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Synthesis of Novel Diflunisal Hydrazide-Hydrazones as Anti-Hepatitis C Virus Agents and Hepatocellular Carcinoma Inhibitors

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ABSTRACT

Hepatitis C virus (HCV) infection is a main cause of chronic liver disease, leading to liver cirrhosis and hepatocellular carcinoma (HCC). The objective of our research was to develop effective agents against viral replication. We have previously identified the hydrazide-hydrazone scaffold as a promising hepatitis C virus (HCV) and hepatocelluler inhibitor. Herein we describe the design a number of 2',4'-difluoro-4-hydroxy-*N'*-(arylmethylidene) biphenyl-3-carbohydrazide **(3a-t)** as anti-HCV and anticancer agents. Results from evaluation of anti-HCV activity indicated that most of the synthesized hydrazone derivatives inhibited viral replication in the Huh7/Rep-Feo1b and Huh 7.5-FGR-JCI-Rluc2A reporter systems. Antiproliferative activities of increasing concentrations of 2',4'-difluoro-4-hydroxy-*N'*-(2-pyridyl methylidene)biphenyl-3-carbohydrazide **3b** and diflunisal (2.5-40 μM) were assessed in liver cancer cell lines (Huh7, HepG2, Hep3B, Mahlavu, FOCUS and SNU-475) with sulforhodamine B assay for 72h. Compound **3b** with 2-pyridinyl group in the hydrazone part exhibited promising cytotoxic activity against all cell lines with IC_{50} values of 10, 10.34 16.21 4.74, 9.29 and 8.33 μM for Huh7, HepG2, Hep3B, Mahlavu, FOCUS and SNU-475cells, respectively, and produced dramatic cell cycle arrest at SubG1/G0 phase as an indicator of apoptotic cell death induction.

Running Title
Synthesis of Diflunisal hydrazones as anti-HCV and anticancer agents

Keywords: Diflunisal, Hydrazide-hydrazone, Hepatitis C, Hepatocellular carcinom, antiviral.

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1. Introduction

Hepatitis C virus (HCV) is an enveloped virus that is classified in the hepacivirus genus of the *Flaviviridae* family [1]. The virus RNA genome encodes a polyprotein, which is posttranslationally processed by host and virus proteases into 10 mature proteins, of which 4 are structural proteins (C, E1, E2, and p7) and 6 nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [2]. HCV is a worldwide infectious pathogen that causes chronic liver diseases, including hepatic fibrosis, hepatic cirrhosis and hepatocellular carcinoma (HCC) [3]. Until recently, HCV-infected patients were treated with a combination of pegylated interferon- α (IFN- α) and the nucleoside analog ribavirin. However, this therapy had limited effectiveness especially in context of patients infected with HCV genotype 1. Furthermore, treatment with interferon is associated with numerous side effects. Recently, new anti-HCV therapies utilizing the direct acting antivirals (DAAs) against the viral proteins HCV NS3-4A protease and NS5B polymerase have been approved. These therapy although more promising have complicated dosing regimens limiting patient compliance [4-7] urther, the selection of HCV drug resistant variants continues to remain a concern [8-9]. On the other hand, acute and chronic liver diseases that are caused by an infection with hepatitis-C virus (HCV), such as hepatocellular carcinoma and liver cirrhosis have received much attention over the past decade. Recently, HCV is believed to act as carcinogen by virtue of the increased risk of hepatocellular carcinoma among persistently infected patients with chronic active hepatitis. Therefore, it is important to develop new, safer and even more effective agents against HCV infection and resistance emergence.

Diflunisal derivatives [10-14] (Fig. 1) have been reported to possess diverse biological activities such as anticancer, anti-HCV, anticonvulsant, antimicrobial and anti-inflammatory properties [15]. In medicinal chemistry, the presence of a hydrazide-hydrazone group in compounds, has usually led to the development of clinically relevant biological molecules with antimicrobial, anticancer [16-17] and antiviral properties [18].

Fig. 1. Diflunisal derivatives

Recently, our group reported the synthesis of novel hydrazide-hydrazone derivatives and their HCV NS5B inhibition effects [19] (Fig. 2).

Fig. 2. Hydrazones of anti-HCV activity.

Diflunisal is a difluorophenyl derivative of salicylic acid and a non-steroidal drug with analgesic, anti-inflammatory and antipyretic properties. It is a peripherally-acting non-narcotic analgesic drug which functions as a prostaglandin synthase inhibitor. In animals, prostaglandins sensitize afferent nerves and potentiate the action of bradykinin in inducing pain. Since prostaglandins are known to be among the mediators of pain and inflammation, the mode of action of diflunisal may be due to a decrease of prostaglandins in peripheral tissues.

Herein, we report our ongoing efforts towards development of more effective anti-HCV agents. We focused our attention on the hydrazide-hydrazone moiety. Thus, a new series of hydrazide-hydrazone derivatives were synthesized from diflunisal and evaluated for their anti-HCV activity *in vitro* and anticancer activity against hepatocellular cancer cell lines.

2. Results and discussion

2.1. Chemistry

Methyl 2′,4′-difluoro-4-hydroxybiphenyl-3-carboxylate **[1]** was prepared by the reaction of diflunisal and methanol in the presence of a few drops of concentrated sulfuric acid. 2′,4′-Difluoro-4-hydroxybiphenyl-3-carboxylic acid hydrazide **[2]** was prepared by heating hydrazine-hydrate and **[1]** in methanol [10]. After condensing hydrazide with substituted aldehydes in ethanol, novel 2',4'-difluoro-4-hydroxy-*N'*-(arylmethylidene) biphenyl-3-carbohydrazide [**3a-t**] were obtained. The synthesis of novel series of hydrazide-hydrazones **3a-t** was performed as outlined in Scheme 1. All synthesized compounds were checked for purity using TLC and HPLC-UV/DAD and were characterized by their melting points, ^{1}H-NMR, ^{13}C-NMR and HR-MS spectral data.

Scheme 1. The synthetic route for the preparation of the target compounds **(3a-t).**

The FT-IR spectra of hydrazones showed absorption bands at 1583-1614 cm^{-1} due to C=N groups. Moreover, C=O absorption bands were observed between 1633-1647 cm^{-1}. The absorption bands associated with other functional groups appeared in the expected regions. In the ^{1}H-NMR spectra of all hydrazones, the signal representing the azomethine CH protons appeared at 8.41-9.11 ppm, whereas a D_2O-exchangeable signal due to NH amidic proton (-CONHN=CH-) and -OH resonated at 11.78-12.28 ppm. The other protons appeared at the expected chemical shifts and integral values. ^{13}C-NMR spectrum of **3a-t** showed the absence of the C=O signal at δ 164.59-165.38 ppm and -N=CH 144.70-149.32 ppm. Besides, the heteronuclear multiple bond correlation (HMBC) spectrum of compound **3o** also confirmed the detection of long-range ^{1}H-^{13}C couplings. HR-EI (for **3j** and **3o**) and DART-MS (for **3d** and **3s**) confirmed the molecular weights and empirical formulae of compounds with less than 1 mmu bias between calculated and experimental m/z values of either molecular or fragment ions. The major fragmentation pattern involved the cleavage of the CONH-NH= amide bond m/z 249. The species m/z 232 and m/z177 in compounds **(3j, 3o)** may results from loss of -NH_2 and subsequent loss of CO in the phenyl ring.

2.2. Biological evaluation

2.2.1. Anti-HCV effect of diflunisal hydrazide-hydrazones

In order to identify potential anti-HCV agents, we employed two reporter cell lines, Huh7/Rep-Feo1b and Huh7.5-FGR-JC1-Rluc2A (Table 1). These cells carry the autonomously replicating HCV RNA of genotype 1b and 2a in the firefly and Renilla luciferase reporters, respectively [20-24]. Therefore levels of their respective luciferase serves as an indicator of HCV RNA replication. The effect of the compounds on cell viability was investigated in the Huh7.5 parental cells by the MTS assay and employed to compute 50% cytotoxicity values (CC_{50}) (Table 1) .

Table 1. Anti-HCV activity of diflunisal hydrazide-hydrazones **(3a-t).**

The nineteen compounds displayed a wide range of cytotoxicity in the parental Huh7.5 cells. Among these **3f**, **3g**, **3i**, **3k**, **3n**, **3o**, **3p** and **3t** exhibited CC_{50} < 50 µM, suggesting that these compounds may be detrimental to cell viability. In contrast, **3c**, **3m** and **3r** with CC_{50} > 200 µM, proved to be the least cytotoxic. The remaining compounds displayed cytotoxicity in the interim range with compounds **3a**, **3d**, **3e**, **3h**, and **3l** exhibiting CC_{50} > 50 µM, while **3b**, **3j** and **3s** displaying CC_{50} > 100 µM.

We next screened the compounds at 50 µM concentration for their anti-HCV activity. With the exception of **3d** which exhibited 33% and 23% inhibition against 1b and 2a replicon reporters respectively, all others displayed ≥58% inhibition. Notably, all compounds with the exception of **3g**, **3h**, **3r** and **3a** exhibited higher inhibition of 2a replicons relative to 1b, suggesting higher antiviral potency of the compounds against HCV genotype 2a. Compounds exhibiting ≥70% inhibition against 1b replicon and CC_{50} ≥ 50 µM were further characterized in terms of their EC_{50} values. This resulted in the identification of 6 compounds **3a**, **3b**, **3c**, **3h**, **3m** and **3r** which met this cut-off criteria and displayed EC_{50} values ranging between 3.9-16.5 µM and selectivity index (SI) between 3-25. Among these, compound **3b**, 2',4'-difluoro-4-hydroxy-*N'*-[(pyridin-2-yl)methylidene]biphenyl-3-carbohydrazide appeared the most promising with an EC_{50} of 3.9 and SI >25.6.

To identify the mechanism of action of these compounds against HCV, we investigated if the compounds target HCV NS5B. Towards this end, we screened the compounds in vitro for their ability to inhibit NS5B RdRp activity. As seen in Table 1, the compounds displayed no inhibition or ≤18% inhibition of HCV NS5B, thus suggesting that the compounds function through a mechanism other than targeting HCV NS5B.

Further studies are in progress to optimize the structures of hydrazide-hydrazone derivatives with the aim of increasing their anti-HCV potency.

2.2.2. Anticancer activity of compound **3b** *against liver cancer cell lines*

In order to determine the potential anticancer activity of the obtained diflunisal hydrazide hydrazone **3b** and diflunisal, we evaluated their cytotoxic activity on liver cancer cell lines (Huh7, HepG2, Hep3B, Mahlavu, FOCUS and SNU-475) with the sulforhodamine B assay (2.5–40 μM) for 72h as described before [25] (Fig. 3). Compound **3b** was bioactive in all six of the cancer cell lines with IC_{50} values in micromolar ranges (Table 2). In this study, diflunisal was included as an experimental control.

Fig. 3. Cytotoxicity induced by the compounds on liver cancer cell lines (Huh7, HepG2, Hep3B, Mahlavu, FOCUS and SNU-475). The cells were treated with increased concentration of the compound (2.5–40 μM) for 72h. NCI-60 SRB assay was then performed. Absorbance values were obtained and normalized to DMSO control. The experiment was conducted in triplicate.

Table 2. IC_{50} values of **3b** on liver cancer cell lines.

Compound **3b,** bearing 2-pyridinyl group in the hydrazone core inhibited cell proliferation with IC_{50} values between 4.74-16.21 μM as shown in Table 2. While Diflunisal showed no inhibition against Huh7, HepG2, Hep3B, and SNU-475 liver cells, compound **3b** by contrast demonstrated anticancer activity against the same cells with IC_{50} values 10.0, 10.34, 16.21 and 8.33 μM, respectively. Compound **3b** exhibited the highest growth inhibitory activity against Mahlavu lines (IC_{50}=4.74 μM).

2.2.2.1. Effect of diflunisal and compound **3b** *on the morphology of Huh 7 and Mahlavu cells*

It is known that a cell that is undergoing apoptosis exhibits nuclear condensation and DNA fragmentation, which can be detected by staining with Hoechst 33258 and fluorescence microscopy. To examine the nature of cell death induced upon compound treatment, we next analyzed the changes in cell morphology with light microscopy. Towards this end, human liver cancer cells were treated with diflunisal and compound **3b** at their IC_{50} values for 72 h (Table 2) and compared against DMSO treated controls. As shown in Fig. 4, compound **3b**

induced cell death with diverse morphologies. In Huh7 cells, treatment with **3b** at IC_{50} concentration, resulted in nuclear condensation and DNA fragmentation in the cells, which was in parallel with their cell cycle analysis but no such morphological change were observed upon diflunisal (Fig. 4).

Fig. 4. Nuclear Hoechst (33258) staining (40x) of liver cancer cells treated with IC_{50} concentrations of the compounds or DMSO control for 72h.

2.2.2.2. Effect of diflunisal and compound **3b** *on cell cycle arrest in Huh 7 and Mahlavu cells*

In order to investigate the effects of the compounds on cell cycle, we treated the liver cancer cells with DMSO or compounds at their IC_{50} concentrations for 72h and stained the cells with propidium iodide. This assay revealed that compound **3b** induced SubG1/Go arrest in Mahlavu cells lines, with the effect being more prounced in Mahlavu cells (Fig. 5).

Fig. 5. Cell cycle distribution of liver cancer cells (Huh7 and Mahlavu) treated with IC_{50} concentrations of the compounds or DMSO control for 72h.

2.2.2.3. Apoptosis induction by compound **3b**

The changes observed in fluorescent microscopy together with SubG1 cell cycle arrest suggested that the compound induced apoptotic cell cycle arrest. In order to confirm this, the presence of cleaved PARP (an indicator of apoptitic induction) was investigated in cells treated with DMSO control, diflunisal or **3b** according to IC_{50} concentrations for 72h. In Huh7 cells, no cleaveage could be observed. On the other hand, compound **3b** caused cleavage of PARP protein indicating that **3b** induced apoptosis in Mahlavu liver cancer cell line (Fig. 6).

Fig. 6. Western blot results of liver cancer cells treated with IC_{50} concentrations of the compounds or DMSO control for 72h.

3. Conclusion

In this study, a series of of 2',4'-difluoro-4-hydroxy-*N'*-(arylmethylidene)biphenyl-3-carbohydrazide **[3a-t]** have been synthesized and evaluated for their anti-HCV and anticancer

activity. The results revealed increase in cleaved PARP (a marker for apoptosis) in Mahlavu cells treated with **3b** indicating induction of apoptosis. In Huh7 cells no cleaved fragment was observed. According to FACS analysis, in both cell lines, treatment with compound **3b** resulted in SubG1 cell cycle arrest. Together, this data indicates that compound **3b** may be a promising lead candidate for further optimization and development as a prospective anti-HCV and hepatocellular carcinoma inhibitory agent.

4. Experimental section

4.1. Chemistry

All reagents and solvents were obtained from commercial suppliers and were used without further purification. Merck silica gel 60 F254 plates were used for analytical TLC. Melting points wee determined using Schmelzpunktbestimmer SMP II apparatus and were uncorrect. ^{1}H and ^{13}C- NMR spectra were recorded on 300 MHz or 500 MHz Varian UNITY INOVA or HD BRUKER 300 MHz Ultrashield TM spectrometer. Elemental analyses were determined by CHNS-932 (LECO). FT-IR spectra were recorded on a Schimadzu FTIR-8400S spectrometer. HR-MS mass spectra were acquired using a Jeol JMS-700 spectrometer. Purity of the synthesized compounds have been demonstrated by HPLC analysis using reversed-phase chromatography. The liquid chromatographic system consists of an Agilent Technologies 1100 series instrument equipped with a quaternary solvent delivery system and a model Agilent series G1315 A photodiode array detector. A Rheodyne syringe loading sample injector with a 50-µL sample loop was used for the injection of the analytes. Chromatographic data were collected and processed using Agilent Chemstation Plus software. The separation was performed at ambient temperature using a reverse phase ACE C_{18} (4.0x100 mm) column. All experiments were performed in gradient mode. ACN/H_2O system was used as gradient system: 50:50 from 0 to 3 min; 75:25 to 50:50 from 3 to 6 min; 100:0 to 75:25 from 6 to 12 min; the flow rate was 1.0 mL/min with monitoring at 254 nm.

4.1.1. Methyl 2',4'-difluoro-4-hydroxybiphenyl-3-carboxylate **(1)** and *4.1.2. 2',4'-Difluoro-4-hydroxybiphenyl-3-carboxylic acid hydrazide* (**2**) were prepared described in the literature [10].

4.1.3. General procedure for the synthesized of the compounds

A mixture of appropriate aldehydes and an equimolar amount of diflunisal hydrazide in ethanol was refluxed for 2h and novel 2',4'-difluoro-4-hydroxy-*N*'(arylmethylidene)biphenyl-3-carbohydrazide [**3a-t**] were obtained.

4.1.3.1. 2',4'-Difluoro-4-hydroxy-N'-[(thiophen-2-yl)methylidene]biphenyl-3-carbohydrazide (**3a**)

Dark brown solid; Yield: 90%; HPLC: t_R (min.): 8.94; Mp 251 ^oC; FTIR (cm^{-1}): 3242 (O-H&N-H), 3072, 3032 (=C-H arom.), 1637 (C=O), 1608 (C=N); ^{1}H-NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7.08 (d, 1H, *J*=8.4 Hz, H_5), 7.22 (t, 1H, H_{11}), 7.37 (t, 1H, H_9), 7.49-7.65 (m, 4H, Ar-H), 7.72 (d, 1H, J=5.1 Hz, H_{14}),7.99 (s, 1H, H_2), 8.67 (s, 1H, CH=N), 11.90 (s, 2H, Ar-OH &CO-NH-N=CH); Anal. Calcd for $C_{18}H_{12}F_2N_2O_2S$: C, 60.33; H, 3.38; N, 7.82; S, 8.95; found C, 59.82; H, 3.36; N, 7.79; S, 9.42.

4.1.3.2. 2',4'-Difluoro-4-hydroxy-N'-[(pyridin-2-yl)methylidene]biphenyl-3-carbohydrazide (**3b**)

Light yellow solid; Yield: 80%; HPLC: t_R (min.): 2.76; Mp 241 ^oC; FTIR (cm^{-1}): 3254 (O-H&N-H), 3076, 3045 (=C-H arom.), 1639 (C=O), 1614 (C=N); ^{1}H-NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7.10 (d, 1H, *J*=8.5 Hz, H_5); 7.22 (t, 1H, H_{11}); 7.35-8.65 (m, 8H, Ar-H); 8.48 (s, 1H, CH=N); 11.85 (s, 1H, Ar-OH); 12,07 (s, 1H, CO-NH-N=CH); Anal. Calcd for $C_{19}H_{13}F_2N_3O_2$: C, 64.59; H, 3.71; N, 11.89; found C, 64.32; H, 3.78; N, 11.80.

.

4.1.3.3. 2',4'-Difluoro-4-hydroxy-N'-[(pyridin-3-yl)methylidene]biphenyl-3-carbohydrazide (**3c**)

Off-white solid; Yield: 88%; HPLC: t_R (min.): 2.39; Mp 277-278 ^oC; FTIR (cm^{-1}): 3242 (O-H&N-H), 3041, 3032 (=C-H arom.), 1629 (C=O), 1608 (C=N); ^{1}H-NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7,06 (d, 1H, *J*=8.7 Hz, H_5); 7,22 (t, 1H, H_{11}); 7,39 (t, 1H, H_9); 7.43-8.65 (m, 6H, Ar-H); 8.02 (s, 1H, H_2); 8.88 (s, 1H, CH=N); 11.92 (s, 1H, Ar-OH); 12.05 (s, 1H, CO-NH-N=CH); Anal. Calcd for $C_{19}H_{13}F_2N_3O_2$: C, 64.59; H, 3.71; N, 11.89; found C, 63.75; H, 3.76; N, 11.74.

4.1.3.4. 2',4'-Difluoro-4-hydroxy-N'-[(pyridin-4-yl)methylidene]biphenyl-3-carbohydrazide (**3d**)

Off-white solid; Yield: 86%; HPLC: t_R (min.): 2.51; Mp 261-263 ^oC; FTIR (cm^{-1}): 3251 (O-H&N-H), 3036 (=C-H arom.), 1646 (C=O), 1608 (C=N); ^{1}H-NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7,11 (d, 1H, *J*=8.7 Hz, H_5); 7.22 (t, 1H, H_{11}); 7.37 (t, 1H, H_9); 7.59-7.71 &8,66-8,68 (m, 6H, Ar-H); 8.02 (s, 1H, H_2); 8.44 (s, 1H, CH=N); 12.12 (s, 2H, Ar-OH &CO-NH-N=CH); DART-MS; (m/z, Calcd./Found): 354,1048/354,1047 $[C_{19}H_{13}F_2N_3O_2+H]^+$; 707.2024/707.2009 $[2(C_{19}H_{13}F_2N_3O_2)+H]^+$; Anal. Calcd for $C_{19}H_{13}F_2N_3O_2$: C, 64.59; H, 3.71; N, 11.89; found C, 64.40; H, 4.06; N, 11.67.

4.1.3.5. 2',4'-Difluoro-4-hydroxy-N'-[(5-methylthiophen-2-yl)methylidene]biphenyl-3-carbohydrazide (**3e**)

Light brown solid; Yield: 95%; HPLC: t_R (min.): 9.03; Mp 242 ^oC; FTIR (cm^{-1}): 3246 (O-H&N-H), 3076, 3047 (=C-H arom.), 1639 (C=O), 1610 (C=N); ^{1}H-NMR (300 MHz), (CD_3COCD_3-d_6/TMS) δ ppm: 2.52 (s, 3H, methyl); 6.87 (d, 1H, *J*=3.6 Hz, H_4, thiophene); 7.08 (d, 1H, *J*=8.7 Hz, H_5); 7.22 (t, 1H, H_{11}); 7.30 (d, 1H, *J*=3.6 Hz, H_3, thiophene); 7.38 (t, 1H, H_9); 7.56-7.64 (m, 2H, Ar-H); 7.99 (s, 1H, H_2); 8.59 (s, 1H, CH=N); 11.78 (s, 1H, Ar-OH); 11.99 (s, 1H, CO-NH-N=CH); ^{13}C-NMR (150 MHz) (DMSO-d_6/TMS) δ ppm: 15.86, 104.93,112.47, 116.79,118.02,124.54, 125.50, 126.90, 129.17, 132.16, 132.39, 134.45, 136.93, 143.95, 144.70 (-N=<u>C</u>H), 158.66&160.31 (C-F), 159.11, 161.14&162.77 (C-F), 164.59 (-<u>C</u>=O); Anal. Calcd for $C_{19}H_{14}F_2N_2O_2S$: C, 61.28; H, 3.79; N, 7.52; S, 8.61; found C, 60.78; H, 3.66; N, 7.33; S, 9.45.

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4.1.3.6. 2',4'-Difluoro-4-hydroxy-N'-[(3-bromophenyl)methylidene]biphenyl-3-carbohydrazide (**3f**)

White solid; Yield: 81%; HPLC: t_R (min.): 4.75; Mp 262 ^oC; FTIR (cm^{-1}): 3232 (O-H&N-H), 3047 (=C-H arom.), 1606 (C=O), 1606 (C=N); ^{1}H-NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7.09 (d, 1H, *J*=8.4Hz, H_5); 7.21 (t, 1H, H_{11}); 7.37 (t, 1H, H_9); 7.42-7.95 (m, 6H, Ar-H); 8.01 (s, 1H, H_2); 8.41 (s, 1H, CH=N); 11.94 (2s, 2H, CO-NH-N=CH&Ar-OH). ^{13}C-NMR (150 MHz) (DMSO-d_6/TMS) δ ppm: 104.92, 112.49, 117.04, 118.08, 122.64, 124.50, 125.61, 126.85, 129.54, 129.77, 131.53, 132.15, 133.30, 134.57, 136.98, 147.49 (-N=CH),

158.65&160.29 (C-F); 158.80, 161.13&162.77 (C-F); 164.79 (-C=O); Anal. Calcd for $C_{20}H_{13}BrF_2N_2O_2$: C, 55.70; H, 3.04; N, 6.50; found C, 55.34; H, 2.93; N, 6.47.

4.1.3.7. 2',4'-Difluoro-4-hydroxy-N'-[(2-chlorophenyl)methylidene]biphenyl-3-carbohydrazide (**3g**)

Light yellow solid; Yield: 86%; HPLC: t_R (min.): 9.65; Mp 269-270 ^oC; FTIR (cm^{-1}): 3261 (O-H&N-H), 3078 (=C-H arom.), 1639 (C=O), 1614 (C=N); ^{1}H-NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7.06 (d, 1H, *J*=8.4Hz, H_5); 7.22 (t, 1H, H_{11}); 7.39 (t, 1H, H_9); 7.33-7.70 (m, 5H, Ar-H); 8.02 (s, 1H, H_2); 8.84 (s, 1H, CH=N); 12.28 (s, 2H, CO-NH-N=CH&Ar-OH). ^{13}C-NMR (75 MHz) (DMSO-d_6/TMS) δ ppm: 104.90, 112.57, 116.73, 118.28, 124.60, 124.77, 125.05, 127.49, 128.13, 129.27, 130.43, 131.88, 132.17, 133.80, 134.60, 144.91 (-N=CH), 157.91&160,33 (C-F), 159.98, 161.20&163.44 (C-F), 165.38 (-C=O); Anal. Calcd for $C_{20}H_{13}ClF_2N_2O_2$: C, 62.11; H, 3.39; N, 7.24; found C, 61.39; H, 3.23; N, 7.08.

4.1.3.8. 2',4'-Difluoro-4-hydroxy-N'-[(2-chloro-6-fluorophenyl)methylidene]biphenyl-3-carbohydrazide (**3h**)

Light yellow solid; Yield: 78%; HPLC: t_R (min.): 9.38; Mp 260 ^oC; FTIR (cm^{-1}): 3227 (O-H&N-H), 3080, 3051 (=C-H arom.), 1647 (C=O), 1602 (C=N); ^{1}H-NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7.09 (d, 1H, *J*=8.4 Hz, H_5); 7.22 (t, 1H, H_{11}); 7.33-7.70 (m, 6H, Ar-H); 8.01 (s, 1H, H_2); 8.69 (s, 1H, CH=N); 11.92 (s, 1H, Ar-OH); 12.11 (s, 1H, CO-NH-N=CH); Anal. Calcd for $C_{20}H_{12}ClF_3N_2O_2$: C, 59.35; H, 2.99; N, 6.92; found C, 58.74; H, 2.92; N, 6.75.

4.1.3.9. 2',4'-Difluoro-4-hydroxy-N'-[(2,6-dichlorophenyl)methylidene]biphenyl-3-carbohydrazide (**3i**)

Dark yellow solid; Yield: 95%; HPLC: t_R (min.): 9.76; Mp 208 ^oC; FTIR (cm^{-1}): 3244 (O-H&N-H), 3074, 3032 (=C-H arom.), 1637 (C=O), 1608 (C=N); ^{1}H-NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7.10 (d, 1H, *J*=8.7 Hz, H_5); 7.22 (t,1H, H_{11}); 7.36 (t, 1H, H_9); 7.42-7.67 (m, 5H, Ar-H); 8.02 (s, 1H, H_2); 8.66 (s, 1H, CH=N); 11.87 (s, 1H, Ar-OH); 12.16 (s, 1H, CO-NH-N=CH); Anal. Calcd for $C_{20}H_{12}Cl_2F_2N_2O_2$: C, 57.03; H, 2.87; N, 6.65; found C, 56.92; H, 2.79; N, 6.62.

4.1.3.10. 2',4'-Difluoro-4-hydroxy-N'-[(2,6-difluorophenyl)methylidene]biphenyl-3-carbohydrazide (**3j**)

Off-white solid; Yield: 90%; HPLC: t_R (min.): 8.89; Mp 280 ^oC; FTIR (cm^{-1}): 3232 (O-H&N-H), 3046, 3022 (=C-H arom.), 1647 (C=O), 1608 (C=N); ^{1}H-NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7.09 (d, 1H, *J*=8.7 Hz, H_5); 7.23-7.66 (m, 7H, Ar-H); 8.01 (s, 1H, H_2); 8.61 (s, 1H, CH=N); 12.16 (s, 2H, Ar-OH&CO-NH-N=CH). HRMS (EI, m/z): monoisotopic mass for $C_{20}H_{12}F_4N_2O_2$ (Calcd. / Found): 388.0834/ 388.0842; Anal. Calcd for $C_{20}H_{12}F_4N_2O_2$: C, 61.86; H, 3.11; N, 7.07; found C, 61.16; H, 2.98; N, 7.07.

4.1.3.11. 2',4'-Difluoro-4-hydroxy-N'-[(3,4-dichlorophenyl)methylidene]biphenyl-3-carbohydrazide (**3k**)

Off-white solid; Yield: 82%; HPLC: t_R (min.): 9.53; Mp 258-260 ^oC; FTIR (cm^{-1}): 3255 (O-H&N-H), 3076, 3037 (=C-H arom.), 1639 (C=O), 1614 (C=N); ^{1}H-NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7.10 (d, 1H, *J*=8.7 Hz, H_5); 7.21 (t, 1H, H_{11}); 7.38 (t, 1H, H_9); 7.57-8.01 (m, 4H, Ar-H); 7.75 (s, 1H, H_{14}); 7.78 (s, 1H, H_2); 8.43 (s, 1H, CH=N); 11.96 (s, 1H, Ar-OH); 12.03 (s, 1H, CO-NH-N=CH); Anal. Calcd for $C_{20}H_{12}Cl_2F_2N_2O_2$: C, 57.03; H, 2.87; N, 6.65; found C, 56.66; H, 3.00; N, 6.63.

4.1.3.12. 2',4'-Difluoro-4-hydroxy-N'-[(5-ethylthiophen-2-yl)methylidene]biphenyl-3-carbohydrazide (**3l**)

Dark brown solid; Yield: 93%; HPLC: t_R (min.): 9.28; Mp 196 ^oC; FTIR (cm^{-1}): 3228, 3213 (O-H&N-H), 3074 (=C-H arom.), 1635 (C=O), 1606 (C=N); ^{1}H-NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 1.25 (t, 3H, methyl); 2.83 (q, 2H, ethyl); 6.89-7.65 (m, 7H, Ar-H); 8.00 (s, 1H, H_2); 8.58 (s, 1H, CH=N); 11.79 (s, 1H, Ar-OH); 12.11 (s, 1H, CO-NH-N=CH); Anal. Calcd for $C_{20}H_{16}F_2N_2O_2S$: C, 62.16; H, 4.17; N, 7.25; S: 8.30; found C, 61.95; H, 4.27; N, 6.90; S, 9.18.

4.1.3.13. 2',4'-Difluoro-4-hydroxy-N'-[(4-cyanophenyl)methylidene]biphenyl-3-carbohydrazide (**3m**)

Light yellow solid; Yield: 82%; HPLC: t_R (min.): 8.23; Mp 284-285 ^oC; FTIR (cm^{-1}): 3227 (O-H&N-H), 3066, 3022 (=C-H arom.), 1633 (C=O), 1612 (C=N); ^{1}H-NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7.11 (d, 1H, *J*=8.4Hz, H_5); 7.22 (t, 1H, H_{11}); 7.39 (t, 1H, H_9); 7.57-7.94 (m, 6H, Ar-H); 8.01 (s, 1H, H_2); 8.52 (s, 1H, CH=N); 12.06 (s, 2H, CO-NH-N=CH&Ar-OH). ^{13}C-NMR (75 MHz) (DMSO-d_6/TMS) δ ppm: 104.95, 112.62, 117.16, 118.01, 119.10, 124.44 (CN), 124.62, 125.64, 128.25, 129.65, 132.10, 133.63, 134.63, 139.02, 147.20 (-N=CH); 157.93&160.26 (C-F); 159.74, 161.21&163.94 (C-10); 164.80 (-C=O); Anal. Calcd for $C_{21}H_{13}F_2N_3O_2$: C, 66.84; H, 3.47; N, 11.14; found C, 66.30; H, 3.44; N, 11.05.

4.1.3.14. 2',4'-Difluoro-4-hydroxy-N'-[(2,2-difluoro-1,3-benzodioxol-5-yl)methylidene] biphenyl-3-carbohydrazide (**3n**)

White solid; Yield: 82%; HPLC: t_R (min.): 9.64; Mp 244-245 ^oC; FTIR (cm^{-1}): 3244 (O-H&N-H), 3080, 3024 (=C-H arom.), 1641 (C=O), 1583 (C=N); ^{1}H-NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7.11 (d, 1H, *J*=8.5Hz, H_5); 7.22 (t, 1H, H_{11}); 7.38 (t, 1H, H_9); 7.51-7.65 (m, 5H, Ar-H); 8.02 (s, 1H, H_2); 8.47 (s, 1H, CH=N); 11.94 (s, 2H, CO-NH-N=CH&Ar-OH). ^{13}C-NMR (150 MHz) (DMSO-d_6/TMS) δ ppm: 104.93, 108.04, 110.95, 112.41, 116.95, 118.07, 124.51, 125.31, 125.45, 129.97& 131.95&133.31 (C-F); 124.55, 131.71, 132.14, 134.52, 143.83, 144.42, 147.33 (-N=CH); 158.66&160.30 (C-F); 159.10, 161.12. 162.80 (C-F), 164.79 (-C=O); Anal. Calcd for $C_{21}H_{12}F_4N_2O_4$: C, 58.34; H, 2.80; N, 6.48; found C, 57.86; H, 2.73; N, 6.37.

4.1.3.15. 2',4'-Difluoro-4-hydroxy-N'-{[3-(trifluoromethyl)methylidene}biphenyl-3-carbohydrazide (**3o**)

White solid; Yield: 92%; HPLC: t_R (min.): 9.72; Mp 221 ^oC; FTIR (cm^{-1}): 3257 (O-H&N-H), 3047 (=C-H arom.), 1641 (C=O), 1608 (C=N); ^{1}H-NMR (500 MHz), (DMSO-d_6/TMS) δ ppm: 7.09 (d, 1H, *J*=8.7 Hz, H_5); 7.20 (t, 1H, H_{11}); 7.35 (t, 1H, H_9); 7.57-8.04 (m, 6H, Ar-H); 8.08 (s, 1H, H_2); 8.54 (s, 1H, CH=N); 12.02 (s, 2H, CO-NH-N=CH&Ar-OH) (exchangeable with D_2O). ^{13}C-NMR (125 MHz) (DMSO-d_6/TMS) δ ppm: 105.16, 112.78, 117.34, 118.25, 121.45&123.62&125.78&127.95 (-CF_3); 123.87, 124.76, 125.86, 127.23, 129.83, 130.28,

130.79, 131.89, 132.38, 134.79, 135.97, 147.71 (-N=CH); 158.74&160.71 (C-F), 159.02, 161.22. 163.18 (C-F), 165.05 (-C=O). HRMS (EI,m/z): monoisotopic mass for $C_{21}H_{13}F_5N_2O_2$ (Calcd./Found): 420.0897/ 420.0894; Anal. Calcd for $C_{21}H_{13}F_5N_2O_2$: C, 60.01; H, 3.12; N, 6.66; found C, 59.69; H, 3.45; N, 6.57.

4.1.3.16. 2',4'-Difluoro-4-hydroxy-N'-{[4-(trifluoromethyl)phenyl]methylidene}biphenyl-3-carbohydrazide (**3p**)

Yellow solid; Yield: 81%; HPLC: t_R (min.): 9.69; Mp 260 ^oC; FTIR (cm^{-1}): 3261 (O-H&N-H), 3074, 3049 (=C-H arom.), 1633 (C=O), 1616 (C=N); ^{1}H-NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7.10 (d, 1H, *J*=8.4Hz, H_5); 7.22 (t, 1H, H_{11}); 7.39 (t, 1H, H_9); 7.57-7.97 (m, 6H, Ar-H); 8.02 (s, 1H, H_2); 8.54 (s, 1H, CH=N); 11.91 (s, 1H, Ar-OH) 12.04 (s, 1H, CO-NH-N=CH); Anal. Calcd for $C_{21}H_{13}F_5N_2O_2$: C, 60.01; H, 3.12; N, 6.66; found C, 59.58; H, 3.01; N, 6.64.

4.1.3.17. 2',4'-Difluoro-4-hydroxy-N'-[(napthalen-1-yl)methylidene]biphenyl-3-carbohydrazide (**3r**)

Yellow solid; Yield: 80%; HPLC: t_R (min.): 9.41; Mp 266-268 ^oC; FTIR (cm^{-1}): 3265 (O-H&N-H), 3078, 3047 (=C-H arom.), 1637 (C=O), 1612 (C=N); ^{1}H-NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7.13 (d, 1H, *J*=8.4Hz, H_5); 7.24 (t, 1H, H_{11}); 7.41 (t, 1H, H_9); 7.60-8.95 (m, 10H, Ar-H); 9.11 (s, 1H, CH=N); 12.01 (s, 2H, Ar-OH&CO-NH-N=CH); Anal. Calcd for $C_{24}H_{16}F_2N_2O_2$: C, 71.64; H, 4.01; N, 6.96; found C, 71.34; H, 4.07; N, 6.98.

4.1.3.18. 2',4'-Difluoro-4-hydroxy-N'-[(napthalen-2-yl)methylidene]biphenyl-3-carbohydrazide (**3s**)

Light yellow solid; Yield: 89%; HPLC: t_R (min.): 9.88; Mp 279 ^oC; FTIR (cm^{-1}): 3244 (O-H&N-H), 3047, 3039 (=C-H arom.), 1651 (C=O), 1606 (C=N); ^{1}H-NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7.10 (d, 1H, *J*=8.4Hz, H_5); 7.22 (t, 1H, H_{11}); 7.38 (t, 1H, H_9); 7.57-8.05 (m, 9H, Ar-H); 8.25(s, 1H, H_2); 8.61 (s, 1H, CH=N); 11.95 (s, 1H, Ar-OH&CO-NH-N=CH). ^{13}C-NMR (150 MHz) (DMSO-d_6/TMS) δ ppm: 104.93, 112.49, 116.99, 118.06,

123.19, 124.52, 127.29, 127.76, 128.27, 128.86, 129.04, 129.41, 129.54, 132.15, 132.21, 132.27, 133.27, 134.32, 134.54, 149.32 (-N=CH), 158.67&160.14, 159.04, 160.31&162.77, 164.87 (-C=O). DART-MS; (m/z, Calcd /Found): 403.1253/403.1258 $[C_{24}H_{16}F_2N_2O_2+H]^+$; 805.2432/805.2445 $[2(C_{24}H_{16}F_2N_2O_2)+H]^+$; Anal. Calcd for $C_{24}H_{16}F_2N_2O_2$: C, 71.64; H, 4.01; N, 6.96; found C, 71.64; H, 3.82; N, 6.86.

4.1.3.19. 2',4'-Difluoro-4-hydroxy-N'-[(3-phenoxyphenyl)methylidene]biphenyl-3-carbohydrazide (**3t**)

Yellow solid; Yield: 85%; HPLC: t_R (min.): 9.21; Mp 198 ^oC; FTIR (cm^{-1}): 3257 (O-H&N-H), 3064, 3020 (=C-H arom.), 1635 (C=O), 1614 (C=N); ^{1}H-NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 6.98-7.64 (m, 14H, Ar-H); 8.01 (s, 1H, H_2); 8.44 (s, 1H, CH=N); 11.82 (s, 1H, Ar-OH); 11.94 (s, 1H, CO-NH-N=CH); Anal. Calcd for $C_{26}H_{18}F_2N_2O_3$: C, 70.26; H, 4.13; N, 6.32; found C, 69.90; H, 4.13; N, 6.32.

4.2. Biological studies

4.2.1.1. Cell Culture

Huh7/Rep-Feo1b and Huh7.5-FGR-JC1-Rluc2A replicon reporter cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 5% antibiotic and 0.5 mg/ mL G418. All cells were cultured at 37^{o}C and 5% CO_2.

4.2.1.2. HCV Replicon Based Luciferase Reporter Assays

The Huh7/Rep-Feo1b and Huh7.5-FGR-JC1-Rluc2A replicon reporter cells have been described previously [20, 21]. To evaluate the anti-HCV activity of the compounds, HCV replicon reporter cells were seeded in 96 well plate at a confluence of $1x10^4$cells/well. Eight hours post seeding, the cells were treated with the individual compounds (100 µM/well) or equivalent amounts of DMSO for 42 h. Cell viability was measured in the parental Huh7.5 cells by the colorimetric MTS assay employing the CellTiter 96AQueous One Solution assay reagent (Promega, USA). The anti-HCV activity of the compounds was evaluated as the relative levels of the luciferase signals in compound treated cells versus DMSO controls.

4.2.1. 3. NS5B inhibition assay

The biological activity of the compounds against NS5B polymerase were evaluated in a reaction buffer containing 20 mM Tris-HCl (pH 7.0),100 mM NaCl, 100 mM sodium glutamate, 0.1 mM DTT, 0.01% BSA,0.01% Tween-20, 5% glycerol, 20 U/mL of RNase Out, 0.25 µM of polyrA/U12, 25 µM UTP, 2 µCi [alpha-^{32}P]UTP, 300 ng of NS5BCΔ21and 1.0 mM MnCl2 with or without inhibitors (100 µM) in a total volume of 25 µL for 1 h at 30^{o}C as previously described [26, 27]. Reactions were terminated by the addition of ice-cold 5% (v/v) trichloroacetic acid(TCA) containing 0.5 mM pyrophosphate. Reaction products were precipitated on GF-B filters and quantified on a liquid scintillation counter. NS5B activity in the presence of DMSO control was set at 100% and that in the presence of the compounds was determined relative to this control.

4.2.2. Anticancer activity against hepatocelluler cell lines

4.2.2.1. Cell culture

Human liver cancer cell lines were grown in the standard growth medium medium (2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 units/mL penicillin, 100 lg/mL streptomycin, 10% FCS in DMEM (Gibco, Invitrogen) or RPMI (Gibco, Invitrogen) and incubated at 37 ^{o}C under 5% CO_2 .

4.2.2. 2. Sulforhodamine B (SRB) assay for cytotoxicity screening

Human liver cancer cells were cultured in 96-well plates (1000-3000 cell/well) and for 24h. They were treated with increasing concentrations of the compounds (2.5–40 µM). Cell were washed with 1xPBS ($CaCl_2$-, $MgCl_2$-free) (Gibco, Invitrogen) at the end of 72h incubation period. Fixation was performed using 10% (v/v) trichloroacetic acid (MERCK). Finally, 0.4% (m/v) of sulforhodamine (Sigma–Aldrich) in 1% acetic acid solution was added to each well for staining process. The bound sulforhodamine B was then solubilized usin 10 mM Tris-base. The absorbance values were obtained at 515 nm.

4.2.2. 3. Hoechst stain

Human liver cancer cells (Huh7 and Mahlavu) were inoculated in 6-well plates for 24h. The cells were treated with IC_{50} concentrations of the compounds for 72 h. Hoescht 33258 (Sigma–Aldrich) staining was used to determine nuclear condensation. Cells were fixed with 1 mL of cold methanol and the samples were incubated with 3 lg/mL of Hoescht, and examined under fluorescent microscopy (40x).

4.2.2.4.Western blotting

Human liver cancer cells were cultured in 100mm culture dish. 24h later, growth medium was replaced and cells were treated with IC_{50} concentrations of the compounds or DMSO controls. At the end of 72h incubation, samples were scraped and collected for western blot analysis. Anti-PARP antibody (Cell Signaling, 9532) and anti-β-actin antibody (Sigma, 5441) were used as primary antibodies. Anti-rabbit (6154) and anti-mouse (0168) secondary antibodies were used.

4.2.2.5. Cell cycle analysis

Cells were treated with IC_{50} concentrations of the compounds or 72h. Then samples were stained with propidium iodide which binds to DNA and analyzed with MUSE Cell Cycle analyzer kit.

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Captions of the Figures

Fig. 1. Diflunisal derivatives .

Fig. 2. Hydrazones of anti-HCV activity.

Fig. 3. Cytotoxicity induced by the compounds on liver cancer cell lines (Huh7, HepG2, Hep3B, Mahlavu, FOCUS and SNU-475). The cells were treated with increased concentration of the compound (2.5–40 μM) for 72h. NCI-60 SRB assay was then performed. Absorbance values were obtained and normalized to DMSO control. The experiment was conducted in triplicate.

Fig. 4. Nuclear Hoechst (33258) staining (40x) of liver cancer cells treated with IC_{50} concentrations of the compounds or DMSO control for 72h.

Fig. 5. Cell cycle distribution of liver cancer cells (Huh7 and Mahlavu) treated with IC_{50} concentrations of the compounds or DMSO control for 72h.

Fig. 6. Western blot results of liver cancer cells treated with IC_{50} concentrations of the compounds or DMSO control for 72h.

Captions of the Tables

Table 1. Anti-HCV activity of diflunisal hydrazide-hydrazones **(3a-t)**

Table 2. IC_{50} values of **3b** on liver cancer cell lines.

Captions of the Schemes

Scheme 1. The synthetic route for the preparation of the target compounds **(3a-t)**

Graphical abstract

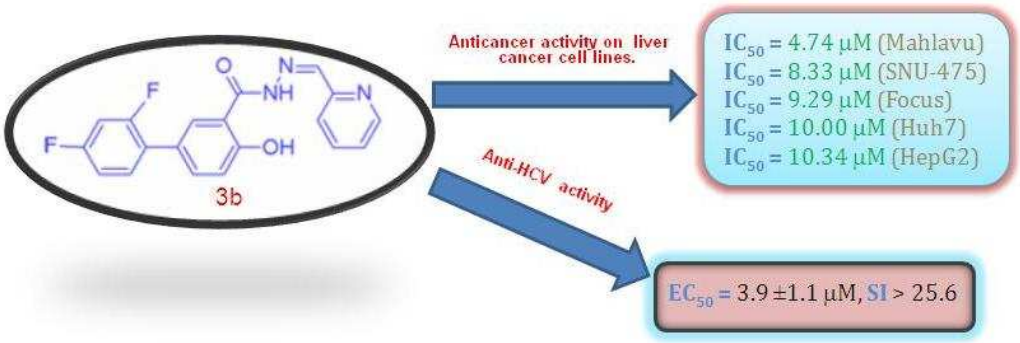


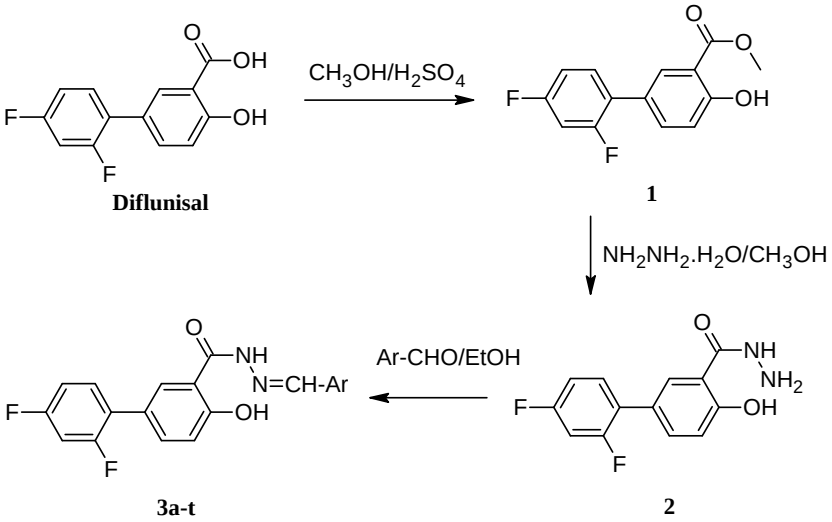
Table 1. Anti-HCV activity of diflunisal hydrazide-hydrazones **(3a-t)**

Comp.	CC_{50} (μM)[a]	Huh7.5-FGR-JC1-Rluc2A (% inhibition)[b]	Huh7/Rep-Feo1b			Anti-NS5B Activity (% Inh., 50 μM)[f]
			(Inhibition%)[c]	EC_{50} (μM)[d]	SI[e]	
3a	>50	98 ± 1	91 ± 2	15.6 ± 1.1	>3.2	17 ± 8
3b	>100	90 ± 3	79 ± 3	3.,9 ± 1.1	>25.6	NI
3c	>200	88 ± 10	83 ± 6	8.,1 ± 3.0	>24.7	8 ± 5
3d	>50	23 ± 13	33 ± 2	ND	ND	7 ± 4
3e	>50	95 ± 2	64 ± 2	ND	ND	8 ± 3
3f	<50	92 ± 5	81 ± 4	ND	ND	9 ± 2
3g	<50	78 ± 11	90 ± 2	ND	ND	7 ± 4
3h	>50	74 ± 14	83 ± 2	3.9 ± 1.,7	>12.8	11 ± 9
3i	<50	96 ± 3	72 ± 5	ND	ND	NI
3j	>100	72 ± 15	69 ± 2	ND	ND	NI
3k	<50	95 ± 2	66 ± 3	ND	ND	NI
3l	>50	96 ± 2	65 ± 2	ND	ND	NI
3m	>200	82 ± 4	77 ± 5	11.8 ± 0.5	>16.9	NI
3n	<50	95 ± 2	65 ± 1	ND	ND	NI
3o	<50	99 ± 1	87 ± 3	ND	ND	18 ± 4
3p	<50	99 ± 1	86 ± 3	ND	ND	12 ± 6
3r	>200	70 ± 10	79 ± 3	16.5 ± 4.2	>6.1	NI
3s	>100	58 ± 11	65 ± 13	ND	ND	NI
3t	<50	99 ± 1	92 ± 2	ND	ND	14 ± 2

[a]CC_{50} values were evaluated in Huh 7.5 parental cells by the MTS assay. Anti-HCV activity of the compounds was determined at 50 μM against Huh7.5-FGR-JC1-Rluc2A[b] and Huh7/Rep-Feo1b[c] replicon reporter cells. [d]EC50 values were computed from dose-response curves at 8-10 concentrations of the compounds employing CalcuSyn V2 software. Cells treated with equal amounts of DMSO served as control in all experiments. [e]SI: selectivity index represents the ratio of CC_{50} to EC_{50}. The values shown are an average of three independent experiments with standard deviation. [f]Percent inhibition of HCV NS5B RdRp activity was determined at 50 μM concentration of the indicated compound. The data represents an average of three independent measurements. NS5B RdRp activity in the absence of the inhibitor was taken as 100 percent after subtraction of residual background activity. NI, no inhibition; ND, not determined.

Table 2. IC_{50} values of **3b** on liver cancer cell lines.

Cell line	Diflunisal	3b
	IC_{50} (μM)	IC_{50} (μM)
Huh7	NO INHIBITION	10
HepG2	NO INHIBITION	10.34
Hep3B	NO INHIBITION	16.21
Mahlavu	51.7	4.74
Focus	29.8	9.29
SNU-475	NO INHIBITION	8.33



Scheme 1. The synthetic route for the preparation of the target compounds **(3a-t)**

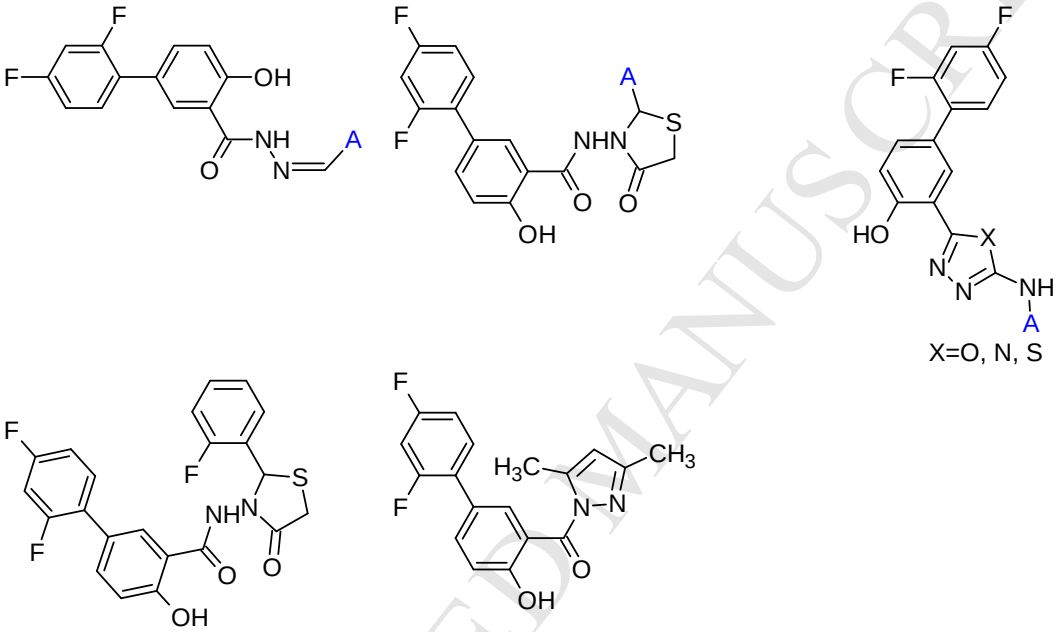


Fig. 1. Diflunisal derivatives

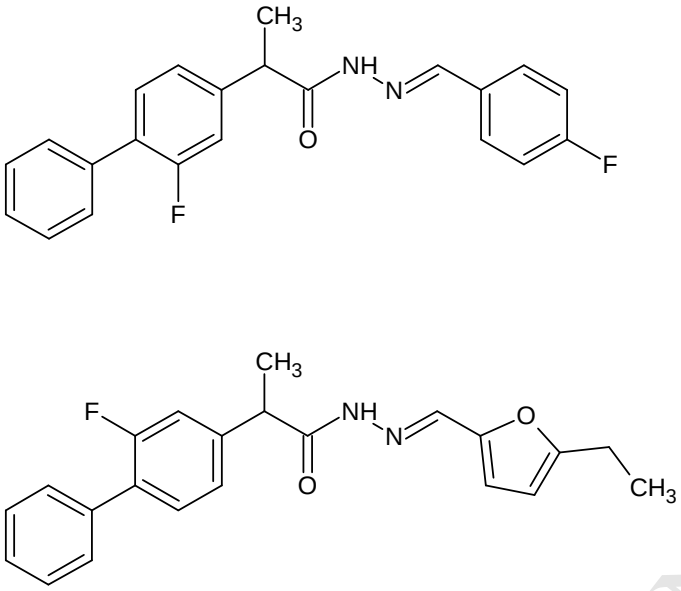


Fig. 2. Hydrazones of anti-HCV activity.

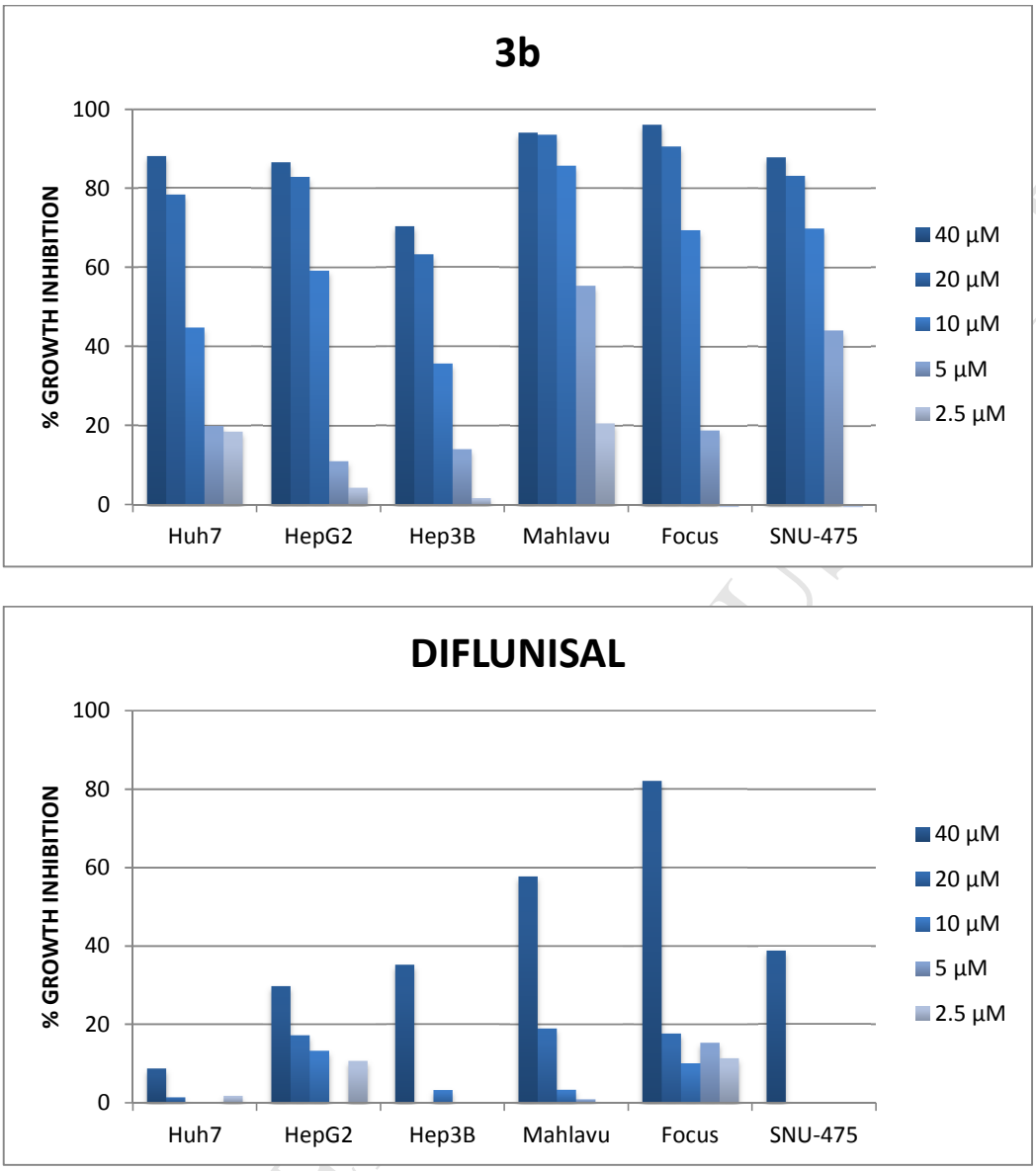


Fig. 3. Cytotoxicity induced by the compounds on liver cancer cell lines (Huh7, HepG2, Hep3B, Mahlavu, FOCUS and SNU-475). The cells were treated with increased concentration of the compound (2.5–40 µM) for 72h. NCI-60 SRB assay was then performed. Absorbance values were obtained and normalized to DMSO control. The experiment was conducted in triplicate.

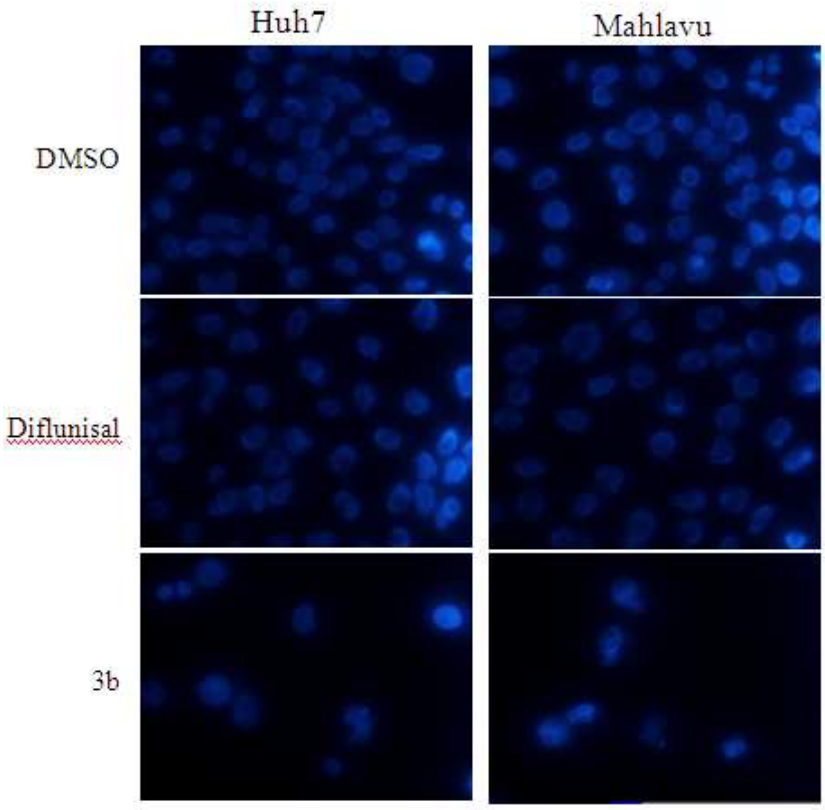


Fig. 4. Nuclear Hoechst (33258) staining (40x) of liver cancer cells treated with IC_{50} concentrations of the compounds or DMSO control for 72h.

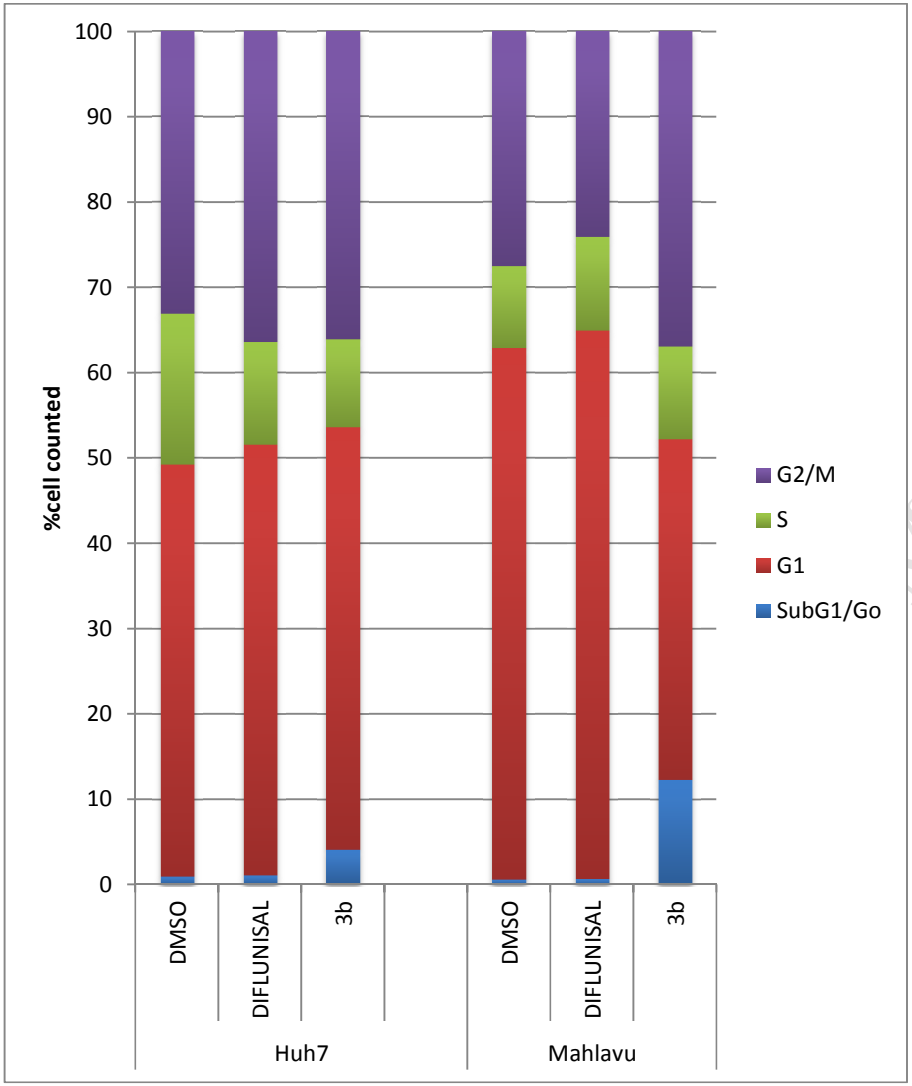


Fig. 5. Cell cycle distribution of liver cancer cells (Huh7 and Mahlavu) treated with IC_{50} concentrations of the compounds or DMSO control for 72h.

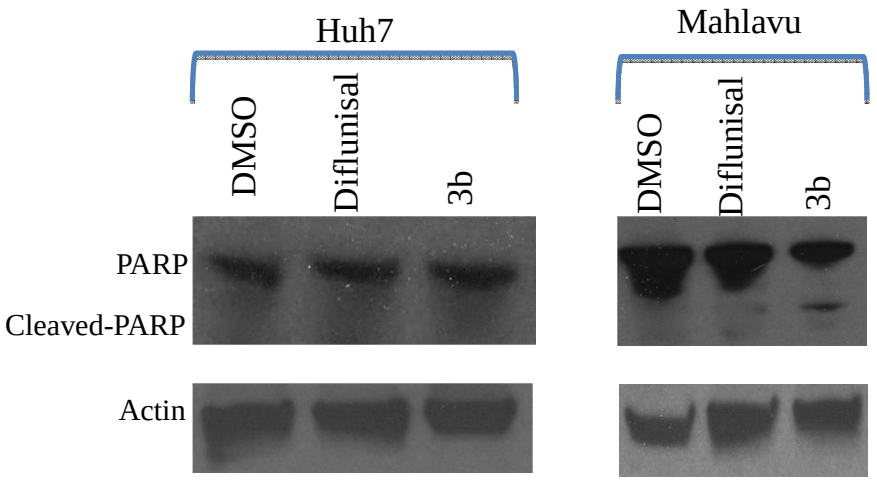


Fig. 6. Western blot results of liver cancer cells treated with IC_{50} concentrations of the compounds or DMSO control for 72h.

Highlights

- We synthesized new diflunisal hydrazide-hydrazones
- We investigated their activity against hepatitis C virus (HCV)
- Some compounds exhibited strong inhibition against HCV 1b and 2a genotypes
- The hydrazide-hydrazone derivatives were shown to inhibit cellular growth of liver cancer cells