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Letter

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# Imidazo[1,2-*a*]pyridines That Directly Interact with Hepatitis C NS4B: Initial Preclinical Characterization

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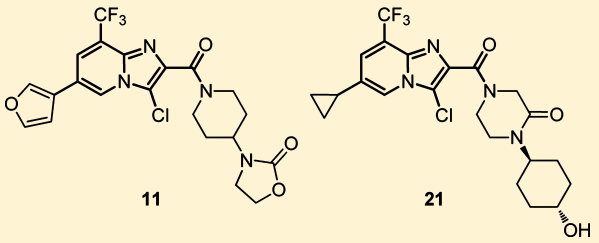
Supporting Information

**ABSTRACT:** A series of imidazo[1,2-*a*]pyridines which directly bind to HCV Non-Structural Protein 4B (NS4B) is described. This series demonstrates potent *in vitro* inhibition of HCV replication ($EC_{50}$ < 10 nM), direct binding to purified NS4B protein ($IC_{50}$ < 20 nM), and an HCV resistance pattern associated with NS4B (H94N/R, V105L/M, F98L) that are unique among reported HCV clinical assets, suggestive of the potential for additive or synergistic combination with other small molecule inhibitors of HCV replication.




**KEYWORDS:** *NS4B, hepatitis C virus, imidazo[1,2-a]pyridines, replicon*

Hepatitis C virus (HCV) is a serious worldwide concern, infecting an estimated 170 million people (3% of the global population), including 4 million people in the United States.[1,2] HCV leads to chronic liver disease, cirrhosis, and hepatocellular carcinoma, and it remains the most common indication for liver transplantation in developed countries. Between 8,000 and 10,000 deaths per year in the United States are attributed to complications of chronic HCV infection.[1,2] The long-time standard of care, a combination of injected interferon and oral ribavirin, is poorly tolerated, contraindicated in a significant portion of the HCV-infected patient population, and affords a sustained virologic response in only half of those treated.[1,2] The addition of one of the two recently approved protease inhibitors to the current standard of care significantly improves the overall cure rate; however, tolerability and drug−drug interactions remain a key concern.[3,4]

Identifying an oral drug cocktail that would both obviate the need for injected interferon and lead to sustained virologic responses over 90% remains the ultimate goal of most clinical development plans. The majority of clinical candidates currently undergoing investigation lead to HCV resistance arising in viral proteins, including well-characterized clinical mutations in the HCV protease, NS5A, and the HCV RNA-dependent RNA polymerase (NS5B).[5,6] This article discloses the initial lead optimization of a series of potent HCV replication inhibitors structurally related to anguizole (**1e**),[7,8] which is reported to directly bind to isolated HCV NS4B protein (Figure 1).[8,9] These small molecules appear to act via a novel mechanism of action, which likely does not suffer from clinical cross-resistance to known NS5B, NS5A, or protease inhibitors currently approved or undergoing clinical trials and represents a potential option for combination therapy.

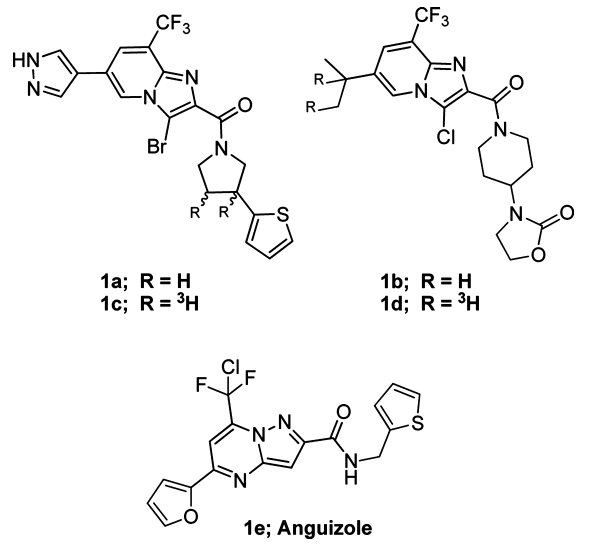



**Figure 1.** Tritiated NS4B probes and anguizole.

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Imidazo[1,2-*a*]pyridines **1a**–**b** are potent inhibitors of HCV in the *in vitro* replicon[10] system, with measured EC50s for wild type (WT) genotype 1a and 1b replicons of <55 nM. Both **1a** and **1b** were recently disclosed in conjunction with other novel chemical matter optimized from a replicon-based HTS screen, with **1b** having been identified following extensive optimization of **1a**.[11−15] Resistance passaging for compounds within this series (including **21** and anguizole[7]) revealed that key mutants arise in the HCV NS4B protein, specifically H94N/R, F98L, and V105L/M (genotype 1b). Imidazo[1,2-*a*]pyridines **1a**–**b** show significant shifts in $EC_{50}$s for replicons bearing these mutations (>20×). Titration of **1c** with purified HCV NS4B protein affords a $K_{app}$ of 76 nM (data not shown) while an identical experiment with **1d** affords a $K_{app}$ of 30 nM (Figure 2). Displacement of **1d**

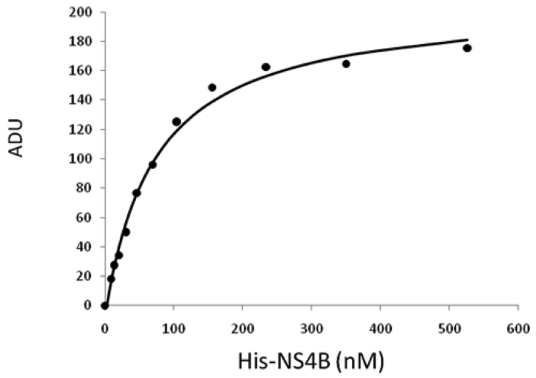



**Figure 2.** Equilibirum binding of **1d** for isolated His-Tagged NS4B. $K_d$ = 30 nM (see Supporting Information).

with **1b** affords a measured $IC_{50}$ = 30 nM (Figure 3) as expected, and direct displacement of **1d** by anguizole (**1e**) affords a

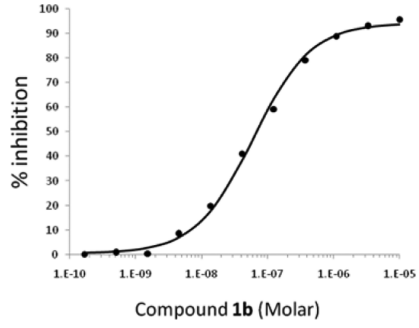


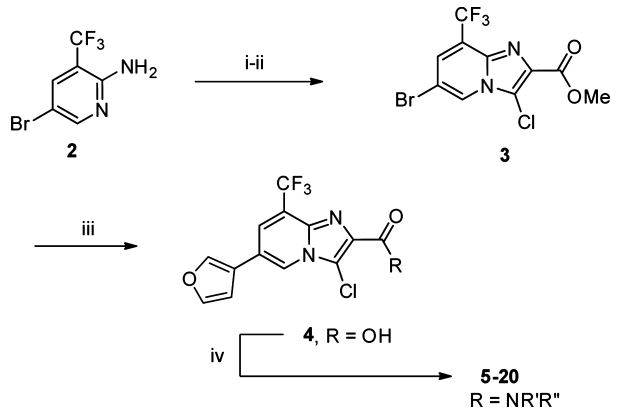

**Figure 3.** $IC_{50}$ determination for **1b** via **1d** displacement from purified NS4B.

measured $IC_{50}$ = 22 nM. The affinities of additional analogues for purified NS4B protein (see Supporting Information) are reported using the direct displacement of **1d** (see NS4B binding in subsequent tables[16]). These data are consistent with a direct interaction between small molecules within this class and the HCV NS4B protein.[17]

The development of initial lead molecule **1a** was complicated by apparent thiophene-related, rapid *in vitro*/*in vivo* metabolism and irreversible trapping of glutathione in *in vitro* reactive metabolite assays. We set out to systematically optimize the DMPK and potency of **1a**.

Versatile imidazo[1,2-*a*]pyridine carboxylic acid **4** could be prepared in four steps from commercially available 2-amino pyridine **2** (Scheme 1). Alkylation/cyclization with α-bromopyruvate,

**Scheme 1. Synthesis of Core and Elaboration**[a]




[a]Conditions: (i) α-bromopyruvate, DMF, 50 °C (83%);[11] (ii) NCS, DMF, 50 °C, 1 h, (91%);[11] (iii) 3-furanylboronic acid, $K_3PO_4$, MeCN, $Pd(dppf)Cl_2$, (82%);[12] (iv) amine, HATU, DIPEA, DMF (20–85%).[11−15]

followed by selective chlorination with NCS and tandem Suzuki cross-coupling/hydrolysis, affords furan **4**. Subsequent HATU mediated amide formation affords novel compounds **5**–**20**. We found that a 3-substituted furan at C(5) and chlorine for bromine replacement at C(3) were well-tolerated relative to **1** (±**6**, Table 1), and we chose to focus first on the optimization of the amide moiety in the context of C(3) chloro and C(5) 3-furyl substituents.

A range of 3-aryl (**5**) and 3-heteroaryl (**6**, **7**) substituted pyrrolidines were well tolerated, giving submicromolar inhibitors of the HCV WT1a and WT1b replicons with retained ability to displace radioligand **1d** from purified NS4B (Table 1). Ring expansion to the corresponding piperidine (i.e., **6** → **8**) gave similarly equipotent HCV replication inhibitor **8**.

Eliminating the chiral center proved most efficient for rapid assessment of novel amides. As such, we chose initially to further pursue the piperidine subseries. Basic pyrrolidine (**9**) exibited no HCV inhibition or NS4B binding, while incorporation of the corresponding cyclic amide (**10**) rescued both to a significant extent. Replacing the cyclic amide with the corresponding oxazolidinone or oxazolidine dione afforded **11** and **12**, respectively. Oxazolidinone **11** was 5–10 times more potent than the corresponding amide (**10**). Direct substitution of the oxazolidinone ring was not well tolerated (see **13** and **14**). In fact, the size, substitution, and electronics of the oxazolidinone ring appeared critical, as dozens of ring-opened and ring-substituted oxazolidinone analogues showed a much reduced ability to inhibit HCV replication *in vitro* and/or to bind directly to purified NS4B.[11−15]

With potent oxazolidinone **11** and oxazolidine dione **12** in hand, we examined standard DMPK properties, including P450 inhibition, reactive metabolite potential, and *in vivo* rat PK. We found **11** had no significant P450 liabilities or reactive metabolite potential in a glutathione trapping assay, but exhibited rapid *in vivo* clearance in rat (Table 2). Importantly, high circulating levels of **12** were observed following IV or PO dosing of **11**. Although dione **12** represented a marginal improvement in *in vivo* clearance, potency, and overall exposure relative to **11**, it was positive in our assessments of reactive metabolite. With these data in hand, we set out to identify a suitable oxazolidinone replacement, which could not be oxidized *in vivo* to reactive oxazolidine diones such as **12**.


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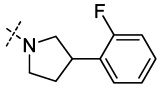
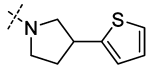
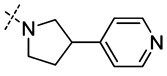
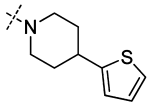
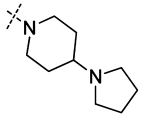
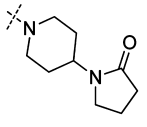
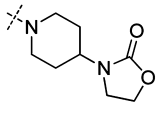
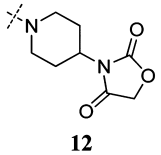
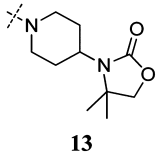
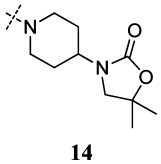
**Table 1. Inhibition of HCV RNA in the Replicon System[a]**

| R | $EC_{50}$ (nM) WT 1a | $EC_{50}$ (nM) WT 1b | $IC_{50}$ (nM) 4B Binding |
|---|---|---|---|
| **1e** | 740 | 450 | 22 |
|  **(±) 5** | 680 | 78 | 5.4 |
|  **(±) 6** | 770 | 67 | 12 |
|  **(±) 7** | 380 | 270 | 25 |
|  **8** | 220 | 110 | 5.3 |
|  **9** | >50000 | >50000 | >50000 |
|  **10** | 260 | 90 | 170 |
|  **11** | 7.4 | 18 | 150 |
|  **12** | 20 | 51 | 35 |
|  **13** | 33000 | 31000 | 9000 |
|  **14** | 9900 | 76000 | 220 |

[a]For **5**–**10**, **12**–**14** WT 1a and 1b replicon data is the average of $n$ = 3; for NS4B binding, data is the average of $n$ = 6. For **11**, WT 1a, 1b, and NS4B binding represent $n$ = 506, 523, and 24, respectively. An analysis of variance (ANOVA) model was fit and used to link values to the compounds (see Supporting Information).

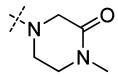
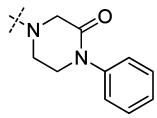
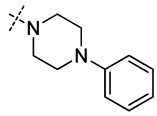
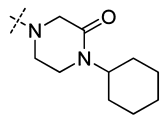
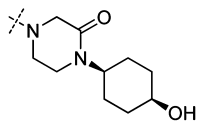
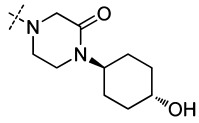
**Table 2. Rat DMPK Profile for 11 and 12[a]**

| | 11 | 12 |
|---|---|---|
| clearance | 72 | 46 |
| Vdss | 2.5 | 3.1 |
| PO $T_{1/2}$ | 1.9 | 3 |
| $AUC_{0-24}$ | 700 | 1100 |
| %F | 60% | 60% |

[a]Dosed as a solution in DMSO/4% HP$\beta$CD in acetic acid (5:95); IV dosed 1 mg/kg; PO dosed 5 mg/kg; clearance (mL/min/kg); Vdss (L/kg); $T_{1/2}$ (h), AUC (ng·h/mL). All $n$ = 3 animals.

Recognizing that the carbonyl moiety was an important pharmacophore (i.e., **9** → **10**, Table 1), we envisioned a number of modifications which might position this critical group in similar space to **10**. Methyl piperazinone **15** exhibited a 1.2 $\mu$M $IC_{50}$ in our radioligand displacement assay, but aryl **16** showed more promising potency, with the addition of the pendant ring affording 50-fold improvements in NS4B affinity and replicon potency. In contrast to the oxazolidinone series, incorporation of a properly placed carbonyl gave a less dramatic (i.e., **17** → **16**) change in replicon potency and NS4B binding affinity. However,

**Table 3. Inhibition of HCV RNA in the Replicon System[a]**

| R | $EC_{50}$ (nM) WT 1a | $EC_{50}$ (nM) WT 1b | $IC_{50}$ (nM) 4B Binding |
|---|---|---|---|
|  **15** | 18000 | 36000 | 1200 |
|  **16** | 430 | 160 | 24 |
|  **17** | 450 | 1600 | 20 |
|  **18** | 11 | 3.5 | 5.6 |
|  **19** | 150 | 300 | 250 |
|  **20** | 0.9 | 9.7 | 43 |

[a]For WT 1a and 1b replicon data is the average of $n$ = 3; for NS4B binding, data is the average of $n$ = 6. $XC_{50}$ determinations performed as for Table 1.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further refinement to cyclohexyl afforded potent HCV replicon inhibitor **18**.

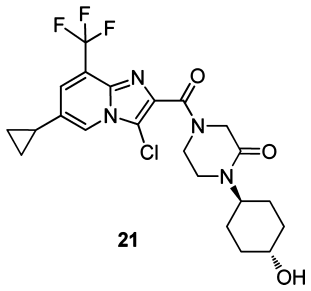
Bare cyclohexyl **18** suffered from rapid *in vitro* and *in vivo* clearance in rat (data not shown, $Cl_{rat} \gg$ hepatic blood flow). Metabolite ID studies with rat/human hepatocytes indicated a principal route of metabolism of **18** was via direct oxidation of the pendant cyclohexyl. Preoxidation of the cyclohexyl was tolerated with respect to NS4B binding and replicon potency, with a *trans* relative configuration of a pendant hydroxyl better tolerated than the corresponding *cis* arrangement (**20** vs **19**, Table 3).

The imidazopyridine analogues, in general, display a reasonable correlation ($R^2$ = 0.48) between direct NS4B binding and cell-based replicon inhibition (see Supporting Information Table SI-4). While permeability for analogues in this series was uniformly high, many of the outliers with reduced potency in the replicon assays (e.g., **5** and **6**) relative to the NS4B binding assay were found to have poor measured solubility (<15 $\mu$M). Precipitation of compound at higher concentrations likely confounds $EC_{50}$ interpretation and reproducibility. Conversely, analogues lacking an extra aromatic ring (**9**−**15**, **18**−**21**) afforded maximum measured solubilities of >500 $\mu$M, were generally well-behaved in the replicon assay, and as such constituted the focus of our efforts.

Overall hydroxycyclohexyl moieties **19** and **20** gave an improved *in vivo* DMPK profile (data not shown) relative to **18**. Despite reductions in *in vivo* clearance, **20** suffered from positive reactive metabolite, apparently now as a result of oxidation of the pendant furan (not observed directly for any previous molecules). A brief survey of less reactive functionality at the furan position revealed that small, alkyl groups in addition to heteroaryl functionality were well tolerated. This survey led to the subsequent identification of **21**, wherein the pendant furan was replaced by a cyclopropyl. The same trends in SAR observed with the furan functionality were observed in the context of the cyclopropyl as well. For example, the cis isomer corresponding to **21** shows significant reductions in both NS4B binding and replicon inhibition (see Table SI-3, Supporting Information).

Compound **21** was equipotent to furan **20** and showed no shift in potency in well-characterized replicon mutants arising in NS5B, NS3, or NS5A (Table 4), consistent with a novel MOA involving direct interaction with NS4B. In contrast, stable replicons bearing NS4B mutations H94N or V105M showed significant resistance to **21** (>100×; see Table 4), while transiently transfected NS4B mutations F98L, H94R, and V105L showed resistance as well. Further, **21** possessed no measurable P450 (3A4, 2C9, 2D6, 1A2, 2C19) or reactive metabolite liabilities. *In vivo* profiling of **21** in rat revealed improved clearance and oral exposure relative to **11**, **18**, and **20** when dosed in rat or dog at 5 mg/kg (Table 5).

**Table 4. Replicon Profile for 21**[a]




**21**

| mutant replicon | gene | $EC_{50}$ (nM) |
|---|---|---|
| NS4B binding | | 60 |
| WT 1a | | 0.81 |
| WT 1b | | 5.4 |
| 1B H94N | NS4B | 200 |
| 1B V105M | NS4B | 800 |
| 1B H94R* | NS4B | 53 |
| 1B V105L* | NS4B | 38 |
| 1B F98L* | NS4B | 27 |
| 1B A156T* | NS3 | 1.0 |
| 1B M414T* | NS5B | 1.0 |
| 1B L31V* | NS5A | 1.0 |

[a]WT 1a and WT 1b data represents $n$ = 137 and 138, respectively; for NS4B binding, data represents $n$ = 6. $XC_{50}$ determinations performed as for Table 1. For 1B replicons H94N ($n$ = 65), for 1B V105M ($n$ = 59), for 1B A156T/M414T/L31 V ($n$ = 2), H94R ($n$ = 30), F98L ($n$ = 20), and V105L ($n$ = 182). * denotes transiently transfected replicons; all other replicons were stable.

**Table 5. Rat and Dog DMPK Profile for 21**

| | rat (**21**)[a] | dog (**21**)[a] |
|---|---|---|
| clearance | 20 | 1.1 |
| Vdss | 4.1 | 0.9 |
| PO $T_{1/2}$ | 3 | 10 |
| $AUC_{0-24}$ | 3800 | 52000 |
| %F | 98% | 65% |

[a]Dosed as a solution in DMSO/Solutol/20% HP$\beta$CD (10:10:80); IV dosed 1 mg/kg; PO dosed 5 mg/kg; clearance (mL/min/kg); Vdss (L/kg); $T_{1/2}$ (h), AUC (ng·h/mL).

Imidazo[1,2-*a*]pyridine (**21**) inhibits HCV replication *in vitro* with $EC_{50}$ < 10 nM for genotype 1a and 1b replicons (Table 4), demonstrates good oral exposure in rodent and dog (Table 5), and maps its resistance to HCV NS4B (Table 4), unique among existing HCV inhibitors undergoing clinical trials. These properties make this class of imidazo[1,2-*a*]pyridines a promising and unique class of HCV replication inhibitors. We will report the full *in vivo* characterization of **21** and other related development candidates in due course.

## ■ ASSOCIATED CONTENT

### Supporting Information

Replicon and NS4B binding assay protocols, synthetic procedures, compound purities, protein purification protocols, in vivo/in vitro DMPK protocols, details of radioligand syntheses, and statistical methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare the following competing financial interest(s): All authors are current or former employees of GlaxoSmithKline.

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