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Discovery and structure-resistance relationship study of new thieno[2,3-*b*] pyridine HCV NS4B inhibitors

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Received December 23, 2018, accepted January 25, 2019

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Pharmazie 74: 321-325 (2019) *doi: 10.1691/ph.2019.8960*

The non-structural protein 4B (NS4B) of hepatitis C virus (HCV) has emerged as a promising target for chronic hepatitis C treatment. The thieno[2,3-*b*]pyridine HCV inhibitor **2** has demonstrated properties as a NS4B inhibitor. Subsequent hybridization of **2** with our recently published imidazo[2,1-*b*]thiazole NS4B inhibitor **3** resulted in the discovery of several more potent compounds with sub-micromolar EC_{50} against HCV genotype 1b replicon. More importantly, the resistant profile study of the new synthesized HCV inhibitors illustrated that the bicyclic scaffold would mediate the resistance of H3R and Q26R mutations, while the piperazinone motif would mediate the resistance of H94R, F98C and V105M mutations, and the C3- amino group would disrupt the interaction between piperazinone motif and NS4B. This structure-resistance relationship detail could help us to develop new NS4B inhibitors with higher resistant barrier in the future.

1. Introduction

As a member of the Flaviviridae family, Hepatitis C Virus (HCV) infects approximately 184 million people worldwide (Thrift et al. 2017), of which 80 percent will develop into chronic hepatitis C (CHC), which is the leading reason for liver fibrosis, cirrhosis and hepatocellular carcinoma (Lauer and Walker 2001).

After first characterized in 1989 (Choo et al. 1989), a series of drugs, from interferon (IFN) and ribavirin (RBV) to recently approved superblockbuster sofosbuvir (Liang and Ghany 2013), have been developed to address HCV. The introduction of direct-acting antivirals (DAAs) in the last few years, including NS3/4A inhibitors, NS5A inhibitors and NS5B inhibitors, have increased the sustained virologic response (SVR) rate and shortened the treatment duration substantially (Kwong 2014). But the emergence of resistance to existing drugs (Sarrazin and Zeuzem 2010) and the relapse after current treatment have also implied the need to develop new anti-HCV drugs, especially ones with novel mechanism of action.

The non-structural protein 4B (NS4B) of HCV is an integral membrane protein which is essential for HCV replication by participating several important stages of its life cycle (Li et al. 2012). Its inhibitor was not reported until recently the arginine rich motif (ARM) in the C-terminal region of NS4B could specifically bind the 3' terminus of the negative strand of HCV, which could be blocked by the H1 histamine receptor antagonist clemizole (Einav et al. 2008). This compound has shown highly synergistic interaction with HCV protease inhibitors like boceprevir and telaprevir (Einav et al. 2010), while the moderate *in vitro* HCV inhibitory activity has limited its development as HCV inhibitor. Subsequent optimization of this chemotype with respect to potency did not yield a better candidate compared with clemizole until now.

The second amphipathic α helix in the N-terminal region of NS4B (4BAH2) could induce vesicle aggregation which is required for HCV genome replication (Cho et al. 2010). Small molecules such as anguizole (**1a**) could inhibit 4BAH2 activity and prevent the replication of HCV by abrogating vesicle aggregation (Bryson et al. 2010). A series of compounds with preferred potency and safety profile as well as similar mechanism of action with anguizole (Shotwell et al. 2012; Miller et al. 2014; Dufner-Beattie et al. 2014; Tai et al. 2014; Graci et al. 2016), which we termed as 4BAH2 inhibitors, have been reported. The *in vivo* efficacy of this kind of HCV inhibitors (**1b**) in PXB mice (Miller et al. 2014) as well as the recently reported synergistic interactions of imidazo[2,1-*b*] thiazole 4BAH2 inhibitors (**3**) with other DAAs (Wang et al. 2015) both suggested that NS4B would be a promising target for HCV treatment (Cannalire et al. 2016).

Resistant profile studies have played an important role in the development of antiviral drugs. It could not only help to find the straight target for a given compound discovered by phenotype-based screening, but also provide detailed transactional information between small molecular and its target. Moreover, it could also guide us to design new compounds with higher resistant barrier. In this study, the resistant profile study of our previously reported thieno[2,3-*b*]pyridine HCV inhibitor (Wang et al. 2014) demonstrated that it would be a new chemotype of NS4B inhibitors, and hybridization strategy with respect to thieno[2,3-b]pyridine scaffold and our imidazo[2,1-b]thiazole 4BAH2 inhibitors led to a new series of NS4B inhibitors. The structure-resistance relationship of this kind of NS4B inhibitors could also help us to design new NS4B inhibitors with higher resistant barrier.

2. Investigations and results

2.1. Resistance profile studies

Our group recently reported the discovery of thieno[2,3-*b*]pyridine derivatives as HCV inhibitors by phenotype-based screening of our privileged small molecule library. Detailed structure-activity relationship study provided us several compounds with low micromolar inhibitory activity against HCV genotype 1b replicon (Wang et al. 2014). To find the possible target of this kind of HCV inhibitors, compound **2** was chosen to test its potency against a panel of representative resistant replicons which contain mutations mapped to NS3/4A protease, NS4B, NS5A, and NS5B polymerase. (Table 1). Interestingly, only two strains (H3R and Q26R) (Rai and Deval

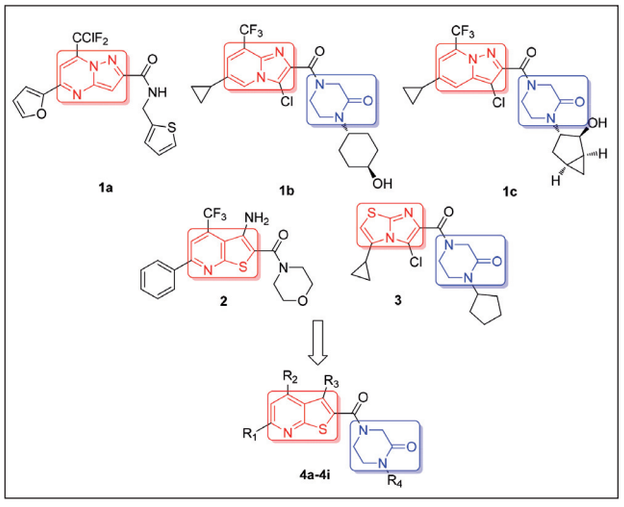


Fig. 1: Reported NS4B inhibitors and compound design

2011) bearing mutations mapped to the N-terminal region of NS4B were resistant to compound **2**, suggesting that this compound would be a NS4B inhibitor. Because H3R and Q26R also endow genotype 1b replicon resistance to imidazo[2,1-*b*]thiazole NS4B inhibitor **3** and imidazo[1,2-*a*]pyridine NS4B inhibitor **1b** (Wang et al. 2015), we hypothesized that both compounds would, at least partially, share a mechanism to inhibit the replicaion of HCV, and some similar structural features in both compounds would mediate the cross-resistance between these compounds. These results thus spurred us to design new compounds by hybridization strategy to find more potent HCV inhibitors and investigate the in-depth structure-resistance relationship of this kind of NS4B inhibitors (Fig. 1).

Table 1: Resistance profile studies of 2

Mutant Replicon	Gene	EC_{50} (μM)	Mutant Replicon	Gene	EC_{50} (μM)
WT (1b)		3.1	A156T	NS3/4A	3.8
H3R	NS4B	>50	Y93H	NS5A	2.5
Q26R		>50	S282T	NS5B(Active Site)	2.9
H94R		3.0	M423I	NS5B(Thumb 2)	2.2
F98C	NS4B	1.8	P495A	NS5B(Thumb 1)	1.6
V105M		2.0	Y448H	NS5B(Palm)	2.3

All the mutation replicons were transiently transfected replicons.

2.2. *Compound design and SAR studies*

Compounds **2** and **3**, as well as a number of other recently reported NS4B inhibitors (**1a-1c**), incorporate a bicyclic ring system with similar patterns of substitution, and a piperazinone side chain seems to improve the genotype **1b** potency significantly (Shotwell et al. 2012). We hypothesized that compounds which hybridize the thieno[2,3-*b*]pyridine scaffold and the piperazinone side chain could yield HCV inhibitors more potent than compound **2**. More importantly, the incorporation of a piperazinone group would also reshape the resistance profile of thieno[2,3-*b*]pyridines.

As shown in Table 2, compounds **4a** and **4c** which retained all of the C3-, C4-, and C6- substituents on the thieno[2,3-*b*]pyridine core were firstly synthesized which just afforded two less potent compounds compared with compound **2**. However, removing of the amino group of both compounds improved the potency significantly, with an EC_{50} of 0.17 μM and 0.45 μM for **4b** and **4d**, respectively. These preliminary attempts have suggested that our hybridization strategy was feasible and the C3- amino group was unfavorable for the HCV inhibitory activity, at least for our new hybridization series.

We next evaluated the influence of C2- and C6- substituents on the potency. When the piperazinone motif was replaced by thiophen-2-ylmethanamine (**4e**) or 1-(cyclopropylsulfonyl)piperazine (**4f**), a sharp loss of potency was observed, implying the importance of piperazinone segment for maintaining the potency. A relatively hydrophilic piperazinone group (**4g**) was also harmful for the potency, which was also observed in the imidazo[2,1-*b*]thiazole series, suggesting that both our new thieno[2,3-*b*]pyridines and imidazo[2,1-*b*]thiazoles would share structure-activity relationships. The replacement of the cyclopropyl group at C6- position by aryls was also detrimental to the potency, as is illustrated by the furan-(**4h**) and thiophene- (**4i**) derivatives, which were 49- and 14-fold less potent than their cyclopropyl analogue **4b**.

So the preliminary SAR study of this new series of HCV inhibitors has provided us several thieno[2,3-*b*]pyridines with improved potency compared with **2**, of which compound **4b** was the most potent one, with an EC_{50} of 0.17 μM against genotype 1b replicon.

2.3. *Structure-resistance relationship studies*

To verify that the hybrids were indeed NS4B inhibitors, we next investigated the cross-resistance between our new hybrids and compounds **2** and **3** (Table 3). Because **4b** and its C3- amino substituted analogue **4a** exhibited distinct potency against genotype 1b replicon, both compounds were selected to test their potency against the aforementioned resistant replicons of NS4B. To our surprise, these two compounds displayed distinct resistant profiles in a way that **4a** exhibited a resistant profile similar to **2** while **4b** was analogous to **3**, although there was only a small structural difference between them. Replicons harboring H3R and Q26R were resistant to both compounds, but the remained replicons, including H94R, F98C and V105M, were only resistant to **4b**. We speculated that the piperazinone group in **4b** and **3** may mediate their deactivation against the classical 4BAH2 resistant replicons (H94R, F98C and V105M) and this motif was also an important contributor for HCV inhibitory activity, while the bicyclic ring like thieno[2,3-*b*]pyridine in **4b** and imidazo[2,1-*b*]thiazole in **3** mediate their deactivation against the H3R and Q26R replicons. Besides, the introduction of an amino group at C3-position of the thieno[2,3-*b*]pyridine core would impair the interaction between piperazinone motif and NS4B (Fig. 2), possibly resulting from its stereospecific blockade effect or because its interaction with the piperazinone motif would change the conformation of this molecular, which could explain the moderate potency of **4a** and its equipotency against the classical 4BAH2 resistant replicons (H94R, F98C and V105M) compared with the wild-type replicon. More importantly, the fold changes of all the five NS4B resistant replicons against **4b** were significantly better than our previous reported imidazo[2,1-*b*]thiazole NS4B inhibitor **3** (Fig. 3)**,** suggesting that the change of the bicyclic system could improve the resistance profile of NS4B inhibitors.

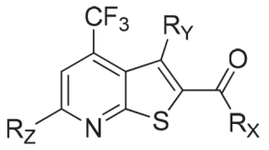
2.3. *Chemistry*

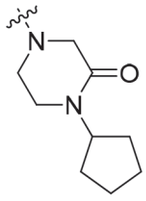
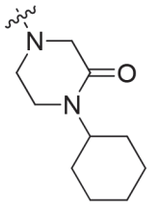
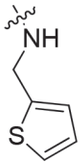
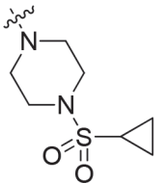
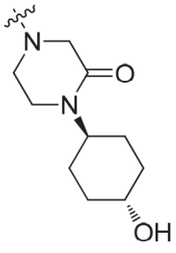
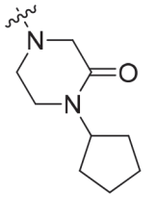
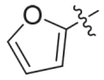
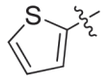
The synthesis of compounds **4a-4i** is similar to our previous study (Wang et al. 2014) as shown in the Scheme. Briefly, commercially available ketones **5a-5c** are condensed with ethyl trifluoroacetate to afford trifluorobutane-1,3-diones **6a-6c;** cyclization with cyanothioacetamide in the presence of DABCO in ethanol provided pyridine-2-(1*H*)-thiones **7a-7c**. These building blocks were subsequently treated with ethyl bromoacetate giving esters **8a-8c**, which were deaminized with *tert.*-butyl nitrite to key intermediates **9a-9c** with relatively low transformation efficiency. The final target compounds **4a-4i** were then prepared by coupling reaction of various amines and hydrolyzation products **10a-10d** (Scheme).

2.4. *Conclusion*

In this study, the resistance profile study of our previously reported thieno[2,3-*b*]pyridine HCV inhibitor **2** has demonstrated that it could be a NS4B inhibitor. Because the two mutant replicons (H3R and Q26R) that were resistant to compound **2** were also

Table 2: Inhibitory effects of thieno[2,3-b]pyridines on HCV Gt 1b replicon replication in Huh 7 cells



Compd.	R_X	R_Y	R_Z	Cytotoxicity CC_{50}(μM)	GT-1b replicon EC_{50}(μM)
4a		-NH_2		>10	8.1
4b		H		>10	0.17
4c		-NH_2		>10	8.6
4d		H		>10	0.45
4e		H		>10	2.2
4f		H		>10	44%@10μM
4g		H		>10	0.95
4h		H		>10	8.4
4i		H		>10	2.4

insensitive to our recently reported imidazo[2,1-*b*]thiazole NS4B inhibitor **3**, hybridization strategy was employed by combining the thieno[2,3-*b*]pyridine core in **2** and the piperazinone side chain in **3** to generate a new series of thieno[2,3-*b*]pyridines, which led to the discovery of several submicromolar HCV inhibitors. Subsequent cross-resistance study has illustrated that the two structurally similar compounds **4a** and **4b** displayed a distinct resistance profile, which suggested that the rigid bicyclic ring mediates the resistance of H3R and Q26R mutations while the piperazinone side chain mediates the resistance of H94R, F98C and V105M mutations, and the amino group at C3- position could influence the interaction between **4a** and its target. This structure-resistance relationship study thus could help us to develop new NS4B inhibitors with higher resistant barrier in the future.

Table 3: Resistance profile studies of 4a and 4b

Mutant Replicon	EC_{50} (μM)	
	4a	**4b**
WT (1b)	8.10	0.17
H3R	>50	2.41
Q26R	>50	2.15
H94R	6.75	2.28
F98C	6.15	9.36
V105M	6.09	7.63

All the mutation replicons were transiently transfected replicons.

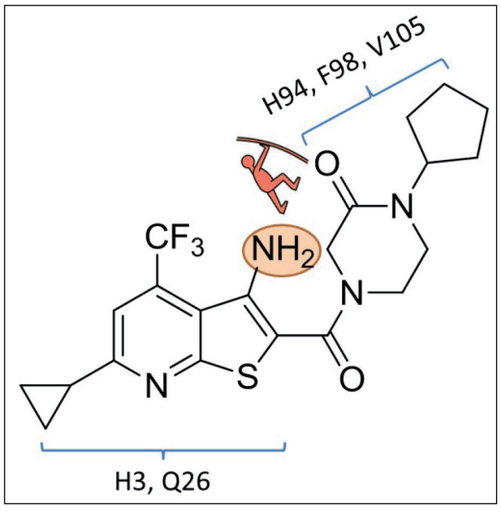


Fig. 2: Supposed structure-resistance relationship of piperazinone NS4B inhibitors

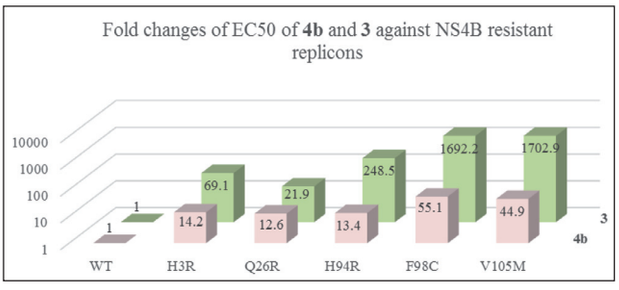


Fig. 3: Comparison of fold changes of EC_{50} of 4b and 3 against NS4B resistant replicons.

3. Experimental

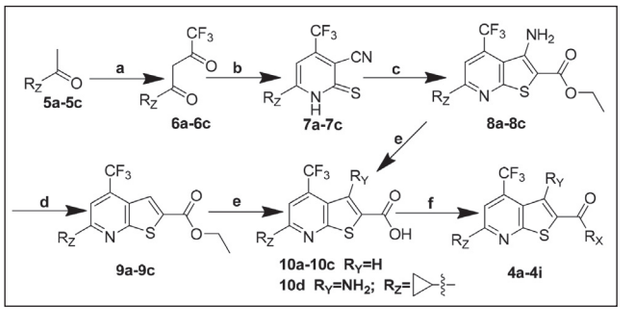
3.1. General chemical methods

Unless otherwise noted, all materials were obtained from commercial suppliers and used without further purification. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE^{m} 400 spectrometer (Bruker Company, Germany) using DMSO-d_6 or $CDCl_3$ as the solvent. Chemical shifts (δ) were reported in ppm relative to Me_4Si (internal standard), and coupling constants (*J*) were reported in Hz. Mass Spectra (MS) were performed on a Waters Q-TOF Premier mass spectrometer (Micromass, Manchester, UK). Thin layer chromatography (TLC) was performed on 0.20 mm Silica Gel F-254 plates (Qingdao Haiyang Chemical, China), and column chromatography were performed using Silica gel 60 of 300-400 mesh (Qingdao Haiyang Chemical, China). The purity of all the title compounds (>95%) was determined on an UltiMate 3000 (Dionex, USA) HPLC system (column, Atlantis dC18, 4.6 mm × 150 mm, 5 μm; mobile phase, methanol (55 %) / water (45 %) or methanol (65 %) / water (35 %); flow rate, 1.0 mL/min; UV wavelength, 190 – 400 nm; temperature, 35 °C; injection volume, 10 μ L).

3.2. Synthetic procedures for thieno[2,3-b]pyridine derivatives

*3.2.1. Synthesis of 1,3-diones **6a-6c***

To a freshly prepared sodium methylate solution (1.5 equiv) in methanol and THF, ethyl trifluoroacetate (1.2 equiv) was added under stirring at 0 °C. The mixture was stirred for 30 min followed by addition of ketone **5a-5c** (1.0 equiv). The reaction mixture was stirred for another 12-24 h until the starting materials were consumed. Then the mixture was concentrated under reduced pressure and the resulted residue was acidified with hydrochloric acid (1 N) and extracted with acetic ether. The combined organic layers were dried ($MgSO_4$), filtered and concentrated to dryness. The product was purified by column chromatography. Yield: 72-84 %



Scheme: Synthesis of thieno[2,3-*b*]pyridines 4a-4i. Reagents and conditions: (a) ethyl trifluoroacetate, sodium methoxide, methanol/THF, r.t., 72-84%; (b) cyanothioacetamide, DABCO, ethanol, reflux, 56-74 %; (c) DMF, KOH (10 %), ethyl bromoacetate, 65-82 %; (d) *tert.*-butyl nitrite, DMF, 60 °C, 26-33 %; (e) LiOH, H_2O/THF/methanol, 70-92 %; (f) EDCI/HOBT, DCM, amines, 65-86 %.

*3.2.2. Synthesis of 2-thioxo-1,2-dihydropyridines **7a-7c***

Cyanothioacetamide (1.5 equiv) and DABCO (1.0 equiv) were added to a solution of the 1, 3-diones **6a-6c** (1.0 equiv) in ethanol at room temperature. The reaction mixture was stirred under reflux for 3-6 h until the starting materials complete transformed. After cooled to room temperature, the mixture was concentrated under reduced pressure and the residue was neutralized with diluted hydrochloric acid (1 N) to precipitate the crude products. The product was collected by filtration after by dried *in vacuo***.** Yield: 56-74 %.

*3.2.3. Synthesis of 3-amino-thieno[2,3-b]pyridines **8a-8c***

To a solution of **7a-7c** (1.0 equiv) in DMF were added aqueous KOH solution (1.8 N, 1.2 equiv) and ethyl 2-bromoacetate (1.2 equiv) at room temperature. The resulting mixture was stirred for 30 min at room temperature followed by the addition of another portion of aqueous KOH solution (1.8 N, 1.2 equiv). The reaction mixture was stirred for additional 2-4 h until the starting materials had completely transformed. Then water was added slowly to the mixture so that the desired product precipitated. The product was collected by filtration, washed with distilled water, and dried under reduced pressure. Yield: 60-90%.

*3.2.4. Synthesis of 3-H-thieno[2,3-b]pyridines **9a-9c***

A solution of **8a-8c** (1.0 equiv) in DMF was added dropwise to a solution of t-BuONO (2.0 equiv) in DMF at 60 °C. Thirty min later, the mixture was cooled to room temperature and poured into hydrochloric acid (1 N) followed by extraction with ethyl acetate. The organic phase was washed with brine and dried over $MgSO_4$. After filtration, the filtrate was concentrated under reduced pressure and separated by column chromatography to give the title compounds. Yield: 26-33 %.

*3.2.5. Synthesis of thieno[2,3-b]pyridine-2-carboxylic acids **10a-10d***

To a solution of **8a** or **9a-9c** (1.0 equiv) in THF/methanol/water (V/V/V = 2/2/1), LiOH (5.0 equiv) was added. The mixture was stirred under room temperature for 2-6 h until the starting materials had completely transformed. Then the solvent was removed under reduced pressure, and the residue was suspended in water after acidification with hydrochloric acid (1 N) so that the desired compounds **10a-10d** precipitated. The products were collected by filtration, washed with distilled water, and dried *in vacuo.* Yield: 70-92 %.

*3.2.6. Synthesis of **4a-4i***

To a suspension solution of **10a-10d** (1.0 equiv) and various amines (1.5 equiv) in dichloromethane was added EDCI (1.5 equiv) and 1-hydroxybenzotriazole (1.2 equiv). The mixture was stirred at room temperature for 6-12 h until the starting materials had completely transformed. Then water was added to the reaction mixture and the organic phase was washed with diluted hydrochloric acid (1 N), saturated K_2CO_3 solution and brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified by column chromatography to give the title compounds. Yield: 65-86 %.

*3.2.7. Spectral data of compounds **4a-4i***

4-(3-Amino-6-cyclopropyl-4-(trifluoromethyl)thieno[2,3-b]pyridine-2-carbonyl)-1-cyclopentylpiperazin-2-one (**4a**): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46 (s, 1H), 5.70 (br s, 2H), 5.06 – 4.86 (m, 1H), 4.44 (s, 2H), 4.05 – 3.86 (m, 2H), 3.53 – 3.29 (m, 2H), 2.23 (s, 1H), 1.97-1.81 (m, 2H), 1.78-1.57 (m, 4H), 1.57-1.43 (m, 2H), 1.25-1.15 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.54, 164.81, 164.40, 161.68, 144.98,

132.01, 122.99, 117.85, 115.19, 99.89, 54.21, 50.10, 42.94, 40.45, 28.06, 24.10, 17.70, 11.84. MS(ESI) *m/z*: 451.2 $[M - H]^-$.

1-Cyclopentyl-4-(6-cyclopropyl-4-(trifluoromethyl)thieno[2,3-b]pyridine-2-carbonyl)piperazin-2-one (**4b**): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60 (d, *J* = 1.4 Hz, 1H), 7.45 (s, 1H), 5.08 – 4.85 (m, 1H), 4.43 (s, 2H), 4.04 – 3.88 (m, 2H), 3.48 – 3.31 (m, 2H), 2.23 - 2.19 (m, 1H), 1.95 – 1.40 (m, 8H), 1.25 – 1.11 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.20, 163.65, 162.81, 144.07, 133.27, 123.00, 121.57, 119.83, 115.34, 54.33, 48.00, 43.08, 40.15, 28.11, 24.08, 17.90, 11.60. MS(ESI) *m/z*: 460.2 $[M + Na]^+$.

4-(3-Amino-6-cyclopropyl-4-(trifluoromethyl)thieno[2,3-b]pyridine-2-carbonyl)-1-cyclohexylpiperazin-2-one (***4c***): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 (s, 1H), 5.71 (br s, 2H), 4.43 (br s, 3H), 3.94 (s, 2H), 3.40 (s, 2H), 2.23 (s, 1H), 1.82 (s, 2H), 1.71 (s, 2H), 1.42-1.09 (m, 10H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.49, 164.39, 164.30, 161.70, 144.93, 131.82, 122.91, 117.80, 115.17, 99.92, 52.54, 50.07, 42.96, 40.44, 29.52, 25.48, 25.44, 17.70, 11.82. MS(ESI) *m/z*: 489.2 $[M + Na]^+$.

1-Cyclohexyl-4-(6-cyclopropyl-4-(trifluoromethyl)thieno[2,3-b]pyridine-2-carbonyl) piperazin-2-one (***4d***): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60 (s, 1H), 7.45 (s, 1H), 4.43 (s, 3H), 3.93 (d, *J* = 5.4 Hz, 2H), 3.40 (s, 2H), 2.22 (s, 1H), 1.82 (s, 2H), 1.76 – 1.37 (m, 8H), 1.27 - 1.16 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.08, 163.54, 162.70, 144.01, 133.18, 122.95, 121.51, 119.81, 115.27, 52.76, 48.02, 43.06, 40.14, 29.50, 25.45, 25.43, 17.91, 11.62. MS(ESI) *m/z*: 452.2 $[M + H]^+$.

6-Cyclopropyl-N-(thiophen-2-ylmethyl)-4-(trifluoromethyl)thieno[2,3-b]pyridine-2-carboxamide (***4e***): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (d, *J* = 1.4 Hz, 1H), 7.42 (s, 1H), 7.32 – 7.20 (m, 1H), 7.08 (d, *J* = 3.2 Hz, 1H), 6.99 (dd, *J* = 5.0, 3.5 Hz, 1H), 6.60 (s, 1H), 4.84 (d, *J* = 5.6 Hz, 2H), 2.30 – 2.12 (m, 1H), 1.26 – 1.06 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.79, 161.30, 139.88, 138.57, 132.93, 127.07, 126.74, 125.72, 125.06, 123.02, 120.40, 115.26, 115.22, 38.93, 17.94, 11.58. MS(ESI) *m/z*: 383.1 $[M + H]^+$.

(6-Cyclopropyl-4-(trifluoromethyl)thieno[2,3-b]pyridin-2-yl)(4-(cyclopropylsulfonyl)piperazin-1-yl)methanone (***4f***): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56 (s, 1H), 7.46 (s, 1H), 3.98 – 3.81 (m, 4H), 3.48 – 3.31 (m, 4H), 2.33 – 2.15 (m, 2H), 1.28 – 1.10 (m, 6H), 1.07 – 0.99 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.49, 163.41, 136.23, 132.72, 123.07, 121.09, 115.30, 46.14, 25.78, 17.86, 11.58, 4.49. MS(ESI) *m/z*: 482.2 $[M + Na]^+$.

4-(6-Cyclopropyl-4-(trifluoromethyl)thieno[2,3-b]pyridine-2-carbonyl)-1-((1r,4r)-4-hydroxycyclohexyl)piperazin-2-one (***4g***): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60 (s, 1H), 7.46 (s, 1H), 4.86 (s, 1H), 4.51 (s, 1H), 4.44 (s, 2H), 3.97 (s, 2H), 3.39 (s, 2H), 2.20 (d, *J* = 13.5 Hz, 3H), 1.87 (d, *J* = 11.2 Hz, 2H), 1.78 – 1.59 (m, 4H), 1.29 – 1.11 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.99, 163.49, 162.60, 144.10, 133.15, 122.93, 121.48, 119.64, 115.30, 69.07, 50.46, 48.25, 44.30, 40.06, 34.32, 27.20, 17.91, 11.60. MS(ESI) *m/z*: 490.2 $[M + Na]^+$.

1-Cyclopentyl-4-(6-(furan-2-yl)-4-(trifluoromethyl)thieno[2,3-b]pyridine-2-carbonyl)piperazin-2-one (***4h***): ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (s, 1H), 7.77 – 7.54 (m, 2H), 7.27 (d, *J* = 3.6 Hz, 1H), 6.62 (dd, *J* = 3.4, 1.7 Hz, 1H), 5.13 – 4.79 (m, 1H), 4.45 (s, 2H), 4.10 – 3.87 (m, 2H), 3.55 – 3.27 (m, 2H), 1.89 (d, *J* = 7.6 Hz, 2H), 1.80 – 1.57 (m, 4H), 1.57 – 1.42 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.50, 163.46, 162.53, 152.22, 148.21, 144.84, 137.93, 133.80, 125.30, 122.87, 121.51, 112.80, 112.31, 111.49, 54.35, 42.73, 40.22, 28.12, 24.08. MS(ESI) *m/z*: 486.2 $[M + Na]^+$.

1-Cyclopentyl-4-(6-(thiophen-2-yl)-4-(trifluoromethyl)thieno[2,3-b]pyridine-2-carbonyl)piperazin-2-one (***4i***): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92 (s, 1H), 7.77 (dd, *J* = 3.7, 1.0 Hz, 1H), 7.64 (d, *J* = 1.5 Hz, 1H), 7.52 (dd, *J* = 5.0, 1.0 Hz, 1H), 7.18 (dd, *J* = 5.0, 3.8 Hz, 1H), 5.04 – 4.84 (m, 1H), 4.45 (s, 2H), 4.02 – 3.91 (m, 2H), 3.48 – 3.34 (m, 2H), 1.96 – 1.45 (m, 8H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.52, 163.35, 162.54, 151.80, 143.02, 137.76, 133.70, 129.82, 128.52, 126.97, 125.30, 122.44, 121.56, 112.59, 54.35, 42.70, 40.34, 28.12, 24.09. MS(ESI) *m/z*: 502.1 $[M + Na]^+$.

3.3. Biological assays

HCV Gt1b replicon assay and mutation replicon assay were performed as previously described. EC_{50} values were calculated by GraphPad Prism and reported as the average of three independent determinations for gt1b wild replicon and two independent determinations for mutation replicons, with a maximum of 2-fold variation. For every test, a positive drug was evaluated parallel as well to validate the credibility of this replicon.

Acknowledgment: This work was supported by The National Science and Technology Major Project of China (2012ZX09103101-079).

Conflicts of interest: None reported.

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