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Preliminary communication

Synthesis and evaluation of hexahydropyrimidines and diamines as novel hepatitis C virus inhibitors

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ABSTRACT

In order to identify novel anti-hepatitis C virus (HCV) agents we devised cell-based strategies and screened phenotypically small molecule chemical libraries with infectious HCV particles, and identified a hit compound (**1**) containing a hexahydropyrimidine (HHP) core. During our cell-based SAR study, we observed a conversion of HHP **1** into a linear diamine (**6**), which is the active component in inhibiting HCV and exhibited comparable antiviral activity to the cyclic HHP **1**. In addition, we engaged into the biological characterization of HHP and demonstrated that HHP does not interfere with HCV RNA replication, but with entry and release of viral particles. Here we report the results of the preliminary SAR and mechanism of action studies with HHP.

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1. Introduction

Approximately 200 million people worldwide are chronically infected with the hepatitis C virus (HCV) [1]. This pathogen is the major cause of acute hepatitis and chronic liver disease including cirrhosis and liver cancer. The HCV genome encodes a single polyprotein of ~3,000 amino acids which is co- and posttranslationally cleaved into structural (C, E1, E2, and p7) and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [2]. Recently, two direct acting antivirals (DAAs), telaprevir and boceprevir inhibiting the viral NS3/4A protease, have been approved by the FDA, but unfortunately, both therapies are expensive and accompanied by severe side effects and still require a combinatorial treatment regimen with pegylated interferon-alpha (PEG-IFN-α) and ribavirin which is prone to side effects, too [3–6]. To date, a number of anti-HCV agents mainly targeting the viral non-structural proteins are in clinical development, e.g. inhibiting NS5A or NS5B and, are awaiting FDA approval [1,7]. Nevertheless, the unpredictability of drug development and clear unmet medical needs encouraged us to screen for new, safer and potent drugs against HCV, ideally with a pan-genomic activity and a high genetic barrier to resistance. By using the recently developed infectious cell culture system for HCV (HCVcc), we devised strategies for a phenotypic high throughput screening (HTS) assay, which enables targeting the entire viral life cycle to identify novel HCV inhibitors [8].

2. Result and discussion

2.1. High throughput screening and hit selection

The screening campaign was conducted at 6 μM final compound concentration in duplicates with quadruplicate measurements. Selected active hits were subjected to 10-point dose response curve

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(DRC) analysis in the primary assay; briefly, serially diluted compounds were incubated with naïve Huh-7 target cells in 384-well microplates, and subsequently inoculated with HCVcc expressing an HCV NS5A-GFP fusion protein at a multiplicity of infection (MOI) of 1. At 72 h post-infection cells were analyzed by automated confocal microscopy (Opera®, PerkinElmer) and the half maximal effective concentration (EC_{50}) to inhibit the viral life cycle was determined. Additionally, to rule out that the observed antiviral effects were due to compound-induced toxicity the cytotoxic concentration (CC_{50}) was determined by fully automated quantification of cell nuclei, which served as a marker for cell proliferation.

The main focus of our small molecule screening campaign using cell culture derived HCV (HCVcc) was to identify compounds with a novel mechanism of action. Therefore, all identified active hits were counter-screened in the HCV replicon system to filter out inhibitors interfering with HCV RNA replication. This strategy reduces competition on already advanced DAAs interfering with viral replication. Following this rational, we identified amongst others a scaffold (**1**, Fig. 1) containing a hexahydropyrimidine (HHP) structure being inactive in the replicon system. This result suggested that HHP **1** interferes with early and/or late steps in the HCV life cycle rather than interfering with viral RNA replication. This drug mechanism accommodated our strategy and encouraged us to initiate a preliminary SAR study to move towards the development of novel HCV interventions. Additionally, to elucidate mode of action (MoA) of HHPs we conducted experiments with HCV pseudoparticles (HCVpp) [9] and analyzed viral secretion to monitor early and late steps in the HCV life cycle, respectively. Here we report the synthesis and cell based SAR study of HHPs and linear diamine analogs in the infectious HCV cell culture system and describe the MoA of HHPs.

2.2. Chemistry

To explore the preliminary SAR study of HHPs, we evaluated the effects of R^1 and R^2 groups. The general scheme for the synthesis of HHPs has been previously described and is briefly outlined in Scheme 1 [9,10]. Diamines **5** were prepared by a condensation of corresponding aldehydes and primary diamines, followed by reduction with $NaBH_4$. The diamines **5** were condensed with aldehydes to obtain the corresponding HHPs **2**.

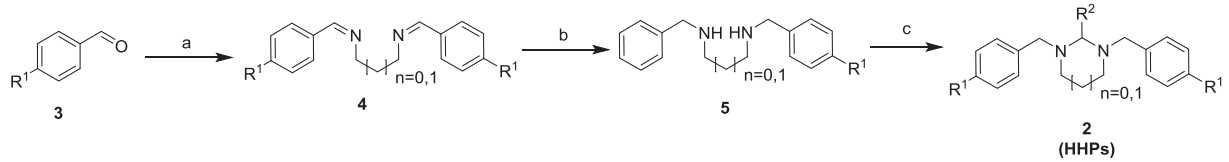
Compound **2f** bearing a ketone moiety in the HHP core was synthesized from commercially available trimethylene urea **31** by treatment of benzyl bromide (Scheme 2). Acylation of compound **6** with acetyl chloride provided compound **9** with an acetamido moiety. Dibenzamide **8** and diphenylacetamide **10** were obtained from propane-1,3-diamine **26** by treatment of the corresponding benzoyl chloride and 2-phenylpropionic acid, respectively. Compound **17** having an aniline moiety was prepared from dibromobutane **27** and aniline. Compounds **24** and **25** bearing a piperidine moiety were synthesized from intermediate **30**, which was synthesized from commercially available compound **28** by *N*-alkylation, followed by deprotection of the *N*-Boc group. Reductive alkylation with *p*-anisaldehyde and CuI-catalyzed C–N bond formation reaction with 4-methoxychlorobenzene gave the corresponding products **24** and **25**, respectively.

2.3. Structure–activity relationship (SAR) study

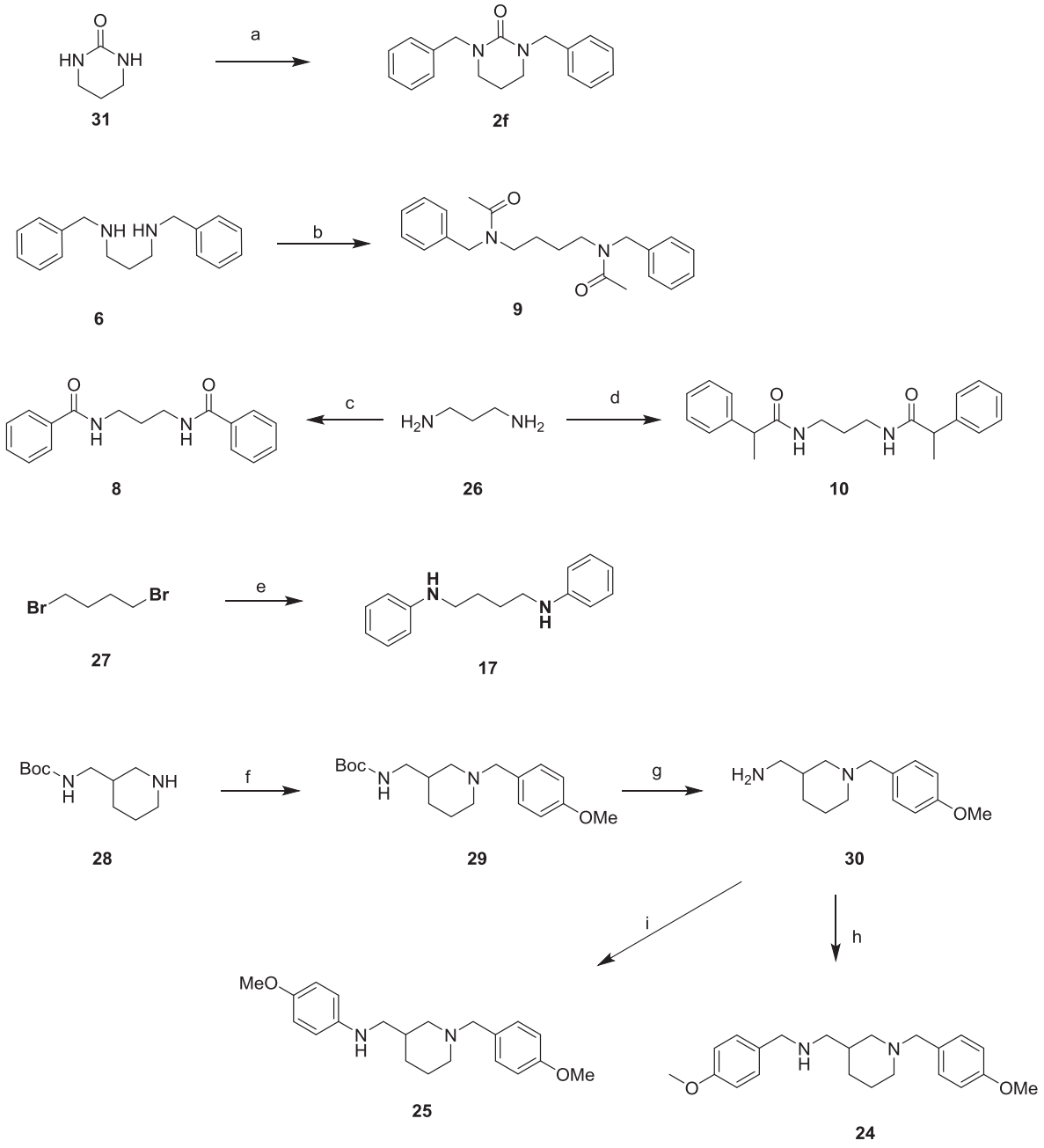
All synthesized HHPs were evaluated for their ability to inhibit the HCV life cycle as described earlier and the inhibitory activities of HHPs are summarized in Table 1. First, we explored R^2 effect on the HHPs (**1** and **2**, Fig. 1). Replacement of the 4-pyridyl group with a 4-Me_2N–Ph group significantly reduced anti-HCV potency (**2a**, EC_{50} = 25 μM), while 3-CF_3–Ph substitution retained the potency (**2b**, EC_{50} = 6.5 μM). Another heteroaryl compound **2c**, furanyl, showed no inhibitory activity (EC_{50} > 50 μM). Interestingly, methyl substituent **2d** was almost equipotent (EC_{50} = 3.7 μM) compared to HHP **1**, whereas no inhibitory potency was observed in unsubstituted compound (**2e**, EC_{50} > 50 μM). Furthermore, ketone **2f** was inactive. In case of R^1 = 4-MeO and 4-Me_2N, most of the compounds (**2g**–**p**) showed similar activity, ranging between 0.83 and 3.2 μM EC_{50} values. Thus no strong electron effect of R_2 by substituents was observed in this series. Next, we examined the effect of the R^1 group. 4-Methoxy- (**2g**) and 4-Me_2N-substituted (**2n**) compounds showed three- and five-fold improved potency (EC_{50} = 1.5 and 0.83 μM, respectively) compared to HHP **1**, but also demonstrated increased cytotoxicity of CC_{50} = 23 and 14 μM, respectively. Likewise, compounds **2i** and **2m** with a 4-MeO group in R^1 exhibited better potency (EC_{50} = 3.2 and 1.3 μM, respectively) in comparison to compounds **2b** and **2a** (EC_{50} = 6.5 and 25 μM, respectively). Asymmetric HHPs (**2q**–**s**) were also evaluated and showed moderate anti-HCV activities (EC_{50} = 4.7, 2.7, 11 μM, respectively). Regarding ring size, five-membered imidazolidine analogs (**2t**–**v**) were evaluated and demonstrated a loss of antiviral activities regardless of the substitution patterns at R^2 (Table 1).

Next, we measured the metabolic stability of HHP (**1**) in the liver microsomal matrix. Interestingly, only molecular weight of diamine (**6**), a ring-cleaved form of HHP, was predominantly detected in the mass spectrum (data not shown), suggesting that HHP was converted to diamine **6** (Fig. 2). It was recently reported that an HHP, named aminal, is reversibly in an equilibrium with diamine **6** in an aqueous environment [11]. Therefore, we examined the chemical stability of HHP in cell culture medium. HHP **1** was incubated with cells in culture medium at 37 °C for two days, followed by analysis of compound **1** in the cell culture supernatant by LC/MS. This revealed that only linear diamine **6** was detected by mass spectrum but not even trace amounts of HHP **1**. We assumed that the linear diamine **6** is most likely to be the active component inhibiting HCV infection and therefore several additional linear diamine compounds were synthesized and characterized. Diamines with different lengths between the two nitrogen atoms and substituted diamines were synthesized as shown in Schemes 1 and 2 and their activities in the HCVcc system are summarized in Table 2.

The anti-HCV efficacy of diamine **6** is comparable to HHP **1** with EC_{50} values of 4.6 μM and 4.3 μM, respectively. This result strongly suggests that the linear diamine is the active component inhibiting HCV infection. Surprisingly, imine compound **7**, the precursor of compound **6**, was inactive. In addition, no inhibition was observed



Scheme 1. General synthetic procedure of diamine **5** and HHPs **2**. Reagents and conditions: (a) diamines, EtOH, room temperature, 2 h; (b) $NaBH_4$, EtOH, room temperature, overnight; (c) aldehydes, H_2O, reflux, overnight.



Scheme 2. Reagents and conditions: (a) Benzyl bromide, NaH, 1,4-dioxane, reflux, 12 h; (b) acetyl chloride, K_2CO_3, DMF, 120 °C, 24 h; (c) benzoyl chloride, TEA, DCM, rt; (d) 2-phenylpropionic acid, EDC, HOBT, DCM, rt; (e) aniline, K_2CO_3, DMF, 120 °C, 24 h; (f) (i) *p*-anisaldehyde, MeOH, 50 °C, 12 h, (ii) $NaBH_4$, rt, 12 h; (g) 4 N HCl in dioxane, rt, 3 h; (h) *p*-anisaldehyde, $NaBH_4$, MeOH; (i) 4-methoxychlorobenzene, CuI, K_2CO_3, L-proline, DMSO, microwave, 130 °C, 20 min.

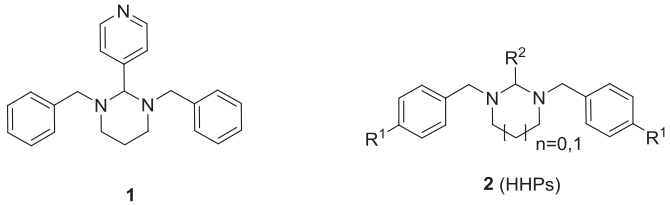


Fig. 1. Hit compound **1** (HHP) and generic structure of HHPs (**2**).

with amide compounds (**8**–**10**). This result suggests that a sp3-hybridized nitrogen is required for anti-HCV potency. Based on this observation, we further explored the effect of various linker lengths between the two nitrogen atoms. Increasing the linker size to greater than three-carbon units improved antiviral potency as shown by compounds **12**–**15** (0.98 for four-carbon, 0.61 for five-carbon, and 0.65 μM for six-carbon), while compound **11** with a shorter linker (two-carbon) completely lost its antiviral activity ($EC_{50} > 50$ μM). This result corroborated our observation that five-membered imidazolidine analogs (**2t**–**v**), having only two carbon units between two nitrogen atoms, were inactive. Insertion of an oxygen atom in the linker slightly reduced the activity (**16**, $EC_{50} = 1.7$ μM). Among longer linkers, four-carbon linker (**12**) provided improved profiling in both increased antiviral potency

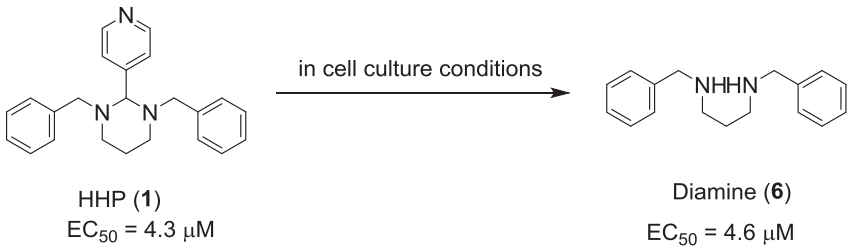
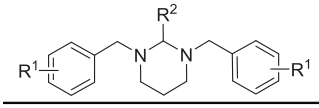
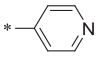
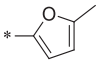
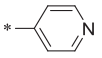
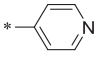
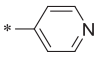
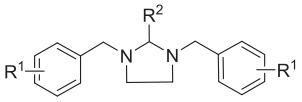
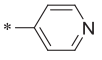


Fig. 2. Conversion of HHP (**1**) to linear diamine in cell culture conditions.

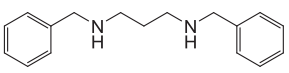
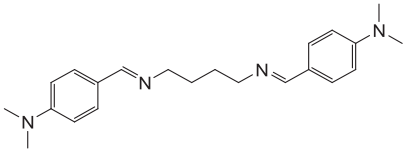
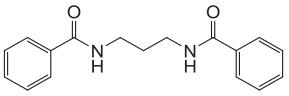
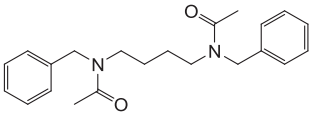
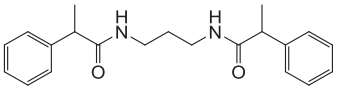
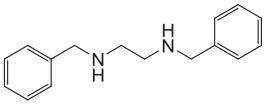
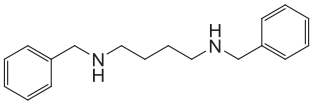
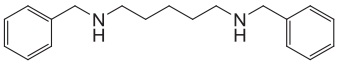
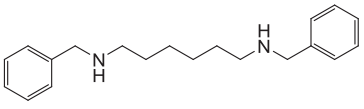
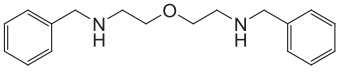
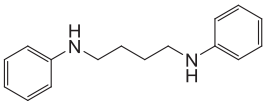
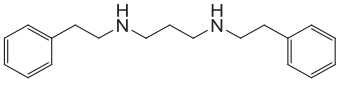
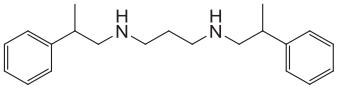
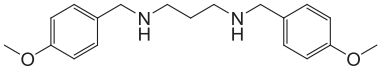
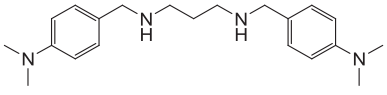
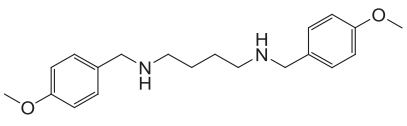
($EC_{50} = 0.98$ μM) and reduced cytotoxicity ($CC_{50} = 42$ μM), thereby resulting in a SI value of 43. Replacement of benzyl amine to aniline resulted in a total loss of potency (**17**, $EC_{50} > 50$ μM), while substitution to phenethylamine improved potency (**18**, $EC_{50} = 1.1$ μM). An alpha-methyl substituted phenethylamine compound (**19**) also gave improved antiviral potency, but both phenethylamine compounds (**18** and **19**) increased cytotoxic effects in parallel. A 4-methoxy or 4-dimethylamino substituent in the HHP structure (**2g** and **2n**) improved the anti-HCV potency by three- and five-fold, respectively. This trend was also observed in linear diamine compounds in which substitution with 4-methoxy (**20** and **22**) and 4-dimethylamino groups (**21** and **23**) on the benzyl moiety showed

Table 1
Activity profiling of HHPs.

No.	R^1	R^2	EC_{50} (μM)	CC_{50} (μM)	SI (CC_{50}/EC_{50})
					
1	H		4.3	>50	>12
2a	H	4-Me_2N–Ph	25	>50	>2.0
2b	H	3-CF_3–Ph	6.5	>50	>7.7
2c	H		>50	>50	N.A.
2d	H	Me	3.7	>50	>14
2e	H	H	>50	>50	N.A.
2f	H	* = O (ketone)	>50	>50	N.A.
2g	4-MeO		1.5	23	15
2h	4-MeO	4-CF_3–Ph	1.6	34	21
2i	4-MeO	3-CF_3–Ph	3.2	43	13
2j	4-MeO	4-CN–Ph	1.5	20	13
2k	4-MeO	4-F–Ph	1.9	38	20
2l	4-MeO	3-F–4-CN–Ph	1.9	29	15
2m	4-MeO	4-Me_2N–Ph	1.3	28	22
2n	4-Me_2N		0.83	14	17
2o	4-Me_2N	4-F–Ph	1.3	15	12
2p	4-Me_2N	4-CF_3–Ph	1.5	23	15
2q	3-MeO/2-F		4.7	>50	>11
2r	3-MeO/2-F	4-CF_3–Ph	2.7	23	8.5
2s	2-Cl/2-F	3-CF_3–Ph	11	>50	>4.5
					
2t	H		>50	>50	N.A.
2u	H	Me	>50	>50	N.A.
2v	H	4-Me_2N–Ph	>50	>50	N.A.

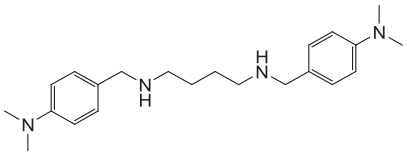
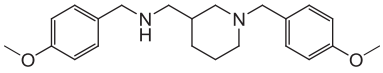
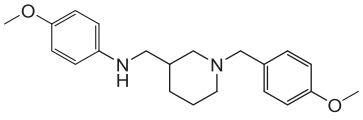
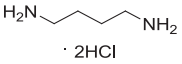
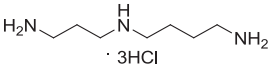
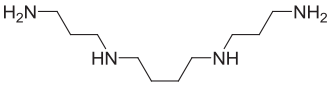
EC_{50} and CC_{50} values were determined by 10-point DRC analysis in duplicates with quadruplicate measurements. N.A. not applicable.

Table 2
Activity profiling of diamines.

No.	Structure	EC_{50} (μM)	CC_{50} (μM)	SI (CC_{50}/EC_{50})
6		4.6	27	5.9
7		>50	>50	N.A.
8		>50	>50	N.A.
9		>50	>50	N.A.
10		>50	>50	N.A.
11		>50	>50	N.A.
12		0.98	42	43
14		0.61	29	48
15		0.65	17	26
16		1.7	28	16
17		>50	>50	N.A.
18		1.1	12	11
19		1.2	18	15
20		1.5	33	22
21		0.88	17	19
22		0.38	32	84

(*continued on next page*)

Table 2 (*continued*)

No.	Structure	EC_{50} (μM)	CC_{50} (μM)	SI (CC_{50}/EC_{50})
23		0.32	11	34
24		1.1	34.5	31
25		11	>50	>4.7
Putrescine		>50	>50	N.A.
Spermidine		>50	>50	N.A.
Spermine		>50	>50	N.A.

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

increased potencies of three to five-fold. Finally, compound **22** with four carbon linker and 4-methoxy group exhibited not only significantly improved antiviral efficacy ($EC_{50} = 0.38$ μM), but also reduced cytotoxicity ($CC_{50} = 32$ μM), resulting in an SI value of 84. Two compounds **24** and **25** with piperidine moiety in a linear diamine were examined. Interestingly, compound **24** with EC_{50} value of 1.1 μM is equipotent with acyclic diamine **20**, whereas compound **25** significantly reduced the activity ($EC_{50} = 11$ μM). This result revealed that the cyclic and rigid form of diamine is comparable to linear diamine but diamines bearing at least an aniline moiety demonstrated poor activity or were inactive (**17** and **25**). Since diamine structures appear similar to polyamines, which play important roles in cells [12–14], we tested polyamines including putrescine, [15] spermidine, [16] and spermine, [17,18] but none of these compounds was active in the infectious HCVcc system.

2.4. Characterization of HHPs by virological assays

After screening >220,000 small chemical molecules, selected hit compounds were re-ordered and re-confirmed in the primary HTS assay followed by counter-screening with HCV replicon cells. Only re-confirmed hit compounds inactive in the replicon system suited our strategy. Among others, HHP **1** did not interfere with HCV RNA replication ($EC_{50} > 50$ μM and $CC_{50} > 50$ μM), which encouraged us to initiate a SAR study. In parallel, we engaged in the characterization of HHPs and performed preliminary MoA studies. The HCVpp system was used to monitor early steps of the viral life cycle, especially binding and internalization of viral particles. Thereby, we observed that HHP interfered with E1/E2-mediated viral entry (HHP **1** EC_{50} 4 μM and $CC_{50} > 50$ μM). Furthermore, in order to analyze late steps of the HCV life cycle, the secretion of virions was determined by a viral supernatant transfer assay [19]. Under this experimental conditions HHP prevented the release of infectious virions into the cell culture supernatant (data not shown). Thus, these results suggested that HHPs have a novel MoA by interfering with early and late steps of the HCV life cycle. Continuous effort will be required to characterize the MoA of HHPs in more detail.

3. Conclusion

We identified a HHP compound in our phenotypic HTS campaign using the infectious HCVcc system. The aim of the present research work was to synthesize novel HHP lead compounds and to elucidate the MoA of HHP. During the cell based SAR study we characterized HHPs and observed a conversion into a linear diamine under cell culture conditions, which is the active component in inhibiting HCV. This observation led to an adjustment of our chemistry strategy and focusing on a systematic analysis of linear diamines. This study revealed that diamines to be potent have to have two crucial substitution patterns containing: (i) more than two carbon units between two nitrogen atoms, (ii) a basic diamine with sp3-hybridized nitrogen.

In parallel to our SAR studies we performed a preliminary MoA study and observed that HHPs exclusively interfere with viral entry and release. Interestingly, a compound similar to our HHP was recently described interfering with entry steps of Marburg virus as demonstrated by using pseudoparticles [20]. Although the exact MoA of this HHP series has yet to be determined, HHPs and diamines can be useful compounds to further investigate the poorly understood HCV life cycle or for future drug development campaigns.

4. Experimental section

4.1. Chemistry

4.1.1. General

All materials were obtained from commercial suppliers and used without further purification. All solvents used were dried using an aluminum oxide column. Thin-layer chromatography was performed on pre-coated silica gel 60 F254 plates. Purification of compounds was carried out by normal phase column chromatography (MPLC, Silica gel 230–400 mesh). NMR spectra were recorded on a Varian 400 MHz. LC/MS data were obtained using a Waters 2695 LC and Micromass ZQ spectrometer. Identity of all final

compounds was confirmed by proton NMR and by mass spectrometry and carbon NMR data of representative compounds **1**, **2n**, **6**, **18**, **22**, and **23** were reported. Yields refer to purified products and are not optimized.

4.1.2. General procedure for the preparation of compound *4*: synthesis of 4,4′-((1E,1′E)-(propane-1,3-diylbis(azanylylidene)) bis(methanylylidene))bis(N,N-dimethylaniline) (*7*)

A solution of propane-1,3-diamine (1 g, 13.491 mmol) and 4-(dimethylamino)benzaldehyde (3.82 g, 25.634 mmol) in anhydrous EtOH (100 mL) was stirred at room temperature for 2 h. After reaction was completed, the precipitate was filtered and washed with *n*-hexane to give the desired product **7** (4.0 g, 91%) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 (s, 2H), 7.57 (d, J = 8.4 Hz, 4H), 6.67 (d, J = 8.4 Hz, 4H), 3.61 (t, J = 6.8 Hz, 4H), 2.99 (s, 12H), 2.05 (p, J = 6.8 Hz, J = 13.7 Hz, 2H); LC/MS (electrospray) m/z $(M + H)^+$ 337.27.

4.1.3. General procedure for the preparation of compound *5*: synthesis of 4-(1,3-bis(4-methoxybenzyl)hexahydropyrimidin-2-yl) benzonitrile (*21*)

To a solution of **7** (2.0 g, 5.944 mmol) in anhydrous EtOH (40 mL) was added $NaBH_4$ (270 mg, 7.132 mmol) and stirred at room temperature for 2 h. The reaction was quenched by the addition of H_2O and concentrated *in vacuo*. The reaction residue was diluted with DCM and washed with water. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give the desired product **21** as white solid (1.51 g, 75%). The product was used for the next reaction without any purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.14 (d, J = 8.4 Hz, 4H), 6.69 (d, J = 8.4 Hz, 4H), 3.66 (s, 4H), 2.91 (s, 12H), 2.66 (t, J = 6.8 Hz, 4H), 1.68 (p, J = 6.8 Hz, J = 13.7 Hz, 2H); LC/MS (electrospray) m/z $(M + H)^+$ 341.27.

4.1.4. General procedure for the preparation of compound *2*: synthesis of 4,4′-((2-(pyridin-4-yl)dihydropyrimidine-1,3(2H,4H)-diyl)bis(methylene))bis(N,N-dimethylaniline) (*2n*)

A solution of **21** (100 mg, 0.293 mmol) and isonicotinaldehyde (27 μL, 0.293 mmol) in H_2O (2 mL) was refluxed for 2 h. The reaction mixture was concentrated *in vacuo* and the precipitate was filtered and washed with ether to give the desired product **2n** (40 mg, Yield 31%) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.59 (d, J = 8.0 Hz, 2H), 7.59 (d, J = 8.0 Hz, 2H), 7.02 (d, J = 8.8 Hz, 4H), 6.61 (d, J = 8.8 Hz, 4H), 3.66 (s, 1H), 3.48 (d, J = 13.2 Hz, 2H), 2.98 (m, 4H), 2.88 (s, 12H), 2.03 (t, J = 11.6 Hz, 2H), 1.69 (m, 1H), 1.48 (m, 1H); LC/MS (electrospray) m/z $(M + H)^+$ 430.38.

4.1.5. Synthesis of 1,3-dibenzyl-2-(pyridin-4-yl) hexahydropyrimidine (*1*)

This compound was synthesized by the procedure described above for the synthesis of compound **2n**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.64 (d, J = 8.0 Hz, 2H), 7.64 (d, J = 8.0 Hz, 2H), 7.21 (m, 10H), 3.75 (s, 1H), 3.59 (d, J = 13.2 Hz, 2H), 3.03 (d, J = 13.2 Hz, 2H), 2.97 (m, 2H), 2.13 (t, J = 11.6 Hz, 2H), 1.79 (m, 1H), 1.56 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 151.5, 150.2, 139.1, 128.7, 128.4, 127.1, 124.9, 86.8, 58.5, 51.0, 23.6; LC/MS (electrospray) m/z $(M + H)^+$ 344.64.

4.1.6. Synthesis of 4-(1,3-dibenzyl-hexahydropyrimidin-2-yl)-N,N-dimethylbenzenamine (*2a*)

This compound was synthesized by the procedure described above for the synthesis of compound **2n**. Yield 63%, white solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 (d, J = 4.8 Hz, 4H), 7.22–7.23 (m, 4H), 7.19–7.21 (m, 4H), 6.57 (d, J = 5.6 Hz, 2H), 3.82 (s, 1H), 3.54 (d, J = 4.8 Hz, 2H), 3.08 (d, J = 7.2 Hz, 2H), 3.04 (s, 6H), 2.42–2.43 (m, 2H), 2.32–2.33 (m, 2H), 1.48–1.52 (m, 2H); LC/MS (electrospray) m/z $(M + H)^+$ 386.50.

4.1.7. Synthesis of 1,3-dibenzyl-2-(3-(trifluoromethyl)phenyl) hexahydropyrimidine (*2b*)

This compound was synthesized by the procedure described above for the synthesis of compound **2n**. Yield 54%, white solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 (s, 1H), 7.86 (d, J = 7.6 Hz, 1H), 7.52 (d, J = 7.6 Hz, 1H), 7.45 (t, J = 7.6 Hz, 1H), 7.21 (m, 10H), 3.68 (s, 1H), 3.51 (d, J = 13.6 Hz, 2H), 2.99 (m, 2H), 2.94 (d, J = 13.6 Hz, 2H), 2.09 (m, 2H), 1.82 (m, 1H), 1.46 (m, 1H); LC/MS (electrospray) m/z $(M + H)^+$ 410.23.

4.1.8. Synthesis of 1,3-dibenzyl-2-(5-methylfuran-2-yl)-hexahydropyrimidine (*2c*)

This compound was synthesized by the procedure described above for the synthesis of compound **2n**. Yield 15%, brown solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27–7.29 (m, 2H), 7.22–7.23 (m, 4H), 7.19–7.21 (m, 4H), 6.27 (d, J = 4.8 Hz, 1H), 6.12 (d, J = 5.6 Hz, 1H), 3.82 (s, 1H), 3.57 (d, J = 13.6 Hz, 2H), 3.11 (d, J = 4.8 Hz, 2H), 2.42–2.43 (m, 2H), 2.32–2.33 (m, 2H), 2.13 (s, 3H), 1.48–1.51 (m, 2H); LC/MS (electrospray) m/z $(M + H)^+$ 347.20.

4.1.9. Synthesis of 1,3-dibenzyl-2-methyl-hexahydropyrimidine (*2d*)

This compound was synthesized by the procedure described above for the synthesis of compound **2n**. Yield 36%, yellow solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27–7.29 (m, 2H), 7.22–7.23 (m, 4H), 7.19–7.21 (m, 4H), 3.82 (d, J = 13.6 Hz, 2H), 3.63 (q, J = 4.8 Hz, 1H), 3.58 (d, J = 6.4 Hz, 2H), 2.42–2.43 (m, 2H), 2.32–2.33 (m, 2H), 1.47–1.49 (m, 2H), 1.28 (d, J = 5.6 Hz, 3H); LC/MS (electrospray) m/z $(M + H)^+$ 281.20.

4.1.10. Synthesis of 1,3-dibenzyl-hexahydropyrimidine (*2e*)

This compound was synthesized by the procedure described above for the synthesis of compound **2n**. Yield 45%, yellow solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27–7.29 (m, 2H), 7.22–7.23 (m, 4H), 7.19–7.21 (m, 4H), 3.86 (q, J = 4.8 Hz, 2H), 3.58 (d, J = 6.4 Hz, 2H), 3.47 (s, 2H), 2.34 (t, J = 5.6 Hz, 4H), 1.50–1.52 (m, 2H); LC/MS (electrospray) m/z $(M + H)^+$ 267.20.

4.1.11. Synthesis of 1,3-dibenzyl-tetrahydropyrimidin-2(1H)-one (*2f*)

To a solution of trimethylene urea (0.30 g, 2.996 mmol) in 1,4-dioxane (3 mL) were sequentially added NaH (0.18 g, 4.494 mmol) and benzyl bromide (1.02 g, 5.992 mmol) at 0 °C. The reaction mixture was refluxed for 12 h and cooled to room temperature. The solvent was removed under reduced pressure, the residue was diluted with ethyl acetate and washed with saturated $NaHCO_3$. The organic layer was dried over Na_2SO_4, concentrated *in vacuo*, and purified by column chromatography (silica gel, gradient 10–50 percent, ethyl acetate in hexane) to give compound **2f** (0.39 g, yield 42%) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37–7.39 (m, 4H), 7.32–7.33 (m, 4H), 7.29–7.30 (m, 2H), 3.84 (q, J = 5.6 Hz, 2H), 3.54 (d, J = 8.8 Hz, 2H), 3.28 (t, J = 4.8 Hz, 4H), 1.81–1.82 (m, 2H); LC/MS (electrospray) m/z $(M + H)^+$ 281.30.

4.1.12. Synthesis of 1,3-bis(4-methoxybenzyl)-2-(pyridin-4-yl) hexahydropyrimidine (*2g*)

This compound was synthesized by the procedure described above for the synthesis of compound **2n**. Yield 56%, white solid, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.60 (d, J = 5.6 Hz, 2H), 7.59 (d, J = 5.6 Hz, 2H), 7.08 (d, J = 4.8 Hz, 4H), 6.78 (d, J = 4.8 Hz, 4H), 3.75 (s, 6H), 3.66 (s, 1H), 3.49 (d, J = 13.2 Hz, 2H), 2.93 (m, 4H), 2.06 (t, J = 11.6 Hz, 2H), 1.72 (m, 1H), 1.52 (m, 1H); LC/MS (electrospray) m/z $(M + H)^+$ 404.26.

4.1.13. Synthesis of 1,3-bis(4-methoxybenzyl)-2-(4-(trifluoromethyl) phenyl)hexahydropyrimidine (*2h*)

This compound was synthesized by the procedure described above for the synthesis of compound **2n**. Yield 30%, white solid; ^{1}H

NMR (400 MHz, $CDCl_3$) δ 7.76 (d, J = 5.6 Hz, 2H), 7.60 (d, J = 5.6 Hz, 2H), 7.07 (d, J = 4.8 Hz, 4H), 6.75 (d, J = 4.8 Hz, 4H), 3.74 (s, 6H), 3.64 (s, 1H), 3.44 (d, J = 13.2 Hz, 2H), 2.97 (m, 2H), 2.83 (d, J = 13.2 Hz, 2H), 2.02 (t, J = 11.6 Hz, 2H), 1.78 (m, 1H), 1.45 (m, 1H); LC/MS (electrospray) m/z $(M + H)^+$ 471.26.

4.1.14. Synthesis of 1,3-bis(4-methoxybenzyl)-2-(3-(trifluoromethyl)phenyl)hexahydropyrimidine (**2i**)

This compound was synthesized by the procedure described above for the synthesis of compound **2n**. Yield 12%, white solid; ^1H NMR (400 MHz, $CDCl_3$) δ 7.94 (s, 1H), 7.85 (d, J = 7.2 Hz, 1H), 7.55 (d, J = 8.0 Hz, 1H), 7.48 (t, J = 7.6 Hz, 1H), 7.08 (d, J = 4.8 Hz, 4H), 6.78 (d, J = 4.8 Hz, 4H), 3.77 (s, 6H), 3.66 (s, 1H), 3.46 (d, J = 13.2 Hz, 2H), 2.99 (d, J = 11.6 Hz, 2H), 2.85 (d, J = 13.2 Hz, 2H), 2.05 (t, J = 11.6 Hz, 2H), 1.82 (m, 1H), 1.50 (m, 1H); LC/MS (electrospray) m/z $(M + H)^+$ 469.22.

4.1.15. Synthesis of 4-(1,3-bis(4-methoxybenzyl) hexahydropyrimidin-2-yl)benzonitrile (**2j**)

This compound was synthesized by the procedure described above for the synthesis of compound **2n**. Yield 33%, white solid; ^1H NMR (400 MHz, $CDCl_3$) δ 7.82 (d, J = 5.6 Hz, 2H), 7.64 (d, J = 5.6 Hz, 2H), 7.05 (d, J = 4.8 Hz, 4H), 6.76 (d, J = 4.8 Hz, 4H), 3.74 (s, 6H), 3.70 (s, 1H), 3.24 (d, J = 13.2 Hz, 2H), 2.95 (m, 2H), 2.87 (d, J = 13.2 Hz, 2H), 2.02 (t, J = 11.6 Hz, 2H), 1.75 (m, 1H), 1.50 (m, 1H); LC/MS (electrospray) m/z $(M + H)^+$ 428.27.

4.1.16. Synthesis of 2-(4-fluorophenyl)-1,3-bis(4-methoxybenzyl) hexahydropyrimidine (**2k**)

This compound was synthesized by the procedure described above for the synthesis of compound **2n**. Yield 14%, white solid; ^1H NMR (400 MHz, $CDCl_3$) δ 7.60 (m, 2H), 7.04 (m, 6H), 6.76 (d, J = 4.8 Hz, 4H), 3.74 (s, 6H), 3.56 (s, 1H), 3.48 (d, J = 13.2 Hz, 2H), 2.94 (d, J = 11.6 Hz, 2H), 2.76 (d, J = 13.2 Hz, 2H), 1.98 (t, J = 11.6 Hz, 2H), 1.78 (m, 1H), 1.43 (m, 1H); LC/MS (electrospray) m/z $(M + H)^+$ 419.15.

4.1.17. Synthesis of 4-(1,3-bis(4-methoxybenzyl) hexahydropyrimidin-2-yl)-2-fluorobenzonitrile (**2l**)

This compound was synthesized by the procedure described above for the synthesis of compound **2n**. Yield 49%, oil; ^1H NMR (400 MHz, $CDCl_3$) δ 7.54 (m, 3H), 7.07 (d, J = 4.8 Hz, 4H), 6.75 (d, J = 4.8 Hz, 4H), 3.75 (s, 6H), 3.72 (s, 1H), 3.46 (d, J = 13.2 Hz, 2H), 2.99 (d, J = 13.2 Hz, 2H), 2.92 (m, 2H), 2.08 (t, J = 11.6 Hz, 2H), 1.68 (m, 1H), 1.56 (m, 1H); LC/MS (electrospray) m/z $(M + H)^+$ 446.30.

4.1.18. Synthesis of 4-(1,3-bis(4-methoxybenzyl) hexahydropyrimidin-2-yl)-N,N-dimethylaniline (**2m**)

This compound was synthesized by the procedure described above for the synthesis of compound **2n**. Yield 56%, white solid; ^1H NMR (400 MHz, $CDCl_3$) δ 7.44 (d, J = 8.0 Hz, 2H), 7.08 (d, J = 8.8 Hz, 4H), 6.76 (d, J = 8.8 Hz, 4H), 6.70 (d, J = 8.0 Hz, 2H), 3.74 (s, 6H), 3.49 (d, J = 13.2 Hz, 2H), 3.42 (s, 1H), 2.92 (m, 8H), 2.74 (d, J = 13.2 Hz, 2H), 1.93 (t, J = 11.6 Hz, 2H), 1.76 (m, 1H), 1.40 (m, 1H); LC/MS (electrospray) m/z $(M + H)^+$ 446.37.

4.1.19. Synthesis of 4,4′-((2-(pyridin-4-yl)dihydropyrimidine-1,3(2H,4H)-diyl)bis(methylene))bis(N,N-dimethylaniline) (**2n**)

This compound was synthesized by the procedure described above for the synthesis of compound **2n**. Yield 31%, white solid; ^1H NMR (400 MHz, $CDCl_3$) δ 8.59 (d, J = 8.0 Hz, 2H), 7.59 (d, J = 8.0 Hz, 2H), 7.02 (d, J = 8.8 Hz, 4H), 6.61 (d, J = 8.8 Hz, 4H), 3.66 (s, 1H), 3.48 (d, J = 13.2 Hz, 2H), 2.98 (m, 4H), 2.88 (s, 12H), 2.03 (t, J = 11.6 Hz, 2H), 1.69 (m, 1H), 1.48 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 150.1, 150.0, 129.7, 126.9, 124.9, 112.6, 86.5, 57.9, 50.6, 40.9, 23.4; LC/MS (electrospray) m/z $(M + H)^+$ 430.38.

4.1.20. Synthesis of 4,4′-((2-(4-fluorophenyl)dihydropyrimidine-1,3(2H,4H)-diyl)bis(methylene))bis(N,N-dimethylaniline) (**2o**)

This compound was synthesized by the procedure described above for the synthesis of compound **2n**. Yield 45%, white solid; ^1H NMR (400 MHz, $CDCl_3$) δ 7.60 (m, 2H), 7.02 (m, 6H), 6.61 (d, J = 4.8 Hz, 4H), 3.51 (s, 1H), 3.46 (d, J = 13.2 Hz, 2H), 2.98 (d, J = 11.6 Hz, 2H), 2.71 (d, J = 13.2 Hz, 2H), 1.97 (t, J = 11.6 Hz, 2H), 1.75 (m, 1H), 1.42 (m, 1H); LC/MS (electrospray) m/z $(M + H)^+$ 447.39.

4.1.21. Synthesis of 4,4′-((2-(4-(trifluoromethyl)phenyl) dihydropyrimidine-1,3(2H,4H)-diyl)bis(methylene))bis(N,N-dimethylaniline) (**2p**)

This compound was synthesized by the procedure described above for the synthesis of compound **2n**. Yield 24%, white solid; ^1H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, J = 8.0 Hz, 2H), 7.59 (d, J = 8.0 Hz, 2H), 7.02 (d, J = 8.8 Hz, 4H), 6.61 (d, J = 8.8 Hz, 4H), 3.62 (s, 1H), 3.43 (d, J = 13.2 Hz, 2H), 2.98 (d, J = 11.6 Hz, 2H), 2.87 (s, 12H), 2.80 (d, J = 13.2 Hz, 2H), 2.00 (t, J = 11.6 Hz, 2H), 1.76 (m, 1H), 1.48 (m, 1H); LC/MS (electrospray) m/z $(M + H)^+$ 497.45.

4.1.22. Synthesis of 4-(1,3-dibenzylimidazolidin-2-yl)pyridine (**2t**)

This compound was synthesized by the procedure described above for the synthesis of compound **2n**. Yield 36%; White solid; ^1H NMR (400 MHz, $CDCl_3$) δ 8.57 (d, J = 8.0 Hz, 2H), 7.35 (d, J = 7.2 Hz, 2H), 7.29–7.30 (m, 2H), 7.22–7.23 (m, 4H), 7.20–7.21 (m, 4H), 3.82 (s, 1H), 3.55 (d, J = 8.4 Hz, 2H), 3.38 (d, J = 6.4 Hz, 2H), 2.51–2.52 (m, 2H), 2.41–2.42 (m, 2H); LC/MS (electrospray) m/z $(M + H)^+$ 330.20.

4.1.23. Synthesis of 1,3-dibenzyl-2-methylimidazolidine (**2u**)

This compound was synthesized by the procedure described above for the synthesis of compound **2n**. Yield 30%, yellow solid; ^1H NMR (400 MHz, $CDCl_3$) δ 7.28–7.29 (m, 2H), 7.22–7.23 (m, 4H), 7.20–7.21 (m, 4H), 3.83 (d, J = 6.4 Hz, 2H), 3.65 (q, J = 4.0 Hz, 1H), 3.60 (d, J = 6.4 Hz, 2H), 2.52–2.53 (m, 2H), 2.42–2.43 (m, 2H), 1.18 (d, J = 5.6 Hz, 3H); LC/MS (electrospray) m/z $(M + H)^+$ 267.10.

4.1.24. Synthesis of 4-(1,3-dibenzylimidazolidin-2-yl)-N,N-dimethylbenzenamine (**2v**)

This compound was synthesized by the procedure described above for the synthesis of compound **2n**. Yield 56%, yellow solid; 1H NMR (400 MHz, $CDCl_3$) δ 7.29–7.31 (d, J = 7.2 Hz, 4H), 7.22–7.23 (m, 4H), 7.20–7.21 (m, 4H), 6.57 (d, J = 5.6 Hz, 2H), 3.83 (s, 1H), 3.54 (d, J = 8.4 Hz, 2H), 3.39 (d, J = 6.4 Hz, 2H), 3.02 (s, 6H), 2.42–2.43 (m, 2H), 2.32–2.33 (m, 2H); LC/MS (electrospray) m/z $(M + H)^+$ 372.30.

4.1.25. Synthesis of N^1,N^3-dibenzylpropane-1,3-diamine (**6**)

This compound was synthesized by the procedure described above for the synthesis of compound **21**. Yield 75%, oil; ^1H NMR (400 MHz, acetone-d_6) δ 7.37 (d, J = 7.2 Hz, 4H), 7.31 (t, J = 5.8 Hz, 4H), 7.23 (t, J = 7.2 Hz, 2H), 3.76 (s, 4H), 2.69 (t, J = 6.8 Hz, 4H), 1.70 (p, J_{12} = 6.8 Hz, J_{13} = 13.7 Hz, 2H); ^{13}C NMR (100 MHz, acetone-d_6) δ 142.1, 128.9, 128.7, 127.2, 54.5, 48.6, 30.9; LC/MS (electrospray) m/z $(M + H)^+$ 255.29.

4.1.26. Synthesis of N,N′-(propane-1,3-diyl)dibenzamide (**8**)

To a stirred solution of diamine (58 mg, 0.65 mmol) in anhydrous dichloromethane (2.2 mL) were added benzoyl chloride (0.23 mL, 1.95 mmol) and triethylamine (0.28 mL, 1.95 mmol) at 0 °C. After 10 min, the reaction mixture was warmed to room temperature and stirred for 5 h. White precipitate was filtered, washed with dichloromethane and purified by flash column chromatography (dichloromethane:methanol = 30:1–10:1) to give the product **8** as white solid (119 mg, yield 62%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.88 (d, J = 8.4 Hz, 4H), 7.49 (m, 6H), 7.19 (NH, 2H),3.56 (t,

J = 6.8 Hz, 4H), 1.82 (p, J = 6.8 Hz, J = 13.7 Hz, 2H); LC/MS (electrospray) m/z $(M + H)^+$ 283.45.

4.1.27. Synthesis of N,N′-(butane-1,4-diyl)bis(N-benzylethanamide) (9)

To a solution of **12** (0.17 g, 0.633 mmol) in dimethylformamide (5 mL) were treated potassium carbonate (0.17 g, 1.852 mmol) and acetyl chloride (0.11 mL, 1.583 mmol) at room temperature. The reaction mixture was heated for 24 h and cooled to room temperature. The reaction mixture was concentrated under reduced pressure, diluted with ethyl acetate, and washed with sat. $NaHCO_3$. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The resulting residue was purified by column chromatography (silica gel, gradient 10–25%, ethyl acetate in hexane) to yield the compound **9** (0.17 g, yield 78%) as pale yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34–7.36 (m, 4H), 7.30–7.33 (m, 4H), 7.28–7.29 (m, 2H), 4.92 (s, 4H), 3.19 (t, J = 4.8 Hz, 4H), 2.42 (s, 6H), 1.52 (t, J = 3.6 Hz, 4H); LC/MS (electrospray) $m/z(M + H)^+$ 353.30.

4.1.28. Synthesis of N,N′-(propane-1,3-diyl)bis(2-phenylpropanamide) (10)

Yield 75%, white solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 (m, 10H), 5.98 (NH, 2H), 3.55 (p, J_{12} = 6.8 Hz, J_{13} = 13.7 Hz, 2H), 3.11 (m, 4H), 1.47 (d, J = 7.2 Hz, 6H), 1.43 (m, 2H); LC/MS (electrospray) m/z $(M + H)^+$ 339.50.

4.1.29. Synthesis of N^1,N^2-dibenzylethane-1,2-diamine (11)

This compound was synthesized by the procedure described above for the synthesis of compound **21**. Yield 80%, yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 (d, J = 4.4 Hz, 8H), 7.23–7.26 (m, 2H), 3.71 (s, 4H), 2.71 (s, 4H); LC/MS (electrospray) m/z $(M + H)^+$ 241.30.

4.1.30. Synthesis of N^1,N^4-dibenzylbutane-1,4-diamine (12)

This compound was synthesized by the procedure described above for the synthesis of compound **21**. Yield 93%, yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38–7.36 (m, 4H), 7.31–7.32 (m, 4H), 7.29–7.30 (m, 2H), 3.78 (s, 4H), 2.52 (t, J = 4.8 Hz, 4H), 1.42 (t, J = 5.6 Hz, 4H); LC/MS (electrospray) m/z $(M + H)^+$ 269.30.

4.1.31. Synthesis of N^1,N^5-dibenzylpentane-1,5-diamine (14)

This compound was synthesized by the procedure described above for the synthesis of compound **21**. Yield 75%, oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.51 (m, 8H), 7.42 (m, 2H), 3.96 (s, 4H), 2.81 (t, J = 6.8 Hz, 4H), 1.71 (p, J = 6.8 Hz, J = 13.7 Hz, 2H), 1.55 (m, 4H); LRMS (electrospray) m/z $(M + H)^+$ 283.38.

4.1.32. Synthesis of N^1,N^6-dibenzylhexane-1,6-diamine (15)

This compound was synthesized by the procedure described above for the synthesis of compound **21**. Yield 71%, oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 (m, 8H), 7.30 (m, 2H), 3.77 (s, 4H), 2.61 (t, J = 6.8 Hz, 4H), 1.51 (p, J_{12} = 6.8 Hz, J_{13} = 13.7 Hz, 4H), 1.35 (m, 4H); LC/MS (electrospray) m/z $(M + H)^+$ 297.40.

4.1.33. Synthesis of 2,2′-oxybis(N-benzylethan-1-amine) (16)

This compound was synthesized by the procedure described above for the synthesis of compound **21**. Yield 68%, oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 (m, 8H), 7.23 (m, 2H), 3.80 (s, 4H), 3.56 (t, J = 5.2 Hz, 4H), 2.80 (t, J = 5.2 Hz, 4H), 1.74 (NH, 2H); LC/MS (electrospray) m/z $(M + H)^+$ 285.42.

4.1.34. Synthesis of N^1,N^4-diphenylbutane-1,4-diamine (17)

To a solution of 1,4-dibromobutane **26** (0.20 g, 0.926 mmol) in dimethylformamide (3 mL) were treated potassium carbonate (0.26 g, 1.852 mmol) and aniline (0.18 mL, 1.945 mmol) at room temperature. The reaction mixture was refluxed for 24 h and cooled to room temperature. The reaction mixture was concentrated under reduced pressure, diluted with ethyl acetate, and washed with sat. $NaHCO_3$. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The resulting residue was purified by column chromatography (silica gel, gradient 5–12.5 percent, ethyl acetate in hexane) to yield the title compound **17** (0.05 g, yield 23%) as pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.07 (t, J = 5.6 Hz, 4H), 6.67 (t, J = 7.2 Hz, 2H), 6.57 (d, J = 8.0 Hz, 4H), 3.32 (t, J = 5.2 Hz, 4H), 1.50 (t, J = 4.8 Hz, 4H); LC/MS (electrospray) m/z $(M + H)^+$ 241.14.

4.1.35. Synthesis of N^1,N^3-diphenethylpropane-1,3-diamine (18)

This compound was synthesized by the procedure described above for the synthesis of compound **21**. Yield 64%, white solid; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.51 (br s, 2H), 7.34–7.38 (m, 4H), 7.26–7.30 (m, 6H), 3.17–3.21 (m, 4H), 3.02–3.06 (m, 4H), 2.90–2.94 (m, 4H), 1.94 (q, J = 7.6 Hz, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 159.1, 137.6, 129.4, 129.3, 127.6, 48.3, 44.7, 32.3, 23.0; LC/MS (electrospray) m/z $(M + H)^+$ 283.

4.1.36. Synthesis of N^1,N^3-bis(2-phenylpropyl)propane-1,3-diamine (19)

This compound was synthesized by the procedure described above for the synthesis of compound **21**. Yield 20%, oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 (m, 4H), 7.19 (m, 6H), 2.89 (m, 2H), 2.69 (d, J = 4.8 Hz, 4H), 2.54 (m, 4H), 1.55 (p, J = 6.8 Hz, J = 13.7 Hz, 4H), 1.22 (d, J = 7.2 Hz, 6H); LC/MS (electrospray) m/z $(M + H)^+$ 311.48.

4.1.37. Synthesis of 4-(1,3-bis(4-methoxybenzyl) hexahydropyrimidin-2-yl)benzonitrile (20)

This compound was synthesized by the procedure described above for the synthesis of compound **21**. Yield 43%, white solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.20 (d, J = 8.4 Hz, 4H), 6.82 (d, J = 8.4 Hz, 4H), 3.76 (s, 6H), 3.69 (s, 4H), 2.66 (t, J = 6.8 Hz, 4H), 1.68 (p, J = 6.8 Hz, J = 13.7 Hz, 2H); LC/MS (electrospray) m/z $(M + H)^+$ 315.27.

4.1.38. Synthesis of 4-(1,3-bis(4-methoxybenzyl) hexahydropyrimidin-2-yl)benzonitrile (22)

This compound was synthesized by the procedure described above for the synthesis of compound **21**. Yield 85%, white solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.21 (d, J = 8.4 Hz, 4H), 6.84 (d, J = 8.4 Hz, 4H), 3.80 (s, 6), 3.71 (s, 4H), 2.62 (t, J = 6.8 Hz, 4H), 1.55 (p, J_{12} = 6.8 Hz, J_{13} = 13.7 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.8, 132.5, 129.5, 114.0, 55.4, 53.5, 49.3, 28.0; LC/MS (electrospray) m/z $(M + H)^+$ 329.50.

4.1.39. Synthesis of 4-(1,3-bis(4-methoxybenzyl) hexahydropyrimidin-2-yl)benzonitrile (23)

This compound was synthesized by the procedure described above for the synthesis of compound **21**. Yield 80%, white solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.16 (d, J = 8.4 Hz, 4H), 6.70 (d, J = 8.4 Hz, 4H), 3.68 (s, 4), 2.92 (s, 12H), 2.62 (t, J = 6.8 Hz, 4H), 1.55 (p, J = 6.8 Hz, J = 13.7 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 150.1, 129.4, 127.9, 112.9, 53.4, 49.1, 40.9, 28.0; LC/MS (electrospray) m/z $(M + H)^+$ 355.56.

4.1.40. Synthesis of compound 29

To a stirred solution of **28** (500 mg, 2.333 mmol) in MeOH (5.0 mL) was added *p*-anisaldehyde (0.3 mL, 2.566 mmol). The mixture was stirred at 50 °C for overnight. After reaction was completed, sodium borohydride (264 mg, 6.999 mmol) was added at 0 °C. The mixture was stirred at room temperature for overnight. After reaction was completed, the reaction mixture was quenched by adding H_2O. The residue was diluted with EtOAc and washed with saturated brine. The organic layer was dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by flash column chromatography to give **29** (yield 49%) as white

solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.20 (d, J = 8.8 Hz, 2H), 6.84 (d, J = 8.4 Hz, 2H), 4.62 (br s, 1H), 3.80 (s, 3H), 3.45–3.37 (m, 2H), 3.00 (br s, 2H), 2.76–2.68 (m, 2H), 1.98–1.93 (m, 1H), 1.73–1.53 (m, 5H), 1.41 (s, 9H), 1.01–0.97 (m, 1H).

4.1.41. Synthesis of compound **30**

To a solution of **29** (184 mg, 0.550 mmol) in dioxane (2 mL) were added a solution of 4 N HCl in dioxane (1.1 mL, 4.400 mmol) and the mixture was stirred at room temperature for 3 h. After reaction was completed, the reaction mixture was diluted with DCM and washed with saturated 2 N NaOH. The organic layer was dried over anhydrous $MgSO_4$ and concentrated to give **30** (yield 71%) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.21 (d, J = 8.4 Hz, 2H), 6.84 (d, J = 8.4 Hz, 2H), 3.79 (s, 3H), 3.43 (q, J = 20.0, 12.8 Hz, 2H), 2.85 (d, J = 9.6 Hz, 1H), 2.76 (d, J = 10.8 Hz, 1H), 2.54 (d, J = 6.0 Hz, 2H), 1.93–1.88 (m, 1H), 1.76–1.53 (m, 5H), 0.92–0.88 (m, 1H).

4.1.42. Synthesis of N-(4-methoxybenzyl)-1-(1-(4-methoxybenzyl) piperidin-3-yl)methanamine (**24**)

To a stirred solution of **30** (42 mg, 0.180 mmol) in MeOH (0.5 mL) was added *p*-anisaldehyde (0.024 mL, 0.200 mmol). The mixture was stirred at 50 °C for overnight. After reaction was completed, sodium borohydride (8.0 mg, 0.200 mmol) was added at 0 °C. The mixture was stirred at room temperature for overnight. After reaction was completed, the reaction mixture was quenched by adding H_2O, diluted with EtOAc, and washed with saturated brine. The organic layer was dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by flash column chromatography to give **3** (yield 52%) as yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.21 (t, J = 8.0 Hz, 4H), 6.85 (dd, J = 8.4, 1.2 Hz, 4H), 3.79 (s, 6H), 3.68 (s, 2H), 3.44 (q, J = 25.6, 12.8 Hz, 2H), 2.88 (d, J = 10.0 Hz, 1H), 2.77 (d, J = 11.2 Hz, 1H), 2.48 (d, J = 6.4 Hz, 2H), 1.95–1.54 (m, 6H), 0.95–0.88 (m, 1H); LCMS (electrospray) m/z $(M + H)^+$ 355.

4.1.43. Synthesis of 4-methoxy-N-((1-(4-methoxybenzyl)piperidin-3-yl)methyl)aniline (**25**)

To a stirred solution of **30** (20 mg, 0.140 mmol) in DMSO (1.0 mL) were added 4-methoxychlorobenzene (49 mg, 0.210 mmol), CuI (2.7 mg, 0.014 mmol), K_2CO_3 (39 mg, 0.280 mmol) and L-proline (3.2 mg, 0.028 mmol). The reaction mixture was heated under microwave at 130 °C for 20 min. After reaction was completed, the reaction mixture was diluted with EtOAc and washed with saturated brine. The organic layer was dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by flash column chromatography to give **25** (yield 28%) as clear oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.26–7.19 (m, 2H), 6.87–6.83 (m, 2H), 6.78–6.74 (m, 2H), 6.55–6.51 (m, 2H), 3.80 (s, 3H), 3.74 (s, 3H), 3.44 (q, J = 12.8 Hz, 2H), 3.01–2.91 (m, 2H), 2.86 (d, J = 9.6 Hz, 1H), 2.74–2.71 (m, 1H), 2.02–1.97 (m, 1H), 1.92–1.78 (m, 3H), 1.71–1.66 (m, 2H), 1.60–1.54 (m, 1H), 1.09–1.00 (m, 1H); LC/MS (electrospray) m/z $(M + H)^+$ 341.

4.2. Biology

4.2.1. HCVcc assay

Naïve Huh-7 cells were plated at 2400 cells/well in 40 μL of culture media in 384-well plates (Greiner bio-one, μ clear black). After overnight incubation, compounds serially diluted in 20 μL of cell culture media were added. At 2 h post compound treatment, cells were inoculated with cell culture adapted HCVcc, expressing a NS5A-GFP fusion protein, at a multiplicity of infection (MOI) of 1. At 72 h post-infection, cells were fixed with 2% paraformaldehyde (PFA) and cell nuclei stained with 10 μg/mL of Hoechst 33342 (Sigma Aldrich). Four cell images per well were taken by automated confocal microscopy (ImageXpress ULTRA, Molecular Devices) and analyzed by in-house software [21]. HCV infection rates and cytotoxicity were determined by GFP expression and nucleus quantification, respectively.

4.2.2. HCV replicon assay

Huh-7 replicon cells were plated at 2000 cells/well in 40 μL of culture media in 384-well plates (Greiner bio-one, μ clear black). After overnight incubation, serially diluted compounds in 20 μL of cell culture media were added. At 72 h post compound treatment, plates with GFP expressing replicon cell lines were fixed with 2% paraformaldehyde (PFA) and applied for nuclei staining with 10 μg/mL of Hoechst 33342 (Sigma Aldrich). Cells were visualized by fully automated confocal microscopy (Opera, PerkinElmer) and analyzed by in-house software. HCV RNA replication was measured by GFP expression and cytotoxicity was measured by nucleus counting. EC_{50} and CC_{50} were calculated by non-linear regression analysis using GraphPad Prism (GraphPad software).

4.2.3. HCV pseudoparticle production and entry inhibition assay

HCV E1/E2-pseudotyped (or VSV-G pseudotyped as a control) lentiviral particles bearing the GFP reporter gene were produced as described previously [9]. Comparable amounts of infectious HCVpp and VSVpp were used in every experiment. Briefly, Huh-7 cells were plated in 384-well plates and treated with compounds. After 1 h incubation at 37 °C cells were inoculated with HCVpp or VSVpp and incubated at 37 °C for 12 h followed by a wash step. At 72 h post inoculation the transduction efficiency was evaluated by measuring GFP reporter gene expression by confocal microscopy at 20× magnification (ImageXpress ULTRA, Molecular Device) and quantified by in-house image processing software. Relative transduction values were obtained using DMSO-treated cells.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.09.055.

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