**2**), and representative structure of 7-aminopyrazolo[1,5-*a*]pyrimidines (7-APPs, **3**).



Scheme 1. Reagents and conditions: (a) Na, EtOH, reflux; (b) hydrazine monohydrate, EtOH, reflux; (c) beta-ketoester (**8**), AcOH, 110 °C, 3 h; (d) $POCl_3$, 100 °C, 1 h; (e) R^1R^2NH, DIPEA, DMF, rt, overnight.

Table 1
Substituent effect on R^1, R^2, R^3, and R^4 for anti-HCV activity



No.	R^1R^2NH	R^3	R^4	Anti-HCVcc (EC_{50}, μM)	Cytotoxicity (CC_{50}, μM)	SI (CC_{50}/EC_{50})
11		H	Me	1.4	30	21
12		H	Et	0.60	8.9	15
13		H	*i*-Pr	1.3	7.2	5.5
14		H	Ph	1.1	3.2	2.9
15		H	CF_3	7.2	15	2.1
16		Me	Me	11	13	1.2
17				2.8	8. 7	3.1

EC_{50} and CC_{50} values were determined by 10 point dose–response curve analysis in duplicates with quadruplicate measurements.

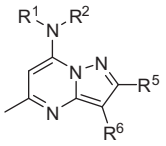
Additionally, the cytotoxic concentration (CC_{50}) was calculated by determining cell growth inhibition to rule out that the observed antiviral effects were due to toxicity induced by compound.

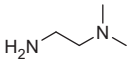
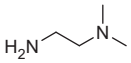
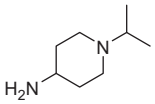
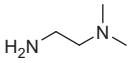
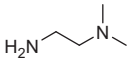
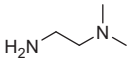
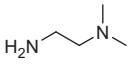
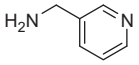
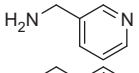
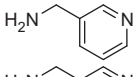
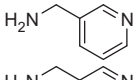
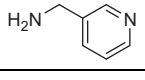
From our ongoing drug discovery program using the phenotypic screening assay as described above, we identified 7-aminopyrazolo[1,5-*a*]pyrimidine (7-APP) as a potent HCV inhibitor. 7-APP has been used as one of prevalent backbones in drugs and biological active compounds, such as antidepressants,[10–12] hypertension,[13,14] cancer,[15–17] anti-obesity,[18,19] ischemic stroke,[20,21] anti-diabetes,[22] etc. Interestingly, the pyrazolo[1,5-*a*]pyrimidine backbone was reported to inhibit RNA replication of subgenomic genotype 1b replicons by interfering with the NS4B-induced rearrangement of cellular membranes into a so-called membranous web(**1**).[23] Furthermore, activity in enzymatic RNA-dependent RNA polymerase (RdRp) assays was described (**2**)[24,25] (Fig. 1). However, its structural features including substitution pattern are apparently different from our initial active compound (**3**). Recently, a 7-APP analog was reported to inhibit phosphatidylinositol 4-kinase III beta (PI4KB) with anti-poliovirus activity, but was inactive in the HCV replicon system.[26] Herein, we report a structure–activity relationship (SAR) study with 7-aminopyrazolo[1,5-*a*]pyrimidine (7-APP) to improve anti-HCV activity. A general scheme for the synthesis of 7-APPs is outlined in Scheme 1.

The 7-APPs **3** were synthesized from aminopyrazoles **7** which were prepared in two-steps from nitriles **4**. Reaction of nitriles **4** with ethyl ester **5** in the presence of sodium in EtOH generated the corresponding alpha-cyanoketone intermediates **6**, which were subsequently cyclized with hydrazine to afford aminopyrazoles **7**. Cyclocondensation of the key intermediates **7** was performed with beta-ketoesters **8** to afford compounds **9** as major products.[12] Compounds **9** were converted to their corresponding chlorinated compounds **10** by $POCl_3$. Subsequent reaction of **10** with various amines provided the target compounds **3**. All synthesized compounds were evaluated for their ability to inhibit the HCV life cycle as described earlier.

Our initial effort toward the SAR study of 7-APP was focused on the exploration of R^3 and R^4 substituents as shown in Table 1. For R^4 substituents, most of compounds (**11–14**) showed moderate activity ranging 0.6–1.4 μM EC_{50} values, except with CF_3-substituted compound (**15**, EC_{50} = 7.2 μM). Especially ethyl substituted compound (**12**) exhibited submicromolar activity (EC_{50} = 0.60 μM). The cytotoxicity of these compounds appeared to be significantly influenced by R^4 substituent. For example, methyl substituted compound (**11**, CC_{50} = 30 μM) showed 10-fold less toxic than phenyl substituent (**14**, CC_{50} = 3.2 μM). Most likely, small groups appear to be more favorable than large groups on

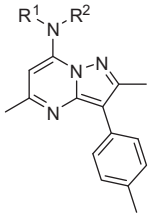
Table 2
Substituent effect on R^5 and R^6

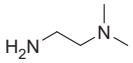
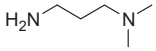
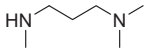
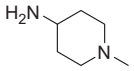
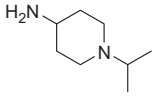
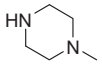
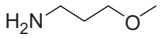
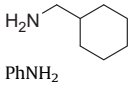
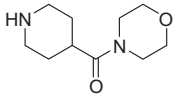
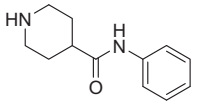


No.	R^1R^2NH	R^5	R^6	Anti-HCVcc (EC_{50}, μM)	Cytotoxicity (CC_{50}, μM)	SI (CC_{50}/EC_{50})
11		Me	4-Me-Ph	1.4	30	21
18		H	H	>20	>20	N.A.
19		H	H	12	12	1.0
20		H	Ph	9.3	>50	>5.4
21		H	4-Cl-Ph	2.0	26	13
22		H	4-F-Ph	2.8	>20	>7.1
23		H	3-Me-Ph	3.1	>20	>6.5
24		4-F-Ph	Me	5.2	30	5.8
25		4-MeO-Ph	Me	2.0	>50	>25
26		4-Me-Ph	4-F-Ph	6.0	8.4	1.4
27		4-F-Ph	4-F-Ph	5.3	6.5	1.2
28		4-MeO-Ph	4-F-Ph	>20	>20	N.A.

EC_{50} and CC_{50} values were determined by 10 point dose–response curve analysis in duplicates with quadruplicate measurements. N.A. not applicable

Table 3
Substituted amine effect



No.	R^1R^2NH	Anti-HCVcc (EC_{50}, μM)	Cytotoxicity (CC_{50}, μM)	SI (CC_{50}/EC_{50})
11		1.4	30	21
29		0.54	22	41
30		0.69	18	26
31		1.0	18	18
32		0.28	8.8	31
33		1.9	16	8.4
34		2.3	19	8.3
35		2.6	17	6.5
36	$PhNH_2$	1.9	17	8.9
37	$BnNH_2$	2.7	10	3.7
38	$PhEtNH_2$	5.3	5.2	1.0
39	Me_2NH	3.4	>20	>5.9
40		12	>20	>1.7
41		13	>50	>3.8

EC_{50} and CC_{50} values were determined by 10 point dose–response curve analysis in duplicates with quadruplicate measurements.

Table 4
Antiviral activities and pharmacological properties of compound **29** and **32**

No.	HCVcc gt2 (EC_{50}, μM)	HCVrp gt2 (EC_{50}, μM)	HCVpp gt1 (at 1 μM)	Solubility (pH = 7.4, μM)	Metabolic stability ($t_{1/2}$, min)	
					Human	Rat
29	0.54	N.D.	Inactive	76	>60	22.4 ± 0.3
32	0.28	Inactive	Inactive	73	>60	>60

N.D. not determined.

R^4, and thus compound **11** displayed the highest selectivity index (SI = 21) among the compounds tested in Table 1. For R^3 substituents, two compounds with methyl (**16**) or fused-cyclohexyl (**17**) were evaluated, and both molecules showed a very narrow selectivity index (1.2 and 3.1, respectively) due to their weak anti-HCV activity (11 μM and 2.8 μM, respectively), suggesting that any substitution at this R^3 position might not be favorable for either anti-HCV activity or cytotoxicity.

Next, we further explored substitution effects of R^5 and R^6 for HCV inhibitory activity and the data were summarized in Table 2. Unsubstituted compounds (**18** and **19**) on both R^5 and R^6 were inactive. Compounds **20**–**23** bearing substituted phenyl group on R^6 showed increased anti-HCV activity compared to unsubstituted compounds (**18** and **19**, R^5/R^6 = H). Compound **24** and **25** having substituted phenyl on R^5 and methyl on R^6 displayed similar activity range (EC_{50} = 5.2 and 2.0 μM, respectively) in comparison with compound **11** (R^5 = Me and R^6 = 4-Me-Ph). However, compound **26**–**28**, disubstituted with phenyl group on both R^5 and R^6, were toxic or inactive. These results indicated that mono substitution with phenyl group on either R^5 or R^6 appears to be tolerable. Among

the compounds in this series, two compounds **11** and **25** exhibited not only good inhibitory activity against HCV but also low cytotoxicity, resulting in high selectivity index values (SI = 21 and 25, respectively).

With preliminary SAR data in hand, we explored R^1 and R^2 substitution effects with various amines. The results are summarized in Table 3. Interestingly, compounds **29**–**32** bearing basic diamine moiety apparently demonstrated good antiviral activity (EC_{50} = 0.28–1.0 μM) and low cytotoxicity (CC_{50} = 8.8–22 μM), resulting in a high selectivity index (SI = 18–41). In addition, these molecules contain three carbons between two nitrogens. However, methylpiperazine (**33**) and compounds containing mono-amine (**34**–**39**) slightly reduced anti-HCV activity. Piperidine–carboxamide compound **40** and **41** even further deteriorated inhibitory activity. Among the compounds tested, isopropyl piperazine **32** turned out to be the most promising in this series.

By using the infectious HCV cell culture system for primary screening which allows targeting the entire viral life cycle, the mechanism of action (MoA) and the targets of potent hit compounds are initially unknown. In order to identify which part of the viral life cycle is inhibited, we devised strategies to elucidate the MoA of the 7-APP scaffold. To discriminate between viral RNA replication and HCV entry mediated by the viral envelope proteins E1 and E2, we conducted experiments with the HCV replicon (HCVrp) and the HCV pseudoparticle (HCVpp) system, respectively. HCV subgenomic replicons express only the nonstructural protein NS3 to NS5B,[27] whereas HCVpp are pseudotyped retroviral particles expressing HCV E1 and E2.[28,29] The inhibitory effect of 7-APP on viral RNA replication was examined in HCVrp cells replicating genotype 2 subgenomes and in the HCVpp system expressing HCV genotype 1 envelope proteins (Table 4). The 7-APPs were inactive in the HCVrp or in the HCVpp assay, suggesting that 7-APP is neither a viral RNA replication inhibitor nor a HCV E1/E2-mediated entry inhibitor. In fact, the result that 7-APP is inactive against HCV replicon corroborates the previous report.[26]

Subsequently, pharmacological properties including solubility and microsomal stability were evaluated. The metabolic stability assay indicated that compound **32** exhibited long half-life in both human and rat ($t_{1/2}$ > 60 min), whereas compound **29** showed relatively shorter half-life in rat microsomes ($t_{1/2}$ = 22.4 min). Both compounds showed good solubility (76 and 73 μM, respectively) in neutral buffer conditions.

In conclusion, we described the discovery and characterization of a novel class of compounds with anti-HCV activity. This series of compounds appears to inhibit HCV life cycle, but does not interfere with either HCV genotype 1 E1/E2-mediated entry or genotype 2 RNA replication of viral. Although the exact mechanism of action of this series remains unclear, the 7-APP scaffold can be utilized as a new tool compound for further anti-HCV studies.

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