Bioorganic & Medicinal Chemistry Letters 20 (2010) 4614–4619

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Cyclic amide bioisosterism: Strategic application to the design and synthesis of HCV NS5B polymerase inhibitors

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ARTICLE INFO

Article history:
Received 4 April 2010
Revised 26 May 2010
Accepted 1 June 2010
Available online 8 June 2010

Keywords:
Amide bioisostere
Modeling
Synthesis
HCV
NS5B polymerase
Thumb
Mutant
3mt5
X-ray structure
PDB
Ruthenium catalyst

ABSTRACT

Conformational modeling has been successfully applied to the design of cyclic bioisosteres used to replace a conformationally rigid amide bond in a series of thiophene carboxylate inhibitors of HCV NS5B polymerase. Select compounds were equipotent with the original amide series. Single-point mutant binding studies, in combination with inhibition structure–activity relationships, suggest this new series interacts at the Thumb-II domain of NS5B. Inhibitor binding at the Thumb-II site was ultimately confirmed by solving a crystal structure of **8b** complexed with NS5B.

Hepatitis C virus (HCV) is a major threat to public health worldwide as an estimated 3% of the world's population is infected. Approximately 15–20% of infected individuals clear the virus without treatment, while the remaining 75–85% become chronically infected.[1] Chronic HCV infection has become a leading cause of cirrhosis, hepatocellular carcinoma and liver transplantation in the US.[2] The current standard of care treatment for HCV infection consists of a combination dose of pegylated interferon-α (PEG-IFNα) along with the broad spectrum antiviral agent Ribavirin for up to 48 weeks. However, only a moderate sustained virological response (SVR) of 42–46% is achieved in patients infected with genotype-1 virus, the genotype which accounts for ~70% of cases in the US. In addition, patient compliance is often limited by severe side effects. Based on these limitations, there is still a significant unmet clinical need for safer and more effective anti-HCV therapies.

HCV contains a positive-sense, single-strand RNA molecule of 9.6 kb with one open reading frame coding for a large polyprotein of ~3000 amino acids. Intracellular processing of the polyprotein yields at least ten individual functional proteins, namely C, E1, E2, P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. Although the best path for HCV treatment has yet to be defined,[3] it will likely require a combination therapy approach targeting multiple viral proteins and/or pathways in the HCV lifecycle in order to effectively suppress resistance.

One particular HCV target protein of interest is NS5B, an RNA-dependent RNA polymerase (RdRp) that is essential for the synthesis and replication of viral RNA. It has a three-dimensional shape similar to other known RNA polymerases and is often described as a right-handed motif, with distinct Palm, Finger, and Thumb domains. Several inhibitors targeting various known binding sites within HCV NS5B polymerase have demonstrated useful efficacy in clinical trials.[4]

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doi:10.1016/j.bmcl.2010.06.008

At Roche Palo Alto we initiated a program to identify new inhibitors targeting the Thumb-II binding site, which is located near the base of the Thumb domain and is approximately 35 Å away from the active site.[4,5] Several NS5B inhibitor chemo types, including phenylalanine **1**,[6] thiophenecarboxylic acid **2**,[5a,b] pyranoindole **3**,[7] 4-hydroxydihydropyranone **4**,[8] and recently 1*H*-benzo[*de*]isoquinoline-1,3-(2*H*)-dione **5**[9] (Fig. 1), have been shown to bind to this hydrophobic pocket. In this Letter, we report the design, synthesis and biological evaluation of a new series of thiophenecarboxylic acid analogs derived from the prototypical compound **2**.

It has been shown that both the amide linkage and the isopropyl group of **2** are important structural elements for enzymatic and replicon potency.[5a] We previously reported the X-ray structure of **2** bound to HCV NS5B polymerase[5c] which shows the amide carbonyl is involved in a single water-mediated hydrogen bond to Trp528, while the isopropyl group is fully exposed to solvent. However, this isopropyl group is believed to play two important conformational roles: (1) orienting the amide bond in a *cis*-like (i.e., relative to the des-isopropyl analog) or *E*-configuration; (2) positioning the plane of the amide bond nearly orthogonal relative to the thiophene ring. A similar conformation has been observed for the corresponding aromatic amides **6** and **7** when complexed to NS5B.[5b] In this conformation, the *trans*-4-methylcyclohexyl group is favorably positioned in a deep hydrophobic pocket and makes extensive interactions with residues Arg422, Met423, Leu474, His475, Tyr477, Lys501, and Trp528.

Bioisosteric replacement is an important tool in medicinal chemistry that can be used to improve the potency, selectivity and pharmacokinetics of key compounds or to generate new intellectual property (IP).[10] In particular, replacement of amide bonds with suitable bioisosteres that maintain similar geometric, electronic, or hydrogen bonding properties have been extensively utilized.[11] Based on our structural analyses of the NS5B complexes with inhibitors **2**, **6**, and **7**,[12] our strategy centered on replacement of the amide bond with a bioisosteric cyclic ring system (Fig. 2). The primary small molecule conformational elements considered were the dihedral angle between the thiophene ring and the amide plane (as defined by atoms C_2–C_3–N_4–C_5), and the exit vector for the hydrophobic 'R' group.

We performed a small molecule conformational analysis on several proposed analogs (**8**–**10**; Table 1) in order to evaluate the impact of aryl or heteroaryl ring substitution on both the key dihedral angle and the positioning of the hydrophobic R-group. Conformational analysis was performed in Maestro[13] using MMFFs parameters in water with a distance dependant dielectric constant. Torsional sampling was performed using 5000 steps of Monte Carlo. We narrowed our analysis to include only those observed conformations which were (a) within 5 kcal/mol of the global minimum conformation; and (b) relevant to binding in the NS5B Thumb-II site. Our results reasonably predicted the observed (via X-ray) dihedral angles for molecules **2**, **6** and **7** to within ±30° (Table 1). Compared to **6** and **7**, the cyclohexyl group of **2** led to a wider distribution of observed relevant conformations. The modeled results for **6** and **7** match closely with what has been previously reported[5a] and we observed a relatively narrow distribution of relevant conformations. Our analysis of the 4-methyl-cyclohexylphenyl analog **8b** showed a very narrow distribution of conformations which strongly suggest a nearly orthogonal relationship (96°) between the phenyl and thiophene rings is to be expected. Examination of the N-linked pyrazalone **9** predicts an angle that is close to that observed for compound **6**; however, this particular conformation is 4.7 kcal/mol higher in energy than the observed global minimum for this structure. Favorable electrostatic interactions between the carboxylate and the pyrazalone-NH in **9** appear to limit the number of binding site-relevant conformations that are accessible in this molecule. The triazole results (**10b**, **d**) match well with comparable analogs in the amide series and **10d** appears to have a somewhat smaller distribution of available relevant conformations. In all cases the general alignment of the R-group exit vectors matched very well with the X-ray data for **2** (Fig. 3).

Encouraged by our initial conformational analysis, we prepared two six-membered ring analogs in which the amide group was replaced by a simple phenyl ring as a preliminary test of our hypothesis (Table 2). Analogs **8a** and **8b** were prepared in a similar fashion

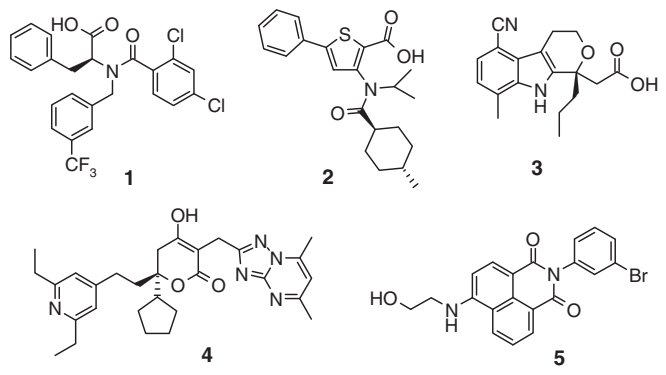
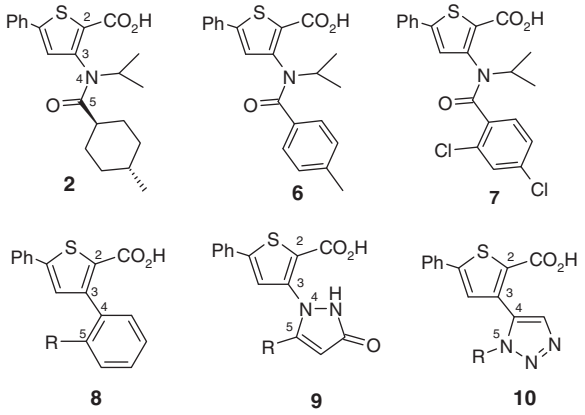


Figure 1. Known Thumb-II domain NS5B inhibitors.

Figure 2. Bioisosteric amide bond replacement strategy.

Table 1
Dihedral angle comparison: X-ray data versus modeling analysis



Compd	R-group	X-ray[a]	Modeled[a,b]
2		93	63
6		75	49
7		80	59
8b	4-Me-cyclohexyl	—	96
9	Tolyl	—	47
10b	Tolyl	—	70
10d	4-Me-cyclohexyl	—	79

[a] Measured dihedral angle: C_2–C_3–N_4–C_5.
[b] Angles noted are for each Thumb-II binding site-relevant lowest energy conformation within 5 kcal/mol of the observed global minimum.

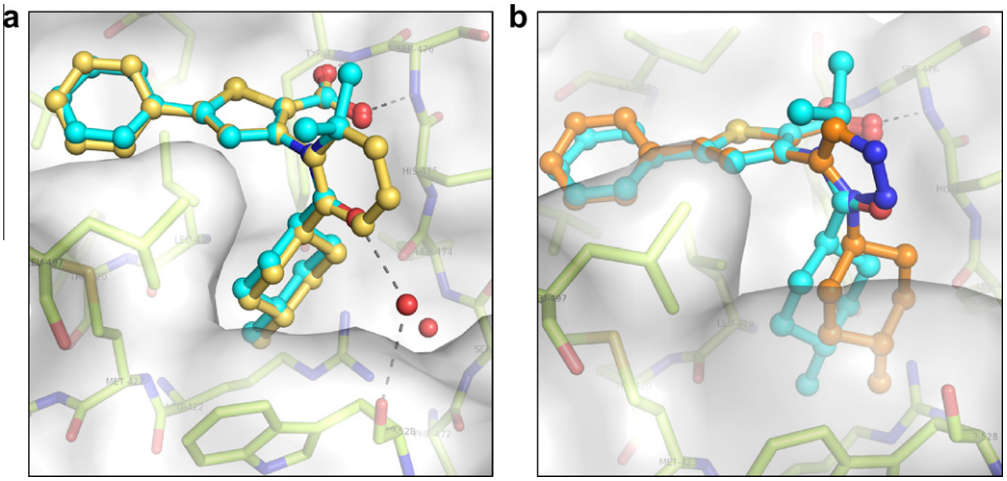
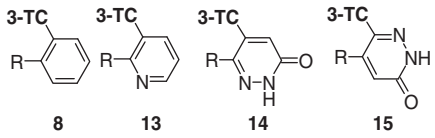


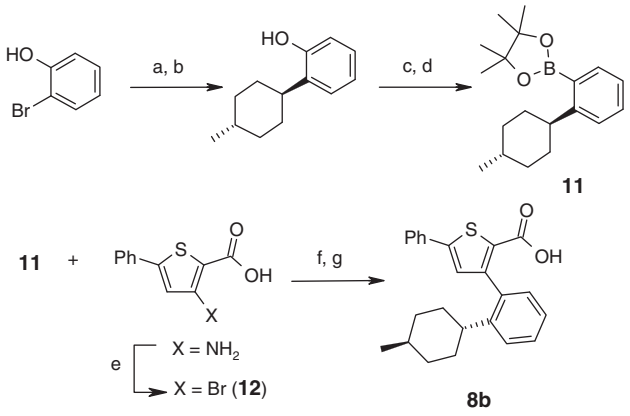
Figure 3. (a) Overlay of **8b** lowest energy conformation (yellow) with X-ray bound structure of **2** (NS5B bound conformation in blue; red spheres are water); (b) overlay of lowest energy relevant conformation of **10b** (orange) with NS5B bound structure of **2**.

Table 2
Sixmembered ring[a] amide replacement structure–activity relationships and inhibitory activities against HCV NS5B



Compd	R-group	NS5B[b]	*Clog P*
2		0.028	7.55
8a	*O-n*-Butyl	16	5.8
8b	*trans*-4-Me-cyclohexyl	0.042	7.67
13a	*trans*-4-Me-cyclohexyl	0.022	6.22
13b	4-Me-cyclohex-1-enyl	0.17	6.21
14	Phenyl	92	2.97
15	4-Tolyl	3.3	3.47

[a] **3-TC** = 3-substituted 5-phenylthiophene-2-carboxylic acid.
[b] GT-1b NS5B inhibition (IC_{50}, μM).

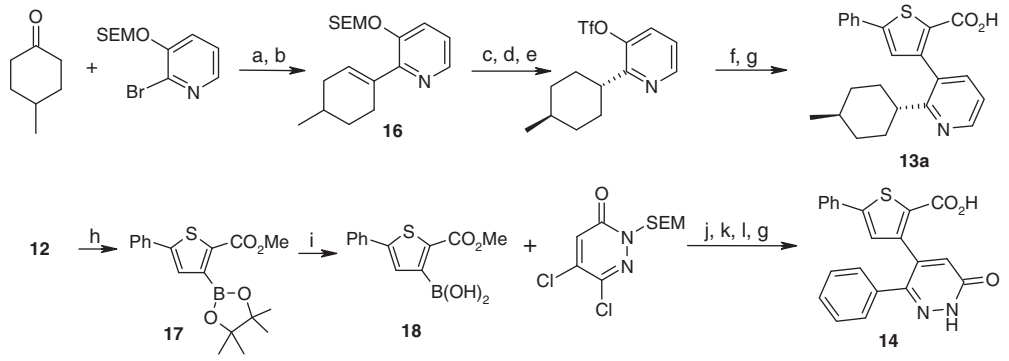


Scheme 1. Reagents and conditions: (a) *n*-BuLi (2 equiv), −78 °C, Et_2O, then *trans*-4-methylcyclohexanone, −78 °C to −5 °C, 1 h (49%); (b) Et_3SiH, TFA, rt, DCM (79%); (c) Tf_2O, pyridine/DCM (89%); (d) bis(pinacolato)diboron (1.1 equiv), $PdCl_2$dppf (0.08 equiv), dppf (0.16 equiv), KOAc (3 equiv), DMF, 100 °C (15%); (e) *t*-BuONO, $CuBr_2$, ACN, 0 °C, 2 h; (f) $Pd(PPh_3)_4$, Na_2CO_3, Bu_4NBr, tol/H_2O, 90 °C (70%); (g) LiOH, THF/MeOH/H_2O.

by coupling bromide **12** with the appropriate aryl boron reagent under Suzuki coupling conditions. The synthesis of **8b** is outlined in Scheme 1. It should be noted that the choice of solvent in the preparation of 4-methylcyclohexyl phenyl boronate **11** was crucial. A useful, albeit low, yield was obtained in DMF, whereas no product was detected in dioxane. The assay results using GT-1b NS5B (20 nM enzyme, poly-A RNA template)[14] are summarized in Table 2. Although the flexible *n*-butyl ether analog **8a** was inactive, we were quite pleased to see that 4-methyl-cyclohexyl substituted **8b** showed inhibitory potency that was nearly equal to that of the prototypical amide **2**, and provided us with a convincing proof of concept for our primary hypothesis.

In addition to using phenyl as a conformational mimic of the amide linkage in **2**, we were also interested in exploring the importance of additional polar group interactions. In particular, specific analogs were designed to mimic the water-mediated hydrogen bond of the amide carbonyl to Trp528, observed in the NS5B X-ray bound complex with **2** (Fig. 3a).[12] Along with the prospect of improved potency, the incorporation of heteroatoms into the linkage provides a method of reducing *Clog P*, thus improving the overall drug-like properties of this series. Towards this end we first examined a set of six-membered heterocyclic analogs (**13–15**; Table 2) capable of mimicking the hydrogen bond acceptor properties of the amide carbonyl. These compounds were prepared using an alternative coupling route highlighted in Scheme 2. Attempts to prepare either the 2-(4-methyl-cyclohexyl)pyridyl-3-boronate or 3-boronic acid precursor to **13a** were unsuccessful, however, we were able to successfully convert bromide **12** into the requisite boronate **17** and boronic acid **18** via palladium catalyzed borylation. Conversion of the unsaturated SEM intermediate **16** to the corresponding triflate, followed by coupling with **17** led to **13b** in good yield. Palladium catalyzed coupling of SEM-protected 3,4-dichloro-6-pyridazinone with **18**, followed by coupling with phenylboronic acid provided **14**. By reversing the order of coupling (i.e., 4-tolylboronic acid first, then **18**), we were able to prepare the isomeric pyridazinone **15** in similar overall yield.

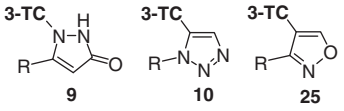
Encouragingly, the 3-pyridyl analog **13a** was equipotent with **2**, and was ~1.3 log units less lipophilic based on *Clog P* calculations[16] (Table 2). On the other hand, the corresponding olefin analog **13b** was eight-fold less active, and the 4-, 5-, and 6-pyridyl variants[17] (data not shown) all had roughly similar potency to **13b**. The notably more polar pyridazinones **14** and **15** were significantly less active. We note that **14** lacks 4-substitution, which has been shown to limit potency in related series.[5a] The isomeric pyridazinone **15** has a 4-tolyl group, but is still two orders of magnitude less active relative to pyridyl **13a**.



Scheme 2. Reagents and conditions: (a) *n*-BuLi, THF, −78 °C (50%); (b) Burgess reagent,[15] benzene, reflux, 3 h (72%); (c) H_2 (1 atm), PtO_2, HOAc/EtOAc (1:6) (75%); (d) dil. H_2SO_4, MeOH, rt, 24 h; (e) Tf_2O, pyridine/DCM (78% for d + e); (f) **17**, $Pd(PPh_3)_4$, K_3PO_4 (2 equiv), dioxane, 85 °C (89%); (g) 2 M LiOH, MeOH/THF, reflux, 1 h (90%; 70% for **14**); (h) $Pd(PPh_3)_4$, Et_3N, THF, pinacolborane, 40 °C, 2 h; (i) AcOH/H_2O/THF (63%); (j) $Pd(PPh_3)_4$, 2 M Na_2CO_3 (aq), MeOH, reflux (65%); (k) phenylboronic acid, $Pd(PPh_3)_4$, 2 M Na_2CO_3 (aq), MeOH, reflux (39%); (l) HCl/MeOH, reflux, 24 h.

Our preliminary small molecule conformational analysis suggested the various five-membered ring exit vectors, though generally similar to those of the six-membered ring analogs, offered subtle variations in the positioning of the hydrophobic R-group. Furthermore, five-membered heterocycles provide additional options for mimicking the amide carbonyl. We were further encouraged by recent results demonstrating a successful bioisosteric exchange of an amide by a triazole ring.[11c] We chose to prepare a limited set of five-membered ring analogs (**9**, **10**, **26**; Table 3) in order to test this hypothesis.

Table 3
Fivemembered ring amide replacements:[a] structure–activity relationships and inhibitory activities against HCV NS5B



Compd	R-group	NS5B[b]	*Clog P*
2		0.028	7.55
9	4-Tolyl	0.62	5.66
10a	Phenyl	17	4.62
10b	4-Tolyl	0.11	5.12
10c	Cyclohexyl	0.34	4.43
10d	*trans*-4-Me-cyclohexyl	0.10	4.95
25a	Phenyl	29	4.36
25b	4-Tolyl	2.5	4.86
25c	Cyclohexyl	0.074	5.18
25d	*trans*-4-Me-cyclohexyl	0.11	5.70
25e	4-Chlorophenyl	2.6	5.07
25f	4-Fluorophenyl	5.7	4.50
27		32	6.21

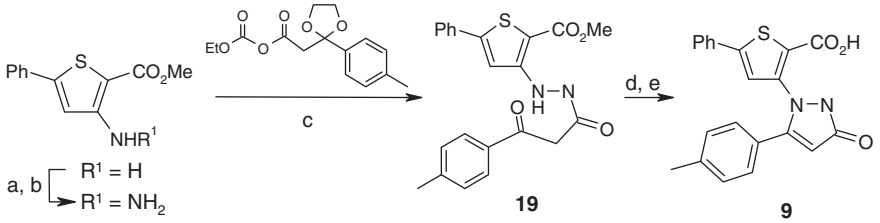
[a] **3-TC** = 3-substituted 5-phenylthiophene-2-carboxylic acid.
[b] GT-1b NS5B inhibition (IC_{50}, μM).

The N-linked 3-pyrazalone **9** was prepared from the corresponding 3-aminothiophene carboxylic ester as summarized in Scheme 3. The mixed anhydride intermediate was readily prepared starting from 4-methyl acetophenone and dimethyl malonate. Mild acid catalyzed cyclization of the coupled hydrazide **19** gave **9** in modest yield following saponification. In comparison to **15** (the six-membered ring homolog), **9** displayed a five-fold enhancement in potency (Table 3, IC_{50} = 0.62 μM).

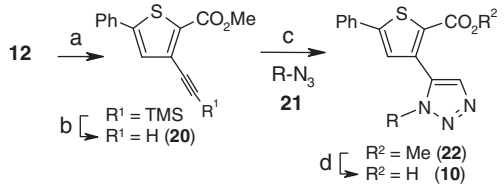
The triazole and isoxazole analogs (**10**, **25**; Table 3) were synthesized in a convergent fashion via a Ru-catalyzed coupling.[18] Specifically, the cycloaddition of alkyne **20** with alkyl or aryl azides **21a**–**d** (see Table 3 for descriptions of **a**–**d**) gave the desired cycloaddition products **22a**–**d** in low to moderate yields as a single regioisomer (Scheme 4). In accordance with the observation in the literature, the less reactive alkyl azides **21c** and **21d** required the use of the more reactive Cp*Ru(COD) catalyst.

In contrast, yields for the isoxazole synthesis shown in Scheme 5 were much higher (55–98%), except in the case of **24d** (13% yield plus 8% of the 1,4-regioisomer **26**). We attributed the low yield and poor selectivity in this case to the impure nature of chlorooxime **23d**, which was prepared in five steps without purification (Scheme 6). We suspect impurities present in **23d** suppressed the catalyzed process by poisoning the catalyst, therefore allowing a background reaction leading to the 1,4-isoxazole regioisomer **26** to become a competing process.

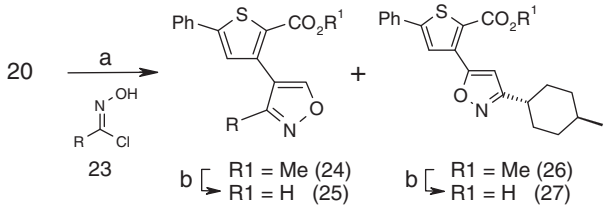
The structure–activity relationships for the triazole and isoxazole series are consistent with what has been observed in the prototypical thiophene amide series: increasing steric bulk of the R-group leads to increased inhibitory potency (Table 3; **10a**, **25a** vs **10b**, **25b**–**e**). Simply adding a 4-fluoro substituent (**25f**) gave a modest five-fold improvement over the phenyl analog (**25a**). However, in contrast to the amide-linked series the addition of a *trans*-4-methyl group to the cyclohexyl ring provided only a three-fold improvement in the triazole series (**10d**), and showed essentially no change in potency in the isoxazole series (**25c**,**d**). Both 4-meth-



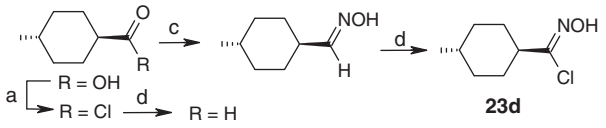
Scheme 3. Reagents and conditions: (a) $NaNO_2$, HCl; (b) $SnCl_2$, H_2O (60%); (c) DCM, rt, then HCl (aq)/THF (43%); (d) *p*-TsOH/benzene, 60 °C; (e) LiOH, THF/MeOH/H_2O, rt (36%).



Scheme 4. Reagents and conditions: (a) trimethylsilyl acetylene, $PdCl_2(PPh_3)_2$ (10 mol %), *i*-Pr_2NEt, CuI, THF, 85 °C, overnight; (b) K_2CO_3, MeOH, rt, (90% for a + b); (c) condition 1: $Cp^*RuCl(PPh_3)_2$ (5 mol %), benzene, 60 °C, overnight, **22a** (17%); **22b** (19%); Condition 2: $Cp^*Ru(COD)$ (2 mol %), toluene, rt, 16 h, **22c** (28%); **22d** (38%); (d) KOH, MeOH or LiOH, THF/H_2O.



Scheme 5. Reagents and conditions: (a) $Cp^*Ru(COD)$, Et_3N, DCE, rt, 16 h; (b) KOH, MeOH or LiOH, THF/H_2O.



Scheme 6. Reagents and conditions: (a) oxalyl chloride, DMF/DCM; (b) 10% Pd/C, 2,6-lutidine; (c) hydroxylamine–HCl, NaOAc, H_2O, rt; (d) NCS, DMF, cat. HCl, rt.

ylcyclohexyl analogs were only four-fold less potent than the prototypical compound **2**. Not surprisingly, the 3,5-isoxazole isomer **27** showed essentially no inhibitory potency.

In order to confirm that this series of NS5B inhibitors were indeed binding at the putative Thumb-II binding site, we performed a structure–activity analysis based on binding affinity with **10b** and **10d** using two well characterized NS5B single-point mutants: NS5B L419M and NS5B M414T.[19,5c] The L419M mutation displays resistance against inhibitors that bind in the Thumb-II region, whereas the M414T mutation displays resistance against inhibitors that bind in the Palm region. The amide-linked analog **2** was used as a positive control. The data shown in Table 4 clearly demonstrate that only the Thumb region mutation significantly impacted binding: both triazole analogs showed ~five-fold reduction in binding affinity relative to WT binding and showed no change in affinity towards the Palm region mutant. The amide control **2** showed a 10-fold loss in affinity for L419M and is similarly unchanged by the M414T mutation.

Further confirmation of our binding hypothesis was provided by a co-crystal structure of the 4-methyl-cyclohexylphenyl analog **8b** complexed to NS5B (Fig. 4), obtained through a crystal soaking experiment.[5c,20] The structure of the protein-inhibitor complex clearly shows **8b** bound at the Thumb-II site with the phenyl ring positioned orthogonal relative to the plane of the thiophene ring

Table 4
Thumb versus Palm region binding affinity (K_D, μM)

Compd	NS5B WT	L419M	M414T
2	0.041 ± 0.011	0.427 ± 0.111	0.033 ± 0.008
10b	4.32 ± 0.555	23.09 ± 2.87	3.994 ± 0.91
10d	0.86 ± 0.365	4.48 ± 0.581	0.597 ± 0.218

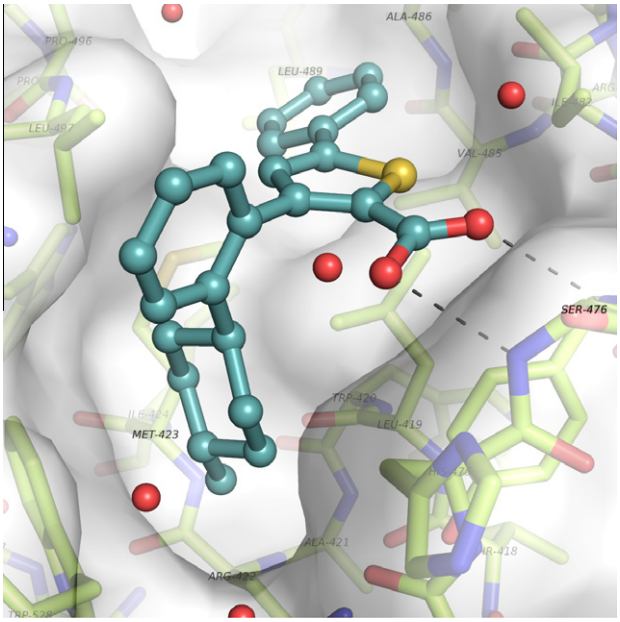


Figure 4. NS5B co-crystal complex with compound **8b**; PDB code 3MF5.

(dihedral angle: 96.7°), nearly identical to our conformational analysis prediction. The bridging water observed in the NS5B structure with amide **2** has been displaced by the linking aryl ring. A new water is observed near the carboxyl binding region (3.0 Å to the nearest carboxyl oxygen). This water is also near His475, but the closest distance is 3.8 Å, suggesting the His475 interaction may be only weakly stabilized through hydrogen bonding. A second water is observed deeper in the lipophilic binding pocket that is involved in a stabilizing hydrogen bond (2.8 Å) with Lys533.

Amide **2** has been reported to show good inhibition in a cell-based replicon assay (EC_{50} = 0.6 μM)[5a] and we have independently confirmed similar potency in our own GT-1b replicon assay[14] (EC_{50} = 0.23 μM; CC_{50} = 48 μM). Regardless of the generally good predicted permeability and a wide range of estimated polarity (i.e., *Clog P*) across our series of NS5B-active amide replacements, selected compounds tested in a GT-1b replicon assay showed generally weak replicon activity and significant cytotoxicity, with only low selectivity indices (CC_{50}/EC_{50} <2). For example, compound **8b** showed an EC_{50} of 12 μM, but this could not be reasonably differentiated from toxicity (CC_{50} = 20 μM). Triazole **10b** was inactive (EC_{50} and CC_{50} >30 μM) and **10d** showed only weak activity (EC_{50} = 15 μM; CC_{50} >50 μM). It has been suggested that within an acidic series, replicon potency is reduced with decreasing pK_a.[21] The measured pK_a of **2** was found to be 3.8, whereas the pK_a of **10d** was 3.6, suggesting that pK_a alone is not a significant contributing factor in explaining the limited replicon potency for this series.

In summary, we describe our efforts towards successful bioisosteric replacements of the rigidly oriented amide bond in a previously reported series of HCV NS5B inhibitors. We performed a preliminary conformational analysis on proposed structures designed to mimic the shape and electronic nature of the amide linkage and prepared a series of *ortho*-substituted aryl and heteroaryl analogs. Members of the series possess excellent inhibitory potency against HCV NS5B and demonstrated structure–activity relationships consistent with the prototypical amide series. Data from both a single-point NS5B mutant binding analysis and an X-ray co-crystal structure confirm that these cyclic amide mimics bind at the NS5B Thumb-II domain.

Acknowledgments

The authors would like to thank Steve Swallow, Connie Oshiro and George Adjabeng for contributions to the design and synthesis of these analogs, and Dave Rotstein and Francisco Talamas for their helpful discussions.

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