Electronic Supplementary Information for Dalton Transactions

Electronic Supplementary Information to:

Kinetic preference for interaction of cisplatin with the G-C-rich wobble basepair region in both $tRNA^{Ala}$ and Mh^{Ala}

Pal Papsai,[a] Jasmin Aldag,[a] Tina Persson[b] and Sofi K. C. Elmroth[a]*

Materials and Methods

Buffers, metal reagents and radionucleotides.

Elution buffer (NaOAc, pH 4.6): Excised bands were eluted in 1.0 M NaOAc (Riedel-de Haen) at pH 4.6.

RNA precipitation buffer (EtOH/NaOAc, pH 5.0): The RNA was EtOH precipitated according to standard protocols (Maniatis) and 20 μg glycogen (MBI Fermentas) as carrier.

Denaturing loading buffer (5x): 2.3 M urea (Fluka), 1x TBE, 66 % (w/v) formamide (Fluka), 0.05 % (w/v) bromphenolblue (Sigma), 0.05 % (w/v) xylene cyanol (Sigma)

Phosphorylation buffer (Tris/HCl, pH 7.6): The reaction buffer provided from MBI Fermentas (50 mM Tris/HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.10 mM spermidine and 0.10 mM EDTA) supplied together with the enzyme was used as received.

Ligation buffer (HEPES): 100 mM HEPES, 10 mM DTT, 15 mM $MgCl_2$, 0.5 mM ATP, 10% (w/v) DMSO and RNase inhibitor (0.5 units/μl, provided by MBI Fermentas).

Alkaline hydrolysis (NaOH, EDTA): Typically, the alkaline hydrolysis reactions were carried out in the presence of 2.5 μg carrier tRNA (baker's yeast, Sigma), 30 mM NaOH and 0.30 mM EDTA in a total reaction volume of 10 μl.

RNase T1 cleavage (Sodium citrate, pH 5.0): RNase T1 (MBI Fermentas) digestion was performed in 20 mM sodium citrate (Fluka), 7 mM urea **(**Fluka**)**, 1 mM EDTA **(**Sigma**)** and 0.5 unit RNase T1 for 30 min at 55 °C (denaturing conditions) or in water with 0.5 - 1 units for 20 min at ambient temperature (native conditions).

Nuclease S1 cleavage: Nuclease S1 (MBI Fermentas) digestion was performed in water containing 4.5 mM $ZnSO_4$ **(**Sigma**)** with 1,5 or 10 units for 20 min at ambient temperature.

Platination buffer (MOPS, pH 6.3): Exposure of $Microhelix^{Ala}$ (Mh^{Ala}) to the platination reagents was performed in a 50 mM MOPS buffer (Sigma) at pH 6.3 adjusted with 140 mM NaOAc (Reidel-de Haen) in the presence of 2.0 mM $Mg(OAc)_2$ (Sigma) (chloride free platination buffer). Full length tRNA ($tRNA^{Ala}$) was platinated under the same conditions except that 2.0 mM $MgCl_2$ was used instead of $Mg(OAc)_2$ (platination buffer).

Cisplatin. The compound *cis*-$Pt(NH_3)_2Cl_2$ (cisplatin) was purchased from Sigma-Aldrich (Sweden). Stock solutions of 1 mM cisplatin in H_2O were freshly prepared and aliquots stored at –20 °C. Aliquots were taken for reactions and not frozen again.

Radionucleotides. Radionucleotides [^{32}P]pCp and [γ-^{32}P]ATP were obtained from Amersham Biosciences (Uppsala, Sweden).

Ribonucleic acids and cleavage reactions

$Microhelix^{Ala}$. The synthetic oligonucleotide 5'-GGGGCUAUAGCUCUAGCUCCACCA-3' (Mh^{Ala}) was synthesised by IBM GmbH (Göttingen, Germany).

Construction of $tRNA^{Ala}$.

Purification of crude RNA. $tRNA^{Ala}$ was synthesized by enzymatic ligation of two chemically synthesized RNA oligomers (IBA, Göttingen); 5'- ACG CAA GAG GUC UGC GGU UCG AUC CCG CAU AGC UCC ACC A-3' (40-mer) and 5'-GGG GCU AUA GCU CAG CUG GGA GAG CGC UUG CUU UGC-3□' (36-mer). The crude oligomers were purified on PAGE (20 %, denaturing conditions

with 8 M urea). The purified RNA was excised and eluted from the gel overnight in 1 M NaOAc at pH 4.7, followed by EtOH precipitation. The resulting RNA was dissolved in 100 µl H_2O.

Ligation. Prior to ligation, 9 nM phosphorylated RNA 40-mer (phosphorylation described below) was heated together with 4.5 nM of the 36-mer at 90 °C for 3 min to allow for denaturation, followed by incubation at 65 °C for 10 min, and slow cooling to room temperature over *ca.* 45 min in ligation buffer. The ligation (mix V_{tot} = 200 µl) was initiated by addition of 140 units T4 RNA ligase (MBI, Fermentas), and the reaction mixture was allowed to incubate overnight at 16 °C. The ligation product was purified on PAGE (12%, denaturing conditions), and collected after extraction as described above (see purification of crude RNA). The final product, *i.e.* synthetically produced full lenght $tRNA^{Ala}$, was dissolved in 100 µl H_2O and stored at –20 °C.

Phosphorylation. Prior to ligation, *ca.* 12 nmol 40-mer oligonucleotide corresponding to the 3'-end of $tRNA^{Ala}$, divided into four portions, were phosphorylated in phosphorylation buffer (see above) using 25 units of T4 polynucleotide kinase (MBI Fermentas), with additional 250 nmol ATP, in a total volume of 50 µl ($C_{ATP} \approx 5$ mM). After initial incubation for 30 min, a second aliquot of 250 nmol ATP was added together with 25 units of T4 polynucleotide kinase, followed by further incubation for 20 min at 37 °C. The phosphorylated RNA was extracted by use of a standard phenol-chloroform-isoamyl alcohol extraction mixture (Fluka, ratio 125:24:1, pH 4.7), followed by ethanol precipitation in the presence of 20 µg glycogen as described above.

3'- and 5'-end labeling. 3'-ends of RNA (15 pmol) were labeled with 1.11 MBq [5'-^{32}P]-pCp using 2 units of T4 RNA ligase (MBI Fermentas) and 150 pmol ATP in T4 RNA ligation buffer (50 mM HEPES-NaOH, pH 8.0, 10 mM $MgCl_2$, 10 mM DTT) (MBI Fermentas) and a total volume of 12 µl at 4°C. The 5'-end labeling was performed in T4 PNK buffer (50 mM Tris/ HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA) with 1.11 MBq [γ-^{32}P]-ATP and 10 units of T4 polynucleotide kinase (MBI Fermentas) in a total volume of 20 µl at 37 °C. After incubation for 30 min, 100 nmol ATP and another 10 units of T4 polynucleotide kinase were added followed by incubation for 30 min at 37 °C. In case of 5'-end labeling, RNA was ethanol precipitated after reaction. The labeled nucleic acids were purified on denaturing polyacrylamide gels (12 % for $tRNA^{Ala}$ and 15 % for Mh^{Ala}) and the bands were visualised by luminoradiography using a Bio-Imager Analyser (Fujifilm), and were then excised and eluted in 1 M NaOAc, pH 4.6. The resulting nucleic acid was precipitated using ethanol and 20 µg glycogen as described above.

Rough platination kinetics of Mh^{Ala}. Radiolabeled Mh^{Ala} (typically with a total activity of 26 – 36 × 10^4 cpm) was dissolved in a chloride-free platination buffer. The RNA was allowed to denature and renature by heating to 90 °C for 2 min followed by slow cooling to room temperature. Aliquots of the resulting labeled Mh^{Ala} (52 – 72 × 10^3 cpm) were added to solutions containing increasing concentrations of cisplatin (0, 0.025, 0.050, 0.10 and 0.15 mM) in chloride-free platination buffer. The resulting reaction mixtures (V_{tot} = 50 µl) were incubated at 37 °C. Aliquots of the reaction mixture (5 µl each) were removed after the time intervals 30 min, 1 h, 2 h, 4 h and after overnight incubation (19 – 23 h). Denaturing loading buffer was added to the aliquots, followed by freezing in liquid nitrogen to quench the reactions. The samples were analyzed on 20 % denaturing polyacrylamide gels immediately after thawing.

Rough platination kinetics of $tRNA^{Ala}$. Radiolabeled $tRNA^{Ala}$ (typically [RNA] ≈ 15 nM and with a total activity of 6 × 10^4 cpm per reaction) was denatured and renatured as described above for Mh^{Ala}. Aliquots of the resulting solution were added to solutions containing increasing concentrations of cisplatin (0, 0.025, 0.050, 0.10 and 0.15 mM) in platination buffer and the mixtures were incubated at 37 °C. Aliquots of the reaction mixture (5 µl each) were removed after the time intervals 30 min, 1 h, 2 h, 4 h, and overnight incubation. The platination was quenched by dilution of the aliquots in 5 µl RNA loading buffer, followed by freezing in liquid nitrogen. If needed, the aliquots were stored overnight at -20 °C prior to PAGE analysis (15 % denaturing gel).

Large scale platination of Mh^{Ala}. Purified, radiolabeled Mh^{Ala} (1.5 – 4.5 × 10^6 cpm) was dissolved in chloride free platination buffer. Prior to platination, the solution was heated to 90 °C for 2 min followed by slow cooling to room temperature. Two thirds of the material, 0.14 µM Mh^{Ala} (typically in a total volume of 100 µl), was then incubated with 0.10 mM cisplatin for 2 h. The remaining one third of the solution was used as the negative control and treated in the same manner as the solution containing cisplatin. After incubation, the reaction mixtures were concentrated either by use of Microcon YM-3 filterunits (Millipore/Amicon) or ethanol precipitation. The different platination products were separated on 15 % denaturing polyacrylamide gels. The product bands were visualised by luminoradiography, excised, eluted in 1 M NaOAc, pH 4.6, and precipitated using EtOH.

Large scale platination of $tRNA^{Ala}$. Purified, radiolabeled $tRNA^{Ala}$ (*ca.* 11 x 10^6 cpm) was dissolved in platination buffer and was then denatured and renatured prior to use, *vide supra*. Two thirds of the material, 0.4 µM $tRNA^{Ala}$, was incubated with 0.050 mM cisplatin at 37 °C (V_{tot} = 100 µl) for 3 h and one third of the material was kept as a negative control. The platination reactions were stopped by EtOH precipitation as described above. The products were gel separated (15 % PAGE, denaturing conditions) and visualized as described above for Mh^{Ala}.

Chemical and enzymatic structural probing of Mh^{Ala} and platinated Mh^{Ala}. Prior to enzymatic or chemical structural probing, end-labeled RNA (typically with an activity of 14 – 50 × 10^3 cpm) was dissolved in chloride free platination buffer and heated to 90 °C for 2 min, followed by cooling to room temperature. All cleavage reactions of RNA, except those with RNase T1 under denaturing conditions, were performed including 2.5 µg of carrier tRNA (bakers yeast, Sigma). Alkaline hydrolysis was performed in 30 mM NaOH and 0.30 mM EDTA for 90 sec at 110 °C. RNase T1 (MBI Fermentas) digestion was performed at pH 5.0 in sodium citrate buffer (denaturing conditions, see above) for 30 min with 0.5 units of enzyme at 55 °C, or in water with 0.05 units for 20 min at ambient temperature (native conditions). Nuclease S1 (MBI Fermentas) digestion was performed in water containing 4.5 mM $ZnSO_4$ (Sigma), with 1 or 5 units for 20

min at ambient temperature. The enzymatic reactions were stopped by addition of 7 µl denaturing loading buffer containing 120 mM EDTA, the alkaline hydrolysis reactions by addition of 5 µl denaturing loading buffer, followed by freezing in liquid nitrogen. The products were analyzed on 25 % denaturing polyacrylamide gels.

Chemical and enzymatic structural probing of $tRNA^{Ala}$ and platinated $tRNA^{Ala}$. Typically, *ca*. 60 pmol tRNA was 5'-labeled with [γ-^{32}P]ATP and purified according to the above described protocols for 5'-labeling. The resulting obtained activity was *ca.* 300 000 cpm/µl. The labeled tRNA was denatured and renatured in platination buffer by heating at 90 °C for 2 min followed by slow cooling to room temperature over ca 20 min prior to cleavage. All cleavage reactions of RNA, except those with RNase T1 under denaturing conditions, were performed including 2.5 µg of carrier tRNA (baker's yeast, Sigma) in total a reaction volume of 6 – 10 µl. The reaction mixtures were incubated for 20 min at 20 °C after addition of the appropriate buffers and enzymes. Alkaline hydrolysis was performed in the presence of 30 mM NaOH and 0.30 mM EDTA, typically for 90 sec at 110 °C, and with a tRNA-activity of *ca.* 150 000 cpm. RNase T1 (MBI Fermentas) digestion was performed at pH 5.0 in sodium citrate buffer (denaturing conditions, see above) for 30 min with 0.5 units of enzyme at 55 °C, or in water with 0.05 units for 20 min at ambient temperature (native conditions). Nuclease S1 (MBI Fermentas) digestion was performed in water containing 4.5 mM $ZnSO_4$ (Sigma), with 1 or 5 units for 20 min at ambient temperature. The enzymatic reactions were stopped by addition of 7 µl denaturing loading buffer containing 120 mM EDTA, the alkaline hydrolysis reactions by addition of 5 µl denaturing loading buffer, followed by freezing in liquid nitrogen. If needed, the stored overnight at -20 °C until further analysis. The cleavage was stopped by addition of 5 µl RNA loading buffer, and were frozen in liquid nitrogen. The samples were were stored overnight at -20 °C until further analysis if needed. The digestion products were analyzed on 20 % denaturing polyacrylamide gels and visualized as described above.