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Short communication

Bicyclic octahydrocyclohepta[*b*]pyrrol-4(1*H*)one derivatives as novel selective anti-hepatitis C virus agents

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ABSTRACT

We report the discovery of the bicyclic octahydrocyclohepta[*b*]pyrrol-4(1*H*)-one scaffold as a new chemotype with anti-HCV activity on genotype 1b and 2a subgenomic replicons. The most potent compound **34** displayed EC_{50} values of 1.8 μM and 4.5 μM in genotype 1b and 2a, respectively, coupled with the absence of any antimetabolic effect (gt 1b SI = 112.4; gt 2a SI = 44.2) in a cell-based assay. Compound **34** did not target HCV NS5B, IRES, NS3 helicase, or selected host factors, and thus future work will involve the unique mechanism of action of these new antiviral compounds.

1. Introduction

The hepatitis C virus (HCV) was discovered in 1989 as the etiological agent causing non-A non-B hepatitis [1,2]. HCV is now understood to be a diverse genus of positive sense single-stranded RNA viruses, which is part of the *Flaviviridae* family. There are presently eleven known HCV genotypes (gt1-gt11) whose RNA sequences differ by up to 30–50% [3]. Within the HCV genotypes, there are also several subtypes (designated as 1a, 1b, 1c, *etc.*) [4]. HCV infection typically causes few symptoms, but about 85% of patients develop chronic infection, which leads to progressive liver damage, fibrosis, cirrhosis, hepatocellular carcinoma, and ultimately liver failure. Presently, 130–170 million people are chronically infected with HCV [5], about 35,000 of whom die each year [6]. Fortunately, unlike other chronic viral diseases like AIDS, antiviral therapy can eliminate detectable HCV in patients. Such a sustained virologic response (SVR) also prevents cirrhosis, hepatocellular carcinoma and related HCV-induced mortality.

Early HCV therapies relied on agents that modulate the host antiviral response like interferon-alpha (IFN-α) and ribavirin (RBV) [7]. INF-α/RBV therapy results in an overall 50%–75% SVR (depending on HCV genotype and numerous other factors), but the therapy is poorly tolerated. INF-α-based therapy has therefore been

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[1] Equal contribution.

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replaced with combinations of antiviral drugs that target viral proteins, called direct-acting antivirals (DAAs). DAA drug targets include the proteins encoded by the 9600 nucleotide HCV viral genome: core, E1, E2 p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B [8]. Core, E1 and E2 constitute the viral particle, p7 and NS2 are involved in virion assembly and release, and the NS3 through NS5B proteins are involved in RNA replication [9,10]. In early stage discovery efforts, DAA candidates are typically tested using recombinant HCV proteins, or RNA replicons that are capable of autonomous replication in cells because they contain portions of the HCV genome [9].

The U.S. Food and Drug Administration (FDA) approved the first two HCV DDAs (telaprevir and boceprevir) in 2011. Both were NS3/4A protease inhibitors, but they were only effective in triple therapies when combined INF-α and RBV [11]. In 2014, the FDA approved the second generation NS3/4A protease inhibitor simeprevir, the NS5B nucleotide inhibitor sofosbuvir, and the NS5A inhibitor daclatasvir. More recently approved HCV DAAs include ledipasvir, ombitasvir, dasabuvir, grazoprevir, and elbasvir [12–15]. Notably, these DAAs can be used in all-oral, INF-free therapies, which cure most HCV patients [12]. Despite this enormous progress toward ultimate HCV eradication, HCV DAA development still needs to address high manufacturing costs, the evolution of drug resistant HCV alleles, and side effects [16]. In this context, we herein report the discovery of new anti-HCV compounds that have an octahydrocyclohepta[*b*]pyrrol-4(1*H*)-one core.

2. Results and discussion

Last year, we reported a new compound class possessing a 2-phenyl-4,5,6,7-tetrahydro-1H-indole core that inhibits HCV genotype 1b and 2a replication in cells [17,18]. Inspired by this success, we tested an additional set of representative heterobicyclic compounds (**1**–**21** [19–22], Fig. S1 Supporting Information) that was available from the EDASA Scientific public repertory of compound stocks (http://www.edasascientific.com/page/catalogue). The compounds were first tested at 50 μM using HCV replicons derived from HCV gt1b and gt2a, which are two widely studied HCV genotypes. Each compound was tested using Huh7/Rep-Feo1b and Huh7.5-FGR-JC1-Rluc2A cells, which carry the autonomously replicating RNA from HCV gt1b and gt2a, and the firefly and *Renilla* luciferase reporters, respectively. The compounds that lowered cellular HCV replicon content more than 50% at 50 μM were further evaluated in concentration-response assays (Table S1 Supporting Information). A selectivity index (SI) was calculated by comparing the concentrations needed to reduce cell viability by 50% (CC_{50}) with EC_{50} values. Five compounds (**6**, **16**, **18**, **19**, and **20**) showed an ability to selectively reduce levels of both gt1b and gt2a replicons in cells with little apparent toxicity (SI > 10). In addition, derivatives **1**, **2**, and **3** reduced cellular levels of gt2a replicons. Compound **6** [22] was the most attractive among all the compounds tested, displaying low cytotoxicity (CC_{50} > 200) and an ability to reduce both genotype 1b (EC_{50} = 7.1 μM) and 2a (EC_{50} = 6.1 μM) HCV cellular replicon levels (Table 1).

Based on the promising data from HCV replicon assays, and the consolidated synthetic procedure to obtain the bicyclic octahydrocyclohepta[*b*]pyrrol-4(1*H*)one scaffold [22], we designed and synthesized eighteen new close derivatives of **6 (22**–**39)**, that have been clustered in five groups accordingly to their chemical modification: a) replacement of the ethyl ester with a carboxylic acid or amides, b) "trans" variation of the ring junction, c) tosyl (Ts) modification and replacement, d) keto group functionalization or reduction, and e) methyl insertion at the bicyclic junction (Fig. 1, Schemes 2 and 3, and Fig. S2 in Supporting Information).

The starting octahydrocyclohepta[*b*]pyrrol-4(1*H*)-ones **40**–**43** [22] were obtained *via* an aza-Cope-Mannich reaction from corresponding amino vinylic alcohols and either glyoxylic acid or ester following previously disclosed conditions (Scheme 1).

The relatively mild, high-yielding and operationally simple conditions of this tandem process, accompanied by an accurate and predictable stereochemical outcome, are the main virtues which provided the permanent access to the requisite bicyclic proline derivatives in desirable quantities for further chemical modifications.

Since compound **6** is an ethyl ester, we also tested the biological effect of its corresponding acid **22** and of a similar acid **23,** and its corresponding ethyl ester **24**, where a methyl group was inserted at 3a position of the octahydrocyclohepta[*b*]pyrrol-4(1*H*)-one scaffold (Scheme 2). The two acid derivatives **22** and **23** were prepared by the tosylation of the appropriate amino acids **40** and **41** [22]. Compound **24** was prepared in a quantitative yield by direct esterification, with acetyl chloride and ethanol, of the corresponding acid **23**.

We also investigated whether changing stereochemical relationship of the ring fusion on "*cis*-" to "*trans*-" might affect the biological behavior of the octahydrocyclohepta[*b*]pyrrol-4(1*H*)-one scaffold. The "*trans*" variation of **6**, compound **25**, was obtained by applying the general procedure for tosylation starting from the amino ester **42** (Scheme 2) [22].

Taking into account that amides are much more stable metabolically than esters, another modification of **6** was achieved by replacing its ester moiety with an amide group. Thus, amides **26**–**29** were synthesized by a HBTU coupling reaction of the corresponding acid **22** and appropriate amine (Scheme 2).

Variations of the tosyl moiety were also investigated by synthesizing compounds **30**–**33,** which were prepared by treating the amino ester **43** with the corresponding acyl or sulfonyl chloride (Scheme 3).

Finally, compounds derived from functionalization (**34**–**37**) or reduction (**38**) of the keto group of compound **6** were also synthesized. Hydrazide **34**, semicarbazide **35** and two oximes (**36**, **37**) were prepared by refluxing compound **6** in ethanol with appropriate nucleophile (Scheme 3). By reducing the keto group of **6** with $NaBH_4$, it was possible to obtain compound **38**, though in low yields. Its further treatment with acetic anhydride furnished compound **39** (Scheme 3).

It should be noted that compounds **22**–**39** contain three stereo centers (all racemic mixtures), two of which are placed near electron withdrawing groups. However, reaction conditions were very mild and no epimerization (based on ^{1}H and ^{13}C NMR data) was observed during the synthesis of compounds **22**–**39.** The relative configuration was assigned based on the parent compounds **40**–**43**.

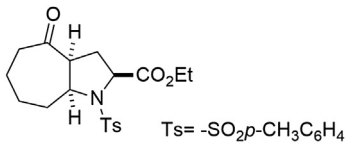
Reduction of **6** led exclusively to one stereoisomer (**38**). The configuration of the newly formed stereocenter was elucidated using X-Ray analysis of derivative **39** (Fig. 2, CCDC 1454446 and Supporting Information).

All the synthesized compounds were then tested in HCV replicon assays using the same protocol described above for the initial set of 21 compounds (Table 2). The new derivatives **22**–**39** that reduced HCV replicon levels in cells more than 50% at 50 μM, were then evaluated for their toxicity (CC_{50}), potency (EC_{50}) and selectivity (SI) (Table 2).

These data highlighted that all the chemical modifications involving the replacement of the ethyl ester with a carboxylic acid (**22** and **23**), methyl insertion (**24**), fused ring stereochemistry variation (**25**), ester-to-amide replacement (**26**–**29**), tosylate residue modification (**30**–**33**) and keto group reduction (**38** and **39**) were generally detrimental, as inactive or less active/selective derivatives than the parent compound **6** were obtained.

Conversely, out of the series of derivatives obtained through the

Table 1
Compound **6** effects on gt 1b and 2a HCV replicon levels and cell viability.



6, BB 0263372

CC_{50} (μM)[a]	Huh7/Rep-Feo1b			Huh7.5-FGR-JC1-Rluc2A		
	Inhibition (%)[b]	EC_{50} (μM)[c]	SI[d]	Inhibition (%)[b]	EC_{50} (μM)[c]	SI[d]
>200	89 ± 2	7.1 ± 0.73	>28.2	84 ± 2	6.1 ± 0.6	>32.8

[a] CC_{50} values were determined in Huh7.5 parental cells by the MTS assay. CC_{50} is the concentration required to reduce the bioreduction of MTS (3-(4,5- dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) into formazan by 50%. The reported value represents the means ± SD of data derived from three independent experiments.
[b] Percent reduction of HCV replicon levels in cells exposed to 50 μM of compound **6** (mean ± SD, n = 3).
[c] EC_{50} is the effective concentration required to reduce HCV replicon levels 50%. The reported values represent the mean (EC_{50}) ± SD obtained from three independent 8–12 point quarter log dilutions.
[d] SI: selectivity index = CC_{50}/EC_{50}.

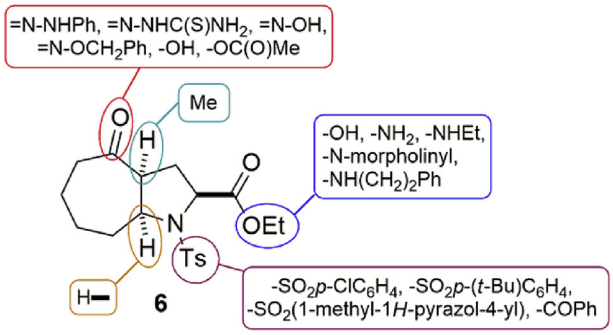
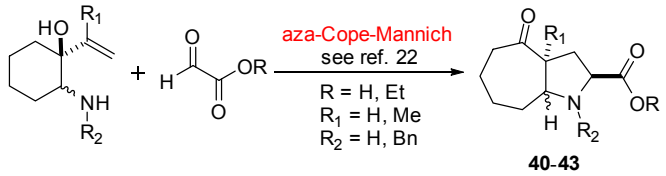


Fig. 1. Summary of the chemical modifications around hit **6**. Colored circles highlight the parts of the molecule that were modified to prepare a library of analogs.

keto group functionalization (**34**–**37**), compound **34** presented an improved activity against both replicons and high SI (EC_{50} = 1.8 μM and SI = 112.4 for gt1b and EC_{50} = 4.5 μM and SI = 44.2 for gt2a).

Since **34** potently reduced levels of both gt 1b and gt 2a replicons, we decided to synthesize and test its corresponding acid, **44.** Compound **44** was synthesized by reacting **22** with phenyl hydrazine in a refluxing pyridine because conventional conditions for **34**–**37** resulted in no product (Scheme 4). Replacement of the ester function with the carboxylate resulted in a total loss of activity, probably due to permeability issue (Table 2).

In order to further validate the anti-HCV activity of these compounds, we investigated the effect of a representative compound (**34**) in a reporter free HCV cell culture system. Towards this goal, MH-14 cells were treated with various concentrations of **34**, and cellular HCV RNA levels were measured using quantitative RT-PCR. Consistent with the above gt1b reporter assay results, **34** inhibited



Scheme 1. Aza-Cope-Mannich rearrangement as a key step in the generation of a bicyclic octahydrocyclohepta[*b*]pyrrol-4(1H)one compound array.

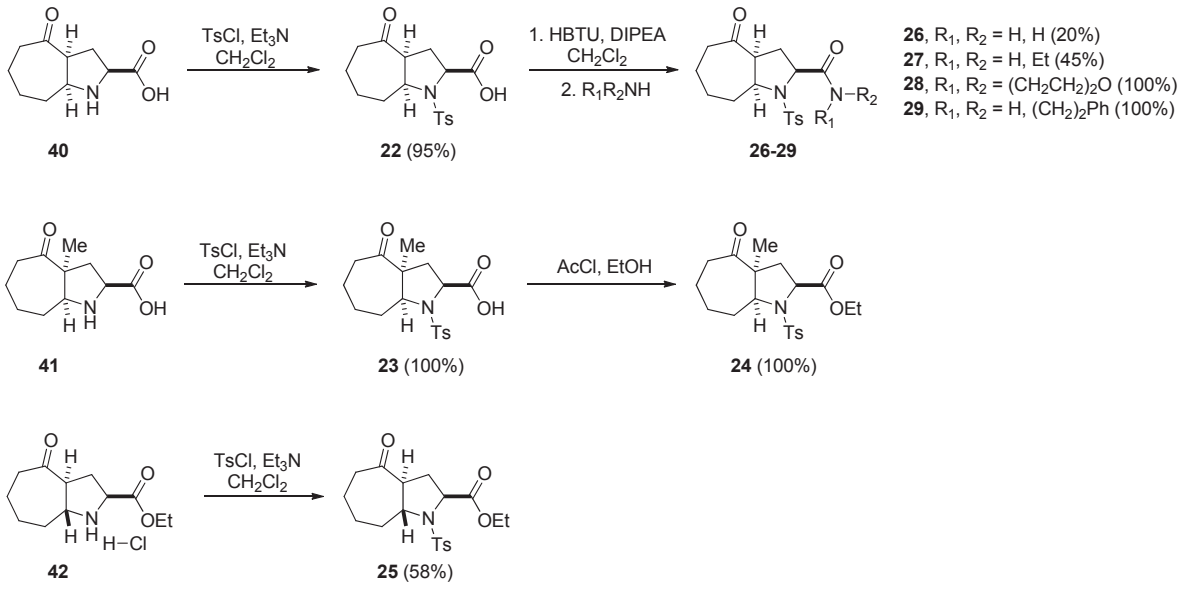
HCV replication in a concentration-dependent manner with an EC_{50} value of 3.8 ± 0.5 (Fig. S3, Supporting Information). These results with autonomously replicating HCV system clearly suggest that compound **34** is a *bona fide* HCV inhibitor.

To better understand the possible mechanism by which the compounds inhibit HCV RNA replication, we investigated the effect of the compounds on the activities of recombinant purified HCV proteins. First, we tested the compounds in standard primer-dependent HCV NS5B polymerase RNA elongation assays [23,24]. All the compounds were tested at 50 μM in NS5B assays, but none of the compounds inhibited the RNA-dependent RNA polymerase activity of HCV NS5B (data not shown). Selected representative compounds were then tested in HCV NS3 helicase assays that monitored the ability of NS3 to unwind a DNA substrate in the presence of each compound. The results revealed that none of the compounds inhibited the NS3 helicase even at 500 μM. These results ruled out HCV NS3 or NS5B as the molecular targets of these compounds.

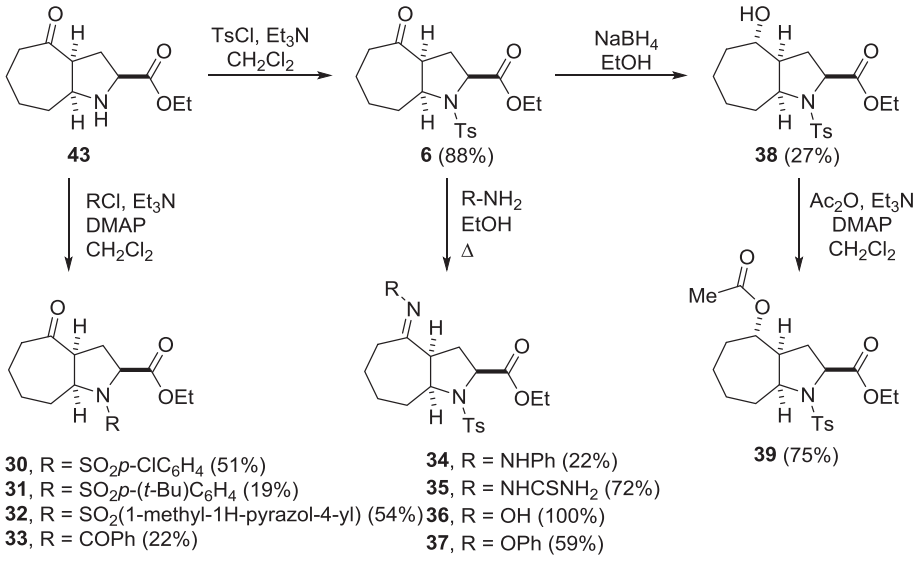
Many DAA candidate small molecules are also known that interfere with the HCV Internal Ribosome Entry Site (IRES)-mediated translation and thus virus replication [25,26]. We therefore investigated two representative compounds (**6** and **34)** for their ability to down-regulate HCV IRES-mediated translation [18,25]. Our results indicated that these compounds had no effect on HCV IRES mediated translation even at a higher (25 μM) concentration than is needed to fully inhibit the HCV replicons (data not shown).

We last tested the possibility that compound **6** and compound **34** could function as potential suppressors or activators of human genes needed to facilitate HCV replication. To this end, we performed cell-based assays using reporter plasmids encoding promotors of either the anti-oxidant response element, cyclooxygenase-2, heme oxygenase-1, or the interferon-stimulated response element. In each plasmid, each promoter was fused to a luciferase reporter protein. Each reporter plasmid was used to transfect cells, and gene expression in transfected cells was compared to the same transfected cells exposed to either 5, 10 or 25 μM of each compound. Neither **6** nor **34** had a significant effect on any gene at any concentration tested, thus ruling out the possibility that the octahydrocyclohepta pyrrolone-based compounds influence the expression of these host factors needed for HCV replication.

Thus, the exact mechanism by which bicyclic octahydrocyclohepta[*b*]pyrrol-4(1*H*)-one derivatives inhibit HCV



Scheme 2. Synthesis of compounds **22**–**29.**



Scheme 3. Synthesis of compounds **30**–**39.**

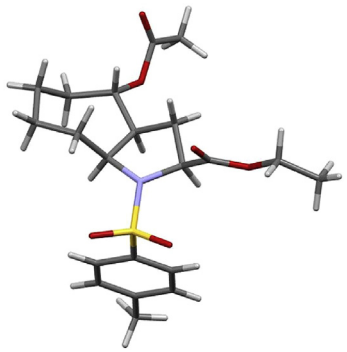


Fig. 2. X-Ray structure of **39**.

replication remains to be clarified.

3. Conclusion

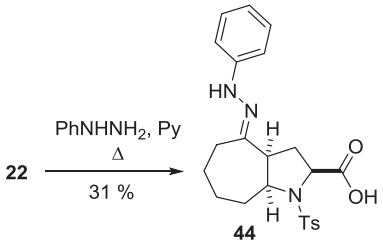
The data presented herein clearly indicate that promising anti-HCV activity coupled with no apparent cytotoxic effect, has been obtained for the bicyclic octahydrocyclohepta[*b*]pyrrol-4(1*H*)-one scaffold. Preliminary structure-activity relationship revealed that the ester function as well as the *cis*-fused ring junction are essential features to gain antiviral potency on both genotype 1b and 2a. Compound **34** was the most potent and selective derivative within the series, exhibiting antiviral activity (gt 1b EC_{50} = 1.8 μM; gt 2a EC_{50} = 4.5 μM) coupled with the absence of any antimetabolic effect (CC_{50} > 200 μM; gt 1b SI = 112.4; gt 2a SI = 44.2) in a cell-based assays.

These promising results make the bicyclic octahydrocyclohepta

Table 2
Effect of compound **6** derivatives on HCV replicons and Huh7.5 cells.

Cpd	CC_{50}[a] (μM)	Huh7/Rep-Feo1b			Huh7.5-FGR-JC1-Rluc2A		
		Inhibition[b] (%)	EC_{50}[c] (μM)	SI[d]	Inhibition[b] (%)	EC_{50}[c] (μM)	SI[d]
6	>200	89 ± 2	7.1 ± 0.73	>28.2	84 ± 2	6.1 ± 0.6	>32.8
22	>200	14 ± 5	ND	ND	42 ± 4	ND	ND
23	>200	41 ± 6	ND	ND	33 ± 11	ND	ND
24	>200	50 ± 4	49.1 ± 3.9	4.1	76 ± 7	14.2 ± 1.5	14.1
25	>200	51 ± 9	59.4 ± 4.8	3.4	91 ± 8	8.7 ± 0.8	22.9
26	>200	NI	ND	ND	38 ± 8	ND	ND
27	>200	15 ± 4	ND	ND	39 ± 11	ND	ND
28	>200	19 ± 4	ND	ND	12 ± 9	ND	ND
29	153 ± 2.2	66 ± 10	28.1 ± 1.1	5.4	48 ± 6	ND	ND
30	>200	32 ± 8	ND	ND	60 ± 1	23.8 ± 1.8	8.4
31	>200	49 ± 5	34.4 ± 1.9	>5.8	94 ± 4	13.0 ± 0.9	>15.4
32	>200	NI	ND	ND	17 ± 7	ND	ND
33	>200	59 ± 1	14.9 ± 1.6	>13.4	95 ± 2	14.3 ± 1.6	>13.9
34	>200	88 ± 1	1.8 ± 0.1	112.4	70 ± 7	4.5 ± 0.8	44.2
35	>200	66 ± 6	14.3 ± 2.5	14.0	58 ± 4	11.6 ± 0.9	17.3
36	>200	63 ± 8	25.3 ± 1.6	7.9	66 ± 5	15.3 ± 2.1	13.1
37	>200	NI	ND	ND	30 ± 10	ND	ND
38	>200	22 ± 2	ND	ND	77 ± 5	27.6 ± 3.6	7.3
39	>200	60 ± 2	27.2 ± 1.6	7.3	73 ± 9	25.8 ± 0.9	7.7
44	>200	25 ± 6	ND	ND	81 ± 7	26.3 ± 3.9	>7.6

[a] CC_{50} values were determined in Huh7.5 parental cells by the MTS assay. CC_{50} is the concentration required to reduce the bioreduction of MTS (3-(4,5- dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) into formazan by 50%. The reported value represents the means ± SD of data derived from three independent experiments.
[b] Percent reduction of HCV replicon levels in cells exposed to 50 μM of compound **6** (mean ± SD, n = 3).
[c] EC_{50} is the effective concentration required to reduce HCV replicon levels 50%. The reported values represent the mean (EC_{50}) ± SD obtained from three independent 8–12 point quarter log dilutions.
[d] SI: selectivity index ratio of CC_{50} to EC_{50}. ND: not determined.



Scheme 4. Synthesis of compound **44.**

[*b*]pyrrol-4(1*H*)one scaffold an attractive starting point for chemistry efforts aimed at further potency and selectivity optimization. In parallel, additional studies are needed to finally elucidate the mechanism of action of these new anti-HCV agents.

4. Experimental section

4.1. General biological methods

Cell culture reagents such as, L-glutamine, Dulbecco's Phosphate Buffered Saline (DPBS), Dulbecco's Modified Eagle's Medium (DMEM), Trypsin-EDTA were purchased from Mediatech Inc. USA. G-418, penicillin, treptomycin and fetal bovine serum (FBS) were from Sigma-Aldrich. To maintain the HCV replicons in cells, the antibiotic G418 was added to all media. All cells were cultured at 37 °C and 5% humidified CO_2. The firefly and *Renilla* luciferase assay kits, and the CellTiter 96® AQueous One Solution Cell Proliferation kit were purchased from Promega, USA. The 96-well cell culture and luminometer plates were obtained from Fisher Scientific. All compounds were stored as 10 mM stock solution in DMSO at 4 °C and diluted just prior to use. The final concentration of DMSO in the cell culture experiments were 1%. EC_{50} and CC_{50} values were calculated using the Calcusyn 2.0 program, Biosoft, UK.

4.2. Replicon reporter assays

HCV replicon reporter cells (Huh7/Rep-Feo1b and Huh7.5-FGR-JC1-Rluc2A) were cultured in DMEM containing 10% FBS, 100 U/ml penicillin/streptomycin, 4 g/ml of L-glutamine. The Huh7/Rep-Feo1b cell carrying HCV replicons of genotype 1b have been well documented [25,26]. The Huh7.5-FGR-JC1-Rluc2A replicon cells were generated in Dr. Hengli Tang's lab (Florida State University, USA) and is described previously [27]. The anti-HCV assays were carried out following the method described previously [28,29]. In brief, the cells were placed in 96 well plates (~1×10^4 cells/well) and 12 h post seeding, the cells were treated either with the compounds or DMSO (control). Following 48 h treatment, the cells were washed three times with PBS and luciferase activities were measured employing the firefly or *Renilla* kits in accordance with the manufacturer's protocol. Percent inhibitions were determined by the relative levels of luciferase activities in compound treated versus DMSO treated cells. Cellular cytotoxicity assays were carried out in Huh7.5 cells in 96 well format as described previously [28,29]. Briefly, after 12 h of seeding, cells were treated with 8–10 dilutions of compounds or DMSO (Control) for 48 h. Cells treated with an equal amount of DMSO served as a control. Cell viability was analyzed using CellTiter 96® AQueous One Solution Cell Proliferation kit (Promega Inc, USA) following manufacturer's recommendation.

4.3. NS5B RdRp inhibition assay

The anti-RdRp activity of compounds were assessed by standard primer dependent elongation assay as described previously [23,24]. In brief, NS5B 1b was used as the source of polymerase in buffer containing 20 mM Tris-HCl (pH 7.0), 100 mM NaCl, 100 mM Na-glutamate, 0.01% BSA, 0.1 mM DTT, 5% glycerol, 0.01% Tween-20, 20 U/mL of RNasin, 20 μM UTP, 1–2 μCi [α-^{32}P]UTP 0.25 μM pre-annealed polyrA/U_{12}, 100 ng NS5BCΔ21. The reactions were

carried out in the presence of the individual compound or equivalent amount of DMSO. The reactions were started by the addition of $MnCl_2$ (1 mM final). After 60 min incubation at 30 °C, the reactions were terminated by adding ice cold 5% trichloroacetic acid containing 0.5 mM sodium pyrophosphate. The precipitated radiolabeled RNAs were filtered on GF-B filters and quantified using scintillation counter. RdRp inhibition by compounds was determined relative to control.

4.4. RT-PCR

MH-14 cells carrying HCV subgenomic replicons were grown in 12 well plates and 12 h post-seeding treated with compounds or DMSO. After 48 h of treatment, total RNA was isolated using RNeasy mini kit (Qiagen). Approximately 0.5 μg of RNA was reverse transcribed using M-MLV reverse transcriptase (Life Technologies) and either HCV specific primers or oligo dT_{18} in accordance with manufacturer's protocol. Fifty nanograms of synthesized cDNA's were used for PCR applications using gene specific primers and Power SYBR green PCR master mix (Applied Biosystems) in a final volume of 25 μl using Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument. The forward and reverse primer sequences for HCV are 5′-CGGGAGAGCCATAGTGG-3′ and 5′-AGTACCACAAGGCCTTTCG-3′. The primer sequence for β-Actin is 5′- AGCGAGCATCCCCCAAAGTT-3′ for forward primer and 5′-GGGCACGAAGGCTCATCATT-3′ for reverse primer, respectively.

4.5. Host gene expression reporter assays

All assays were carried out in Huh7.5 or in MH-14 cells as indicated. Transfections were performed using LipoD293 reagent (SignaGen, USA). The effect of compounds on HCV IRES mediated translation was studied in Huh7.5 using a dual luciferase reporter construct (pClneo- Rluc-IRES-Fluc) as reported earlier [25]. Sixteen h post-transfection, the cells were treated with compounds or DMSO and Luciferase activity was evaluated using Dual-Glo Luciferase Assay Kit. To investigation host-factors as potential targets, MH-14 cells were transfected with 300 ng of gene specific reporter plasmid p3xARE-Luc [30], pCOX-2-FLuc [29,31–33], pHO-1-Luc [34], pISRE-Luc [35], and 16 h post transfection cells were treated with compounds or DMSO (control) for 48 h. Transfection efficiencies were normalized by Renilla luciferase expression and evaluated employing Dual-Glo Luciferase Assay Kit.

4.6. NS3 assays

Truncated C-terminally His-tagged NS3 protein lacking the N-terminal protease (NS3h) from the con1 strain of genotype 1b [Genbank accession AB114136], was expressed and purified as previously described [36,37]. Molecular beacon-based NS3 helicase assays were performed as described by Hanson et al. [38]. Reactions contained 25 mM MOPS pH 6.5, 1.25 mM $MgCl_2$, 5% DMSO, 5 μg/ml BSA, 0.01% (v/v) Tween20, 0.05 mM DTT, 5 nM florescent DNA substrate, 12.5 nM NS3h, and 1 mM ATP. HCV Helicase-catalyzed ATP hydrolysis in the absence of RNA was monitored in reactions containing 50 nM HCV NS3h, 25 mM MOPS pH 6.5, 1.25 mM $MgCl_2$, 1 mM ATP, 33 μg/ml BSA, 0.07% (v/v) Tween 20, 0.3 mM DTT, and 10% v/v DMSO. Reactions in the presence of polyU RNA were performed with 4 nM HCV NS3h in the same buffer with 1 μM PolyU (Sigma, expressed and nucleotide concentration) was added to each reaction.

4.7. Synthesis of the target compounds **22-39**

Experimental procedures for the synthesis of compounds **22**–**39** are reported in the Supporting Information.

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Appendix B. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.06.041.

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