A Modular Approach to Synthetic RNA Binders of the Hepatitis C Virus Internal Ribosome Entry Site

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Natural products that target the RNA components of bacterial ribosomes and thereby act as antibiotics that shut down microbial protein synthesis, have provided a rich source of inspiration for the design and synthesis of small-molecule ligands directed at RNA targets.[1,2] A prominent example of a privileged scaffold for RNA recognition occurring in natural aminoglycoside antibiotics is 2-deoxystreptamine (2-DOS)[3] which contains a rigid framework of hydrogen-bond donors among which the rigid cis-1,3 arrangement of amino groups is responsible for selective interaction with structural motifs in RNA targets (Figure 1).[4] In an approach to reduce the complexity of chemical library synthesis that involves the highly functionalized 2-DOS scaffold, we have recently developed the 3,5-diaminopiperidine heterocycle (DAP) as a structural mimetic of the RNA-recognizing pharmacophore of the 2-DOS scaffold (Figure 1). Structure-guided design had been applied to discover a series of antibacterial DAP-triazine derivatives that act on the same ribosomal RNA target as the natural aminoglycoside antibiotics, which initially served as the inspiration for the conception of the DAP compounds.[5,6]

Here, we describe the synthesis of a novel class of modular ligands (1) that contain the DAP scaffold as the key moiety for RNA recognition in nonribosomal targets (Figure 1).[7] Screening of modular DAP ligands against the subdomain IIa, an RNA target in the internal ribosome entry site (IRES) of hepatitis C virus (HCV) (Figure 1), revealed a set of N-amido substituted amino acid conjugates of DAP (2) as micromolar binders of this RNA. We had previously shown that ligand-induced conformational change in the subdomain IIa RNA disrupts the function of the IRES and blocks viral protein synthesis; this ultimately leads to inhibition of HCV in infected cells.[8]

The common building block 4 used in the synthesis of the modular DAP compounds 2 was obtained from 2-chloro-3,5-dinitro-pyridine (3) following an established route,[9] which required, in the last step, high-pressure hydrogenation to reduce the pyridine (Scheme 1). As the yield in this transformation critically depends on the hydrogen pressure (> 1000 psi), we sought to employ an alternative reduction procedure. Among the explored methods, neither transformation of the pyridine to an N-oxide followed by ammonium formate treatment[10] nor reduction by lithium triethylborohydride[11] afforded the desired piperidine, which led us to pursue the original route to the building block 4. Synthesis of the DAP compounds 2 commenced with coupling of 4 with N-Cbz and side-chain protected α-amino acids 5 (Scheme 2). Commercially available pro-
tected amino acids were used except for fully Boc-protected L-arginine, which was synthesized by alkylation of N-Cbz-L-ornithine with N,N'-Boc-(5)-methyliothiourea. Selective deprotection of the coupling products gave DAP-amino acid conjugates 6, which were further treated with carboxylic acids 7 to furnish the final products 2 after deprotection of the intermediates 8 and HPLC purification.

RNA binding of the modular DAP compounds 2 was tested in two different fluorescence assays that we had previously developed for the subdomain IIa target in the HCV IRES. In the first assay, an oligonucleotide construct was used in which an adenine residue at position 54 in the internal loop of IIa was replaced by the fluorescent base analogue 2-aminopurine (2AP) as a sensitive probe of the RNA folding state. These compounds bind to the IIa RNA at the internal loop region, where 2AP54 is located, and thereby shield the fluorescent label from solvent exposure. The fact that a residual signal was observed even at the highest concentrations tested suggested that the 2AP labeled target remained in solution and that the fluorescence decrease was not due to nonspecific RNA aggregation. Binding was in competition with salt; this indicates electrostatic contributions to the ligand-RNA interaction.

Table 1. Structure-activity relationships for modular DAP compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Component</th>
<th>2AP assay [^{[a]}] EC50 [μM]</th>
<th>FRET assay [^{[b]}] EC50 [μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>2aa</td>
<td>5a</td>
<td>7a</td>
<td>n.a.</td>
</tr>
<tr>
<td>2ab</td>
<td>5b</td>
<td>7b</td>
<td>6.3 ± 0.6</td>
</tr>
<tr>
<td>2ac</td>
<td>5a</td>
<td>7c</td>
<td>n.a.</td>
</tr>
<tr>
<td>2ba</td>
<td>5b</td>
<td>7a</td>
<td>n.a.</td>
</tr>
<tr>
<td>2bb</td>
<td>5c</td>
<td>7b</td>
<td>7.2 ± 1.2</td>
</tr>
<tr>
<td>2bc</td>
<td>5c</td>
<td>7c</td>
<td>n.a.</td>
</tr>
<tr>
<td>2dc</td>
<td>5d</td>
<td>7c</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

[a] 2AP EC50 concentration required for 50% of the 2AP fluorescence decrease triggered by compound interaction with 2AP54 labeled IIa RNA, in the presence of 100 μM Mg2+. b) FRET EC50, relative change of the FRET intensity of Cy5/Cy5 5'-labeled IIa RNA in the presence of 100 or 1000 μM compound, measured in an assay that was described previously. Compounds were tested for optical interference with the fluorescent labels for both the 2AP and FRET assays and no significant interference was found. n.a. = no activity; n.d. = not determined.

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toward the ribosomal E site in proximity of the active site. [16] In-
structure that directs the highly conserved terminal hairpin IIb
the domain II located at one end, and adopting a hook-like
0000
[Image 62x605 to 275x775]

works of amino groups, which are positively charged under
physiological conditions, play an important role for target rec-
ognition of aminoglycoside antibiotics that interact with RNA
often by exploiting structural electrostatic complementarity
with divalent metal-ion binding sites. [14] Similarly, binding data
-derived from the 2AP54 assay suggest that the DAP scaffold in
the modular ligands described here participates in interactions
with Mg\(^{2+}\) sites at the IIa target.

The IIa-binding DAP compounds 2ab, 2bb and 2cb were in-
active in the FRET assay at micromolar concentrations in which
binding occurred in the 2AP assay (Table 1); this indicates that
these ligands interact with the RNA target without inducing a
conformational change. For two of the ligands, 2ab and 2bb,
both of which contain a basic amino acid (Lys or Arg), a small
increase of the FRET signal was observed at millimolar concen-
trations. As optical interference of the DAP compounds with
the fluorescent cyanine dyes was ruled out, the increased FRET
intensity was likely due to immobilization of the IIa RNA target
in the L-shaped fold upon ligand binding. Comparison of struc-
tural data from an NMR study of the IRES domain II [15] and crys-
tal structure analysis [13] suggests that the bent subdomain IIa
shows some flexibility, attested by an interhelical angle that
averaes at 90° in solution and that is fixed at 90° in the
crystal. The DAP ligands are able to bind and arrest the IIa RNA
in the 90° bent state in solution, thereby increasing the overall
intensity of the FRET signal. This immobilizing action of the
modular DAP ligands might provide a mechanistic basis for a
novel class of HCV IRES inhibitors (Figure 3). [7]

Figure 2. Titrations of 2AP54 labeled IIa RNA with DAP compound 2ab in the
absence and presence of salt. The assay was performed as previously de-
scribed [12] For the titration in 100 mM NaCl, 100 μM Mg\(^{2+}\) was present as
well. Error bars represent ± 1σ of triplicate titrations. EC\(_{50}\) values for all
tested DAP compounds are listed in Table 1. \(i_F\) = relative fluorescence intensi-
ty.

Cyro-electron microscopy investigations of the HCV IRES
bound to the 40S ribosomal subunit [16] and whole 80S ribo-
somes [17] revealed an overall extended shape of the RNA with
the domain II located at one end, and adopting a hook-like
structure that directs the highly conserved terminal hairpin IIb
toward the ribosomal E site in proximity of the active site. [16] In-
teraction of domain II with the ribosome induces a conforma-
tional change in the 40S head [17] that leads to closure of the
mRNA binding cleft. [16] Correct positioning of the IIb hairpin
and the viral mRNA at the ribosome depends critically on the
L-shaped fold of the domain II. [18] Intrinsic flexibility in the sub-
domain IIa might be required for release of the ribosome from
the IRES during translation initiation. We have previously dem-
strated that IRES-binding benzimidazole inhibitors of the
HCV replicon act by conformational induction of a widened in-
terhelical angle in the subdomain IIa (Figure 3), which facil-
titates the undocking of the hairpin IIb from the ribosome and
ultimately leads to inhibition of IRES-driven translation in HCV-
infected cells. [8] In a distinct mode of action, the IIa RNA-bind-
ing modular DAP ligands described here might represent a
second class of novel IRES inhibitors that affect HCV translation
by arresting subdomain IIa in a 90° bent state and thereby in-
terfering with translation initiation (Figure 3).

Preliminary testing of one of the IIa RNA-binding DAP com-
pounds in a cell based HCV replicon assay revealed that the
ligand 2ab induced dose-dependent reduction in HCV IRES-
driven translation of a luciferase reporter (Figure 4). The inhibi-
tion of reporter expression in infected human cells was specific
for the replicon construct and not due to cytotoxicity. Future
work will be focused on the iterative optimization of the mod-
ular DAP compounds to improve RNA target binding and HCV
replicon inhibition.

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Figure 4. Biological activity of DAP compound 2ab in human Huh-7.5 cells. A) Dose-dependent translation inhibition of luciferase reporter under HCV IRES control in the BM4-5 FEO HCV replicon.[20] B) Cytotoxicity as determined in a colorimetric viability assay. The luciferase reporter replicon assay and cytotoxicity testing were performed as described before.[8] $I_L$ = relative luminescence intensity.


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DAPper ligands: The 3,5-diaminopiperidine (DAP) heterocycle has been developed as a structural mimetic of the RNA-recognizing pharmacophore of the 2-DOS scaffold. Here we describe the synthesis of novel modular DAP ligands that bind to a conformational target in the internal ribosome entry site RNA of the hepatitis C virus.