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# 2-Heteroarylimino-5-arylidene-4-thiazolidinones as a new class of non-nucleoside inhibitors of HCV NS5B polymerase

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ABSTRACT

Hepatitis C virus (HCV) NS5B polymerase is an important and attractive target for the development of anti-HCV drugs. Here we report on the design, synthesis and evaluation of twenty-four novel allosteric inhibitors bearing the 4-thiazolidinone scaffold as inhibitors of HCV NS5B polymerase. Eleven compounds tested were found to inhibit HCV NS5B with $IC_{50}$ values ranging between 19.8 and 64.9 μM. Compound **24** was the most active of this series with an $IC_{50}$ of 5.6 μM. A number of these derivatives further exhibited strong inhibition against HCV 1b and 2a genotypes in cell based antiviral assays. Molecular docking analysis predicted that the thiazolidinone derivatives bind to the NS5B thumb pocket-II (TP-II). Our results suggest that further optimization of the thiazolidinone scaffold may be possible to yield new derivatives with improved enzyme- and cell-based activity.



## 1. Introduction

Hepatitis C virus (HCV) is an important human pathogen of global public health significance with an estimated 3% of the world population infected [1,2]. The enveloped, ~9.6 kb positive-stranded RNA virus encodes for a large polyprotein of ~3010 amino acids in length [3,4]. Persistent HCV infection is the leading cause of severe hepatitis that often progresses to cirrhosis, steatosis, and hepatocellular carcinoma [5–7]. HCV is also a lymphotropic virus causing several lymphoproliferative disorders such as cryoglobulinemia, B-cell non-Hodgkin's lymphoma and monoclonal gammopathies [8].

To date, there is no prophylactic vaccine against HCV. The emergence of FDA approved HCV protease inhibitors Boceprevir (Victrelis®) and Telaprevir (Incivek®) has significantly improved sustained virological response in treating HCV genotype 1 infections [9–11]. These HCV protease inhibitors block the enzymatic activity of the NS3-4A viral protease, thereby inhibiting the protease from cleaving the HCV polyprotein into individual replicase components [9–11]. However, these inhibitors must be used in combination with pegylated-interferon (IFN) and ribavirin to avoid selection for protease resistant HCV variants. Both IFN-related side effects and a complicated dosing regimen that may limit patient compliance remain major concerns in current HCV therapeutics [9–11]. Therefore, the search for novel directly acting antivirals with reduced side-effects and high therapeutic index that target multiple HCV genotypes continues to be an area of high priority.

Most small molecule inhibitor approaches to HCV have focused upon inhibition of essential viral targets, such as the NS3-4A protease (analogous to HIV protease) [12]. Additionally, the HCV non-structural protein 5B (NS5B) represents an attractive target for anti-HCV agents for several reasons. NS5B encodes the viral RNA dependent RNA polymerase (RdRp) and is a critical component of the viral replication machinery [13–15]. Pharmacologic agents that inhibit NS5B function block replication of viral RNA in tissue culture [16–18]. Additionally, mammalian cells lack proteins with RdRp activity. Therefore, molecules that target NS5B are not likely to exhibit toxicity in host cells. The structure of the 66 kDa NS5B has been well characterized. NS5B has the characteristic "right hand" structure of the polymerase family and contains a catalytic site in

*Abbreviations:* AP, allosteric pocket; HCV, hepatitis C virus; NI, nucleoside inhibitor; NNI, non-nucleoside inhibitor; PP-I, palm pocket-I; RdRp, RNA-dependent RNA polymerase; SP, subpocket; TP, thumb pocket.

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the palm domain and RNA interacting regions of the RdRp in the finger and thumb domains [19–22]. Current NS5B inhibitors have been grouped into two broad categories. Nucleoside inhibitors (NIs) mimic rNTPs substrates resulting in chain termination when incorporated into elongating HCV RNA. Non-nucleoside inhibitors (NNIs) are a diverse set of small molecule inhibitors that function by binding NS5B in one of five allosteric pockets located on the thumb and palm domains [12,17,18]. Presumably, the binding of NNIs to allosteric pockets results in a conformational change that inhibits NS5B enzymatic activity. However, current research cannot rule out the possibility that these inhibitors have additional mechanisms that block NS5B function.

Thiazolidinone compounds possess versatile biological activity including antiproliferative, antibacterial, antifungal, and anti-mycobacterial functions [23–27]. These compounds are also known to target cyclooxygenase (COX) enzymes making thiazolidinone compounds excellent candidates to develop as anti-tumor and anti-inflammatory agents [25,26]. Further, thiazolidone compounds have been shown to inhibit the HIV-1 reverse transcriptase [28–30]. Because these compounds showed antiviral activity, we originally investigated thiazolidinone compounds for the ability to inhibit the HCV NS5B polymerase. We reported compounds bearing the thiazolidinone motif that non-competitively inhibited HCV NS5B through binding to an allosteric site within the viral RdRp [31]. We concluded that these compounds have the potential to be developed into more potent HCV NS5B NNIs.

A literature survey on HCV NS5B polymerase inhibitors clearly indicates that 4-thiazolidinones could inhibit this enzyme and might be promising candidates for the development of novel antiviral agents against HCV. The 4-thiazolidinone scaffold appears in the form of 2,3-diaryl/2-aryl-3-acylamino-4-thiazolidinones ($A_1$, $A_2$) [31,32]; 2-amino-5-arylidene-4-thiazolinones ($B_1$, $B_2$) [33,34]; or 3-substituted-2-thioxo-4-thiazolidinones ($C_1$, $C_2$) (Fig. 1) [35,36]. As can be seen from Fig. 1, 5-arylidene derivatives (B and C type compounds) inhibit NS5B at lower $IC_{50}$ values. These findings encouraged us to go further with our ongoing studies on 4-thiazolidinones derivatives. In this study, we designed a new class of potential inhibitors by the modification of the compounds presented in Fig. 1. Design strategy included: *i.* protection of the 4-thiazolidinone core; *ii.* introduction of an arylidene moiety at C5 position of the core; *iii.* introduction of arylimino moiety at C2 position of the core instead of aryl, amino or thioxo functionality; and *iv.* keeping the N3 position of the core unsubstituted. Here, we report the synthesis, anti-NS5B activity and anti-HCV activity of these new derivatives. We employed molecular modeling to predict the binding site of the thiazolidinone compounds on NS5B polymerase. This study represents an important step in developing these compounds as NS5B inhibitors.

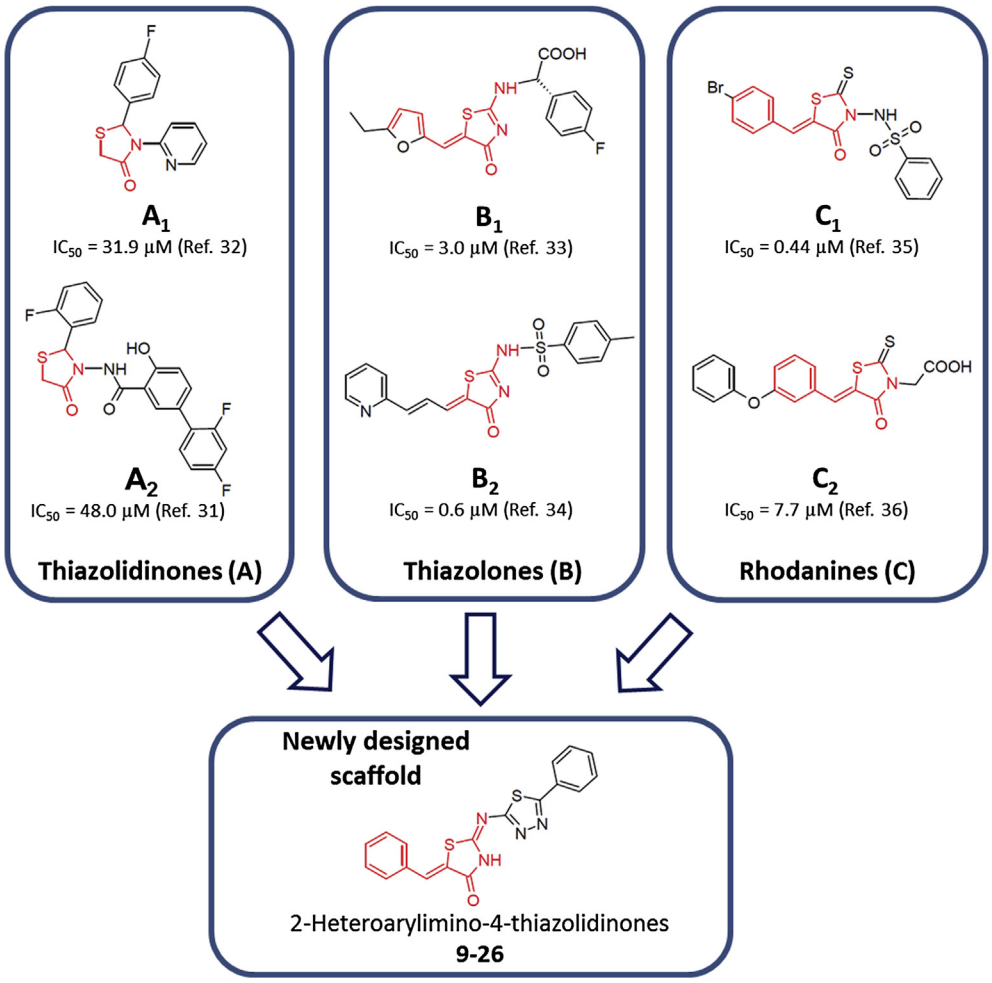



**Fig. 1.** Structures of existing thiazolidinones (A), thiazolones (B) and rhodanines (C) as inhibitors of HCV NS5B and the newly designed 5-arylidene-2-heteroarylimino-4-thiazolidinones.

## 2. Results and discussion

### 2.1. Chemistry

The target compounds **9**–**26** were synthesized using a stepwise reaction protocol starting from thiosemicarbazide and 4-chloro/4-fluorobenzoyl chloride at first step (Scheme 1). Resulting 1-aroylthiosemicarbazides **1** and **2** were then subjected to cyclization using concentrated sulfuric acid to give 2-amino-5-aryl-1,3,4-thiadiazoles **3** and **4**. Synthesis of 2-chloro-*N*-[5-(4-chloro/fluorophenyl)-1,3,4-thiadiazol-2-yl]acetamides **5** and **6** were carried out by the reaction of the appropriate amines **3** and **4** with chloroacetylchloride in the presence of TEA and DCM at room temperature. Cyclization of chloroacetamides **5** and **6** in the presence of ammonium thiocyanate in refluxing ethanol afforded 2-[[5-aryl-1,3,4-thiadiazol-2-yl]imino]-1,3-thiazolidine-4-ones **7** and **8** in good yield. The target compounds **9**–**26** were obtained refluxing a methanolic solution of 4-thiazolidinones **7** or **8** with commercially available aldehydes in the presence of sodium methoxide. This method was different from the one described by Vicini et al. who reacted 4-thiazolidinones with aldehydes in acetic acid buffered medium with sodium acetate [37]. However, use of sodium methoxide in methanolic medium instead of sodium acetate in acetic acid, provided shorter reaction times [38].

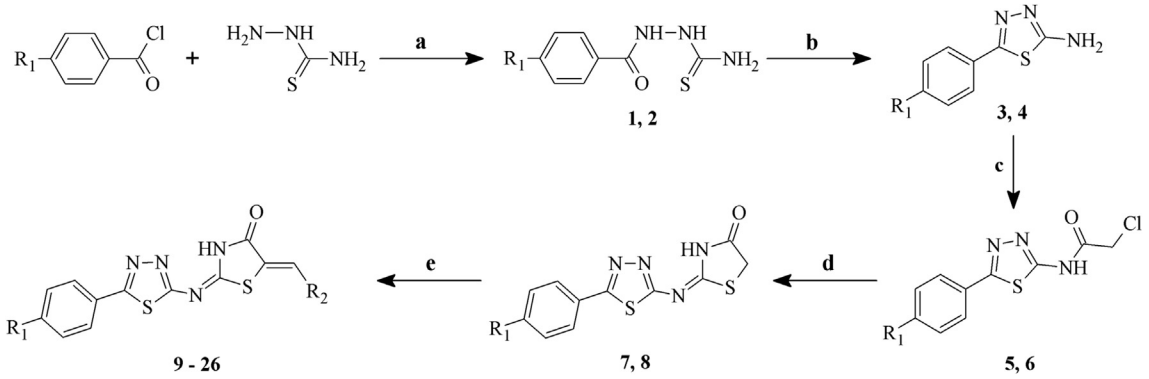
All synthesized compounds were checked for purity using HPLC–UV/DAD and elemental analysis. The new compounds **7**–**26** were characterized by their melting points and spectral data ($^1$H NMR, $^{13}$C NMR and HR-MS). Absorption bands at 1720 cm$^{-1}$ were attributed to the C=O stretching bands of both 1,3-thiazolidine-4-one compounds **7** and **8** provided confirmatory evidence for ring closure [24]. $^1$H NMR chemical shifts were recorded within expected regions for all synthesized compounds. Support for 4-thiazolidinone ring closure was the detection of signals at 4.12 and 4.04 ppm, due to the presence of endocyclic –S–$CH_2$– protons [39]. Determination of endocyclic –NH– protons of compounds **7** and **8** at 12.22 and 12.38 ppm influenced in favor of lactam proton owing to the fact that an imine proton resonates around 9.70 ppm [37,40]. Disappearance of signals of endocyclic –S–$CH_2$– protons in the spectra of compounds **9**–**26** indicates that active methylene group of 4-thiazolidinones **7**–**8** reacted with the selected aldehydes to yield 5-arylidene-1,3-thiazolidine-4-one derivatives **9**–**26**. Assignment of NH peaks of compounds **7**–**26** at 12.07–13.66 ppm as one signal may impute to predominant existence of only one isomer on C=N imino exocyclic double bond [27]. Compounds **9**–**26** exhibited arylidene Ar–CH=C< resonances at 7.70–7.98 ppm. A survey of literature revealed that; Ar–CH=C< proton, deshielded by the adjacent C=O, was detected at 7.63–7.97 ppm in $^1$H NMR spectra in respect of thermodynamically stable *Z* configuration of exocyclic C=C bond [25,38,41–43]. $^{13}$C NMR data of the thiazolidinone compounds **7** and **8** have also supported the carbon framework [24,44]. The carbonyl carbon of the thiazolidinone ring appeared at 174.57 ppm, the signal of the thiazolidinone $C_5$ appeared at about 36 ppm and the signal at about 166 ppm is attributed to $C_2$ of thiazolidinone ring. Earlier reports reveal that, distinctive methylidene carbon of arylidene moiety showed resonance around 120–130 ppm and the mentioned carbon was evaluated between 133 and 145 ppm for our representative compounds **9**–**11**, **14**, **17**–**20**, **24**–**25** [45–47].

### 2.2. Structure–activity relationship

The HCV NS5B RdRp inhibitory activity of the synthesized compounds **3**–**26** is shown in Table 1. Synthetic precursors 1,3,4-thiadiazole-2-amines **3**–**4** and chloroacetamides **5**–**6** showed very low inhibition (15.4–20.0%) and their $IC_{50}$ values were not determined. This clearly indicates the necessity of 4-thiazolidinone ring attached to 1,3,4-thiadiazole ring with imino linkage. Compounds **7** and **8**, which bear C5 unsubstituted thiazolidinone ring, showed modest activity profiles. However, the most active compounds were observed to have an arylidene moiety at C5 of thiazolidinone ring. This clearly indicates the contribution of these arylidene groups for stabilizing the binding mode to NS5B active site.

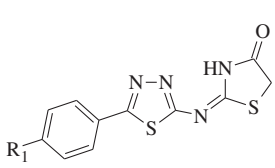
Thiadiazolylimino-4-thiazolidinone backbone was chosen as template for the development of new HCV NS5B inhibitors. We preferred 4-fluorophenyl and 4-chlorophenyl moieties at C5 of 1,3,4-thiadiazoles **7**–**8** as a starting point. The first 4-thiazolidinone compounds **7**–**8** were identified as NS5B inhibitors with $IC_{50}$ values of 38.6 and 42.5 μM, respectively. Therefore, we decided to perform a SAR survey around **9**–**26** to explore the contribution of benzylidene moiety introduced at C5 of 4-thiazolidione ring. Compounds **9**–**17** had a 4-fluorophenyl moiety whereas this fluorine atom was replaced with chlorine in **18**–**26**. Most of the 2-thiadiazolylimino-5-arylidene-4-thiazolidinones **9**–**26** exhibited appreciable inhibition of HCV NS5B polymerase at 100 μM ranging between 11.5 and 82.0 percent. Among the compounds tested, eleven derivatives were shown to exhibit greater than 50% NS5B RdRp inhibition at 100 μM concentration and $IC_{50}$ values of these derivatives were determined.

Among **9**–**17** series, four representatives exhibited higher than 50% inhibition; $IC_{50}$ values of these derivatives ranged between 19.8

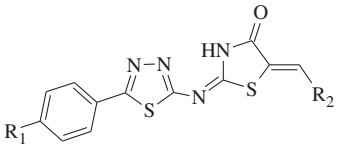



**Scheme 1.** General procedure for the synthesis of compounds **1**–**26**. Reagents and conditions: (a) $NaHCO_3$, acetone, 0 °C to rt; (b) conc. $H_2SO_4$, rt, 4 h; (c) $ClCOCH_2Cl$, TEA, DCM, 50 °C, 2 h; (d) $NH_4SCN$, ethanol, reflux, 6 h; (e) $R_2$-CHO, $CH_3ONa$, MeOH, reflux, 4 h.

**Table 1**
Anti-NS5B RdRp activity and calculated lipophilicity values of compounds **3**–**26**.



**7-8**



**9-26**

| Compound | $R_1$ | $R_2$ | Log *P*[a] | % Inhibition[b] | $IC_{50}$ (μM) |
|---|---|---|---|---|---|
| **3** | F | – | 1.75 | 15.4 | n.d. |
| **4** | Cl | – | 2.34 | 16.8 | n.d. |
| **5** | F | – | 2.38 | 16 | n.d. |
| **6** | Cl | – | 2.6 | 20 | n.d. |
| **7** | F | – | 1.69 | 76.1 | 38.6 ± 0.9 |
| **8** | Cl | – | 2.24 | 74.4 | 42.5 ± 1.6 |
| **9** | F | 2-Fluorophenyl | 3.99 | 62.4 | 51.1 ± 1.3 |
| **10** | F | 3-Fluorophenyl | 3.9 | 72 | 34.6 ± 1.6 |
| **11** | F | 4-Fluorophenyl[c] | 3.88 | n.d. | n.d. |
| **12** | F | 2-Chloro-6-fluorophenyl | 4.45 | 28.1 | n.d. |
| **13** | F | 2-Chlorophenyl | 4.28 | 53.9 | 64.9 ± 0.7 |
| **14** | F | 2,4-Dichlorophenyl | 4.59 | 11.5 | n.d. |
| **15** | F | 2,6-Dichlorophenyl | 4.67 | 74.1 | 19.8 ± 1.5 |
| **16** | F | 2,6-Dimethoxyphenyl | 3.9 | 19.7 | n.d. |
| **17** | F | 4-Nitrophenyl | 3.73 | 24.1 | n.d. |
| **18** | Cl | 2-Fluorophenyl | 4.25 | 56.2 | 56.2 ± 4.9 |
| **19** | Cl | 3-Fluorophenyl | 4.26 | 65.5 | 48.2 ± 1.3 |
| **20** | Cl | 4-Fluorophenyl | 4.27 | 25 | n.d. |
| **21** | Cl | 2-Chloro-6-fluorophenyl | 4.85 | 60.3 | 45.3 ± 1.1 |
| **22** | Cl | 2-Chlorophenyl | 4.68 | 73.6 | 33.8 ± 1.9 |
| **23** | Cl | 2,4-Dichlorophenyl | 4.9 | 25.7 | n.d. |
| **24** | Cl | 2,6-Dichlorophenyl | 4.95 | 82 | 5.6 ± 1.1 |
| **25** | Cl | 2,6-Dimethoxyphenyl | 4.26 | 24.1 | n.d. |
| **26** | Cl | 4-Nitrophenyl | 4.2 | 33 | n.d. |

[a] Log *P* values were calculated using ALOGPS 2.102 Log *P*/Log *S* calculation software http://www.vcclab.org.

[b] Percent inhibition was determined at 100 μM concentration of the indicated compound and represents an average of at least two independent measurements in duplicate. NS5B RdRp activity in the absence of the inhibitor was taken as 100 percent after subtraction of residual background activity. The $IC_{50}$ values of the compounds were determined from dose–response curves using 8–12 concentrations of each compound in duplicate in two independent experiments. Curves were fitted to data points using nonlinear regression analysis and $IC_{50}$ values were interpolated from the resulting curves using GraphPad Prism 3.03 software. n.d., not determined.

[c] Exhibited solubility issues.

and 64.9 μM. Of the compounds **18**–**26**, five compounds exceeded 50% inhibition and their $IC_{50}$ values ranged between 5.6 and 56.2 μM. Fluorine substitution of the benzylidene moiety gave the best result in compound **10** which had a 4-fluorophenyl substitution at C5 of 1,3,4-thiadiazole ring with an $IC_{50}$ value of 34.6 μM. In both **9**–**17** and **18**–**26** series, a shift of fluorine atom to 2 or 4-position resulted in decrease in activity. Simultaneous fluorine and chlorine substitution of the benzylidene moiety resulted in zero or marginal inhibition of NS5B. Three types of chlorine substitution were attempted in compounds **13**–**15** and **22**–**24** and best positioning was observed to be 2,6-dichloro substitution as evidenced by compound **24**, which had an $IC_{50}$ value of 5.6 μM. This compound was the most potent representative of all the synthesized compounds **7**–**26**. Replacing the 4-chlorophenyl of compound **24** with 4-fluorophenyl whilst keeping 2,6-dichlorobenzylidene constant resulted in the second most active compound of this study which had an $IC_{50}$ value of 19.8 μM.

Introduction of benzylidene groups with 2,6-dimethoxy or 4-nitro substitution resulted in marginal or complete loss of activity. It was also noteworthy that the most active derivatives **15** and **24** were the ones with highest Log *P* values of their series **9**–**17** ($R_1$ = F) and **18**–**26** ($R_2$ = F), respectively. These studies revealed the influence of two different 4-halogenophenyl groups at C5 position of thiadiazole and several benzylidene moieties at C5 position of 4-thiazolidinone ring. Compounds **15** and **24** possessing 2,6-dichlorobenzylidene moiety were proven as promising lead compounds for further development.

### 2.3. Correlation between Log P and $IC_{50}$ values

The lipophilicity of a molecule represented by its Log *P* value is a valuable index utilized in rational drug design to predict the physicochemical properties of the molecule in terms of drug absorption, bioavailability, and hydrophobic drug–receptor interactions. In order to gain insight into the lipophilicity of the 4-thiazolidinone derivatives, we calculated their Log *P* values using ALOGPS 2.102 Log *P*/Log *S* calculation software (http://www.vcclab.org) as previously described [48,49] and then examined the relationship between the 4-thiazolidinone-mediated inhibition of HCV NS5B ($IC_{50}$ values) and their Log *P* values (Table 1). Compounds **3** to **8** exhibited low lipophilicity with Log *P* values between 1.7 and 2.4, compounds **9**, **10**, **11**, **16** and **17** with Log *P* between 3.7 and 4.0 exhibited moderate lipophilicity, while the remaining 4-thiazolidinone derivatives exhibited relatively higher lipophilicity with Log *P* values ranging from 4.3 to 4.9. Compound **24**, the most active compound of this series ($IC_{50}$ = 5.6 μM) exhibited the highest lipophilicity, while paradoxically, compound **23** with similar lipophilicity was a poor inhibitor of HCV NS5B exhibiting only 26% inhibition at 100 μM. Similarly, while compound **7**, the least lipophilic compound of this series (Log *P* = 1.7) displayed modest inhibition of HCV NS5B ($IC_{50}$ = 38.6 μM), compound **3** with similar Log *P* of 1.7 was the least active NS5B inhibitor among these derivatives. Thus, no clear cut correlation emerged between the lipophilicity of these compounds and their properties as NS5B inhibitors.

### 2.4. High throughput cell-based screening of compounds

Three reporter cell lines BHK-NS5B-FRLuc, Huh7/Rep-Feo1b and Huh7.5-FGR-JC1-Rluc2A were employed in a preliminary screen to investigate the cytotoxicity and anti-HCV activities of the compounds at 100 μM (Table 2) [50–53]. The BHK-NS5B-FRLuc carrying a bicistronic reporter gene (+)FLuc-(−)UTR-RLuc and stably transfected NS5B, permitted the measure of cellular viability by the firefly luciferase luminescence, and intracellular HCV NS5B RdRp activity as a ratio of *Renilla to* firefly luciferase luminescence, thus facilitating the simultaneous identification of potent non-toxic HCV NS5B RdRp inhibitors [50]. Further screening of these compounds as anti-HCV agents was carried out in the Huh7/Rep-Feo1b and Huh7.5-FGR-JC1-Rluc2A cells, which carry the autonomously replicating HCV RNA of genotype 1b and 2a in the firefly and *Renilla* luciferase reporters, respectively [51].

In the BHK-NS5B-FRLuc reporter cells, the twenty-three compounds displayed a wide range of cytotoxicity (0–100%) and inhibition of intracellular NS5B RdRp activity (0–85%) at 100 μM concentration (Table 2). Of these, four compounds (**14**, **21**–**23**) displayed no cytotoxicity or inhibition, suggesting that these compounds may not be cell permeable, while five others (compounds **5**, **6**, **12**, **24** and **25**) displayed >85% cytotoxicity and were not investigated for their inhibition parameters. Compounds **16** and **20** exhibited a similar pattern with ~70% cytotoxicity and no inhibition, while **9** and **15** displayed a somewhat similar trend in terms of inhibition of 11–14%, though the cytotoxicity varied between 56 and 72%. Compound **4** displayed ~29% cytotoxicity but did not inhibit, while **3** and **17** exhibited ~10% inhibition and low toxicity of 0–15%. Compounds **10** and

**Table 2**
Anti-HCV effects in cell based reporter assays.

| Compound | BHK-NS5B FR Luc[a] | | Huh7.5 | 1b Replicon reporter[b] | 2a Replicon reporter[c] |
|---|---|---|---|---|---|
| | Cytotoxicity (%) | Inhibition (%) | Cytotoxicity (%) | Inhibition (%) | Inhibition (%) |
| **3** | 0.0 | 9.4 ± 3.1 | 18.6 ± 1.0 | 46.8 ± 4.2 | 52.4 ± 14.3 |
| **4** | 28.6 ± 5.7 | n.i. | 32.4 ± 0.4 | 37.4 ± 8.7 | 72.8 ± 5.1 |
| **5** | 98.3 ± 0.6 | n.d. | 84.8 ± 6.4 | 99.0 ± 0.3 | 99.4 ± 0.2 |
| **6** | 98.9 ± 0.5 | n.d. | 81.9 ± 7.0 | 99.1 ± 0.1 | 99.3 ± 0.1 |
| **7** | 0.0 | 57.9 ± 7.7 | 14.0 ± 4.1 | 70.0 ± 4.2 | 72.8 ± 5.0 |
| **8** | 15.2 ± 0.8 | 65.7 ± 5.3 | 13.3 ± 1.4 | 54.1 ± 0.8 | 89.8 ± 3.5 |
| **9** | 55.7 ± 8.2 | 11.0 ± 2.9 | 59.9 ± 1.1 | 97.4 ± 0.4 | 96.1 ± 0.6 |
| **10** | 55.1 ± 6.1 | 85.9 ± 4.4 | 59.7 ± 4.5 | 95.5 ± 1.1 | 98.0 ± 0.8 |
| **11**[d] | n.d. | n.d. | n.d. | n.d. | n.d. |
| **12** | 89.8 ± 0.4 | n.d. | 74.6 ± 4.7 | 99.4 ± 0.0 | 98.5 ± 0.3 |
| **13** | 11.4 ± 3.9 | 61.0 ± 1.9 | 15.6 ± 5.1 | 98.0 ± 0.8 | 95.3 ± 4.9 |
| **14** | 0.0 | n.i | 4.2 ± 1.8 | 29.4 ± 2.6 | 54.7 ± 14.1 |
| **15** | 72.2 ± 7.5 | 13.6 ± 3.7 | 73.2 ± 1.0 | 98.8 ± 0.3 | 98.9 ± 0.5 |
| **16** | 73.7 ± 8.6 | n.i | 63.0 ± 4.4 | 87.7 ± 1.4 | 93.2 ± 1.7 |
| **17** | 14.6 ± 5.7 | 7.0 ± 1.8 | 13.9 ± 1.6 | 51.2 ± 6.3 | 50.4 ± 5.2 |
| **18** | 12.8 ± 5.6 | 65.6 ± 5.1 | 13.1 ± 2.8 | 96.4 ± 0.2 | 98.5 ± 0.8 |
| **19** | 58.1 ± 3.3 | 63.1 ± 5.7 | 60.3 ± 1.0 | 92.8 ± 0.4 | 95.6 ± 1.6 |
| **20** | 73.1 ± 5.7 | n.i | 64.2 ± 3.7 | 82.2 ± 6.6 | 94.9 ± 3.6 |
| **21** | 0.0 | n.i. | 0.0 | n.i. | n.i. |
| **22** | 0.0 | n.i. | 0.0 | n.i. | n.i. |
| **23** | 0.0 | n.i. | 0.0 | n.i. | n.i. |
| **24** | 94.9 ± 1.9 | n.d. | 76.0 ± 4.7 | 98.5 ± 0.7 | 98.1 ± 0.7 |
| **25** | 87.4 ± 8.1 | n.d. | 80.4 ± 5.6 | 97.7 ± 0.5 | 98.9 ± 0.5 |

[a] BHK-NS5B-FRLuc; [b] Huh7/Rep-Feo1b; and [c] Huh7.5-FGR-JC1-Rluc2A reporter cells were treated with the indicated compounds at 100 μM concentration for 42 h. Cell viability in the BHK-NS5B-FRLuc reporter was estimated as the relative levels of Firefly luciferase in compound treated cells versus DMSO controls, while that in the Huh7/Rep-Feo1b cells and Huh7.5-FGR-JC1-Rluc2A was evaluated by the MTS assay. The inhibitory effect of the compounds on NS5B RdRp activity and HCV RNA replication is presented as percent of DMSO treated controls. Data represents an average of two independent experiments in duplicate. n.d., not determined; n.i., no inhibition.; [d] Displayed solubility issues.

**19** displayed 60–86% inhibition though the ~55% cytotoxicity remained a concern. The remaining four compounds **7**, **8**, **13**, and **18** emerged the most promising, displaying negligible cytotoxicity (0–15%) and 58–66% inhibition of intracellular NS5B RdRp activity.

In the parental Huh7.5 cells, all compounds exhibited a remarkably similar trend of cytotoxicity (Table 2) to that observed in the BHK cells, thus suggesting that the permeability of the compounds remained similar in these two cell types. Notably, compounds **21**–**23** which displayed no cytotoxicity or inhibition in the BHK-NS5B reporter, displayed an identical pattern in the two replicon reporters, thus reiterating the lack of cell permeability of these compounds. The other compounds exhibited an overall trend of higher inhibition in the two replicon reporters compared to the BHK-NS5B reporter, thus suggesting that the compounds in addition to targeting NS5B polymerase may potentially target other host factors required by HCV for its replication. This was true for all compounds except **8**, which showed a somewhat reduced inhibition in the 1b replicon reporter compared to the NS5B reporter, thus suggesting that the inhibition observed in the 1b replicon reporter may be attributed to inhibition of NS5B polymerase activity alone in these cells. Furthermore, the inhibition of HCV RNA replication was similar in the two replicon reporters for all compounds, except **4**, **8** and **14**, which exhibited 1.6–2-fold higher inhibition of HCV RNA replication in the 2a versus 1b replicon reporter. Interestingly, both **4** and **14** do not inhibit NS5B1b as is evident from the BHK-NS5B reporter data, while **8** inhibits NS5B1b to a comparable extent in the 1b replicon. Therefore, one may speculate that **4** and **14** potentially inhibit the 1b replicon by predominantly targeting some essential host factors required by HCV 1b. In case of 2a replicon, **4** and **14** may in addition display different sensitivities to the NS5B2a polymerase. For compounds **5**, **6**, **12**, **15**, **24** and **25** which exhibit >70% cytotoxicity and near complete inhibition of HCV RNA replication in both reporters, the inhibition observed may be partly attributed to the cellular toxicity effects of these compounds.

These results suggest that the 4-thiazolidinone derivatives inhibit HCV RNA replication by either targeting the NS5B polymerase and/or other host factors required by HCV for its replication. While the exact mechanism needs to be elucidated, the present study provides a platform to further optimize this scaffold and develop them into potent anti-HCV agents.

### 2.5. Molecular modeling

Since some thiazolone-based non-nucleoside inhibitors of HCV NS5B were reported to bind to allosteric pocket located on the thumb domain of NS5B [33], we preferably performed docking studies using thumb pocket-II of NS5B. Glide predicted binding model of the most potent compound **24** within the thumb pocket-II of NS5B polymerase using the co-crystal structure of PF-868554-NS5B (PDB ID: 3FRZ) is shown in Fig. 2 [54]. The 4-chlorophenyl ring is stabilized by hydrophobic interactions with the side chains of Leu419, Met423, Tyr477 and Leu497 and through offset type of aromatic–aromatic interaction with the indole ring of Trp528. The 4-chloro group was found to be involved in electrostatic interactions with the guanidine group of Arg422. The 1,3,4-thiadiazole ring forms aromatic–aromatic interaction with Tyr477. The terminal 2,6-dichlorobenzylidene moiety extends to a hydrophobic channel defined by residues Ile482, Ala486, Leu489, Pro496 and Leu497. One of the chloro atoms on the benzylidene ring seems to be stabilized through the electrostatic interaction with the guanidine group of Arg490. The thiazolidinone ring is anchored by Ile482. In addition to hydrophobic interactions, the N3 and N4 atoms of the thiadiazole ring were found to be located within hydrogen bonding distances from the backbone –NHs of Tyr477 and Ser476, respectively.

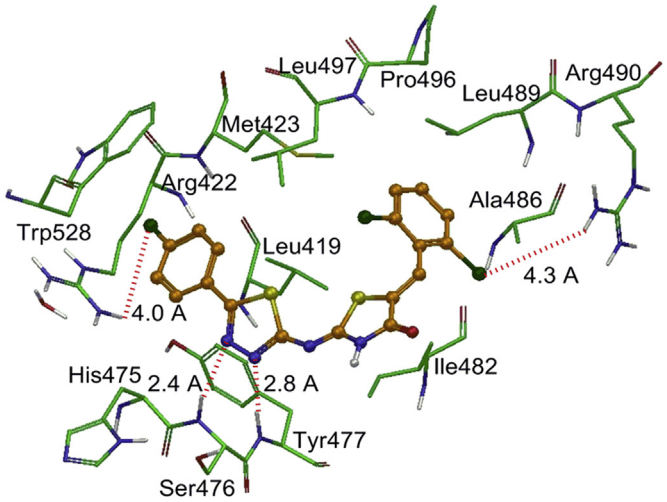



**Fig. 2.** XP-Glide predicted binding model of compound **24** with residues of NS5B TP-II site. Compound **24** is shown as ball and stick model whereas amino acids are shown as stick model. Dotted red lines show electrostatic contacts with distances in Å. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

These binding interactions suggested scope for further optimization of thiazolidinones at the terminal phenyl rings. For example, these phenyl rings may be substituted with di and/or tri-fluoro groups to enhance electrostatic contacts with the guanidine group of Arg422 and Arg490.

## 3. Conclusions

Our efforts to develop potent inhibitors of HCV NS5B polymerase is a preeminent activity in the search to find novel therapies for treating HCV infection. A novel series of 5-arylidene-4-thiazolidinones were synthesized and evaluated as HCV NS5B polymerase inhibitors. Of the twenty-four compounds, eleven derivatives exhibited modest inhibition of NS5B RdRp activity resulting in the identification of 2-{[5-(4-chlorophenyl)-1,3,4-thiadiazol-2-yl]imino}-5-(2,6-dichlorobenzylidene)-1,3-thiazolidin-4-one (**24**) with an $IC_{50}$ value of 5.6 μM as the most active of this series. A number of these derivatives further exhibited strong inhibition against HCV 1b and 2a genotypes in cell based antiviral assays. Binding model of compound **24** with thumb pocket-II of NS5B was also studied. Biological data and molecular modeling outcomes revealed the substantial influence of 5-arylidene moiety upon high inhibition of HCV NS5B enzyme activity. Currently we are working on optimization strategy for anti-NS5B activity of lead compounds which involves (i) modification of 1,3,4-thiadiazole ring of compounds **9**–**26** or replacement of the same ring with other heteroaryl rings and (ii) attachment of various arylmethylidene groups on thiazolidinone ring. These efforts should lead to new analogs with potentially high affinity towards NS5B and low cellular toxicity.

## 4. Experimental

### 4.1. Chemistry

All solvents and reagents were obtained from commercial sources and used without purification. All melting points (°C, uncorrected) were determined using Kleinfeld SMP-II basic model melting point apparatus. Elemental analyses were obtained using Leco CHNS-932 and are consistent with the assigned structures. Infrared spectra were recorded on a Shimadzu FTIR 8400S and data are expressed in wavenumber $\nu$ ($cm^{-1}$). NMR spectra were recorded on Bruker AVANCE-DPX 400 at 400 MHz for $^1H$ NMR and 100 MHz for $^{13}C$ NMR (DEPT and Decoupled), the chemical shifts were expressed in $\delta$ (ppm) downfield from tetramethylsilane (TMS) using DMSO-$d_6$ as solvent. 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 by using a reversed phase Nova-Pak C18 (3.9 × 150 mm, 5 μm particle size) column. All experiments were performed in gradient mode. The mobile phase was prepared by mixing acetonitrile and TEA-pH: 4.51 phosphate buffer (50:50 v/v during 0–3 min, 75:25 v/v during 3–6 min, 100:0 v/v during 6–9 min, 100:0 v/v during 9–15 min, 75:25 v/v during 15–18 min, 50:50 v/v during 18–20 min) and filtered through a 0.45 μm pore filter and subsequently degassed by ultrasonication, prior to use. Solvent delivery was employed at a flow rate of 1 ml $min^{-1}$. Detection of the analytes was carried out at 230, 254, 270, 310 nm.

SMILES were generated from the structures using the ACD/ChemSketch version 12.0 molecular editor (http://www.acdlabs.com) and then Log *P* values were calculated using ALOGPS 2.102 Log *P*/Log *S* calculation software [48,49]. The calculated Log *P* values for all the compounds are given in Table 1.

#### 4.1.1. Synthesis of 1-aroylthiosemicarbazides *1* and *2*

The solution of thiosemicarbazide (10 mmol) in 20 ml acetone was reacted with 4-fluorobenzoyl chloride or 4-clorobenzoyl chloride (10 mmol) in the presence of sodium bicarbonate (10 mmol) with continuous stirring in an ice bath. Acetone was evaporated under vacuo and the solid precipitated was filtered and washed with water and then recrystallized.

*4.1.1.1. 4-Fluorobenzoylthiosemicarbazide* ***1***. It was obtained as a white solid and was recrystallized from ethanol. HPLC $t_R$ (min.): 2.77, Mp: 180 °C (lit. 172 °C) [55].

*4.1.1.2. 4-Chlorobenzoylthiosemicarbazide* ***2***. It was obtained as a white solid and was recrystallized from ethanol. HPLC $t_R$ (min.): 2.91, Mp: 214 °C (lit. 218–220 °C) [55].

#### 4.1.2. Synthesis of 2-amino-5-aryl-1,3,4-thiadiazoles *3* and *4*

1-Aroylthiosemicarbazide (compounds **1** or **2**) was stirred in concentrated $H_2SO_4$ at room temperature for 4 h and the crude product was precipitated by using NaOH solution (10%) and the solid precipitated was filtered and washed with water and then recrystallized.

*4.1.2.1. 2-Amino-5-(4-fluorophenyl)-1,3,4-thiadiazole* ***3***. It was obtained as a white solid and was recrystallized from ethanol. HPLC $t_R$ (min.): 4.09, Mp: 240 °C (lit. 232–234 °C [56]; 230 °C [57]).

*4.1.2.2. 2-Amino-5-(4-chlorophenyl)-1,3,4-thiadiazole* ***4***. It was obtained as a white solid and was recrystallized from ethanol. HPLC $t_R$ (min.): 5.57, Mp: 230 °C (lit. 214–216 °C [56]/226–227 °C [58]).

#### 4.1.3. Synthesis of 2-chloro-N-[5-(4-chloro/fluorophenyl)-1,3,4-thiadiazol-2-yl]acetamides *5* and *6*

The solution of 5-(4-chloro/fluorophenyl)-1,3,4-thiadiazol-2-amine (5 mmol) in 20 ml dichloromethane and 1 ml triethylamine was reacted with chloroacetylchloride (10 mmol) at 50 °C for 2 h. Dichloromethane was evaporated under vacuo and the solid precipitated was filtered and washed with water and then recrystallized.

*4.1.3.1. 2-Chloro-N-[5-(4-fluorophenyl)-1,3,4-thiadiazol-2-yl]acetamide* ***5***. It was obtained as a white solid and was recrystallized from ethanol. HPLC $t_R$ (min.): 6.21, Mp: 252 °C, yield 62%. FTIR ($cm^{-1}$): 3185 (N–H), 3050 (=C–H arom), 1705 (C=O), 1576 (C=N). $^1H$ NMR (300 MHz, DMSO-$d_6$): $\delta$ 4.49 (s, 2H, $-CH_2-$), 7.34–7.41 (m, 2H, Ar–H), 7.98–8.04 (m, 2H, Ar–H), 13.08 (s, 1H, NH) ppm. Anal. Calcd. for $C_{10}H_7ClFN_3OS$: C, 44.21; H, 2.60; N, 15.47; S, 11,80. Found: C, 44.63; H, 2.75; N, 14.92; S, 11.73.

*4.1.3.2. 2-Chloro-N-[5-(4-chlorophenyl)-1,3,4-thiadiazol-2-yl]acetamide* ***6***. It was obtained as a white solid and was recrystallized from ethanol HPLC $t_R$ (min.): 7.59, Mp: 251 °C (lit.243–244 °C [59]).

#### 4.1.4. Synthesis of 2-[[5-(4-chloro/fluorophenyl)-1,3,4-thiadiazol-2-yl]imino]-1,3-thiazolidine-4-ones *7* and *8*

A solution of 2-chloro-*N*-[5-(4-chloro/fluorophenyl)-1,3,4-thiadiazol-2-yl]acetamide (5 mmol) and ammonium thiocyanate (10 mmol) in 20 ml absolute ethanol were refluxed for 6 h and allowed to stand overnight. The precipitate was filtered and washed with water and then recrystallized.

*4.1.4.1. 2-[[5-(4-Fluorophenyl)-1,3,4-thiadiazol-2-yl]imino]-1,3-thiazolidine-4-one* **7**. It was obtained as a brown solid from compound **5** and was recrystallized from ethanol. HPLC $t_R$ (min.): 4.37, Mp: 232 °C, yield 87%. FTIR ($cm^{-1}$): 3128 (N–H lactam), 3057 (=C–H arom), 1720 (C=O lactam), 1591 (C=N). $^1$H NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ 4.04 (s, 2H, thiazolidinone–S**CH$_2$**–), 7.56–7.63 (m, 2H, Ar–H), 8.16–8.21 (m, 2H, Ar–H), 12.22 (s, 1H, thiazolidinone NH) ppm. $^{13}$C NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ ppm 36.44 (thiazolidinone **C$_5$**), 117.03 and 117.33, 127.38, 130.08 and 130.19, 162.55 (Ar–**C**), 163.87 (thiadiazole **C$_2$**), 166.93 (thiazolidinone **C$_2$**), 170.83 (thiadiazole **C$_5$**), 174.57 (thiazolidinone **C$_4$**). Anal. Calcd. for $C_{11}H_7FN_4OS_2$: C, 44.89; H, 2.40; N, 19.04. Found: C, 45.171; H, 2.52; N, 18.71.

*4.1.4.2. 2-[[5-(4-Chlorophenyl)-1,3,4-thiadiazol-2-yl]imino]-1,3-thiazolidine-4-one* ***8***. It was obtained as a brown solid from compound **6** and was recrystallized from ethanol. HPLC $t_R$ (min.): 5.62, Mp: 297 °C, yield 89%. FTIR ($cm^{-1}$): 3105 (N–H lactam), 3055 (=C–H arom), 1720 (C=O lactam), 1591 (C=N). $^1$H NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ 4.12 (s, 2H, thiazolidinone–S**CH$_2$**–), 7.58 (d, $J$ = 8.22 Hz, 2H, Ar–H), 7.91 (d, $J$ = 8.51 Hz, 2H, Ar–H), 12.38 (s, 1H, thiazolidinone NH) ppm. $^{13}$C NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ ppm 36.23 (thiazolidinone **C$_5$**), 129.20, 129.39, 129.91, 136.01 (Ar–**C**), 163.58 (thiadiazole **C$_2$**), 166.89 (thiazolidinone **C$_2$**), 170.83 (thiadiazole **C$_5$**), 174.57 (thiazolidinone **C$_4$**). LCMS (ESI-negative ion): 308, 310 [M − 2H]$^-$. Anal. Calcd. for $C_{11}H_7ClN_4OS_2$: C, 42.51; H, 2.27; N, 18.03. Found: C, 43.11; H, 2.37; N, 18.09.

*4.1.5. Synthesis of 2-[[5-(4-chloro/fluorophenyl)-1,3,4-thiadiazol-2-yl]imino]-5-(substituted benzylidene)-1,3-thiazolidin-4-ones* ***9–26***

Compound **7** or **8** (2 mmol) was dissolved in methanolic solution of sodium methoxide (2 mmol). After dissolution by heating, 2–3 mmol corresponding aldehyde was added and reaction medium was refluxed for 4 h. At the end of reaction, the product was precipitated by pouring the medium into ice-cold water and neutralizing by acetic acid. Crude product was filtered and washed with water and then recrystallized from ethanol.

*4.1.5.1. 2-[[5-(4-Fluorophenyl)-1,3,4-thiadiazol-2-yl]imino]-5-(2-fluorobenzylidene)-1,3-thiazolidin-4-one* ***9***. It was obtained as a brown solid from compound **7** and was recrystallized from ethanol. HPLC $t_R$ (min.): 8.32, Mp: 279 °C (d), yield 68%. FTIR ($cm^{-1}$): 3128 (N–H lactam), 3057 and 3010 (=C–H arom), 1720 (C=O lactam), 1591 (C=N). $^1$H NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ 7.34–7.57 (m, 4H, Ar–H), 7.61–7.66 (m, 2H, Ar–H), 7.75 (s, 1H, =CH–Ar), 7.94–7.99 (m, 2H, Ar–H), 12.07 (s, 1H, thiazolidinone NH) ppm. $^{13}$C NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ ppm 116.60 and 116.84, 117.13, 121.38 and 121.59, 124.14, 125.95, 126.99 and 127.10, 129.89 and 130.01, 138.21, 139.44, 152.22, 154.71, 158.86, 159.34, 162.43, 164.38 (Ar–**C**), 133.28 (thiazolidinone **C$_5$**), 142.09 (=**CH**–Ar), 165.74 (thiadiazole **C$_2$**), 167.16 (thiazolidinone **C$_2$**), 170.11 (thiadiazole **C$_5$**), 172.52 (thiazolidinone **C$_4$**). LCMS (ESI-negative ion): 399, 400 [M − H]$^-$. Anal. Calcd. for $C_{18}H_{10}F_2N_4OS_2$: C, 53.99; H, 2.52; N, 13.99. Found: C, 54.06; H, 2.57; N, 13.73.

*4.1.5.2. 2-[[5-(4-Fluorophenyl)-1,3,4-thiadiazol-2-yl]imino]-5-(3-fluorobenzylidene)-1,3-thiazolidin-4-one* ***10***. It was obtained as a brown solid from compound **7** and was recrystallized from ethanol. HPLC $t_R$ (min.): 8.39, Mp: 276–277 °C (d), yield 77%. FTIR ($cm^{-1}$): 3112 (N–H lactam), 3066 and 3022 (=C–H arom), 1720 (C=O lactam), 1602 and 1593 (C=N). $^1$H NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ 7.32–7.66 (m, 6H, Ar–H), 7.76 (s, 1H, =CH–Ar), 7.95–7.99 (m, 2H, Ar–H), 13.05 (s, 1H, thiazolidinone NH) ppm. $^{13}$C NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ ppm 116.84, 117.13, 117.31 and 117.60, 126.10 and 126.97, 129.91 and 130.02, 135.86, 158.90, 161.11, 162.43 (Ar–**C**), 131.89 (thiazolidinone **C$_5$**), 138.58 (=**CH**–Ar), 164.35 (thiadiazole **C$_2$**), 165.73 (thiazolidinone **C$_2$**), 167.26 (thiadiazole **C$_5$**), 170.12 (thiazolidinone **C$_4$**). LCMS (ESI-negative ion): 399, 400 [M − H]$^-$. Anal. Calcd. for $C_{18}H_{10}F_2N_4OS_2$: C, 53.99; H, 2.52; N, 13.99. Found: C, 53.27; H, 2.83; N, 14.23.

*4.1.5.3. 2-[[5-(4-Fluorophenyl)-1,3,4-thiadiazol-2-yl]imino]-5-(4-fluorobenzylidene)-1,3-thiazolidin-4-one* ***11***. It was obtained as a dark green solid from compound **7** and was recrystallized from ethanol. HPLC $t_R$ (min.): 8.58, Mp: 305 °C (d), yield 69%. FTIR ($cm^{-1}$): 3113 (N–H lactam), 3057 and 3012 (=C–H arom), 1716 (C=O lactam), 1585 (C=N). $^1$H NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ 7.35–7.45 (m, 4H, Ar–H), 7.71–7.75 (m, 2H, Ar–H), 7.78 (s, 1H, =CH–Ar), 7.96–8.00 (m, 2H, Ar–H), 12.07 (s, 1H, thiazolidinone NH) ppm. $^{13}$C NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ ppm 116.84 and 116.92, 117.14 and 117.21, 124.07, 127.01, 129.91, 130.02 and 130.22, 132.32, 138.47 and 138.52, 139.18, 159.13 (Ar–**C**), 133.23 (thiazolidinone **C$_5$**), 139.18 (=**CH**–Ar), 164.25 (thiadiazole **C$_2$**), 165.73 (thiazolidinone **C$_2$**), 167.40 (thiadiazole **C$_5$**), 170.19 (thiazolidinone **C$_4$**). LCMS (ESI-negative ion): 399, 400 [M − H]$^-$. Anal. Calcd. for $C_{18}H_{10}F_2N_4OS_2$: C, 53.99; H, 2.52; N, 13.99. Found: C, 54.62; H, 2.49; N, 13.89.

*4.1.5.4. 2-[[5-(4-Fluorophenyl)-1,3,4-thiadiazol-2-yl]imino]-5-(2-chloro-6-fluoro-benzylidene)-1,3-thiazolidin-4-one* ***12***. It was obtained as a brown solid from compound **7** and was recrystallized from ethanol. HPLC $t_R$ (min.): 8.52, Mp: 287 °C (d), yield 76%. FTIR ($cm^{-1}$): 3120 (N–H lactam), 3068 and 3018 (=C–H arom), 1722 (C=O lactam), 1591 (C=N). $^1$H NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ 7.37–7.62 (m, 5H, Ar–H), 7.71 (s, 1H, =CH–Ar), 7.97–8.01 (m, 2H, Ar–H), 13.11 (s, 1H, thiazolidinone NH) ppm. LCMS (ESI-negative ion): 433, 434 [M − H]$^-$. Anal. Calcd. for $C_{18}H_9ClF_2N_4OS_2$ C, 49.71; H, 2.09; N, 12.88. Found: C, 50.08; H, 2.26; N, 12.58.

*4.1.5.5. 2-[[5-(4-Fluorophenyl)-1,3,4-thiadiazol-2-yl]imino]-5-(2-chlorobenzylidene)-1,3-thiazolidin-4-one* ***13***. It was obtained as a black solid from compound **7** and was recrystallized from ethanol. HPLC $t_R$ (min.): 9.14, Mp: 299 °C (d), yield 61%. FTIR ($cm^{-1}$): 3147 (N–H lactam), 3076 and 3036 (=C–H arom), 1720 (C=O lactam), 1599 and 1589 (C=N). $^1$H NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ 7.36–7.53 (m, 2H, Ar–H), 7.55–7.69 (m, 4H, Ar–H), 7.71 (s, 1H, =CH–Ar), 7.92–8.02 (m, 2H, Ar–H), 13.13 (s, 1H, thiazolidinone NH) ppm. LCMS (ESI-negative ion): 415, 416 [M − H]$^-$. Anal. Calcd. for $C_{18}H_{10}ClFN_4OS_2$ C, 51.86; H, 2.42; N, 13.44. Found: C, 52.28; H, 2.44; N, 13.09.

*4.1.5.6. 2-[[5-(4-Fluorophenyl)-1,3,4-thiadiazol-2-yl]imino]-5-(2,4-dichlorobenzylidene)-1,3-thiazolidin-4-one* ***14***. It was obtained as a dark brown solid from compound **7** and was recrystallized from ethanol. HPLC $t_R$ (min.): 9.72, Mp: 318 °C (d), yield 70%. FTIR ($cm^{-1}$): 3129 (N–H lactam), 3064 and 3028 (=C–H arom), 1720 (C=O lactam), 1602 and 1577 (C=N). $^1$H NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ 7.41 (t, $J$ = 8.4 Hz, $J$ = 8.4 Hz, 2H, Ar–H), 7.71 (s, 2H, =CH–Ar, Ar–H), 7.85 (d, $J$ = 5.7 Hz, 2H, Ar–H), 7.98–8.03 (q, 2H, Ar–H), 13.22 (s, 1H, thiazolidinone NH) ppm. LCMS (ESI-negative ion): 449, 451 [M − 2H]$^-$. Anal. Calcd. for $C_{18}H_9Cl_2FN_4OS_2$ C, 47.90; H, 2.01; N, 12.41. Found: C, 48.58; H, 2.10; N, 12.36.

*4.1.5.7. 2-[[5-(4-Fluorophenyl)-1,3,4-thiadiazol-2-yl]imino]-5-(2,6-dichlorobenzylidene)-1,3-thiazolidin-4-one* ***15***. It was obtained as a dark brown solid from compound **7** and was recrystallized from ethanol. HPLC $t_R$ (min.): 8.75, Mp: 289 °C (d), yield 64%. FTIR ($cm^{-1}$): 3130 (N–H lactam), 3084 and 3020 (=C–H arom), 1718 (C=O lactam), 1593 (C=N). $^1$H NMR (300 MHz, $CDCl_3$-DMSO-$d_6$):

δ 7.35–7.65 (m, 5H, Ar–H), 7.73 (s, 1H, =CH–Ar), 7.94–7.98 (m, 2H, Ar–H), 13.14 (s, 1H, thiazolidinone NH) ppm. $^{13}$C NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ ppm 116.88, 117.77, 126.94, 128.99, 129.30 and 129.96, 130.08, 131.95, 158.41 (Ar–**C**), 132.37 (thiazolidinone **$C_5$**), 133.56 (=**CH**–Ar), 164.56 (thiadiazole **$C_2$**), 165.98 (thiazolidinone **$C_2$**), 170.14 (thiadiazole **$C_5$**, thiazolidinone **$C_4$**). LCMS (ESI-negative ion): 449, 451 $[M - 2H]^-$. Anal. Calcd. for $C_{18}H_9Cl_2FN_4OS_2$ C, 47.90; H, 2.01; N, 12.41. Found: C, 48.35; H, 2.09; N, 12.27.

*4.1.5.8. 2-[[5-(4-Fluorophenyl)-1,3,4-thiadiazol-2-yl]imino]-5-(4-nitrobenzylidene)-1,3-thiazolidin-4-one* ***16***. It was obtained as a black solid from compound **7** and was recrystallized from ethanol. HPLC $t_R$ (min.): 7.64, Mp: 338 °C (d), yield 71%. FTIR ($cm^{-1}$): 3111 (N–H lactam), 3068 and 3024 (=C–H arom), 1718 (C=O lactam), 1589 (C=N). $^1$H NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): 7.39 (t, $J$ = 8.4 Hz, $J$ = 8.4 Hz, 2H, Ar–H), 7.89–8.02 (m, 5H, =CH–Ar, Ar–H), 8.37–8.56 (m, 2H, Ar–H), 13.66 (s, 1H, thiazolidinone NH) ppm. LCMS (ESI-negative ion): 425, 427 $[M - 2H]^-$. Anal. Calcd. for $C_{18}H_{10}FN_5O_3S_2$ C, 50.58; H, 2.36; N, 16.38. Found: C, 50.67; H, 2.38; N, 15.90.

*4.1.5.9. 2-[[5-(4-Fluorophenyl)-1,3,4-thiadiazol-2-yl]imino]-5-(2,6-dimethoxybenzylidene)-1,3-thiazolidin-4-one* ***17***. It was obtained as a brown solid from compound **7** and was recrystallized from ethanol. Mp: 273 °C (d), yield 65%. FTIR ($cm^{-1}$): 3120 (N–H lactam), 3057 (=C–H arom), 1716 (C=O lactam), 1595 (C=N). $^1$H NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ 4.13 (s, 6H, $-CH_3$), 7.02 (d, $J$ = 8.4 Hz, 2H, Ar–H), 7.59–7.71 (m, 2H, Ar–H), 7.73 (s, 1H, =CH–Ar–H), 8.15–8.25 (m, 3H, Ar–H), 12.95 (s, 1H, thiazolidinone–NH–) ppm. $^{13}$C NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ ppm 56.50 ($-O-CH_3$), 110.61, 117.04 and 117.33, 125.85, 127.18, 127.37 and 127.41, 158.91 (Ar–**C**), 130.26 (thiazolidinone **$C_5$**), 134.04 (=**CH**–Ar), 164.13 (thiadiazole **$C_2$**), 165.89 (thiazolidinone **$C_2$**), 168.08 (thiadiazole **$C_5$**), 170.56 (thiazolidinone **$C_4$**). LCMS (ESI-negative ion): 440, 441 $[M - H]^-$. Anal. Calcd. for $C_{20}H_{15}FN_4O_3S_2$ C, 54.29; H, 3.42; N, 12.66. Found: C, 54.22; H, 3.45; N, 12.20.

*4.1.5.10. 2-[[5-(4-Chlorophenyl)-1,3,4-thiadiazol-2-yl]imino]-5-(2-fluorobenzylidene)-1,3-thiazolidin-4-one* ***18***. It was obtained as a brown solid from compound **8** and was recrystallized from ethanol. HPLC $t_R$(min.): 9.46, Mp: 284 °C (d), yield 78%. FTIR ($cm^{-1}$): 3117 (N–H lactam), 3068 and 3016 (=C–H arom), 1722 (C=O lactam), 1599 (C=N). $^1$H NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ 7.36 (t, $J$ = 7.6 Hz, $J$ = 7.6 Hz, 1H, Ar–H), 7.49 (d, $J$ = 7.1 Hz, 2H, Ar–H), 7.52–7.68 (m, 3H, Ar–H), 7.79 (s, 1H, =CH–Ar), 7.93–7.98 (m, 2H, Ar–H), 13.09 (s, 1H, thiazolidinone NH) ppm. $^{13}$C NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ 117.36, 117.65 and 117.94, 126.11, 129.28 and 129.97, 136.05 and 136.24, 159.14 and 161.13 (Ar–**C**), 132.05 (thiazolidinone **$C_5$**), 145.55 (=**CH**–Ar), 164.37 (thiadiazole **$C_2$**), 167.31 (thiazolidinone **$C_2$**), 170.41 (thiadiazole **$C_5$**), 171.41 (thiazolidinone **$C_4$**) ppm. LCMS (ESI-negative ion): 414, 416 $[M - 2H]^-$. Anal. Calcd. for $C_{18}H_{10}ClFN_4OS_2$: C, 51.86; H, 2.42; N, 13.44. Found: C, 52.43; H, 2.41; N, 13.00.

*4.1.5.11. 2-[[5-(4-Chlorophenyl)-1,3,4-thiadiazol-2-yl]imino]-5-(3-fluorobenzylidene)-1,3-thiazolidin-4-one* ***19***. It was obtained as a brown solid from compound **8** and was recrystallized from ethanol. HPLC $t_R$ (min.): 8.91, Mp: 284 °C (d), yield 78%. FTIR ($cm^{-1}$): 3117 (N–H lactam), 3068 and 3016 (=C–H arom), 1722 (C=O lactam), 1599 (C=N). $^1$H NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ 7.36 (t, $J$ = 7.8 Hz, $J$ = 7.8 Hz, 1H, Ar–H), 7.49 (d, $J$ = 7.2 Hz, 2H, Ar–H), 7.59–7.68 (m, 3H, Ar–H), 7.79 (s, 1H, =CH–Ar), 7.95–7.98 (m, 2H, Ar–H), 13.09 (s, 1H, thiazolidinone NH) ppm. $^{13}$C NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ 117.36, 117.65 and 117.94, 126.11, 129.28 and 129.97, 136.05 and 136.24, 159.14 and 161.13 (Ar–**C**), 132.05 (thiazolidinone **$C_5$**), 145.55 (=**CH**–Ar), 164.37 (thiadiazole **$C_2$**), 167.31 (thiazolidinone **$C_2$**), 170.41 (thiadiazole **$C_5$**), 171.41 (thiazolidinone **$C_4$**) ppm. LCMS (ESI-negative ion): 414, 416 $[M - 2H]^-$. Anal. Calcd. for $C_{18}H_{10}ClFN_4OS_2$. ½ mol $H_2O$: C, 50.76; H, 2.60; N, 13.16. Found: C, 50.60; H, 2.44; N, 13.11.

*4.1.5.12. 2-[[5-(4-Chlorophenyl)-1,3,4-thiadiazol-2-yl]imino]-5-(4-fluorobenzylidene)-1,3-thiazolidin-4-one* ***20***. It was obtained as a brown solid from compound **8** and was recrystallized from ethanol. HPLC $t_R$ (min.): 9.49, Mp: 318 °C (d), yield 78%. FTIR ($cm^{-1}$): 3144 (N–H lactam), 3076 and 3026 (=C–H arom), 1716 (C=O lactam). $^1$H NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ 7.43 (t, $J$ = 6.4 Hz, $J$ = 6.4 Hz, 2H, Ar–H), 7.59 (d, $J$ = 6.38 Hz, 2H, Ar–H), 7.75 (t, $J$ = 5.1 Hz, $J$ = 5.1 Hz, 2H, Ar–H), 7.81 (s, 1H, =CH–Ar), 7.92–7.96 (m, 2H, Ar–H), 13.03 (s, 1H, thiazolidinone NH) ppm. $^{13}$C NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ 116.96 and 117.25, 124.09, 129.26 and 129.97, 130.24, 136.21, 159.35 (Ar–**C**), 133.26 (thiazolidinone **$C_5$**), 137.73 (=**CH**–Ar), 164.82 (thiadiazole **$C_2$**), 167.43 (thiazolidinone **$C_2$**), 169.65 (thiadiazole **$C_5$**, thiazolidinone **$C_4$**) ppm. LCMS (ESI-negative ion): 414, 416 $[M - 2H]^-$. Anal. Calcd. for $C_{18}H_{10}ClFN_4OS_2$: C, 51.86; H, 2.42; N, 13.44. Found: C, 51.32; H, 2.49; N, 13.36.

*4.1.5.13. 2-[[5-(4-Chlorophenyl)-1,3,4-thiadiazol-2-yl]imino]-5-(2-chloro-6-fluoro-benzylidene)-1,3-thiazolidin-4-one* ***21***. It was obtained as a yellow solid from compound **8** and was recrystallized from ethanol. HPLC $t_R$ (min.): 9.31, Mp: 300 °C (d), yield 70%. FTIR ($cm^{-1}$): 3120 (N–H lactam), 3068 and 3020 (=C–H arom), 1726 (C=O lactam), 1597 (C=N). $^1$H NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ 7.41–7.62 (m, 5H, Ar–H), 7.69 (s, 1H, =CH–Ar), 7.93 (d, $J$ = 8.4 Hz, 2H, Ar–H), 13.32 (s, 1H, thiazolidinone NH) ppm. LCMS (ESI-negative ion): 448, 451 [M − 3H] and 450, 451 $[M - H]^-$. Anal. Calcd. for $C_{18}H_{19}Cl_2FN_4OS_2$: C, 47.90; H, 2.01; N, 12.41. Found: C, 47.72; H, 2.28; N, 12.00.

*4.1.5.14. 2-[[5-(4-Chlorophenyl)-1,3,4-thiadiazol-2-yl]imino]-5-(2-chlorobenzylidene)-1,3-thiazolidin-4-one* ***22***. It was obtained as a yellow solid from compound **8** and was recrystallized from ethanol. HPLC $t_R$ (min.): 9.79, Mp: 337 °C (d), yield 67%. FTIR ($cm^{-1}$): 3147 (N–H lactam), 3086 and 3034 (=C–H arom), 1720 (C=O lactam), 1597 (C=N). $^1$H NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ 7.61–7.70 (m, 6H, Ar–H, =CH–Ar), 7.96 (d, $J$ = 8.1 Hz, 3H, Ar–H) 13.15 (s, 1H, thiazolidinone NH) ppm. LCMS (ESI-negative ion): 430, 433 $[M - 3H]^-$ and 432, 433 $[M - H]^-$. Anal. Calcd. for $C_{18}H_{10}Cl_2N_4OS_2$: C, 49.89; H, 2.33; N, 12.93. Found: C, 49.89; H, 2.36; N, 12.70.

*4.1.5.15. 2-[[5-(4-Chlorophenyl)-1,3,4-thiadiazol-2-yl]imino]-5-(2,4-dichlorobenzylidene)-1,3-thiazolidin-4-one* ***23***. It was obtained as an orange solid from compound **8** and was recrystallized from ethanol. HPLC $t_R$ (min.): 11.32, Mp: 324 °C (d), yield 69%. FTIR ($cm^{-1}$): 3122 (N–H lactam), 3064 and 3024 (=C–H arom), 1716 (C=O lactam), 1595 (C=N). $^1$H NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): δ 7.43–7.70 (m, 5H, Ar–H), 7.82 and 7.84 (s, 1H, =CH–Ar), 7.95 (d, $J$ = 8.4 Hz, 2H, Ar–H), 12.85 (s, 1H, thiazolidinone NH) ppm. LCMS (ESI-negative ion): 467, 468 $[M - H]^-$. Anal. Calcd. for $C_{18}H_9Cl_3N_4OS_2$: C, 46.22; H, 1.94; N, 11.98. Found: C, 46.08; H, 2.11; N, 11.81.

*4.1.5.16. 2-[[5-(4-Chlorophenyl)-1,3,4-thiadiazol-2-yl]imino]-5-(2,6-dichlorobenzylidene)-1,3-thiazolidin-4-one* ***24***. It was obtained as an orange solid from compound **8** and was recrystallized from ethanol. HPLC $t_R$ (min.): 9.46, Mp: 324 °C (d), yield 69%. FTIR ($cm^{-1}$): 3126 (N–H lactam), 3070 and 3016 (=C–H arom), 1718 (C=O lactam), 1585 (C=N). $^1$H NMR (400 MHz, $CDCl_3$-DMSO-$d_6$): δ 7.48–7.63 (m, 5H, Ar–H), 7.72 (s, 1H, =CH–Ar–H), 7.88 (d, $J$ = 8.7 Hz, 2H, Ar–H), 13.13 (s, 1H, thiazolidinone **N**H) ppm. $^{13}$C NMR (400 MHz, $CDCl_3$-DMSO-$d_6$): δ 129.00, 129.10, 129.23, 129.27 and 129.92, 130.44, 131.91, 132.34, 135.08, 136.26 (Ar–**C**), 133.54 (thiazolidinone **$C_5$**),

138.55 (=CH–Ar), 164.44 (thiadiazole **$C_2$**), 165.96 (thiazolidinone **$C_2$**), 170.29 (thiadiazole **$C_5$**), 172.50 (thiazolidinone **$C_4$**) ppm. LCMS (ESI-negative ion): 464, 468 $[M-4H]^-$ and 467, 468 $[M-H]^-$. Anal. Calcd. for $C_{18}H_9Cl_3N_4OS_2$: C, 46.22; H, 1.94; N, 11.98. Found: C, 46.09; H, 2.15; N, 11.47.

*4.1.5.17. 2-[[5-(4-Chlorophenyl)-1,3,4-thiadiazol-2-yl]imino]-5-(2,6-dimethoxy-benzylidene)-1,3-thiazolidin-4-one* ***25***. It was obtained as a yellow solid from compound **8** and was recrystallized from ethanol. HPLC $t_R$ (min.): 11.22, Mp: 276 °C (d), yield 68%. FTIR ($cm^{-1}$): 3115 (N–H lactam), 3049 and 3010 (=C–H arom), 1716 (C=O lactam). $^1$H NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): $\delta$ 3.88 (s, 6H, Ar–$CH_3$), 6.77 (d, $J$ = 8.4 Hz, 2H, Ar–H), 7.46 (t, 1H, $J$ = 11.2 Hz, $J$ = 11.2 Hz, Ar–H), 7.58 (d, 2H, $J$ = 9.2 Hz, Ar–H), 7.91 (s, 1H, =CH–Ar–H), 7.93 (d, $J$ = 11.6 Hz 2H, Ar–H) 12.73 (s, 1H, thiazolidinone NH). $^{13}$C NMR (400 MHz, $CDCl_3$-DMSO-$d_6$): $\delta$ 56.27 (–$OCH_3$) 110.35, 125.58, 127.01, 129.26, 129.37, 129.92 and 158.68 (Ar–**C**), 133.82 (thiazolidinone **$C_5$**), 136.05 (=**CH**–Ar), 163.81 (thiadiazole **$C_2$**), 167.85 (thiazolidinone **$C_2$**), 170.55 (thiadiazole **$C_5$**, thiazolidinone **$C_4$**). LCMS (ESI-negative ion): 456, 457 $[M-H]^-$. Anal. Calcd. For $C_{20}H_{15}ClN_4O_3S_2$ C, 52.34; H, 3.29; N, 12.21. Found C, 51.95; H, 3.33; N, 11.97.

*4.1.5.18. 2-[[5-(4-Chlorophenyl)-1,3,4-thiadiazol-2-yl]imino]-5-(4-nitrobenzylidene)-1,3-thiazolidin-4-one* ***26***. It was obtained as a yellow solid from compound **8** and was recrystallized from ethanol. HPLC $t_R$ (min.): 8.22, Mp: 319 °C (d), yield 76%. FTIR ($cm^{-1}$): 3171 (N–H lactam), 3100 and 3020 (=C–H arom), 1710 (C=O lactam), 1583 (C=N), 1550 and 1350 (N–O $NO_2$). $^1$H NMR (300 MHz, $CDCl_3$-DMSO-$d_6$): $\delta$ 7.61 (d, $J$ = 8.7 Hz, 2H, Ar–H), 7.94 (t, $J$ = 8.7 Hz, $J$ = 8.7 Hz, 4H, Ar–H), 7.98 (s, 1H, =CH–Ar–H), 8.38 (d, $J$ = 8.4 Hz, 2H, Ar–H), 13.17 (s, 1H, thiazolidinone NH) ppm. LCMS (ESI-negative ion): 441, 443 $[M-2H]^-$. Anal. Calcd. for $C_{18}H_{10}ClN_5O_3S_2$: C, 48.70; H, 2.27; N, 15.78. Found: C, 48.84; H, 2.36; N, 15.

### 4.2. Biological studies

#### 4.2.1. 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/$U_{12}$, 25 μM UTP, 2 μCi [alpha-$^{32}$P]UTP, 300 ng of NS5BCΔ21and 1.0 mM $MnCl_2$ with or without inhibitors (100 μM) in a total volume of 25 μL for 1 h at 30 °C as previously described [31,60]. 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. 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. BHK-NS5B-FRLuc reporter cells were grown in DMEM with 10% fetal bovine serum, 5% antibiotic-antimycotic, 5% nonessential amino acid, 1 mg/mL G418 and 10 μg/mL blasticidin. All cells were cultured at 37 °C and 5% $CO_2$.

#### 4.2.3. BHK-NS5B-FRLuc reporter assay

The effect of the extracts on the intracellular NS5B RdRp activity was evaluated employing the BHK-NS5B-FRLuc reporter as described previously [50,52,53]. Briefly, the cells were seeded at a density of $1 \times 10^4$ cells/well in a 96 well plate and 8 h later treated with the individual compound at 100 μM concentration per well. Cells were lysed 42 h post treatment using passive lysis buffer and luciferase expression was measured using the Dual-Glo Luciferase Assay Kit (Promega, USA). The effect of the compounds on cell viability was estimated as the relative levels of firefly luciferase in compound treated cells versus DMSO controls. The inhibitory effect of the compounds on the intracellular NS5B RdRp activity was evaluated from the percent reduction in RLuc to FLuc luminescence signal in extract treated cells versus DMSO controls.

#### 4.2.4. HCV replicon based luciferase reporter assays

The Huh7/Rep-Feo1b replicon reporter cells have been described previously [51–53]. The Huh7.5-FGR-JC1-Rluc2A replicon cells were generated in Dr. Hengli Tang's lab as described herein. Briefly, the 2623 base pair (b.p.) AgeI-BsiWI fragment from the full length HCV 2a plasmid pFGR-JFH-1 was used to replace the similarly digested 1190 b.p. fragment from the p7-Rluc2A plasmid, such that the JFH-1 UTR, a neomycin resistance gene, and most of the JFH-1 E1 was incorporated into the p7Rluc2a plasmid. The resulting 14,806 b.p. construct was linearized with XbaI for *in vitro* transcription of the viral RNA, and electroporated (10 μg RNA) into Huh7.5 cells. Cells expressing the HCV proteins and the *Renilla* luciferase reporter were selected over a period of 3–4 weeks with 0.5 mg/mL G418.

To evaluate the anti-HCV activity of the compounds, HCV replicon reporter cells were seeded in a 96 well plate at a confluence of $1 \times 10^4$ 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 96AQ$_{ueous}$ 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.

### 4.3. Molecular modeling

Compounds were built and subjected to LigPrep as per previous reports using Maestro 9.0 and Macromodel program v9.7 (Schrödinger, Inc., New York, NY, 2009) [61]. The output obtained from the LigPrep run was used as input for docking simulations. The X-ray co-crystal structure of HCV NS5B-PF868554 (PDB ID: 3FRZ) was used for docking into thumb pocket-II [54]. Protein refinement without crystallographic water molecules, energy grid generation using bound ligand as a reference, and "Extra Precision" (XP) Glide docking v5.0 (Schrödinger, Inc., New York, NY, 2009) was performed with the default parameters. The top scoring pose of compound **24** within the TP-II was used for graphical analysis. All computations were carried out on a Dell Precision 470n dual processor with the Linux OS (Red Hat Enterprise WS 4.0).

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.08.043.

## References


[1] C.W. Shepard, L. Finelli, M.J. Alter, Global epidemiology of hepatitis C virus infection, Lancet Infect. Dis. 5 (2005) 558–567.
[2] M.J. Alter, Epidemiology of hepatitis C virus infection, World J. Gastroenterol. 13 (2007) 2436–2441.
[3] Q.L. Choo, G. Kuo, A.J. Weiner, L.R. Overby, D.W. Bradley, M. Houghton, Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome, Science 244 (1989) 359–362.
[4] V. Brass, D. Moradpour, H.E. Blum, Molecular virology of hepatitis C virus (HCV): 2006 update, Int. J. Med. Sci. 3 (2006) 29–34.
[5] G.M. Lauer, B.D. Walker, Hepatitis C virus infection, N. Engl. J. Med. 345 (2001) 41–52.
[6] I. Saito, T. Miyamura, A. Ohbayashi, H. Harada, T. Katayama, S. Kikuchi, Y. Watanabe, S. Koi, M. Onji, Y. Ohta, et al., Hepatitis C virus infection is associated with the development of hepatocellular carcinoma, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 6547–6549.
[7] T.J. Liang, L.J. Jeffers, K.R. Reddy, M. De Medina, I.T. Parker, H. Cheinquer, V. Idrovo, A. Rabassa, E.R. Schiff, Viral pathogenesis of hepatocellular carcinoma in the United States, Hepatology 18 (1993) 1326–1333.
[8] A.L. Zignego, L. Gragnani, C. Giannini, G. Laffi, The hepatitis C virus infection as a systemic disease, Intern. Emerg. Med. 7 (Suppl. 3) (2012) S201–S208.
[9] C. Rice, Perspective: miles to go before we sleep, Nature 474 (2011) S8.
[10] C. Sheridan, New Merck and Vertex drugs raise standard of care in hepatitis C, Nat. Biotechnol. 29 (2011) 553–554.
[11] K. Garber, Hepatitis C: move over interferon, Nat. Biotechnol. 29 (2011) 963–966.
[12] J. Vermehren, C. Sarrazin, New HCV therapies on the horizon, Clin. Microbiol. Infect. 17 (2011) 122–134.
[13] S.E. Behrens, L. Tomei, R. De Francesco, Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus, EMBO J. 15 (1996) 12–22.
[14] D. Moradpour, V. Brass, E. Bieck, P. Friebe, R. Gosert, H.E. Blum, R. Bartenschlager, F. Penin, V. Lohmann, Membrane association of the RNA-dependent RNA polymerase is essential for hepatitis C virus RNA replication, J. Virol. 78 (2004) 13278–13284.
[15] A.A. Kolykhalov, K. Mihalik, S.M. Feinstone, C.M. Rice, Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3′ nontranslated region are essential for virus replication in vivo, J. Virol. 74 (2000) 2046–2051.
[16] J.F. Dillon, Hepatitis C: what is the best treatment? J. Viral. Hepat. 11 (Suppl. 1) (2004) 23–27.
[17] Z.J. Ni, A.S. Wagman, Progress and development of small molecule HCV antivirals, Curr. Opin. Drug Discovery Dev. 7 (2004) 446–459.
[18] M.L. Barreca, N. Iraci, G. Manfroni, V. Cecchetti, Allosteric inhibition of the hepatitis C virus NS5B polymerase: in silico strategies for drug discovery and development, Future Med. Chem. 3 (2011) 1027–1055.
[19] H. Ago, T. Adachi, A. Yoshida, M. Yamamoto, N. Habuka, K. Yatsunami, M. Miyano, Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus, Structure 7 (1999) 1417–1426.
[20] S. Bressanelli, L. Tomei, F.A. Rey, R. De Francesco, Structural analysis of the hepatitis C virus RNA polymerase in complex with ribonucleotides, J. Virol. 76 (2002) 3482–3492.
[21] S. Bressanelli, L. Tomei, A. Roussel, I. Incitti, R.L. Vitale, M. Mathieu, R. De Francesco, F.A. Rey, Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 13034–13039.
[22] C.A. Lesburg, M.B. Cable, E. Ferrari, Z. Hong, A.F. Mannarino, P.C. Weber, Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site, Nat. Struct. Biol. 6 (1999) 937–943.
[23] K. Babaoglu, M.A. Page, V.C. Jones, M.R. McNeil, C. Dong, J.H. Naismith, R.E. Lee, Novel inhibitors of an emerging target in *Mycobacterium tuberculosis*; substituted thiazolidinones as inhibitors of dTDP-rhamnose synthesis, Bioorg. Med. Chem. Lett. 13 (2003) 3227–3230.
[24] G. Küçükgüzel, A. Kocatepe, E. De Clercq, F. Sahin, M. Güllüce, Synthesis and biological activity of 4-thiazolidinones, thiosemicarbazides derived from diflunisal hydrazide, Eur. J. Med. Chem. 41 (2006) 353–359.
[25] R. Ottana, S. Carotti, R. Maccari, I. Landini, G. Chiricosta, B. Caciagli, M.G. Vigorita, E. Mini, In vitro antiproliferative activity against human colon cancer cell lines of representative 4-thiazolidinones. Part I, Bioorg. Med. Chem. Lett. 15 (2005) 3930–3933.
[26] A. Kumar, C.S. Rajput, S.K. Bhati, Synthesis of 3-[4′-(p-chlorophenyl)-thiazol-2′-yl]-2-[(substituted azetidinone/thiazolidinone)-aminomethyl]-6-bromoquinazolin-4-ones as anti-inflammatory agent, Bioorg. Med. Chem. Lett. 15 (2007) 3089–3096.
[27] K. Omar, A. Geronikaki, P. Zoumpoulakis, C. Camoutsis, M. Sokovic, A. Ciric, J. Glamoclija, Novel 4-thiazolidinone derivatives as potential antifungal and antibacterial drugs, Bioorg. Med. Chem. Lett. 18 (2010) 426–432.
[28] M.L. Barreca, A. Chimirri, E. De Clercq, L. De Luca, A.M. Monforte, P. Monforte, A. Rao, M. Zappala, Anti-HIV agents: design and discovery of new potent RT inhibitors, Farmaco 58 (2003) 259–263.
[29] A. Rao, A. Carbone, A. Chimirri, E. De Clercq, A.M. Monforte, P. Monforte, C. Pannecouque, M. Zappala, Synthesis and anti-HIV activity of 2,3-diaryl-1,3-thiazolidin-4-ones, Farmaco 58 (2003) 115–120.
[30] R.K. Rawal, R. Tripathi, S.B. Katti, C. Pannecouque, E. De Clercq, Design, synthesis, and evaluation of 2-aryl-3-heteroaryl-1,3-thiazolidin-4-ones as anti-HIV agents, Bioorg. Med. Chem. 15 (2007) 1725–1731.
[31] N. Kaushik-Basu, A. Bopda-Waffo, T.T. Talele, A. Basu, Y. Chen, S.G. Küçükgüzel, 4-Thiazolidinones: a novel class of hepatitis C virus NS5B polymerase inhibitors, Front. Biosci. 13 (2008) 3857–3868.
[32] R.K. Rawal, S.B. Katti, N. Kaushik-Basu, P. Arora, Z. Pan, Non-nucleoside inhibitors of the hepatitis C virus NS5B RNA-dependant RNA polymerase: 2-aryl-3-heteroaryl-1,3-thiazolidin-4-one derivatives, Bioorg. Med. Chem. Lett. 18 (2008) 6110–6114.
[33] S. Yan, G. Larson, J.Z. Wu, T. Appleby, Y. Ding, R. Hamatake, Z. Hong, N. Yao, Novel thiazolones as HCV NS5B polymerase allosteric inhibitors: further designs, SAR, and X-ray complex structure, Bioorg. Med. Chem. Lett. 17 (2007) 63–67.
[34] Y. Ding, K.L. Smith, C.V. Varaprasad, E. Chang, J. Alexander, N. Yao, Synthesis of thiazolone-based sulfonamides as inhibitors of HCV NS5B polymerase, Bioorg. Med. Chem. Lett. 17 (2007) 841–845.
[35] G. Lee, D.E. Piper, Z. Wang, J. Anzola, J. Powers, N. Walker, Y. Li, Novel inhibitors of hepatitis C virus RNA-dependent RNA polymerases, J. Mol. Biol. 357 (2006) 1051–1057.
[36] T.T. Talele, P. Arora, S.S. Kulkarni, M.R. Patel, S. Singh, M. Chudayeu, N. Kaushik-Basu, Structure-based virtual screening, synthesis and SAR of novel inhibitors of hepatitis C virus NS5B polymerase, Bioorg. Med. Chem. 18 (2010) 4630–4638.
[37] P. Vicini, A. Geronikaki, K. Anastasia, M. Incerti, F. Zani, Synthesis and antimicrobial activity of novel 2-thiazolylimino-5-arylidene-4-thiazolidinones, Bioorg. Med. Chem. 14 (2006) 3859–3864.
[38] E. Tatar, İ. Küçükgüzel, E. De Clercq, F. Şahin, M. Güllüce, Synthesis, characterization and screening of antimicrobial, antituberculosis, antiviral and anticancer activity of novel 1,3-thiazolidine-4-ones derived from 1-[2-(benzoylamino)-4-(methylthio)butyryl]-4-alkyl/arylalkyl thiosemicarbazides, ARKIVOC 14 (2008) 191–210.
[39] E. Tatar, İ. Küçükgüzel, E. De Clercq, R. Krishnan, N. Kaushik-Basu, Synthesis, characterization and antiviral evaluation of 1,3-thiazolidine-4-one derivatives bearing L-Valine side chain, Marmara Pharm. J. 16 (2012) 181–193.
[40] P. Vicini, A. Geronikaki, M. Incerti, F. Zani, J. Dearden, M. Hewitt, 2-Heteroarylimino-5-benzylidene-4-thiazolidinones analogues of 2-thiazolylimino-5-benzylidene-4-thiazolidinones with antimicrobial activity: synthesis and structure–activity relationship, Bioorg. Med. Chem. 16 (2008) 3714–3724.
[41] O. Bozdag-Dundar, O. Ozgen, A. Mentese, N. Altanlar, O. Atli, E. Kendi, R. Ertan, Synthesis and antimicrobial activity of some new thiazolyl thiazolidine-2,4-dione derivatives, Bioorg. Med. Chem. 15 (2007) 6012–6017.
[42] E. Tatar, İ. Küçükgüzel, Ş.G. Küçükgüzel, F. Yılmaz-Demircan, E. De Clercq, G. Andrei, R. Snoeck, C. Pannecouque, F. Şahin, Ö.F. Bayrak, Synthesis, anti-tuberculosis and antiviral activity of novel 2-isonicotinoylhydrazono-5-arylidene-4-thiazolidinones, Int. J. Drug Des. Discovery 1 (2010) 19–32.
[43] S. Wang, Y. Zhao, G. Zhang, Y. Lv, N. Zhang, P. Gong, Design, synthesis and biological evaluation of novel 4-thiazolidinones containing indolin-2-one moiety as potential antitumor agent, Eur. J. Med. Chem. 46 (2011) 3509–3518.
[44] G. Aridoss, S. Amirthaganesan, M.S. Kim, J.T. Kim, Y.T. Jeong, Synthesis, spectral and biological evaluation of some new thiazolidinones and thiazoles based on t-3-alkyl-r-2, c-6-diarylpiperidin-4-ones, Eur. J. Med. Chem. 44 (2009) 4199–4210.
[45] R. Ottana, R. Maccari, R. Ciurleo, M.G. Vigorita, A.M. Panico, V. Cardile, F. Garufi, S. Ronsisvalle, Synthesis and in vitro evaluation of 5-arylidene-3-hydroxyalkyl-2-phenylimino-4-thiazolidinones with antidegenerative activity on human chondrocyte cultures, Bioorg. Med. Chem. 15 (2007) 7618–7625.
[46] R. Ottana, R. Maccari, R. Ciurleo, P. Paoli, M. Jacomelli, G. Manao, G. Camici, C. Laggner, T. Langer, 5-Arylidene-2-phenylimino-4-thiazolidinones as PTP1B and LMW-PTP inhibitors, Bioorg. Med. Chem. 17 (2009) 1928–1937.
[47] R. Maccari, R. Ciurleo, M. Giglio, M. Cappiello, R. Moschini, A.D. Corso, U. Mura, R. Ottana, Identification of new non-carboxylic acid containing inhibitors of aldose reductase, Bioorg. Med. Chem. 18 (2010) 4049–4055.
[48] I.V. Tetko, J. Gasteiger, R. Todeschini, A. Mauri, D. Livingstone, P. Ertl, V.A. Palyulin, E.V. Radchenko, N.S. Zefirov, A.S. Makarenko, V.Y. Tanchuk, V.V. Prokopenko, Virtual computational chemistry laboratory – design and description, J. Comput. Aided Mol. Des. 19 (2005) 453–463.
[49] VCCLAB, Virtual Computational Chemistry Laboratory, 2005. March 9, 2013, http://www.vcclab.org.
[50] J.C. Lee, C.K. Tseng, K.J. Chen, K.J. Huang, C.K. Lin, Y.T. Lin, A cell-based reporter assay for inhibitor screening of hepatitis C virus RNA-dependent RNA polymerase, Anal. Biochem. 403 (2010) 52–62.
[51] Y. Itsui, N. Sakamoto, S. Kakinuma, M. Nakagawa, Y. Sekine-Osajima, M. Tasaka-Fujita, Y. Nishimura-Sakurai, G. Suda, Y. Karakama, K. Mishima, M. Yamamoto, T. Watanabe, M. Ueyama, Y. Funaoka, S. Azuma, M. Watanabe, Antiviral effects of the interferon-induced protein guanylate binding protein 1

and its interaction with the hepatitis C virus NS5B protein, Hepatology 50 (2009) 1727–1737.
[52] D.B. Nichols, G. Fournet, K.R. Gurukumar, A. Basu, J.C. Lee, N. Sakamoto, F. Kozielski, I. Musmuca, B. Joseph, R. Ragno, N. Kaushik-Basu, Inhibition of hepatitis C virus NS5B polymerase by S-trityl-L-cysteine derivatives, Eur. J. Med. Chem. 49 (2012) 191–199.
[53] A.G. Golub, K.R. Gurukumar, A. Basu, V.G. Bdzhola, Y. Bilokin, S.M. Yarmoluk, J.C. Lee, T.T. Talele, D.B. Nichols, N. Kaushik-Basu, Discovery of new scaffolds for rational design of HCV NS5B polymerase inhibitors, Eur. J. Med. Chem. 58 (2012) 258–264.
[54] H. Li, J. Tatlock, A. Linton, J. Gonzalez, T. Jewell, L. Patel, S. Ludlum, M. Drowns, S.V. Rahavendran, H. Skor, R. Hunter, S.T. Shi, K.J. Herlihy, H. Parge, M. Hickey, X. Yu, F. Chau, J. Nonomiya, C. Lewis, Discovery of (*R*)-6-cyclopentyl-6-(2-(2,6-diethylpyridin-4-yl)ethyl)-3-((5,7-dimethyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-yl)methyl)-4-hydroxy-5,6-dihydropyran-2-one (PF-00868554) as a potent and orally available hepatitis C virus polymerase inhibitor, J. Med. Chem. 52 (2009) 1255–1258.
[55] A. Plumitallo, M.C. Cardia, S. Distinto, A. DeLogu, E. Maccioni, Synthesis and antimicrobial activity evaluation of some new 1-benzoyl-isothiosemicarbazides, Farmaco 59 (2004) 945–952.
[56] G. Tu, S. Li, H. Huang, G. Li, F. Xiong, X. Mai, H. Zhu, B. Kuang, W.F. Xu, Novel aminopeptidase N inhibitors derived from 1,3,4-thiadiazole scaffold, Bioorg. Med. Chem. 16 (2008) 6663–6668.
[57] S.H. Li, G. Li, H.M. Huang, F. Xiong, C.M. Liu, G.G. Tu, The synthesis and preliminary activity assay in vitro of peptide-like derivatives as APN inhibitors, Arch. Pharm. Res. 31 (2008) 1231–1239.
[58] H. Rajak, R. Deshmukh, N. Aggarwal, S. Kashaw, M.D. Kharya, P. Mishra, Synthesis of novel 2,5-disubstituted 1,3,4-thiadiazoles for their potential anticonvulsant activity: pharmacophoric model studies, Arch. Pharm. (Weinheim) 342 (2009) 453–461.
[59] P. Mullick, S.A. Khan, S. Verma, O. Alam, Synthesis, characterization and antimicrobial activity of new thiadiazole derivatives, Bull. Korean Chem. Soc. 32 (2011) 1011–1016.
[60] N. Kaushik-Basu, A. Bopda-Waffo, T.T. Talele, A. Basu, P.R. Costa, A.J. da Silva, S.G. Sarafianos, F. Noel, Identification and characterization of coumestans as novel HCV NS5B polymerase inhibitors, Nucleic Acids Res. 36 (2008) 1482–1496.
[61] D.B. Nichols, R.A.C. Leao, A. Basu, P.d.F. de Moraes, T.T. Talele, P.R.R. Costa, Evaluation of coumarin and neoflavone derivatives as HCV NS5B polymerase inhibitors, Chem. Biol. Drug Des. 81 (2013) 607–614.