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2-((4-Arylpiperazin-1-yl)methyl)benzonitrile Derivatives as Orally Available Inhibitors of HCV With a Novel Mechanism of Action

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KEYWORDS 2-((4-arylpiperazin-1-yl)methyl)benzonitrile, SAR, HCV entry inhibitor, synergistic effect, bioavailability.

ABSTRACT: Although the direct-acting antivirals revolutionized the HCV infection treatment in last decade, more efforts are need to reach the elimination of HCV in the absence of vaccine. 4-(Piperazin-1-yl)-2-((p-tolylamino)methyl)-benzonitrile (1) is a modest HCV inhibitor identified from an in-house screening using HCV-infected Huh7.5 cells culture. Starting from it, the chemical optimization afforded a new 2-((4-arylpiperazin-1-yl)methyl)benzonitrile scaffold with significantly increased antiviral activity against HCV. A highly effective HCV inhibitor **35** (**L0909**, EC_{50} = 0.022 μM, SI > 600) was identified by the SAR study. The biological study revealed that **L0909** could block HCV replication by acting on the HCV entry stage. The high sensitivity to clinical resistant HCV mutants and synergistic effect with clinical drugs were observed for this compound. The further pharmaceutical studies demonstrated **L0909** is long-lasting, oral available and low toxic *in vivo*. These results endowed **L0909** a promising HCV entry inhibitor for single or combinational therapeutic potential.

INTRODUCTION

Hepatitis C virus (HCV) is an enveloped plus-stranded RNA virus, which belongs to the family Flaviviridae and genus *Hepacivirus*. HCV infection is a major cause of chronic liver disease, which may develop into chronic liver fibrosis, cirrhosis and even to hepatocellular carcinoma. There are still 71 million HCV-infected patients globally and 1.75 million individuals are newly infected with HCV in 2015 and an estimated 390,000 people died from HCV infection.[1] The availability of all-oral and interferon-free direct-acting antivirals (DAAs) revolutionized the HCV infection treatment in last decade.[2, 3] Peptidomimetics, benzimidazole dimerics, and nucleoside analogs are major chemotypes that targeting HCV nonstructural (NS) protein 3/4A, 5A or 5B, respectively.[4-6] For each type of DAAs, there have been multiple new drugs (Table 1) approved by the US Food and Drug Adiministration (FDA) and European Medicines Agency (EMA).[7] The HCV DAA combinations strongly inhibited HCV replication with over 95% of sustained virologic response (SVR) rates and higher resistant barrier.[8-10] However, a prophylactic vaccine against HCV infection is still unavailable now.[11] Due to the error-prone nature of the HCV RNA polymerase, HCV will unavoidably develop drug-resistance during the treatment with DAAs, even with the highly effective and pan-genotypic fixed-dose combination of drugs.[12, 13] Moreover, virus clearance by DAAs could not produce adaptive immunity to HCV reinfection, which increased the risk of reinfection among people who inject drugs prisoners, and HIV-infected individuals.[14] Therefore, development of new antivirals with different antiviral mechanisms of action is still important to optimize current therapeutic

regimens for increasing the overall SVR in the Real World and accelerating the global eradication of HCV.

HCV subgenomic replicon system with deletion of HCV structural genes made an important contribution to high-throughput screening of HCV replication inhibitors and the success of DAAs.[15-17] In comparison, the virus-infected cell-based approach contains a full virus infectious cycle and allows the discovery of new molecules which targeting the virus entry, package or release steps. Based on the spontaneous and efficient replication of the full-length HCV genome JFH-1, the high-titer infectious HCV cell culture (HCVcc) system was established for research and development of both vaccines and drugs against HCV.[18, 19] JFH-1 became the standard clone for *in vitro* studies of HCV, and then several chimeras of other genotypes were constructed based on the backbone of JFH-1.[20] Besides of GS4.3 cells, a human hepatoma Huh-7 cell line carrying an HCV subgenomic replicon, we also used the HCVcc system with the chimeric genotype 2a (J6/JFH-1) to investigate the anti-HCV activity of synthetic compounds with expectation to find some molecules with novel mechanism of action (MoA) from that of DAAs.[21, 22] 4-(piperazin-1-yl)-2-((p-tolylamino)methyl)-benzonitrile (**1**, Figure 2) was identified from our in-house screening as a moderate HCV inhibitor in HCVcc system assay while it was not active in GS4.3 replicon assay. This indicated that its antiviral activity depends on the different MoA from the existing HCV drugs. Encouraged by this feature, we further performed a deep chemical optimization taken **1** as a hit. Here we described the design, synthesis, and SAR (structure-activity relationship) study of a new class of 2-((4-arylpiperazin-1-yl)methyl)benzonitriles

against HCV propagation for the first time. Meanwhile, the pharmacological and preclinical evaluation led to the identification of a highly effective, low toxic, and oral available HCV entry inhibitor.

RESULTS AND DISCUSSION

Design of new 2-((4-arylpiperazin-1-yl)methyl)benzonitrile scaffold with antiviral activity against HCV in HCVcc system. In order to evaluate the substitution effect of hit compound **1** on the anti-HCV activity, a small set of 4-substituted-2-(phenylaminomethyl)benzonitrile analogs **8**–**13** (Figure 1) was firstly designed and synthesized by bioisosteres replacement strategy. The 50% effective concentration (EC_{50}) of these compounds was initially calculated by determining the HCV RNA level in HCVcc using a real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) method. The 50% cytotoxic concentration (CC_{50}) was determined by MTT method in naïve Huh7.5 cells culture. As shown in Figure 1, N^4-methylation of piperazine ring afforded the comparable antiviral activity (**8**, EC_{50} = 1.62 μM) to the naked NH group (**1**, EC_{50} = 1.68 μM). However, when the piperazin-1-yl was changed into the morpholin-4-yl (**9**) or piperidin-1-yl (**10**), the antiviral activity was lost. These results indicated the N^4 atom of piperazine made the obvious contribution to the antiviral activity. In addition, *para*-chloro substitution on aniline ring (**12**, EC_{50} = 0.42 μM) is more active than the methyl (**8**, EC_{50} = 1.62 μM) or methoxy substitution (**11**, EC_{50} = 2.14 μM). Meanwhile, comparing to **8**, both antiviral activity and the selectivity index (SI) was dramatically increased when the NH group of aniline was methylated (**13a**, EC_{50} = 0.89 μM, SI = 92) and the piperazine

was replaced by a flexible chain of alkyldiamine (**13b**, EC_{50} = 0.18 μM, SI = 86). Therefore, both halogen substituent on *para*-position and alkylation of NH group of aniline are beneficial to antiviral potency.

Unfortunately, poor bioavailability (F < 5%) was observed for **8** and **13b** when either of which was administered to rats by oral gavage (Supporting information). We estimated that the low bioavailability was resulted from the scaffold but not the substituted groups. Therefore, we attempted to insert a piperazine between benzonitrile and aniline group of **13b** to afford a new 2-((4-arylpiperazin-1-yl)methyl)benzonitrile scaffold (Figure 1). In the meanwhile, a SAR (structure-activity relationship) study upon the new scaffold was performed to discover novel preclinical candidate compounds.

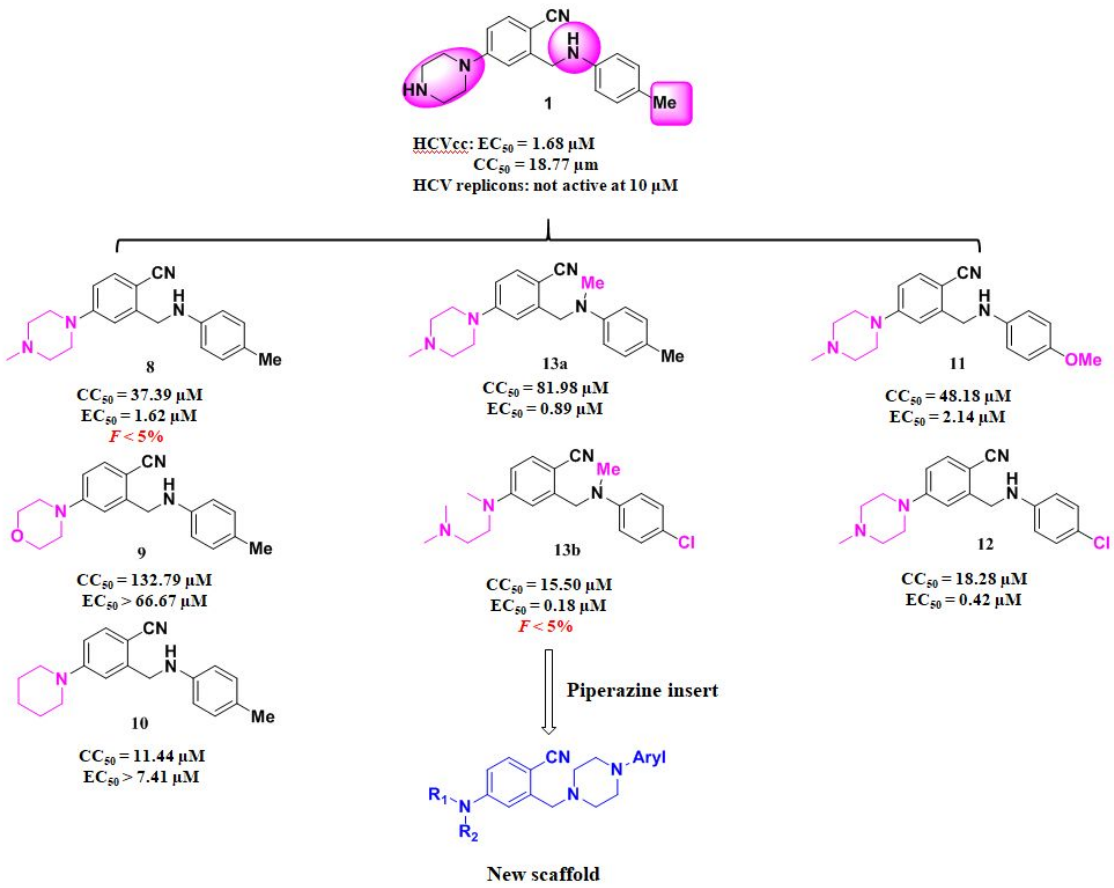
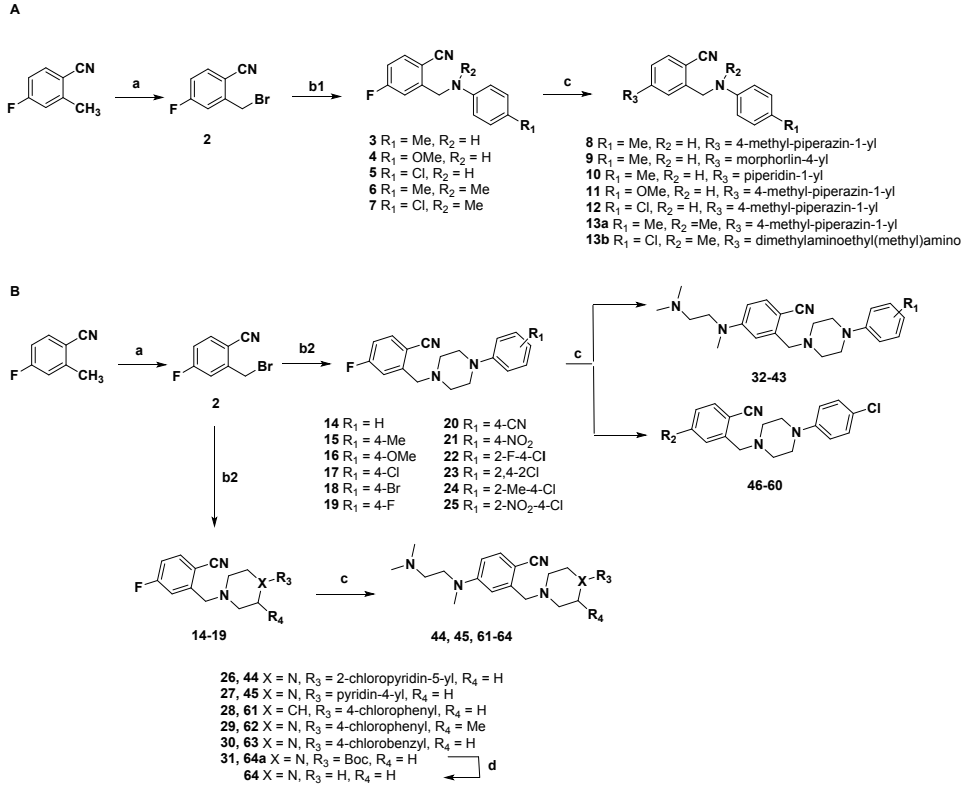


Figure 1. The chemical optimization from hit compound **1** led to a new 2-((4-arylpiperazin-1-yl)methyl)benzonitrile scaffold. The antiviral activity was determined in HCV-infected Huh7.5 cells. EC_{50} was calculated at RNA level detected with qRT-PCR and CC_{50} was detected with MTT assay. *F*, bioavailability.

Chemistry. As shown in Scheme 1A, starting from commercially available 4-fluoro-2-methylbenzonitrile, bromination of methyl group was done using N-Bromosuccinimide (NBS) in the presence of catalytic amount of *p*-toluenesulfonic acid (*p*-TSA) in CCl_4 to afford benzyl bromide intermediate **2** by yield above 80%. Then potassium carbonate (K_2CO_3) mediated nucleophilic substitution of **2** with different aromatic amine in dimethyl

sulfoxide (DMSO) at room temperature afforded the intermediate **3–7**. The resulted mixture was directly underwent nucleophilic aromatic substitution (SNAr) reaction in one-pot with corresponding alkylamine to afford 4-substituted- 2-(phenylaminomethyl)benzonitrile analogs **8–13** in the presence of K_2CO_3 in DMSO at 120°C by yield range of 42–64% (Synthesis procedure was shown in Supporting information).

Scheme 1. Preparation procedure of compounds 8–13 and 32–64[a]

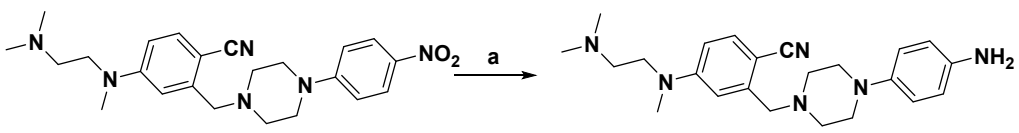


[a]Reagents and conditions: (a) NBS, p-TSA, CCl_4, reflux; (b1) substituted aniline, K_2CO_3, DMSO; (b2) substituted piperazine or piperidine, K_2CO_3, Acetone or ACN; (c) primary or second amine, K_2CO_3, DMSO, 120-140°C; (d) 2M HCl in ether, methanol.

New 2-((4-arylpiperazin-1-yl)methyl)benzonitrile compounds were easily prepared

from **2** by the similar method in Scheme 1A. Firstly, different substituted piperazine or piperidine was reacted with **2** in the presence of K_2CO_3 in acetone or acetonitrile (ACN) to afford the intermediates **14**–**31** in satisfied yield about 70%. Then, the SNAr reaction of intermediates **14**–**27** with N^1,N^1,N^2-trimethyl-1,2-ethyldiamine was performed under heating or microwave irradiation at 120–140°C in the presence of K_2CO_3 in DMSO to afford diverse analogs **32**–**45** in the moderate yield about 40% (Scheme 1B). These analogs were designed and synthesized to investigate the effect of R_1 at benzene ring or benzene self on anti-HCV activity. Similarly, compounds **46**–**60** were prepared by reaction of intermediate **17** with different nucleophilic amines in similar yield as **32**–**45** in order to compare the superiority of R_2. In addition, analog **61** and **62** were designed and synthesized through intermediates **28** and **29** in which the piperazine ring was replaced by piperidine or 3-methyl-piperazine, respectively. Compound **63** was correspondingly obtained from the reaction of **2** with 4-chlorobenzylpiperazine and the following SNAr reaction through intermediate **30** with N^1,N^1,N^2-trimethyl-1,2-ethyldiamine. Title compounds **64** were designed to investigate the contribution of phenyl group to the antiviral potency. Compound **64** was obtained in the presence of hydrochloride acid in methanol from the deprotection of Boc group from **64a**, which was prepared through the intermediate **31**.

Scheme 2. Synthesis of compound 65[a]

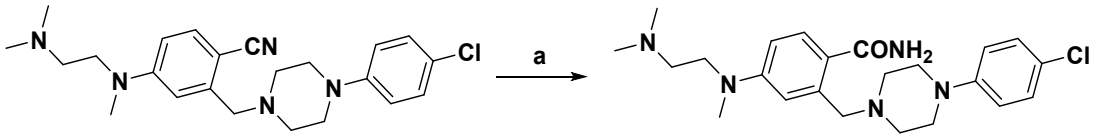


39 **65**

[a]Reagents and conditions: (a) $SnCl_2$, HCl, methanol.

In Scheme 2, reduction of nitro compound **39** conveniently afforded corresponding amino product **65** in the presence of stannous chloride and hydrochloric acid in methanol. In Scheme 3, compound **35** was hydrolyzed in the presence of hydrogen peroxide and sodium hydroxide in ethanol to afford amide derivative **66** by yield of 36%.

Scheme 3. Synthesis of compound 66[a]

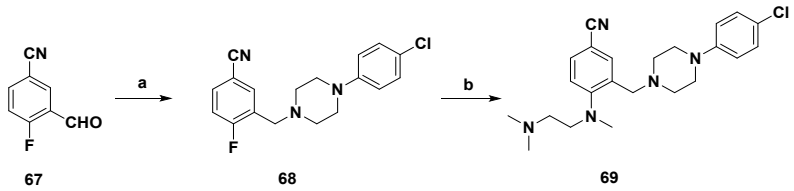


35 66

[a]Reagents and conditions: (a) NaOH, H_2O_2, ethanol/DMSO.

Taking 4-fluoro-3-formylbenzonitrile (**67**) as start material, $NaBH_3CN$ mediated reductive amination of aldehyde with 1-(4-chlorophenyl)piperazine afforded intermediate **68** by yield of 78%. The followed SNAr reaction of **68** with N^1,N^1,N^2-trimethyl-1,2-ethyldiamine afforded a 3-(4-arylpiperazin-1-yl)methylbenzonitrile derivative **69** by yield of 36.1% (Scheme 4).[23].In Scheme 5, reaction of 1-(bromomethyl)-3-iodobenzene (**70**) with 1-(4-chlorophenyl)piperazine easily afforded the intermediate **71** by yield of 79%, which reacted with N^1,N^1,N^2-trimethyl-1,2-ethyldiamine by Ullmann coupling in the presence of K_2CO_3, L-proline and CuI afforded cyano-absent compound **72** by yield of 41%.[24]

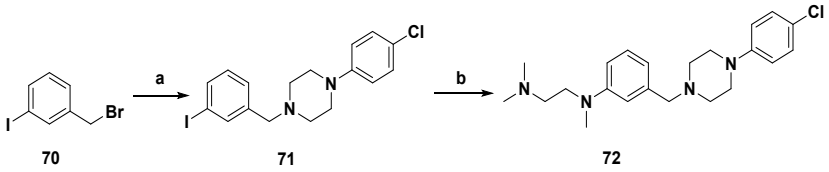
Scheme 4. Synthesis of compound 69[a]



67 68 69

[a]Reagents and conditions: (a) 1-(4-chlorophenyl)piperazine, $NaBH_3CN$, acetic acid, methanol; (b) N^1,N^1,N^2-trimethylethane-1,2-diamine, K_2CO_3, DMSO, 120°C.

Scheme 5. Synthesis of compound 72[a]



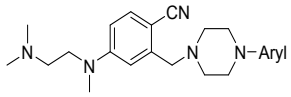
70 71 72

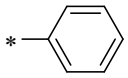
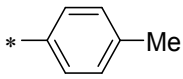
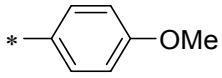
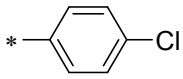
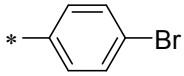
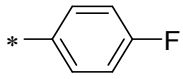
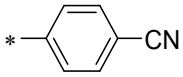
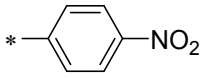
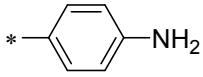
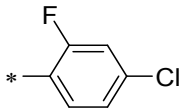
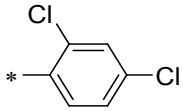
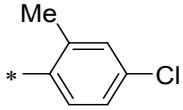
[a]Reagents and conditions: (a) 1-(4-chlorophenyl)piperazine, K_2CO_3, acetone; (b) N^1,N^1,N^2-trimethylethane-1,2-diamine, K_2CO_3, L-proline, CuI, DMSO, 90°C.

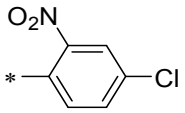
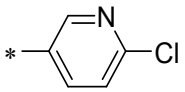
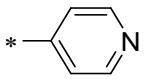
SAR study of 2-((4-arylpiperazin-1-yl)methyl)benzonitrile derivatives. In order to facilitate the rapid screening of a large number of compounds, we employed an In-Cell Western analysis instrument, which could automatically quantify the intracellular HCV protein level in 96-well plate, to determine the inhibitory effect of newly synthetic compounds on viral replication in HCVcc assay.[25] In-Cell Western analysis is much more labor-saving and cost-effective than classical qRT-PCR method. Both telaprevir (VX-950) and sofosbuvir was taken as positive drugs in this assay. Novel 2-((4-substitutedphenylpiperazin-1-yl)methyl)benzonitrile derivatives **32**–**45** as well as **65** were firstly designed and synthesized to investigate the substituent effect of Aryl group on antiviral potency when Aryl is benzene ring. These derivatives maintained the same

dimethylaminoethyl(methyl)amino side chain on the *para*-site of benzonitrile ring.

Table 1. *In vitro* inhibitory activity of 2-cyanobenzyl piperazine derivatives against HCV replication in Huh7.5 cells[a]



Compd.	Aryl	EC_{50} (μM)	CC_{50} (μM)	SI
13b	/	0.506±0.220	15.498±2.538	30.6
32		0.902±0.315	50.219±31.293	55
33		0.399±0.169	36.195±50.873	90
34		1.435±0.294	45.536±25.585	31
35		0.083±0.085	12.761±4.908	154
36		0.104±0.047	13.315±3.362	128
37		0.749±0.156	52.104±34.318	70
38		1.869±1.454	9.054±6.815	4
39		0.314±0.253	12.831±4.810	40
65		6.427±3.566	55.231±41.138	8
40		0.205±0.102	13.154±4.443	64
41		0.166±0.131	9.849±6.073	59
42		0.203±0.173	10.091±5.664	49

43		0.217±0.292	12.538±4.483	57
44		2.511±0.486	52.618±33.381	21
45		2.737±0.653	44.708±26.494	16
VX-950		0.146±0.040	55.024±27.164	377
Sofosbuvir		0.104±0.031	>200	>1917

[a] The EC_{50} and CC_{50} values were indicated as mean ± SD values which were calculated from three independent experiments. The EC_{50} was determined by In-cell western method with treatment time of 72 h.

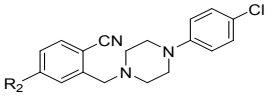
As shown in Table 1, lipophilic substituents such as methyl (**33**, EC_{50} = 0.399 μM) and halogen substitution (EC_{50} = 0.082, 0.104, and 0.749 μM for **35**-chloro, **36**-bromo, and **37**-fluoro, respectively) afforded enhanced activity comparing to unsubstituted derivative **32** (EC_{50} = 0.902 μM). Among them, **35** demonstrated the most superior selectivity index (SI) of 154. Moreover, the obvious decreased activity of 4-fluoro derivatives than chloro and bromo-substitution indicated that the halogen bond played a role to the antiviral potency. Conversely, those derivatives with lipophobic substituents like methoxy, cyano, or amino group exhibited weaker potency (EC_{50} = 1.435, 1.869, and 6.427 μM for **32**, **38**, and **65**, respectively) in comparison to **32**. Surprisingly, compound **39** with substitution of strong electron withdrawing nitro group exerted comparable strong antiviral activity (EC_{50} = 0.314 μM) as methyl substitution compound **33**. When the benzene was changed into pyridine, the antiviral activity of corresponding derivatives was decreased of more than 20-

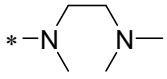
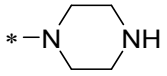
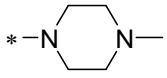
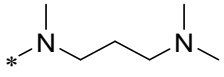
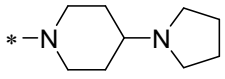
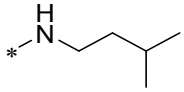
fold comparing to **35** no matter the chloro-substitution was reserved (**44**) or not (**45**). Based on above results, we designed to introduce another substituent into C2-position of the 4-chlorophenyl fragment with expectation to further increase the efficacy. However, the higher EC_{50} and lower SI values of these derivatives (**40**–**43**) were observed comparing to **35** although the selected substituent was validated as beneficial one in *para*-position of benzene ring. Therefore, we intended to maintain the 4-chlorophenyl for Aryl group within the chemical structure of subsequent compounds.

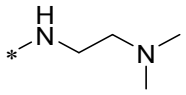
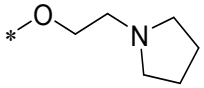
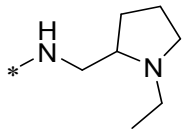
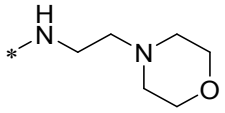
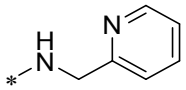
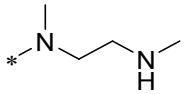
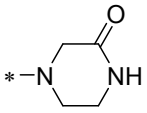
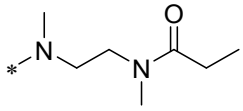
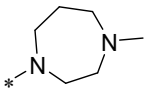
When we fixed the scaffold as shown in Table 2, a series of novel 2-((4-chlorophenyl)piperazin-1-yl)methylbenzonitrile derivatives (**46**–**60**) was designed and synthesized to explore the influence of *para*-substituent on the benzonitrile on the antiviral activity. Comparing to dimethylaminoethyl(methyl)amino group of **35**, more rigid cyclic piperazine group (**46**, **47**) decreased the activity by 5–6 folds. When ethyl-1,2-diamine was lengthened into propyl-1,3-diamine (**48**), a 3-fold more decreased antiviral activity was observed. However, the activity of compound **49** is between **35** and **48**, which probably indicated a requirement for the length between two nitrogen atoms of R_2 group. The activity was almost lost when ethyl-1,2-diamino was replaced by dimethylamino **50** or methylbutylamino **51**. This indicated that the terminal N atom of alkyldiamino group is important for antiviral potency. The removal of methyl group from the initial N of ethyl-1,2-diamine (**52)** or replacing this N atom with O atom (**53**) resulted in a folds of decreasing in both antiviral activity and SI value, which might reveal the role of basicity of this N atom. Meanwhile, the comparison among **54**–**56** demonstrated that the bulky substituents

on the terminal N atom would damage the biological activity. The removal of methyl substitution from the terminal N atom of ethyl-1,2-diamine led to a slightly decreased potency (**57**, EC_{50} = 0.289±0.156 μM) against HCV replication in Huh7.5 cell culture. However, modifying the terminal N atom of R_2 into the amide (**58**, **59**) significantly reduced the antiviral activity. Hence, we referred that the nucleophilicity of terminal N atom is beneficial to the drug-target interaction. Interestingly, the antiviral activity was regained when six-membered piperazine (**47**, EC_{50} = 0.783±0.292 μM) was replaced with seven-membered diazacycle (**60**, EC_{50} = 0.153±0.057 μM).

Table 2. *In vitro* inhibitory activity of 4-substituted 2-((4-(4-chlorophenyl)piperazin-1-yl)methyl)benzonitriles against HCV replication in Huh7.5 cells[a]



Compd.	R_2	EC_{50} (μM)	CC_{50} (μM)	SI
35		0.125±0.094	13.918±3.923	111
46		0.657±0.401	13.749±4.889	20
47		0.783±0.292	16.103±3.262	20
48		0.423±0.078	15.576±2.199	36
49		0.318±0.042	13.477±4.017	42
50		57.519±26.727	>200	>3
51		118.954±31.514	>200	>1

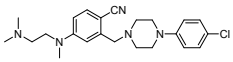
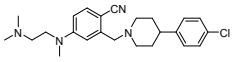
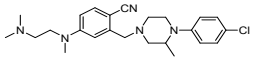
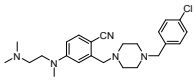
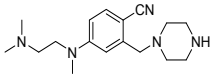
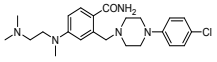
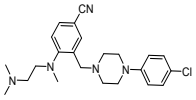
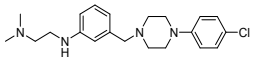
52		0.952±0.102	14.819±4.735	15
53		0.814±0.193	16.607±2.170	20
54		0.454±0.224	7.641±2.997	16
55		4.694±1.141	76.309±34.987	16
56		13.705±6.934	>200	>14
57		0.289±0.156	15.806±3.003	54
58		79.380±36.266	>200	>2
59		7.293±2.295	40.006±16.199	5
60		0.153±0.057	12.501±5.547	81

[a] The EC_{50} and CC_{50} values were indicated as mean ± SD values which were calculated from three independent experiments. The EC_{50} was determined by In-cell western method with treatment time of 72 h.

Replacement of the piperazine with either piperidine (**61**) or 3-methylpiperazine (**62**) didn't afford a favorable potency comparing to the corresponding **35** (Table 3). In addition, the obvious decreased antiviral activity of compound **63** and **64** comparing to **35** also demonstrated that 4-phenylpiperazine fragment was crucial to the high potency against

HCV. Alternatively, hydrolysis of cyano into amide (**66**) and the removal of cyano (**72**) led to a dramatic drop of antiviral potency. As we predicted, both the activity and SI of compound **69** was low when the 4-phenylpiperazine fragment was shifted from *ortho*- into *meta*-position of benzonitrile.

Table 3. *In vitro* inhibitory activity of compounds 61–64, 66, 69, and 72 against HCV replication in Huh7.5 cells[a]

Compd.	Chemical structure	EC_{50} (μM)	CC_{50} (μM)	SI
35		0.125±0.094	13.918±3.923	111
61		0.142±0.115	3.750±1.650	26
62		0.321±0.166	13.727±4.368	42
63		1.051±0.354	15.638±3.331	14
64		35.011±11.124	>200	>5
66		1.155±0.872	15.485±1.936	13
69		1.326±0.468	14.612±2.373	11
72		0.936 ±0.408	13.552±4.912	14

[a] The EC_{50} and CC_{50} values were indicated as mean ± SD values which were calculated from three independent experiments. The EC_{50} was determined by In-cell western method with treatment time of 72 h.

The inhibitory effect on HCV RNA level in HCVcc assay. To further evaluate the anti-HCV potential of this new chemotype, the intracellular HCV RNAs were quantified with qRT-PCR method after treatment of the HCVcc cells with active compounds **35** and

36. As shown in Table 4, the calculated EC_{50} against HCV RNA content was 22, 31 and 46 nM for compound **35**, **36**, and positive drug sofosbuvir, respectively. **35** exhibited the most potent activity among the three test compounds. However, a dramatically higher SI was presented for sofosbuvir than our synthetic compounds because the cytotoxicity of sofosbuvir was overwhelmingly low (CC_{50} > 200 μM). Notably, compound **35** and **36** significantly inhibited HCV RNA synthesis in a dose-dependent manner (Figure 2). Moreover, both of them exerted the stronger suppression on HCV RNA at lower concentration level than sofosbuvir. In view of drug-like ability, we chose compound **35** as a potential candidate for further study.

Table 4. The effect of compound 35 and 36 at HCV RNA level in Huh7.5 cells[a]

Compd.	CC_{50} (μM)	EC_{50} (μM)	SI
35	14.77±2.07	0.022±0.005	671
36	15.60±1.00	0.031±0.007	503
Sofosbuvir	>200	0.046±0.014	> 4347

[a] The EC_{50} and CC_{50} values were indicated as mean ± SD values which were calculated from three independent experiments. The EC_{50} was determined by qRT-PCR method with treatment time of 72 h.

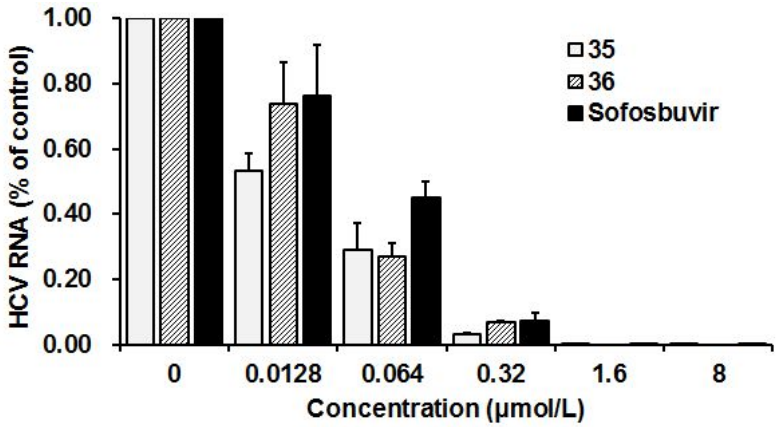


Figure 2. Compound **35** and **36** possessed high potency against HCV RNA replication in Huh7.5 cells. Intracellular HCV RNA was extracted and detected by qRT-PCR method after treatment of HCV-infected cells for 72 h with test compounds or reference drug sofosbuvir.

Investigation the effect of compound 35 (L0909) on HCV life cycle in Huh7.5 cells. The altered scaffold of compound **35** (named **L0909** hereafter) from original hit **1** made us perform a further investigation to confirm its effectiveness in HCV replicon system. When we treated GS4.3 HCV replicon cells with **L0909** in a serial of concentrations for 72 h, no inhibition was observed for all treatment groups, even at a concentration of 5.0 μM while the reference drug VX-950 (telaprevir), an HCV NS3 protease inhibitor, showed a strong inhibitory effect (Figure 3A). Moreover, **L0909** was ineffective in HCV replicon assay even though it was treated for 14 days at concentration of 0.1 or 0.5 μM (Figure 3B). These results suggested that **L0909** might not interrupt the intracellular HCV RNA replication in the replicon GS4.3 cells. The mechanism of **L0909** against HCV replication is different for those of known DAAs, and might involve the viral attachment / entry steps or late stages

in the HCV life cycle.

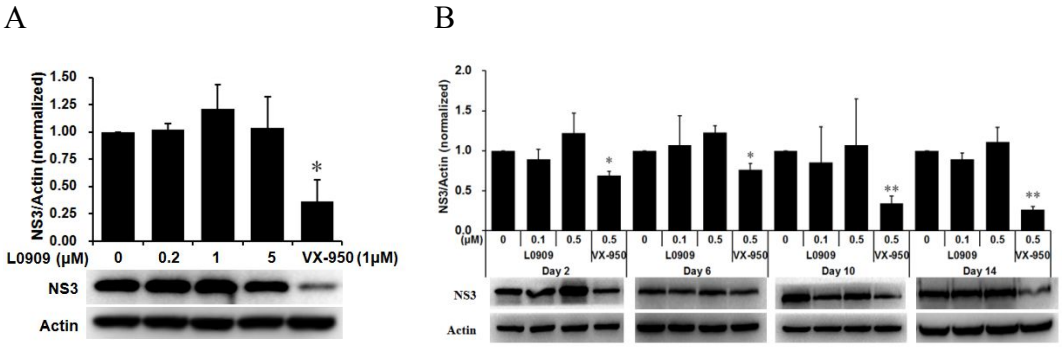


Figure 3. **L0909** was ineffective against HCV replication in HCV replicon (GS4.3) cells after exposure time for 72 h (A) or 14 days (B). Intracellular proteins were extracted and detected with Western Blot assay, and VX-950 was taken as a positive control. The protein bands showed the results of a representative experiment. The data presented are mean ± standard deviation. Student's *t-test* was used. **p*< 0.05 and ***p* < 0.01 *vs* solvent control.

For further investigating which stages of HCV life cycle was interrupted by **L0909**, Huh7.5 cells were treated with **L0909** prior to, during or after HCV viral incubation (Figure 4A). At 72 h, the proteins were detected with Western Blot. Firstly, pre-treatment of **L0909** is ineffective (Figure 4B). The most dramatic anti-HCV activity was observed when **L0909** was simultaneously administered during the inoculation of HCV (Figure 4C). Comparatively, post-treatment only induced relative weak effect on HCV replication (Figure 4D). The results suggested that **L0909** might act on the early stage of the viral life cycle or virus entry stage.

A

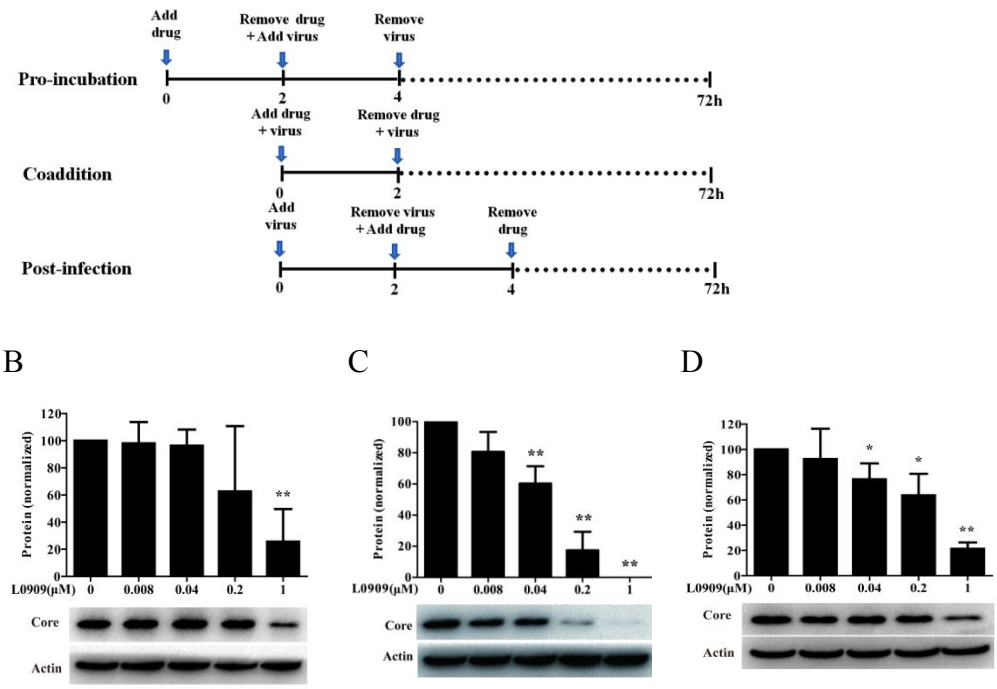


Figure 4. Time-of-addition experiment of **L0909** in HCV-infected Huh7.5 cells. (A) Schematic illustration of the experiment. (B-D) Huh7.5 cells were treated with **L0909** at 2 h prior to infection with HCV (B), during the inoculation of HCV (C), or 2 h after HCV infection (D). The proteins were detected with Western Blot at 72 h. The protein bands showed the results of a representative experiment. The data presented are mean ± standard deviation. Student's *t-test* was used. **p*< 0.05 and ***p* < 0.01 *vs* solvent control.

To further confirm above putative target step, the antiviral effect was investigated when Huh7.5 cells were infected with HCV and simultaneously treated with **L0909** for 4 h. Consequently, **L0909** exerted a significant anti-HCV efficacy in a dose-dependent manner (Figure 5A). However, VX-950 did not inhibit HCV replication. Furthermore, when shortened the incubation time less than 2 h to 15 minutes, **L0909** (1.0 μM) still displayed strong anti-HCV activity (Figure 5B). This further suggested that **L0909** might block the

HCV replication at the stage of viral entry into cells.

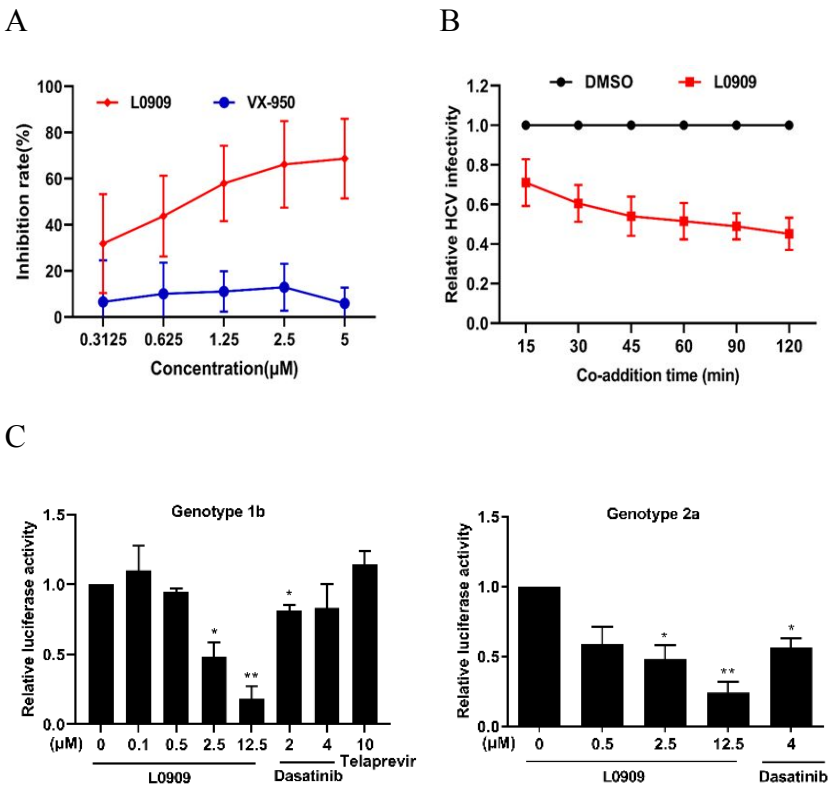


Figure 5. L0909 interrupted HCV replication at viral entry stage. Huh7.5 cells were infected with HCV and simultaneously incubated with the corresponding compound for 4 h (A) or incubated with compound (1.0 μM) for the indicated time (B). The supernatant was removed and replaced by fresh culture medium at the indicated time, and intracellular proteins were detected by In-Cell Western at 72 h. **L0909** inhibited genotype 1b (C, left) and 2a (C, right) HCVpp entry into Huh7.5 cells in a dose-dependent manner. *$p < 0.05$ and **$p < 0.01$ vs solvent control.

We also tested the inhibitory activity of **L0909** in HCV pseudoparticle (HCVpp) model. HCVpp is a viral-like particle with HCV glycoproteins incorporated into the viral envelope

and thus only the early steps of virus entry are similar to that in the HCVcc system.[26] Dasatinib is an HCV entry inhibitor targeting EphA2 protein.[27] Dasatinib showed slight inhibitory activity against genotype 1b and 2a HCVpp (Figure 5C) while the negative control telaprevir (VX-950) showed no activity in the HCVpp system even at a concentration of 10 μM (Figure 5C, left). **L0909** exhibited the inhibitory effect on HCVpp infection in a dose-dependent manner in two HCVpp assays (Figure 5C, left and right), suggesting that **L0909** indeed is an HCV entry inhibitor.

To explore whether **L0909** would directly affect the cell-free virions to abolish subsequent HCV infection, HCV viral stock was pre-incubated with **L0909** for 2 h and then incubated with Huh7.5 cells, intracellular proteins were extracted and quantified by western blot at 72 h. However, the additional pre-incubation of **L0909** with HCV virions did not significantly affect the HCV infectivity (Figure 6A), suggesting that **L0909** might not directly inactivate the viral particles.

Besides, to further investigate the mechanism of action of **L0909**, we carried out a resistance selection experiment.[28] Wild-type (WT) HCV-infected cells were treated with **L0909** by the stepwise increasing concentrations from 0.02 μM to 5.0 μM. Over a period of 10 weeks passages, **L0909**-resistant HCV was induced. After sequencing, we found a potential mutant F291V (T1211G in genome) in HCV structural glycoprotein E1 (Figure 6B), which plays a key role in the process of HCV entry into cells. Meanwhile, synonymous nucleotide mutation in Core G511A and E2 G2000C, T2246C in genome were also detected. In order to precisely identify whether the F291V mutant is associated with drug-

resistance, F291V site mutant plasmid was constructed accordingly and the mutant infectious virus was prepared. Then the antiviral activity of **L0909** was detected using qRT-PCR. Indeed, **L0909** displayed an approximate 20-fold increasing of EC_{50} value against mutant virus comparing to that against WT virus (Figure 6C), suggesting that F291V mutation will impact the antiviral activity of **L0909**. The results indicated that **L0909** inhibited the HCV glycoprotein E1-mediated entry.

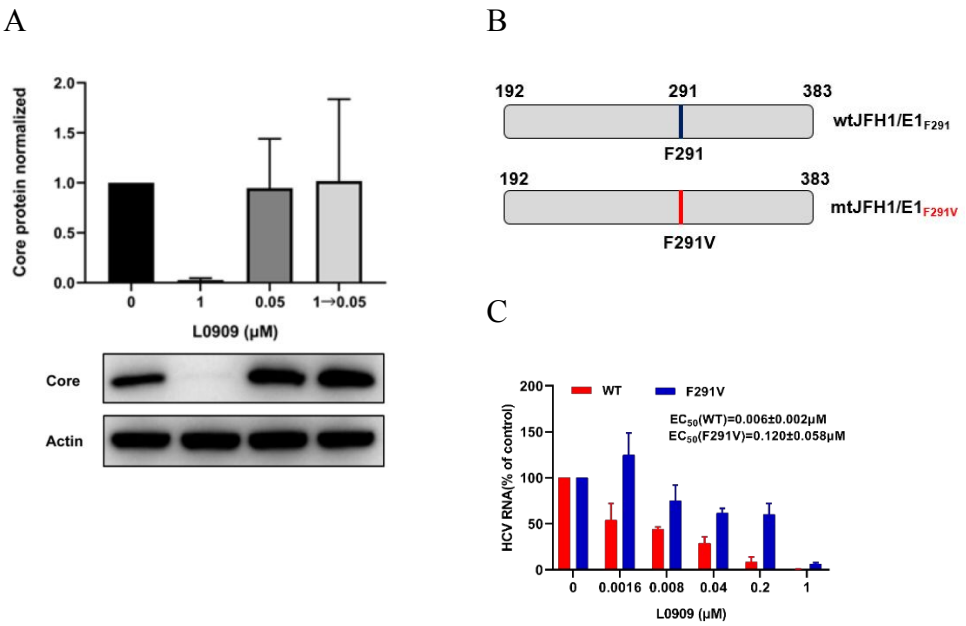


Figure 6. L0909 inhibited HCV glycoprotein E1-mediated entry. (A) **L0909** did not inactive HCV particle. Huh7.5 cells were incubated with HCV viral stock that was pre-treated with 1 μM of **L0909** for 2 h following diluted 20 times leading to a concentration of 0.05 μM of **L0909**. As controls, HCV viral stock was treated with 0.05 μM or 1 μM of **L0909**. Intracellular proteins were extracted and quantified by western blot at 72 h. (B) Schematic diagram of amino acid residues in glycoprotein E1 in wild type ($wtJFH1/E1_{F291}$)

and **L0909**-resistant (mtJFH1/E1$_{F291V}$) virus. (C) Huh7.5 cells were incubated with wild-type (WT) or F291V mutant virus and simultaneously treated with **L0909**, the intracellular RNA was detected with qRT-PCR at 72 h.

To determine whether **L0909** is a broad-spectrum antiviral agent or not, we tested the inhibitory activity of **L0909** towards other viruses, such as Influenza virus, Coxsakie virus, and Zika virus, of all which infection process include viral entry steps in cell culture models. As Table 5 indicated, **L0909** only displayed modest antiviral potency against Coxsakie and Zika viruses at micromole concentration. Moreover, no inhibition was found when **L0909** was administered to Influenza virus. The data indicated that **L0909** is a specific HCV entry inhibitor.

Table 5. Inhibitory activity of L0909 against Influenza, Coxsakie, and Zika virus *in vitro*[a]

Virus	Compound	CC_{50} (μM)	EC_{50} (μM)	SI
Influenza A	**L0909**	4.28	> 2.47	/
	Oseltamivir	> 640	2.14	> 299
Coxsakie B3	**L0909**	22.22	7.41	3
	Pleconaril	>1.05	0.002	> 525
Zika	**L0909**	18.39	5.15	3
	Ribavirin	> 200	33	> 6

[a] The EC_{50} was detected with CPE method and calculated with Reed-Muench method.

Inhibitory effect on mutant HCV variants. Distinct mechanism of action from DAAs might endow **L0909** less inclination of cross-resistance with current DAAs. We further confirmed this assumption by measuring the EC_{50} value of **L0909** in both wildtype and drug-resistant HCV infected cell cultures. A156T and D168V mutants in the NS3 protein, and S282T mutant in the NS5B protein are commonly HCV variants resistant to VX-950, simeprevir, and sofosbuvir, respectively. As expected, **L0909** kept similar potency against these drug-resistant viruses to the wild-type one **(**Table 6).

Table 6. The high potency of L0909 against drug-resistant HCV mutants[a].

Drugs	EC_{50} (μM)			
	WT	**A156T**	**D168V**	**S282T**
L0909	0.003	0.0019	0.03	0.0044
VX-950	0.0078	1.213		
Simeprevir	0.0092		>1	
Sofosbuvir	0.0294			0.1479

[a] the inhibitory activity was detected with qRT-PCR and calculated with Reed-Muench method. WT, Wild-Type.

Synergistic efficacy of L0909 with DAAs. The antiviral therapy needs the combination use of different type of antiviral agents due to high frequency of viral mutation. As a viral entry inhibitor, the antiviral mechanism of **L0909** is different from current DAAs. Taking simeprevir (NS3/4A protease inhibitor), daclatasvir (NS5A inhibitor) and sofosbuvir (NS5B polymerase inhibitor) as the representatives for current DAA types, we investigated

their antiviral activity of both single use of **L0909** (0.1 μM) and combining with these DAAs by measuring the HCV core protein level. The q value calculated by Webb method between single-drug treatment and combinational treatment was 1.05, 1.27 and 1.08 for simeprevir, daclatasvir and sofosbuvir, respectively (Figure 7). It suggested that **L0909** possessed the synergistic anti-HCV activity when combining with these DAAs.

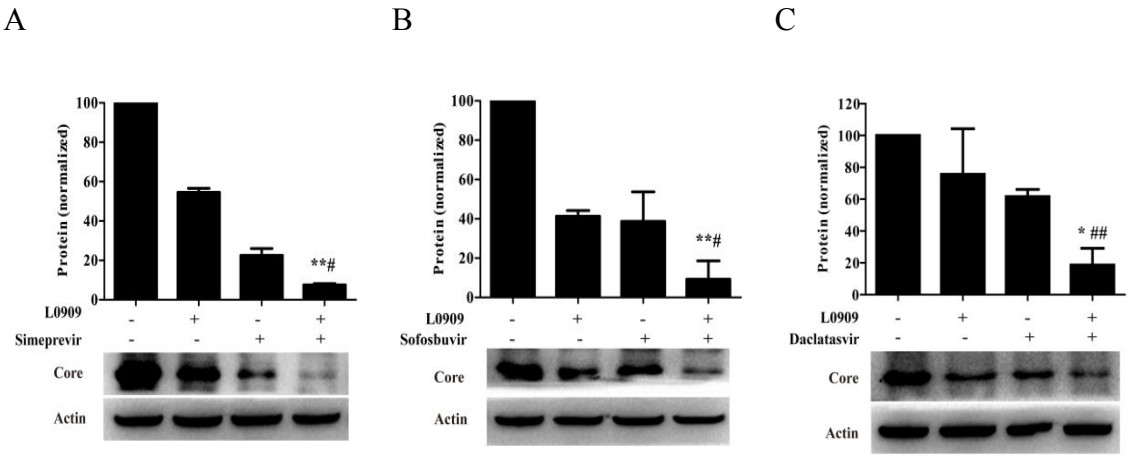


Figure 7. L0909 enhanced the inhibitory activities against HCV of DAAs. HCV-infected Huh7.5 cells were treated with **L0909** (0.1 μM) combined with 0.025 μM of simeprevir (A), 0.1 μM of sofosbuvir (B), or 0.016 nM of daclatasvir (C). At 72 h, intracellular proteins were analyzed with Western Blot assay. *p<0.05, **p<0.01, combination group *vs* single **L0909** group; $^{\#}p$<0.05, $^{\#\#}p$<0.01, combination group *vs* single positive drug group.

***In vivo* pharmacokinetic study.** To investigate the pharmacokinetics (PK) of **L0909** *in vivo*, we determined the plasma drug concentration at a serial of time points after single dose of **L0909** were administered to Sprague−Dawley (SD) rats or beagle dogs by vein injection (iv) or oral gavage (po) (Figure 8). **L0909** could be well absorbed by both rats

and dogs with T_{max} of 3 h after oral dose (Table 7). The oral C_{max} value of **L0909** after administration at 15 mg/kg dose to rats or dogs, was 500.72 ng/mL and 94.51 ng/mL, respectively, which is 54 or 10-fold of the antiviral EC_{50} value of **L0909** (EC_{50} = 22 nM) *in vitro*. Meanwhile, the *in vivo* elimination of **L0909** was slow with long half-life time ($T_{1/2}$) of more than 10 h for both rats and dogs. Moreover, the clearance (Cl) of **L0909** was significantly slower in rats than in dogs. Although the clearance of **L0909** was much quicker in the dogs comparing to in rats, the AUC value in dogs was acceptable due to its higher V_z value. The bioavailability (*F*) of **L0909** was calculated as 59% and 39%, respectively, for rats and dogs (Table 7). To be noted, the plasma concentration of **L0909** in both rats and dogs was still higher than the *in vitro* EC_{50} value at 24 h after oral administration. Moreover, **L0909** was still observed in rats with a concentration (C_{72} = 13.66 ng/mL) comparable to the *in vitro* EC_{50} value at 72 h after treatment.

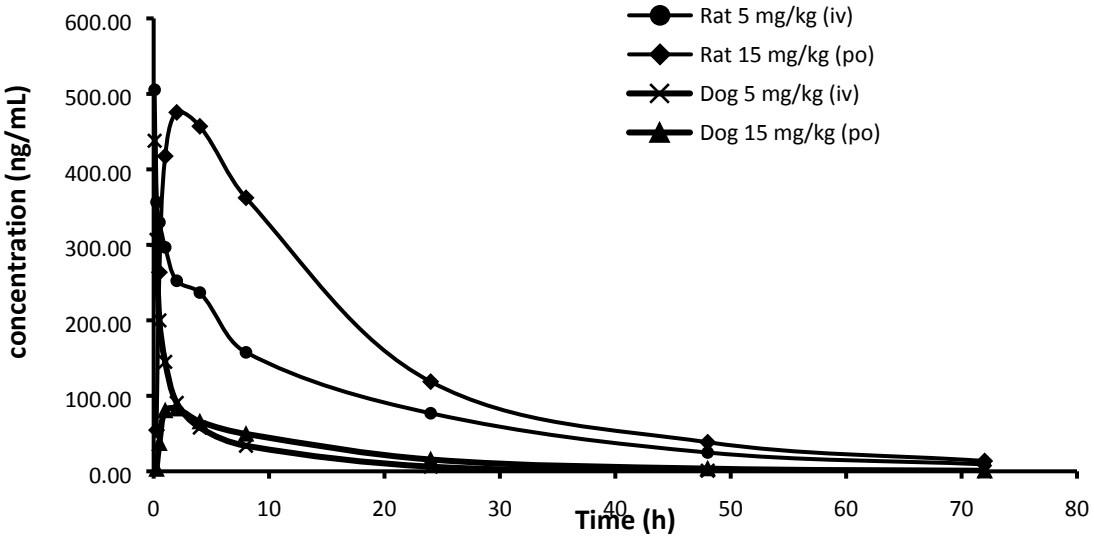


Figure 8. The concentration-time curve of **L0909** in SD rats and beagle dogs after

administration by tail vein injection (iv) or oral gavage (po).

Table 7. PK parameters of L0909 in rats and dogs[a]

Animal		**Rats (n = 12)**		**Dogs (n = 6)**	
Parameters	**Unit**	**iv**	**po**	**iv**	**po**
Dose	mg/kg	5	15	5	15
$\mathbf{T_{1/2}}$	h	14.34	12.83	7.28	12.72
$\mathbf{T_{max}}$	h	/	3.08	/	3
$\mathbf{C_{max}}$	ng/mL	616.99	500.72	531.93	94.51
$\mathbf{AUC_{(0\text{-}72h)}}$	h × ng/mL	5415.2	9589.46	1072.46	1275.09
$\mathbf{V_z}$	mL/kg	20057.05	34834.07	49181.42	238116.97
CL	mL/h/kg	1088.11	2246.3	4752.98	12025.86
$\mathbf{C_{24h}}$	ng/mL	76.96	118.78	6.16	15.62
$\mathbf{C_{72h}}$	ng/mL	9.2	13.66	/	0.69
F	%	59		39	

[a] the parameters were calculated according to non-compartment model. *F* is bioavailability.

***In vivo* toxicity.** To determine the safety of **L0909** *in vivo*, normal Kunming mice were divided into 4 groups (n = 6) and treated with blank solvent or single dose (250, 500, or 1000 mg/kg) of **L0909** by oral gavage for each group and were followed up for 7 days. None of the mice died and the body weight did not change in the **L0909** treated groups (Figure 9A). Blood samples were collected at the end of the experiment and examined for

liver and kidney functions. No significant abnormality was found in blood aspartate transaminase (AST), alanine transaminase (ALT), blood urea nitrogen (UREA) and creatine (CRE) after **L0909** administration, even at dose of 1000 mg/kg (Figure 9B). It appeared that the maximal tolerated dosage of **L0909** is above 1000 mg/kg.

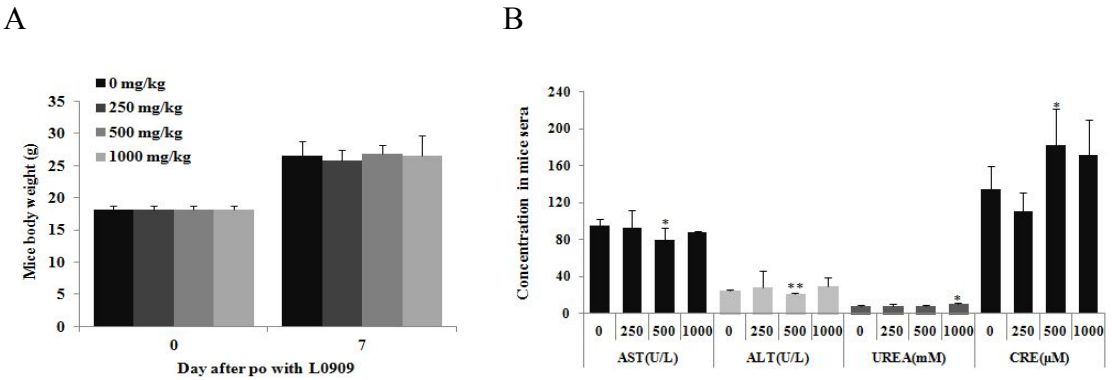


Figure 9. L0909 is low toxic *in vivo* (n = 6). Effect of intragastrical administration with **L0909** on mice survival and body weight (A), as well as liver and kidney functions (B). AST, aspartate transaminase (U/L); ALT, alanine transaminase (U/L); UREA, blood urea nitrogen (mM); CRE, creatinine (μM). *p <0.05, **p<0.01, *vs.* control group.

CONCLUSIONS

By introducing a piperazine into the structural center of 2-(arylamino)methylbenzonitrile hit, a new class of 2-((4-arylpiperazin-1-yl)methyl)benzonitrile derivatives was designed, synthesized, and evaluated for their inhibitory activity against HCV replication. SAR study revealed a highly potent preclinical candidate **L0909** which could inhibit HCV replication at nanomolar level in HCVcc system while no comparable antiviral activity observed in HCV replicon assay. Further virological

study demonstrated **L0909** inhibited the HCV replication by acting on the early stage of virus entry into host cells. Meanwhile, **L0909** and DAA combination produced the synergic efficacy against HCV replication. High sensitivity to both wild-type HCV and drug-resistant HCV variants was also observed for **L0909** at nanomolar concentration. *In vivo* pharmacokinetics and toxicity studies demonstrated that **L0909** was an oral absorbable, long-lasting, and low toxic HCV entry inhibitor. Moreover, the effective drug concentration was maintained to more than 24 h after **L0909** was administered into rats or dogs by single dose of 15 mg/kg. Although its exact MoA is unclear and the target-fishing study is ongoing now, current results have endowed **L0909** the single or combinational therapeutic potential for further development.

EXPERIMENTAL SECTION

Chemical materials and methods. All reagents and solvents used here are commercially available and used without further purification. The reaction was monitored by TLC silica gel 60 F254 aluminum sheets or LC-MS. Purification was performed on a CombiflashRf+ instrument (Teledyne, Lincoln, USA) with silica gel column. The purity of final compounds for antiviral assay is above 95% by UFLC-MS analysis. UPLC–MS instrument is a Shimadzu LC–MS 2020 system (Kyoto, Japan) equipped with s Shim-pack VP-ODS column (2.0 mm × 150 mm, 5 μm) and DAD detector, an electrospray ionization source (ESI) and a single-quadrupole mass analyzer. Two different methods were adopted to perform the UFLC separation at a flow rate of 0.4 mL/min: one method was an isocratic elution with 70% of solvent B (0.3% trifluoroacetic acid in methanol) and 30% of solvent

A (0.3% trifluoroacetic acid in water) for compound **45**, **64**, and **65** at a flow rate of 0.5 mL/min; another was a gradient elution of 10–90% of solvent B (0.1% formic acid in acetonitrile) in solvent A (0.1% formic acid in water) over 7 min. The DAD detector wavelength was set on 280 nm for compound **64** and 254 nm for other compounds, respectively. ^{1}H and ^{13}C NMR spectra were recorded in solution of Chloroform-*d*, Methanol-d_4, or DMSO-d_6 by a Bruker advance Ⅲ 400 MHz or 600 MHz spectrometer or WNMR-1 500MHz spectrometer (Wuhan Zhongke-Niujin, Wuhan, China). High-resolution mass spectra (HRMS) were recorded on a TripleTOF 5600+LC/MS/MS system (CADM-YQ-086) with an ESI mass selective detector.

*Preparation of 2-bromomethyl-4-fluorobenzonitrile (**2**).* To a solution of 4-fluoro-2-methylbenzonitrile (1.35 g, 10 mmol) in CCl4 (20 mL) was added 2.15g of NBS (12 mmol) and 0.25 g of *p*-TSA (0.015 mmol). The reaction mixture was stirred under reflux until 4-fluoro-2-methylbenzonitrile was absent. The reaction was cooled down and quenched with saturated NH_4Cl solution (20 mL). The organic phase was washed with deionized water and brine sequentially, dried over anhydrous $NaSO_4$, concentrated *in vacuo*. The crude was purified with column chromatography eluted with 10-30% of EA in petroleum to afford **2** (1.84 g, 86%) in colorless crystal. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.73 (dd, J = 8.6, 5.3 Hz, 1H), 7.43 – 7.27 (m, 1H), 7.17 (td, J = 8.1, 2.5 Hz, 1H), 4.64 (s, 2H).

*General procedure for preparation of intermediates **14–31**:* to a solution of commercially available 4-(hetero)arylsubstituted piperazine or piperidine (2.0 mmol) and potassium carbonate (2.5 mmol) in acetone or acetonitrile (10 mL), **2** (2.0 mmol) was added.

The reaction mixture was stirred at room temperature until **2** was absent. The reaction was filtered and the filtrate was concentrated *in vacuo*. The residue was extracted with ethyl acetate and deionized water (×3). The organic phase was washed with brine, dried over anhydrous $NaSO_4$, concentrated *in vacuo* to afford crude intermediates **26–31** and **14–25**.

*4-fluoro-2-((4-phenylpiperazin-1-yl)methyl)benzonitrile (**14**).* ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.71 (dd, *J* = 8.6, 5.3 Hz, 1H), 7.42 (d, *J* = 9.3 Hz, 1H), 7.32 (t, *J* = 7.8 Hz, 2H), 7.12 (td, *J* = 8.3, 2.7 Hz, 1H), 6.98 (d, *J* = 8.1 Hz, 2H), 6.91 (t, *J* = 7.3 Hz, 1H), 3.82 (s, 2H, CH_2), 3.28 (t, *J* = 4.9 Hz, 4H), 2.74 (t, *J* = 4.8 Hz, 4H).

*4-fluoro-2-((4-(4-methylphenyl)piperazin-1-yl)methyl)benzonitrile (**15**).* ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.71 (s, 1H), 7.41 (s, 1H), 7.13 (m, 3H), 6.90 (m, 2H), 3.82 (s, 2H), 3.24 (m, 4H), 2.75 (br, 4H), 2.32 (s, 3H).

*4-fluoro-2-((4-(4-methoxyphenyl)piperazin-1-yl)methyl)benzonitrile (**16**).* ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.70 (dd, *J* = 8.5, 5.4 Hz, 1H), 7.41 (d, *J* = 8.0 Hz, 1H), 7.11 (td, *J* = 8.2, 2.1 Hz, 1H), 6.95 (d, *J* = 8.9 Hz, 2H), 6.88 (d, *J* = 9.0 Hz, 2H), 3.81 (s, 5H), 3.16 (s, 4H), 2.74 (s, 4H).

*4-fluoro-2-((4-(4-chlorophenyl)piperazin-1-yl)methyl)benzonitrile (**17**).* ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.71 (dd, *J* = 8.6, 5.3 Hz, 1H), 7.41 (d, *J* = 10.0 Hz, 1H), 7.25 (d, *J* = 8.5 Hz, 2H), 7.12 (td, *J* = 8.1, 2.7 Hz, 1H), 6.88 (d, *J* = 8.8 Hz, 2H), 3.81 (s, 2H), 3.23 (t, *J* = 4.8 Hz, 4H), 2.73 (t, *J* = 5.0 Hz, 4H).

*4-fluoro-2-((4-(4-bromophenyl)piperazin-1-yl)methyl)benzonitrile (**18**).* ^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.02 – 7.93 (m, 1H), 7.51 (d, *J* = 9.3 Hz, 1H), 7.38 (dd, *J* = 20.0, 8.4

Hz, 3H), 6.91 (d, *J* = 8.4 Hz, 2H), 3.74 (s, 2H), 3.17 (br, 4H), 2.59 (br, 4H).

*4-fluoro-2-((4-(4-fluorophenyl)piperazin-1-yl)methyl)benzonitrile (**19**)*. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.71 (dd, *J* = 8.6, 5.3 Hz, 1H), 7.41 (d, *J* = 9.1 Hz, 1H), 7.12 (td, *J* = 8.3, 2.6 Hz, 1H), 7.01 (t, *J* = 8.5 Hz, 2H), 6.93 (dd, *J* = 9.0, 4.6 Hz, 2H), 3.82 (s, 2H), 3.19 (t, *J* = 4.8 Hz, 4H), 2.74 (t, *J* = 5.0 Hz, 4H).

*4-fluoro-2-((4-(4-cyanophenyl)piperazin-1-yl)methyl)benzonitrile (**20**)*. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.72 (dd, *J* = 8.3, 5.5 Hz, 1H), 7.55 (d, *J* = 8.7 Hz, 2H), 7.39 (d, *J* = 8.6 Hz, 1H), 7.14 (t, *J* = 7.2 Hz, 1H), 6.91 (d, *J* = 8.7 Hz, 2H), 3.81 (s, 2H), 3.40 (br, 4H), 2.72 (br, 4H).

*4-fluoro-2-((4-(4-nitrophenyl)piperazin-1-yl)methyl)benzonitrile (**21**)*. ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.16 – 8.10 (m, 2H), 7.69 (d, *J* = 2.5 Hz, 1H), 7.36 (s, 1H), 7.10 (d, *J* = 14.1 Hz, 1H), 6.83 (d, *J* = 9.4 Hz, 2H), 5.30 (s, 2H), 3.47 (s, 4H), 2.70 (s, 4H).

*4-fluoro-2-((4-(2-fluoro-4-chlorophenyl)piperazin-1-yl)methyl)benzonitrile (**22**)*. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.77 – 7.66 (m, 1H), 7.41 (s, 1H), 7.10 (dd, *J* = 13.9, 11.5 Hz, 3H), 6.90 (t, *J* = 8.7 Hz, 1H), 3.84 (s, 2H), 3.16 (s, 4H), 2.77 (s, 4H).

*4-fluoro-2-((4-(2,4-dichlorophenyl)piperazin-1-yl)methyl)benzonitrile (**23**)*. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.71 (t, *J* = 6.9 Hz, 1H), 7.39 (d, *J* = 11.5 Hz, 2H), 7.11 (s, 1H), 7.01 (d, *J* = 8.5 Hz, 1H), 3.82 (s, 2H), 3.10 (s, 4H), 2.75 (s, 4H).

*4-fluoro-2-((4-(2-methyl-4-chlorophenyl)piperazin-1-yl)methyl)benzonitrile (**24**)*. ^{1}H NMR (600 MHz, Methanol-d_4) δ 7.70 (dd, *J* = 8.6, 5.4 Hz, 1H), 7.34 (dd, *J* = 9.4, 2.4 Hz, 1H), 7.12 (td, *J* = 8.4, 2.3 Hz, 1H), 7.05 (d, *J* = 2.3 Hz, 1H), 7.01 (dd, *J* = 8.5, 2.4 Hz, 1H),

6.91 (d, *J* = 8.5 Hz, 1H), 3.71 (s, 2H), 2.88 – 2.76 (m, 4H), 2.61 (s, 4H), 2.18 (s, 3H).

4-fluoro-2-((4-(2-nitro-4-chlorophenyl)piperazin-1-yl)methyl)benzonitrile ***(25)***. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.82 (s, 1H), 7.77 – 7.66 (m, 1H), 7.49 (d, *J* = 8.7 Hz, 1H), 7.36 (d, *J* = 9.4 Hz, 1H), 7.12 (d, *J* = 10.5 Hz, 2H), 3.81 (s, 2H), 3.13 (t, *J* = 4.6 Hz, 4H), 2.73 (d, *J* = 4.7 Hz, 4H).

2-((4-(6-chloropyridin-3-yl)piperazin-1-yl)methyl)-4-fluorobenzonitrile ***(26)***. ^{1}H NMR (600 MHz, Methanol-d_4) δ 7.89 (d, *J* = 3.1 Hz, 1H), 7.70 (dd, *J* = 8.6, 5.4 Hz, 1H), 7.38 – 7.26 (m, 2H), 7.13 (ddd, *J* = 12.4, 11.0, 5.7 Hz, 2H), 3.68 (s, 2H), 3.19 – 3.13 (m, 4H), 2.60 (m, 4H).

4-fluoro-2-((4-(pyridin-4-yl)piperazin-1-yl)methyl)benzonitrile ***(27)***. ^{1}H NMR (500 MHz, DMSO-d6) δ 8.18 (d, *J* = 5.0 Hz, 2H), 8.04 – 7.92 (m, 1H), 7.52 (d, *J* = 9.4 Hz, 1H), 7.40 (t, *J* = 7.7 Hz, 1H), 6.85 (d, *J* = 5.2 Hz, 2H), 3.75 (s, 2H), 2.57 (m, 8H).

2-((4-(4-chlorophenyl)piperidin-1-yl)methyl)-4-fluorobenzonitrile ***(28)***. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.72 (dd, *J* = 8.6, 5.4 Hz, 1H), 7.40 (dd, *J* = 9.2, 2.7 Hz, 1H), 7.13 (td, *J* = 8.2, 2.6 Hz, 1H), 7.00 (d, *J* = 2.9 Hz, 1H), 6.78 (dd, *J* = 8.9, 2.8 Hz, 1H), 3.81 (s, 2H), 3.24 (t, *J* = 4.9 Hz, 4H), 2.72 (t, *J* = 4.8 Hz, 4H), 1.60 (s, 1H).

2-((4-(4-chlorophenyl)-3-methylpiperazin-1-yl)methyl)-4-fluorobenzonitrile ***(29)***. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.64 (q, *J* = 5.9, 5.3 Hz, 1H), 7.39 (d, *J* = 12.0 Hz, 1H), 7.18 (d, *J* = 9.6 Hz, 2H), 7.08 – 6.99 (m, 1H), 6.81 (d, *J* = 10.0 Hz, 2H), 4.15 (d, *J* = 14.9 Hz, 1H), 3.50 (d, *J* = 14.9 Hz, 1H), 3.38 (d, *J* = 9.5 Hz, 1H), 3.30 (d, *J* = 11.7 Hz, 1H), 2.91 (dd, *J* = 13.4, 11.0 Hz, 1H), 2.81 – 2.66 (m, 3H), 2.44 (dd, *J* = 13.5, 10.9 Hz, 1H), 1.19 (s,

3H).

*2-((4-(4-chlorobenzyl)piperazin-1-yl)methyl)-4-fluorobenzonitrile (**30**).* ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.68 (m, 1H), 7.41 – 7.24 (m, 5H), 7.09 (m, 1H), 3.75 (s, 2H), 3.52 (s, 2H), 2.59 (s, 4H), 2.52 (s, 4H).

*tert-butyl 4-(2-cyano-5-fluorobenzyl)piperazine-1-carboxylate (**31**).* ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.70 (dd, *J* = 8.6, 5.3 Hz, 1H), 7.37 (s, 1H), 7.11 (t, *J* = 8.4 Hz, 1H), 3.75 (s, 2H), 3.50 (s, 4H), 2.51 (s, 4H), 1.50 (s, 9H).

*Synthesis procedure of 2-((4-(4-chlorophenyl)piperazin-1-yl)methyl)-4-((2-(dimethylamino)ethyl)(methyl)amino)benzonitrile (**35**, **L0909**).* To a solution of intermediate **17** (4.0 g, 12.1 mmol) in DMSO (10 mL) was added N^1,N^1,N^2-trimethylethane-1,2-diamine (3.0 mL, 36 mmol) and K_2CO_3 (12 g, 87 mmol) in a sealed tube. The reaction mixture was stirred at 120 °C for 12 h until **17** was absent. The reaction was poured into deionized water (15mL), extracted with ethyl acetate (30 mL × 3). The organic phase was washed with water and brine, dried over anhydrous $NaSO_4$, concentrated *in vacuo*. The residue was purified with column chromatography eluted with 1–10% of methanol in DCM to afford **35** (2.4 g, yield: 48%) as white-like powder. ^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.44 (d, *J* = 8.7 Hz, 1H), 7.17 (t, *J* = 6.1 Hz, 2H), 6.88 – 6.77 (m, 3H), 6.56 (dd, *J* = 8.8, 2.6 Hz, 1H), 3.68 (s, 2H), 3.55 – 3.46 (m, 2H), 3.20 – 3.12 (m, 4H), 3.03 (s, 3H), 2.70 – 2.63 (m, 4H), 2.52 – 2.43 (m, 2H), 2.29 (s, 6H). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 151.52, 149.95, 143.15, 134.24, 128.86 (2C), 124.31, 119.57, 117.15 (2C), 111.90, 110.13, 98.12, 60.72, 55.88, 52.78 (2C), 50.62, 49.15 (2C), 45.89 (2C), 38.59.

HRMS (ESI$^+$) *m/z*: calcd for $C_{23}H_{31}ClN_5$ $[M + H]^+$ 412.2268, found 412.2258. UFLC retention time: 3.36 min, purity > 95%.

Compounds **32–34, 36–58**, **60–63,** and **64a**, were prepared using the same method as compound **35**.

*4-((2-(dimethylamino)ethyl)(methyl)amino)-2-((4-phenylpiperazin-1-yl)methyl)benzonitrile (**32**)*. **32** was prepared from intermediate **14** in the yield of 60% as white solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.49 (d, *J* = 8.7 Hz, 1H), 7.31 (d, *J* = 7.4 Hz, 2H), 6.97 (d, *J* = 8.0 Hz, 2H), 6.89 (s, 2H), 6.61 (d, *J* = 8.6 Hz, 1H), 3.74 (s, 2H), 3.56 (t, *J* = 7.5 Hz, 2H), 3.26 (t, *J* = 4.6 Hz, 4H), 3.08 (s, 3H), 2.74 (t, *J* = 4.5 Hz, 4H), 2.53 (t, J = 7.5 Hz, 2H), 2.35 (s, 6H). HRMS (ESI$^+$) *m/z*: calcd for $C_{23}H_{32}N_5$ $[M + H]^+$ 378.2658, found 378.2667. UFLC retention time: 2.97 min, purity > 95%.

*4-((2-(dimethylamino)ethyl)(methyl)amino)-2-((4-(p-tolyl)piperazin-1-yl)methyl)benzonitrile (**33**)*. **33** was prepared from intermediate **15** in the yield of 35% as white solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.48 (d, *J* = 8.7 Hz, 1H), 7.11 (d, *J* = 8.0 Hz, 2H), 6.88 (d, *J* = 8.1 Hz, 3H), 6.60 (d, *J* = 7.1 Hz, 1H), 3.74 (s, 2H), 3.55 (t, *J* = 7.3 Hz, 2H), 3.20 (s, 4H), 3.08 (s, 3H), 2.73 (s, 4H), 2.53 (t, *J* = 7.3 Hz, 2H), 2.34 (s, 6H), 2.31 (s, 3H). HRMS (ESI$^+$) *m/z*: calcd for $C_{24}H_{34}N_5$ $[M+H]^+$ 392.2809, found 392.2806. UFLC retention time: 3.26 min, purity > 95%.

*4-((2-(dimethylamino)ethyl)(methyl)amino)-2-((4-(4-methoxyphenyl)piperazin-1-yl)methyl)benzonitrile (**34**)*. **34** was prepared from intermediate **16** in the yield of 31% as white solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.89 (s, 1H), 7.61 (d, *J* = 8.7 Hz, 1H),

7.25 (s, 2H), 6.93 (d, *J* = 8.4 Hz, 2H), 6.79 (d, *J* = 8.9 Hz, 1H), 4.50 (s, 2H), 4.11 (t, *J* = 7.4 Hz, 2H), 3.99 (br, 2H), 3.84 (br, 4H), 3.73 (m, 5H), 3.56 (d, *J* = 13.1 Hz, 2H), 3.23 (s, 3H), 3.04 (s, 6H). HRMS (ESI$^+$) *m/z*: calcd for $C_{24}H_{34}N_5O$ $[M+H]^+$ 408.2763, found 408.2768. UFLC retention time: 3.00 min, purity > 95%.

*2-((4-(4-bromophenyl)piperazin-1-yl)methyl)-4-((2-(dimethylamino)ethyl)(methyl)amino)benzonitrile (**36**)*. **36** was prepared from intermediate **18** in the yield of 41% as white solid. ^{1}H NMR (600 MHz, Methanol-d_4) δ 7.68 (d, *J* = 8.9 Hz, 1H), 7.58 (d, *J* = 2.6 Hz, 1H), 7.39 (d, *J* = 9.0 Hz, 2H), 6.99 (dd, *J* = 8.9, 2.6 Hz, 1H), 6.95 (d, *J* = 9.1 Hz, 2H), 4.56 (s, 2H), 3.97 – 3.92 (m, 2H), 3.81 (br, 2H), 3.65 (br, 2H), 3.44 (m, 6H), 3.15 (s, 3H), 2.98 (s, 6H). HRMS (ESI$^+$) *m/z*: calcd for $C_{23}H_{31}BrN_5$ $[M+H]^+$ 456.1757, found 456.1761. UFLC retention time: 3.52 min, purity > 95%.

*4-((2-(dimethylamino)ethyl)(methyl)amino)-2-((4-(4-fluorophenyl)piperazin-1-yl)methyl)benzonitrile (**37**)*. **37** was prepared from intermediate **19** in the yield of 18% as white solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.48 (t, J = 7.0 Hz, 1H), 6.98 (d, J = 7.7 Hz, 2H), 6.93 – 6.81 (m, 3H), 6.60 (d, J = 8.7 Hz, 1H), 3.73 (d, J = 5.3 Hz, 2H), 3.56 (t, J = 7.1 Hz, 2H), 3.16 (q, J = 5.0 Hz, 4H), 3.07 (d, J = 5.2 Hz, 3H), 2.72 (q, J = 5.0 Hz, 4H), 2.54 (t, J = 7.1 Hz, 2H), 2.34 (d, J = 5.3 Hz, 6H). HRMS (ESI$^+$) *m/z*: calcd for $C_{23}H_{31}FN_5$ $[M + H]^+$ 396.2563, found 396.2573. UFLC retention time: 3.10 min, purity > 95%.

*2-((4-(4-cyanophenyl)piperazin-1-yl)methyl)-4-((2-(dimethylamino)ethyl)(methyl)amino)benzonitrile (**38**)*. **38** was prepared from intermediate **20** in the yield of 46% as white solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.51 (dd, *J* =

15.5, 8.8 Hz, 3H), 6.88 (d, *J* = 8.8 Hz, 2H), 6.82 (s, 1H), 6.62 (d, *J* = 2.0 Hz, 1H), 3.71 (s, 2H), 3.54 (dd, *J* = 15.2, 7.9 Hz, 2H), 3.43 – 3.31 (m, 4H), 3.08 (s, 3H), 2.74 – 2.68 (m, 4H), 2.52 (t, *J* = 7.1 Hz, 2H), 2.34 (s, 6H). HRMS (ESI$^+$) *m/z*: calcd for $C_{24}H_{31}N_6$ $[M+H]^+$ 403.2605, found 403.2599. UFLC retention time: 3.05 min, purity > 95%.

*4-((2-(dimethylamino)ethyl)(methyl)amino)-2-((4-(4-nitrophenyl)piperazin-1-yl)methyl)benzonitrile (**39**)*. **39** was prepared from intermediate **21** in the yield of 46% as yellow solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.16 (d, J = 9.2 Hz, 2H), 7.50 (d, *J* = 8.8 Hz, 1H), 6.94 – 6.75 (m, 3H), 6.63 (dd, J = 8.8, 2.7 Hz, 1H), 3.73 (s, 2H), 3.59 (t, J = 7.4 Hz, 2H), 3.48 (t, J = 5.0 Hz, 4H), 3.09 (s, 3H), 2.71 (t, J = 5.0 Hz, 4H), 2.57 (t, J = 7.1 Hz, 2H), 2.38 (s, 6H). HRMS (ESI$^+$) *m/z*: calcd for $C_{23}H_{31}N_6O_2$ $[M + H]^+$ 423.2508, found 423.2522. UFLC retention time: 3.21 min, purity > 95%.

*2-((4-(4-chloro-2-fluorophenyl)piperazin-1-yl)methyl)-4-((2-(dimethylamino)ethyl)(methyl)amino)benzonitrile (**40**)*. **40** was prepared from intermediate **22** in the yield of 38% as faint yellow solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.50 (d, *J* = 8.7 Hz, 1H), 7.07 (d, *J* = 11.2 Hz, 2H), 6.88 (dd, *J* = 17.7, 8.7 Hz, 2H), 6.61 (dd, *J* = 8.7, 2.7 Hz, 1H), 3.74 (s, 2H), 3.60 (t, *J* = 7.5 Hz, 2H), 3.13 (m, 4H), 3.09 (s, 3H), 2.75 (t, *J* = 4.9 Hz, 4H), 2.58 (m, 2H), 2.39 (s, 6H). HRMS (ESI$^+$) *m/z*: calcd for $C_{23}H_{30}ClFN_5$ $[M + H]^+$ 430.2174, found 430.2154. UFLC retention time: 3.57 min, purity > 95%.

*2-((4-(2,4-dichlorophenyl)piperazin-1-yl)methyl)-4-((2-(dimethylamino)ethyl)(methyl)amino)benzonitrile (**41**)*. **41** was prepared from intermediate **23** in the yield of 33% as sticky solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.49 (d, *J* =

8.7 Hz, 1H), 7.39 (d, *J* = 2.6 Hz, 1H), 7.27 – 7.16 (m, 1H), 6.99 (d, *J* = 8.6 Hz, 1H), 6.84 (d, *J* = 2.7 Hz, 1H), 6.60 (dd, *J* = 8.8, 2.7 Hz, 1H), 3.74 (s, 2H), 3.57 (t, *J* = 7.5 Hz, 2H), 3.08 (m, 7H), 2.75 (m, 4H), 2.54 (t, *J* = 7.5 Hz, 2H), 2.36 (s, 6H). HRMS (ESI^+) *m/z*: calcd for $C_{23}H_{30}Cl_2N_5$ $[M + H]^+$ 446.1878, found 446.1873. UFLC retention time: 3.68 min, purity > 95%.

*2-((4-(4-chloro-2-methylphenyl)piperazin-1-yl)methyl)-4-((2-(dimethylamino)ethyl)(methyl)amino)benzonitrile (**42**)*. **42** was prepared from intermediate **24** in the yield of 34% as yellow oil. ^1H NMR (500 MHz, DMSO-d_6) δ 7.53 (d, *J* = 8.7 Hz, 1H), 7.24 (d, *J* = 2.6 Hz, 1H), 7.23 – 7.16 (m, 1H), 7.04 (d, *J* = 8.5 Hz, 1H), 6.85 (d, *J* = 2.6 Hz, 1H), 6.69 (dd, *J* = 8.8, 2.6 Hz, 1H), 3.64 (s, 2H), 3.52 (t, *J* = 7.1 Hz, 2H), 3.02 (s, 3H), 2.86 (t, *J* = 4.7 Hz, 4H), 2.61(s, 4H), 2.41 (t, *J* = 7.0 Hz), 2.26 (s, 3H), 2.22 (s, 6H). HRMS (ESI^+) *m/z*: calcd for $C_{24}H_{33}ClN_5$ $[M + H]^+$ 426.2424, found 426.2424. UFLC retention time: 3.63 min, purity > 95%.

*2-((4-(4-chloro-2-nitrophenyl)piperazin-1-yl)methyl)-4-((2-(dimethylamino)ethyl)(methyl)amino)benzonitrile (**43**)*. **43** was prepared from intermediate **25** in the yield of 29% as brown oil. ^1H NMR (500 MHz, Chloroform-*d*) δ 7.79 (d, *J* = 2.6 Hz, 1H), 7.53 – 7.43 (m, 2H), 7.12 (d, *J* = 8.8 Hz, 1H), 6.78 (d, *J* = 2.6 Hz, 1H), 6.61 (dd, *J* = 8.7, 2.7 Hz, 1H), 3.72 (s, 2H), 3.57 (t, *J* = 7.4 Hz, 2H), 3.14 – 3.09 (m, 4H), 3.08 (s, 3H), 2.71 (t, *J* = 4.5 Hz, 4H), 2.55(s, 2H), 2.37 (s, 6H). HRMS (ESI^+) *m/z*: calcd for $C_{23}H_{30}ClN_6O_2$ $[M + H]^+$ 457.2119, found 457.2107. UFLC retention time: 3.19 min, purity > 95%.

*2-((4-(6-chloropyridin-3-yl)piperazin-1-yl)methyl)-4-((2-(dimethylamino)ethyl)(methyl)amino)benzonitrile (**44**)*. **44** was prepared from intermediate **26** in the yield of 15% as white solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.04 (s, 1H), 7.50 (d, *J* = 8.8 Hz, 1H), 7.20 (s, 2H), 6.85 (s, 1H), 6.63 (d, *J* = 8.9 Hz, 1H), 3.73 (s, 2H), 3.60 (t, *J* = 7.4 Hz, 2H), 3.25 (t, *J* = 4.7 Hz, 4H), 3.09 (s, 3H), 2.73 (t, *J* = 4.8 Hz, 4H), 2.58 (t, *J* = 7.5 Hz, 2H), 2.38 (s, 6H). HRMS (ESI$^+$) *m/z*: calcd for $C_{22}H_{30}ClN_6$ $[M + H]^+$ 413.2220, found 413.2210. UFLC retention time: 2.94 min, purity > 95%.

*4-((2-(dimethylamino)ethyl)(methyl)amino)-2-((4-(pyridin-4-yl)piperazin-1-yl)methyl)benzonitrile (**45**)*. **45** was prepared from intermediate **27** in the yield of 25% as yellow oil. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.27 (d, *J* = 4.1 Hz, 2H), 7.49 (d, *J* = 8.7 Hz, 1H), 6.82 (d, *J* = 1.3 Hz, 1H), 6.74 (d, *J* = 5.4 Hz, 2H), 6.66 – 6.55 (m, 1H), 4.57 (s, 2H), 3.71 (s, 2H), 3.58 (t, *J* = 7.3 Hz, 2H), 3.46 (s, 4H), 3.08 (s, 3H), 2.69 (s, 4H), 2.57 (t, *J* = 7.3 Hz, 2H), 2.37 (s, 6H). HRMS (ESI$^+$) *m/z*: calcd for $C_{22}H_{31}N_6$ $[M+H]^+$ 379.2605, found 379.2599. UFLC retention time: 2.48 min, purity > 95%.

*2-((4-(4-chlorophenyl)piperazin-1-yl)methyl)-4-(piperazin-1-yl)benzonitrile (**46**)*. **46** was prepared from intermediate **17** in the yield of 27% as faint yellow solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.57 (d, *J* = 8.8 Hz, 1H), 7.24 (d, *J* = 8.5 Hz, 2H), 7.06 (d, *J* = 2.5 Hz, 1H), 6.95 (t, *J* = 9.4 Hz, 3H), 3.62 (s, 2H), 3.26 (t, *J* = 4.9 Hz, 4H), 3.16 (t, *J* = 4.6 Hz, 4H), 2.84 (t, *J* = 5.0 Hz, 4H), 2.58 (t, *J* = 5.0 Hz, 4H), 2.53 (s, 1H). HRMS (ESI$^+$) *m/z*: calcd for $C_{22}H_{26}ClN_5$ $[M + H]^+$ 396.1955, found 396.1956. UFLC retention time: 3.36 min, purity > 95%.

*2-((4-(4-chlorophenyl)piperazin-1-yl)methyl)-4-(4-methylpiperazin-1-yl)benzonitrile (**47**)*. **47** was prepared in hydrochloride salt form from intermediate **17** in the yield of 27% as white solid. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 12.03 (s, 1H), 11.30 (s, 1H), 7.92 (s, 1H), 7.78 (d, *J* = 8.8 Hz, 1H), 7.32 (d, *J* = 8.8 Hz, 2H), 7.22 (d, *J* = 8.7 Hz, 1H), 7.03 (d, *J* = 8.8 Hz, 2H), 4.78 (s, 8H), 4.49 (s, 3H), 4.27 (d, *J* = 13.8 Hz, 2H), 3.85 (d, *J* = 11.7 Hz, 2H), 3.44 (dt, *J* = 27.4, 12.7 Hz, 10H), 3.15 (t, *J* = 10.8 Hz, 2H), 2.81 (d, *J* = 4.3 Hz, 4H). HRMS (ESI$^+$) *m/z*: calcd for $C_{23}H_{29}ClN_5$ $[M + H]^+$ 410.2106, found 410.2102. UFLC retention time: 3.36 min, purity > 95%

*2-((4-(4-chlorophenyl)piperazin-1-yl)methyl)-4-((3-(dimethylamino)propyl)(methyl)amino)benzonitrile (**48**)*. **48** was prepared in hydrochloride salt form from intermediate **17** in the yield of 23% as white-like solid. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 7.72 – 7.64 (m, 1H), 7.63 (s, 1H), 7.36 – 7.26 (m, 2H), 7.07 – 6.97 (m, 2H), 6.94 – 6.85 (m, 1H), 4.47 (s, 2H), 3.90 – 3.79 (m, 2H), 3.62 – 3.53 (m, 2H), 3.51 – 3.43 (m, 2H), 3.43 – 3.33 (m, 2H), 3.33 – 3.23 (m, 2H), 3.15 (s, 2H), 3.08 (s, 3H), 2.79 – 2.70 (m, 6H), 2.00 (m, 2H). HRMS (ESI$^+$) *m/z*: calcd for $C_{24}H_{33}ClN_5$ $[M + H]^+$ 426.2424, found 426.2409. UFLC retention time: 3. 46 min, purity > 95%.

*2-((4-(4-chlorophenyl)piperazin-1-yl)methyl)-4-(4-(pyrrolidin-1-yl)piperidin-1-yl)benzonitrile (**49**)*. **49** was prepared from intermediate **17** in the yield of 17% as faint yellow solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.50 (d, *J* = 8.6 Hz, 1H), 7.24 (d, *J* = 8.5 Hz, 2H), 7.05 (s, 1H), 6.87 (d, *J* = 8.6 Hz, 2H), 6.81(d, *J* = 8.6 Hz, 1H),3.89 (d, *J* = 12.8 Hz, 2H), 3.72 (s, 2H), 3.22 (t, *J* = 4.6 Hz, 4H), 2.98 (t, *J* = 12.4 Hz, 2H), 2.71 (m, 8H),

2.33 (s, 1H), 2.06 (d, J = 12.7 Hz, 2H), 1.88 (m, 4H), 1.76 (m, 2H). HRMS (ESI^+) *m/z*: calcd for $C_{27}H_{35}ClN_5$ $[M + H]^+$ 464.2581, found 464.2578.

*2-((4-(4-chlorophenyl)piperazin-1-yl)methyl)-4-(dimethylamino)benzonitrile (**50**).* **50** was prepared from intermediate **17** in the yield of 36% as faint yellow solid. ^1H NMR (500 MHz, DMSO-d_6) δ 7.55 (d, J = 8.7 Hz, 1H), 7.25 (d, J = 8.5 Hz, 2H), 6.96 (d, J = 8.4 Hz, 2H), 6.84 (s, 1H), 6.71 (d, J = 8.7 Hz, 1H), 3.34 (s, 2H), 3.16 (t, J = 4.8 Hz, 4H), 3.03 (s, 6H), 2.59 (t, J = 4.8 Hz, 4H). HRMS (ESI^+) *m/z*: calcd for $C_{20}H_{24}ClN_4$ $[M + H]^+$ 355.1689, found 355.1680. UFLC retention time: 4.36 min, purity > 95%.

*2-((4-(4-chlorophenyl)piperazin-1-yl)methyl)-4-(isopentylamino)benzonitrile (**51**).* **51** was prepared from intermediate **17** in the yield of 54% as white solid. ^1H NMR (400 MHz, Chloroform-*d*) δ 7.45 (d, J = 8.5 Hz, 1H), 7.23 (d, J = 8.9 Hz, 2H), 6.87 (d, J = 9.0 Hz, 2H), 6.74 (d, J = 1.9 Hz, 1H), 6.50 (dd, J = 8.5, 2.3 Hz, 1H), 3.70 (s, 2H), 3.21 (dd, J = 11.1, 5.9 Hz, 6H), 2.82 – 2.58 (m, 4H), 1.75 (td, J = 13.3, 6.6 Hz, 1H), 1.57 (dd, J = 14.5, 7.1 Hz, 2H), 1.00 (d, J = 6.6 Hz, 7H). HRMS (ESI^+) *m/z*: calcd for $C_{23}H_{30}ClN_4$ $[M + H]^+$ 397.2153, found 397.2144. UFLC retention time: 5.11 min, purity > 95%.

*2-((4-(4-chlorophenyl)piperazin-1-yl)methyl)-4-((2-(dimethylamino)ethyl)amino)benzonitrile (**52**).* **52** was prepared from intermediate **17** in the yield of 36% as faint yellow solid. ^1H NMR (500 MHz, DMSO-d_6) δ 7.60 (d, J = 8.8 Hz, 1H), 7.25 (d, J = 8.7 Hz, 2H), 6.97 (d, J = 8.6 Hz, 2H), 6.91 (d, J = 2.6 Hz, 1H), 6.82 (dd, J = 9.0, 2.7 Hz, 1H), 3.71 (t, J = 6.9 Hz, 2H), 3.64 (s, 2H), 3.32 (s, 1H), 3.17 (t, J = 4.8 Hz, 4H), 3.09 (t, J = 6.7 Hz, 2H), 3.04 (s, 3H), 2.60 (d, J = 4.7 Hz, 7H). HRMS (ESI^+)

m/z: calcd for $C_{22}H_{29}ClN_5$ $[M + H]^+$ 398.2111, found 398.2102. UFLC retention time: 3.28 min, purity > 95%.

*2-((4-(4-chlorophenyl)piperazin-1-yl)methyl)-4-(2-(pyrrolidin-1-yl)ethoxy)benzonitrile (**53**)*. **53** was prepared from intermediate **17** in the yield of 13% as yellow solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.50 (d, *J* = 8.6 Hz, 1H), 7.24 (d, *J* = 8.5 Hz, 2H), 7.05 (s, 1H), 6.87 (d, *J* = 8.6 Hz, 2H), 6.81(d, *J* = 8.6 Hz, 1H),3.89 (d, *J* = 12.8 Hz, 2H), 3.72 (s, 2H), 3.22 (t, *J* = 4.6 Hz, 4H), 2.98 (t, *J* = 12.4 Hz, 2H), 2.71 (t, *J* = 5.1 Hz, 8H), 2.33 (s, 1H), 2.06 (d, *J* = 12.7 Hz, 2H), 1.88 (s, 4H), 1.76 (s, 2H). HRMS (ESI$^+$) *m/z*: calcd for $C_{24}H_{30}ClN_4O$ $[M + H]^+$ 425.2108, found 425.2131. UFLC retention time: 3.44 min, purity > 95%.

*2-((4-(4-chlorophenyl)piperazin-1-yl)methyl)-4-(((1-ethylpyrrolidin-2-yl)methyl)amino)benzonitrile (**54**)*. **54** was prepared from intermediate **17** in the yield of 27% as yellow oil. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.45 (d, *J* = 8.4 Hz, 1H), 7.23 (s, 2H), 6.88 (d, *J* = 8.4 Hz, 2H), 6.76 (s, 1H), 6.52 (d, *J* = 8.5 Hz, 1H), 5.00 (s, 1H), 3.70 (s, 2H), 3.22 (d, *J* = 4.6 Hz, 8H), 2.84 (dt, *J* = 14.4, 7.1 Hz, 1H), 2.72 (t, *J* = 5.0 Hz, 6H), 2.26 (m, 2H), 1.97(m, 1H), 1.30（s, 1H)， 1.15 (t, *J* = 7.3 Hz, 3H). HRMS (ESI$^+$) *m/z*: calcd for $C_{25}H_{32}ClN_5$ $[M + H]^+$ 438.2424, found 438.2421. UFLC retention time: 3.39 min, purity > 95%.

*2-((4-(4-chlorophenyl)piperazin-1-yl)methyl)-4-((2-morpholinoethyl)amino)benzonitrile (**55**)*. **55** was prepared from intermediate **17** in the yield of 60% as white solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.47 (d, *J* = 8.5 Hz, 1H),

7.24 (d, *J* = 8.5 Hz, 2H), 6.87 (d, *J* = 8.5 Hz, 2H), 6.77 (s, 1H), 6.54 (d, *J* = 8.4 Hz, 1H), 4.95 (s, 1H), 3.77 (t, *J* = 4.7 Hz, 4H), 3.71 (s, 2H), 3.25 (d, *J* = 5.6 Hz, 2H), 3.22 (t, *J* = 4.8 Hz, 4H), 2.73 (s, 6H), 2.53 (s, 4H). HRMS (ESI^+) *m/z*: calcd for $C_{24}H_{31}ClN_5O$ $[M + H]^+$ 440.2217, found 440.2217. UFLC retention time: 3.42 min, purity > 95%.

*2-((4-(4-chlorophenyl)piperazin-1-yl)methyl)-4-((pyridin-2-ylmethyl)amino)benzonitrile (**56**).* **56** was prepared from intermediate **17** in the yield of 14% as white solid. ^1H NMR (500 MHz, DMSO-d_6) δ 8.55 (d, *J* = 4.8 Hz, 1H), 7.78 (t, *J* = 7.8 Hz, 1H), 7.45 (d, *J* = 8.6 Hz, 1H), 7.39 (s, 1H), 7.35 (d, *J* = 7.8 Hz, 1H), 7.32 – 7.28 (m, 1H), 7.26 (d, *J* = 8.6 Hz, 2H), 6.96 (d, *J* = 8.7 Hz, 2H), 6.77 (s, 1H), 6.61 (d, *J* = 8.6 Hz, 1H), 4.47 (d, *J* = 6.0 Hz, 2H), 3.55 (s, 2H), 3.10 (t, *J* = 5.0 Hz,4H), 2.50 (t, *J* = 5.0 Hz,4H). HRMS (ESI^+) *m/z*: Calcd for $C_{24}H_{25}ClN_5$ $[M + H]^+$ 418.1798, found 418.1815. UFLC retention time: 3.92 min, purity > 95%.

*2-((4-(4-chlorophenyl)piperazin-1-yl)methyl)-4-(methyl(2-(methylamino)ethyl)amino)benzonitrile (**57**).* **57** was prepared from intermediate **17** in the yield of 26% as white solid. ^1H NMR (400 MHz, Chloroform-*d*) δ 7.48 (d, *J* = 8.4 Hz, 1H), 7.22 (d, *J* = 8.0 Hz, 2H), 6.94 – 6.80 (m, 3H), 6.66 (d, *J* = 7.7 Hz, 1H), 3.72 (s, 2H), 3.60 (s, 2H), 3.20 (s, 4H), 3.09 (s, 3H), 2.88 (s, 2H), 2.71 (s, 4H), 2.53 (s, 4H). HRMS (ESI^+) *m/z*: calcd for $C_{22}H_{29}ClN_5$ $[M+H]^+$ 398.2106, found 398.2107. UFLC retention time: 3.41 min, purity > 95%.

*2-((4-(4-chlorophenyl)piperazin-1-yl)methyl)-4-(3-oxopiperazin-1-yl)benzonitrile (**58**).* **58** was prepared from intermediate **17** in the yield of 6% as white solid. ^1H NMR (500 MHz,

Chloroform-*d*) δ 7.59 (d, *J* = 8.6 Hz, 1H) , 7.25 (d, *J* = 8.5 Hz, 2H), 7.04 (s, 1H), 6.88 (d, *J* = 8.8 Hz, 2H), 6.78 (d, *J* = 8.5 Hz, 1H), 6.18 (br, 1H), 4.07 (s, $2H_2$), 3.76 (s, 2H), 3.69 – 3.60 (m, 4H), 3.22 (t, *J* = 4.9 Hz,4H), 2.72 (t, *J* = 4.9 Hz, 4H). HRMS (ESI^+) *m/z*: calcd for $C_{22}H_{25}ClN_5O$ $[M+H]^+$ 410.1748, found 410.1750. UFLC retention time: 3.90 min, purity > 95%.

*2-((4-(4-chlorophenyl)piperazin-1-yl)methyl)-4-(4-methyl-1,4-diazepan-1-yl)benzonitrile (**60**)*. **60** was prepared from intermediate **17** in the yield of 40% as white solid. ^1H NMR (400 MHz, D_2O) δ 7.76 (d, *J* = 8.9 Hz, 1H), 7.42 – 7.33 (m, 2H), 7.15 – 6.98 (m, 4H), 4.55 (s, 2H), 4.08 – 3.83 (m, 2H), 3.83 – 3.42 (m, 10H), 3.37 (m, 4H), 2.98 (s, 3H), 2.45 – 2.23 (m, 2H). UFLC retention time: 3.24 min, purity > 95%.

*2-((4-(4-chlorophenyl)piperidin-1-yl)methyl)-4-((2-(dimethylamino)ethyl)(methyl)amino)benzonitrile (**61**)*. **61** was prepared from the reaction of intermediate **28** with N1,N1,N2-trimethylethane-1,2-diamine in the yield of 23% as white solid. ^1H NMR (500 MHz, DMSO-d_6) δ 7.52 (d, *J* = 8.6 Hz, 1H), 7.37 (d, *J* = 8.1 Hz, 2H), 7.30 (d, *J* = 8.2 Hz, 2H), 6.96 (s, 1H),6.68 (d, *J* = 8.8 Hz, 1H), 3.60 (s, 2H), 3.53 (t, *J* = 7.2 Hz, 2H), 3.02 (s, 3H), 2.96 (d, *J* = 10.9 Hz, 2H), 2.58 (s, 1H), 2.42 (s, 2H), 2.23 (s, 6H), 2.17 (t, *J* = 11.3 Hz, 2H), 1.77 (d, *J* = 12.4 Hz, 2H), 1.72 – 1.60 (m, 2H). HRMS (ESI^+) *m/z*: calcd for $C_{24}H_{32}ClN_4$ $[M + H]^+$ 411.2315, found 411.2309. UFLC retention time: 3.42 min, purity > 95%.

*2-((4-(4-chlorophenyl)-3-methylpiperazin-1-yl)methyl)-4-((2-(dimethylamino)ethyl)(methyl)amino)benzonitrile (**62**)*. **62** was prepared from the reaction

of intermediate **29** with N1,N1,N2-trimethylethane-1,2-diamine in the yield of 27% as faint yellow oil. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.43 (d, *J* = 9.0 Hz, 1H), 7.17 (d, *J* = 8.3 Hz, 2H), 6.91 (s, 1H), 6.80 (d, *J* = 8.5 Hz, 2H), 6.56 (d, *J* = 8.7 Hz, 1H), 4.13 (d, *J* = 14.1 Hz, 1H), 3.59 (s, 2H), 3.45 – 3.33 (m, 2H), 3.30 (d, *J* = 11.8 Hz, 1H), 3.03 (s, 3H), 2.87 (dd, *J* = 33.1, 11.4 Hz, 2H), 2.75 (s, 2H), 2.68 – 2.53 (m, 2H), 2.44 (d, *J* = 11.0 Hz, 1H), 2.39 (s, 6H),1.23 (s, 3H). HRMS (ESI$^+$) *m/z*: calcd for $C_{24}H_{33}ClN_5$ $[M + H]^+$ 426.2424, found 426.2422. UFLC retention time: 3.42 min, purity > 95%.

*2-((4-(4-chlorobenzyl)piperazin-1-yl)methyl)-4-((2-(dimethylamino)ethyl)(methyl)amino)benzonitrile (**63**)*. **63** was prepared from intermediate **30** in the yield of 41% as white solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.46 (d, J = 8.6 Hz, 1H), 7.30 (s, 4H), 6.82 (d, J = 2.6 Hz, 1H), 6.57 (dd, J = 8.8, 2.7 Hz, 1H), 3.67 (s, 2H), 3.54 (m, 2H), 3.51 (s, 2H), 3.07 (s, 3H), 2.59 (m, 4H), 2.51 (m, 6H), 2.34 (s, 6H). HRMS (ESI$^+$) *m/z*: calcd for $C_{24}H_{33}N_5Cl$ $[M+H]^+$ 426.2419, found 426.2415. UFLC retention time: 2.98 min, purity > 95%.

*tert-butyl 4-(2-cyano-5-((2-(dimethylamino)ethyl)(methyl)amino)benzyl)piperazine-1-carboxylate (**64a**)*. **64a** was prepared from intermediate **31** in the yield of 45% as white solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.70 (dd, *J* = 8.6, 5.3 Hz, 1H), 7.37 (s, 1H), 7.11 (t, J = 8.3 Hz, 1H),3.75 (s, 2H), 3.50 (m, 4H), 2.51 (m, 4H), 1.50 (s, 9H).

*N-(2-((3-((4-(4-chlorophenyl)piperazin-1-yl)methyl)-4-cyanophenyl)(methyl)amino)ethyl)-N-methylpropionamide (**59**)*. To a solution of **57** (50 mg) in DCM (5 mL) was added with propionyl chloride (11 μL) in the presence of N,N-

diisopropyl-ethylamine (DIPEA, 21μL). The reaction was stirred for 2 h. The resulted mixture was extracted with DCM and water. The organic phase was dried with anhydrous $NaSO_4$ and purified with column chromatography eluted with 1–10% of methanol in DCM to give title compound **59** (37 mg) as white-like solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.52 (dd, J = 18.8, 8.7 Hz, 1H), 7.25 (s, 2H), 6.87 (d, J = 9.1 Hz, 3H), 6.72 (s, 1H), 3.74 (s, 2H), 3.62 (d, J = 7.1 Hz, 2H), 3.57 (t, J = 6.8 Hz, 2H), 3.22 (s, 4H), 3.09 (s, 3H), 3.03 (d, J = 8.0 Hz, 3H), 2.72 (s, 4H), 2.35 – 2.20 (m, 2H), 1.15 (t, J = 7.4 Hz, 3H). HRMS (ESI$^+$) *m/z*: calcd for $C_{25}H_{33}ClN_5O$ $[M + H]^+$ 454.2374, found 454.2361. UFLC retention time: 4.30 min, purity > 95%.

*4-((2-(dimethylamino)ethyl)(methyl)amino)-2-(piperazin-1-ylmethyl)benzonitrile (**64**).* To a solution of **64a** (40 mg, 0.1 mmol) in methanol (2 mL) was added with concentrated hydrochloride acid (0.1 mL). The reaction was stirred until **64a** was absent. The mixture was filtered and residue was washed with ethanol to afford compound **64** (37mg, yield: 89%) in salt form as white solid. ^{1}H NMR (500 MHz, Methanol-d_4) δ 7.63 (d, J = 8.7 Hz, 1H), 7.16 (s, 1H), 6.95–6.87 (m, 1H), 4.00 (s, 2H), 3.93 (t, J = 7.6 Hz, 2H), 3.42 (q, J = 6.9, 5.4 Hz, 6H), 3.15 (s, 3H), 3.05 (s, 4H), 3.01 (s, 6H), 1.41 (t, J = 6.0 Hz, 1H). HRMS (ESI$^+$) calcd for $C_{17}H_{28}N_5$ $[M + H]^+$ 302.2345, found 302.2325. UFLC retention time: 2.47 min, purity > 95%.

*4-((2-(dimethylamino)ethyl)(methyl)amino)-2-((4-(4-aminophenyl)piperazin-1-yl)methyl)benzonitrile (**65**).* To solution of compound **39** (100mg) in methanol (10 mL) 226mg of $SnCl_2$ was added. Then concentrated hydrochloride acid (50 μL) was added

dropwise in ice-bath. The mixture was recovered to room temperature and stirred overnight. The reaction was concentrated *in vacuo* and the residue was dissolved in water and adjusted pH to basic condition with diluted sodium hydroxide aqueous solution. The water phase was extracted with dichloromethane twice. The organic phase was combined and purified by Combiflash to afford **65** (56mg) as grey solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.49 (d, *J* = 8.7 Hz, 1H), 6.90 (s, 1H), 6.85 (d, *J* = 8.5 Hz, 2H), 6.69 (d, *J* = 8.6 Hz, 2H), 6.60 (d, *J* = 8.6 Hz, 1H), 3.75 (s, 2H), 3.58 (s, 2H), 3.55 – 3.32 (br, 2H), 3.12 (s, 4H), 3.08 (s, 3H), 2.74 (s, 4H), 2.56 (s, 2H), 2.37 (s, 6H). HRMS (ESI$^+$) calcd for $C_{23}H_{33}N_6$ $[M + H]^+$ 393.2761, found 393.2760. UFLC retention time: 2.46 min, purity > 95%.

*2-((4-(4-chlorophenyl)piperazin-1-yl)methyl)-4-((2-(dimethylamino)ethyl)(methyl)amino)benzamide (**66**)*. **35** (200mg) was suspended in mixed solvent of DMSO (3 mL) and ethanol (7 mL). Then 2.5 mL of 30% H_2O_2 aqueous solution and sodium hydroxide (52 mg) were added. The resulted mixture was stirred at 70 °C for 12 h. The reaction was concentrated in vacuo and the residue was extracted with DCM and deionized water (×3). The organic phase was washed with brine, dried over anhydrous $NaSO_4$, concentrated *in vacuo*, and purified by gel silica column to afford **66** (30 mg) as white solid. ^{1}H NMR (500 MHz, Methanol-d_4) δ 7.78 (d, *J* = 8.7 Hz, 1H), 7.22 (d, *J* = 8.7 Hz, 2H), 6.95 (d, *J* = 8.8 Hz, 2H), 6.84 – 6.73 (m, 1H), 6.67 (s, 1H), 3.69 (s, 2H), 3.65 – 3.55 (m, 2H), 3.37 (d, *J* = 19.4 Hz, 2H), 3.17 (s, 4H), 3.07 (s, 3H), 2.71 (s, 4H), 2.62 – 2.52 (m, 2H), 2.36 (s, 6H). HRMS (ESI$^+$) calcd for $C_{23}H_{33}ClN_5O$ $[M + H]^+$ 430.2374, found 430.2366. UFLC retention time: 3.14 min, purity > 95%.

*2-((4-(4-chlorophenyl)piperazin-1-yl)methyl)-3-((2-(dimethylamino)ethyl)(methyl)amino)benzonitrile (**69**).* 600 mg of 4-fluoro-3-formylbenzonitrile (**67**) and 1-(4-chlorophenyl)piperazine (1.6 g) were dissolved in methanol (10 mL). Sodium cyanoborohydride (754 mg) and acetic acid (0.5 mL) were added into above solution. The reaction was stirred overnight. Resulted mixture was concentrated in vacuo and extracted with ethyl acetate and water. The organic phase was washed with brine, dried over anhydrous $NaSO_4$, concentrated *in vacuo*, and purified by gel silica column eluted with 8% methanol in DCM to afford 3-((4-(4-chlorophenyl)piperazin-1-yl)methyl)-4-fluorobenzonitrile (**68**) as white solid (1.0 g). To solution of **68** (500 mg) in DMSO (5 mL) K_2CO_3 (630 mg) and N^1,N^1,N^2-trimethylethane-1,2-diamine (0.6 mL) were added. The reaction was done under microwave irradiation at 140 °C for 2 h. The resulted mixture was extracted with ethyl acetate and water. The organic phase was washed with brine, dried over anhydrous $NaSO_4$, concentrated *in vacuo*, and purified by gel silica column eluted with 8% methanol in DCM to afford **69** as white solid (225 mg) in yield of 36%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.47 (s, 1H), 7.93 (d, J = 8.5 Hz, 1H), 7.49 (d, J = 8.7 Hz, 1H), 7.32 (d, J = 8.5 Hz, 2H), 7.01 (d, J = 8.6 Hz, 2H), 4.71 (s, 2H), 3.81 (d, J = 13.0 Hz, 2H), 3.42 (s, 3H), 3.37 (s, 4H), 3.22 (s, 2H), 2.75 (d, J = 4.1 Hz, 7H), 2.71 (s, 3H). HRMS (ESI$^+$) calcd for $C_{23}H_{31}ClN_5$ $[M + H]^+$ 412.2268, found 412.2252. UFLC retention time: 3.37 min, purity > 95%.

*N^1-(3-((4-(4-chlorophenyl)piperazin-1-yl)methyl)phenyl)-N^1,N^2,N^2-trimethylethane-1,2-diamine hydrochloride salt (**72**).* To solution of 1-(bromomethyl)-3-iodobenzene (600

mg) in aceton (10 mL) anhydrous K_2CO_3 (426 mg) and 1-(4-chlorophenyl)piperazine (400 mg) were added. The reaction was stirred overnight. The mixture was filtered, the filtrate was concentrated in vacuo and extracted with ethyl acetate and water. The organic phase was washed with brine, dried over anhydrous $NaSO_4$, concentrated *in vacuo*. The residue was suspended in ether (5 mL) and filtered to afford 1-(4-chlorophenyl)-4-(3-iodobenzyl)piperazine (**71**, 740mg) as white solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.77 (s, 1H), 7.65 (d, *J* = 7.8 Hz, 1H), 7.36 (d, *J* = 7.5 Hz, 1H), 7.24 (d, *J* = 8.9 Hz, 2H), 7.11 (t, *J* = 7.7 Hz, 1H), 6.87 (d, *J* = 8.9 Hz, 2H), 3.54 (s, 2H), 3.27 – 3.15 (m, 4H), 2.69 – 2.56 (m, 4H). To a solution of **71** (700 mg) and N^1,N^1,N^2-trimethylethane-1,2-diamine (0.43 mL) in DMSO (5 mL) CuI (32 mg), L-proline (30 mg) and anhydrous K_2CO_3 (472 mg) were added. The reaction was processed under microwave irradiation at 140 °C for 2 h. The resulted mixture was extracted with ethyl acetate and water. The organic phase was washed with brine, dried over anhydrous $NaSO_4$, concentrated *in vacuo*, and purified by gel silica column eluted with 1-5% methanol in DCM to afford **72** as oil (55 mg) in yield of 8%. To solution of **72** in DCM (2.0 mL) 2M hydrochloride acid (mL) was added and stirred for 1.5 h. The mixture was concentrated in vacuo and suspended in ethyl acetate (2.0 mL). The mixture was filtered to afford hydrochloride salt of **72** as whiter solid (45 mg). ^{1}H NMR (500 MHz, DMSO-*d6*) δ 11.44 (br, 1H), 10.71 (br, 1H), 7.28 (m, 4H), 7.00 (d, *J* = 7.9 Hz, 2H), 6.86 (d, *J* = 7.4 Hz, 2H), 4.31 (s, 2H), 3.82-3.57 (m, 8H), 3.36 (d, *J* = 10.5 Hz, 2H), 3.16 (m, 2H), 2.97 (s, 3H), 2.83 (s, 6H). HRMS (ESI$^+$) calcd for $C_{22}H_{32}ClN_4$ $[M + H]^+$ 387.2315, found 387.2295. UFLC retention time: 3.01 min, purity > 95%.

Cell culture, HCV infection, agents, and drug exposure. Huh7.5 cells and the plasmid pFL-J6/JFH/JC1 containing the full-length chimeric HCV complementary DNA (cDNA) were kindly provided by the Vertex Pharmaceuticals Inc. (Boston, USA). Huh7.5 cells were maintained in Dulbecco's modified eagle medium supplemented with 10% heat inactivated fetal bovine serum, penicillin-streptomycin. GS4.3 replicon cells, a human hepatoma Huh7 cell line carrying an HCV subgenomic replicon I377-3′del.S, were cultured in Dulbecco's modified eagle medium supplemented with 10% heat inactivated fetal bovine serum, 100 μg/mL of penicillin-streptomycin, and 400 μg/mL of G418 disulphate salt solution.

The S282T, D168V, and A156T mutant HCV variants were prepared with plasmids pHCV-S282T, pHCV-D168V, and pHCV-A156T derived from the plasmid pFL-J6/JFH/JC1, respectively. Wild-type and mutant HCV virus stock were prepared as described.[29]

Telaprevir (HY-10235), sofosbuvir (HY-15005), daclatasvir (HY-10466), and simeprevir (HY-10241) were purchased from the MedChemExpress (Princeton, NJ). The mAbs to HCV Core (ab2740) and to HCV NS3 (ab13830) were from Abcam, Co. Ltd. The mAb to β-actin (3700S) and anti-mouse secondary antibody (7076S) were from the Cell Signaling Technology, Inc. The pAbs to GAPDH(10494-1-AP) was from Protein Tech Inc.

All test compounds were supplied in 100% DMSO. Compound serial dilutions were performed in DMEM culture medium. For EC_{50} and CC_{50} determinations, test compounds were serially diluted in eight steps of 1:5 dilutions in 96-well plates. All serial dilutions

were performed in three replicates per compound within the same 96-well plate. The EC_{50} and CC_{50} were calculated with Reed & Muench methods.[30]

RNA extraction and qRT-PCR. Huh7.5 cells were seeded at a density of $3 \times 10^4/cm^2$ in 96 wells. After 24 h incubation, the Huh7.5 cells were incubated with wild-type or mutant HCV virus stock and simultaneously treated with compound or solvent control. After 72 h, intracellular RNA was extracted with RNeasy Mini Kit, RNA was analyzed by qRT-PCR. It was performed on a 7500 Fast Real-Time PCR system (Applied Biosystems, Singapore) using an AgPath-ID One-Step RT-PCR Kit (Applied Biosystems, Foster, CA, USA) according to the manufacturer's instructions. The levels of HCV RNA were calculated with $2^{-\triangle\triangle CT}$ method. And all quantifications were normalized to the level of the internal control gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Cell cytotoxicity. The Huh7.5 cells were seeded into a plate of 96-well at density of $3\times10^4/cm^2$, and then treated with fresh medium containing test compounds or solvent control at 24 h. Cytotoxicity was evaluated with the tetrazolium-based MTT assay at 96 h.

Western Blot Assay: Briefly, after SDS-PAGE and transmembrane, the target proteins were accordingly probed with antibodies. After an incubation with the corresponding HRP-conjugated secondary antibody, the signal of the target proteins was detected using the ChemiDo XRS gel imager system (Bio-Rad), with an enhanced chemiluminescence (ECL) kit (GE Healthcare Life Sciences, Pittsburgh, PA, USA) and was scanned with the Gelpro32 software. The ratio of the protein of interest to the internal control protein Actin was calculated and normalized as 1.00 for the control group.

In-cell western assay for antiviral activity. Huh7.5 cells infected with HCVcc (MOI=0.1) in a 96-well plate were simultaneously treated with drugs. After 72 h, the cells are fixed with 4% paraformaldehyde (PFA). Then, the cells were permeabilized with 0.3% Triton X-100 for 20 min at room temperature (RT) and blocked with LI-COR Odyssey Blocking Solution (LI-COR Biosciences) for 30 min. The cells were incubated HCV Core anti-mouse antibody and a rabbit IgG antibody against GAPDH(1:1000 dilution, Protein Tech Inc., Wuhan, China) at 4°C overnight . After three washes with TBST, the cells were stained with a goat anti-mouse IgG IRDye™ 800 antibody (1:1,000 dilution, LI-COR Biosciences) and a goat anti-rabbit IgG IRDye™ 680 antibody (1:1,000 dilution, LI-COR Biosciences) at RT for 1 h. Again three washes with TBST, the microplates were scanned with the Odyssey CLx Infrared Imaging System (LI-COR Biosciences), and the integrated fluorescence intensities representing the protein expression levels were acquired using the software provided with the imager station (Odyssey Software Version 3.0, LI-COR Biosciences). The relative amount of HCV Core protein was obtained by normalizing to GAPDH in all experiments. the images were obtained using an Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE, USA) according to the manufacturer's instructions.

Time of addition assay. Test compound was added for 2 h before 2 h of viral inoculation (pro-incubation), during 2 h of viral inoculation (co-addition) and after 2 h of viral inoculation (post-infection) in Huh7.5 cells at 37℃, respectively. After 72 h post infection, cells were lysed and HCV infectivity was measured by western blot. Infectivity is expressed as a percentage relative to the ratio of HCV Core protein to β-actin measured in

controls not treated with test compound.

Generation of HCV pseudoparticles and the inhibitory activity assay. Genotype 1b and 2a HCV pseudoparticles (HCVpp) were generated as described previously.[31] HEK 293T cells were co-transfected with pcDNA3.1-CE1E2 and pNL4.3-R$^-$E$^-$Luc plasmids. At 48 h post-transfection, the culture supernatant was harvested, filtered through 0.45 μm filter (Millipore, USA) and tested for luciferase activity to standardize the viral input for the subsequent inhibition analysis. Huh7.5 cells were co-incubated with compounds and HCVpp for 4 h at 37℃, then the cells were washed with PBS for twice following fresh medium addition. The luciferase activity was measured by luciferase assay system (Promega, USA) at 72 h.

Inactivation of HCV particles. The experiment was carried out as described previously.[32] Huh7.5 cells seeded in a 12-well plate were inoculated with HCV viral stock that was pre-treated with 1.0 μM of **L0909** for 2 h following the dilution by 20 times to the final concentration of 0.05 μM of **L0909**. As controls, HCV viral stock was treated with 0.05 μM or 1.0 μM of **L0909**. Intracellular proteins were extracted and detected with western blot at 72 h.

Induction and isolation of drug-resistant HCV *in vitro*. Huh 7.5 cells were infected with wild-type HCV virus stock (MOI = 0.7) and simultaneously treated with **L0909** at the initial concentration of 20 nM. Either infected cells or supernatants were passaged under drug selective pressure every 3 or 4 days as previously reported.[28] The process was repeated with stepwise (2-fold) increasing of **L0909** concentration in 10 weeks. After the cells were

treated with **L0909** at concentration of 5.0 μM, the viral RNA was extracted with Qiagen RNA extraction kit and reversed into cDNA by Reverse Transcription Kit (Promega, USA). The PCR products were then sequenced. Amino acid substitutions arose under **L0909** treatment pressure were identified by analyzing the sequence differences between the drug-treatment and control passages in the absence of drug.

The antiviral activity assay for drug combination. Huh 7.5 cells seeded in a 6-well plate were infected with HCV virus stock in the presence of test compound (0.1 μM) combined with 0.025 μM of simeprevir, 0.1 μM of sofosbuvir, or 0.016 nM of daclatasvir. Intracellular proteins were extracted and detected with Western Blot assay at 72 h. The q values to evaluate the combined effect were calculated with Webb's method, and $q < 1$, $=1$, and >1 indicating antagonism, addition and synergy, respectively.[33]

Statistical analysis. Data shown in the histogram were mean ± standard deviation of over 3 independent experiments. Data were analyzed using ANOVA analysis and Student's t-test. The level of significance was set at $p < 0.05$.

***In vivo* pharmacokinetics evaluation.** SD rats and beagle dogs were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Each type of animal was divided into two groups (n = 12, 6 males, 6 females for rats and n = 6, 3 males, 3 females for dogs) for orally and intravenously treatment, respectively. Test compound was homogeneously suspended in a 0.5% CMC-Na solution in certain concentration for oral administration while the citric acid buffer (pH5.0) containing 2.5% of DMSO was used to dissolve the sample for intravenous dosing. The dose was 5 mg/kg for intravenously

treatment or 15 mg/kg for oral treatment. The animals were housed under standard conditions, fastened for 12 h before the treatment. They had free access to water and consumed a standard laboratory diet throughout the experiments. Blood samples were collected from the jugular vein and collected into test tubes coated by sodium heparin at the time points 0.08, 0.25, 0.50, 1.0, 2.0, 4.0, 8.0, 24, 48 and 72 h after intravenous administration and 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 24, 48 and 72 h after oral administration. Plasma was centrifuged with 4000 rpm for 10 minutes at 2-8ºC and stored at -70 ºC until analysis. Plasma samples were analyzed by LC-MS/MS (Waters UPLC-Xevo TQ-S with ESI ion resource) equipped with a Waters ACQUITY UPLC® Peptide CSHTM C18 column (2.1*50mm, 1.7μm) to determine the plasma drug concentration. The pharmacokinetic curves were described as concentration *vs.* time plots. The non-compartmental model analysis was used for calculating pharmacokinetic parameters with WinNolin (8.0) program. All the experiments were approved by the Animal Care and Use Committee of People's Republic of China.

***In vivo* toxicity:** Kunming mice were randomly divided into 4 groups (n = 6, 3 male and 3 female in each group). The mice were fastened from 12 h before to 1h after administration. Then mice was individually given with 0.5% carboxymethyl cellulose sodium solvent control or different doses of compound by single oral gavage for each group. Body weight was monitored before treatment or on 7 days after treatment. And blood samples were collected to measure the function of liver and kidney on day 7 after treatment.

ASSOCIATED CONTENT

Supporting information

Supporting Information Available: ^{1}H NMR and UFLC-MS spectrum of compounds **32**–**66**, **68**, and **72**; synthesis procedures and ^{1}H NMR and ^{13}C NMR spectrum of compounds **8**–**13**; antiviral assays against Influenza virus, Coxsakie virus, and Zika virus; the pharmacokinetics study data of compound **8** and **13b**. This material is available free of charge via the Internet at http://pubs.acs.org.

Molecular formula strings and antiviral activity data (CSV)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

HCV, hepatitis C virus; DAAs, direct-acting antivirals; NS, nonstructural; SVR, sustained virologic response; qRT-PCR, real-time quantitative reverse-transcription polymerase chain reaction; MoA, mode of action; HCVcc, HCV cells culture; SAR, structure-activity relationship; EC_{50}, 50% effective concentration; CC_{50}, 50% cytotoxic concentration; SI, selectivity index; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; VX-950, telaprevir; PK, pharmacokinetics; AUC, area under curve; CL, clearance; V_z, apparent volume of distribution; AST, aspartate transaminase; ALT, alanine transaminase; UREA, blood urea nitrogen; CRE, creatinine.

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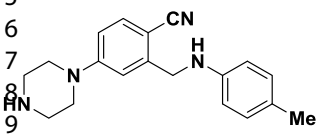


Hit Compound 1

HCVcc: EC_{50} = 1,680 nM
CC_{50} = 18,770 nM
SI = 11

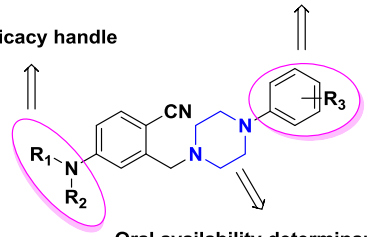
Chemical Optimization

Increased Antiviral Activity

Poor bioavailability *F* < 5% in rats

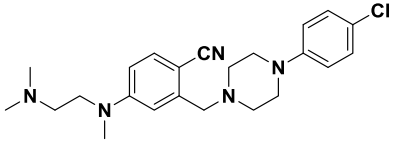
Efficacy handle

Metabolic stability



Oral availability determinant

New Chemotype

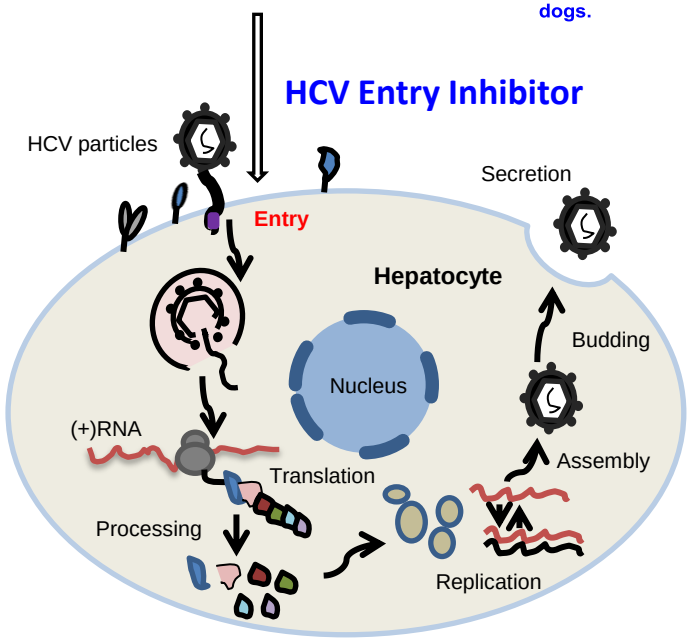


Candidate 35 (L0909)

HCVcc: EC_{50} = 22 nM
CC_{50} = 14,770 nM
SI > 600

Good bioavailability
***F* = 59% in rats, 39% in dogs.**
$T_{1/2}$ (oral) > 12 h in rats and dogs.

Note: The antiviral activity was determined by qRT-PCR method.



HCV life cycle