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Discovery of novel dialkyl substituted thiophene inhibitors of HCV by in silico screening of the NS5B RdRp

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Abstract—A novel 5,4-dialkyl substituted thiophene was discovered by in silico screening of the 3D polymerase crystal structure (1GX6) that demonstrated single digit micromolar HCV inhibition activity in the replicon assay and dose-dependent inhibition in the replicase complex assay. Subsequently, SAR was explored with a small set of dialkyl and tetrahydro-benzo thiophenes. Since these thiophenes inhibit synthesis of both, single- and double-stranded RNAs, their mechanism of action is distinct from other known HCV inhibitors.

In the USA alone there are an estimated four million carriers of the RNA virus Hepatitis C, 80% of which will develop chronic symptoms and 20–30% will progress on to the end-stage liver diseases. Current treatment results in a sustained response rate in about 50% patients.[1] The need for a drug that is well tolerated and targets HCV directly motivates continued drug discovery efforts such as the one presented here.

Hepatitis C is a member of the Flaviviridae family and exists in six known genotypes and over 12 subtypes.[2] The HCV RNA dependent RNA polymerase (RdRp, NS5B) is a key enzyme in the viral life cycle and catalyzes RNA polymerization.[3] Recent clinical studies of HCV NS5B inhibition by non-nucleoside and active site nucleoside analogs have validated the HCV RdRp as a therapeutic target.[4]

Multiple crystallographic structures of HCV NS5B have been solved to date[5–10] and reveal a common polymerase 3D structure that resembles a right hand made up of fingers, thumb, and palm domains (Fig. 1). It includes an active site that is enclosed by the finger and thumb domains. In addition, a β-hairpin loop that is unique to HCV NS5B protrudes into the active site binding pocket which likely aids in positioning the 3′ end of the RNA template.[11] The hydrophobic C-terminal tail is believed to act as a membrane anchor and help in modulating the RNA synthesis as well.[12] Two divalent metal ions coordinated in the palm region drive the polymerization reaction.[13–15] One ion stabilizes the triphosphate group of the incoming nucleotide, while the other is positioned to activate the 3′ hydroxyl group of the growing RNA chain for nucleophilic attack. This polymerization process can proceed in the absence of a primer and from a single nucleotide in vitro.[16,17]

X-ray crystallography and mutational mapping studies provide evidence for multiple non-nucleoside inhibitor binding sites in and around the thumb domain.[18–23] A shallow binding pocket that binds at least three distinct chemotypes is found at the outer base of the thumb domain, 30 Å away from the active site. Binding of disubstituted phenyl-alanines, thiophene-2-carboxylic acids, and cyclopentyl dihydropyran-ones is found to be mutationally sensitive to Met423. Two distinct benzimidazole analogs interact with a highly conserved Pro495 and share another allosteric, low affinity GTP binding site, at the top of the thumb domain. Benzothiadiazines and proline sulfonamides are found to bind in a pocket at the inner surface of the thumb and mutations to Met414 confer resistance to the benzothiadiazine series. Substituted benzofurans are found to bind on the interior of the RdRp palm region interacting with

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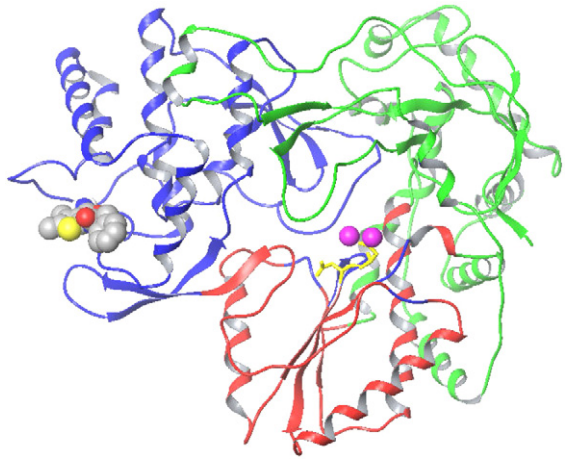

Figure 1. HCV NS5B polymerase: thumb, palm, and fingers domains are color coded with red, blue, and green colors, respectively. The active site residues Asp 318 and 319 are displayed with yellow sticks and catalytic metal ions are in magenta CPK. The predicted binding pose of compound **1** is also shown in CPK.

Arg200 and loop residues 364–368, a hinge region that is closed in the apo form of the enzyme but open upon binding of the inhibitor. Resistance to the benzofurans is conferred by mutations to Val201 and Cys316. Finally, pyrophosphate mimics bind in the palm region and presumably interact with bound metal ions to disrupt binding of incoming RNA nucleotides or activation of the elongating RNA chain. It is noteworthy that, unlike nucleosides that are chain elongation inhibitors, all the known non-nucleoside HCV polymerase ligands are chain initiation inhibitors.

We used virtual screening to dock a library of available lead-like compounds in the thumb domain of HCV NS5B for selection and purchase of novel putative ligands. In order to identify HCV/NS5B inhibitors, the purchased compounds were evaluated in an HCV replicase complex (RC) assay[24,25] (cell-free) and HCV replicon assay[26] (cell-based). As opposed to the use of recombinant NS5B in the biochemical assay, RC assay utilizes NS5B within the replicon complex that is isolated from the replicon containing cells. This assay has been used to determine NS5B inhibition potency of several compounds.[26–30] Subsequently, one of the active compounds identified was followed-up by applying substructure and similarity searches and in-house synthesis of analogs.

A database of lead-like compounds used for virtual screening was derived from an initial pool of about two million compounds from the Specs & BioSpecs, Bionet, Microsource, Available Chemicals Directory (ACD), Nanoscale, ChemDiv, Orion, Asinex, Interbioscreen, Timtec, and Chembridge databases. Tripos SLN Filters[32] were used to remove duplicates and reactive functionalities and to select compounds according to the following criteria; compounds containing at least one ring, $\leqslant 1$ chiral center, $\leqslant 8$ rotatable bonds, 275–400 molecular weight, and $C \log P \leqslant 3.5$. This resulted in a library of 90,000 molecules. In preparation of the molecules for docking, Schrodinger utilities were used to strip salts, neutralize, and ionize compounds at the physiological pH before energy minimization to a low energy conformer.

The 3D structure of HCV NS5B (1GX6)[6] was prepared for virtual screening by deleting the UTP ligand and mutating seleno-methionine residues to methionines. This was followed by energy minimization of the model with the impref utility of Schrodinger suite of software.[33] The PRCG method was used with a maximum number of iterations set to 10,000 and gradient convergence criteria set at 0.05 kJ/A mol. The force field used was OPLS-AA with the GB/SA implicit solvent treatment at a constant dielectric of 1.0. Next, Glide[34,35] was used to calculate grids with a 20 Å bounding box centered on residues 421, 422, and 426 and an enclosing box to fit ligands with up to 30 Å length that enclosed the entire thumb region.

Compounds were docked flexibly using Glide, allowing generation of up to 5000 initial conformations and refinement of 400 poses per ligand with the best scoring pose retained for each ligand. The virtual screening

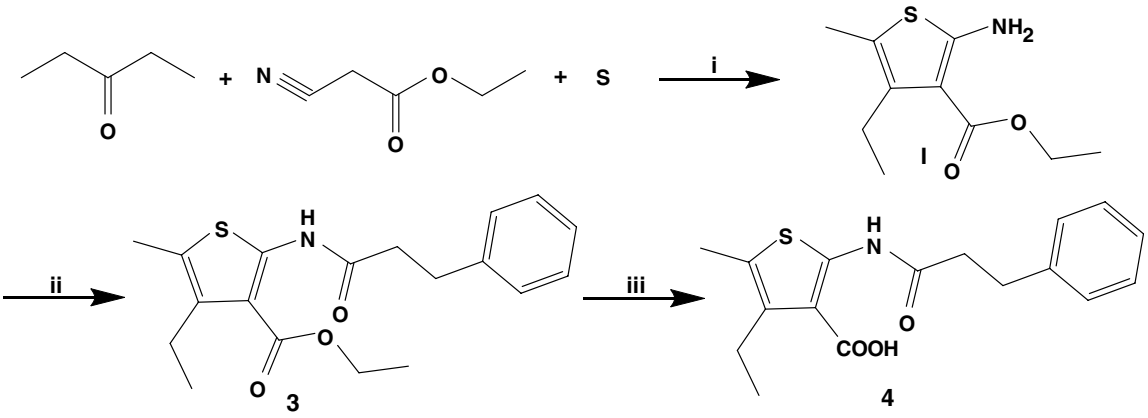

Figure 2. Literature procedure for synthesis of amino thiophenes.

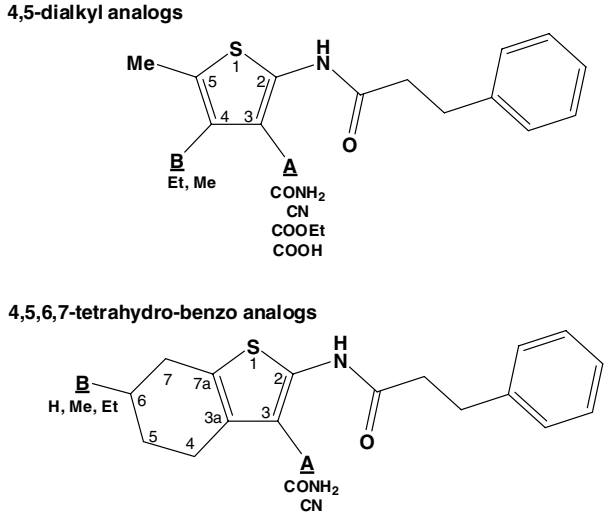

Figure 3. Dialkyl substituted thiophene inhibitors of HCV.

calculation was performed with impact-v20012 on a Linux cluster of four dual processor computers.

Docked ligands were found to cluster in the three known binding sites of the thumb domain. The top 1318 ligands that scored −7.17 (glide score) or better were visually inspected to select and purchase a structurally diverse set of 50 putative ligands, encompassing the known binding sites. Subsequently, purchased compounds were tested in the RC and replicon assays.

The previously described RC assay[24,25] was modified as following: various concentrations of compounds were incubated with membrane fractions containing HCV replicase complexes isolated from HCV replicon cells in the reaction buffer at 30 °C for 30 min. Viral RNA synthesis was then initiated by adding ATP, GTP, UTP, and [α-^{32}P]CTP. After incorporation of nucleotides at 30 °C for 60 min, the reaction was stopped and RNA was isolated with TRIzol LS Reagent (Invitrogen). The isolated RNA was resolved by electrophoresis on a 1% agarose gel, dried, and visualized by autoradiography (Fig. 4).

Previously described replicon assay[31] was modified to use firefly luciferase as the reporter. Serially diluted compounds were added to the replicon cells seeded in 96-well plates. The 72 h long treatment was followed by measuring anti-HCV activity of the tested compounds with Blight-Glo luciferase assay kit (Promega) and the cellular toxicity was measured with CellTiter 96 Aqueous One Solution (Promega) Table 1.

The most active hit identified (compound **1**) caused a dose-dependent inhibition in the RC assay (IC_{50} 50–100 μM). Following up the initial hit, available analogs of compound **1** were purchased to explore electronic properties of the substituents at thiophene 3-position and steric properties of substituents at thiophene 4- and 5-positions. In addition, two aminothiophenes, **3** and **4**, were synthesized in-house following the literature procedure.[36] 3-Pentanone, sulfur, and ethylcyanoacetate

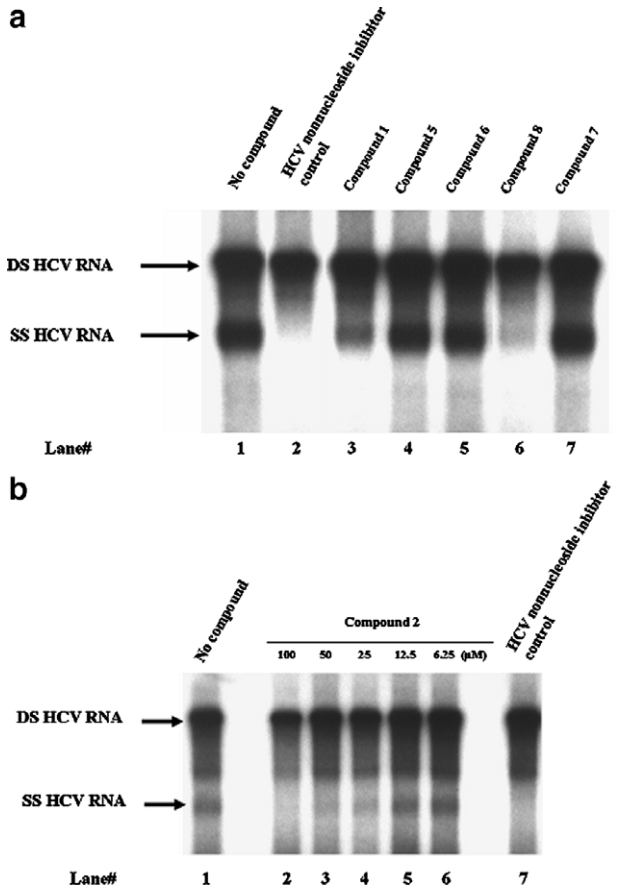

Figure 4. Inhibition of HCV single-stranded RNA synthesis in a RC assay. (a) Five compounds tested at a concentration of 100 μM displayed different inhibitory effects on HCV RNA synthesis. The positive control was a benzothiadiazine derivative, a known HCV non-nucleoside inhibitor (testing concentration was 5 μM). DS, double-stranded; SS, single-stranded. (b) Dose–response of inhibition of the compound *2* on single-stranded RNA synthesis. The compound was twofold diluted in DMSO serially before mixing with the reaction mixture. The controls were the same as (a).

were refluxed together in ethanol in the presence of pyrrolidine for 6 h (Fig. 2). All the volatiles were evaporated and the residue was purified by chromatography on silica gel to give the aminothiophene **I**. The amino group was then converted to the amide using 3-phenylpropionylchloride to give compound **3** followed by hydrolysis of the ester group to give the corresponding carboxylic acid, compound **4**.

The resulting follow-up set led to limited SAR data captured in eight additional compounds (Fig. 3). Replacement of the amide function in compound **1** by a cyano (compound **2**) results in >2× improvement in potency in the RC assay. Because of their structural similarity to known thiophene-2-carboxylic acid inhibitors of HCV NS5B,[37] in addition to the amide and cyano compounds available for purchase, the ethyl ester (compound **3**) and carboxylic acid (compound **4**) at the 3-position were synthesized in-house. Surprisingly, the acid and ethyl ester were found to be inactive in the RC assay. These results imply that the dialkyl substituted

Table 1. Substituents, HCV replicon, and cell-free assay EC_{50} results for compounds **1–5** and **6–9**

Compound	A	B	HCV replicon EC_{50} (μM) mean (SD)[a]	HCV replicase complex IC_{50} (μM)
4,5-Dialkyl analogs				
1	$CONH_2$	Et	0.92 (0.67)	50–100[b]
2	CN	Et	1.66 (0.79)	25[c]
3	COOEt	Et	ND	>100
4	COOH	Et	ND	>100
5	$CONH_2$	Me	ND	>100
4,5,6,7-Tetrahydro-benzo analogs				
6	$CONH_2$	H	ND	>100
7	$CONH_2$	Me	ND	>100
8	$CONH_2$	Et	1.95 (0.90)	30–50[b]
9	CN	Et	ND	>100

HCV replicon assay. ND, not determined.

[a] Values are means of three experiments. HCV RC assay: single point (100 μM) experiment, IC_{50} estimated from single-strand band intensity reduction on gel; 50% → ~100 μM, 75% → ~50 μM, 92.5% → ~25 μM.

[b] Ranges reflect $N = 2$.

[c] Five point 2× dilution experiment; 100–6.25 μM.

thiophenes have a distinct binding mode or location from that of the previously reported thiophene-2-carboxylic acid HCV NS5B inhibitors.

The inhibition activity of the thiophenes displays a remarkable sensitivity to the aliphatic substituent changes. Loss of activity was found for simple substitutions of a methyl group for an ethyl at the 4 (acyclic) or 6 (cyclic) position (compounds **1/5** and **7/8**), while activities of the 4-Et/5-Me (acyclic) and 6-Et (cyclic) substituted analogs were comparable (compounds **1** and **8**) in the RC assay. Perhaps, this could be explained by a shape complementarity between aliphatic substituents and a hydrophobic region of the binding pocket.

It is noteworthy that in addition to inhibiting single-stranded (SS) RNA synthesis, these dialkyl thiophenes inhibited double-stranded (DS) RNA synthesis. We have previously reported that the benzothiadiazine and benzimidazole NS5B non-nucleoside inhibitors, binding to two different sites, block SS RNA synthesis and have rather little effect on DS RNA synthesis.[25] Therefore, as expected, benzothiadiazine (the positive control) in the current experiment completely inhibited SS RNA synthesis with very little effect on the DS RNA synthesis (Fig. 4, compare lane 2 to lane 1 in (a) and compare lane 7 to lane 1 in (b)). Among six amino-thiophenes tested, two (compounds **8** and **2**) showed a nearly complete inhibition of SS RNA at the highest concentration tested. However, unlike benzothiadiazine, these thiophenes inhibited DS RNA synthesis as well (Fig. 4a and b, compare lane 6 to lane 2 in (a) and compare lane 2 to lane 7 in (b)). These results suggest that dialkyl thiophenes inhibit NS5B via a mechanism different from the other two chemotypes, warranting for further studies.

The compounds active in the RC assay were also evaluated in the HCV replicon assay (Table 1). Lower than expected EC_{50} values are partly derived from the cellular toxicity because the therapeutic window (CC_{50}/EC_{50}) for these compounds is less than 10 (3–9).

The observed activity of dialkyl thiophenes in the replicon and RC assays suggests inhibition of HCV/NS5B and the limited SAR explored further supports interaction of compounds with a well-defined binding site. In addition, inhibition of both single- and double-stranded RNA synthesis represents a unique mechanism for the potential treatment of HCV infection. These novel inhibitors are amenable to parallel synthesis exploration and offer a reasonable starting point for further drug discovery efforts.

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