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Biological Evaluation of Hepatitis C Virus Helicase Inhibitors

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Abstract—A small chemical library has been synthesized and assayed for inhibition of HCV helicase activity. This study provides the structure–activity relationship of the reported inhibitors, with emphasis placed on the aminophenylbenzimidazole moiety and the linkers. Our data highlight the importance of preserving the aminophenylbenzimidazole core and the hydrophobic linkers for biological activity. The development of a robust HCV helicase assay is also described.

Introduction

Hepatitis C virus (HCV) infection is a major health concern worldwide, with about 3% of the world population infected.[1] HCV is the principal pathogen of transfusion-associated non-A non-B hepatitis.[2] Infection with HCV results in acute or chronic hepatitis, which could lead to liver cirrhosis and hepatocellular carcinoma.[3] There is no vaccine against HCV at present. The current therapy using α-interferon alone or in combination with ribavirin is not highly efficacious.[4] Hence, there is an urgent drive towards the discovery of other therapeutic targets and potent inhibitors for development into anti-HCV drugs.

The HCV genome encodes a polyprotein which is processed into 10 distinct structural and non-structural (NS) proteins.[4] Recently, much attention has been paid to the NS3 protein which possesses serine proteolytic activity at the N-terminus, helicase and nucleotide triphosphatase activities at the C-terminus. The helicase unwinds the nucleic acid duplexes, playing a crucial role during the viral replication cycle. Intervention of the helicase activity with chemical ligands has the potential of terminating the HCV proliferation.

There are only a few HCV helicase inhibitors reported so far. ViroPharma Inc. patented two series of compounds containing aminophenylbenzimidazole (**1a**–**g**) and benzimidazole-like moieties (**2**) attached to symmetrical linkers of different lengths (Fig. 1).[5] The IC_{50}'s of these inhibitors are in the low micromolar range. Vertex Pharmaceuticals Inc. reported several aminothiadiazoliums which also inhibit helicase activity but with lower potency.[6]

The SAR study of the ViroPharma's inhibitors has not been described. It is imperative to identify the critical pharmacophores to aid the design of potent helicase inhibitors. Reported herein are the results of our preliminary SAR study with emphasis placed on the

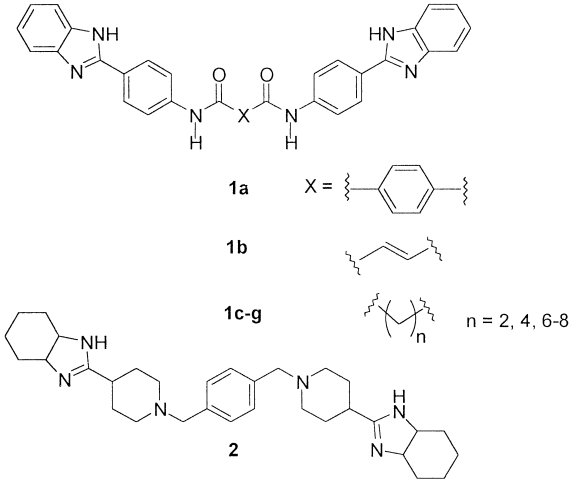

Figure 1. The HCV helicase inhibitors reported by ViroPharma Inc.

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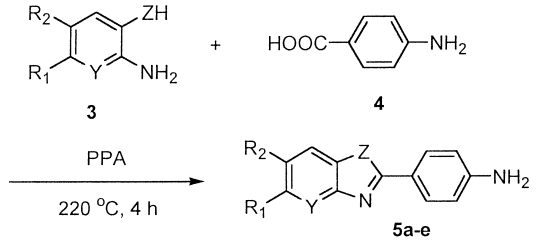
aminophenylbenzimidazole moiety and the linkers. The development of a robust helicase assay is also described.

Synthesis

The necessity of having benzimidazole was firstly evaluated by replacement with benzoxazoles, benzothiazoles and a pyridinoxazole. Five linkers with aromatic ring, double bond, and aliphatic chains of various lengths are selected for the study. The synthesis of these analogues is outlined in Scheme 1. Aminophenols and thiophenols reacted easily with *p*-aminobenzoic acid in the presence of polyphosphoric acid to afford the corresponding aminophenylbenzoxazoles and aminophenylbenzothiazoles **5** in 64–84% yields after recrystallization from MeOH–H_2O (Table 1).[7]

Subsequent coupling of aminophenylbenzothiazoles **5** with acid dichlorides furnished the products **6** (Fig. 2) which precipitated spontaneously. The precipitates were isolated by centrifugation. In order to assess the contribution of benzene ring within the aminophenylbenzimidazole core to biological activity, a few diamides **7** were prepared by reacting the commercially available 2-aminobenzimidazole with acid dichlorides. We are also interested in probing whether the bioactivity could be affected by changes in the linkers. This question was addressed by preparing the aminophenylbenzimidazole-derived diureas **8**.[8]

In addition, other heterocycles such as furfurylamine, tryptamine, 3-(aminomethyl)pyridine, aminobenzothiazoles and aminothiazoles shown in Figure 3 were also reacted with acid dichlorides to provide the diamides. Precipitation of products was induced by adding water in some cases (amines **13**, **16**, and **18**). Higher purity (>95%) was observed in products that precipitated spontaneously.[9] Those products with purity higher than 85% were tested in the bioassay.



Scheme 1. Synthesis of the aminophenylbenzothiazoles **5**.

Table 1. The yields of compounds **5a–e**

Compound	Z	Y	R_1	R_2	% Yield
5a	O	CH	H	H	67
5b	S	CH	H	H	84
5c	O	N	H	H	64
5d	O	CH	CH_3	H	81
5e	S	CH	H	CH_3	Commercially available

Biological Assay

The bacterial expression and purification of HCV NS3 helicase was carried out as reported in the literature.[10] The helicase was found to be more than 95% pure as determined by SDS-PAGE with Coomassie Blue staining. A double-stranded DNA template was prepared[11] and used as the helicase substrate in our assay due to its ease of handling. The helicase activity was determined by monitoring the unwinding of the double-stranded DNA template that resulted in the presence of single-stranded DNA.[12] Within 10 min, over 75% of the template was observed as single-stranded DNA. In the absence of enzyme, only double-stranded DNA was observed.

The compounds were assayed in duplicate for helicase inhibition at a concentration of 25 μg/mL. Four known inhibitors (**1a**, **b, d**, and **e**) were synthesized and included in the assay as positive controls. They exhibited 75–93% inhibition at the assay concentration of 25 μg/mL.

Results and Discussion

Of 66 compounds screened, there was no inhibition with the majority of the aminophenylbenzoxazoles- and

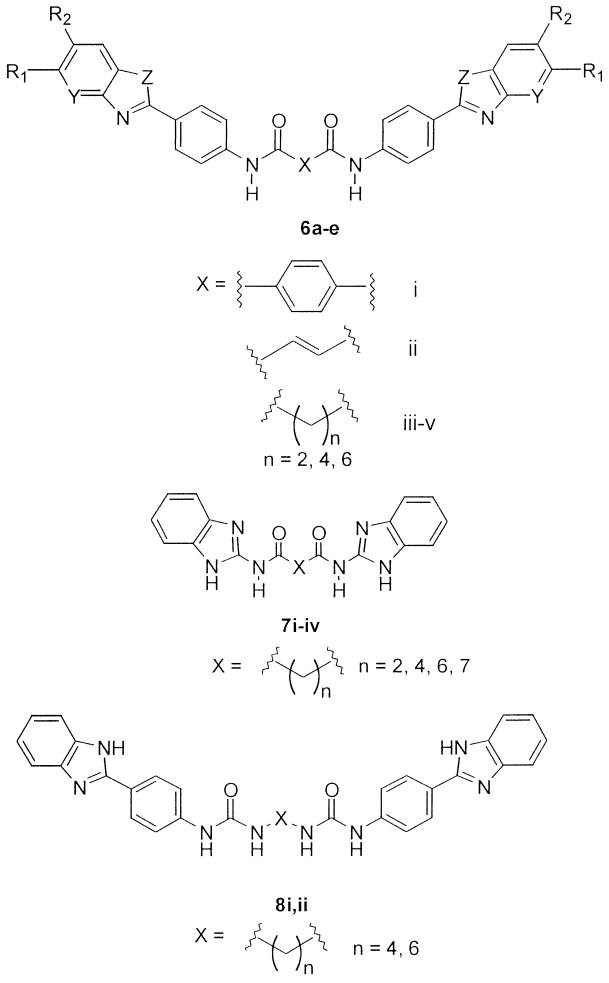


Figure 2. The diamides **6**, aminobenzimidazole-derived diamides **7** and aminophenylbenzimidazole-derived diureas **8**.

aminophenylbenzothiazoles-containing compounds **6**. The aminobenzimidazole-derived diamides **7** showed 6–13% inhibition whereas the aminophenylbenzimidazole-derived diureas **8** ($n=4$ and 6) have 20 and 28% inhibitory activity, respectively. Compounds **19** and **20** (Fig. 4) also displayed 14 and 10% inhibition, respectively.

There is a drastic decrease of potency after replacing the benzimidazole moiety (**1a**–**e**) with benzoxazole **6a(i**, **ii**, **iv**, and **v**) and benzothiazole **6b(i**–**v**) moieties. Similarly, the biological activity is also reduced after deletion of the benzene ring. Inhibition of compounds **7i**–**iv** decreases tremendously as compared to the ViroPharma's inhibitors **1c**–**f**. The linker also plays an important role as replacement of the diamide linkage (**1e**,**g**) with diurea (**8i**–**ii**) leads to diminished potency of the inhibitors.

The information derived from our SAR study shows that the NH group within the benzimidazole ring, the benzene group at the C-2 position of benzimidazole, and the nature of the linker are essential for inhibitory activity. Although the exact mechanism of the inhibition is still not clear, it has been suggested that ViroPharma's inhibitors might compete with nucleic acids for the substrate's binding site. Thus, the NH group could possibly interact with the enzyme through hydrogen bonding while the benzene ring might be interacting through hydrophobic interaction. It is hoped that the biological evaluation carried out in this work would provide further insight to the design of HCV helicase inhibitors.

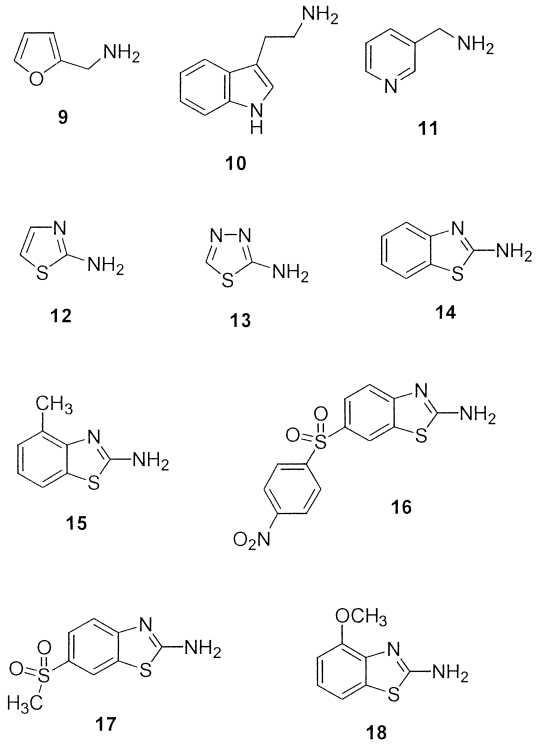


Figure 3. Amine building blocks employed in the library synthesis.

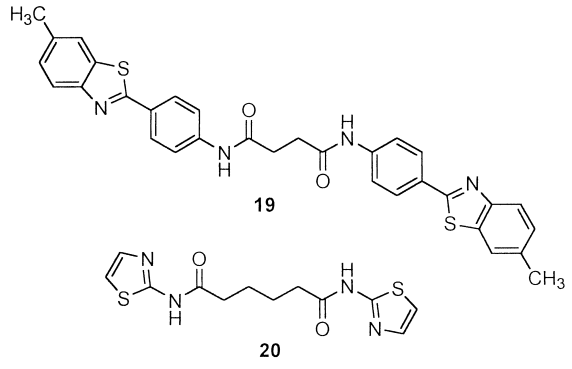


Figure 4.

Acknowledgements

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References and Notes

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7. Representative procedure for the synthesis of compound **6**: To a mixture of aminophenol or aminothiophenol (6.5 mmol) and *p*-aminobenzoic acid (7 mmol) was added polyphosphoric acid (10 g). The mixture was stirred vigorously at 220 °C for 4 h, cooled and poured into 10% Na_2CO_3 solution. The suspension was stirred until gas evolution ceased and then filtered. The solid collected was washed with H_2O (3×50 mL), and recrystallized from MeOH–H_2O to afford the product **5**. To a solution of compound **5** (0.6 mmol) in anhydrous CH_2Cl_2 or DMF (5 mL) containing *N*,*N*-diisopropylethylamine (0.105 mL, 0.6 mmol) was added acid dichloride (0.2 mmol). The mixture was stirred at room temperature for 18 h. The suspension was centrifuged and the supernatant was discarded. The pellet was resuspended with CH_2Cl_2 or DMF–H_2O and centrifuged. This washing procedure was carried out twice. Product **6** was then dried in vacuo.

Compound **19**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.44 (6H, s), 2.72 (4H, s), 7.32 (2H, d, $J=8.3$ Hz), 7.77 (4H, d, $J=8.6$ Hz), 7.88 (2H, d, $J=8.3$ Hz), 7.89 (2H, s), 8.00 (4H, d, $J=8.6$ Hz), 10.35 (2H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 20.5, 28.4, 118.6, 121.2, 121.6, 126.9, 127.3, 127.5, 133.8, 134.5, 141.4, 151.2, 170.3; *m*/*z* (ESI) $C_{32}H_{27}N_4O_2S_2$ $(M+H)^+$calcd 563.1575, found 563.1563.

Compound **20**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.59 (4H, br s), 2.43 (4 H, br s), 7.16 (2H, d, $J=3.5$ Hz), 7.43 (2H, d, $J=3.5$ Hz), 12.07 (2H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 23.7, 34.0, 112.7, 137.0, 157.4, 170.5; *m*/*z* (ESI) $C_{12}H_{15}N_4O_2S_2$ $(M+H)^+$ calcd 311.0636, found 311.0646.

8. Compound **8** was purified by preparative TLC (CH_2Cl_2–MeOH 7:1, developed twice).

Compound **8i** ($n=4$): ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.47 (4H, m), 3.16 (4H, m), 6.45 (2H, br t, $J=5.7$ Hz), 7.14 (4H, br d, $J=5.5$ Hz), 7.46 (2H, br s), 7.55 (4H, d, $J=8.7$ Hz), 7.56 (2H, br s), 8.01 (4H, d, $J=8.7$ Hz), 8.87 (2H, s), 12.70 (2H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 27.7, 39.2, 111.5, 117.9, 118.9, 121.9, 122.5, 123.1, 127.6, 135.4, 142.8, 144.4, 152.0, 155.6; *m*/*z* (ESI) $C_{32}H_{32}N_8O_2$ $(M+2H)^{2+}$ calcd 280.1324, found 280.1325.

Compound **8ii** ($n=6$): ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.32 (4H, m), 1.44 (4H, m), 3.09 (4H, m), 6.52 (2H, br s), 7.14 (4H, br d, $J=4.3$ Hz), 7.46 (2H, br s), 7.55 (4H, dd, $J=8.7$, 2.5 Hz), 7.56 (2H, br s), 8.00 (4H, d, $J=8.7$ Hz), 8.95 (2H, s), 12.70 (2H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 26.6, 30.1, 39.5, 111.5, 117.9, 118.8, 122.0, 122.6, 122.9, 127.6, 135.3, 142.8, 144.2, 152.0, 155.6; *m*/*z* (ESI) $C_{34}H_{36}N_8O_2$ $(M+2H)^{2+}$ calcd 294.1481, found 294.1479.

9. The purities of the compounds were assessed by observation of ^{1}H NMR spectra.

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11. Preparation of the helicase substrate: Two oligos, OLG 54 (5′-GTC AGT TGA GTG GCA GGC GGC ACA CAT TAT AGT GTC GTA GGC TTC-3′) and OLG 55 (5′- GTG TGC CGC CTG CCA CTC AAC TGA CTC AAC TAC TGT CTT GGG CAT CGG CA-3′) were used to prepare a double-stranded DNA template for the helicase assay. 75 pmol of OLG54 was radiolabeled with [γ-^{32}P]-ATP (100 nCi) using polynucleotide kinase (NEB) in a final volume of 30 μL. The labeling reaction was performed at 37 °C for 1 h. The unbound radioisotope was removed using Qiagen nucleotide removal kit. The radiolabeled OLG 54 was incubated with OLG 55 at a 1:3 molar ratio and annealed by cooling the mixture from 100 °C to rt gradually.

12. Helicase assay: To a 6 μL of drug solution (DMSO–H_2O 1:5) was added 9 μL of helicase stock (which contains helicase, 5× assay buffer, and water in a ratio of 1:3:5). The mixture was left at rt for 3 min before 5 μL of DNA solution (which contains 1 pmol/μL of radiolabeled double-stranded DNA, 5× assay buffer, and water in a ratio of 1:5:19) was introduced. These make up to a final mixture that contains 0.2 μg of helicase and 0.2 pmol of dsDNA. The mixture was incubated at 37 °C for 10 min. The helicase activity was stopped by adding 5 μL of 5× loading buffer. The single- and double-stranded DNA were separated on a 10% native polyacrylamide gel. The gel was dried in vacuo and the single- and double-stranded DNA were detected by autoradiography and quantitated on a densitometer (Bio-Rad). The percentage of single-stranded DNA was calculated as the percentage of radioactivity of the single-stranded DNA from the total radioactivity in each reaction. Calculation of inhibition is based on the formula:

$$\left\{\left[\%\mathrm{ssDNA}_{\mathrm{Ref}} - \%\mathrm{ssDNA}_{\mathrm{Drug}}\right]/\%\mathrm{ssDNA}_{\mathrm{Ref}}\right\} \times 100$$

Composition of 5× assay buffer: 100 mM Hepes-KOH pH 7.3, 10 mM DTT, 7.5 mM $MnCl_2$, 12.5 mM ATP, 0.5 μg/μL BSA.

Composition of 5× loading buffer: 500 mM Tris-Cl pH 7.4, 50 mM EDTA, 0.25% SDS, 0.05% bromophenol blue, 25% glycerol.

The reference control well contains enzyme, substrate, and 1 μL of DMSO in assay buffer.