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Exploring the importance of zinc binding and steric/hydrophobic factors in novel HCV replication inhibitors

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ABSTRACT

Several novel compounds have been identified that inhibit the replication of hepatitis C virus in a replicon assay with EC_{50} values as low as 0.6 μM. Lead compounds were modified to investigate the possible role that zinc binding may play in inhibitor efficacy. In addition, the structure–activity relationship was explored to increase inhibitor efficacy and possibly identify favorable interactions within the currently unknown inhibitor binding pocket. The rationale for inhibitor design and biological results are presented herein.

Estimates suggest that approximately 150 million people are currently infected with the hepatitis C virus (HCV) and as many as 500,000 die each year from HCV related ailments.[1] The first interferon-free regimens, consisting of two or more direct-acting antivirals (DAAs) in combination therapy, were approved for treatment of HCV in late 2014. The first all-oral treatment approved is a combination of the NS5B RNA polymerase inhibiting compound sofosbuvir and the NS3/4A targeting drug simeprevir.[2] Shortly after, Harvoni, a combination of sofosbuvir and the NS5A inhibiting drug ledipasvir, was approved.[3] In December of 2014 Abbvie's four drug combination therapy Viekira Pak received FDA approval. This treatment consists of the NS5A inhibitor ombitasvir, the NS3/4A protease inhibitor paritaprevir, the non-nucleoside NS5B RNA polymerase inhibitor dasabuvir, and ritonavir, which was a previously approved drug shown to increase the bioavailability of paritaprevir.[4] Depending on the HCV subtype and the state of the patient's liver, ribavirin may still be required with Viekira Pak. Although early results for all-oral treatment regimens seem promising, the estimated treatment costs range from approximately $84,000 to $189,000, depending on the drug and the required treatment time (12–24 weeks).[5,6] Early cases of resistant strains have already been reported for each individual DAA.[7–11] The elusive nature of the error-prone NS5B polymerase and the extremely high viral replication rate are sufficient to generate skepticism that the HCV problem has been solved. Research toward additional HCV therapeutics will hopefully decrease the cost and length of treatment, while providing additional modes of action in halting viral replication and decreasing resistance.

The HCV genome encodes a single polypeptide of approximately 3000 amino acids, that is, translated into ten viral proteins.[12–16] The four structural proteins core, E1, E2, and p7 are located in the amino terminal third of the peptide and the remaining two-thirds is composed of the non-structural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B.[15] The non-structural proteins function as a complex and are responsible for the synthesis of viral RNA, and thus viral replication.[16]

In a recent communication, we reported several structural analogs of the natural product UK-1 as novel inhibitors of HCV replication in an HCV replicon assay.[17] A subset of these inhibit the non-structural protein 3 (NS3) helicase, while others act via an undetermined mechanism.[18] Similar to UK-1, lead compound **1a** (Fig. 1) is ineffective against the NS3 helicase and has been shown to bind zinc, presumably via the Lewis-basic carbonyl oxygen, phenolic hydroxyl group, and heterocyclic nitrogen.[18] Within the HCV genome, at least three non-structural proteins (NS2, NS3, and NS5A) contain/require a structural zinc ion and all three proteins are required for viral replication.[19–25] Disruption of zinc binding within any of the three proteins severely hinders or eliminates protein function, thus inhibiting viral proliferation.[20,23,25] It is therefore hypothesized that the activity of **1a** is related to the coordination of one or more protein-bound structural zinc ions, causing a perturbation of protein structure that diminishes protein activity, thereby inhibiting viral replication.

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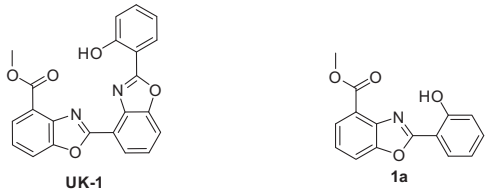


Figure 1. Bacterial natural product UK-1 and lead compound **1a**.

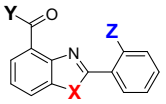
NS2 is a novel dimeric cysteine protease located at the 5′ end of the NS proteins. NS2 is responsible for the intramolecular cleavage from adjacent NS3, which remains attached to the downstream NS proteins.[26] The N-terminal domain of NS3, which acts as a cofactor for NS2, contains a zinc-binding region and increases NS2 protease activity. It has been hypothesized that the NS3 zinc-binding domain promotes the correct positioning of the NS2/NS3 scissile bond.[20] Consistent with these predictions, NS2 protease activity is inhibited by the zinc chelator 1,10-phenanthroline, albeit weakly.[21] Furthermore, point mutations to residues involved in zinc binding completely eliminate NS2 activity.[21] Prior to NS2 cleavage from NS3, it has been suggested that the structural zinc ion is coordinated by four cysteine residues, three within NS3 and one in the C-terminus of NS2. Following NS2 cleavage, a change in coordination occurs and the zinc ion becomes bound completely within the N-terminus of NS3. This change in conformation has been hypothesized to activate the NS3 protease.[20]

An active NS3 protease is required for viral replication and it has been shown that the structural zinc bound in the N-terminus of NS3 is required for protease activity.[21–23,27,28] The amino-terminal domain of NS3 and adjacent NS4A function as a dimeric serine protease, that is, responsible for the intramolecular cleavage of NS3 from NS4A, as well as the intermolecular cleavage between all downstream NS proteins.[29] Similar to NS2, NS3 protease activity is weakly inhibited by 1,10-phenanthroline and point mutations to zinc binding residues significantly diminish NS3 protease activity.[21,22,28]

NS5A is yet another protein required for viral replication that relies on a structural zinc ion for proper function.[25,30] NS5A has been shown to play roles in RNA replication, virion assembly, host cell protein modification, and other factors crucial to the viral lifecycle.[31,32] This protein has three domains. Domain one, which is the most conserved among genotypes, contains a novel fold coordinating a structural zinc ion.[24,25] It has been shown via mutational analysis that the structural zinc is required for viral replication.[25]

To investigate whether or not zinc binding plays a role in the activity of the HCV replication inhibitor **1a**, a series of analogs have been synthesized with structural modifications expected to increase zinc affinity (Fig. 2, left). First, the benzoxazole heterocycle has been replaced with a benzimidazole scaffold. It has been shown in UK-1 that replacement of one of the two benzoxazole heterocycles with a benzimidazole increases zinc affinity by approximately a factor of two.[33] Secondly, the 2-position phenolic hydroxyl of **1a** has been replaced with a sulfhydryl group.

Table 1
Inhibition of HCV replication data and Zn^{2+} dissociation constants for compound **1a** and analogs **1b–c**, **2a**, and **3a**



Compound ID	X=	Y=	Z=	K_d[a] (μM)	EC_{50}[b] (μM)	CC_{50}[c] (μM)	SI[d]
1a	O	OCH_3	OH	65	40	71	1.8
1b	O	OH	OH	0.50	4.9	25	5.1
1c	O	NHMe	OH	53	117	>186	1.6
2a	NH	OCH_3	OH	190	106	132	1.3
3a	O	OCH_3	SH	0.23	2.5	4.1	1.6

[a] Zinc dissociation constants measured via equilibrium titration experiments monitored by UV–VIS spectroscopy in 50:50 methanol/water. Results are an average of three independent trials.
[b] The concentration of compound that inhibits 50% of viral RNA replication, measured in the HCV subgenomic replicon assay using a luciferase reporter. This assay was conducted using Huh 5-2 cells containing the hepatitis C virus genotype **1b** I389luc-ubi-neo/NS3-3′/5.1 replicon.
[c] The concentration of compound at which the metabolic activity of the cells is reduced by 50% as compared to untreated cells.
[d] The ratio of CC_{50} to EC_{50}.

In addition to modifications expected to increase zinc affinity, a structure–activity relationship (SAR) study was performed to explore the effects of sterics/hydrophobicity and hydrogen bonding on inhibitor efficacy (Fig. 2, right). These modifications include variation of the 4-position carbonyl-containing moiety, N-benzylation of the benzimidazole nitrogen, and addition of steric/hydrophobic bulk about the phenolic ring via various naphthylene- and quinoline-containing analogs.

The majority of compounds included were synthesized using slightly modified previously reported methodology.[33–35] Benzoxazole amides **1d–1g** (Table 2) and benzimidazole **2c** (Table 3) were prepared via acid activation of **1b** (Table 1) or **2b** (Table 3), respectively, and coupling to the appropriate amine. Thiol analog **3a** (Table 1) was prepared from commercially available 2,2′-dithiodibenzoic acid, whereby protection of the potentially reactive thiol was achieved via a symmetrical disulfide. Schemes and methods can be found in the Supplementary material.

All compounds were evaluated for selective antiviral activity on the replication of HCV in a HCV replicon assay using either a luciferase reporter or via quantitative analysis of viral RNA production (Tables 1–5).[17] In addition, Zn^{2+} dissociation constants were measured for lead **1a** and five representative analogs to investigate a correlation between zinc affinity and inhibitor efficacy (Table 1). A representative zinc-binding titration can be found in Figure 3. Among methyl ester **1a**, acid **1b**, and methyl amide **1c**, the acid has the highest affinity for zinc as well as the lowest EC_{50} value.

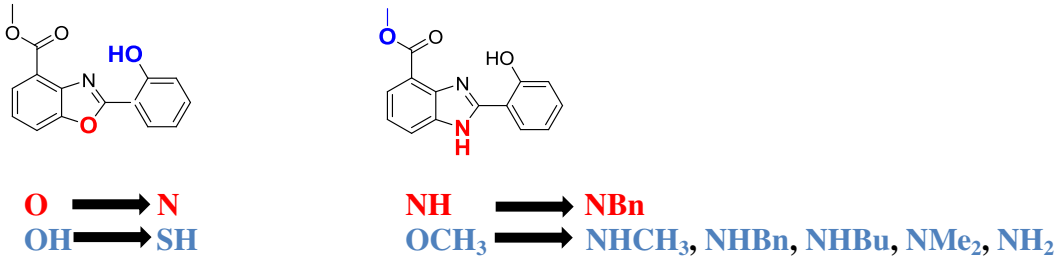


Figure 2. (Left) Modifications expected to increase zinc affinity. (Right) Modifications to explore the effects of sterics/hydrophobicity and hydrogen bonding.