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Identification of a series of 1,3,4-trisubstituted pyrazoles as novel hepatitis C virus entry inhibitors

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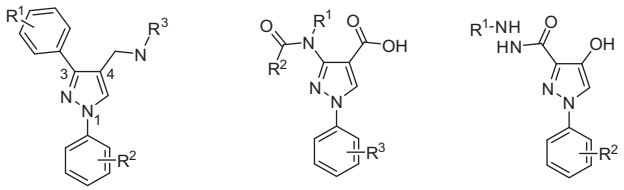
ABSTRACT

In this report we describe the identification of novel pyrazole analogs as potent hepatitis C virus (HCV) entry inhibitor. The pyrazoles were identified by our phenotypic high-throughput screening using infectious HCV. A series of pyrazole derivatives was synthesized and evaluated for inhibitory activity against HCV in the infectious cell culture system. Through evaluation of selected compounds we observed that the pyrazoles did not interfere with HCV RNA replication but with viral entry as shown by experiments with HCV replicons and HCV pseudo particles, respectively.

Hepatitis C virus (HCV) is a global health concern, affecting more than 3% of the world's population by causing chronic liver disease.[1] Viral genome replication is prone to high error rates leading to a large diversity of HCV genotypes and subtypes[2] with differences in susceptibility to current treatment and outcome of disease. To date, the standard of care (SOC), a combination of pegylated interferon-alpha (PEG-IFNα) and ribavirin (RBV), results frequently in unsatisfactory sustained virologic response rates (SVR) of only 45–70% for patients infected with HCV genotype (gt) 1 and about 70–80% for those infected with gt 2 or 3.[3,4] Unfortunately, this treatment is associated with side effects responsible for low adherence to therapy.[5–8] More recently, two direct-acting antiviral agents (DAAs) were introduced to the clinics increasing the SVR rates in HCV gt1 infected patients, but are unfortunately accompanied by severe side effects.[9] This emerging clinical data encouraged us to develop a high-throughput screening (HTS) assay to identify novel antiviral targets. By devising screening strategies using infectious HCV expressing a fluorescent marker protein, we screened phenotypically the entire viral life cycle.[10,11] Identified inhibitors were triaged in HCV replicon and HCV pseudo particle (HCVpp) systems to monitor viral RNA replication and viral entry, respectively. The antiviral activity of hit compounds was determined by 10-point dose response curve (DRC) analysis. Briefly, naïve Huh-7 target cells which were plated in 384-well microplates, incubated with serially diluted compounds, and inoculated with cell culture derived HCV (HCVcc) at a multiplicity of infection (MOI) 1. At 72 h post-infection live cells were analyzed and the half maximal effective concentration (EC_{50}) to inhibit viral replication was determined. In parallel, to rule out that the observed antiviral effects were due to toxicity induced by compounds, the cytotoxic concentration (CC_{50}) was calculated by automatically counting cells of each individual well treated with antivirals.

In our phenotypic target-free HTS campaign for the discovery of novel anti-HCV chemical entities we identified among others



1 2 3

Figure 1. Structure of 1,3,4-trisubstituted pyrazoles exhibiting anti-HCV activity (**1**) and HCV RNA replication inhibitor (**2** and **3**).

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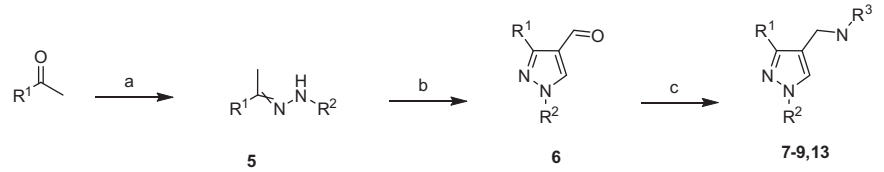
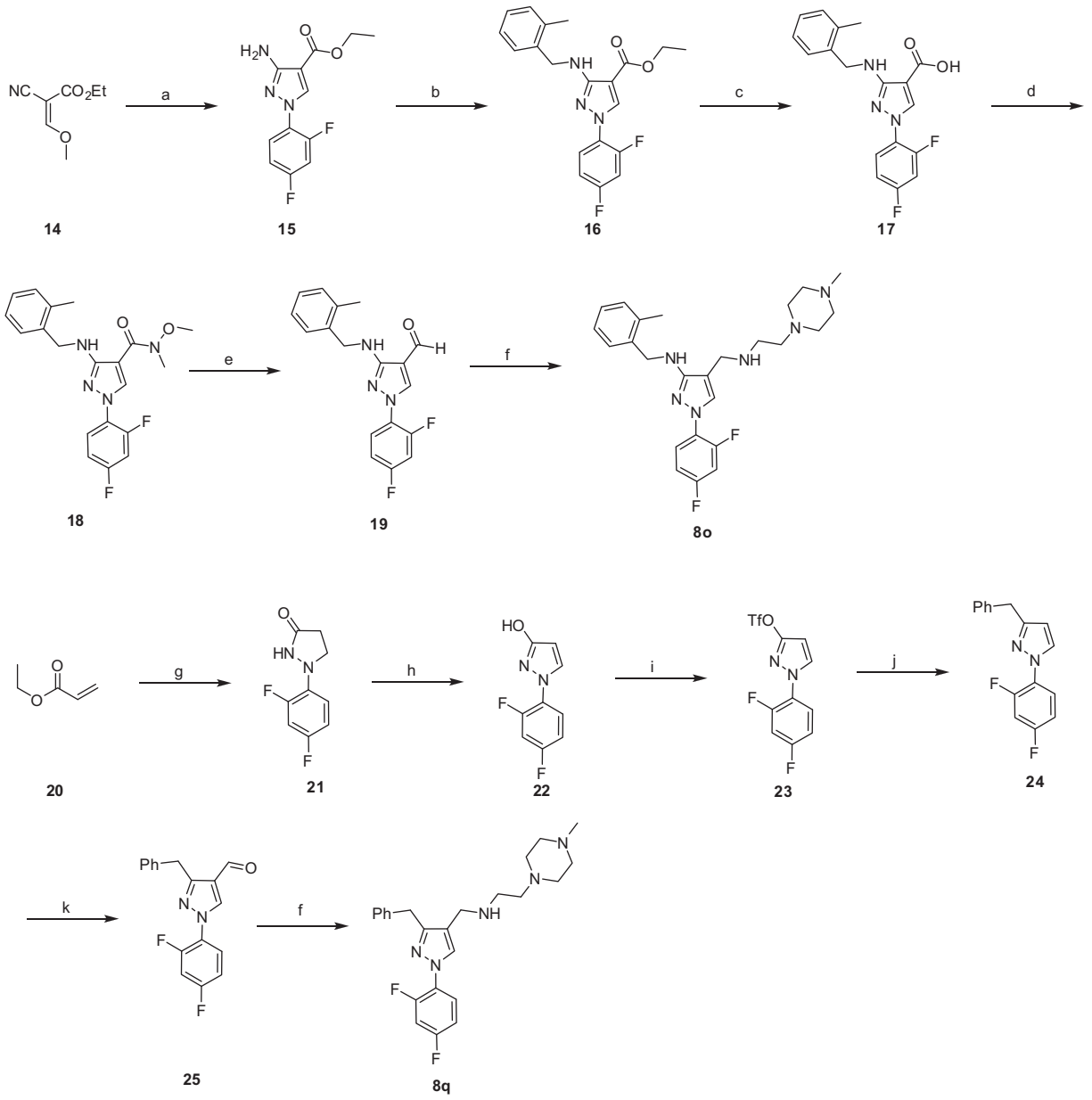
active compounds containing a 1,3,4-trisubstituted pyrazole core (Fig. 1 and **1**). Pyrazoles **1** were counter-screened in the HCV replicon system to filter out viral replication inhibitor. The pyrazole core is well-known structure with broad biological spectrum in drug discovery. Two pyrazole structures (**2**[12,13] and **3**[14]) have been previously reported to inhibit HCV RNA replication. However, pyrazoles **1** do not interfere with HCV RNA replication, indicating a novel mechanism of action (MoA), which led us to pursue extensive medicinal chemistry work to improve anti-HCV potency and also to characterize the MoA of pyrazoles. Here, we describe the identification of a series of pyrazole derivatives by conducting structure-activity relationship (SAR) studies with the pyrazole core

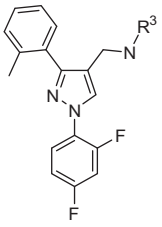


Scheme 1. Reagents and conditions: (a) R^2NHNH_2, NaOAc, EtOH, microwave, 130 °C, 1 h; (b) $POCl_3$, DMF, 80 °C, 3 h; (c) R^3NH, $NaBH(OAc)_3$, DCM, rt.

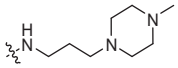
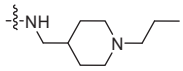
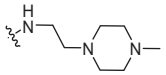
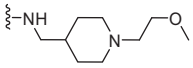
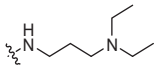
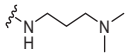
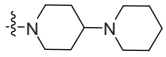
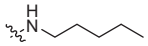
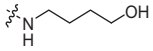
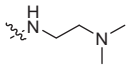
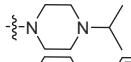
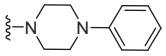
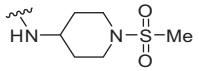
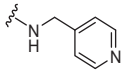
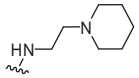
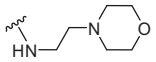


Scheme 2. Reagents and conditions: (a) 2,4-difluorophenyl hydrazine, NaOAc, AcOH, H_2O, microwave, 130 °C, 30 min, 86%; (b) 2-MeBnBr, NaH, DMF, rt, 4 h, 69%; (c) LiOH, H_2O/THF (1:3, v/v), rt, overnight, 77%; (d) *N*,*O*-dimethylhydroxyamine hydrochloride, EDC·HCl, HOBt, DMF, rt, overnight, 93%; (e) LAH, THF, −40 to 0 °C, 5 h, 86%; (f) 2-(4-methylpiperazin-1-yl)ethanamine, $NaBH(OAc)_3$, DCM, rt; (g) 2,4-difluorophenyl hydrazine, NaOAc, AcOH, H_2O, microwave, 130 °C, 30 min, 58%; (h) $FeCl_3$, air, DMF, 80 °C, 6 h, 96%; (i) Tf_2O, TEA, DCM, rt, 98%; (j) BnZnBr, $Pd(OAc)_2$, X-Phos, THF, 50 °C, overnight, 94%; (k) $POCl_3$, DMF, 80 °C, 3 h, 70%.

Table 1
Effect of amine on anti-HCV activity



7a-7q

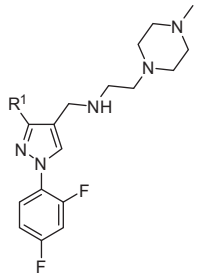
No.	NR^3	Anti-HCVcc (EC_{50}, μM)	Cytotoxicity (CC_{50}, μM)	SI (CC_{50}/EC_{50})
7a		2.2 ± 0.1	>20	>9
7b		2.3 ± 0.1	>20	>8.8
7c		2.8 ± 0.1	>20	>7.2
7d		3.0 ± 0.1	>20	>6.7
7e		4.3 ± 0.1	>20	>4.6
7f		6.7 ± 0.2	>20	>3.0
7g		7.5 ± 0.6	>20	>2.7
7h		18 ± 1	17 ± 1	1
7i		>20	>20	N.A.
7j		>20	>20	N.A.
7k		>20	>20	N.A.
7l		>20	>20	N.A.
7m		>20	>20	N.A.
7n		>20	>20	N.A.
7o		20	>20	>1
7p		>20	>20	N.A.

EC_{50} and CC_{50} values were determined by 10-point DRC analysis in duplicates with quadruplicate measurements. N.A.: not applicable.

to characterize its anti-HCV activity in the infectious HCV cell culture system.

To evaluate anti-HCV activity of pyrazoles **1**, we explored the effect of R^1, R^2, and R^3 group. A general procedure for the synthesis of 1,3,4-trisubstituted pyrazoles **1** is depicted in Scheme 1. Commercially available alkyl or aryl ketones were condensed with various hydrazines under microwave-assistant conditions to afford hydrazones **5**.[15] Subsequent cyclization–formylation of hydrazones **5** under Vilsmeier–Haack conditions gave pyrazole aldehydes **6** in good yield.[16] Reductive amination of the aldehyde **6** with various amines provided the desired 1,3,4-trisubstituted pyrazole derivatives **7**–**9** and **13**.

Table 2
Substituent effect on R^1

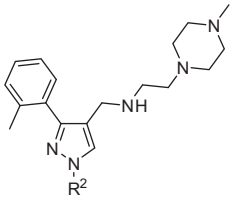


8

No.	R^1	Anti-HCVcc (EC_{50}, μM)	Cytotoxicity (CC_{50}, μM)	SI (CC_{50}/EC_{50})
8a	4-Cl–Ph	0.38 ± 0.70	19 ± 1	50
8b	4-CF_3O–Ph	0.36 ± 0.04	18 ± 1	50
8c	4-CF_3–Ph	0.54 ± 0.03	20 ± 1	37
8d	3-Cl–Ph	0.58 ± 0.04	15 ± 1	26
8e	4-Me–Ph	0.81 ± 0.02	>20	>25
8f	Cyclohexyl	1.2 ± 0.1	>20	>17
8g	4-Me_2N–Ph	1.3 ± 0.1	>20	>15
8h	Ph	1.6 ± 0.2	>20	>13
8i	3-Me–Ph	1.7 ± 0.2	>20	>12
8j	4-(Me_2NSO_2)–Ph	1.8 ± 0.1	>20	>11
8k	4-MeO–Ph	2.1 ± 0. 1	>20	>9.5
7c	2-MePh	2.8 ± 0.2	>20	>7.2
8l	3-Me_2N–Ph	3.2 ± 0.2	>20	>6.3
8m	2-Cl–Ph	3.3 ± 0.2	>20	>6.1
8n	4-(MeO_2C)–Ph	3.8 ± 0.1	>20	>5.3
8o	2-MeBnNH	4.3 ± 0.3	>20	>4.7
8p	*tert*-Bu	11 ± 1	>20	>1.8
8q	Bn	16 ± 1	>20	>1.3

EC_{50} and CC_{50} values were determined by 10-point DRC analysis in duplicates with quadruplicate measurements.

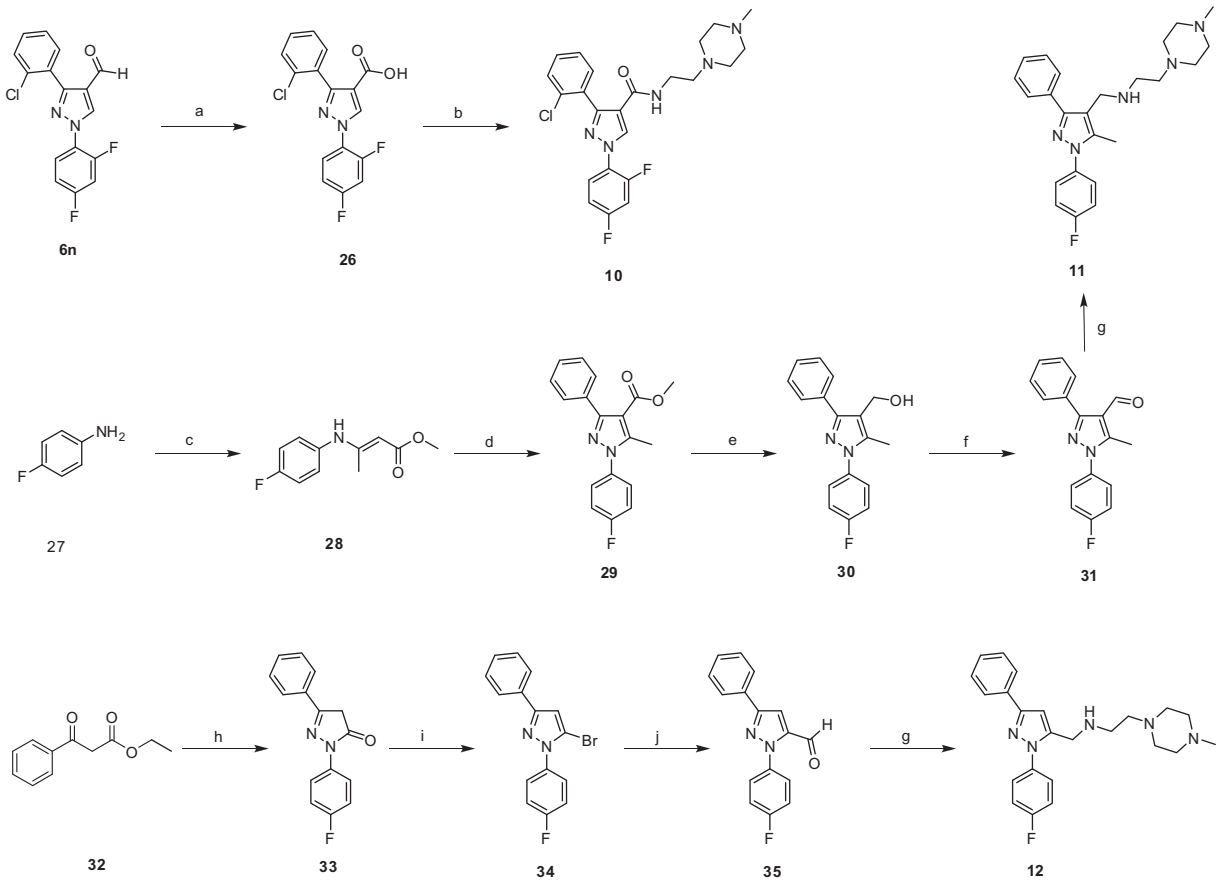
Table 3
R^2 effect on anti-HCV activity



9

No.	R^2	Anti-HCVcc (EC_{50}, μM)	Cytotoxicity (CC_{50}, μM)	SI (CC_{50}/EC_{50})
9a	4-CF_3–Ph	0.46 ± 0.04	11 ± 1	24
9b	4-NO_2–Ph	0.49 ± 0.05	>20	>41
9c	4-Cl–Ph	0.65 ± 0.04	17 ± 1	26
9d	4-MeS–Ph	0.67 ± 0.06	17 ± 1	25
9e	4-CF_3O–Ph	0.90 ± 0.75	17 ± 1	19
9f	3-CF_3–Ph	0.91 ± 0.02	18 ± 1	20
9g	3-Cl–Ph	1.0 ± 0.1	16 ± 1	16
9h	4-Br–Ph	1.2 ± 0.1	9.6 ± 1	8.0
9i	2,4-Me_2–Ph	2.4 ± 0.1	>20	>8.3
9j	2,5-F_2–Ph	2.5 ± 0.1	>20	>8.0
9k	3,4-F_2–Ph	2.6 ± 0.1	>20	>7.7
6c	2,4-F_2–Ph	2.8 ± 0.2	>20	>7.1
9l	4-MeO–Ph	2.8 ± 0.1	>20	>7.1
9m	4-F–Ph	3.7 ± 0.2	>20	>5.4
9n	2-Cl–Ph	3.8 ± 0.2	>20	>5.3
9o	Bn	7.5 ± 0.8	>20	>2.3
9p	4-NH_2–Ph	10 ± 0.2	>20	>2
9q	4-F–Bn	10 ± 1	>20	>2
9r	*i*-Pr	>20	>20	N.A.
9s	EtOAc	>20	>20	N.A.

EC_{50} and CC_{50} values were determined by 10-point DRC analysis in duplicates with quadruplicate measurements. N.A.: not applicable.



Scheme 3. Reagents and conditions: (a) $NaClO_2$, sulfamic acid, H_2O, acetone, 0 °C, 3 h, 80%; (b) 2-(4-methylpiperazin-1-yl)ethanamine, EDC·HCl, HOBt, DMF, rt; (c) methyl acetoacetate, $InBr_3$, 4 Å molecular sieves, MeOH, rt,18 h, 91%; (d) benzonitrile, $Cu(OAc)_2$, air, 120 °C, microwave, 1 h, 64%; (e) LAH, THF, 0 °C to rt, 3 h, 89%; (g) IBX, DMSO, 80 °C, 2 h, 64%; (g) 2-(4-methylpiperazin-1-yl)ethanamine, $NaBH(OAc)_3$, DCM, rt; (h) NaOAc, EtOH, microwave, 150 °C, 30 min, 62%; (i) PBr_3, MeCN, microwave, 150 °C, 77%; (j) *n*BuLi/THF, DMF (cat.), −78 °C to rt, 1 h, 79%.

Compound **8o** bearing 2-methylbenzylamine moiety at 3-position of the pyrazole was synthesized from intermediate **15** which was prepared by condensation of 2-cyano-3-methoxyacrylate **14** and 2,4-difluorophenylhydrazine under microwave-assistant condition.[17] Intermediate **15** was alkylated with 2-methylbenzyl bromide to give pyrazole **16**. Subsequent reactions of pyrazole **16** through hydrolysis, Weinreb amide formation, and reduction gave pyrazole-4-aldehyde **19**. The aldehyde **19** was subjected to reductive amination condition to afford the desired compound **8o**.

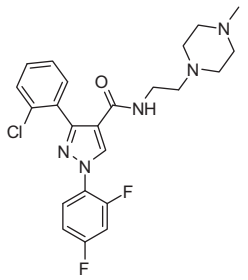
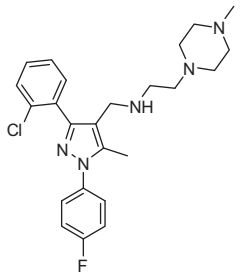
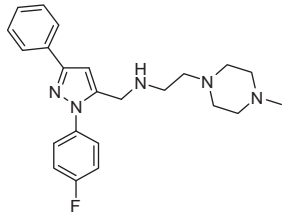
Compound **8q** was synthesized from 3-hydroxypyrazole **22** in four steps. 3-Hydroxypyrazole **22** was prepared from ethyl acrylate **20** by condensation with hydrazine under microwave assistance, followed by oxidation with $FeCl_3$ and air.[18] Hydroxyl **22** was converted to the corresponding triflate **23** by reaction with triflic anhydride. Following palladium-catalyzed Negishi coupling condition[19] of pyrazole triflate **23** with benzyl zinc bromide generated pyrazole **24**, which was then subjected to sequential Vilsmeier formylation and reductive amination step to give the desired product **8q** (Scheme 2).

Pyrazole-4-carboxamide **10** was prepared by oxidation of carboxaldehyde **6n**, followed by amide coupling condition. 5-Methyl substituted pyrazole **11** was generated by following Neumann et al.[20] Enamine **28** prepared from aniline and methyl acetoacetate with catalytic InBr was coupled with benzene nitrile under copper acetate, air, and microwave irradiation condition to give carboxylic ester pyrazole **29**. The ester group of pyrazole **29** was reduced by LAH to give alcohol **30**, which was afterwards oxidized by IBX to afford aldehyde pyrazole **31** as a precursor of compound **11** generated by the following reductive amination. 5-(Aminomethyl)-pyrazole **12** was prepared from ethyl benzoylacetate **32** in four steps. Benzoylacetate **32** was condensed with hydrazine to give pyrazol-one **33**.[21] Subsequent bromination with PBr_3[22] and formylation by nBuLi[23] generated pyrazole-5-carboaldehyde **35** which was converted to the desired product **12** by the following reductive amination.

All synthesized compounds were evaluated for their ability to inhibit the HCV life cycle and cytotoxicity as described earlier. First we evaluated anti-HCV activity depending on NR^3 group with various amines (Table 1). Several compounds (**7a**–**7g**) showed a single-digit micromolar (μM) antiviral activity (EC_{50} = 2.2−7.5 μM) without cytotoxicity in the testing range (CC_{50} >20 μM). Among them compounds **7a** and **7b** were the most potent molecules with 2.2 and 2.3 μM EC_{50} value, respectively. The anti-HCV activity of this series appeared to be significantly influenced by NR^3 substitutions. With the exception of compound **7n** bearing a pyridine moiety, only amines that have two or three basic nitrogen atoms and at least three carbon units between two terminal nitrogen atoms showed antiviral activity.

We further investigated the effect of R^1 group on pyrazoles; therefore, we used a 2,4-difluorophenyl for the R^2 group and a (4-methylpiperazino)ethylamine for the NR^3 group (Table 2). In this series, most of compounds (**7c** and **8a**–**8o**) showed good activities with EC_{50} values below 10 μM, except the *tert*-Bu and Bn substituent (**8p** and **8q**). In addition, electron-withdrawing groups (EWG) at *para*-position in phenyl ring are more favorable than electron-donating groups (EDG) (4-Cl, 4-CF_3O, 4-CF_3 > 4-Me >

Table 4
Anti-HCV activity of compounds **10**–**12**

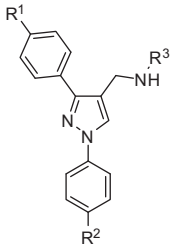
No.	Structure	Anti-HCVcc (EC_{50}, μM)	Cytotoxicity (CC_{50}, μM)
10		>20	>20
11		8.5 ± 0.8	>20
12		13 ± 1	>20

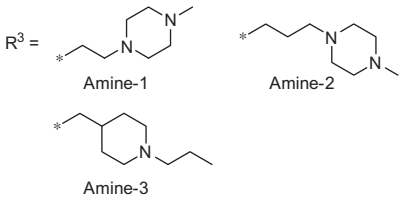
4-MeO). Compound **8j** and **8n** slightly reduced antiviral activities although having a strong EWG at *para*-position. Cyclohexyl compound **8f** was almost equipotent with phenyl compound **8h**, indicating that the cyclic system is favorable in comparison of *tert*-butyl **8p**, but the pi–pi interaction is not essential. Among this series tested, compounds **8a** and **8b** exhibited good inhibitory activity against HCV with acceptable selectivity index values (SI = 50)

Additionally, further substitution effects of R^2 on HCV inhibitory activities were explored and the data were summarized in Table 3. In this series, we fixed 2-methylphenyl for R^1 group and a (4-methylpiperazino)ethylamine for the NR^3 group. A set of pyrazoles in this series showed a broad activity range depending on substituents of R^2 group. Among mono-substituents, no clear correlation between antiviral activity and electronic effect (σ value) or hydrophobicity effect (π value) by substituents was observed.[24] However, the electron-withdrawing group is likely to be more favorable than the electron-donating group for antiviral activity. Among halides at 4-position in phenyl ring, the anti-HCV activity are Cl > Br > F in order. Benzyl substituted pyrazoles **9o** and **9q** reduced antiviral activity and no inhibitory activities were observed in aliphatic alkyl pyrazoles **9r**–**9s** (*i*-Pr and ethylacetate). Reducing nitro group **9b** to amine **9p** decreased anti-HCV activity by 20-fold.

With this preliminary SAR data in hand, we designed and synthesized additional pyrazole derivatives to elucidate essential elements in the pyrazole to be active against HCV (Scheme 3 and Table 4). To address the importance of methylene amine moiety ($–CH_2NR^3$) in pyrazoles, pyrazole-amide **10** was evaluated, and it was inactive in the testing range (EC_{50} >20 μM). Methyl substituted compound **11** at 5-postion of pyrazole slightly reduced anti-HCV activity (EC_{50} = 8.5 μM) compared to compound **6c**. Compound **12** bearing a methylene amine moiety at 5-position of the pyrazole also showed reduced antiviral activity with 13 μM EC_{50} value.

Table 5
SAR of 1,3,4-trisubstituted pyrazoles

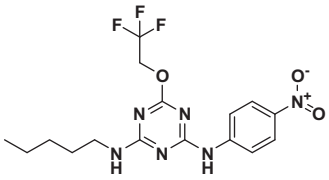
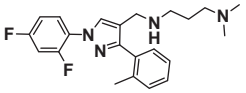
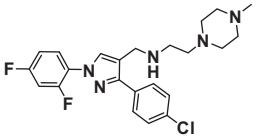




No.	R^1	R^2	R^3	Anti-HCVcc (EC_{50}, μM)	Cytotoxicity (CC_{50}, μM)	SI (CC_{50}/EC_{50})
12m	CF_3	NO_2	Amine 1	0.11 ± 0.02	3.2 ± 0.1	29
12n	CF_3	NO_2	Amine 2	0.12 ± 0.03	4.9 ± 0.1	41
12b	Cl	NO_2	Amine 2	0.13 ± 0.02	3.7 ± 0.1	28
12o	CF_3	NO_2	Amine 3	0.15±0.02	2.3±0.1	15
12a	Cl	NO_2	Amine 1	0.29±0.05	5.0±0.1	17
12f	Cl	CF_3	Amine 3	0.29±0.04	1.9±0.1	6.7
12c	Cl	NO_2	Amine 3	0.30±0.01	11±1	37
12r	CF_3	CF_3	Amine 3	0.33±0.06	1.2±0.6	3.6
12h	CF_3O	NO_2	Amine 2	0.34±0.04	1.6±0.3	4.6
12d	Cl	CF_3	Amine 1	0.36±0.03	3.0±0.1	8.4
12e	Cl	CF_3	Amine 2	0.36±0.03	2.5±0.1	6.9
12q	CF_3	CF_3	Amine 2	0.38±0.07	2.0±0.1	5.3
12g	CF_3O	NO_2	Amine 1	0.4±0.04	4.4±0.2	11
12l	CF_3O	CF_3	Amine 3	0.41±0.05	2.2±0.1	5.3
12j	CF_3O	CF_3	Amine 1	0.44±0.07	3.0±0.4	6.9
12p	CF_3	CF_3	Amine 1	0.49±0.05	1.5±0.1	3.1
12i	CF_3O	NO_2	Amine 3	0.60±0.06	18±1	30
12k	CF_3O	CF_3	Amine 2	0.75±0.03	2.3±0.1	3.1

EC_{50} and CC_{50} values were determined by 10-point DRC analysis in duplicates with quadruplicate measurements. Compounds were sorted by EC_{50} values.

Table 6
Determination of antiviral activity of compound **7c** and **8a** in the HCVpp system

No.	Structure	Anti-HCVpp gt-1 (EC_{50}, μM)	Cytotoxicity (CC_{50}, μM)	VSVg-pp
EI-1		0.040	>25	Inactive
7f		6.1	>25	Inactive
8a		1.9	9.1	Inactive

EC_{50} and CC_{50} values were determined by 5-point DRC analysis with duplicate measurements.

On the basis of the evaluation of the initial series of pyrazoles, we further designed and synthesized a limited set of pyrazole compounds to improve anti-HCV activity and selectivity index. This study focused on a substitution pattern containing (i) 4-Cl or 4-CF_3 for R^1 group, (ii) 4-NO_2 or 4-CF_3 for R^2 group, and (iii) three selected amines as shown in Table 5. This set of compounds was synthesized as described in Scheme 1 and evaluated for antiviral activity and summarized in Table 5.

All compound synthesized in this series demonstrated significantly improved anti-HCV potency with sub-micromolar ranges. Among them compound **12m** was the most potent compound with 0.11 μM EC_{50}. However, the majority of compounds in this series was accompanied by increased cytotoxicity, thereby providing poor selectivity index values. The exceptions were compound **12n** and **12c**, showing moderate selectivity index values of 41 and 37, respectively.

By using the infectious HCV cell culture system during primary screening, facilitating to target the entire viral life cycle, the MoA and the targets of hit compounds are initially unknown. In order to identify which step of the viral life cycle is inhibited, we devised strategies to elucidate the MoA of the 1,3,4-trisubstituted pyrazole scaffold. We demonstrated that these pyrazoles do not interfere with HCV RNA replication by counter-screening in HCV replicon cell lines (pyrazole **1** EC_{50} >50 μM and CC_{50} >50 μM). In order to monitor the early entry steps in the HCV life cycle, inhibitory effects of pyrazoles were determined by using the HCV pseudo particle (HCVpp) system. HCVpp are pseudotyped retroviral particles expressing HCV envelope proteins E1 and E2.[25] In order to discriminate between non-specific entry and HCV specific entry inhibition pseudo particle expressing the vesicular stomatitis virus glycoprotein (VSVg-pp) were utilized. A previously described HCV entry inhibitor (EI-1)[26] was used as a positive control and the data were summarized in Table 6. Two selected compounds **7f** and **8a** were tested in the HCVpp system to demonstrate that the viral E1/E2 proteins are involved in the inhibitory effect of pyrazoles. Both compounds exhibited moderate inhibitory activity in the HCVpp assay with EC_{50} values of 6.1 and 1.9 μM, respectively, while these were inactive in the VSVg-pp assay, suggesting that these pyrazoles interfere specifically with HCV E1/E2-mediated viral entry.

In conclusion, a series of 1,3,4-trisubstituted pyrazoles was identified by our phenotypic HTS campaign using infectious HCVcc. Intensive SAR studies indicated that 1,3-diphenyl substitution with EWGs increased antiviral activity. In addition, amine side chains that contain two or three basic nitrogen atoms and at least three carbon units between two terminal nitrogen atoms are crucial for antiviral efficacy. Furthermore, based on HCVpp experiments, this pyrazole series appears to inhibit early entry steps in the viral life cycle as demonstrated by HCVpp experiments. Although anti-HCV potency of this series might be potentially further improved, the 1,3,4-trisubstituted pyrazole scaffold can be utilized as a novel entry inhibitor to elucidate the HCV life cycle.

Acknowledgments

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