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C-6 aryl substituted 4-quinolone-3-carboxylic acids as inhibitors of hepatitis C virus

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ABSTRACT

Quinolone-3-carboxylic acid represents a highly privileged chemotype in medicinal chemistry and has been extensively explored as antibiotics and antivirals targeting human immunodeficiency virus (HIV) integrase (IN). Herein we describe the synthesis and anti-hepatitis C virus (HCV) profile of a series of C-6 aryl substituted 4-quinlone-3-carboxylic acid analogues. Significant inhibition was observed with a few analogues at low micromolar range against HCV replicon in cell culture and a reduction in replicon RNA was confirmed through an RT-qPCR assay. Interestingly, evaluation of analogues as inhibitors of NS5B in a biochemical assay yielded only modest inhibitory activities, suggesting that a different mechanism of action could operate in cell culture.

1. Introduction

HCV[1] infects an estimated 180 million people globally[2] and approximately 4 million in the USA.[3] Chronic HCV infection progresses through liver injury, fibrosis, cirrhosis and can lead to hepatocellular carcinoma.[4,5] Until the recent approval of two protease inhibitors, the standard of care (SOC) for treating HCV infection has been the combination of subcutaneous pegylated interferon (peg-IFN) alpha and oral ribavirin (RBV).[6] By boosting the host immune system this SOC has proved only moderately successful in patients infected with HCV genotype 1,[6,7] the predominant genotype in North America and Europe, with a 40–50% sustained virological response (SVR) rates. In addition, the peg-IFN/RBV regimen is associated with severe adverse effects including fatigue, hemolytic anemia, depression, and flu like symptoms.[8] Developing novel HCV chemotherapy with better efficacy and tolerability continues to be an endeavor of tremendous interest from both academia and industry. Direct-acting antivirals (DAAs) specifically targeting HCV replication have consistently yielded improved antiviral profiles and would provide better treatments for HCV infection.[9]

HCV is an enveloped, positive-sense and single-stranded RNA virus belonging to the *Hepacivirus* genus of the *Flaviviridae* family. Its genomic RNA encodes a polyprotein precursor of about 3000 amino acids, which is processed first by host peptidases at the N-terminus to release four structural proteins (core, E1, E2 and P7), and then by the virally encoded NS2 zinc dependent protease and NS3-4A serine proteases to yield six nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B).[10] Apparently the NS3-4A serine-protease is essential for viral replication and represents an important antiviral target[11] as exemplified by the successful development of telaprevir (**1**)[12,13] and boceprevir (**2**)[14,15] (Fig. 1), the two recently approved protease inhibitors. Combined with PEG-IFN/RBV, these protease inhibitors treat HCV infection with significantly improved SVR rates and reduced duration, though the response is largely limited to genotype 1 HCV infection and the regimens are susceptible to resistance development.[10] Second-generation HCV protease inhibitors with improved efficacies against resistant viral strains are being actively pursued.[16–21] NS5B is the RNA dependent RNA polymerase, thus another major target for HCV chemotherapy development. Numerous nucleoside inhibitors (NIs)[10,22] and non-nucleoside inhibitors (NNIs)[23–25] of NS5B are in current HCV antiviral pipeline. The NIs such as the uridine analogue PSI-7977 (**3**, Fig. 2)[26] and the guanosine analogue PSI-938 (**4**, Fig 2),[27] target the highly conserved active site, thus pose a high genetic barrier to resistance development and confer a pan-genotypic antiviral activity. By contrast, the NNIs bind to mutable allosteric sites and are more susceptible to resistance mutations. GS-9190[28] (**5**, Fig. 2) isan established NNI with exceptional in vitro antiviral activityA third HCV-encoded protein, NS5A, is also becoming an increasingly important target for HCV drug discovery.[29–31] NS5A is a phosphoprotein associated with

Abbreviations: HIV, human immunodeficiency virus; IN, integrase; HCV, hepatitis C virus; SOC, standard of care; peg-INF, peglylated interferon; RBV, ribavirin; SVR, sustained virological response; DAA, direct-acting antiviral; NNI, non-nucleoside inhibitor; EVG, elvitegravir; Luc, luciferase; 2′-C-Me-A, 2′-C-methyl adenosine; SAR, structure-activity-relationship; RT-qPCR, reverse transcription and quantitative polymerase chain reaction.

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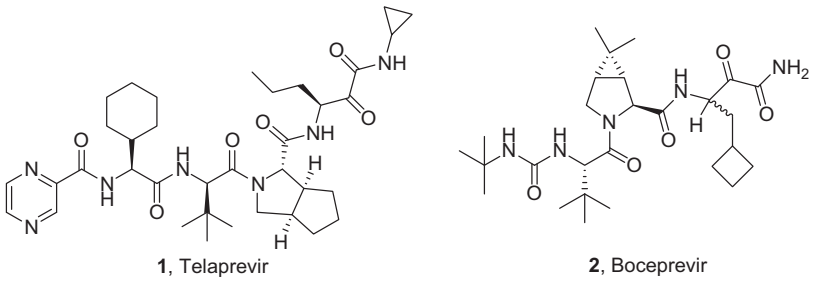


Figure 1. The first FDA-approved DAAs: protease inhibitors telaprevir (**1**) and boceprevir (**2**)

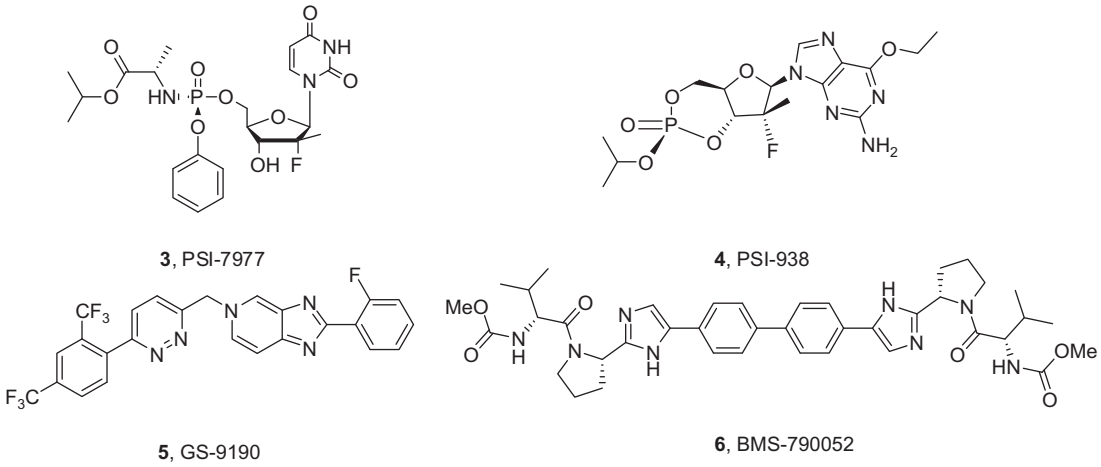


Figure 2. Representative HCV inhibitors in clinical development: **3**, non-nucleoside polymerase inhibitor; **4** and **5**, nucleoside polymerase inhibitors; **6**, NS5A inhibitor.

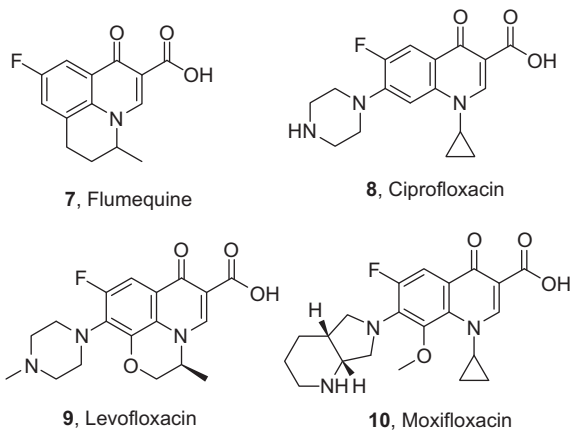


Figure 3. Four generations of clinical quinolone antibiotics, all featuring a quinolone-3-carboxylic acid core: **7**, first generation; **8**, second generation; **9**, third generation; and **10**, fourth generation.

host cell membranes without apparent enzymatic activity. By binding directly to the NS5B polymerase, NS5A constitutes an essential component of the HCV replication complex and is believed to modulate the activity of NS5B.[32,33] BMS-790052 (**6**),[29,31] the first-in-class NS5A inhibitor is currently under clinical development. These experimental drugs and many others in the pipeline have the great potential to enhance current regimens. However, the challenges of HCV chemotherapy in achieving pan-genotypic efficacy, large barrier to resistance selection, interferon-free regimen and efficacy in hard-to-treat populations necessitate continued efforts in search for novel inhibitors. We report herein the design, chemical synthesis and biological evaluations of quinolone-3-carboxylic acid analogues as HCV inhibitors.

Quinolones are among the most privileged molecular scaffolds in medicinal chemistry.[34] Of particular importance is the 4-quinolone-3-carboxylic acid chemotype that constitutes a major class of antibacterial agents. Four generations of quinolone-3-carboxylic acid antibiotics (Fig. 3) have been developed for Gram-positive, Gram-negative and anaerobic bacterial infections.[35] Obviously this chemotype possesses highly favorable pharmacokinetic properties and provides an attractive scaffold for designing inhibitors of other medicinally interesting targets, a notable example being HIV IN inhibitor elvitegravir (EVG, **13**, Fig. 4).[36] EVG builds on the 4-quinolone-3-carboxylic acid core and mimics the canonical diketoacid pharmacophore for chelating Mg^{2+} ions.[37] Significantly, HCV NS5B polymerase shares a similar active site fold to HIV IN,[38] which is manifested by the resemblance between their inhibitor pharmacophores (Fig. 4). Numerous chemotypes including the 5-hydroxy-pyrimidinones (**11**[39] and **14**[40]) and 2-hydroxyisoquinoline-1,3-diones (**12**,[41–43] **15**–**16**[44]) have shown potent inhibition against both HIV IN and HCV NS5B. The major pharmacophore difference, however, is that IN inhibition requires a flexible terminal benzyl group whereas a rigid aromatic group, typically a thiophene or furan moiety,[23] is immediately connected to the core of NS5B inhibitors (Fig. 4). Naturally we have decided to synthesize and test scaffold **17** as HCV inhibitors. During the course of our work, a quinolone-3-carboxylic acid ester series was reported to inhibit HCV through targeting NS5B.[45]

2. Results and discussion

2.1. Chemistry

Synthesis of quinolone scaffold is well documented.[46] In our case, the key quinolone-3-carboxylic intermediates **23**–**24** were synthesized via the classic Gould–Jacobs approach.[47] This reaction

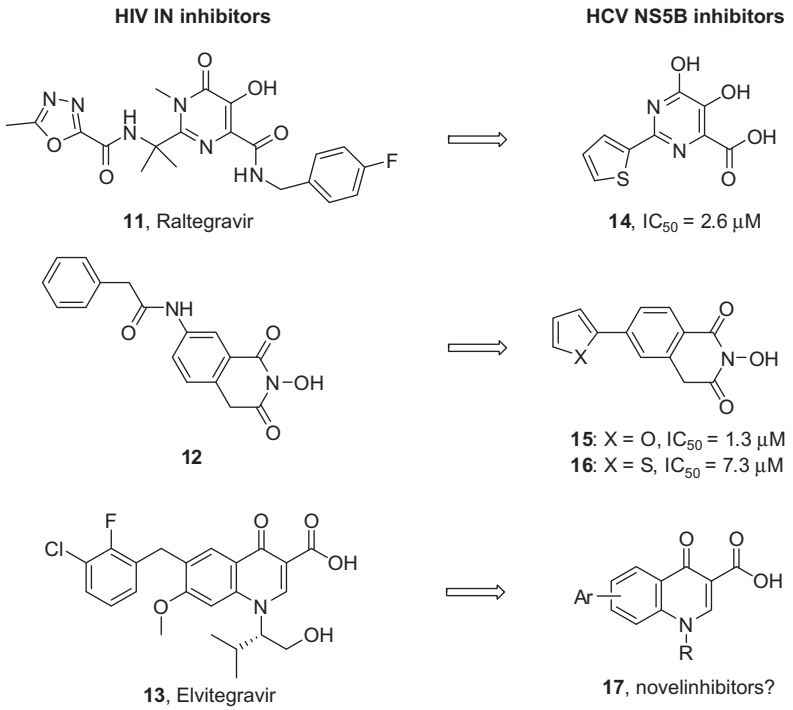
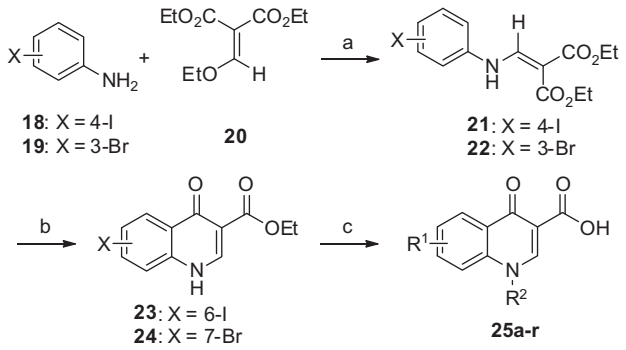


Figure 4. Design of novel NS5B inhibitor scaffold **17** based on the pharmacophore resemblance between HIV IN inhibitors and HCV NS5B inhibitors.



Scheme 1. Synthesis of C-6/C-7 substituted quinolone-3-carboxylic acids. Reagents and conditions: (a) toluene, reflux, 58–91%; (b) Ph_2O, reflux, 42–96%; (c) (i) K_2CO_3, DMF, 100 °C oil bath, 30 min, then alkylating reagent (R^2-Br), μW 110 °C, 2.8 h; (ii) $R^1B(OH)_2$, K_2CO_3, $PdCl_2$, μW 130 °C, 35 min; (iii) *p*TSA/MeOH (*O*-THP protection) or K_2CO_3/H_2O (*O*-Ac protection), 11–99% over three steps.

sequence began with an addition-elimination reaction between halogen substituted anilines **18**–**19** and ethoxy ethylenemalonate **20** to produce malonate intermediates **21**–**22**, which upon intramolecular cyclization yielded the desired quinolone-3-carboxylic intermediates **23**–**24**. The halogen in these intermediates serves as a synthetic handle for the introduction of a panel of aromatic rings onto the C-6 or C-7 site. This was achieved by first alkylating the N-1 position followed by a Suzuki coupling reaction[48] with commercial aromatic boronic acids. The alkylating reagents containing a hydroxyl group are preferably protected by THP for optimal Suzuki coupling step. *O*-Ac protection also allowed the Suzuki coupling reaction, albeit with a lower yield. The resulting esters were converted to acid analogues **25a–r** via removal of *O*-protection and saponification.

2.2. HCV replicon assay

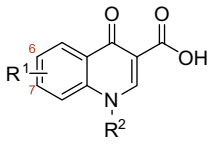
To evaluate the ability of quinolone-3-carboxylic acid analogues to inhibit HCV replication, an HCV genotype 1b replicon stably maintained in Huh7 human liver cells (Huh-7/HCV1b-Rluc) was used. HCV subgenomic viral RNAs (replicons) capable of self-replication in human liver cells are commonly used to identify and characterize inhibitors of HCV replicative enzymes.[49–51] The replicon RNA expresses renilla luciferase (Luc) thereby permitting the measurement of Luc activity as an indication of the level of replicon RNA.

The initial testing of all quinolone-3-carboxylic acid analogues in the Huh7 replicon assay was at a single concentration of 10 μM. RBV and 2′-*C*-methyl adenosine (2′-C-Me-A)[52] were included in each experiment as control compounds and known inhibitors at 10 μM and 0.5 μM, respectively. Replicon cells were also treated with vehicle alone (DMSO) and cell culture medium in each assay. Compounds showing apparent Luc reduction (⩾20%) were tested for cell viability using an MTS-based colorimetric assay. Results from these testings are summarized in Table 1.

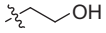
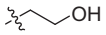
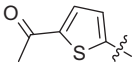
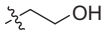
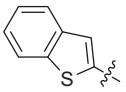
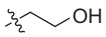
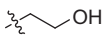
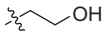
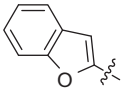
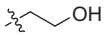
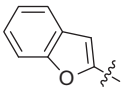
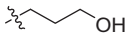
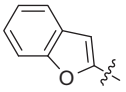
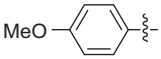
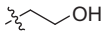
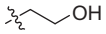
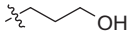
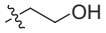
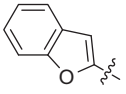
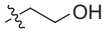
These preliminary screening results revealed a few discernible structure–activity-relationship (SAR) trends. First of all, the substitution position of the aromatic ring appears to greatly impact potency as all C7 analogues were inactive (**25o–r**, Table 1) whereas a number of C6 isomers showed significant activity (**25a**, **25c**, **25e**, **25g**, **25i**, **25k** and **25l**, Table 1). This trend could reflect the influence of different molecular shapes between C6 and C7 isomers on target binding. Secondly, the N-1 substituent also appears crucial for inhibition, with unsubstituted (**25d**, **25h**) and simple alkyl substituted (**25m**) analogues showing no activity while hydroxyethyl and hydroxypropyl groups are substantially better for inhibition. Thirdly, for the aromatic substituent both 2-thiophene (**25e**) and 2-furan (**25i**) seem to be tolerated. However, the best inhibition was achieved with 2-benzothiophene (**25g**) and 2-benzofuran (**25k**, **25l**) substituted analogues. Other aromatic substituents, including 5-acylthiophene (**25f**), 3-furan (**25j**) and para-methoxyphenyl (**25n**), failed to yield appreciable inhibitory activity against HCV replicon.

With the preliminary results, four compounds with significant inhibition (⩾40%) were then selected for further testing in dose response fashion. Both RBV and 2′-C-Me-A were used as controls for this assay. The results are shown in Figure 5 and summarized in

Table 1
Preliminary screening results of compounds **25a-r** against HCV replicon



25a-r

Compd	C6/C7	R^1	R^2	Luc reduction% (10 μM)[a]	Cell viability% (10 μM)
25a	6	I	H	22	100
25b	6	I	Bn	0	99
25c	6	I		21	100
25d	6		H	0	100
25e	6			40	100
25f	6			0	100
25g	6			58	100
25h	6		H	0	100
25i	6			20	100
25j	6			0	100
25k	6			63	100
25l	6			44	100
25m	6			4	91
25n	6			0	95
25o	7			14	100
25p	7			12	97
25q	7			15	100
25r	7			14	94
RBV	—	—	—	44	92
2′-C-Me-A	—	—	—	98	87

[a] Reduction of Luc activity, indicating inhibitory activity of a compound.

Table 2. Notably compounds with a benzo fused aromatic substituent (**25g** and **25k**) were found to inhibit HCV replicon at low micromolar range, while the 2-thiophene analogue (**25e**) and the N-1 hydroxypropyl substituted analogue (**25l**) were considerably less potent. This finding is consistent with the SAR trends observed from the preliminary screening. Nevertheless, the two best compounds (**25g** and **25k**) inhibit HCV clearly better than RBV, suggesting that quinolone-3-carboxylic acid scaffold has the potential for antiviral discovery against HCV. Future work needs to address the toxicity and the low therapeutic indices associated with these

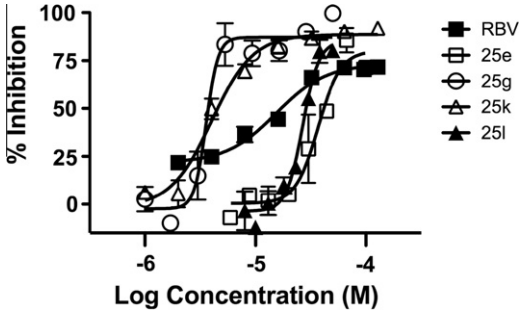


Figure 5. Representative dose–response curves for compound inhibition of HCV replicon-containing cells. The percent (%) inhibition of each compound compared to DMSO alone is shown. Doses were performed in triplicate and repeated 2–3 independent times. Average values for each dose plus standard deviation for one representative experiment are shown.

Table 2
Dose response antiviral results of selected compounds against HCV replicon

Compd	EC_{50} (μM)[a]	CC_{50} (μM)[b]	TI[c]
25e	36 ± 3.5	>50	>1.4
25g	5.9 ± 2.2	15 ± 3.5	2.5
25k	5.8 ± 1.7	34 ± 22	5.9
25l	23 ± 4.2	25 ± 2.1	1.1
RBV[d]	14 ± 4.5	32	2.3
2′-C-Me-A[d]	0.20 ± 0.11	55 ± 15	270

[a] Concentration inhibiting virus replication by 50%, mean value ± standard deviation from at least two independent determinations.
[b] Concentration resulting in 50% cell death.
[c] Therapeutic index, defined by CC_{50}/EC_{50}.
[d] Ref.[44]

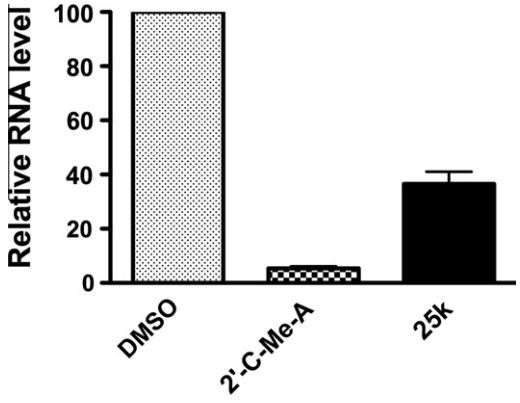


Figure 6. Compound **25k** inhibits HCV replicon RNA production. Relative HCV replicon RNA level for cells treated with control compound 2′-C-Me-A (0.5 μM), **25k** (10 μM) or DMSO alone for 3 days prior to RNA isolation and RT-qPCR.

compounds, which might reflect their ability to bind to DNA topoisomerase, the underlying mechanism of action for quinolone antibiotics.[35]

2.3. RT-qPCR assay

To further confirm that these quinolone compounds actually inhibit HCV sub-genomic replication, we have also performed an assay to directly detect the viral replicon RNA level using RT-qPCR. This was done by lysing cells in a 96-well dish, converting RNA in that lysate to cDNA, and conducting real-time PCR to quantify cDNA levels. Given the high cost of this assay, a representative compound **25k** was chosen and the relative level of replicon RNA for compound-treated cells compared to DMSO-treated cells was

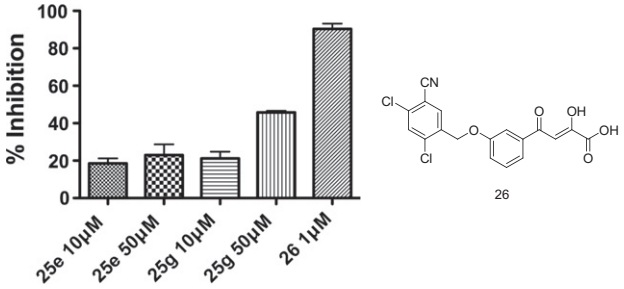


Figure 7. Evaluation of compounds as inhibitors of NS5B primer-dependent RNA polymerase activity. Primer-dependent incorporation of [^{3}H]UTP on a poly(A) template by recombinant NS5BΔ55 was measured in duplicate samples in the presence of compound or DMSO and mean value compared to that of DMSO. The mean value and standard deviation of two independent experiments are shown. A known NS5B inhibitor **26**[54] was used as a control.

measured using the TaqMan® Gene Expression Cells-to-CT system (Applied Biosystems) and TaqMan® primers/probes. The primers/probes to measure HCV replicon cDNA were custom synthesized to NS5B (Applied Biosystems). The control primers/probe recognize GAPDH cDNA (Applied Biosystems). Parallel wells of lysate were analyzed for either HCV replicon RNA or GAPDH RNA and relative levels of HCV RNA calculated using the comparative method.[53] The percent reduction in replicon RNA for cells treated with **25k** compared to cells treated with DMSO alone is shown in Figure 6. Compound **25k** was tested at 10 μM and reduced replicon RNA approximately 70%. 2′-C-Me-A treatment (0.5 μM) reduced RNA levels approximately 95%. We conclude from this data that **25k** inhibits replicon RNA production as expected.

2.4. Biochemical NS5B assay

Quinolone-3-carboxylic acid scaffold is known to inhibit HIV IN based on its ability to chelate Mg^{2+} ions at the enzyme active site.[36,37] HCV NS5B catalysis also involves Mg^{2+} chelation as well as a similar fold of active conformation. Therefore, we had hypothesized that our quinolone analogues will inhibit NS5B, likely via binding to the active site. To test this hypothesis, two compounds (**25e** and **25g**) active in the replicon assay were selected for study in the biochemical NS5B assay measuring primer-dependent RNA polymerase activity from a heterologous template. A known potent DKA NS5B inhibitor **26**[54] was used as a control compound. The assay results are shown in Figure 7. Notably while the control compound inhibits NS5B effectively at 1 μM in our assay, our HCV inhibitors showed only modest inhibition at concentrations up to 50 μM. In addition, the inhibition observed at 10 μM and 50 μM did not appear to occur in a strict dose-dependent fashion for either **25e** or **25g**, especially **25e** where the increase in dose did not significantly improve the percent inhibition. Furthermore, virtually no inhibition was observed with compound **25k** at 10 μM and 50 μM doses (data not shown). The lack of robust and dose-dependent inhibition of NS5B in this assay may indicate that these analogues are not NS5B inhibitors. There are, however, established NS5B inhibitors that do not demonstrate inhibition in biochemical assays measuring recombinant NS5B polymerase activity.[55] Such inhibitors may require NS5B expression in the cell with other viral and cellular proteins to manifest inhibition. Therefore further experimentation is required to provide a better indication of mechanism of action.

3. Conclusions

Quinolone-3-carboxylic acid is a highly privileged molecular scaffold, particularly as antibacterial agents and HIV IN inhibitors. Based on the similar fold of active site shared by HIV IN and HCV

NS5B and the pharmacophore resemblance between their inhibitors, we designed C-6 aromatic substituted quinolone-3-carboxylic acids as a novel inhibitor scaffold for HCV. These analogues were synthesized following a classic Gould-Jacobs reaction sequence and featuring a Suzuki coupling reaction. All synthetic analogues were tested in a primary screening assay using HCV replicon 1b Huh-7/HCV1b-Rluc cells. Four active compounds were further tested in a dose response fashion, which identified **25k** as the best HCV inhibitor of this series (EC_{50} = 5.8 μM, CC_{50} = 34 μM). The ability of **25k** to inhibit HCV viral RNA production was verified through an RT-qPCR assay. However, the biochemical NS5B polymerase assay yielded poor inhibition suggesting that a mechanism of action other than NS5B inhibition is possible.

4. Experimental

4.1. Chemistry

4.1.1. General procedures

All commercial chemicals were used as supplied unless otherwise indicated. Dry solvents (THF, Et_2O, CH_2Cl_2 and DMF) were dispensed under argon from an anhydrous solvent system with two packed columns of neutral alumina or molecular sieves. Flash chromatography was performed on a Teledyne Combiflash RF-200 with RediSep columns (silica) and indicated mobile phase. All reactions were performed under inert atmosphere of ultra-pure argon with oven-dried glassware. ^1H and ^{13}C NMR spectra were recorded on a Varian 600 MHz or a Varian 400 MHz spectrometer. Mass data were acquired on an Agilent TOF II TOS/MS spectrometer capable of ESI and APCI ion sources. Analysis of sample purity was performed on a Varian Prepstar SD-1 HPLC system with a Varian Microsorb-MW 100–5 C18 column (250 × 4.6 mm). HPLC conditions: solvent A = H_2O, solvent B = MeCN; flow rate = 1.0 mL/min; Gradient (B%): 0–13 min (10–95); 13–20 min (95); 20–23 min (95–10); 23–25 min (10). All tested compounds have a purity ⩾ 96%.

4.1.2. Ethyl α-carbethoxy-β-*p*-iodoanilinoacrylate (21)[56]

21.9 g (0.1 mol) of 4-iodoaniline and 21.6 g (0.1 mol) of diethyl ethoxymethylene malonate were dissolved in 150 mL of toluene and refluxed for 1.0 h. The mixture was then evaporated to dryness. The residue was adsorbed on silica gel and purified by standard flash chromatography method with EtOAc and hexanes to give 22.7 g (0.058 mol) of product **21** (58%) as a light grey crystal. R_f 0.58 (EtOAc:hexanes = 1:2); ^1H NMR (400 MHz, $CDCl_3$) δ 10.96 (d, *J* = 13.5 Hz, 1H, NH), 8.44 (d, *J* = 13.5 Hz, 1H, H_{alkene}), 7.68–7.61 (m, 2H, H_{aryl}), 6.92–6.84 (m, 2H, H_{aryl}), 4.26 (pseudo-dq, *J* = 21.6, 7.1 Hz, 4H, Et), 1.36 (t, *J* = 7.1 Hz, 3H, Et), 1.31 (t, *J* = 7.1 Hz, 3H, Et). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.23(C≡O), 165.82(C≡O), 151.47(N–C≡C), 139.35(2C, $C_{t,aryl}$), 139.01(2C, $C_{t,aryl}$), 119.21($C_{q,aryl}$), 94.73(N–C≡C), 88.23(C $C_{q,aryl}$), 60.83(Et), 60.53(Et), 14.72(Et), 14.58(Et); HRMS (ESI+) calcd for $C_{14}H_{17}INO_4^+$(M+H$^+$) 390.0197, found 390.0202; HPLC t_R 16.2 min; Mp: 110–111 °C.

4.1.3. Ethyl α-carbethoxy-β-m-bromoanilinoacrylate (22)[56]

Starting from 8.6 g (50 mmol) of 3-bromoaniline and 11.0 g (51 mmol) of diethyl ethoxymethylene malonate, the same procedure as described for the preparation of compound **(21)** gave 15.58 g (45.7 mmol) of white solid **22** (91%). R_f 0.40 (EtOAc:hexanes = 1:5); ^1H NMR (400 MHz, $CDCl_3$) δ 11.23 (d, *J* = 13.2 Hz, 1H, NH), 8.46 (d, *J* = 13.3 Hz, 1H, H_{alkene}), 7.55 (dd, *J* = 8.0, 1.2 Hz, 1H, H_{aryl}), 7.37–7.20 (m, 2H, H_{aryl}), 6.97 (td, *J* = 8.0, 1.5 Hz, 1H, H_{aryl}), 4.32 (q, *J* = 7.1 Hz, 2H, Et), 4.23 (q, *J* = 7.1 Hz, 2H, Et), 1.36 (t, *J* = 7.1 Hz, 3H, Et), 1.31 (t, *J* = 7.1 Hz, 3H, Et). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.31(C≡O), 165.57(C≡O), 150.46(N-C=C), 137.67 ($C_{q,aryl}$), 133.42($C_{t,aryl}$), 128.71($C_{t,aryl}$), 125.35($C_{t,aryl}$), 115.90($C_{t,aryl}$), 113.66($C_{q,aryl}$), 95.50(N-C=C), 60.57(Et), 60.25(Et), 14.38(Et), 14.32(Et); HRMS (ESI+) calcd for $C_{14}H_{17}BrNO_4^+$(M+H$^+$) 342.0335, found 342.0310; HPLC t_R 15.9 min; Mp: 61–62 °C.

4.1.4. Ethyl 6-iodo-4-oxo-1*H*-quinoline-3-carboxylate (23)[56]

3 mL of diphenyl ether was vigorously stirred in a test tube and heated to reflux with a sand bath, and to which, 1.0 g (2.6 mmol) of starting material **(21)** was added. The resulting solution was refluxed for another 30 min, cooled to rt, and triturated with diethyl ether to give the product as a precipitation, which was collected by filtration. The solid was washed with diethyl ether for several times to give 0.86 g (2.5 mmol) of grey powder **23** (96%). ^1H NMR (400 MHz, DMSO-d_6, measured at 100 °C due to solubility issue) δ 12.39 (s, 1H, NH), 8.55 (s, 1H, H-2), 8.41 (d, *J* = 2.0 Hz, 1H, H_{aryl}), 7.97 (dd, *J* = 8.6, 2.1 Hz, 1H, H_{aryl}), 7.42 (d, *J* = 8.6 Hz, 1H, H_{aryl}), 4.20 (q, *J* = 7.1 Hz, 2H, Et), 1.26 (t, *J* = 7.1 Hz, 3H, Et). ^{13}C NMR (100 MHz, DMSO-d_6, measured at 100 °C due to solubility issue) δ 172.40(C≡O), 164.97(C≡O), 145.64(C-2), 140.93(CH_{aryl}), 139.02 ($C_{q,aryl}$), 134.51(CH_{aryl}), 129.36($C_{q,aryl}$), 121.65(CH_{aryl}), 110.72 ($C_{q,aryl}$), 90.09($C_{q,aryl}$), 60.13(Et), 14.76(Et); HRMS (ESI-) calcd for C12H9INO3$^-$(M-H$^+$) 341.9633, found 341.9641; HPLC: Solubility is too poor to make an HPLC analysis; Mp: 322–324 °C (decomp.).

4.1.5. Ethyl 7-bromo-4-oxo-1*H*-quinoline-3-carboxylate (24)[56]

3 mL of diphenyl ether was vigorously stirred in a test tube and heated to reflux with a sand bath, and to which, 800 mg (2.35 mmol) of starting material **(22)** was added. The resulting solution was refluxed for another 30 min, cooled to rt, and triturated with diethyl ether to give the product as a precipitation, which was collected by filtration. The solid was washed with diethyl ether for several times to give 497 mg of white powder as the crude product, which is sufficiently pure for the further coupling reactions. For analytical purpose, the crude product was washed with 20% MeOH in DCM for three times by means of centrifuge. The resulting solid was dissolved in a minimum amount of boiling DMSO, and then cooled to rt to give the pure product as a white powder **24** (291 mg, 0.99 mmol, 42%). ^1H NMR (400 MHz, DMSO-d_6, measured at 100 °C due to solubility issue) δ 11.88 (s, 1H, NH), 8.45 (s, 1H, H-2), 8.07 (d, *J* = 8.6 Hz, 1H, H_{aryl}), 7.80 (d, *J* = 1.7 Hz, 1H, H_{aryl}), 7.51 (dd, *J* = 8.6, 1.7 Hz, 1H, H_{aryl}), 4.24 (q, *J* = 7.1 Hz, 2H, Et), 1.29 (t, *J* = 7.1 Hz, 3H, Et). ^{13}C NMR (100 MHz, DMSO-d_6, measured at 100 °C due to solubility issue) δ 173.17 (C≡O), 164.99(C≡O), 145.27(C-2), 140.86($C_{q,aryl}$), 128.36 (CH_{aryl}), 127.94(CH_{aryl}), 126.76($C_{q,aryl}$), 126.00($C_{q,aryl}$), 121.55 (CH_{aryl}), 112.01($C_{q,aryl}$), 60.03(Et), 14.62(Et); HRMS (ESI-) calcd for C12H9BrNO3$^-$(M-H$^+$) 293.9771, found 293.9772; HPLC: Solubility is too poor to make an HPLC analysis; Mp: 334–335 °C (decomp., recrystallized from DMSO).

4.2. General procedure for preparation of compounds 25a–r[36]

(a) Alkylation and Suzuki coupling in one pot: A mixture of 0.58 mmol of halogen derivative **23** or **24**, 100 mg (0.72 mmol, 1.2 equiv) of K_2CO_3 and 3 mL of DMF was stirred in a 100 °C oil bath for 30 min, cooled to rt, and treated with alkylating reagent (1.2 equiv to halogen derivative). The resulting mixture was subjected to microwave reactor at 110 °C for 10,000 s, cooled to rt, charged with a mixture of boronic acid (1.2 equiv to halogen derivative), 115 mg of K_2CO_3 (1.2 equiv to boronic acid), and 15 mg of $PdCl_2$, and again subjected to microwave reactor at 130 °C for 35 min. The resulting suspension was evaporated to dryness. The residue was adsorbed on silica gel and purified by standard flash chromatography method with MeOH and DCM to give the crude product (bearing protected and unprotected hydroxyls) as a mixture.

(b) Removal of *O*-THP and saponification in one pot: In the case of using *O*-THP alkylating reagents, the crude product was mixed with 150 mg of *p*TSA hydrate in 3 mL of MeOH. The mixture was stirred at rt until TLC indicated disappearance of the protected compound. All volatile components were removed under vacuum. The residue was mixed with 1.2 g of K_2CO_3 and 3 mL of water and subjected to microwave reactor at 130 °C for 35 min. The resulting mixture (at ca. 70–90 °C) was filtered through glass wool, which was washed with boiling aq. K_2CO_3 (10% w/v) for several times. Combined filtrates were cooled to rt and acidified (to pH = 1) with 2 N HCl to give the product **25a–r** as a precipitation, which was collected by filtration, washed with water for several times, and dried at rt overnight.

(c) Removal of *O*-Ac, if applicable, and saponification in one pot: In the case of using *O*-Ac alkylating reagents or alkylating reagent without protected hydroxyls, The residue was directly mixed with 1.0 g of K_2CO_3 and 3 mL of water and subjected to microwave reactor at 130 °C for 35 min. The resulting mixture (at ca. 70–90 °C) was filtered through glass wool, which was washed with boiling aq K_2CO_3 (10% w/v) for several times. Combined filtrates were cooled to rt and acidified (to pH = 1) with 2 N HCl to give the product **25a–r** as a precipitation, which was collected by filtration, washed with water for several times, and dried at rt overnight.

4.2.1. 6-Iodo-4-oxo-quinoline-3-carboxylic acid (25a)

Prepared from saponification of **23**, white solid, yield: 99%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.44 (s, 1H, -NH), 8.88 (s, 1H, H-2), 8.51 (d, J = 2.0 Hz, 1H, H_{aryl}), 8.12 (dd, J = 8.7, 2.0 Hz, 1H, H_{aryl}), 7.58 (d, J = 8.7 Hz, 1H, H_{aryl}). ^{13}C NMR (100 MHz, DMSO-d_6) δ 177.35(C≡O), 166.45(C≡O), 146.05(C-2), 142.34(CH_{aryl}), 139.23($C_{q,aryl}$), 133.77(CH_{aryl}), 126.51($C_{q,aryl}$), 122.28(CH_{aryl}), 108.54($C_{q,aryl}$), 91.97($C_{q,aryl}$); HRMS (ESI-) calcd for C10H5INO3$^-$(M-H$^+$) 313.9320, found 313.9311; HPLC t_R 9.3 min; Mp: 335–340 °C (decomp.).

4.2.2. 1-Benzyl-6-iodo-4-oxo-quinoline-3-carboxylic acid (25b)

Prepared from (1) BnBr and **23**, and (2) saponification, white solid, yield: 61% for two steps. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.26 (s, 1H, H-2), 8.60 (d, J = 2.1 Hz, 1H, H_{aryl}), 8.11 (dd, J = 9.1, 2.1 Hz, 1H, H_{aryl}), 7.62 (d, J = 9.1 Hz, 1H, H_{aryl}), 7.36–7.20 (m, 5H, Bn), 5.83 (s, 2H, Bn). ^{13}C NMR (101 MHz, DMSO-d_6) δ 177.06(C≡O), 166.06(C≡O), 150.92(C-2), 142.53(CH_{aryl}), 139.34($C_{q,aryl}$), 135.61($C_{q,aryl}$), 134.63(CH_{aryl}), 129.40(2C, CH_{Bn}), 128.51(CH_{Bn}), 127.82($C_{q,aryl}$), 127.00(2C, CH_{Bn}), 121.24(CH_{aryl}), 109.04($C_{q,aryl}$), 92.57($C_{q,aryl}$), 56.82(CH_{2Bn}); HRMS (ESI-) calcd for C17H11INO3$^-$(M-H$^+$) 403.9789, found 403.9808; HPLC t_R 13.9 min; Mp: 277–280 °C.

4.2.3. 1-(2-Hydroxyethyl)-6-iodo-4-oxo-quinoline-3-carboxylic acid (25c)

Prepared from (1) $AcOCH_2CH_2Br$ and **23**, and (2) removal of *O*-Ac and saponification, white solid yield: 57% for two steps. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.88 (s, 1H, H-2), 8.60 (d, J = 2.1 Hz, 1H, H_{aryl}), 8.18 (dd, J = 9.0, 2.1 Hz, 1H, H_{aryl}), 7.85 (d, J = 9.0 Hz, 1H, H_{aryl}), 5.00 (br s, 1H, –OH), 4.58 (t, J = 4.8 Hz, 2H, NC$H_2$$CH_2$OH), 3.72 (m, 2H, NCH_2CH_2OH). ^{13}C NMR (101 MHz, DMSO-d_6) δ 176.89(C≡O), 166.21(C≡O), 151.06(C-2), 142.40(CH_{aryl}), 139.35($C_{q,aryl}$), 134.52(CH_{aryl}), 127.60($C_{q,aryl}$), 120.96(CH_{aryl}), 108.08($C_{q,aryl}$), 92.35($C_{q,aryl}$), 58.93(NCH_2CH_2OH), 56.37(NCH_2CH_2OH); HRMS (ESI-) calcd for $C_{12}H_9INO_4^-$(M−H$^+$) 357.9582, found 357.9573; HPLC t_R 9.0 min; Mp:251–254 °C (decomp.).

4.2.4. 6-(Thiophen-2-yl)-4-oxo-quinoline-3-carboxylic acid (25d)

Prepared from (1) corresponding boronic acid and **23**, and (2) saponification, light yellow solid, yield: 39% for two steps. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.44 (s, 1H, NH), 8.84 (s, 1H, H-2), 8.36 (d, J = 2.1 Hz, 1H, H_{aryl}), 8.17 (dd, J = 8.7, 2.1 Hz, 1H, H_{aryl}), 7.82 (d, J = 8.7 Hz, 1H, H_{aryl}), 7.64 (m, 2H, 2x $H_{thiophene}$), 7.17 (dd, J = 5.1, 3.6 Hz, 1H, $H_{thiophene}$). ^{13}C NMR (101 MHz, DMSO-d_6) δ 178.41(C≡O), 166.76(C≡O), 145.43(C-2), 142.11($C_{q,aryl}$), 139.16($C_{q,aryl}$), 132.04($C_{q,aryl}$), 131.53(CH_{aryl}), 129.32($CH_{thiophene}$), 127.36($CH_{thiophene}$), 125.52($CH_{thiophene}$), 125.27($C_{q,aryl}$), 121.25(CH_{aryl}), 120.65(CH_{aryl}), 108.23($C_{q,aryl}$); HRMS (ESI-) calcd for $C_{14}H_8NO_3S^-$(M−H$^+$) 270.0230, found 270.0234; HPLC t_R 10.0 min; Mp: >400 °C.

4.2.5. 1-(2-Hydroxyethyl)-6-(thiophen-2-yl)-4-oxo-quinoline-3-carboxylic acid (25e)

Prepared from (1) $THPOCH_2CH_2Br$ and **24**, (2) corresponding boronic acid, and (3) removal of *O*-THP and saponification, yellow powder, yield: 51% for three steps. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.87 (s, 1H, H-2), 8.35 (d, J = 8.5 Hz, 1H, H_{aryl}), 8.13 (s, 1H, H_{aryl}), 7.90 (d, J = 3.0 Hz, 1H, $H_{thiophene}$), 7.86 (dd, J = 8.5, 1.2 Hz, 1H, H_{aryl}), 7.75 (d, J = 4.3 Hz, 1H, $H_{thiophene}$), 7.24 (dd, J = 4.3, 3.0 Hz, 1H, $H_{thiophene}$), 5.07 (br s, 1H, -OH), 4.70 (t, J = 4.5 Hz, 2H, NC$H_2$$CH_2$OH), 3.80 (br s, 2H, NCH_2CH_2OH). ^{13}C NMR (100 MHz, DMSO-d_6) δ 177.70(C≡O), 166.56(C≡O), 151.20(C-2), 141.72($C_{q,aryl}$), 140.63($C_{q,aryl}$), 139.47($C_{q,aryl}$), 129.41($CH_{thiophene}$), 129.06($CH_{thiophene}$), 127.54(CH_{aryl}), 127.44($CH_{thiophene}$), 124.70($C_{q,aryl}$), 124.17(CH_{aryl}), 113.87(CH_{aryl}), 107.68($C_{q,aryl}$), 59.10(NCH_2CH_2OH), 56.31(NCH_2CH_2OH); HRMS (ESI-) calcd for $C_{16}H_{12}NO_4S^-$(M−H$^+$) 314.0493, found 314.0486; HPLC t_R 10.0 min; Mp: 237–240 °C.

4.2.6. 1-(2-Hydroxyethyl)-6-(4-acetylthiophen-2-yl)-4-oxo-quinoline-3-carboxylic acid (25f)

Prepared from (1) $THPOCH_2CH_2Br$ and **23**, (2) corresponding boronic acid, and (3) removal of *O*-THP and saponification, yellow solid, yield: 81% for three steps. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.86 (s, 1H, H-2), 8.52 (d, J = 2.2 Hz, 1H, H_{aryl}), 8.25 (m, 1H, H_{aryl}), 8.10 (d, J = 9.0 Hz, 1H, H_{aryl}), 7.95 (d, J = 3.9 Hz, 1H, $H_{thiophene}$), 7.81 (d, J = 3.9 Hz, 1H, $H_{thiophene}$), 5.04 (t, J = 5.2 Hz, 1H, -CH_2OH), 4.62 (d, J = 4.6 Hz, 2H, NC$H_2$$CH_2$OH), 3.77 (m, 2H, NCH_2CH_2OH), 2.54 (s, 3H, MeC≡O). ^{13}C NMR (101 MHz, DMSO-d_6) δ 191.07(MeC≡O), 177.90(C≡O), 166.34(C≡O), 150.82(C-2), 149.26 ($C_{q,aryl}$), 144.18($C_{q,aryl}$), 139.89($C_{q,aryl}$), 135.63($CH_{thiophene}$), 131.69 (CH_{aryl}), 130.79($C_{q,aryl}$), 126.94($CH_{thiophene}$), 126.37($C_{q,aryl}$), 122.54 (CH_{aryl}), 119.99(CH_{aryl}), 107.98($C_{q,aryl}$), 59.05(NCH_2CH_2OH), 56.42 (NCH_2CH_2OH), 26.85(*Me*C≡O); HRMS (ESI-) calcd for $C_{18}H_{14}NO_5S^-$(M−H$^+$) 356.0598, found 356.0584; HPLC t_R 10.0 min; Mp: 286–288 °C.

4.2.7. 1-(2-Hydroxyethyl)-6-(benzo[b]thiophen-2-yl)-4-oxo-quinoline-3-carboxylic acid (25g)

Prepared from (1) $THPOCH_2CH_2Br$ and **23**, (2) corresponding boronic acid, and (3) removal of *O*-THP and saponification, yellow powder, yield: 49% for three steps. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.89 (s, 1H, H-2), 8.58 (d, J = 2.3 Hz, 1H, H_{aryl}), 8.36 (dd, J = 9.0, 2.3 Hz, 1H, H_{aryl}), 8.17 (d, J = 9.0 Hz, 1H, H_{aryl}), 8.11 (s, 1H, H_{aryl}), 8.04–7.97 (m, 1H, H_{aryl}), 7.91–7.85 (m, 1H, H_{aryl}), 7.45–7.35 (m, 2H, H_{aryl}), 4.66 (t, J = 5.0 Hz, 2H, NC$H_2$$CH_2$OH), 3.79 (t, J = 5.0 Hz, 2H, NCH_2CH_2OH). ^{13}C NMR (150 MHz, DMSO-d_6) δ 177.21(C≡O), 167.11(C≡O), 165.69($C_{q,aryl}$), 150.00($C_{t,aryl}$), 140.68($C_{q,aryl}$), 140.12($C_{q,aryl}$), 138.89($C_{q,aryl}$), 138.57($C_{q,aryl}$), 131.02($C_{q,aryl}$), 125.71($C_{t,aryl}$), 124.96($C_{t,aryl}$), 124.78($C_{q,aryl}$), 123.86($C_{t,aryl}$), 122.31($C_{t,aryl}$), 121.75($C_{t,aryl}$), 121.68($C_{t,aryl}$), 119.26($C_{t,aryl}$), 107.17($C_{t,aryl}$), 58.29(NCH_2CH_2OH), 55.72(NCH_2CH_2OH); HRMS (ESI-) calcd for $C_{20}H_{14}NO_4S^-$(M−H$^+$) 364.0649, found 364.0648; HPLC t_R 13.2 min; Mp: 238–245 °C (decomp.).

4.2.8. 6-(Furan-2-yl)-4-oxo-quinoline-3-carboxylic acid (25h)

Prepared from (1) $THPOCH_2CH_2Br$ and **23**, (2) corresponding boronic acid, and (3) removal of *O*-THP and saponification, light

grey powder, yield: 47% for three steps. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.45 (s, 1H, –NH), 8.84 (s, 1H, H-2), 8.44 (d, J = 1.9 Hz, 1H, H_{aryl}), 8.16 (dd, J = 8.6, 1.9 Hz, 1H, H_{aryl}), 7.81 (m, 2H, H_{aryl}, H_{furan}), 7.13 (d, J = 3.3 Hz, 1H, H_{furan}), 6.63 (dd, J = 3.3, 1.8 Hz, 1H, H_{furan}). ^{13}C NMR (101 MHz, DMSO-d_6) δ 178.52(C≡O), 166.75(C≡O), 152.05($C_{q,aryl}$), 145.28(C-2), 144.34(CH_{furan}), 138.96 ($C_{q,aryl}$), 129.74(CH_{aryl}), 128.49($C_{q,aryl}$), 125.23($C_{q,aryl}$), 121.04(CH_{aryl}), 118.79(CH_{aryl}), 112.88(CH_{furan}), 108.21($C_{q,aryl}$), 108.05(CH_{furan}); HRMS (ESI-) calcd for $C_{14}H_8NO_4^-$(M−H$^+$) 254.0459, found 254.0468; HPLC t_R 9.7 min; Mp: 300–304 °C (decomp.).

4.2.9. 1-(2-Hydroxyethyl)-6-(furan-2-yl)-4-oxo-quinoline-3-carboxylic acid (25i)

Prepared from (1) THPOCH$_2$CH$_2$Br and **24**, (2) corresponding boronic acid, and (3) removal of *O*-THP and saponification, yellow powder, yield: 60% for three steps. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.86 (s, 1H, H-2), 8.34 (d, J = 8.5 Hz, 1H, H_{aryl}), 8.10 (s, 1H, H_{aryl}), 7.92 (d, J = 8.5 Hz, 1H, H_{aryl}), 7.89 (br s, 1H, H_{furan}), 7.38 (d, J = 3.3 Hz, 1H, H_{furan}), 6.70 (m, 1H, H_{furan}), 5.07 (br s, 1H, -OH), 4.65 (d, J = 4.3 Hz, 2H, NCH_2CH$_2$OH), 3.79 (br s, 2H, NCH$_2$CH_2OH). ^{13}C NMR (100 MHz, DMSO-d_6) δ 177.66(C≡O), 166.56(C≡O), 151.78($C_{q,aryl}$), 151.09(C-2), 145.40(CH_{furan}), 140.59($C_{q,aryl}$), 135.45($C_{q,aryl}$), 127.24(CH_{aryl}), 124.53($C_{q,aryl}$), 121.88(CH_{aryl}), 113.21(CH_{furan}), 111.76(CH_{aryl}), 110.72(CH_{furan}), 107.69($C_{q,aryl}$), 59.03(NCH$_2$*C*H$_2$OH), 56.27(NCH$_2$CH$_2$OH); HRMS (ESI-) calcd for $C_{16}H_{12}NO_5^-$(M−H$^+$) 298.0721, found 298.0729; HPLC t_R 9.9 min; Mp: 260–262 °C.

4.2.10. 1-(2-Hydroxyethyl)-6-(furan-3-yl)-4-oxo-quinoline-3-carboxylic acid (25j)

Prepared from (1) THPOCH$_2$CH$_2$Br and **23**, (2) corresponding boronic acid, and (3) removal of *O*-THP and saponification, yellow powder, yield: 40% for three steps. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.85 (s, 1H, H-2), 8.49 (d, J = 2.1 Hz, 1H, H_{aryl}), 8.43 (dd, J = 1.4, 0.9 Hz, 1H, H_{furan}), 8.17 (dd, J = 9.0, 2.1 Hz, 1H, H_{aryl}), 8.07 (d, J = 9.0 Hz, 1H, H_{aryl}), 7.83–7.78 (m, 1H, H_{furan}), 7.13 (dd, J = 1.9, 0.9 Hz, 1H, H_{furan}), 5.04 (br s, 1H, –CH$_2$OH), 4.63 (t, J = 4.7 Hz, 2H, NCH_2CH$_2$OH), 3.77 (dd, J = 9.2, 4.7 Hz, 2H, NCH$_2$CH_2OH). ^{13}C NMR (101 MHz, DMSO-d_6) δ 178.08(C≡O), 166.65(C≡O), 150.29(C-2), 145.28(CH_{furan}), 141.19(CH_{furan}), 138.73($C_{q,aryl}$), 132.00(CH_{aryl}), 130.54($C_{q,aryl}$), 126.44($C_{q,aryl}$), 124.79($C_{q,aryl}$), 121.75(CH_{aryl}), 119.44(CH_{aryl}), 109.08(CH_{furan}), 107.54($C_{q,aryl}$), 59.01(NCH$_2$CH$_2$OH), 56.42(NCH$_2$CH$_2$OH); HRMS (ESI-) calcd for $C_{16}H_{12}NO_5^-$(M−H$^+$) 298.0721, found 298.0710; HPLC t_R 10.0 min; Mp: 193–194 °C.

4.2.11. 1-(2-Hydroxyethyl)-6-(benzo[B]furan-2-yl)-4-oxo-quinoline-3-carboxylic acid (25k)

Prepared from (1) THPOCH$_2$CH$_2$Br and **24**, (2) corresponding boronic acid, and (3) removal of *O*-THP and saponification, yellow powder, yield: 13% for three steps. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.93 (s, 1H, H-2), 8.47 (d, J = 8.5 Hz, 1H, H_{aryl}), 8.40 (*pseudo*-s, 1H, H_{aryl}), 8.17 (dd, J = 8.5, 1.3 Hz, 1H, H_{aryl}), 7.91 (s, 1H, $H_{benzofuran}$), 7.75 (d, J = 7.6 Hz, 1H, $H_{benzofuran}$), 7.70 (d, J = 8.2 Hz, 1H, $H_{benzofuran}$), 7.44–7.38 (m, 1H, $H_{benzofuran}$), 7.31 (t, J = 7.6 Hz, 1H, $H_{benzofuran}$), 5.08 (br s, 1H, -OH), 4.74 (t, J = 4.9 Hz, 2H, NCH_2CH$_2$OH), 3.83 (t, J = 4.9 Hz, 2H, NCH$_2$CH_2OH). ^{13}C NMR (101 MHz, DMSO-d_6) δ 177.71(C≡O), 166.48(C≡O), 155.18($C_{q,aryl}$), 153.74($C_{q,aryl}$), 151.34 (C-2), 140.58($C_{q,aryl}$), 135.10($C_{q,aryl}$), 128.89($C_{q,aryl}$), 127.39(CH_{aryl}), 126.34($CH_{benzofuran}$), 125.53($C_{q,aryl}$), 124.12($CH_{benzofuran}$), 122.77 (CH_{aryl}), 122.29($CH_{benzofuran}$), 113.69(CH_{aryl}), 111.91($CH_{benzofuran}$), 107.88 $C_{q,aryl}$), 106.70($CH_{benzofuran}$), 59.13(NCH$_2$*C*H$_2$OH), 56.32 (NCH$_2$CH$_2$OH); HRMS (ESI-) calcd for C20H14NO5$^-$(M-H$^+$) 348.0877, found 348.0869; HPLC t_R 12.5 min; Mp: 346–355 °C (decomp.).

4.2.12. 1-(3-Hydroxypropyl)-6-(benzo[B]furan-2-yl)-4-oxo-quinoline-3-carboxylic acid (25l)

Prepared from (1) THPOCH$_2$CH$_2$Br and **23**, (2) corresponding boronic acid, and (3) removal of *O*-THP and saponification, yellow powder, yield: 48% for three steps. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.99 (s, 1H, H-2), 8.77 (d, J = 1.4 Hz, 1H, $H_{benzofuran}$), 8.46 (d, J = 7.9 Hz, 1H, H_{aryl}), 8.15 (d, J = 9.0 Hz, 1H, H_{aryl}), 7.69 (br s, 3H, 2 × $H_{benzofuran}$, 1xH_{aryl}), 7.37 (t, J = 7.6 Hz, 1H, $H_{benzofuran}$), 7.29 (t, J = 7.4 Hz, 1H, $H_{benzofuran}$), 4.75 (br s, 1H, -OH), 4.64 (t, J = 6.9 Hz, 2H, NCH_2CH$_2$CH$_2$OH), 3.48 (br s, 2H, NCH$_2$CH$_2$CH_2OH), 2.07–1.91 (m, 2H, NCH$_2$CH_2CH$_2$OH). ^{13}C NMR (100 MHz, DMSO-d_6) δ 177.91(C≡O), 166.29(C≡O), 154.91($C_{q,aryl}$), 153.72($C_{q,aryl}$), 150.00 (C-2), 139.49($C_{q,aryl}$), 130.74(CH_{aryl}), 129.04($C_{q,aryl}$), 127.78($C_{q,aryl}$), 126.34($C_{q,aryl}$), 125.67($CH_{benzofuran}$), 123.88($CH_{benzofuran}$), 121.88 (CH_{aryl}), 121.15($CH_{benzofuran}$), 119.59(CH_{aryl}), 111.72(CH_{aryl}), 108.34 ($C_{q,aryl}$), 104.39(CH_{aryl}), 57.84(NCH$_2$CH$_2$*C*H$_2$OH), 51.74 (NCH$_2$CH$_2$CH$_2$OH), 31.88(NCH$_2$*C*H$_2$CH$_2$OH); HRMS (ESI-) calcd for $C_{21}H_{16}NO_5^-$(M−H$^+$) 362.1034, found 362.1025; HPLC t_R 11.3 min; Mp: 211–214 °C.

4.2.13. 1-Propyl-6-(benzo[B]furan-2-yl)-4-oxo-quinoline-3-carboxylic acid (25bm)

Prepared from (1) CH$_3$CH$_2$CH$_2$Br and **23**, (2) corresponding boronic acid, and (3) saponification, light grey powder, yield: 52% for three steps. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.94 (s, 1H, H-2), 8.77 (d, J = 1.9 Hz, 1H, $H_{benzofuran}$), 8.37 (dd, J = 9.0, 2.0 Hz, 1H, H_{aryl}), 8.08 (d, J = 9.0 Hz, 1H, H_{aryl}), 7.66 (dd, J = 10.8, 8.2 Hz, 2H, H_{aryl}, $H_{benzofuran}$), 7.56 (s, 1H, $H_{benzofuran}$), 7.35 (t, J = 7.3 Hz, 1H, $H_{benzofuran}$), 7.27 (t, J = 7.4 Hz, 1H, $H_{benzofuran}$), 4.52 (t, J = 7.3 Hz, 2H, NCH_2CH$_2$CH$_3$), 3.03 (s, 1H, –OH), 1.97–1.81 (m, 2H, NCH$_2$CH_2CH$_3$), 0.96 (t, J = 7.3 Hz, 3H, NCH$_2$CH$_2$CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.01(C≡O), 166.05(C≡O), 155.19($C_{q,aryl}$), 153.95($C_{q,aryl}$), 149.67 (C-2), 139.71($C_{q,aryl}$), 130.73(CH_{aryl}), 129.20($C_{q,aryl}$), 128.03($C_{q,aryl}$), 126.64($C_{q,aryl}$), 125.60($CH_{benzofuran}$), 123.81($CH_{benzofuran}$), 121.86 (CH_{aryl}), 121.65($CH_{benzofuran}$), 119.50(CH_{aryl}), 111.64(CH_{aryl}), 108.73 ($C_{q,aryl}$), 104.30($CH_{benzofuran}$), 55.58(NCH$_2$CH$_2$CH$_3$), 22.53 (NCH$_2$CH$_2$CH$_3$), 10.86(NCH$_2$CH$_2$CH$_3$); HRMS (ESI-) calcd for $C_{21}H_{16}NO_4^-$(M−H$^+$) 346.1085, found 346.1083; HPLC t_R 15.6 min; Mp: 250–254 °C.

4.2.14. 1-(2-Hydroxyethyl)-6-(*p*-methoxyphenyl)-4-oxo-quinoline-3-carboxylic acid (25n)

Prepared from (1) THPOCH$_2$CH$_2$Br and **23**, (2) corresponding boronic acid, and (3) removal of *O*-THP and saponification, white solid, yield: 67% for three steps. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.88 (s, 1H, H-2), 8.54 (d, J = 2.1 Hz, 1H, H_{aryl}), 8.23 (dd, J = 8.9, 2.1 Hz, 1H, H_{aryl}), 8.11 (d, J = 8.9 Hz, 1H, H_{aryl}), 7.42 (m, 1H, $H_{methoxyphenyl}$), 7.33 (m, 1H, $H_{methoxyphenyl}$), 7.30–7.26 (m, 1H, $H_{methoxyphenyl}$), 6.99 (ddd, J = 8.2, 2.5, 0.8 Hz, 1H, $H_{methoxyphenyl}$), 5.05 (t, J = 5.5 Hz, 1H, -OH), 4.70–4.60 (m, 2H, NCH_2CH$_2$OH), 3.83 (d, J = 2.8 Hz, 3H, OMe), 3.81–3.74 (m, 2H, NCH$_2$CH_2OH). ^{13}C NMR (101 MHz, DMSO-d_6) δ 178.22(C≡O), 166.59(C≡O), 160.35($C_{q,aryl}$), 150.62(C-2), 140.06($C_{q,aryl}$), 139.25($C_{q,aryl}$), 138.11($C_{q,aryl}$), 133.07(CH_{aryl}), 130.77($CH_{methoxyphenyl}$), 126.29($C_{q,aryl}$), 123.43(CH_{aryl}), 119.71 ($CH_{methoxyphenyl}$), 119.46(CH_{aryl}), 114.32($CH_{methoxyphenyl}$), 112.85 ($CH_{methoxyphenyl}$), 107.64($C_{q,aryl}$), 59.00(NCH$_2$*C*H$_2$OH), 56.43 (NCH$_2$CH$_2$OH), 55.68(OMe); HRMS (ESI-) calcd for $C_{19}H_{16}NO_5^-$(M−H$^+$) 338.1034, found 338.1039; HPLC t_R 13.4 min; Mp: 202–203 °C.

4.2.15. 1-(2-Hydroxyethyl)-7-(thiphen-2-yl)-4-oxo-quinoline-3-carboxylic acid (25o)

Prepared from (1) AcOCH$_2$CH$_2$Br and **23**, (2) corresponding boronic acid, and (3) removal of *O*-Ac and saponification, yellow powder, yield: 11% for three steps. ^{1}H NMR (400 MHz, DMSO-d_6)

δ 8.86 (s, 1H, H-2), 8.48 (d, J = 2.1 Hz, 1H, H_{aryl}), 8.23 (m, 1H, H_{aryl}), 8.09 (m, 1H, H_{aryl}), 7.73 (m, 1H, $H_{thiophene}$), 7.65 (dd, J = 5.0, 0.8 Hz, 1H, $H_{thiophene}$), 7.18 (dt, J = 13.8, 6.9 Hz, 1H, $H_{thiophene}$), 5.04 (t, J = 5.3 Hz, 1H, -OH), 4.63 (br s, 2H, NCH_2CH_2OH), 3.77 (d, J = 4.2 Hz, 2H, NCH_2CH_2OH). ^{13}C NMR (101 MHz, DMSO-d_6) δ 177.96(C≡O), 166.50(C≡O), 150.49(C-2), 141.66($C_{q,aryl}$), 139.01($C_{q,aryl}$), 132.08($C_{q,aryl}$), 131.47(CH_{aryl}), 129.40($CH_{thiophene}$), 127.63($CH_{thiophene}$), 126.47($C_{q,aryl}$), 125.88($CH_{thiophene}$), 121.42 (CH_{aryl}), 119.84(CH_{aryl}), 107.71($C_{q,aryl}$), 59.02(NCH_2CH_2OH), 56.45 (NCH_2CH_2OH); HRMS (ESI-) calcd for $C_{16}H_{12}NO_4S^-$(M−H$^+$) 314.0493, found 314.0499; HPLC t_R 9.7 min; Mp: 209–211 °C.

4.2.16. 1-(3-Hydroxypropyl)-7-(thiophen-2-yl)-4-oxo-quinoline-3-carboxylic acid (25p)

Prepared from (1) $THPOCH_2CH_2Br$ and **24**, (2) corresponding boronic acid, and (3) removal of *O*-THP and saponification, yellow powder, yield: 47% for three steps. ^1H NMR (400 MHz, DMSO-d_6) δ 8.96 (s, 1H, H-2), 8.32 (d, J = 8.5 Hz, 1H, H_{aryl}), 8.12 (d, J = 1.2 Hz, 1H, H_{aryl}), 7.89–7.82 (m, 2H, H_{aryl}, $H_{thiophene}$), 7.74 (dd, J = 5.0, 1.0 Hz, 1H, $H_{thiophene}$), 7.23 (dd, J = 5.0, 3.7 Hz, 1H, $H_{thiophene}$), 4.82 (t, J = 4.6 Hz, 1H, –OH), 4.66 (t, J = 7.0 Hz, 2H, $NCH_2CH_2CH_2OH$), 3.47 (dd, J = 10.1, 5.4 Hz, 2H, $NCH_2CH_2CH_2OH$), 2.05–1.88 (m, 2H, $NCH_2CH_2CH_2OH$). ^{13}C NMR (100 MHz, DMSO-d_6) δ 177.60(C≡O), 166.43(C≡O), 150.53 (C-2), 141.71($C_{q,aryl}$), 140.37($C_{q,aryl}$), 139.53($C_{q,aryl}$), 129.44 ($CH_{thiophene}$), 129.07($CH_{thiophene}$), 127.48(CH_{aryl}), 127.38(CH_{aryl}), 124.67($C_{q,aryl}$), 124.10($CH_{thiophene}$), 113.74(CH_{aryl}), 108.10($C_{q,aryl}$), 57.82($NCH_2CH_2CH_2OH$), 51.41($NCH_2CH_2CH_2OH$), 31.87 ($NCH_2CH_2CH_2OH$); HRMS (ESI-) calcd for $_{C17}H_{14}NO_4S^-$(M−H$^+$) 328.0649, found 328.0638; HPLC t_R 11.4 min; Mp: 192–195 °C (decomp.).

4.2.17. 1-(2-Hydroxyethyl)-7-(furan-2-yl)-4-oxo-quinoline-3-carboxylic acid (25q)

Prepared from (1) $AcOCH_2CH_2Br$ and **23**, (2) corresponding boronic acid, and (3) removal of *O*-Ac and saponification, yellow powder, yield: 63% for three steps. ^1H NMR (400 MHz, DMSO-d_6) δ 8.85 (s, 1H, H-2), 8.55 (d, J = 2.1 Hz, 1H, H_{aryl}), 8.22 (dd, J = 9.0, 2.1 Hz, 1H, H_{aryl}), 8.09 (d, J = 9.1 Hz, 1H, H_{aryl}), 7.84 (dd, J = 1.7, 0.6 Hz, 1H, H_{furan}), 7.21 (d, J = 3.2 Hz, 1H, H_{furan}), 6.65 (dd, J = 3.2, 1.7 Hz, 1H, H_{furan}), 5.05 (br s, 1H, –CH_2OH), 4.62 (t, J = 4.8 Hz, 2H, NCH_2CH_2OH), 3.77 (br s, 2H, NCH_2CH_2OH). ^{13}C NMR (101 MHz, DMSO-d_6) δ 178.03(C≡O), 166.54(C≡O), 151.75($C_{q,aryl}$), 150.41 (C-2), 144.52(CH_{furan}), 138.87($C_{q,aryl}$), 129.71(CH_{aryl}), 128.46($C_{q,aryl}$), 126.44($C_{q,aryl}$), 119.67(CH_{aryl}), 119.52(CH_{aryl}), 112.94(CH_{furan}), 108.44(CH_{furan}), 107.68($C_{q,aryl}$), 59.01(NCH_2CH_2OH), 56.45 (NCH_2CH_2OH); HRMS (ESI-) calcd for $C_{16}H_{12}NO_5^-$(M−H$^+$) 298.0721, found 298.0712; HPLC t_R 10.3 min; Mp: 233–235 °C.

4.2.18. 1-(2-Hydroxyethyl)-7-(benzo[B]furan-2-yl)-4-oxo-quinoline-3-carboxylic acid (25r)

Prepared from (1) $AcOCH_2CH_2Br$ and **23**, (2) corresponding boronic acid, and (3) removal of *O*-Ac and saponification, yellow powder, yield: 23% for three steps. ^1H NMR (400 MHz, DMSO-d_6) δ 8.90 (s, 1H, H-2), 8.78 (d, J = 2.1 Hz, 1H, H_{aryl}), 8.43 (dd, J = 9.1, 2.2 Hz, 1H, H_{aryl}), 8.18 (d, J = 9.1 Hz, 1H, H_{aryl}), 7.75–7.65 (m, 3H, 3x$H_{benzofuran}$), 7.42–7.34 (m, 1H, $H_{benzofuran}$), 7.30 (m, 1H, $H_{benzofuran}$), 5.09 (br s, 1H, -OH), 4.67 (t, J = 4.8 Hz, 2H, NCH_2CH_2OH), 3.81 (br s, 2H, NCH_2CH_2OH).^{13}C NMR (101 MHz, DMSO-d_6) δ 178.04(C≡O), 166.43(C≡O), 154.94($C_{q,aryl}$), 153.76($C_{q,aryl}$), 150.71(C-2), 139.75 ($C_{q,aryl}$), 130.64(CH_{aryl}), 129.08($C_{q,aryl}$), 127.81($C_{q,aryl}$), 126.38($C_{q,aryl}$), 125.71($CH_{benzofuran}$), 123.92($CH_{benzofuran}$), 121.92($CH_{benzofuran}$), 121.12 (CH_{aryl}), 119.83(CH_{aryl}), 111.75($CH_{benzofuran}$), 107.95($C_{q,aryl}$), 104.46 ($CH_{benzofuran}$), 59.04(NCH_2CH_2OH), 56.48 (NCH_2CH_2OH); HRMS (ESI-) calcd for $C_{20}H_{14}NO_5^-$(M−H$^+$) 348.0877, found 348.0881; HPLC t_R 12.6 min; Mp: 247–251 °C (decomp.).

4.3. Biology

4.3.1. Cells and recombinant protein

The HCV genotype 1b replicon-containing cells, Huh-7/HCV1b-Rluc cells (Y-L Chen, et al, Bioorg Med Chem, 2012) (obtained from G. Luo, University of Kentucky), were maintained in DME supplemented with 10% fetal bovine serum, 500 μgm/ml G418 (Invitrogen), 100 IU streptomycin and 100 IU penicillin, 1× non-essential amino acids (Invitrogen), and 1 mM sodium pyruvate (Invitrogen). The HCV genotype 1b NS5B polymerase missing the C-terminal 55 amino acids (NS5BΔ55) was expressed from the plasmid pET-21d(+)-NS5BΔ55 and purified, excluding the heparin-agarose chromatography, from bacteria as described.[57] The enzyme was stored at −80 °C in storage buffer (50 mM Tris pH 7.5, 50% glycerol, 1 mM β-ME).

4.3.2. HCV replicon assay

Approximately 6×10^3 replicon-containg cells per well were plated in an opaque 96-well tissue culture plate (BD Falcon) in the absence of G418. Cells were exposed to culture medium containing compound (dissolved in DMSO), DMSO alone, or nothing added the next day and incubated at 37 °C/5% CO_2 for three days. The culture medium was removed and renilla luciferase activity measured using ViVi-Ren Live Cell Substrate (Promega) as described.[44] Compounds and controls were performed in triplicate and each experiment repeated independently at least twice. For the intial 10 μM compound evaluation, ribavirin (10 μM) and 2′-C-Me-A (0.5 μM) were included as controls. The concentration of compound that reduced luciferase activity by 50% was defined as the 50% effective concentration (EC_{50}). The EC_{50} was determined by comparing luciferase activity for eight serial dilutions of compound and vehicle treated cells using GraphPad Prism software.

4.3.3. Cell proliferation assay

Approximately 6×10^3 replicon-containg cells per well were plated in a clear 96-well tissue culture plate (Corning) in the absence of G418. Cells were exposed to culture medium containing compound (dissolved in DMSO), DMSO alone, or nothing added the next day and incubated at 37 °C/5% CO_2 for three days. CellTiter 96 AQ_{ueous} One Solution Cell Proliferation reagent (Promega) was added and measured according to manufacturer's instructions by spectrometry at 450 nm with a SpectraMax E5 (Molecular Devices). Compounds and controls were performed in triplicate and each experiment repeated independently at least twice. For the intial 10 μM compound evaluation, ribavirin (10 μM) and 2′-C-Me-A (0.5 μM) were included as controls. The concentration of compound that reduced cell proliferation by 50% was defined as the 50% cytotoxic concentration (CC_{50}). The CC_{50} was determined by comparing absorbance readings from eight serial dilutions of compound and vehicle treated cells using GraphPad Prism software.

4.3.4. RT-qPCR Assay

Approximately 6×10^3 replicon-containg cells per well were plated in a clear 96-well tissue culture plate (Corning) in the absence of G418. Cells were exposed to culture medium containing compound (dissolved in DMSO) or DMSO alone and incubated at 37 °C/5% CO_2 for three days. The relative level of replicon RNA for compound-treated cells compared to DMSO-treated cells was measured using the TaqMan® Gene Expression Cells-to-CT system (Applied Biosystems) and TaqMan® primers/probes as per manufacturers instructions. The cells were lysed in the wells and stored at -80 °C. The primers/probes to measure HCV replicon cDNA were custom synthesized to NS5B (Applied Biosystems), LC609-NS5F 5′ CCCCACATTCGGCCAGATC, LC609-NSR 5′ GATAGGTTCCGGACGTCCTTTG, FAM probe 5′ CCCCATAGCCAAATTT. The control primers/probe recognize GAPDH cDNA (Applied

Biosystems, catalog number 402869). The samples were analyzed in triplicate per experiment and the experiment performed two independent times. Samples were processed using a Mastercycler ep *realplex*^{2} gradient S (Eppendorf) with cycling parameters of 50 °C for 2 min, 95 °C for ten min, 95 °C for 15 s, 60 °C for 1 min, repeat latter two steps 39 more times. Parallel wells of lysate were analyzed for either HCV replicon RNA or GAPDH RNA and relative levels of HCV RNA calculated using the comparative method.[53] The PCR efficiency for the two sets of primers/probe was determined using 5-fold serial dilutions (over 3 logs) of lysate from replicon cells as described[53] and was 100% (R^2 0.999) and 110% (R^2 0.997) for NS5B primers/probe and GAPDH primers/probe, respectively.

4.3.5. HCV NS5B assay

Primer-dependent NS5BΔ55 polymerase activity was measured using a scintillation proximity assay (SPA) as described.[44] Briefly, purified NS5BΔ55 (2 nM) was added to Mix 1 (20 mM Tris pH 7.5, 50 mM NaCl, 5 mM KCl, 0.5 mM $MnCl_2$, 4U RNasin (Promega), 1 mM DTT, 1 μL compound in DMSO or 1 μL DMSO alone) and Mix 2 was added (450tnM Oligo-U12 primer, 30 nM UTP, 75 nM Poly(A) template, 0.2 μi [^{3}H]UTP). The 41 μL reaction was incubated in 0.7 mL tubes at room temperature for 3 h and stopped by adding 350 μL of 1.2 mg streptavidin-coated SPA beads (Perkin-Elmer) in 0.15 M EDTA. The samples were analyzed immediately for 1 min in a scintillation counter. Each compound concentration was performed in duplicate and repeated at least two independent times. (see Scheme 1)

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.05.066.

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