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Quinolones as HCV NS5B polymerase inhibitors

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ABSTRACT

Hepatitis C virus (HCV) infection is treated with a combination of peginterferon alfa-2a/b and ribavirin. To address the limitations of this therapy, numerous small molecule agents are in development, which act by directly affecting key steps in the viral life-cycle. Herein we describe our discovery of quinolone derivatives, novel small-molecules that inhibit NS5b polymerase, a key enzyme of the viral life-cycle. A crystal structure of a quinoline analog bound to NS5B reveals that this class of compounds binds to allosteric site-II (non-nucleoside inhibitor-site 2, NNI-2) of this protein.

Hepatitis C virus (HCV), a member of the *Flaviviridae* family, is estimated to infect 170 million individuals worldwide.[1,2] The viral genome, approximately 9.6 kb in size, is a single-stranded, positive-sense RNA that encodes structural and non-structural proteins.[3] Chronic infection by HCV often leads to severe liver disease including hepatocellular carcinoma, cirrhosis, and liver failure.[4,5] The current standard of care is a combination of peginterferon alfa-2a/b and Ribavirin, which successfully eradicates the virus in only about 50% of the patients infected with genotype 1.[6–8] Consequently, considerable pharmaceutical resources have been focused upon the discovery of novel therapies, particularly direct-acting agents specifically targeting essential viral proteins.[9]

The viral RNA dependent RNA polymerase (RdRp) encoded by the non-structural protein 5b (NS5B) is essential for viral replication.[10] Several specific NS5B polymerase inhibitors have shown promising results in clinical trials[11–13] and the polymerase is considered a well validated drug target. Reported inhibitors are either nucleoside analogs which target the active site or non-nucleoside inhibitors (NNIs) that bind to one of four allosteric sites (Fig. 1). Two of these allosteric sites are located in the thumb region (NNI-1 and NNI-2) and two are in the palm region (NNI-3 and NNI-4) as determined by X-ray crystallography and in vitro resistance studies.[14–16]

Recognizing the value of additional therapies, we initiated a program directed towards the discovery of novel inhibitors of NS5B polymerase. This Letter describes our discovery of small-molecules that inhibit HCV replication by impairing the function of NS5B polymerase.

We employed a SPA (Scintillation Proximity Assay)[18a] format for our HTS campaign to screen for inhibitors of NS5B polymerase. Among our confirmed 'hits' was compound **1** (Fig. 2), which inhibited NS5B polymerase enzymatic function with an IC_{50} of 1.15 μM against the wild-type (genotype 1b) enzyme. Upon testing this

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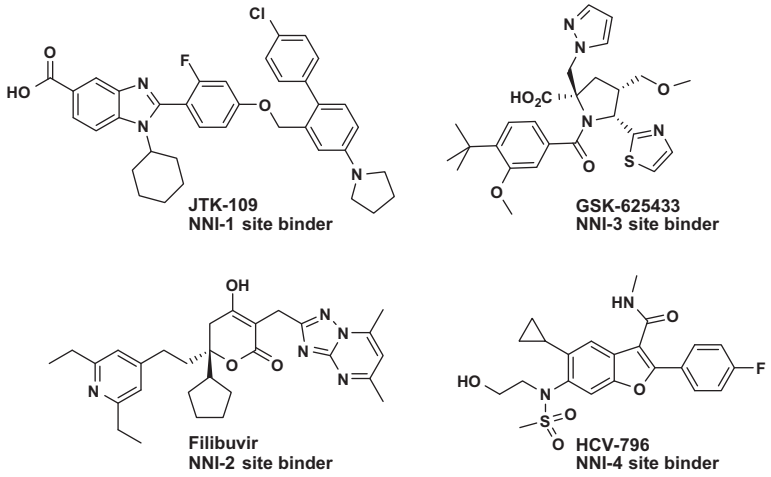
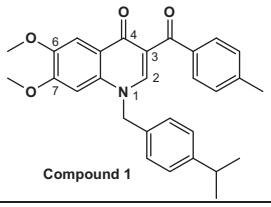


Figure 1. Representative non-nucleoside HCV polymerase inhibitors that have progressed to clinical trials and their respective allosteric binding sites.



WT IC_{50} (μM)	Fold shift in IC_{50} Measured for Mutant NS5b Relative to the Wild Type Value		
	P495L/WT (NNI-1)	M423F/WT (NNI-2)	M414T/WT (NNI-3)
1.15	1	> 200	2

Figure 2. Analysis of hit compound **1** through site-directed mutagenesis and IC_{50} shifts led to the conclusion that this class of compound binds at allosteric site II of NS5B polymerase.

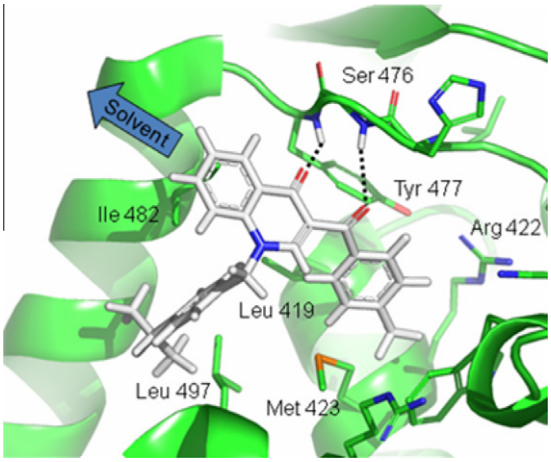


Figure 3. Model of **1** bound to NNI-2 site observed for X-ray structure 1NHU. Potential hydrogen bonding interactions are shown as dashed lines. Solvent accessible region is marked by an arrow.

compound against a panel of recombinant NS5B enzymes designed with targeted mutations in allosteric sites NNI-1, NNI-2, and NNI-3 and evaluating the fold-change in enzyme inhibition over wild-type, we were able to conclude that compound **1** was inhibiting enzymatic activity by binding at site NNI-2 of the polymerase.

Two previously reported crystal structures of NNI-2 allosteric site binders (PDB codes 1NHU[19] and 1OS5[20]) were used to develop a binding mode hypothesis for compound **1**. These X-ray structures revealed the presence of two critical recognition elements in the binding pocket: (1) a conserved hydrogen bonding motif to the backbone amide NH of Ser 476 and/or Tyr 477 and (2) a hydrophobic pocket formed predominantly by Leu 419, Met 423 and Trp 528. Manual docking of compound **1**, while maintaining these interactions, suggested a reasonable fit (Fig. 3). The C-4 carbonyl may form a hydrogen bond to the backbone amide NH of Tyr 477, the C-3 ketone carbonyl forms a hydrogen bond to Ser 476, and the primary hydrophobic pocket is occupied by the phenyl of the C-3 phenyl ketone group. This pose would place the 6- and 7-position substituents of the quinolone oriented towards solvent. To improve potency by optimizing hydrophobic interactions, we began our SAR investigations at the 1- and 3-positions of the quinolone ring.

In addition to determining the NS5b inhibition potential, compounds were also evaluated in a cell-based viral replication surrogate assay known as the replicon system.[17,18b] In parallel, compounds were also evaluated for their cytotoxic effects in the same cell line. Table 1 shows selected examples of analogs at the 1-position of the quinolone ring. Para-substitution on the benzyl analogs (**2**) was important, with the bulky *tert*-butyl and methyl sulfone derivatives bringing the binding potency down to sub-micromolar levels. A polar amine substituent at the para-position results in loss of potency, while adding a 2- or 3-substituent to a para-substituted benzyl analog is well tolerated (compounds **3** and **4**). This SAR is consistent with the hypothesis that 1-position substituents fit in a small hydrophobic pocket. Among the non-aromatic substituents, the cyclopropyl-methyl compound **5** is inactive with potency being regained to 9 μM with the larger cyclohexyl methyl group in compound **6**. The data obtained with the phenethyl substitution (compound **7**) suggests that the vector into the pocket is quite narrow and requires a more linear substituent as potency is regained with a phenylpropyl substitution (compound **8**).

The poor replicon potency observed for compounds in Table 1 underscored the need to improve the binding potency and physical properties of the series. Therefore, the 3-position phenyl ketone was subjected to an exploratory SAR analysis. The proposed binding mode of compound **1** suggested that the ketone carbonyl formed an important hydrogen bond to Ser 476. Analogs were synthesized that retained this critical hydrogen bond acceptor, but varied the distance and geometry between the quinolone ring H-bond acceptor, and the terminal phenyl ring (Table 2).

The highlight of this SAR exploration was the discovery of the 3-position benzyl ester which imparted improved binding potency.

Table 1
1-Position SAR of quinolone series

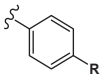
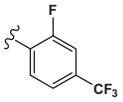
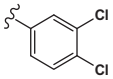
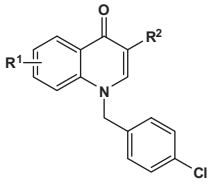
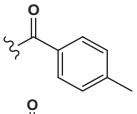
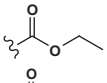
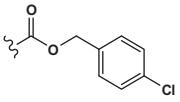
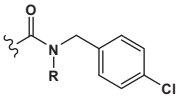
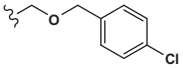
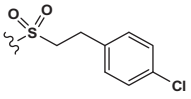
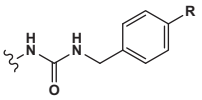
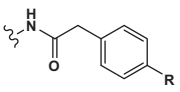
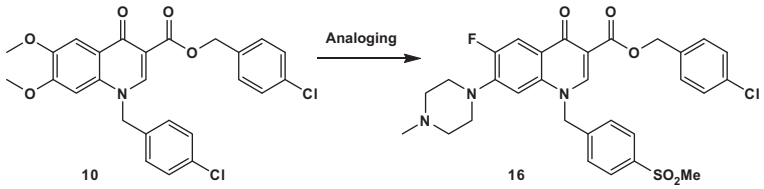
#			IC_{50} (μM)	EC_{50}[18b,c] (μM)	GI_{50}[18b,c] (μM)
2		–H	170	na	na
		–Cl	1.1	11.7	15.5
		–*t*-Bu	0.68	9.5	>25
		$-NH_2$	140	na	na
		$-SO_2Me$	0.5	25.3	>50
		$-SO_2NH_2$	0.85	>50	>25
3			0.41	>12	>25
4			1.1	na	na
5			120	na	na
6			9	38	22
7			110	na	na
8			2.2	na	na

Table 2
3-Position SAR of quinolone series; discovery of benzyl esters

#			IC_{50} (μM)	EC_{50} (μM)	GI_{50}(μM)
	R^1	R^2			
1		6,7-Dimethoxy	1.7	11.7	15.5
9		6,7-Dimethoxy	16	>15	>15
10		6,7-Dimethoxy	0.02	1.2	28.3
11		6-Fluoro, 7-morpholinyl	16 (R = H) 330 (R = Me)	na	na
12		6,7-Dimethoxy	100	na	na
13		6,7-Dimethoxy	> 150	na	na
14		6-Fluoro, 7-*N*-methyl piperazinyl	26 (R = H) >150 (R = Cl)	na	na
15		6-Fluoro, 7-*N*-methyl piperazinyl	4.6 (R = H) 2 (R = Cl)	na	na

The 4-chlorobenzyl ester (compound **10**) with a binding potency of 20 nM provided us with a lead compound, notwithstanding the presumed in vivo metabolic liability of the ester functionality. The 4-chlorobenzyl group apparently occupies a critical pocket of the protein as the smaller ethyl ester (compound **9**, IC_{50} 16 μM)) had poor activity. We explored alternative ways to access this putative pocket using linkers other than an ester group. Selected examples shown in Table 2 include amide, reverse amide, sulfone, ether, and urea linkers (**11**–**15**). Exploration of alternative ways to access this putative pocket using linkers other than an ester group resulted in potency losses of >100-fold and was not pursued further.

The benzyl ester **10** proved to have poor solubility in physiological media. Introducing a methyl-piperazine group at the 7-position afforded compound **16** (IC_{50} 0.016 μM) with improved binding affinity and solubility. The structure of this compound was solved in complex with a construct of NS5B from which the C-terminal 21 residues had been removed.



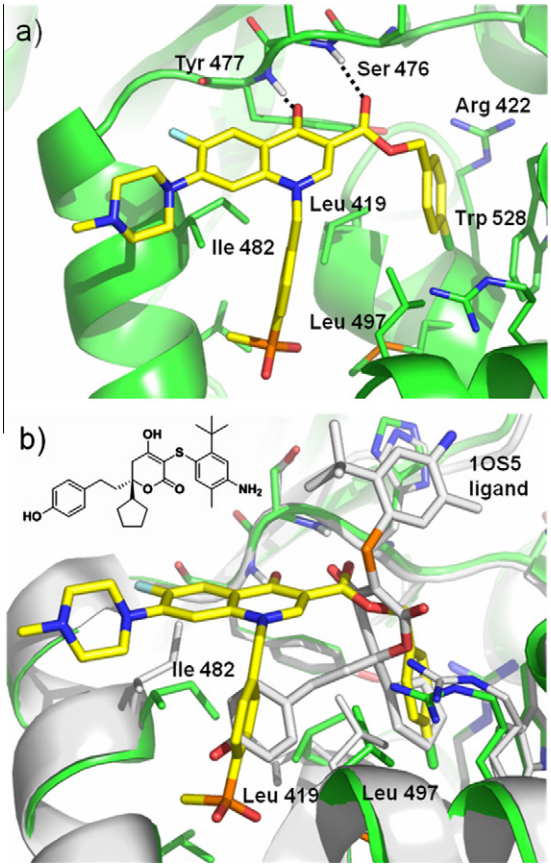
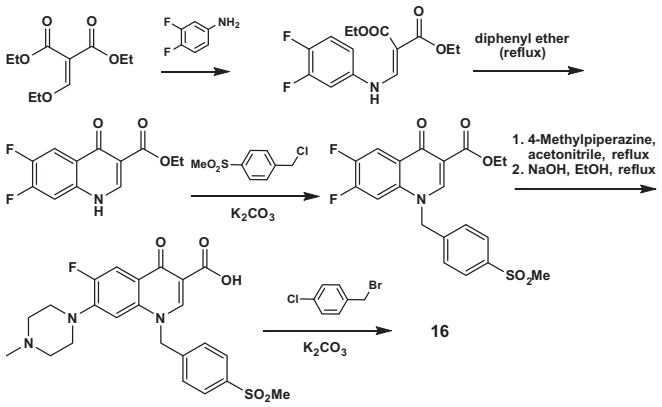
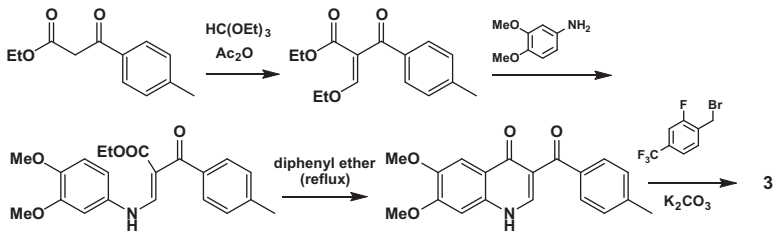


Figure 4. (a) X-ray structure of **16** bound to NNI-2 site (PDB accession code: 3PHE). (b) Overlay with 1OS5 (white, also drawn in 2-D) showing overlapping regions of the two ligands, conserved backbone conformation and rotamer differences of Ile 482 and Leu 419.

The structure was refined using data to 2.2 Å resolution (R = 0.219; FreeR = 0.264; RMS_{bonds} = 0.005 Å; RMS_{angles} = 0.74°, PDB accession code: 3PHE). There were four independent molecules in the asymmetric unit and each showed clear electron density for the inhibitor. The crystal structure revealed a binding mode consistent with our earlier hypothesis. The ligand occupies the NNI-2 binding site located in the thumb region (Fig. 4a). Overall, the protein structure is very similar to that observed for 1OS5 and 1NHU. Two hydrogen bonds exist between the ligand carbonyl oxygen atoms and the backbone amide NH atoms of Ser 476 and Tyr 477. The benzyl ester occupies a hydrophobic pocket lined by Leu 419, Met 423, and Trp 528. Residues Leu 497 and Met 423 adopt conformations similar to those observed in 1OS5, not 1NHU (Fig. 4b). This allows for a pocket to accommodate the

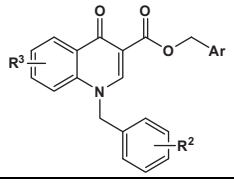


Scheme 2.



Scheme 1.

Table 3
Optimization of benzyl ester series

#				IC_{50} (μM)	EC_{50} (μM)	GI_{50} (μM)
	Ar	R^2	R^3			
10	4-Cl-phenyl	4-Cl	6,7-Dimethoxy	0.02	1.2	28.3
17	4-Cl-phenyl	4-Cl	6-Fluoro, 7-morpholinyl	0.020	1.7	>12
18	4-Cl-phenyl	4-Cl	6-Fluoro, 7-piperazinyl	0.010	0.12[a]	1.1
19	4-Tolyl	2-F, 4-CF_3	6,7-Dimethoxy	0.008	0.23	20
20	2-Tolyl	2-F, 4-CF_3	6,7-Dimethoxy	0.015	0.71	31
21	3-Tolyl	2-F, 4-CF_3	6,7-Dimethoxy	0.06	1.2	7.3

[a] 1a replicon EC_{50} = 0.41 μM.

methyl sulfone, which makes mostly hydrophobic interactions with Val 485, Leu 489, and Leu 419. Leu 419 and Ile 482 adopt different rotamers than seen in the 1OS5 structure, allowing for better hydrophobic packing with the quinolone ring. Finally, the piperazine is largely solvent exposed, confirming our earlier hypothesis that the 6- and 7-positions project towards solvent.

A final round of optimization led to improvement in replicon potency. With evidence that the 6,7-positions of the quinolone scaffold were indeed solvent exposed, we sought to improve the replicon potency of our lead benzyl ester compounds by attaching a variety of polar substituents in this region of the molecule. The 7-morpholinyl substituted analog (**17**) did not offer improvement in replicon potency but the charged 7-piperazinyl substituted analog (**18**) improved replicon potency to 0.12 μM, albeit with a compromised safety window. This compound was also tested in the genotype 1a replicon and had a comparable potency of 0.41 μM. Improvement in binding affinity also led to improved replicon potency. This is exemplified in analogs with 2-fluoro, 4-trifluoromethyl benzyl substitution at the 1-position (**19–21**). Compound **19** with the most improved enzyme potency at 8 nM also had improved replicon potency (replicon 1b EC_{50} = 0.23 μM) and a 100-fold safety window. The 2-, 3- and 4-tolyl substitution off the benzyl ester (compounds **19–21**) showed that 4-substituted benzyl ester has the best binding potency, while 2- and 3-substitution results in a relative potency loss.

Scheme 1 describes the preparation of compound **3**, which exemplifies the synthesis of keto-quinolone compounds shown in Table 1.[21]

Scheme 2 describes the preparation of the benzyl ester **16** which serves to exemplify syntheses of key quinolone compounds with a benzyl ester substituent at the 3-position. The details for synthesis of this and other key compounds in Tables 2 and 3 have been described previously.[18c]

In conclusion, we have described the discovery of a novel class of compounds that inhibit HCV viral replication. This class of compounds binds to the allosteric site NNI-2 of NS5B polymerase as confirmed through a co-crystal structure of compound **16** to NS5B. Preliminary optimization led to attractive lead compounds with sub-micromolar replicon potency in genotypes 1a and 1b. Recognizing the possible in vivo liability of the key benzyl ester substitution in these lead compounds, we initiated SAR directed towards replacing this ester functionality. Our efforts in this area will be the subject of a future disclosure.

Acknowledgment

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18a. As described in: McKercher, G.; Beaulieu, P. L.; Lamarre, D.; LaPlante, S.; Lefebvre, S.; Pellerin, C.; Thauvette, L.; Kukolj, G. *Nuclic Acids Res.* **2004**, *32*, 422; scintillation proximity assays (SPA) were performed at ambient temperature (22 °C) in 96-well plates using 50 nM of enzyme (a C-terminal 21 residue deletion mutant of NS5b from genotype 1b BK isolate). Reaction components included 20 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, 5% DMSO, 0.01% BSA and 25 mM KCl. 80 μl reactions were initiated by adding a mixture of 0.5 μCi of [^{3}H]UTP, 1 μM UTP, 250 nM 5′-biotinylated oligo(rU_{12}) and 0.8 μg/ml poly(rA) to assay wells containing enzyme preincubated with inhibitor. Reactions were terminated after 120 min by the addition of 20 μl of stop solution containing 150 μg/ml tRNA and 10 mg/ml streptavidin-coated SPA beads (Amersham) in 20 mM Tris–HCl pH 7.5, 25 mM KCl, 0.5 M EDTA and 0.025% sodium azide. After incubation for 30 min, 65 μl of 5 M cesium chloride were added to the wells and further incubated for 1 h before top-counting in a Microbeta Trilux plate reader.
18b. *Replicon assay*: HCV replicon-containing cells (Huh7/Clone A, genotype 1b) were maintained in growth medium (DMEM medium, Invitrogen), supplemented with 10% Fetal Bovine Serum, non essential amino acids and 1 mg/mL G418) (Blight, K. J.; Kolykhalov, A. A.; Rice, C. M. *Science* **2000**, *290*, 1972–1974). For the HCV replicon assay, Huh7/Clone A cells were trypsinized from culture flasks, seeded in 1 ml of Clone A growth medium without G418 at 40,000 cells per well in 24-well plates and incubated at 37 °C in a humidified CO_2 (5%) incubator overnight. Following overnight incubation, test compound was serially diluted in DMSO and added to the test system such that the final

concentration of DMSO was 0.5% in each well. For IC_{50} determinations, compounds were tested at seven concentrations in triplicates. Plates were incubated at 37 °C for 48 h. After incubation, cells were harvested, transferred to 96-well plates, and subjected to total RNA extraction using the RNA Isolation Kit (RNeasy 96, Qiagen). TaqMan quantitative PCR (RT-qPCR) was used to quantify the amount of HCV replicon RNA in each sample. The samples without compound treatment served as a control and the HCV replicon RNA level from untreated cells was defined as 100%. Compound inhibitory activity was determined as the ratio of the normalized HCV RNA amount in treated samples relative to the untreated control. Compound IC_{50}'s were calculated using a standard 4 parameter curve fit model.

18c. Kumar, D. V.; Rai, R. WO 2007/005779, 2007.

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21. 3-Oxo-3-*p*-tolyl propionic acid ethyl ester was heated under reflux (160 °C) with 1.6 equiv of triethylorthoformate and acetic anhydride (2.5 equiv) for 4 h, then concentrated and dried to afford the substituted acrylic ester intermediate shown. This was dissolved in isopropanol and refluxed with an equimolar amount of 3,4-dimethoxyaniline. Purification by silica gel chromatography afforded (*Z*)-3-(3,4-dimethoxy-phenylamino)-2-(4-methyl-benzoyl)-acrylic acid ethyl ester in 60% yield over two steps. This compound was added as a solid to refluxing diphenyl ether and the mixture heated at reflux for 1 h. Upon cooling 6,7-dimethoxy-3-(4-methyl-benzoyl)-1*H*-quinolin-4-one crystallized out. This compound was dissolved in DMF and alkylated by treatment with 1-bromomethyl-2-fluoro-4-methyl-benzene (1.5 equiv) in presence of K_2CO_3 (3 equiv) to afford compound **3** in 70% yield.