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Discovery of the 2-phenyl-4,5,6,7-Tetrahydro-1*H*-indole as a novel anti-hepatitis C virus targeting scaffold

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Abstract

Although all-oral direct-acting antiviral (DAA) therapy for hepatitis C virus (HCV) treatment is now a reality, today's HCV drugs are expensive, and more affordable drugs are still urgently needed. In this work, we report the identification of the 2-phenyl-4,5,6,7-Tetrahydro-1*H*-indole chemical scaffold that inhibits cellular replication of HCV genotype 1b and 2a subgenomic replicons. The anti-HCV genotype 1b and 2a profiling and effects on cell viability of a selected representative set of derivatives as well as their chemical synthesis are described herein. The most potent compound **39** displayed EC_{50} values of 7.9 and 2.6 μM in genotype 1b and 2a, respectively. Biochemical assays showed that derivative **39** had no effect on HCV NS5B polymerase, NS3 helicase, IRES mediated translation and selected host factors. Thus, future work will involve both the chemical optimization and target identification of 2-phenyl-4,5,6,7-Tetrahydro-1*H*-indoles as new anti-HCV agents.

Keywords

Hepatitis C virus; 4,5,6,7-Tetrahydro-1*H*-indole; Anti-HCV agents

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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.2015.04.022.

1. Introduction

Hepatitis C virus (HCV) infection represents a global health problem that has an associated high risk for serious liver diseases. On the basis of annual World Health Organization (WHO) reports, more than 130–150 million people are infected and more than 350,000–500,000 individuals die from HCV-related liver pathologies each year [1]. To date, at least eleven HCV genotypes (gt) have been identified. These genotypes can be divided into multiple subtypes. The global distribution of HCV genotypes varies depending on the particular geographical area. HCV gt 1 is the most common in North and South America, Europe and Australia [2]. HCV gt 2 is widespread in America and Europe, while gt 3 is common in Central Asia and Middle East. Finally, HCV gt 4 and gt 5 are found almost exclusively in Africa, and HCV gt 6 is endemic in East and Southeast Asia [2]. Gt 1 and gt 4 are the hardest to treat and are associated with a particularly aggressive form of the disease.

HCV was discovered in 1989, and until recently all treatments included some combination of pegylated interferon-α (pegIFN-α) and ribavirin (RBV), both of which cause debilitating side effects often worse than HCV symptoms. PEG-IFN/RBV treatment alone has been moderately successful and is genotype-dependent as only 40–50% of gt 1 and gt 4 patients have achieved a sustained virological response (SVR) indicative of a cure [3]. This treatment regimen remained the standard-of-care (SOC) until 2011 for gt 1, and until 2014 for the other genotypes. Over the past 20 years, a combination of developments of new models and tools have been able to reveal the different steps of the HCV life cycle and tremendous drug discovery efforts have allowed the development of direct-acting antivirals (DAAs) that specifically target HCV proteins. Since 2011, the new SOC for patients infected with gt 1 is based on a combination of pegIFN-α and RBV with the first-generation HCV protease inhibitors telaprevir or boceprevir (Fig. 1). Although the cure rates have improved (SVR = 60–80%), the new SOC provides only limited clinical benefit against HCV gt 2–6 and has resulted in some serious side effects in clinical trials [4,5]. Consequently, two new HCV DAAs, simeprevir and sofosbuvir (Fig. 1), have been approved in December 2013 in the United States and in the first half of 2014 in Europe [6–8]. Simeprevir is a second-generation protease inhibitor that is endowed with a broader genotypic coverage (gt 1, 2 and 4). Its combination with pegIFN-α and RBV has shown improved SVR and a better tolerance profile [6]. Sofosbuvir, the first nucleotide inhibitor of NS5B polymerase approved by FDA, has paved the way for all-oral IFN-free therapies, two of which were approved in 2014: Viekira Pak (ombitasvir, paritaprevir, ritonavir and dasabuvir), and Harvoni (ledipasvir and sofosbuvir) (Fig. 1) [9–11]. Viekira Pak and Harvoni are both approved only for adult HCV patients with gt1 infection; they have displayed >90% SVR and are also effective against other genotype in clinical trials.

There are currently many similar HCV DAAs in development, and most target the NS3 protease, NS5B polymerase and NS5A protein. They are undergoing late stages of clinical development and are close to approval. An up-to-date status of the clinical trials along with comprehensive overviews of the continued and discontinued HCV-specific DAAs have been recently described [12].

The main drawback is that the newly approved drugs and/or regimens are very expensive, thus restricting access for most HCV-infected patients to the new anti-HCV therapies. Another serious medical issue is the high mutation rate of HCV coupled with the rapid emergence of drug resistance to the DAAs [13–15]. These observations serve to encourage continuing research in the field of HCV drug discovery that will lead to the identification of new antiviral agents effective against HCV.

Thus, it is within this context that we herein report the discovery of a new chemical class of anti-HCV compounds that have a 2-phenyl-4,5,6,7-Tetrahydro-1*H*-indole core.

2. Results and discussion

2.1. Cell-based screening of EDASA compounds: hit identification

Compounds **1–33** (Fig. 2), representative chemotypes of the EDASA Scientific public compound library (http://www.edasascientific.com/page/catalogue), have been screened for their possible anti-HCV activity using HCV replicons based on the two most widely studied HCV genotypes (gt 1b and gt 2a) (Table 1). All compounds, except **24**, are racemates.

Gt 1b was studied for many years because it is one of the most resistant to pegIFN-α/RBV therapy, and the gt 1b (con1) strain was used in the first subgenomic HCV replicons [16]. Gt 2a exhibits a greater sensitivity than gt 1 to pegIFN-α/RBV treatment, but it was the first to be replicated in a robust cell culture model [17]. Taking this into consideration for our discovery campaign, we decided to screen the compounds against both the HCV genotypes.

The compounds were evaluated against Huh7/Rep-Feo1b and Huh7.5-FGR-JC1-Rluc2A cells, which carry the autonomously replicating HCV RNA of gt 1b and 2a in the firefly and Renilla luciferase reporters, respectively [18]. During initial screening, the 33 EDASA Scientific compounds were assayed at 50 μM against both the HCV replicons in reporter assays. The compounds that inhibited HCV replication by > 50% in the primary assays were then further evaluated in concentration-response assays. The ability of each compound to inhibit activity in gt 1b and 2a replicons, and their effect on cell viability are shown in Table 1. The selectivity index (SI) was calculated as well to estimate the therapeutic potential of the compounds in this system. Only two compounds (**28** and **31**) were found to be active against gt 1b (displaying EC_{50} values of 24.3 and 12.4 μM, respectively), although they showed poor SI (<10). In contrast, a total of 16 compounds were active against gt 2a, with associated EC_{50} values in the range from 4.9 to 28.1 μM and moderate to good SI. Compound **25** was the most potent among all the tested compounds and showed EC_{50} value of 4.9 μM with a SI > 41. Interestingly, the only two compounds found to be active on gt 1b replicons (**28** and **31**) were also active against gt 2a replicons and exhibited EC_{50} values of 6.0 and 8.7 μM, respectively, with SI values > 10.

Overall, compound **31**, having a 2-phenyl-4,5,6,7-Tetrahydro-1*H*-indole scaffold, emerged as a hit compound, displaying low cytotoxicity (CC_{50} = 109.9 μM) and promising anti-HCV activity in replicon reporter cells of both the genotype 1b (EC_{50} =12.4 μM) and 2a (EC_{50} = 8.7 μM). Following on from this, we had eleven more analogues of **31** available at EDASA Scientific that we decided to further evaluate for their anti-HCV activities (**34–44**, Fig. 3).

2.2. Synthesis of derivatives 31, 34–44

Recently, we have developed a two-step one-pot synthetic methodology, which leads to 4,5,6,7-Tetrahydro-1*H*-indoles with a wide range of substituents, including chiral moieties, both at C-2 and at the *N*-1 positions [19]. This synthetic sequence was successfully applied to achieve derivatives **31, 34–44** (Scheme 1).

This one-pot Sonogashira cross-coupling/5-endo-dig cyclization procedure was used as a flexible and versatile synthetic approach. Thus, the trans-stereoselective and highly regioselective nucleophilic epoxide ring opening of **45** with different amines was followed by a subsequent one-pot Pd-catalyzed arylation/cyclization. This short sequence allowed the variation of substituents both at the nitrogen atom and at the C-2 position of the pyrrole ring, along with a judicial design and a fast preparation of the most promising tetrahydroindole derivatives. Furthermore, it utilized mild conditions and inexpensive catalysts, being highly tolerant to a range of functional groups and readily scalable to provide sufficient amounts of tetrahydroindoles on gram scales in a good to excellent yields to effectively assemble the tetrahydroindole compound array for further screening. The full report on the synthetic sequence as well as compound characterization is presented in Supporting Information.

2.3. Cell-based assays of compounds 34–44

The anti-HCV activities of the new analogues of **31** (**34–44**) are shown in Table 2. The cell-based assays revealed that, out of the eleven compounds tested, eight derivatives in gt 1b and ten compounds in gt 2a showed >60% inhibition during preliminary screening.

All these compounds except one were then evaluated for their EC_{50} and SI values; in fact, derivative **36** exerted its HCV replication inhibition at toxic concentration (CC_{50} < 25 μM) and thus it was not submitted to EC_{50} evaluation.

Taking into account the obtained biological data (Table 2), some preliminary SAR can be proposed for this new class of anti-HCV agents.

Derivatives **39, 40** and **42**, all having a *N*-benzyl substitution at the tetrahydroindole core, showed the higher anti-HCV activities with SI values ranging from 10 to 13 for gt 1b, and from 27 to 32 for gt 2a. Among them, compound **39** was found to be the most potent in both gts displaying EC_{50} values of 7.9 μM (1b) and 2.6 μM (2a). Compared to **39**, derivative **34**, having a *para*-fluorophenyl group at the nitrogen atom, showed a nearly 3.7 and 4.7 fold reduction in anti-HCV activity for gt 1b and 2a replicon reporter assays, respectively. Furthermore, when the *N*-benzyl substituent of the tetrahydroindole nucleus was replaced with non-aromatic groups, the anti-HCV activity on gt 1b was completely lost (**37, 38**, and **41**) or a non selective antiviral effect (i.e. low SI value) was obtained (**44**); the analysis on gt 2a provided similar conclusions with the exception of derivative **37** which turned out to be active.

When analyzing the biological data for the whole subset of *N*-benzyl derivatives (i.e. compounds **31, 35, 39, 40, 42** and **43**), the key role of the aryl substituent at the C-2 position became evident. An unsubstitued phenyl (**39**) as well as a *para*-substituted phenyl (i.e. **31**: NH_2, **40**: NO_2 and **42**: OCH_3) were both well tolerated; conversely, the presence of either

meta-disubstituents (**35**) or *ortho*-OH (**43**) substituent led to compounds endowed with high toxicity. Moreover, the replacement of the phenyl (**39**) with a 3-pyridinyl ring (**36**) was also responsible for the increased cytoxicity (CC_{50} = 80.8 μM vs CC_{50} < 25 μM, respectively).

In order to further validate the anti-HCV activity of these compounds, hit **39** was selected and tested as a representative candidate against a reporter free cell culture system. To achieve this, we treated MH-14 cells carrying stably replicating HCV sub genomic replicon gt 1b with compound **39** and the HCV RNA was quantitated using standard quantitative RT-PCR methods. Notably, **39** inhibited the HCV replication in a dose-dependent manner and exhibited EC_{50} value of 3.13 μM (Fig. 4), which was quite similar to the value obtained in the replicon reporter cells (i.e. EC_{50} = 7.9 μM).

Overall, the results clearly indicated that promising anti-HCV activity coupled with no apparent cytotoxic effects were obtained when the 2-phenyl-4,5,6,7-Tetrahydro-1*H*-indole scaffold was properly functionalized.

2.4. Molecular target investigation

Next, we carried out target investigation for the most active tetrahydro-1*H*-indoles (i.e., **31, 34, 39, 40** and **42**). Towards this end, we tested the compounds for their ability to inhibit the activity of two HCV viral proteins, i.e. NS5B polymerase and NS3 helicase. These two targets were chosen as first choice because indole derivatives have been reported in literature as both HCV NS5B polymerase and NS3 helicase inhibitors [20,21].

We utilized a standard primer-dependent elongation assay to test whether the compounds possessed anti-NS5B RNA-dependent RNA polymerase (RdRp) activity [22,23]. The compounds were investigated at 50 μM concentration in the preliminary assay. The results clearly revealed that none of the compounds was inhibitory to NS5B RdRp activity (data not shown), thus ruling out the possibility of possessing anti-HCV activity by targeting this protein.

The five compounds were also tested in HCV NS3 helicase assays as described previously [24]. None of the compounds inhibited the ability of the NS3 helicase to unwind a DNA substrate even at concentrations as high as 500 μM (data not shown). However, high concentrations of compound **31** inhibited the ability of NS3 helicase to cleave ATP in the presence of RNA. About 420 μM of **31** inhibited HCV helicase catalyzed ATP hydrolysis by 50% (see Fig. S1 Supporting Information).

Apart from targeting HCV proteins, small molecules known to interfere with HCV Internal Ribosome Entry Site (IRES)-mediated translation have been documented [25,26]. We therefore investigated if the observed anti-HCV activity of the 2-phenyl-tetrahydro-1*H*-indole scaffold could be due to the down-regulation of HCV IRES-mediated translation. Using compound **39** as representative, our results displayed that this compound had no effect on HCV IRES mediated translation (data not shown).

We also tested the possibility that compound **39** could function as a potential activator or suppressor of host-factor's that facilitate HCV replication. Towards this end, we carried-out

cell based assays in which reporter plasmids of cyclooxygenase-2, heme oxygenase-1, interferon-stimulated response element or anti-oxidant response element were transfected, and the ability of derivative **39** to modulate the activation or suppression of the corresponding host-factors at three varying compound concentrations (5, 10 and 25 μM) were investigated. Our results revealed that **39** had no effect in these reporter mediated assays, thus ruling out the specified host factors as targets of the 2-phenyl-4,5,6,7-Tetrahydro-1*H*-indole core.

3. Conclusion

Overall, these results highlight the identification of 2-phenyl-4,5,6,7-Tetrahydro-1*H*-indole scaffold as a new anti-HCV chemotype.

Preliminary SAR highlighted the key role of both the substituents on the 2-phenyl ring and the *N*-1 benzyl moiety in modulating cytotoxicity and activity, respectively, with derivatives **39, 40** and **42** being the best hits within this first series of 2-phenyl-4,5,6,7-Tetrahydro-1*H*-indoles.

While the present study has revealed a novel chemotype worthy of further investigation, the exact mechanism by which these derivatives inhibit HCV replication remains to be clarified.

4. Experimental section

4.1. Cell culture

Huh7/Rep-Feo1b and Huh7.5-FGR-JC1-Rluc2A replicon reporter cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 5% antibiotic and 0.5 mg/mL G418. Huh 7.5 cells were grown similarly as above without G418. All cells were cultured at 37 °C in 5% humidified CO_2.

4.2. NS5B RdRp assay

Recombinant HCV NS5B bearing hexa-histidine tag at N-terminal was expressed in *Escherichia coli* and purified as previously described [23,27]. The anti-NS5B RdRp activity of the compounds was evaluated by using a primer-dependent elongation assay as reported earlier [28]. In brief, the reaction buffer containing 20 mM Tris–HCl (pH 7.0), 100 mM Na-glutamate, 100 mM NaCl, 0.01% BSA, 0.01% Tween-20, 0.1 mM DTT, 5% glycerol, 20 U/mL of RNasin, 20 μM UTP, 1 μCi [α-^{32}P]UTP, 0.25 μM polyrA/U_{12}, 100 ng NS5BCΔ21 was incubated with compounds and the polymerase reaction was started by addition of 1 mM $MnCl_2$ in a final volume of 20 μl. The reactions were incubated at 30 °C for 60 min, and then stopped by adding 5% trichloroacetic acid containing 0.5 mM sodium pyrophosphate, filtered through GF-B filters, and successively washed with water and ethanol. The amount of radiolabeled RNA was quantified using liquid scintillation counter. The activity of NS5B in the presence of an equal amount of DMSO was set at 100% and that in the presence of the compounds was determined relative to this control.

4.3. Huh7/Rep-Feo1b, Huh7.5-FGR-JC1-Rluc2A reporter system and cellular viability assay

The anti-HCV activity of compounds was measured using the Huh7/Rep-Feo1b and Huh7.5-FGR-JC1-Rluc2A replicon reporter cells as described earlier [29,30]. In short, approximately 1×10^4 cells were plated in 96 well plates and treated with compounds or DMSO for 48 h. The concentration of DMSO in cell culture was kept constant at 1.0%. The luciferase activities were measured by following the manufacturer's protocol (Promega Inc, USA). The activity of the compounds was evaluated as the comparative levels of the luciferase signals in compound-treated cells versus DMSO-treated controls. The cellular cytotoxicity assays were conducted in 96 well plate format using parental Huh7.5 cells. Briefly, cells treated at 6-8 doses of compounds for 48 h were evaluated employing the CellTiter 96® AQueous One Solution Cell Proliferation kit (Promega Inc, USA). The luciferase activities of the cells treated with an equal amount of DMSO served as control.

4.4. Target identification reporter assays

The effect of compound **39** on HCV IRES mediated translation was studied using a dual luciferase reporter construct (pClneo-Rluc-IRES-Fluc) in which Rluc was translated in a cap-dependent manner and Fluc was translated via HCV IRES-mediated initiation, as described previously [29]. Transfections were carried our using LipoD293 reagent in Huh7.5 cells. Sixteen h post-transfection, the cells were treated with compound or DMSO and Luciferase activity assay was performed using Dual-Glo Luciferase Assay Kit.

For investigation host-factors as potential targets, hepatoma cells carrying HCV subgenomic replicons (MH-14) were transfected with 300 ng of gene specific reporter plasmid pCOX-2-FLuc [31–34], pHO-1-Luc [35], pISRE-Luc [36], or p3xARE-Luc [37]. Sixteen h post-transfection, cells were treated with compound **39** or DMSO (control) for 48 h and luciferase activities were measured as described above. Transfection efficiencies were normalized by Renilla luciferase expression.

4.5. RT-PCR

Total RNA was isolated using an RNeasy mini kit (Qiagen) and quantified using NanoDrop (ND1000, NanoDrop Technologies). Approximately 500 ng of RNA was reverse transcribed using MMLV reverse transcriptase (Life Technologies) and either oligo dT_{18} or HCV specific primers in a final volume of 20 μl. Approximately 50 ng 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. The PCR was performed on Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument. The forward and reverse primer sequence for β-Actin was 5′-AGCGAGCATCCCCCAAAGTT-3′ and 5′-GGGCACGAAGGCTCATCATT-3′, respectively. The HCV primer sequence was 5′-CGGGAGAGCCATAGTGG-3′ for forward and 5′-AGTACCACAAGGCCTTTCG-3′ for the reverse primer.

4.6. NS3 helicase assay

4.6.1. Chemicals and reagents—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 [38,39].

4.6.2. Molecular beacon based helicase assays—Molecular beacon-based NS3 helicase assays were performed as described by Hanson et al. [49] 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.

4.6.3. ATP hydrolysis (ATPase) assays—A modified malachite green-based assay was used to measure helicase-catalyzed ATP hydrolysis (Sweeney et al., 2013). The colorimetric reagent was prepared fresh by mixing 3 volumes of 0.045% (w/v) malachite green, with 1 volume 4.2% ammonium molybdate in 4 N HCl, and 0.05 volumes of 20% Tween 20. Reactions (30 μL) were initiated by adding ATP, incubated for 15 min at 37 °C, and terminated by adding 200 μL of the malachite green reagent, followed by 30 μL of 35% sodium citrate. The color was allowed to develop for 30 min and an absorbance at 630 nm was observed.

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.

To determine the compound concentration, it was necessary to reduce helicase-catalyzed ATP hydrolysis by 50% (IC_{50}). Reactions were performed in duplicate through a two-fold dilution series so that final compound concentrations ranged from 0.5 mM to 0.78 μM. Data obtained from all reactions within the linear range of the colorimetric assay as determined with a phosphate standard curve were normalized to controls lacking an inhibitor (100%) and controls lacking an enzyme (0%), and fitted to a normalized dose response equation with a variable Hill slope using GraphPad Prism (v. 6). Reactions were performed in duplicate and each titration conformed to the above concentration response equation. Average IC_{50} values ± standard deviations were reported. In another set of controls, 100 μM of inorganic phosphate was titrated with each compound, followed by the addition of a malachite green reagent. None of the compounds affected the absorbance of the colorimetric reaction products in these controls.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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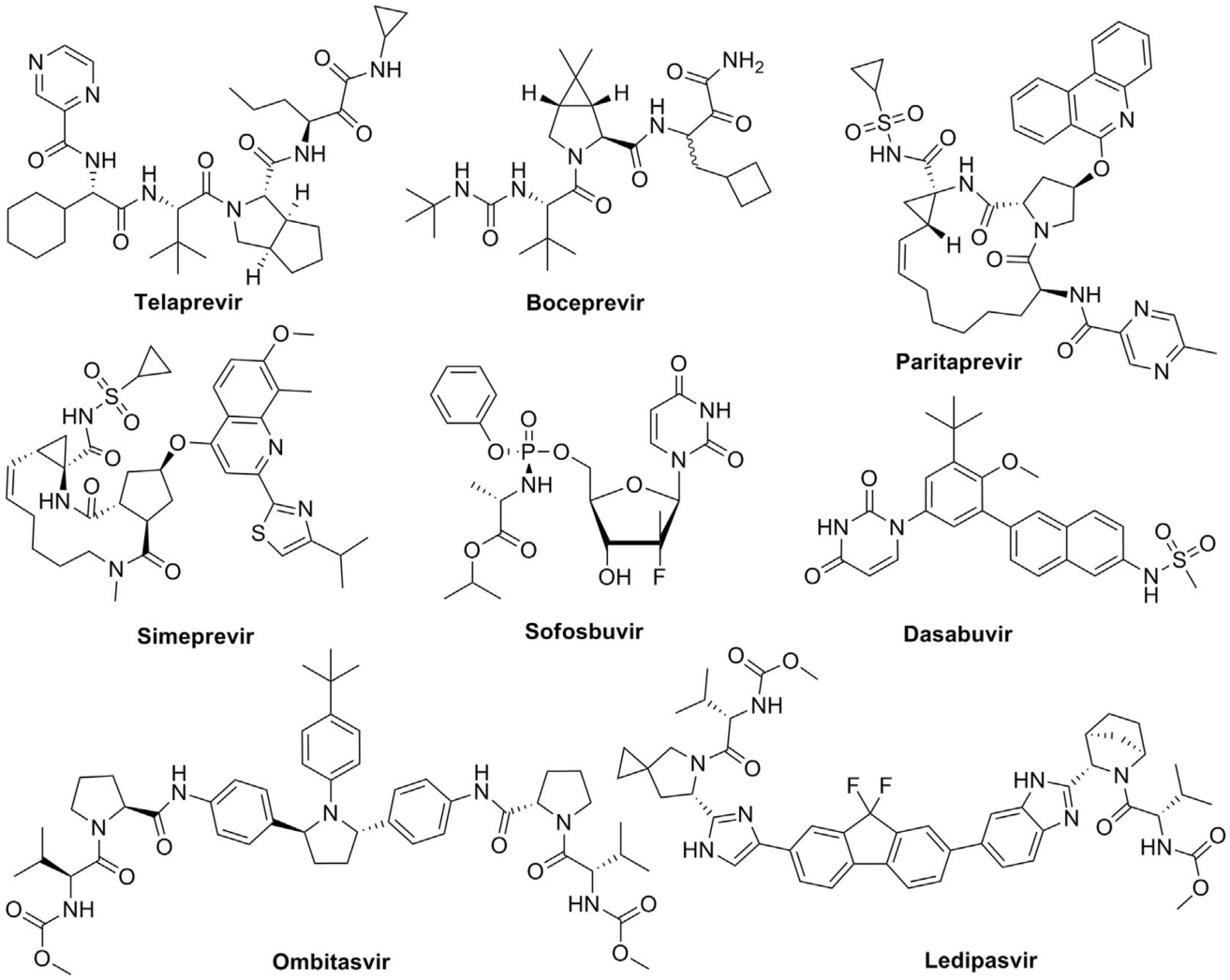


Fig. 1.
DAAs – FDA approved drugs for the treatment of Hepatitis C.

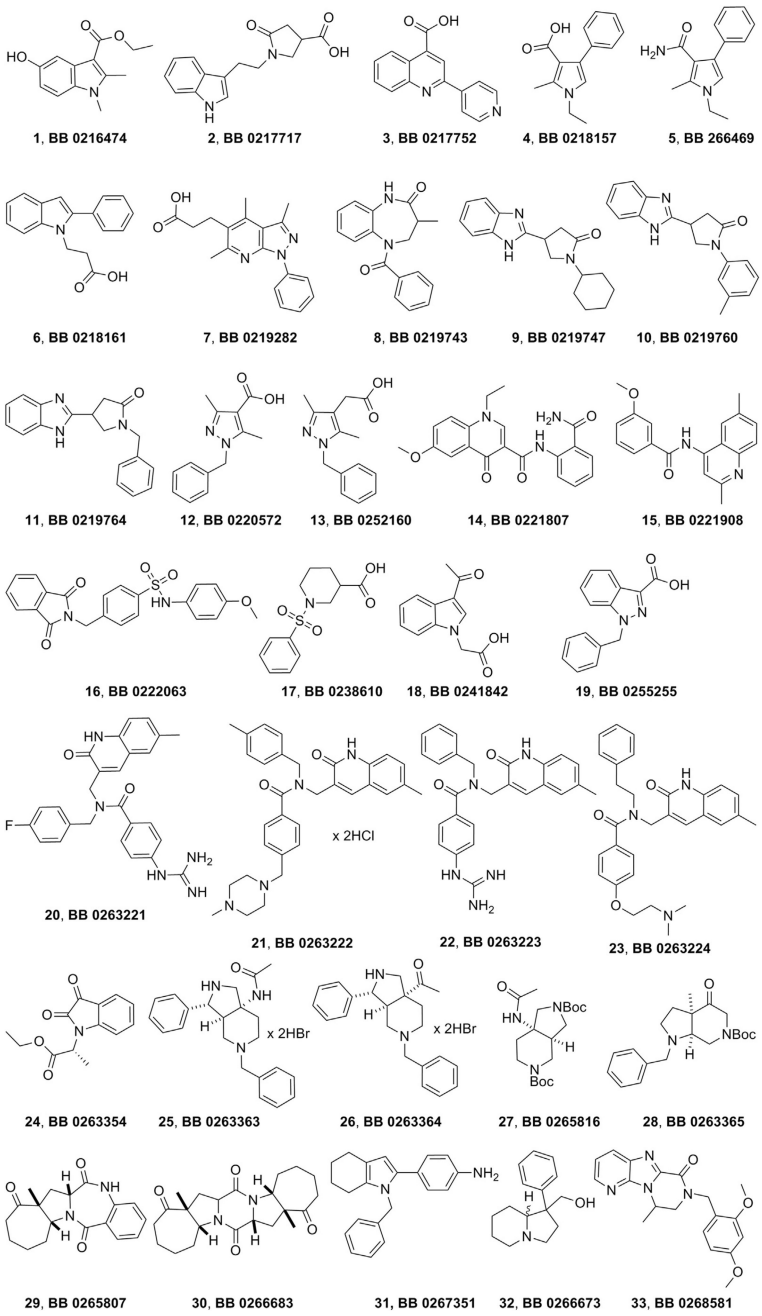


Fig. 2.

Chemical structures and internal EDASA Scientific codes of the first set of compounds that underwent biological evaluation.

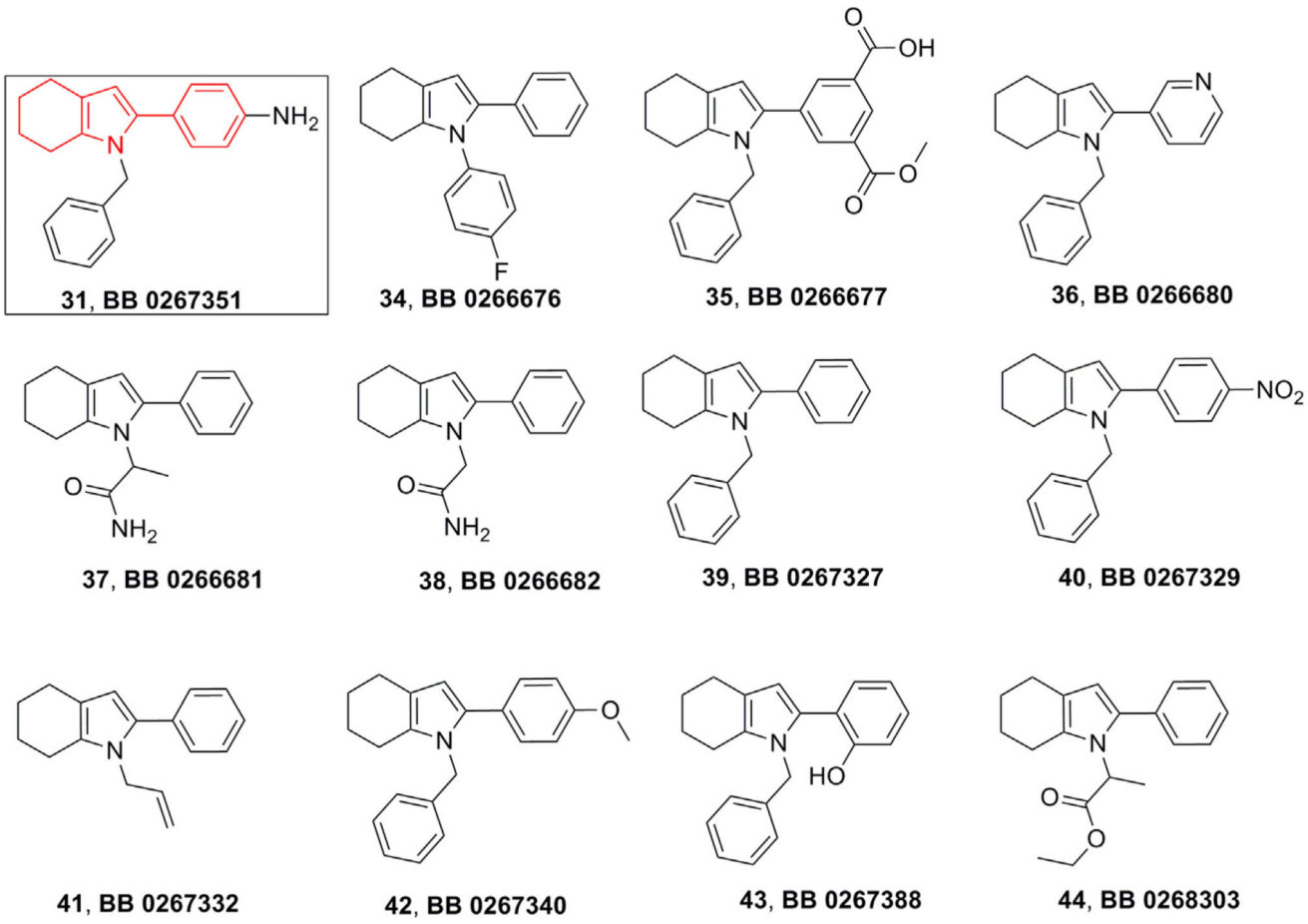


Fig. 3.

Structures of EDASA analogues of 2-phenyl-4,5,6,7-Tetrahydro-1*H*-indole **31**.

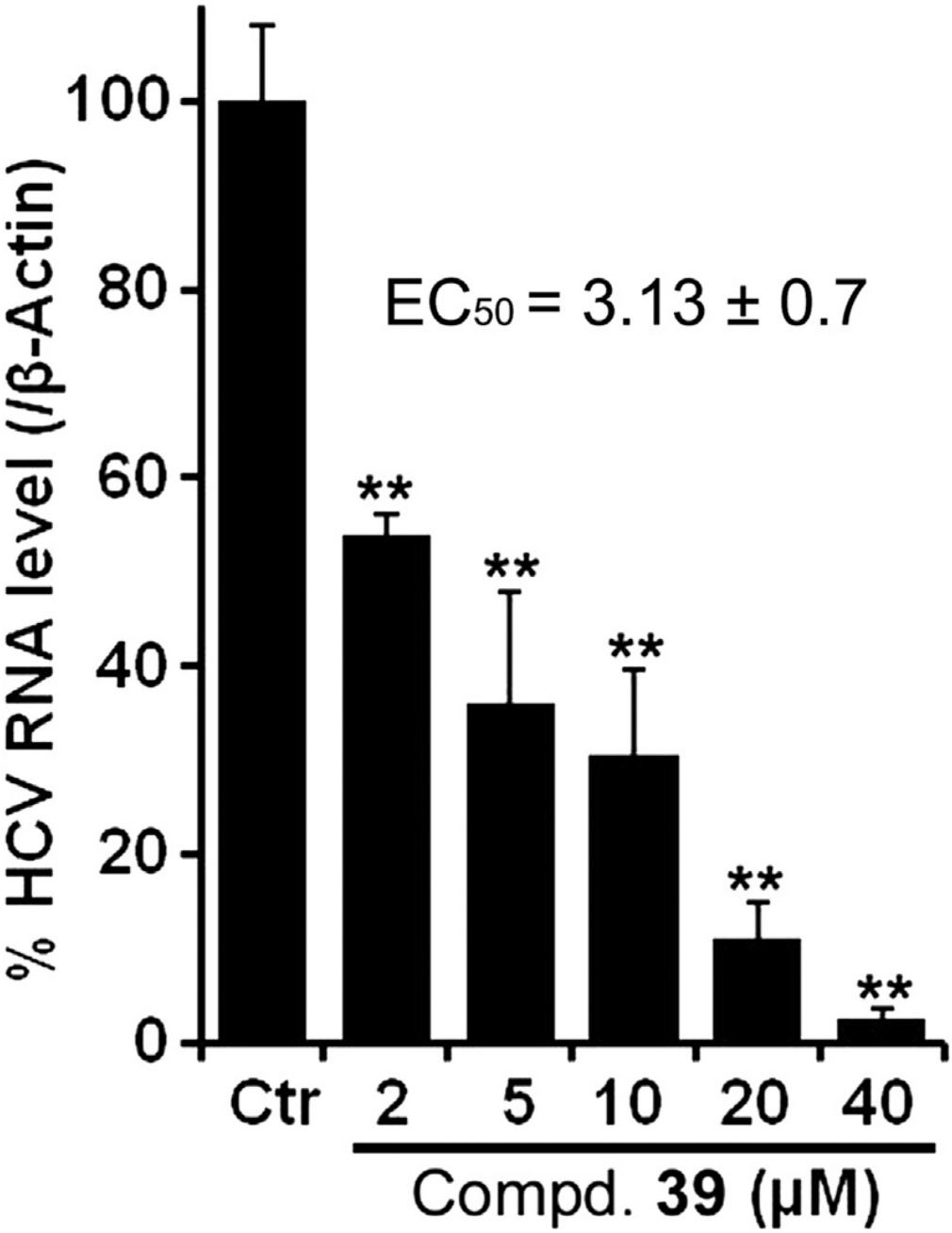
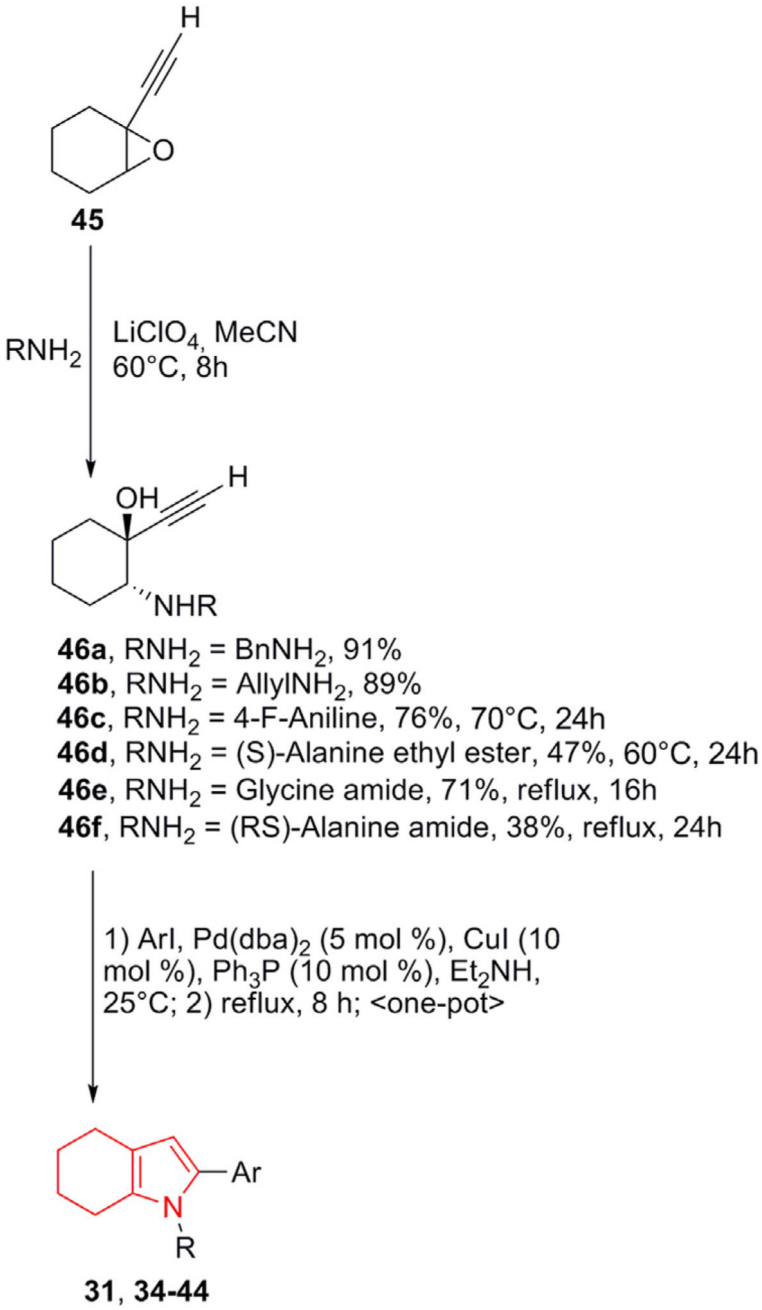


Fig. 4.

Dose-dependent response of compound **39** assayed in MH-14 cells. **p < 0.01.



Scheme. 1.

Synthesis of 2-aryl-4,5,6,7–1H-tetrahydroindoles. The explicit structures of compounds **31** and **34–44** are reported in Fig. 3.

Table 1

Anti-HCV activities and cytotoxicity of the first 33 EDASA Scientific compounds evaluated on gt 1b and 2a.

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]
1	>200	19 ± 6	ND	ND	80 ± 5	11.1 ± 0.9	>18
2	>200	22 ± 9	ND	ND	51 ± 8	48.5 ± 3.9	>4
3	>200	NI	ND	ND	49 ± 6	ND	ND
4	>200	NI	ND	ND	36 ± 10	ND	ND
5	>200	49 ± 12	ND	ND	66 ± 7	23.6 ± 4.0	>8
6	ND	26 ± 1	ND	ND	54 ± 4	ND	ND
7	>200	NI	ND	ND	78 ± 3	11.7 ± 0.9	>17
8	>200	21 ± 2	ND	ND	77 ± 7	14.1 ± 1.9	>14
9	>200	19 ± 10	ND	ND	72 ± 3	15.6 ± 3.7	>13
10	>200	21 ± 2	ND	ND	77 ± 7	17.3 ± 3.2	>12
11	>200	NI	ND	ND	67 ± 5	21.1 ± 4.4	>9
12	>200	19 ± 8	ND	ND	NI	ND	ND
13	>200	NI	ND	ND	14 ± 8	ND	ND
14	85.6 ± 5.9	17 ± 3	ND	ND	65 ± 5	20.6 ± 2.9	4
15	<25	88 ± 2	ND	ND	99 ± 1	ND	ND
16	>200	39 ± 4	ND	ND	60 ± 6	22.5 ± 3.8	>9
17	>200	NI	ND	ND	62 ± 1	28.1 ± 4.8	>7
18	>200	NI	ND	ND	55 ± 9	ND	ND
19	>200	NI	ND	ND	46 ± 8	ND	ND
20	>200	54 ± 8	ND	ND	29 ± 8	ND	ND
21	<25	92 ± 1	ND	ND	99 ± 1	ND	ND
22	>200	NI	ND	ND	44 ± 9	ND	ND
23	>200	45 ± 6	ND	ND	61 ± 9	24.8 ± 4.5	>8
24	>200	19 ± 9	ND	ND	85 ± 9	7.3 ± 0.5	>27
25	>200	NI	ND	ND	73 ± 3	4.9 ± 0.4	>41
26	>200	NI	ND	ND	76 ± 7	17.4 ± 0.8	>11
27	>200	NI	ND	ND	43 ± 10	ND	ND

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]
28	114.7 ± 14.6	73 ± 9	24.3 ± 1.2	5	98 ± 2	6.0 ± 1.0	>19
29	>200	NI	ND	ND	NI	ND	ND
30	>200	NI	ND	ND	28 ± 4	ND	ND
31	109.9 ± 2.9	66 ± 9	12.4 ± 1.0	9	88 ± 8	8.7 ± 1.9	13
32	>200	50 ± 4	ND	ND	25 ± 5	ND	ND
33	>200	45 ± 2	ND	ND	48 ± 11	ND	ND

[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)-2H-tetrazolium) into formazan by 50%. The reported value represents the means ± SD of data derived from three independent experiments.

[b] Anti-HCV activity of the compounds were carried out at 50 μM in preliminary screening.

[c] The inhibition data from 8 to 12 quarter log dilutions were used to generate the dose response curves. EC_{50} = the effective concentration required to inhibit virus induced cytopathic effect by 50%. The reported values represent the means ± SD of data derived from three independent experiments.

[d] SI: selectivity index ratio of CC_{50} to EC_{50}. ND: not determined. NI: no inhibition.

Table 2

Anti-HCV activities and cytotoxicity of analogues of **31** evaluated on gt 1b and 2a.

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]
34	>200	71 ± 6	29.2 ± 1.2	>7	66 ± 10	12.3 ± 1.0	>16
35	45.6 ± 6.1	81 ± 3	35.8 ± 3.4	>1	96 ± 3	9.9 ± 1.6	>5
36	<25	92 ± 1	ND	ND	99 ± 1	ND	ND
37	155.6 ± 11	50 ± 8	ND	ND	83 ± 3	15.4 ± 3.9	>10
38	>200	37 ± 18	ND	ND	48 ± 11	ND	ND
39	80.8 ± 3.1	95 ± 4	7.9 ± 0.5	10	99 ± 1	2.6 ± 0.4	32
40	>200	75 ± 7	15.0 ± 1.3	13	98 ± 2	7.3 ± 1.4	27
41	118.8 ± 2.8	35 ± 6	ND	ND	69 ± 2	32.1 ± 4.1	4
42	137.4 ± 1.0	99 ± 1	11.8 ± 0.6	12	96 ± 2	4.9 ± 0.3	28
43	48.9 ± 1.7	96 ± 2	9.2 ± 0.6	5	99 ± 1	6.5 ± 0.6	8
44	84.3 ± 1.3	74 ± 4	13.2 ± 1.4	6	95 ± 3	13.7 ± 2.1	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)-2H-tetrazolium) into formazan by 50%. The reported value represents the means ± SD of data derived from three independent experiments.

[b] Anti-HCV activity of the compounds were carried out at 50 μM in preliminary screening.

[c] The inhibition data from 8 to 12 quarter log dilutions were used to generate the dose response curves. EC_{50} = the effective concentration required to inhibit virus induced cytopathic effect by 50%. The reported values represent the means ± SD of data derived from three independent experiments.

[d] SI: selectivity index ratio of CC_{50} to EC_{50}. ND: not determined.