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Time dependence of cisplatin-induced duplex dissociation of 15-mer RNAs and mature miR-146a†

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The kinetics for the binding of cisplatin to duplex RNAs, two fully complementary model systems and mature miR-146a, exhibits a linear dependence on cisplatin concentration and results in duplex dissociation at 38 °C.

Our knowledge regarding the use of small non-coding RNAs (ncRNAs) as regulators of gene expression is currently under rapid expansion.1 Various types of both single- and double-stranded RNAs have been identified today, ranging from the relatively short ca. 20-mer duplexes of mature microRNAs (miRs), to the much longer transcripts of e.g. long and circular non-coding RNAs (lncRNAs and circRNAs).2,3 A common feature of all these seems to be their involvement in the cellular machinery as regulators, and a role in fine-tuning protein production as their main function through interaction with the RNA induced silencing complex (RISC).4,6 In the developing human cell, the proper functioning of ncRNAs is known to be crucial for key events such as early differentiation and organ development.7 Consequently, dysregulation of ncRNAs is recognized today as a reliable signature feature of many common diseases, with diabetes and cancer as well documented examples.8–11

In the case of cancer, clinically used treatment regimens involve the use of several different types of drugs, often including alkylating ones, i.e. molecules able to covalently modify nucleic acids. Here, the use of cis-Pt(NH₃)₂Cl₂ (cisplatin, 1) has been particularly successful for the treatment of testicular cancer, with well documented efficacy also against other types, e.g. ovarian- and head-and-neck cancers.12 With respect to the intracellular mode of action, the reaction path involving the binding of cisplatin to nuclear DNA – with induction of apoptosis and necrosis as downstream biological consequences – has so far been subjected to the most thorough investigations.13 However, since the ncRNAs are already present in the cytosol, where activation of cisplatin by formation of the corresponding mono-aquo complex cis-[Pt(NH₃)₂Cl(OH)]⁺ (1a) takes place, RNA binding is also prone to occur.14–18 To address the latter phenomenon, our laboratory has initiated research aiming at investigating the ability of platinum-based drugs to interfere with the RISC function.4,6,19,20 We have previously been able to show that fully complementary RNAs (siRNAs) processed by RISC maintained their function after platination,21–23 but with a typical overall trend of a moderately reduced silencing ability when compared to their corresponding unplatinated siRNAs. Due to the multistep process leading to silencing, the origin of the Pt-induced modulation could be a sum of several contributions.24 For example, adducts located at crucial recognition sites during loading might lead to impaired functioning.25,26 Once loaded, the presence of a platinum-adduct in the biologically active guide strand might also interfere with the silencing ability by thermal destabilization of the duplex formed between the si- or miRNA and its complementary target mRNA.27–29

To gain further insights into the endogenous response following e.g. clinical treatment with cisplatin, we present here an initial study illustrating a methodology that allows for documentation of the reactivity of cisplatin towards endogenous miRs. Since adduct profiles of nucleic acids are likely to be under kinetic rather than thermodynamic control,30 we believe that such information serves as a guide to the identification of particularly reactive metal binding sites in the RNA environment. Towards this goal we here – to our knowledge for the first time – present data that allow for a comparison of the reactivity of model duplex RNAs (RNA-1 and RNA-2) with that of an endogenous mature miRNA (miR-146a), see Scheme 1. Highly reproducible kinetics were obtained using UV/vis spectroscopy, and allowed for the establishment of a reactivity trend of RNA-1 > miR-146a > RNA-2 at physiologically relevant salt concentrations.
As can be seen in Scheme 1, the fully complementary 15-mer RNAs both contain a single, centrally located preferred platination site in one of the strands: the r(GG)- or r(GUG)-sequence in RNA-1 and RNA-2, respectively. Both these duplexes have recently been characterized with respect to thermodynamic properties and the accessibility of G-N7 binding sites underlined and the seed sequence of miR-146a is shown in italics.

**Scheme 1** RNA oligonucleotides used in the present study. Tentative G-N7 binding sites are underlined and the seed sequence of miR-146a is shown in italics.

Table 1 Summary of melting temperatures ($T_{m}$) and reaction rate constants ($k_{obs}$ and $k_{2,app}$) for RNA-1, RNA-2 and mir-146a

<table>
<thead>
<tr>
<th>Duplex</th>
<th>$T_{m}$ (°C)</th>
<th>$C_{Pt}$ (µM) 7.5</th>
<th>$C_{Pt}$ (µM) 15.0</th>
<th>$C_{Pt}$ (µM) 22.5</th>
<th>$C_{Pt}$ (µM) 30.0</th>
<th>$C_{Pt}$ (µM) 45.0</th>
<th>$k_{2,app}$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-146a</td>
<td>5’-CCU CUG AGU UCU UCA G-3’</td>
<td>0.54</td>
<td>0.39</td>
<td>0.66</td>
<td>0.90</td>
<td>1.2</td>
<td>2.2</td>
</tr>
<tr>
<td>miR-146a</td>
<td>5’-UU GUG UCU AAG UCA AGA GRG-5’</td>
<td>0.50</td>
<td>0.83</td>
<td>1.2</td>
<td>1.5</td>
<td>2.2</td>
<td>4.51</td>
</tr>
</tbody>
</table>

*Measurements were conducted with an individual RNA strand concentration of 1.5 µM, resulting in a total strand concentration ($C_{T,RNA}$) of 3.0 µM. Measurements performed in triplicates. *Indicated errors correspond to the standard error.

**Fig. 1** Spectral changes as a function of time and related kinetic traces together with fits to an exponential function obtained after addition of 1a to RNA-1 in buffered solution at 38 °C. (A) $C_{Pt} = 7.5$ µM, $C_{T,RNA} = 3.0$ µM, $C_{Na^+} = 29$ mM, and pH = 5.7. (B) $C_{Pt} = 7.5, 15.0, 22.5, 30.0$ and 45.0 µM, $C_{T,RNA} = 3.0$ µM, $C_{Na^+} = 29$ mM, and pH = 5.7. (C) $C_{Pt} = 7.5, 15.0, 22.5, 30.0$ and 45.0 µM, $C_{T,RNA} = 3.0$ µM, $C_{Na^+} = 129$ mM, and pH = 5.7.
conducted in which the absorbance change at $\lambda = 260$ nm was followed as a function of time. The reaction was studied at two different salt concentrations, at $C_{Na^+} = 29$ and 129 mM, with increasing concentrations of 1a in the interval 7.5–45.0 $\mu$M. The resulting experimental data obtained after exposure of RNA-1 to 1a, together with a fit of data to single exponential functions, are illustrated in Fig. 1B and 1C (see Fig. S2 and S3† for the corresponding data for RNA-2 and miR-146a). As can be seen here, the nature of the absorbance change is clearly dependent on the concentration of added 1a, and also well described by an exponential function under all investigated reaction conditions. The present kinetic data are thus indicative of the formation of a mono-platinated duplex as the step initiating melting.\textsuperscript{35} Further, a visual comparison of the 1st reactive formation of a mono-platinated duplex as the step reaction conditions. The present kinetic data are thus indicative of the formation of a mono-platinated duplex as the step initiating melting.\textsuperscript{35} Further, a visual comparison of the 1st half-life for similar platinum concentrations shows that the reaction is salt dependent, with a pronounced higher reactivity at the lower salt concentration employed. For example, $t_{1/2}$ increases from ca. 90 to 250 min with a change of $C_{Na^+}$ from 29 to 129 mM at the lowest platinum concentration employed: [1a] = 7.5 $\mu$M, compare the bottom curves in Fig. 1B and 1C.

This observation is in line with the expected behaviour of a system involving charge neutralization as the rate determining step, and of a magnitude similar to earlier reports on salt-dependent metalation kinetics.\textsuperscript{16,31} Further, for all RNA duplexes, a linear relationship between $k_{obs}$ and [1a] was observed, thus allowing for determination of $k_{2,app}$ directly from the slope (see Fig. S4† for an illustration of data obtained for RNA-1, RNA-2 and miR-146a). The obtained values for $k_{obs}$ and $k_{2,app}$ are summarized in Table 1. The data reveal that the three target RNAs studied here have similar reactivity at the lower salt concentration employed, with $k_{2,app}$ ca. 20 M$^{-1}$ s$^{-1}$, with only a slight tendency for a higher reactivity of the GG-containing RNA-1 ($k_{2,app} = 22.1 \pm 1.7$ M$^{-1}$ s$^{-1}$). At the higher salt concentration however, the reactivity varies significantly between the duplexes, revealing RNA-1 to be the most reactive one, with $k_{2,app}$ ca. 7.7 M$^{-1}$ s$^{-1}$, followed in reactivity by miR-146a ($k_{2,app}$ ca. 4.5 M$^{-1}$ s$^{-1}$) and RNA-2 ($k_{2,app}$ ca. 3.5 M$^{-1}$ s$^{-1}$). The latter data thus seem to suggest that the monoaquated form of cisplatin is able to discriminate between RNA target sites at the salt concentrations relevant for biological systems. The sequence of reactivity obtained in the present investigation suggests an order according to: $k_{2,app}(GG) > k_{2,app}(GUG)$, with centrally located consecutive GGs as the most reactive site.

Conclusions

In summary, we have presented here a method allowing for direct monitoring of metal binding to duplex RNA. The method relies on the ability of the monoaquated form of cisplatin (1a) to initiate duplex melting, and was applied here to two fully complementary 15-mer model RNAs and a mature version of the endogenous miR-146a. The reactivity trends reveal the miR to be as reactive as the model RNAs, indicating a half-life for interaction with 1a in the hour-range at physiologically relevant salt concentrations.

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Notes and references

33 For RNA-1, RNA-2 and miR-146a at $C_{Na^+} = 129$ mM the absorbance change is due to conversion of predominantly duplex RNA to single-stranded oligonucleotides. For miR-146a at $C_{Na^+} = 29$ mM, the reacting RNA-pool is a mixture of duplex- and corresponding single-stranded material thus resulting in a reduction of the measured $\Delta A$-value by ca. 50%.
35 Characterization of adduct type(s) is currently ongoing in our laboratory, C. Polonyi and S. K. C. Elmroth, private communication.