Mildly inducible and selective cross-link methodology for RNA duplexes

Lieselot L. G. Carrette, Ellen Gyssels, Joke Loncke and Annemieke Madder

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## OVERVIEW OF THE SEQUENCES

Table S1. Overview of the sequences

<table>
<thead>
<tr>
<th>ON</th>
<th>2'OMe RNA</th>
<th>5'-CUG ACG GUG UGC-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>ON1</td>
<td>2'OMe RNA</td>
<td>5'-CUG ACG G1G UGC-3’</td>
</tr>
<tr>
<td>ON2</td>
<td>2’OMe RNA</td>
<td>5’-CUG ACG G2G UGC-3’</td>
</tr>
<tr>
<td>ODN</td>
<td>DNA</td>
<td>5'-CTG ACG GTG TGC-3’</td>
</tr>
<tr>
<td>ODN1</td>
<td>DNA</td>
<td>5’-CTG ACG G1G TGC-3’</td>
</tr>
<tr>
<td>ODN2</td>
<td>DNA</td>
<td>5’-CTG ACG G2G TGC-3’</td>
</tr>
<tr>
<td>RNA1</td>
<td>RNA</td>
<td>5’-GCA CCC CGU CAG-3’</td>
</tr>
<tr>
<td>RNA2</td>
<td>RNA</td>
<td>5’-GCA CUC CGU CAG-3’</td>
</tr>
<tr>
<td>RNA3</td>
<td>RNA</td>
<td>5’-ACG CCC GAC UGC-3’</td>
</tr>
<tr>
<td>DNA1</td>
<td>DNA</td>
<td>5’-GCA CCC CGT CAG-3’</td>
</tr>
</tbody>
</table>
SUMMARY OF THE MELTING TEMPERATURES OF THE DIFFERENT DUPLEXES

Table S2. Melting temperatures of the different duplexes

<table>
<thead>
<tr>
<th>probe</th>
<th>target</th>
<th>RNA1</th>
<th>DNA1</th>
<th>RNA2</th>
<th>DNA2</th>
<th>RNA3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T&lt;sub&gt;m&lt;/sub&gt; (°C)</td>
<td>ΔT&lt;sub&gt;m&lt;/sub&gt; (°C)</td>
<td>T&lt;sub&gt;m&lt;/sub&gt; (°C)</td>
<td>ΔT&lt;sub&gt;m&lt;/sub&gt; (°C)</td>
<td>T&lt;sub&gt;m&lt;/sub&gt; (°C)</td>
</tr>
<tr>
<td>ON</td>
<td>5’-CUG ACG GUG UGC-3’</td>
<td>82</td>
<td>n.d.</td>
<td>73</td>
<td>n.d.</td>
<td>39</td>
</tr>
<tr>
<td>ON1</td>
<td>5’-CUG ACG G1G UGC-3’</td>
<td>51</td>
<td>-31</td>
<td>53</td>
<td>-20</td>
<td>n.d.</td>
</tr>
<tr>
<td>ON2</td>
<td>5’-CUG ACG G2G UGC-3’</td>
<td>60</td>
<td>-22</td>
<td>n.d.</td>
<td>59</td>
<td>-14</td>
</tr>
<tr>
<td>ODN</td>
<td>5’-CTG ACG GTG TGC-3’</td>
<td>39</td>
<td>41</td>
<td>40</td>
<td>44</td>
<td>n.d.</td>
</tr>
<tr>
<td>ODN1</td>
<td>5’-CTG ACG G1G TGC-3’</td>
<td>31</td>
<td>-8</td>
<td>37</td>
<td>-4</td>
<td>34</td>
</tr>
<tr>
<td>ODN2</td>
<td>5’-CTG ACG G2G TGC-3’</td>
<td>37</td>
<td>-2</td>
<td>49</td>
<td>+8</td>
<td>38</td>
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</tbody>
</table>

n.d. = not determined

x = no duplex formation

Figure S1. Melting curve of the very stable duplex ON-RNA1
### SUMMARY OF ALL OBTAINED CROSS-LINK YIELDS

Table S3. Cross-link yields

<table>
<thead>
<tr>
<th>Sequences</th>
<th>NBS 25°C</th>
<th>NBS 37°C</th>
<th>$^{1}O_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA - DNA</td>
<td>ODN1 DNA1</td>
<td>17</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>ODN2 DNA1</td>
<td>36*</td>
<td>28</td>
</tr>
<tr>
<td>DNA - RNA</td>
<td>ODN1 RNA1</td>
<td>35</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>ODN2 RNA1</td>
<td>18</td>
<td>n.d.</td>
</tr>
<tr>
<td>2′OMeRNA - RNA</td>
<td>ON1 RNA1</td>
<td>34</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>ON2 RNA1</td>
<td>19</td>
<td>24</td>
</tr>
</tbody>
</table>

* Determined based on HPLC analysis at 50°C
n.d. = not determined
SUPPORTING SPECTRAL DATA

RP-HPLC Chromatograms of the synthesized ODNs and ONs

Synthesized sequences:
ODN1: 5’-CTG ACG G1G TGC-3’
ODN2: 5’-CTG ACG G2G TGC-3’
ON1: 5’-C*U*G* A*C*G* G*1G* U*G*C*-3’ (*2’OMe-RNA)
ON2: 5’-C*U*G* A*C*G* G*2G* U*G*C*-3’ (*2’OMe-RNA)

Figure S2. RP-HPLC chromatograms of the synthesized ODN1, ODN2, ON1 and ON2
Mass spectra of the formed cross-links

Figure S3. Maldi Mass spectrum of ICL: ON1-RNA1

Figure S4. Maldi Mass spectrum of ICL: ON2-RNA1
PAGE images and RP-HPLC Chromatograms of the cross-link reactions

Cross-linking of ON1 (PAGE)

37°C

25°C

<table>
<thead>
<tr>
<th>Probe</th>
<th>ON1</th>
<th>RNA1</th>
<th>RNA2</th>
<th>RNA3</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBS</td>
<td>-</td>
<td>4 eq</td>
<td>4 eq</td>
<td>4 eq</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Probe</th>
<th>ON1</th>
<th>RNA1</th>
<th>RNA2</th>
<th>RNA3</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBS</td>
<td>-</td>
<td>4 eq</td>
<td>4 eq</td>
<td>4 eq</td>
</tr>
</tbody>
</table>

Figure S5. PAGE of ON1 and RNA targets. Left: cross-link temperature = 37°C. Right: cross-link temperature = 25°C. Ladder consist of a mixture of 4 ODNs with masses 7182, 6457, 4319, 2096 Da

Cross-linking of ON1 to RNA1 (RP-HPLC)

- With NBS

37°C

25°C

Figure S6. RP-HPLC chromatograms of cross-link reaction of ON1 with RNA1 as target. Top: cross-link temperature = 37°C. Bottom: cross-link temperature = 25°C. ICL indicates the cross-linked species (analysis by MS analysis vide supra).
• With singlet oxygen

Figure S7. PAGE of cross-link reaction with singlet oxygen of ON1 and RNA1 as target.

25°C

Figure S8. RP-HPLC chromatograms of cross-link reaction with singlet oxygen of ON1 with RNA1 as target.
Cross-linking of ON1 to RNA2 (RP-HPLC)

- With NBS

37°C

![HPLC chromatogram for ON1 to RNA2 at 37°C](image)

25°C

![HPLC chromatogram for ON1 to RNA2 at 25°C](image)

Figure S9. RP-HPLC chromatograms of cross-link reaction of ON1 with RNA2 as target. Top: cross-link temperature = 37°C. Bottom: cross-link temperature = 25°C. None of the visible peaks could be correlated with a cross-linked species, but correspond to degradation products.

Cross-linking of ON1 to RNA3 (RP-HPLC)

- With NBS

37°C

![HPLC chromatogram for ON1 to RNA3 at 37°C](image)

25°C

![HPLC chromatogram for ON1 to RNA3 at 25°C](image)

Figure S10. RP-HPLC chromatograms of cross-link reaction of ON1 with RNA3 as target. Top: cross-link temperature = 37°C. Bottom: cross-link temperature = 25°C. None of the visible peaks could be correlated with a cross-linked species, but correspond to degradation products.
Cross-linking of ON2 (PAGE)

37°C

<table>
<thead>
<tr>
<th>Ladder (Daion)</th>
<th>ON2</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA1</td>
<td>RNA2</td>
</tr>
<tr>
<td>-</td>
<td>4 eq</td>
</tr>
</tbody>
</table>

25°C

<table>
<thead>
<tr>
<th>Ladder (Daion)</th>
<th>ON2</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA1</td>
<td>RNA2</td>
</tr>
<tr>
<td>-</td>
<td>4 eq</td>
</tr>
</tbody>
</table>

Figure S11. PAGE of ON2 and RNA targets. Left: cross-link temperature =37°C. Right: cross-link T=25°C
Ladder consist of a mixture of 4 ODNs with masses 7182, 6457, 4319, 2096 Da

Cross-linking of ON2 to RNA1 (RP-HPLC)

- With NBS

37°C

Figure S12. RP-HPLC chromatograms of cross-link reaction of ON2 with RNA1 as target. Top: cross-link temperature = 37°C. Bottom: cross-link temperature = 25°C. ICL indicates the cross-linked species and Br indicates the brominated side product (analysis by MS analysis vide supra).
• With singlet oxygen

### Figure S13

PAGE of cross-link reaction with singlet oxygen of ON2 and RNA1 as target.

25°C

### Figure S14

RP-HPLC chromatograms of cross-link reaction with singlet oxygen of ON2 with RNA1 as target.
Cross-linking of ON2 to RNA2 (RP-HPLC)

- With NBS

37°C

Figure S15. RP-HPLC chromatograms of cross-link reaction of ON2 with RNA2 as target. Top: cross-link temperature = 37°C. Bottom: cross-link temperature = 25°C. Br indicates the brominated side product (analysis by MS analysis vide supra), none of the other visible peaks could be correlated with a cross-linked species, but correspond to degradation products.

Cross-linking of ON2 to RNA3 (RP-HPLC)

- With NBS

37°C

Figure S16. RP-HPLC chromatograms of cross-link reaction of ON2 with RNA3 as target. Top: cross-link temperature = 37°C. Bottom: cross-link temperature = 25°C. Br indicates the brominated side product (analysis by MS analysis vide supra), none of the other visible peaks could be correlated with a cross-linked species, but correspond to degradation products.
ADDITIONAL SUPPORTING EXPERIMENTS

Initial screen

A range of furan modified nucleosides has been developed and or tested by our group for DNA ICL formation. The furan moiety has been used to replace the sugar of the nucleoside or has been attached to it, on the 1’ or 2’ position, through different linkers (amide, urea and ether) and combined with different bases (uracil and adenine). The furan moiety can also be attached to the base. (Ref 15-19)

Because an RNA-RNA duplex is structurally different from the DNA-DNA duplex, the properties of the different furan modified building blocks cannot be assumed to be simply transferable. For the selection of the 2 most promising furan modified nucleosides to be used in this study, an initial screen was performed with 5 furan modified nucleosides, depicted in figure S15 incorporated in the DNA sequence 5’-CTG ACG GXG TGC-3’ (available from previous experiments) and targeting RNA1 (5’-GCA CCC CGU CAG-3’). The combination of DNA and RNA in a helix forms an intermediate duplex between A and B and can give an indication of the behavior in pure RNA duplex.

The cross-link reaction was performed at 0°C (initial mild conditions) by addition of 4 equiv. of NBS as described before. Figure S15 shows the RP-HPLC chromatograms before and after oxidation with NBS. The 2nd and 5th furan modified nucleosides were identified as the most promising and therefore selected for further study in this context.

Figure S17. Illustration of the initial screen to identify the furan modified nucleosides most suited for use in RNA ICL formation.
Cross-link tests from DNA to the RNA targets

*Cross-linking of ODN1 (PAGE)*

25°C

<table>
<thead>
<tr>
<th>Probe</th>
<th>ODN1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target</td>
<td>RNA1</td>
</tr>
<tr>
<td>NBS</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure S18. PAGE of ODN1 and RNA targets.

*Cross-linking of ODN1 to RNA1 (RP-HPLC)*

- With NBS

25°C

Figure S19. RP-HPLC chromatograms of cross-link reaction of ODN1 with RNA1 as target. ICL indicates the cross-linked species, which consists of 2 pairs of pseudo enantiomers (analysis by MS analysis vide supra).
Cross-linking of ODN1 to RNA2 (RP-HPLC)

- With NBS

25°C

Figure S20. RP-HPLC chromatograms of cross-link reaction of ODN1 with RNA2 as target. Only remaining RNA2 can be observed after NBS treatment.

Cross-linking of ODN1 to RNA3 (RP-HPLC)

- With NBS

25°C

Figure S21. RP-HPLC chromatograms of cross-link reaction of ODN1 with RNA3 as target. Only remaining RNA2 can be observed after NBS treatment.
**Cross-linking of ODN2 to RNA (PAGE)**

25°C

<table>
<thead>
<tr>
<th>Probe</th>
<th>ODN2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target</td>
<td>RNA1</td>
</tr>
<tr>
<td>NBS</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure S2. PAGE of ODN2 and RNA targets.

**Cross-linking of ODN2 to RNA1 (RP-HPLC)**

- With NBS

25°C

Figure S23. RP-HPLC chromatograms of cross-link reaction of ODN2 with RNA1 as target. ICL indicates the cross-linked species and Br indicates the brominated side product (analysis by MS analysis vide supra).
Cross-linking of ODN2 to RNA2 (RP-HPLC)

- With NBS
  25°C

Figure S24. RP-HPLC chromatograms of cross-link reaction of ODN2 with RNA2 as target. Br indicates the brominated side product (analysis by MS analysis vide supra), none of the other visible peaks could be correlated with a cross-linked species, but correspond to degradation products.

Cross-linking of ODN2 to RNA3 (RP-HPLC)

- With NBS
  25°C

Figure S25. RP-HPLC chromatograms of cross-link reaction of ODN2 with RNA3 as target. Br indicates the brominated side product (analysis by MS analysis vide supra), none of the other visible peaks could be correlated with a cross-linked species, but correspond to degradation products.
DNA ICL tests at 37°C

Cross-linking of ODN1 to DNA1 (RP-HPLC)

- With NBS

37°C

Figure S26. RP-HPLC chromatograms of cross-link reaction of ODN1 with DNA1 as target. Top: cross-link temperature = 37°C. Bottom: cross-link temperature = 25°C. ICL indicates the cross-linked species, which consists of 2 pairs of pseudo enantiomers (analysis by MS analysis vide supra).

Cross-linking of ODN2 to DNA1 (RP-HPLC)

- With NBS

37°C

Figure S27. RP-HPLC chromatograms of cross-link reaction of ODN2 with DNA1 as target. ICL indicates the cross-linked species and Br indicates the brominated side product (analysis by MS analysis vide supra).
Influence of column temperature in RP-HPLC

Depending on the column temperature during an RP-HPLC analysis, the area of the cross-link and consequently the calculated yield is different. This is illustrated in Figure S26 for the cross-link reaction of ODN1 and DNA1 with 4 equiv NBS at 25°C, analyzed at 60°C, 50°C and 40°C. The area decreases with increasing temperature, probably due to instability of the formed crosslink. All HPLC chromatograms and yields used throughout the article and supporting information are based on analysis at 60°C, unless mentioned otherwise.

Figure S28. RP-HPLC chromatograms of cross-link reaction of ODN1 with DNA1 as target, at 25°C and with 4 equiv of NBS. Top: Analysis Temperature = 60°C. Middle: Analysis Temperature = 50°C. Bottom: Analysis Temperature = 40°C.