New 1-Phenyl-5-(1*H*-pyrrol-1-yl)-1*H*-pyrazole-3-carboxamides Inhibit Hepatitis C Virus Replication via Suppression of Cyclooxygenase-2

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ABSTRACT

We report here the synthesis and mechanism of inhibition of pyrazolecarboxamide derivatives as a new class of HCV inhibitors. Compounds **5**, **7**, **11**, and **12** inhibited the subgenomic HCV replicon 1b genotype at EC₅₀ values between 5 to 8 μM and displayed an even higher potency against the infectious Jc1 HCV 2a genotype. Compound **12** exhibited an EC₅₀ of 6.7 μM and selectivity index of 23 against HCV 1b, and reduced the RNA copies of the infectious Jc1 chimeric 2a clone by 82% at 7 μM. Evaluation of the mode of anti-HCV activity of **12** revealed that it suppressed HCV-induced COX-2 mRNA and protein expression, displaying an IC₅₀ of 3.2 μM in COX-2 promoter-linked luciferase reporter assay. Conversely, the anti-HCV activity of **12** was abrogated upon over-expression of COX-2. These findings suggest that these pyrazolecarboxamides function as anti-HCV agents via targeting COX-2 at both the transcription and translation levels.

INTRODUCTION

Hepatitis C virus (HCV), a single stranded positive sense RNA virus, is a causative agent of hepatitis C, a disease that affects 3% of the world population, an estimated 200 million people worldwide. The global incidence of HCV infection is underestimated as the acute infection is generally asymptomatic. Eighty percent of HCV infections lead to chronic liver disease that have the potential to evolve into fibrosis, cirrhosis and hepatocellular carcinoma.¹⁻³ Both prophylaxis of HCV infection and vaccine development have been quite unsuccessful so far.⁴

Treatment of HCV infections with peg-IFN, wherein INF- α is covalently bound to polyethylene glycol, in combination with the synthetic guanosine analogue Ribavirin, has demonstrated severe adverse effects in a large number of patients. The sustained viral response rate to this treatment was also very low in patients infected with HCV genotype 1, the predominant genotype in the western countries.^{5,6} In 2011, the FDA approved the first direct-acting antiviral agents (DAAs) Boceprevir and Telaprevir for the treatment of chronic hepatitis C genotype 1 infection in combination with pegIFN-2 α or -2 β , and Ribavirin.^{7,8} However, despite improved response rates, combination treatments including either Boceprevir or Telaprevir show the rapid selection of drug-resistant viral mutants,⁹ limited antiviral efficacy in patients with HCV genotypes 2-6 infections and long-term toxic effects.^{8,10} Therefore, new HCV agents are under investigation towards either non-structural proteins or host factors.

We have developed a reference model for the anti-HCV agents (Chart 1). Consistent with this model, a built-in 5-membered heterocyclic core (A) sustains two nearby cyclic substituents (B, C), and a short linker (D), also represented by an aromatic ring, bearing an optional non-hydrophobic group (E). In continuation of our studies on pyrazole agents,¹¹ we observed that *N*,1-diphenyl-5-(1*H*-pyrrol-1-yl)-1*H*-pyrazole-3-carboxamide (**4**), a pyrazolecarboxamide previously synthesized by us, as well as some known HCV inhibitors (i.e. **1**,¹² **2**¹³ and **3**¹⁴), well matched the requirements of this reference model.

Chart 1. Reference Model and Structures of Compounds 1-21.^a

^a4-21: R₁ = H, 4-Cl; R₂ = H, 4-Cl, 4,3-Cl₂; R₃ = H, CN, Me.

In a preliminary screening, compound **4** showed significant inhibition of the HCV replicon 1b (Con 1 strain) in Huh 5-2 cells with an EC₅₀ of 31.7 μ M (Table 1), and it was predicted to have drug-like properties by QikProp software (Table 2). These results prompted the synthesis of the new 1-phenyl-5-(1*H*-pyrrol-1-yl)pyrazolecarboxamides **5-21**, in order to analyse the potential for productive modifications of **4**, in a quest to discover new potent anti-HCV agents.

Scheme 1. Synthesis of pyrazole-3-carboxamides 4-19 and pyrazole-4-carboxamides 20,21.^{a,b}

^aCarboxamides **4-21**, see Table 1 for R₁, R₂ and R₃. Carboxylic acids: **22**, R₁ = R₃ = H; **23**, R₁ = H, R₃ = CN; **24**, R₁ = 4-Cl, R₃ = H; **25**, R₁ = 4-Cl, R₃ = CN; **26**, R₁ = H, R₃ = Me; **27**, R₁ = 4-Cl, R₃ = Me; **28**, R₁ = 4-Cl, R₃ = H.

^bReagents and conditions. (a): (**5-19**) (i) 1,1'-carbonyldiimidazole, anhydrous THF, 25 °C, 3 h; (ii) aniline (**6,10,12,14**), 4-chloroaniline (**5,7,9,11,13,15**) or 3,4-dichloroaniline (**16-19**), 25 °C, 24 h, 10-83%; (b): (**4, 20, 21**) aniline (**4, 20**), 4-chloroaniline (**21**) BOP reagent, triethylamine, anhydrous DMF, 25 °C, 12 h, 13-70%.

CHEMISTRY

Carboxamides **5-19** were obtained by reaction of carboxylic acids **22-27** with 1,1'-carbonyldiimidazole in anhydrous THF at 25 °C, and subsequent treatment of the imidazolide intermediates with appropriate anilines (Scheme 1). Carboxamides **4, 20** and **21** were obtained by reaction of acids **22** or **28** and with the appropriate anilines in the presence of BOP reagent and triethylamine in anhydrous DMF at 25 °C. The corresponding carboxylic acids, **22-27**,^{11c} and **28**¹⁵ were synthesized by following our earlier reported procedures.

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Table 1. Anti-HCV activities of compound 4-21.

Compd	R ₁	R ₂	R ₃	Replicon 1b, Con 1 strain, Huh 5-2 Cells (μM) ^a			
				CC ₅₀ ^b	EC ₅₀ ^c	EC ₉₀ ^d	SI ^e
4	H	H	H	>228	31.7 ± 7.3	nd ^f	>3.1
5	H	4-Cl	H	120.2	5.9 ± 1.3	nd	20.4
6	H	H	CN	>247.6	39.6 ± 7.6	>241	>6.25
7	H	4-Cl	CN	137.7	7.8 ± 0.3	nd	17.6
8	4-Cl	H	H	>241.1	26.9 ± 13.1	>206.7	>8.9
9	4-Cl	4-Cl	H	>220.3	35.4 ± 6.7	170.9	>6.2
10	4-Cl	H	CN	202.5	26.2 ± 6.5	nd	7.7
11	4-Cl	4-Cl	CN	151.7	5.5 ± 1.1	129.5	27.3
12	H	H	Me	>153.8	6.7 ± 1.7	nd	23
13	H	4-Cl	Me	193.7	16.5 ± 6.0	61.8	11.7
14	4-Cl	H	Me	>232.2	16.7 ± 2.2	57.1	13.8
15	4-Cl	4-Cl	Me	>160.1	13.6 ± 2.4	nd	>11.8
16	H	3,4-Cl ₂	H	>220.3	39.8 ± 3.2	nd	>5.5
17	4-Cl	3,4-Cl ₂	H	>202.7	>159.0	nd	>1.3
18	H	3,4-Cl ₂	CN	>207.2	108.1 ± 13.4	nd	>1.9
19	4-Cl	3,4-Cl ₂	CN	>257.3	50.5 ± 1.5	nd	5.1
20	4-Cl	H	H	>241.2	>241.2	nd	nd
21	4-Cl	4-Cl	H	62.6	13.7 ± 0.9	nd	4.7

^aThe data represent an average of at least two independent measurements in duplicate ± SD. ^bCC₅₀: concentration of compound that reduces the overall cellular metabolic activity by 50% (MTS-based assay). ^cEC₅₀, ^dEC₉₀: effective concentration of compound that inhibits the virus replication by 50% or 90%, respectively (luciferase-based assay). ^eSI: selectivity index, CC₅₀/EC₅₀ ratio. ^fnd: not determined.

RESULTS AND DISCUSSION

Inhibition of HCV Replicon 1b in Huh 5-2 Cells. Compared to **4**, compound **5** ($EC_{50} = 5.9$ μ M) bearing a chlorine atom at position 4 of the 3-(phenylaminocarbonyl)pyrazole moiety, yielded a 5.4-fold improvement of the antiviral activity. Similarly, the 3-[(4-chlorophenyl)aminocarbonyl]-4-cyanopyrazole derivative **7** ($EC_{50} = 7.8$ μ M) was 5.1-fold more potent than the parent compound **6** ($EC_{50} = 39.6$ μ M). In contrast, when a 3,4-dichlorophenyl moiety was introduced (compounds **16-19**), a reduction in the antiviral activity was observed. Introduction of a chlorine atom at the pendant 1-phenyl group of **7**, to give the corresponding 1-(4-chlorophenyl)pyrazole derivative **11** resulted in a strong replicon inhibition with $EC_{50} = 5.5$ μ M.

Replacement of the cyano substituent at position 4 of the pyrazole with the methyl group (**12**, $EC_{50} = 6.7$ μ M), yielded 5.9- and 4.7-fold improvements in the antiviral activity relative to **6** and **4**, respectively. Furthermore, when the phenylaminocarbonyl moiety was shifted from position 3 to 4 of the pyrazole nucleus, a dramatic reduction (>9-fold) of the HCV replicon activity was observed (compare **20** with **8**). However, the inhibitory activity was restored when the (4-chlorophenyl)aminocarbonyl moiety was placed at position 4 of the pyrazole (**21**, $EC_{50} = 13.7$ μ M).

In summary, the most pronounced antiviral activity was obtained when a chlorine atom was introduced at position 4 of the phenylaminocarbonyl moiety (compounds **5**, **7**, **11**). In comparing position 3 with 4 of the pyrazole, the phenylaminocarbonyl moiety at position 3 resulted in higher selectivity (compare **8** and **9** with **20** and **21**). Compounds **5**, **7**, **11** and **12** were the most potent inhibitors of the HCV replicon within this series with inhibitory concentrations (EC_{50} values) in the lowest micromolar range of concentration. The most active derivative **11** also elicited the highest selectivity index (**11**: $EC_{50} = 5.5$ μ M; SI = 27.3).

Figure 1. Antiviral activity of compounds **4**, **5** and **12** on HCV Jc1 infected cells. Huh 7.5 cells were infected at 1 m.o.i. in 12 wells plates. Six h post-infection, cells were treated for 72 h with either DMSO (control) or compounds **4** (10 μ M), **5** (6 μ M) or **12** (7 μ M). Cells were harvested and processed for total RNA isolation and one step qRT-PCR. * $p < 0.05$; ** $p < 0.01$.

Inhibition of HCV-Jc1 virus infected Huh 7.5 cells. We investigated the antiviral activity of three representative compounds, **4**, **5** and **12**, in HCV infected Huh 7.5 cells, using the most advanced HCV infectious system based on RNA transfection and subsequent infection of Huh7.5 cell line with HCV genotype 2a infectious chimeric clone Jc1.¹⁶ In the Jc1 system, the compounds were initially screened at the EC₅₀ values obtained in Huh 5-2 replicon 1b cells. Compounds **4**, **5** and **12** at concentrations of 10 μ M, 6 μ M and 7 μ M, respectively, reduced the intracellular RNA copies of HCV genome by 70%, 68% and 82% (Figure 1), thus exhibiting higher potency in the Jc1 system than in the replicon 1b cells. These results suggest that treatment with compound **4** as well as with its analogues **5** and **12** effectively inhibited HCV infection in vitro.

Inhibition of HCV NS5B RdRp. To investigate whether the anti-HCV activity of compounds **4-21** is mediated through targeting HCV NS5B, we evaluated NS5B RdRp activity by the standard

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3 primer-dependent elongation reaction employing poly rA/rU₁₂ template-primer and recombinant HCV
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5 NS5BCΔ21.¹⁷ However, either no inhibition or considerably poor inhibition (data not shown) of RdRp
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7 activity of NS5B was observed, suggesting that the observed anti-HCV effect was not due to the direct
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9 inhibition of HCV NS5B.
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12 **HCV IRES Activity.** We next investigated whether the anti-HCV effects of these compounds
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14 could be correlated to down regulation of HCV IRES activity. Huh 7.5 cells were transfected with the
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16 pCIneo-Rluc-IRES-Fluc reporter plasmid,¹⁸ in which Rluc was translated in a cap-dependent manner
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18 and Fluc was translated via HCV IRES-mediated initiation, and treated with increasing concentrations
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20 of compound **12**, as a representative of this series. The results (data not shown) clearly indicated that **12**
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22 had no effect on the HCV IRES mediated translation, thus ruling out interference with this mechanism
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24 of action.
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29 **HCV modulates COX-2 expression.** HCV has been reported to stimulate COX-2 expres-
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31 sion.¹⁹ We therefore investigated the levels of COX-2 in MH-14 cells, which autonomously replicate
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33 the HCV subgenomic replicon 1b.²⁰ In agreement with previous findings, we noted >5-fold upregula-
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35 tion of COX-2 promoter-linked luciferase reporter activity in MH-14 cells relative to their parental
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37 counterparts Huh-7 and Huh-7.5 cells (Figure 2A). Concomitantly, the COX-2 mRNA and protein lev-
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39 els were quantitatively increased by ≥4-fold in MH-14 cells compared to Huh-7.5 cells (Figures 2B and
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41 2C). This data supports the up-regulation of COX-2 by HCV.
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Figure 2. HCV induces COX-2 expression. (A) HCV activates COX-2 gene promoter activity. The pCOX-2-Luc reporter plasmid encoding firefly luciferase under the control of the COX-2 promoter was transfected in Huh-7, Huh7.5 and MH-14 cells and 48 h post-transfection cell lysates were subjected to luciferase activity assay. Fold increase of relative luciferase activity in Huh7.5 and MH-14 cells in comparison to Huh-7 cells is shown. (B) COX-2 protein and mRNA levels in HCV replicon. Huh7.5 and MH-14 cells were cultured and processed for analysis of COX-2 protein and mRNA levels respectively by western blotting (WB) and RT-PCR. β -Actin detection was used as to normalizer. NS5A and NS5B immunoblotting detection and HCV RNA PCR amplification were considered as markers for HCV replication in MH-14 cell line. and (C). Densitometric quantitation of Cox-2 immunoblotting and PCR amplified bands analysed by ImageJ software. Data are the average of three independent experiments performed in duplicate. ** $P < 0.01$.

Compound 12 suppresses COX-2 expression. To investigate the effect of compound **12** on HCV-induced COX-2 expression, we analyzed the transcription and translation levels of COX-2 in compound **12**-treated MH-14 cells. The COX-2 promoter-linked luciferase reporter assay revealed a dramatic suppression of the COX-2 promoter reporter in a concentration-dependent manner (Figure 3A). This corresponded to 35% and 68% suppression at 2.8 μ M and 4.4 μ M, respectively of compound **12** concentration, and near complete abolition above 14 μ M, thus yielding an inhibitory concentration (IC_{50}) = 3.2 μ M. These results indicate that **12** may mediate its antiviral effects through inhibition of COX-2 mRNA transcripts.

Figure 3. Effect of **12** on HCV-induced COX-2 expression (A to C) Concentration-dependent inhibition of COX-2 promoter activity (A), protein synthesis (B) and RNA transcription (C) by **12**. For the promoter activity assay (A), MH-14 cells were transiently transfected with the COX-2 promoter reporter plasmid pCOX-2-Luc and treated for 48 h with DMSO (ctr) or the indicated concentrations of **12**. The percent decrease of relative luciferase activity (RLU) in **12**-treated MH-14 cells relative to DMSO-treated cells, arbitrarily set at 100% for reference is shown. The RLU of pCOX-2-Luc-transfected-Huh-7 cells is shown for comparison. For protein) and RNA analysis (B, and C), MH-14 cells were incubated with increasing concentrations of **12** for 48 h. Total cell lysates were extracted and analyzed by Western blotting with anti-COX-2 and anti- β -Actin (loading control) antibodies. Total RNA in **12**-treated MH-14 cells was extracted and quantified by qRT-PCR for relative levels of COX-2 mRNA and HCV RNA, normalized against β -Actin mRNA. (C) Densitometric analysis of HCV RNA and COX-2 protein levels. Values reported are percent fold change with respect to control cells. Error bars represent SD from three independent experiments performed in duplicate. *P<0.05; **P<0.01.

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Further, the specificity of compound **12** on COX-2 promoter was evident from our findings that at 50 μ M concentration, **12** displayed no effect on the promoter-linked luciferase reporter assay for HO-1, Nrf2 or ISGs in MH-14 cells (data not shown), thus ruling out the transcriptional regulation of these host factors as the potential mechanism of this compound scaffold.

We next investigated the effect of **12** on COX-2 mRNA and protein synthesis in relation to HCV replication inhibition. Towards this aim, MH-14 cells were incubated with **12** at increasing doses for 48 h and subjected to analysis of endogenous levels of COX-2 protein and mRNA expression and concomitant HCV RNA levels evaluation (Figures 3B and 3C). We observed that the synthesis of COX-2 protein was suppressed in a concentration dependent manner in **12**-treated MH-14 cells (Figure 3B). This was reflected by ~50% decrease of endogenous COX-2 expression at 5 μ M and near complete abolition above 15 μ M, consistent with the trend seen with the COX-2 promoter-linked luciferase reporter assay (Figure 3A).

The **12**-mediated suppression of COX-2 appeared to be specific as deduced from the expression levels of β -actin under similar treatment conditions. Notably, **12**-mediated suppression of COX-2 protein expression (Figure 3B) exhibited a correlative trend with levels of COX-2 mRNA and a concomitant decrease in HCV RNA (Figure 3C). This data indicates that compound **12** inhibits HCV RNA replication via attenuating COX-2 mRNA transcription and consequent suppression of COX-2 protein synthesis.

Figure 4. Dose dependent restoration of 12-mediated suppression of HCV replication upon exogenous COX-2 expression. MH14 cells were transfected with the indicated amounts of pCMV-COX-2-Myc plasmid encoding *cox-2* and 6 h post-transfection, treated with either DMSO (control) or 25 μ M compound **12** for 72 h. Cell lysates were analysed by Western blotting (WB) for expression of COX-2 and β -Actin as loading control (A, bottom two panels). In another set, total RNAs were subjected to qRT-PCR to evaluate HCV RNA levels (A, top two panels). B depicts the quantification of HCV RNA and COX-2 expression level with respect to control. The values represent the average $\pm$ SD from three independent experiments.

Exogenous expression of COX-2 abrogates 12-mediated inhibition of HCV RNA replica-

tion. To further investigate at the molecular level the role of COX-2 down-regulation in **12**-mediated inhibition of HCV replication, we resorted to transient overexpression of COX-2 and analyzed the anti-HCV effects of compound **12** under these conditions. MH-14 cells were transfected with either a control vector (pcDNA4/*myc*-His-A) or increasing concentrations of pCMV-COX-2-Myc plasmid and subsequently treated with **12** at 25 μ M, a concentration that inhibited HCV-induced COX-2 expression and HCV replication by >90% (Figure 3C). RT-PCR and Western blotting analysis revealed a complete knock-down of HCV RNA (top) and COX-2 expression (bottom), in **12**-treated cells relative to DMSO treated control cells (Figure 3A, compare lane 2 to 1). A dose-dependent increase of exogenous COX-2-Myc resulted in a gradual restoration of HCV RNA levels in **12**-treated cells (Figure 4A, lanes 3-6). This corresponded to quantitative recovery of HCV RNA levels by 50% and 100% in **12**-treated cells at 0.4 μ g and above 0.8 μ g of pCMV-COX-2-Myc plasmid, respectively (Figure 4B).

Table 2. Prediction of drug-like properties of 4, 5, 7, 11 and 12.

Compd	MW ^a	SASA ^b	Vol ^c	Donor HB ^d	Accept HB ^e	QP polrz ^f	Qplog C16 ^g	Qplo Poct ^h	Qplo Pw ⁱ	Qplo Po/w ^j	QPlogS ^k	PHOA ^l
4	328.37	634.61	1084.9	1	3.5	41.45	12.96	17.79	10.03	4.85	-6.235	100
5	362.37	660.62	1133.4	1	3.5	42.95	13.62	18.78	9.81	5.38	-7.018	100
7	387.82	685.95	1187.6	1	5	44.27	14.38	21.36	11.30	4.74	-7.749	100
11	422.27	692.02	1215.7	1	5	44.78	14.65	20.41	10.90	5.12	-8.173	100
12	342.40	651.35	1133.8	1	3.5	43.06	13.18	18.07	9.82	5.13	-6.538	100

^aMW, molecular weight; ^bSASA, total solvent accessible surface area (range, 300–1000 Å²); ^cVolume, total solvent accessible area (range: 500–2000 Å³); ^dH-bond donors; ^eH-bond acceptors; ^fQPpolrz, Predicted polarizability (range, 13–70 Å³); ^gQplogPC16, predicted log of hexadecane /gas partition coefficient (range, 4–18); ^hQplogPoct, predicted log of octanol /gas partition coefficient (range, 8–43); ⁱQplogPw, predicted log of water / gas partition coefficient (range, 5–48); ^jQplogPo /w predicted log of octanol/water partition coefficient (range -2 to 6); ^kQplogS, predicted log of aqueous solubility (range, -6 to 0.5 M); ^lPHOS per cent human oral absorption.

This data indicates that exogenous COX-2 expression abrogates **12**-mediated anti-HCV activity. Taken together, our findings suggest that **12** mediates its anti-HCV effects via targeting COX-2. This supports targeting COX-2 as a viable strategy for anti-HCV inhibitor development and is in agreement with some previous studies.²¹⁻²³

Prediction of drug-like properties. Drug likeness involves molecular features that mainly describe whether the molecule resembles the properties of known drugs. The drug-like properties of our compounds (Table 2) as predicted employing QikProp software,²⁴ fall within the reference ranges (Table 2 caption) of the 95% of used drugs, with the only violation of the QplogS values. These data highlight an interesting drug-like profile of these HCV agents representing a good basis for further development.

CONCLUSION

The present study describes the synthesis and biological evaluation of a series of 1-phenyl-5-(1*H*-pyrrol-1-yl)pyrazolecarboxamides as a new class of anti-HCV agents. Our results show that the pyrazolecarboxamides inhibited HCV replication without causing host cellular toxicity. Significantly, four compounds bearing this scaffold exerted strong antiviral effects on both HCV genotype 1b and 2a. Of these, compound **12** proved to be the most promising, exhibiting an EC₅₀ value of 6.7 μ M and selectivity index of 23 against HCV 1b, and reducing the RNA copies of the infectious Jc1 chimeric 2a clone by 82% at 7 μ M. The anti-HCV activity of compound **12** was identified to be mediated by COX-2 inhibition, at both the transcriptional and translational levels. The broad range and strong antiviral activities against HCV genotype 1b and 2a of the pyrazolecarboxamide compounds, the interesting molecular mechanism of anti-HCV activity, that targets host factor dysregulated during viral infection, together with their drug likeness properties, serve as a strong basis for further optimization of this new chemical class of HCV inhibitors.

EXPERIMENTAL SECTION

Chemistry. Melting points (mp) were determined on a Stuart Scientific SMP1 apparatus and are uncorrected. Infrared spectra (IR) were run on a Perkin-Elmer SpectrumOne FT-ATR spectrophotometer. Band position and absorption ranges are given in cm^{-1} . Proton nuclear magnetic resonance (^1H NMR) spectra were recorded on a Bruker 400 MHz FT spectrometer in the indicated solvent and corresponding fid files processed by MestreLab Research S.L. MestreReNova 6.2.1-769 software. Chemical shifts are expressed in δ units (ppm) from tetramethylsilane. Column chromatography was performed on columns packed with alumina from Merck (70–230 mesh) or silica gel from Macherey-Nagel (70–230 mesh). Aluminum oxide thin layer chromatography (TLC) cards from Fluka (aluminum oxide precoat-

ed aluminum cards with fluorescent indicator detectable at 254 nm) and silica gel TLC cards from Macherey-Nagel (silica gel precoated aluminum cards with fluorescent indicator detectable at 254 nm) were used for TLC. Developed plates were visualized by a Spectroline ENF 260C/FE UV apparatus. Reagents and solvents from commercial sources were used without further purification. Organic solutions were dried over anhydrous Na₂SO₄. Evaporation of the solvents was carried out on a Büchi rotavapor R-210 equipped with a Büchi V-855 vacuum controller and a Büchi V-700 or V-710 vacuum pump. Elemental analyses of the compounds were found within $\pm 0.4\%$ of the theoretical values. The purity of tested compounds was $>95\%$. For RdRp assays, compounds were dissolved in DMSO as 10 mM stocks and serially diluted in DMSO immediately prior to the use.

General Procedure for the Synthesis of Carboxamides 5-19. *Example. N-(4-Chlorophenyl)-1-phenyl-5-(1H-pyrrol-1-yl)-1H-pyrazole-3-carboxamide (5).* To a mixture of 1-phenyl-5-(1H-pyrrol-1-yl)-1H-pyrazole-3-carboxylic acid (0.25 g, 1.0 mmol) (**22**),^{11c} in anhydrous THF (14 mL) was added 1,1'-carbonyldiimidazole (0.18 g, 1.0 mmol) under Ar stream. The reaction mixture was stirred for 2 h at room temperature. 4-Chloroaniline (0.20 g, 1.5 mmol) was added and the reaction was stirred at room temperature for 24 h. The mixture was diluted with water and extracted with ethyl acetate. The organic layer was washed with brine, dried and filtered. Removal of the solvent gave a residue that was purified by column chromatography (silica gel, ethyl acetate/*n*-hexane 3:7 as eluent) to provide **5** (0.27 g, 75%), mp 140-145 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 6.21-6.23 (m, 2H), 6.87-6.89 (m, 2H), 7.10 (s, 1H), 7.29-7.31 (m, 2H), 7.39-7.46 (m, 5H), 7.85-7.88 (dd, *J* = 9.2 and 1.8 Hz, 2H), 10.40 ppm (br s, disappeared on treatment with D₂O, 1H). IR: ν 1678, 3382 cm⁻¹. Anal. (C₂₀H₁₅ClN₄O (362.81)) C, H, N, Cl

4-Cyano-N,1-diphenyl-5-(1H-pyrrol-1-yl)-1H-pyrazole-3-carboxamide (6). Was synthesized as **5** from 4-cyano-1-phenyl-5-(1H-pyrrol-1-yl)-1H-pyrazole-3-carboxylic acid (**23**) and aniline. Yield 44%, mp 180-185 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 6.29-6.31 (m, 2H), 6.99-7.01 (m, 2H),

7.14 (t, $J = 6.9$ Hz, 1H), 7.35-7.40 (m, 4H), 7.49-7.51 (m, 3H), 7.81-7.83 (d, $J = 7.8$ Hz, 2H), 10.60 ppm (br s, disappeared on treatment with D₂O, 1H). IR: ν 1684, 3299 cm⁻¹. Anal. (C₂₁H₁₅N₅O (353.38)) C, H, N.

N-(4-Chlorophenyl)-4-cyano-1-phenyl-5-(1*H*-pyrrol-1-yl)-1*H*-pyrazole-3-carboxamide (7).

Was synthesized as **5** from **23** and 4-chloroaniline. Yield 42%, mp 197-201 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 6.29-6.31 (m, 2H), 6.98-7.01 (m, 2H), 7.38-7.44 (m, 4H), 7.48-7.52 (m, 3H), 7.86-7.88 (d, $J = 8.6$ Hz, 2H), 10.75 ppm (br s, disappeared on treatment with D₂O, 1H). IR: ν 1678, 3318 cm⁻¹. Anal. (C₂₁H₁₄ClN₅O (387.82)) C, H, N, Cl.

1-(4-Chlorophenyl)-*N*-phenyl-5-(1*H*-pyrrol-1-yl)-1*H*-pyrazole-3-carboxamide (8). Was synthesized as **5** from 1-(4-chlorophenyl)-5-(1*H*-pyrrol-1-yl)-1*H*-pyrazole-3-carboxylic acid (**24**) and aniline. Yield 44%, mp 190-195 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 6.23-6.26 (m, 2H), 6.89-6.91 (m, 2H), 7.08-7.12 (m, 2H), 7.28-7.37 (m, 4H), 7.52-7.54 (d, $J = 8.2$ Hz, 2H), 7.80-7.82 (d, $J = 8.0$ Hz, 2H), 10.24 ppm (br s, disappeared on treatment with D₂O, 1H). IR: ν 1675, 3391 cm⁻¹. Anal. (C₂₀H₁₅ClN₄O (362.81)) C, H, N, Cl.

N,1-bis(4-Chlorophenyl)-5-(1*H*-pyrrol-1-yl)-1*H*-pyrazole-3-carboxamide (9). Was synthesized as **5** from **24** and 4-chloroaniline. Yield 44%, mp 198-200 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 6.24-6.26 (m, 2H), 6.90-6.92 (m, 2H), 7.11 (s, 1H), 7.28-7.30 (d, $J = 8.7$ Hz, 2H), 7.39-7.42 (d, $J = 8.5$ Hz, 2H), 7.52-7.54 (d, $J = 8.0$ Hz, 2H), 7.85-7.87 (d, $J = 8.8$ Hz, 2H), 10.42 ppm (br s, disappeared on treatment with D₂O, 1H). IR: ν 1685, 3395 cm⁻¹. Anal. (C₂₀H₁₄Cl₂N₄O (397.26)) C, H, N, Cl.

1-(4-Chlorophenyl)-4-cyano-*N*-phenyl-5-(1*H*-pyrrol-1-yl)-1*H*-pyrazole-3-carboxamide (10).

Was synthesized as **7** from 1-(4-chlorophenyl)-4-cyano-5-(1*H*-pyrrol-1-yl)-1*H*-pyrazole-3-carboxylic acid (**25**) and aniline. Yield 40% mp 234-238 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 6.31-6.34 (m, 2H), 7.00-7.02 (m, 2H), 7.15 (t, $J = 7.08$ Hz, 1H), 7.35-7.40 (m, 4H), 7.59-7.61 (d, $J = 8.08$ Hz, 2H),

7.81-7.83 (d, $J = 7.68$ Hz, 2H), 10.61 ppm (br s, disappeared on treatment with D₂O, 1H). IR: ν 1685, 3337 cm⁻¹. Anal. (C₂₁H₁₄ClN₅O (387.82)) C, H, N, Cl.

N,1-bis(4-chlorophenyl)-4-cyano-5-(1*H*-pyrrol-1-yl)-1*H*-pyrazole-3-carboxamide (**11**). Was synthesized as **5** from **25** and 4-chloroaniline. Yield 47%, mp 255-258 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 6.31-6.33 (m, 2H), 6.99-7.01 (m, 2H), 7.38-7.44 (m, 4H), 7.58-7.61 (d, $J = 8.7$ Hz, 2H), 7.85-7.87 (d, $J = 8.7$ Hz, 2H), 10.78 ppm (br s, disappeared on treatment with D₂O, 1H). IR: ν 1685, 3323 cm⁻¹. Anal. (C₂₁H₁₃Cl₂N₅O (422.27)) C, H, N, Cl.

N,1-diphenyl-5-(1*H*-pyrrol-1-yl)-4-methyl-1*H*-pyrazole-3-carboxamide (**12**). Was synthesized as **7** from 4-methyl-1-phenyl-5-(1*H*-pyrrol-1-yl)-1*H*-pyrazole-3-carboxylic acid (**26**) and aniline. Yield 62% mp 147-150 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 2.15 (s, 3H), 6.26-6.28 (m, 2H), 6.90-6.92 (m, 2H), 7.10 (t, $J = 7.2$ Hz, 1H), 7.24-7.26 (d, $J = 6.7$, 2H), 7.32-7.43 (m, 5H), 7.81-7.83 (d, $J = 8.4$ Hz, 2H), 10.16 ppm (br s, disappeared on treatment with D₂O, 1H). IR: ν 1666, 3362 cm⁻¹. Anal. (C₂₁H₁₈N₄O (342.39)) C, H, N.

N-(4-chlorophenyl)-4-methyl-1-phenyl-5-(1*H*-pyrrol-1-yl)-1*H*-pyrazole-3-carboxamide (**13**). Was synthesized as **5** from **26** and 4-chloroaniline. Yield 12%, mp 147-150 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 2.15 (s, 3H), 6.25-6.27 (m, 2H), 6.89-6.91 (m, 2H), 7.23-7.25 (m, 2H), 7.37-7.41 (m, 5H), 7.86-7.88 (d, $J = 8.8$ Hz, 2H), 10.34 ppm (br s, disappeared on treatment with D₂O, 1H). IR: ν 1667, 3357 cm⁻¹. Anal. (C₂₁H₁₇ClN₄O (376.84)) C, H, N, Cl.

1-(4-Chlorophenyl)-4-methyl-*N*-phenyl-5-(1*H*-pyrrol-1-yl)-1*H*-pyrazole-3-carboxamide (**14**). Was synthesized as **5** from 1-(4-chlorophenyl)-4-methyl-5-(1*H*-pyrrol-1-yl)-1*H*-pyrazole-3-carboxylic acid (**27**) and aniline. Yield 83% mp 155-158 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 2.14 (s, 3H), 6.28-6.30 (m, 2H), 6.91-6.93 (m, 2H), 7.10 (t, $J = 6.7$ Hz, 1H), 7.22-7.25 (m, 2H), 7.32-7.36 (m, 2H),

7.47-7.50 (m, 2H), 7.80-7.82 (d, $J = 7.9$ Hz, 2H), 10.19 ppm (br s, disappeared on treatment with D₂O, 1H). IR: ν 1680, 3355 cm⁻¹. Anal. (C₂₁H₁₇ClN₄O (376.84)) C, H, N, Cl.

N,1-bis (4-Chlorophenyl)-4-methyl-5-(1H-pyrrol-1-yl)-1H-pyrazole-3-carboxamide (15). Was synthesized as **5** from 1-(4-chlorophenyl)-4-methyl-5-(1H-pyrrol-1-yl)-1H-pyrazole-3-carboxylic acid (**29**) and 4-chloroaniline. Yield 11%, mp 179-182 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 2.14 (s, 3H), 6.28-6.30 (m, 2H), 6.91-6.93 (m, 2H), 7.22-7.24 (d, $J = 8.8$ Hz, 2H), 7.39-7.41 (d, $J = 8.8$ Hz, 2H), 7.48-7.51 (d, $J = 8.8$ Hz, 2H), 7.85-7.87 (d, $J = 8.8$ Hz, 2H), 10.36 ppm (br s, disappeared on treatment with D₂O, 1H). IR: ν 1669, 3367 cm⁻¹. Anal. (C₂₁H₁₆Cl₂N₄O (411.28)) C, H, N, Cl.

N-(3,4-Dichlorophenyl)-1-phenyl-5-(1H-pyrrol-1-yl)-1H-pyrazole-3-carboxamide (16). Was synthesized as **5** from **22** and 3,4-dichloroaniline. Yield 51%, mp 142-146 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 6.20-6.22 (m, 2H), 6.87-6.89 (m, 2H), 7.11(s, 1H), 7.30-7.32 (m, 2H), 7.44-7.46 (m, 3H), 7.60-7.62 (d, $J = 8.8$ Hz, 1H), 7.84-7.86 (dd, $J = 2.2$ and 8.6 Hz, 1H), 8.20 (s, 1H), 10.57 ppm (br s, disappeared on treatment with D₂O, 1H). IR: ν 1693, 3367 cm⁻¹. Anal. (C₂₀H₁₄Cl₂N₄O (397.26)) C, H, N, Cl.

1-(4-Chlorophenyl)-N-(3,4-dichlorophenyl)-5-(1H-pyrrol-1-yl)-1H-pyrazole-3-carboxamide (17). Was synthesized as **5** from **24** and 3,4-dichloroaniline. Yield 53%, mp 165-171°C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 6.23-6.25 (m, 2H), 6.89-6.91 (m, 2H), 7.12 (s, 1H), 7.29-7.31 (d, $J = 8.8$ Hz, 2H), 7.53-7.55 (d, $J = 8.8$ Hz, 2H), 7.60-7.63 (d, $J = 8.8$ Hz, 1H), 7.83-7.86 (dd, $J = 2.4$ and 8.8 Hz, 1H), 8.20 (s, 1H), 10.59 ppm (br s, disappeared on treatment with D₂O, 1H). IR: ν 1687, 3380 cm⁻¹. Anal. (C₂₀H₁₃Cl₃N₄O (431.70)) C, H, N, Cl.

4-Cyano-N-(3,4-dichlorophenyl)-1-phenyl-5-(1H-pyrrol-1-yl)-1H-pyrazole-3-carboxamide (18). Was synthesized as **5** from **23** and 3,4-dichloroaniline. Yield 10%, mp 213-219 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 6.29-6.31 (m, 2H), 6.99-7.01 (m, 2H), 7.38-7.40 (m, 2H), 7.49-7.51 (m, 3H),

7.63-7.65 (d, $J = 8.4$ Hz, 1H), 7.84-7.86 (d, $J = 8.8$ Hz, 1H), 8.21 (s, 1H), 10.92 ppm (br s, disappeared on treatment with D₂O, 1H). IR: ν 1692, 3351 cm⁻¹. Anal. (C₂₁H₁₃Cl₂N₅O (422.27)) C, H, N, Cl.

1-(4-Chlorophenyl)-4-cyano-N-(3,4-dichlorophenyl)-5-(1H-pyrrol-1-yl)-1H-pyrazole-3-carboxamide (19). Was synthesized as **5** from **25** and 3,4-dichloroaniline. Yield 11%, mp 237-244 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 6.31-6.33 (m, 2H), 6.99-7.01 (m, 2H), 7.38-7.40 (d, $J = 8.4$ Hz, 2H), 7.59-7.65 (m, 3H), 7.82-7.85 (dd, $J = 2.4$ and 8.8 Hz, 1H), 8.19 (s, 1H), 10.93 ppm (br s, disappeared on treatment with D₂O, 1H). IR: ν 1693, 3346 cm⁻¹. Anal. (C₂₁H₁₂Cl₃N₅O (456.71)) C, H, N, Cl.

General Procedure for the Synthesis of Carboxamides 4, 20 and 21. *Example. 1-(4-Chlorophenyl)-N-phenyl-5-(1H-pyrrol-1-yl)-1H-pyrazole-4-carboxamide (20)*. A mixture of 1-(4-chlorophenyl)-5-(1H-pyrrol-1-yl)-1H-pyrazole-4-carboxylic acid (**28**)¹⁵ (0.25 g, 0.87 mmol), aniline (0.23 g, 0.24 mL, 2.61 mmol), BOP reagent (0.35 g, 0.87 mmol) and triethylamine (0.26 g, 0.36 mL, 2.61 mol) in anhydrous DMF (8 mL) was stirred at room temperature overnight. The reaction mixture was diluted with brine and extracted with ethyl acetate. The organic layer was washed with brine, dried and filtered. Removal of the solvent gave a residue that was purified by column chromatography (silica gel, *n*-hexane/ethyl acetate 2:1 as eluent) to provide **20** (40 mg, 13%), mp 181-185 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 6.21-6.23 (m, 2H), 6.90-6.92 (m, 2H), 7.06 (t, $J = 7.4$ Hz, 1H), 7.10-7.13 (dd, $J = 1.8$ and 8.7 Hz, 2H), 7.29-7.32 (m, 2H), 7.46-7.48 (dd, $J = 2.1$ and 8.9 Hz, 2H), 7.60-7.61 (d, $J = 7.08$, 2H), 8.43 (s, 1H), 9.77 ppm (br s, disappeared on treatment with D₂O, 1H). IR: ν 1665, 3386 cm⁻¹. Anal. (C₂₀H₁₅ClN₄O (362.81)) C, H, N, Cl.

N,1-bis(4-Chlorophenyl)-5-(1H-pyrrol-1-yl)-1H-pyrazole-4-carboxamide (21). Was synthesized as **20** from **28** and 4-chloroaniline. Yield 59%, mp 177-181 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 6.20-6.22 (m, 2H), 6.88-6.90 (m, 2H), 7.11-7.13 (d, $J = 8.6$ Hz, 2H), 7.36-7.38 (d, $J = 8.9$ Hz, 2H),

7.46-7.48 (d, $J = 8.6$ Hz, 2H), 7.65-7.67(d, $J = 8.9$ Hz, 2H), 8.42 (s, 1H), 10.00 ppm (br s, disappeared on treatment with D₂O, 1H). IR: ν 1670, 3388 cm⁻¹. Anal. (C₂₀H₁₄Cl₂N₄O (397.26)) C, H, N, Cl.

N,1-Diphenyl-5-(1H-pyrrol-1-yl)-1H-pyrazole-3-carboxamide (4). Was synthesized as **20** from **22** and aniline. Yield 70%, mp 114-115 °C (from ethanol). ¹H NMR (CDCl₃): δ 6.29-6.31 (m, 2H), 6.67-6.69 (m, 2H), 7.03-7.04 (m, 1H), 7.15-7.20 (m, 1H), 7.23-7.29 (m, 2H), 7.38-7.43 (m, 5H), 7.77-7.75 (m, 2H), 8.80 ppm (br s, disappeared on treatment with D₂O, 1H). IR: ν 1672, 3377 cm⁻¹. Anal. (C₂₀H₁₆N₄O (328.37)) C, H, N.

Biology. Cell Culture. MH-14 cells expressing the HCV 1b subgenomic replicon, a kind gift from Dr. Kunitada Shimotohno, were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine and 0.5 mg/ mL G418. Huh 5.2 cells bearing the HCV 1b I389luc-ubineo/NS3-3'/5.1 replicon were cultured in DMEM supplemented with 10% FCS, nonessential amino acids, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine and 0.5 mg/ mL G418. Huh7.5, kindly provided by Apath were grown in DMEM supplemented with 10% FBS, 0.3 mg/ml L-glutamine, 100U/ml penicillin, 100 mg/ml streptomycin and non essential amino acids. All cells were cultured at 37 °C in 5% CO₂ atmosphere at 95-99% relative humidity.

HCV Jc1 infection assay. The inhibitory effect of compounds **4**, **6** and **12** on HCV infection was assayed employing the HCV genotype 2a infectious chimeric clone Jc1 (generous gift of C. Rice). Briefly, HCV-Jc1 virus stock of 10⁵ TCID₅₀/mL was used to infect Huh7.5 cell lines at 1 m.o.i., in 12 multi wells plates. Six hours post viral adsorption, cells were washed with PBS, supplemented with fresh media and incubated with the compounds at concentration specified in figure1 legend. Seventy-two hours post-treatment, cells were harvested and processed for total RNA extraction employing RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's protocol.

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HCV RNA quantitation by qRT-PCR. Quantitative One Step RT-PCR was carried out using an ABI 7000 real Time PCR System (Applied Biosystem). 5 μ l of total RNA was mixed with 2x TaqMan One Step RT-PCR Master Mix (Applied Biosystem and 1 μ M forward (TCCCGGGAGAGCCATAGT), 1 μ M reverse primers (CCCAGTCTTCCCGGCAATT) and 200 nM probe (FAM- CACCGGTTCCGCAGACC-TAMRA). In vitro transcribed genotype 2a RNA was used as a standard to quantify the copy numbers of viral RNA.

Cell based inhibition and cytotoxicity assays. Inhibitory potency (EC₅₀, EC₉₀ values) and cytotoxicity (CC₅₀ values) of the compounds were evaluated in Huh 5.2 cells as described previously.²⁵

NS5B RdRp inhibition assay. The effect of the compounds on in vitro NS5B RdRp activity was investigated employing recombinant NS5B Δ 21 1b (HC-J4) and poly rA-U₁₂ template-primer as described previously.²⁸ NS5B activity was determined relative to DMSO treated control that was set at 100%

Transfection and luciferase reporter assay. MH-14 cells were seeded in 48 well plate (1x10⁵ cells/ well) and 12 h post seeding, transfected with 300 ng of gene specific reporter plasmid pCOX-2-FLuc,^{21,25,26} pHO-1-Luc,²⁷ pISRE-Luc²⁸ or p3xARE-Luc²⁹ using LipoD293 transfection reagent in accordance with the manufacturer's protocol (SignaGen Lab, USA). Each transfection complex contained 30 ng of *Renilla* luciferase reporter plasmid (pRL-SV40) as internal control for normalization of the transfection efficiency. Six hours post-transfection, cells were incubated with increasing concentrations of **12** (quarter log dilutions) or DMSO (control) for 48 hours. Luciferase activity assay was performed using the Dual-Glo Luciferase Assay Kit (Promega, USA) in accordance with the manufacturer's instructions.

To study the effect of **12** on HCV IRES mediated translation, Huh7.5 cells were seeded in 48 well plate and transfected with pCneo-Rluc-IRES-Fluc (300 ng/well) reporter plasmid, in which Rluc was translated in a cap-dependent manner and Fluc was translated via HCV IRES-mediated initiation.¹⁸

Six hours post-transfection, the cells were incubated with increasing concentrations of compound **12** or DMSO (control) for 48 hours. Luciferase activity assay was performed employing the Dual-Glo Luciferase Assay Kit.

Western blotting. Equal amounts of whole cell lysates (Dc protein assay, Bio-Rad) were resolved over 8% SDS-PAGE and gels were transferred to PVDF membrane by Trans Blot semi-dry transfer apparatus (Bio-Rad) and blots were blocked in 3% BSA for 1 h and probed with anti-NS5A, anti-NS5B, anti-Cox-2 and β -Actin antibodies.

RT-PCR: Total RNA was isolated either using Triazol reagent (Life Technologies) or RNeasy mini kit (Promega Corp.). Isolated RNA's were quantified using NanoDrop (ND1000, NanoDrop Technologies), and equal amount of RNA's were reverse transcribed using Superscript II reverse transcriptase (Life Technologies) using HCV specific or oligo dT₁₈ as a primer by following manufacturer's protocol in a final volume of 20 μ l. Two micro liters of synthesized cDNA's were used for downstream PCR applications using gene specific primers and Taq DNA polymerase (Sigma-Aldrich) in a final volume of 50 μ l by following manufacturer's protocol. For Cox-2 forward primer was 5'-TGCAAGTTCTCCCGCTCC-3' and reverse primer was 5'-CAGCATTTTGCCATCTTGTGAC-3'. The forward and reverse primer sequences for β -Actin was 5'-AGCGAGCATCCCCCAAAGTT-3' and 5'-GGGCACGAAGGCTCATCATT-3', respectively. The HCV primers sequences were 5'-CGGGAGAGCCATAGTGG-3' for forward and 5'-AGTACCACAAGGCCTTTCG-3' for reverse primer.

ASSOCIATED CONTENT

Supporting Information Available. Additional chemical information is available free of charge via the internet at <http://pubs.acs.org>.

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Notes. The manuscript was written by N. K.-B. and R. S. All authors have given approval to the final version of the manuscript.

ABBREVIATIONS USED

HCV, hepatitis C virus; RdRp, RNA-dependent RNA polymerase; DAAs, direct-acting antiviral agents; COX-2, Cyclooxygenase-2; NS5B, Nonstructural protein 5B; m.o.i.: multiplicity of infection; DMEM, Dulbecco's modified eagle medium; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium.

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