Novel symmetrical phenylenediamines as potential anti-hepatitis C virus agents

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Abstract
Background: Despite the great progress made in the last 10 years, alternative strategies might help improving definitive treatment options against hepatitis C virus infection.

Methods: With the aim of identifying novel inhibitors of the hepatitis C virus-1b replication targeting the viral NS3 helicase, the structures of previously reported symmetrical inhibitors of this enzyme were rationally modified, and according to docking-based studies, four novel scaffolds were selected for synthesis and evaluation in the hepatitis C virus-1b subgenomic replicon assay.

Results: Among the newly designed compounds, one new structural family was found to inhibit the hepatitis C virus-1b replication in the micromolar range. This scaffold was chosen for further exploration and different novel analogues were synthesised and evaluated.

Conclusions: Different new inhibitors of the hepatitis C virus genotype 1b replication were identified. Some of the new compounds show mild inhibition of the NS3 helicase enzyme.

Keywords
Hepatitis C virus, inhibitors

Introduction
Hepatitis C virus (HCV) is a primary cause of chronic liver disease and it affects 3% of the global population.1 The infection, which becomes chronic in 60–85% of cases, leads to hepatic steatosis, fibrosis, cirrhosis and hepatocellular carcinoma.2,3 A vaccine is currently not available, while the standard of care was for long a combination of pegylated interferon and ribavirin.4 HCV genome encodes six non-structural proteins, essential for the viral replication.5 In the past 10 years, safe and highly potent inhibitors of the HCV replication have been developed, providing the basis for a definitive cure against this infection; their main targets are the NS3 protease,6,7 the NS5B polymerase,8 the NS4B protein9 and the NS5A protein.10 Nevertheless, the available combination treatments with these agents are associated with high costs,11,12 and for each of them resistant viral strains have been reported.13–17

Despite the abundance of structural information on the HCV NS3 helicase,18 none of the few inhibitors of this enzyme reported so far has been taken into clinical development.19 This protein, essential for the viral replication,20,21 is responsible for the ATP-dependent unwinding of double-stranded RNA sequences, an intermediate in the viral nucleic acid synthesis.22,23

The NS3 helicase was chosen as target for the rational design and synthesis of novel potential agents against genotype 1b HCV. The structures of previously reported symmetrical inhibitors of the HCV NS3 helicase24,25 were rationally modified into four novel scaffolds, which were then synthesised and evaluated in the genotype 1b subgenomic replicon assay.
Results and discussion

Rational design and synthesis

The HCV NS3 helicase is formed by three domains and occupies the C-terminal portion of the NS3 protein. It presents multiple ligand-binding regions, the main ones being an ATP-binding site in the cleft separating domain 1 from domain 2 and a single-stranded nucleic acid-binding site at the interface of the three domains, as depicted in Figure 1.

The potential interference with the known nucleic acid-binding cleft was evaluated for the design of novel inhibitors of the HCV replication. To achieve this result, the structures of previously reported symmetrical inhibitors of the HCV NS3 helicase were chosen as a starting point for the design of novel potential anti-HCV agents. Due to their bulky

![HCV NS3 helicase domains and main nucleic acid-binding cleft in the 3KQH crystal structure.](Image)

**Figure 1.** HCV NS3 helicase domains and main nucleic acid-binding cleft in the 3KQH crystal structure. HCV: hepatitis C virus.

![Structure of the known symmetrical HCV NS3 helicase inhibitors considered and their proposed modifications.](Image)

**Figure 2.** Structure of the known symmetrical HCV NS3 helicase inhibitors considered and their proposed modifications. HCV: hepatitis C virus.
occupational volumes, the most likely site of interference with the enzyme is, for all these known compounds, the main RNA-binding pocket. In particular, as shown in Figure 1, the nucleic acid substrate occupies this pocket in an extended conformation in the high-affinity open structure of the enzyme, making interactions with residues belonging to all three helicase domains. Given their potential to interfere with the binding of the nucleic acid substrate of the enzyme, the structures of the known helicase inhibitors were rationally modified in order to combine their central linkers with two equal aromatic sulphonamide lateral groups. This last feature has been identified as important for the antiviral activity of a different series of agents against genotype 1b HCV recently found in our research group (unpublished data). The newly designed potential anti-HCV-1b scaffolds are described in Figure 2, along with the structures of the parent symmetrical helicase inhibitors.

All four new scaffolds were analysed with molecular docking simulations with the Glide SP module in Maestro. A 12 Å docking grid was generated from the 3KQH crystal structure, using as centroid the area defined by residues Arg393, Glu493 and Trp501. The presence of the newly inserted hydrophobic sulphonamides should maximise the potential interactions with the target site, as indicated by docking results (Figure 3(a) to (d)).

The predicted binding mode found for all the structures suggests a good spatial occupation of the target site of the HCV NS3 helicase, with an optimal fitting of the region at the interface of the three main domains, and with the potential of forming hydrophobic and hydrogen bond interactions with several residues, including Trp501, Arg393, Glu493, Thr411, Ser287, Asn556 and Phe557.

As docking results support the potential interference with the target site of the enzyme, all four new scaffolds were selected for synthetic development.

The new symmetrical structures were synthesised according to two different procedures, a general one for scaffolds [3]–[5], and a second one for [6]. In the case of structural families [3]–[5], different hydrophobic substituents in the para position of the sulphonamide aromatic rings were explored. The presence of a hydrophobic group in this position should facilitate the interaction of the molecule with Trp501.

The common synthetic strategy applied for the scaffolds of [3]–[5] is shown in Scheme 1, along with the procedure to obtain [6].

Common intermediates [9a–h] were obtained through a nucleophilic displacement between the different sulfonyl chlorides [7a–h] and para-phenylenediamine [8], performed at 0°C in anhydrous environment using Diisopropylethylamine (DIPEA) and Dichloromethane (DCM) as base/solvent system. A second nucleophilic substitution reaction between [9a–h], used in excess, and the different symmetrical acyl chlorides [10]–[12] gave the desired symmetrical products [3a–b], [4a–c] and [5a–h], isolated in
variable yield after chromatographic purification or recrystallisation.

Derivative [6] was obtained with a two-step synthesis involving two nucleophilic displacement reactions, the first one between sulfonyl chloride [7a] and ethylenediamine [13], from which intermediate [14] was obtained in good yield after stirring the reaction mixture in dichloromethane at room temperature for 2 h in the presence of triethylamine as base, and the second one between alkyl amine [14] and succinyl chloride [12]. Desired product [6] was obtained in good yield after chromatographic purification.

**Biological activity**

All the newly synthesised compounds were evaluated for their potential effect against genotype 1b HCV replication in the Huh7-2 replicon system (Table 1). The HCV protease inhibitor Telaprevir (VX-950) was included as positive control. For most of the analogues prepared, their potential interference with the HCV NS3 helicase unwinding activity was also evaluated (Table 1). Primuline and aurintricarboxylic acid were included as positive controls.

Among the four new structural families designed, the most interesting compound was initially found to be [5a], which is associated with an antiviral effect against the genotype 1b HCV replication in the micromolar range. The activity pattern observed for its analogues [5b] and [5c] indicates retention of some antiviral activity both with a para-methyl substituent in the sulphonamide phenyl ring ([5b]) and with an unsubstituted phenyl sulphonamide ([5c]). Some antiviral activity can be observed also for [3b] and [4b], both showing a 4-methyl substitution in the aromatic sulphonamide, but their close analogues [3a], [4a] and [4c] are associated with a complete loss of activity. In order to further investigate the antiviral potential associated with [5a], its scaffold was further modified with the exploration of different hydrophobic groups in the para position of the phenyl sulphonamide ([5d–f]) and with the increase of the occupational volume of this group with the insertion of a condensed aromatic ring ([5g, h]). The most successful modification found so far is a tert-butyl substituent in the para position of the phenylsulphonamide group ([5d]), which shows improved EC50 and EC90 values and a good selectivity index > 10. The presence of a biphenylsulphonamide substituent ([5f]) is associated with activity retention, while the remaining three modifications attempted ([5e], [5g], [5h]) are associated with loss of activity, suggesting a negative influence of a bicyclic or heterocyclic aromatic ring for the activity of this scaffold.

Compounds [3a], [3b], [4c] and [5b] also show some inhibition of the HCV NS3 helicase unwinding activity at high concentrations, but a trend for this effect is not evident from the data obtained so far. While [3a] and [4c] do not show any antiviral activity in the replicon assay, possibly due to issues in the entrance to the cellular environment, [3b] and [5b] are showing some biological activity in both the assays, cellular and enzymatic. The observed IC50 values in the helicase assay for these two compounds are significantly higher than the range of activities found in the replicon system. The lack of correlation between the two sets of data suggests that the antiviral effect of the new
Table 1. Antiviral effect of the test compounds on genotype 1b hepatitis C virus replication in the Huh5-2 replicon system.

<table>
<thead>
<tr>
<th>Comp.</th>
<th>Ar</th>
<th>X</th>
<th>Y</th>
<th>EC₅₀ (µM)ᵇᶜ</th>
<th>EC₉₀ (µM)ᵇᶜ</th>
<th>CC₅₀ (µM)ᵇᵈ</th>
<th>SIᵉ</th>
<th>Helicase IC₅₀ (µM)ᵈ</th>
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<tbody>
<tr>
<td>[3a]</td>
<td>4-Cl-Ph</td>
<td>p-Ph</td>
<td>p-Ph</td>
<td>&gt;180</td>
<td>–</td>
<td>&gt;180</td>
<td>–</td>
<td>362</td>
</tr>
<tr>
<td>[3b]</td>
<td>4-Me-Ph</td>
<td>p-Ph</td>
<td>p-Ph</td>
<td>39.9±20.6</td>
<td>&gt;153</td>
<td>&gt;153</td>
<td>&gt;3.8</td>
<td>515</td>
</tr>
<tr>
<td>[4a]</td>
<td>4-Cl-Ph</td>
<td>CH=CH</td>
<td>p-Ph</td>
<td>&gt;155</td>
<td>&gt;155</td>
<td>&gt;155</td>
<td>–</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>[4b]</td>
<td>4-Me-Ph</td>
<td>CH=CH</td>
<td>p-Ph</td>
<td>53.1±13.8</td>
<td>&gt;124</td>
<td>&gt;124</td>
<td>&gt;2.3</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>[4c]</td>
<td>Ph</td>
<td>CH=CH</td>
<td>p-Ph</td>
<td>&gt;173</td>
<td>&gt;173</td>
<td>&gt;173</td>
<td>–</td>
<td>508</td>
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<td>[5a]</td>
<td>4-Cl-Ph</td>
<td>(CH₂)₂</td>
<td>p-Ph</td>
<td>20.4±12</td>
<td>141</td>
<td>&gt;154</td>
<td>&gt;7.5</td>
<td>&gt;1000</td>
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<tr>
<td>[5b]</td>
<td>4-Me-Ph</td>
<td>(CH₂)₂</td>
<td>p-Ph</td>
<td>66.8±37.8</td>
<td>&gt;165</td>
<td>113</td>
<td>1.7</td>
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<tr>
<td>[5c]</td>
<td>Ph</td>
<td>(CH₂)₂</td>
<td>p-Ph</td>
<td>34.3±3.9</td>
<td>&gt;130</td>
<td>&gt;173</td>
<td>&gt;5</td>
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<tr>
<td>[5d]</td>
<td>4-tBu-Ph</td>
<td>(CH₂)₂</td>
<td>p-Ph</td>
<td>12.8±3.3</td>
<td>24.1±14.1</td>
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<td>&gt;11.3</td>
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<td>[5e]</td>
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<td>p-Ph</td>
<td>54±46</td>
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<td>13.3±1.6</td>
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<td>[5g]</td>
<td>1-Naphthyl</td>
<td>(CH₂)₂</td>
<td>p-Ph</td>
<td>33.6±7.9</td>
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<td>&gt;4.4</td>
<td>&gt;1000</td>
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<tr>
<td>[5h]</td>
<td>8-Quinoline</td>
<td>(CH₂)₂</td>
<td>p-Ph</td>
<td>&gt;147</td>
<td>&gt;147</td>
<td>&gt;147</td>
<td>–</td>
<td>&gt;1000</td>
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<tr>
<td>[6]</td>
<td>4-Cl-Ph</td>
<td>(CH₂)₂</td>
<td>(CH₂)₂</td>
<td>&gt;181</td>
<td>181</td>
<td>&gt;181</td>
<td>&gt;181</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>(VX-950)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.8±0.2</td>
<td>47</td>
<td>58.8</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Primuline</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>10±2</td>
<td>–</td>
<td>0.3±0.1</td>
<td></td>
</tr>
<tr>
<td>Aurintricarboxylic acid</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.3±0.1</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

*EC₅₀ = 50% effective concentration (concentration at which 50% inhibition of virus replication is observed).
*EC₉₀ = 90% effective concentration (concentration at which 90% inhibition of virus replication is observed).
*CC₅₀ = 50% cytostatic/cytotoxic concentration (concentration at which 50% adverse effect is observed on the host cell).
*SI = the ratio of CC₅₀ to EC₅₀.

structures presented in this study might be due to the interference with a different target, viral or cellular, other than the HCV NS3 helicase.

Conclusions

Starting from a rational approach, four new structural scaffolds were designed as potential inhibitors of the genotype 1b-HCV replication targeting the viral NS3 helicase. The newly designed compounds show the presence of the two equal phenylsulphonamide lateral groups, linked together with different symmetrical amide functions. For each new structural family, a small series of new derivatives was synthesised and evaluated in the HCV-1b subgenomic replicon and the helicase unwinding assay. One of these scaffolds showed antiviral activity in the micromolar range and was chosen for further development. Data found so far suggest that antiviral activity for these structures is associated with the presence of two equal phenylenediamine rings in the linker, connected by a central succinamide group. A hydrophobic substituent in the para position of the two terminal phenyl groups is important for activity retention. The best new succinamide analogues found, [5d] and [5g], bear a 4-tert-butylphenyl and a 4-biphenyl substituent on the two sulphonamide groups, respectively, and were found to inhibit HCV-1b replication with EC₅₀ values in the range of the low micromolar.

Preliminary enzymatic evaluations suggest that the antiviral effect associated with the newly prepared compounds is mainly unrelated to the interference with the NS3 helicase activity, with other viral or cellular targets likely involved.

Experimental

All experimental procedures are described in detail in the Supporting Information, along with compound characterisation.

Declaration of conflicting interests

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