SUPPLEMENTARY INFORMATION

Amino-acid-linked platinum(II) analogues have altered specificity for RNA compared to cisplatin

Keshab Rijal, Xun Bao, and Christine S. Chow*

Materials and Methods

General

Potassium tetrachloroplatinate (K_2PtCl_4) was purchased from Strem Chemicals, Inc. (Newburyport, MA). L-Ornithine hydrochloride, L-lysine, L-arginine and L-aspartic acid were purchased from Alfa Aesar (Ward Hill, MA). All other chemicals and reagents were purchased from Sigma-Aldrich or Fisher, unless otherwise stated. RNase-free, distilled, deionized ddH_2O (Millipore water) was used for all experiments.

Synthesis of amino-acid-linked platinum(II) complexes

Amino-acid-linked platinum(II) complexes were synthesized as previously described in the literature.[1, 2] For the synthesis of [Pt(L-lysine)Cl_2] (Kplatin), K_2PtCl_4 (0.21 g, 0.50 mmol) was dissolved in 500 µL of ddH_2O and the solution was vortexed until the crystals were completely dissolved. The L-lysine monohydrochloride (0.18 g, 1.0 mmol) was dissolved in 500 µL of ddH_2O and vortexed. The pH of the solution was maintained between values of 5 and 6. The platinum and amino acid solutions were combined in a 10 mL round-bottom flask and stirred, leading to a reddish-colored solution. The solution was refluxed at 80 ºC for 2 h until the solution turned to a yellow color. The solution was cooled and filtered. The filtrate was kept in the dark at

RT until yellow crystals formed (1 to 3 days). The crystals were collected and washed with cold ddH_2O, ethanol, and ether. The yellowish-orange crystals, [Pt(Lys)Cl_2] (0.11 g, 46% yield), were dried under vacuum. The final product was confirmed by mass spectrometry. ESI-MS (H_2O/methanol, ES^+) calculated for $C_6H_{14}Cl_2N_2O_2Pt$, 411.01; found 433.99 ($M+Na^+$); NMR data match those of previous reports.[2]

The syntheses of [Pt(L-ornithine)Cl_2] (Oplatin) and [Pt(L-arginine)Cl_2] (Rplatin) were the same as for Kplatin, except that 0.091 g (0.5 mmol) of L-ornithine or 0.087 g (0.5 mmol) of L-arginine were used instead of L-lysine. The yield for Oplatin (yellow crystals) was 56%. ESI-MS (H_2O/methanol, ES^+) calculated for $C_5H_{12}Cl_2N_2O_2Pt$, 396.99; found 419.98 ($M+Na^+$). Rplatin was obtained as light yellow crystals with a 38% yield. ESI-MS (H_2O/methanol, ES^+) calculated for $C_6H_{14}Cl_2N_4O_2Pt$, 439.01; found 462.00 ($M+Na^+$). Similarly, the yield of Dplatin was 35% with ESI-MS (H_2O/methanol, ES^-) calculated for $C_4H_7Cl_2NO_4Pt$, 397.94; found 396.93 ($M-H^+$).

Isolation of 16S rRNA

The 70S ribosomes and 30S subunits were isolated from *E. coli* MRE600 by the sucrose gradient method as described previously.[3, 4] *E. coli* was grown to 0.6 OD_{600nm} and cooled on ice for 20 min, followed by centrifugation at 7,000 rpm for 15 min. The cell pellet was resuspended in buffer A (50 mM Tris·HCl, 100 mM NH_4Cl, 10 mM $MgCl_2$, and 1 mM EDTA). The cells were lysed on a French Press and centrifuged at 15,000 rpm for 20 min to remove cellular debris. About 2/3 of the supernatant was transferred to a new tube and centrifuged again. The supernatant was transferred to a new tube and the NH_4Cl concentration was increased to 200 mM. The ribosomes were pelleted in an ultracentrifuge at 42,000 rpm for 4 h. The supernatant was discarded and the pellet was washed with buffer B (50 mM Tris·HCl, 100 mM NH_4Cl, 10 mM $MgCl_2$). The 70S ribosome pellet was resuspended in buffer B by shaking overnight at 4 °C.

The ribosome concentration was determined spectrophotometrically. To obtain pure 70S ribosomes or individual 30S subunits, the crude ribosome mixture was layered in a 10 to 30% sucrose gradient in buffer B with 5 mM $MgCl_2$, and centrifuged in a swinging bucket rotor at 18,000 rpm for 16 h. Fractions corresponding to the 70S ribosomes, 50S subunits, and 30S subunits were pooled and the Mg^{2+} concentration was increased to 10 mM. Sucrose was removed by centrifugation in an ultracentrifuge at 42,000 rpm for 18 h, the pellet was dissolved in buffer E (10 mM HEPES pH 7.2, 30 mM NH_4Cl, and 10 mM $MgCl_2$), quickly frozen, and stored at -80 °C. The purity of the 30S subunits and 70S ribosomes was confirmed by agarose gel electrophoresis of the extracted rRNA(s). The 16S rRNA was isolated from 30S subunits by extraction three times with equal volumes of phenol (phenol: chloroform: isoamyl alcohol; 25:24:1), followed by extraction twice with chloroform in the presence of 6 mM EDTA. Finally, samples were ethanol precipitated with 0.1 volume 3.0 M sodium acetate, pH 5.3, and 2.5 volumes ethanol. The isolated 16S rRNA was renatured in 20 mM HEPES (pH 7.5) and 25 mM NaCl by heating to 90 °C for 2 min and slowly cooling to room temperature.

Platination reaction with 16S rRNA

All amino-acid-linked platinum(II) complexes were converted to the corresponding monoaquated forms by reaction with a 1:1 molar ratio of $AgNO_3$ in dimethyl formamide (DMF) as described previously.[5] Platinum-DMF complex stock solutions were stored at -20 °C for up to one week and diluted as required just prior to use. The platination reaction with 16S rRNA was performed with 50 μM monoaquated platinum(II) complex. Prior to platination, the 16S rRNA was renatured as described above. The reaction was carried out in 20 mM HEPES, pH 6.5, 20 mM K_2SO_4, and 10 mM $MgSO_4$ and incubated at 37 °C for 5 h. For reactions with 16S rRNA, the molar ratio was 1:20 (16S rRNA: complex) or 75:1 (nucleotides: complex). After the reaction

was quenched with 0.2 M NaCl, unreacted complex was removed by ethanol precipitation and the platinum coordination sites on 16S rRNA were mapped by primer extension analysis as described below.

Primer extension with 16S rRNA

Primer extension was carried out with 2 pmol of 16S rRNA after reaction with platinum(II) analogues. Next, 2 μL of the appropriate 5'-^{32}P end-labeled primer (200,000 cpm) and 2 μL of 1 pmol/μL reacted 16S rRNA were mixed with 6 μL ddH_2O, heated to 90 °C, and slowly cooled to room temperature for hybridization. The extension mix was prepared by mixing 1× reverse transcriptase buffer (Promega), 2 mM $MgCl_2$, and 0.5 mM of each dNTPs (dATP, dCTP, dGTP, and dTTP), and 1 μL reverse transcriptase (Promega) in a 20 μL final volume. For sequencing, 0.5 mM of each dNTP and 2.5 mM of the appropriate ddNTPs were used. Unmodified RNA (no platinum treatment) was used as a template for sequencing and for control lanes to monitor artifacts of the reverse transcriptase. Extension of the primer was carried out at 42 °C for 1 h and terminated by heating at 80 °C for 15 min. To each sample, 2 μL of loading buffer was added and the products were denatured by heating to 95 °C for 2 min followed by rapid cooling on ice. To the sequencing gel (0.4 mm thick, 8% polyacrylamide, acrylamide: bisacrylamide 19:1, 1× TBE, 7 M urea), 60,000 cpm per sample were loaded. The gel was run at 1500 V for approximately 2 h until the bromophenol blue dye migrated off the gel. The products were quantified on a Typhoon 9200 using Image Quant^{TM} Software (GE Healthcare).

Gel quantification

The intensity of the each band was quantified and normalized by calculating the percent intensity at the corresponding stop with respect to intensity of the total lane. For each band, the

background was subtracted by calculating the percent intensity of the control lane at the same position. Single-hit conditions were desired such that all reactive sites within a 100-nucleotide region could be observed on the gel. Accumulation of higher read-through transcripts at the top of the gels made the percent intensity relatively low (≤5%) at the corresponding bands after normalization (**Figure S1**).

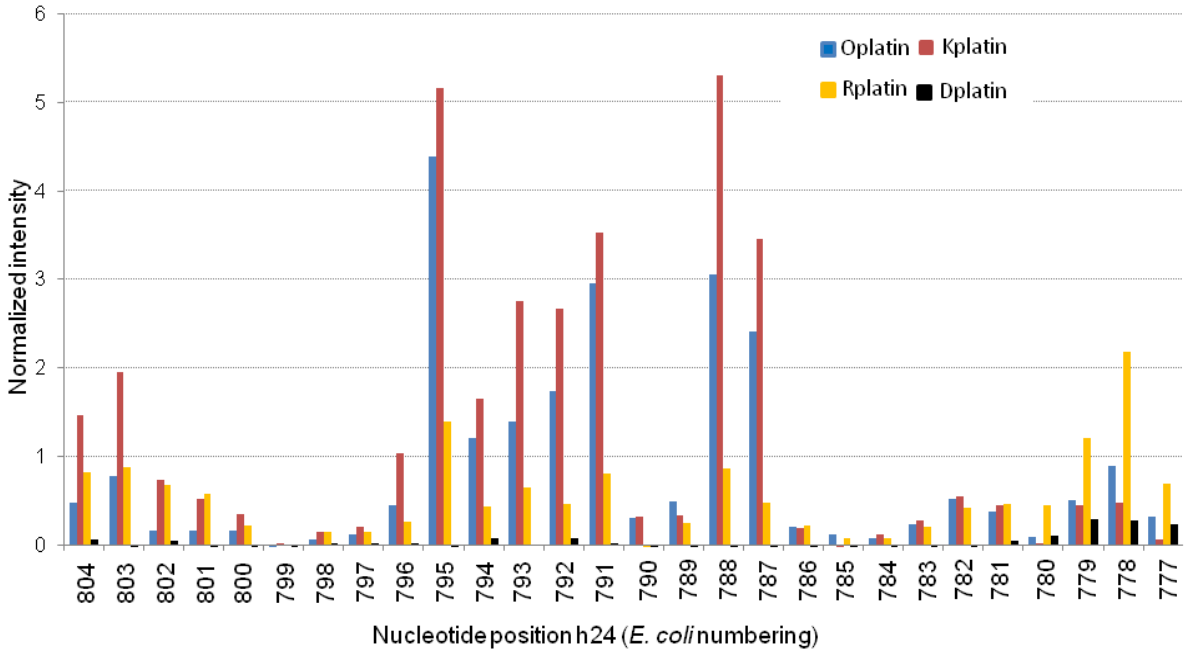

Figure S1. Quantification of the autoradiogram (**Figure 2**) for platination reactions on 16S rRNA with various cisplatin analogues, Oplatin (blue), Kplatin (red), Rplatin (orange), and Dplatin (black). Normalized intensities for reactive sites were determined by calculating the percent intensity at the corresponding stop site with respect to the total intensity of the lane minus the intensities of non-specific primer-extension stop sites in the control lane.

Platination reaction with nucleosides

Single RNA nucleosides, guanosine and adenosine, were purchased from Sigma-Aldrich. Each nucleoside was dissolved in ddH_2O at room temperature to make a stock solution. The concentration of each solution was determined by UV-visible spectroscopy and Beer's Law with

the following extinction coefficients: 14,900 $M^{-1}cm^{-1}$ (260 nm, pH 6) for adenosine and 13,600 $M^{-1}cm^{-1}$ (253 nm, pH 6) for guanosine.[6] Phosphate buffer (pH 6, 100 mM) was used in the dilution step. The difference in UV absorbance between phosphate buffer and water was less than 0.5%. Dilutions were carried out so that the UV readings were in the range of 0.1 to 1 OD.[2] All samples were freshly prepared.

High performance liquid chromatography (HPLC)

Samples were analyzed by reverse-phase HPLC with a Supelco Discovery C18 column (5 μm particle diameter; 4.6 mm i.d. × 250 mm, Sigma-Aldrich, St. Louis, MO, USA) on a Waters 600 LC with a 717 autosampler (Waters, Milford, MA) and UV-detector. The buffer contained 40 mM NH_4OAc, pH 6.5, and 40% acetonitrile was used as eluent. The column was equilibrated with 38 mM NH_4OAc and 2% (v/v) acetonitrile, and then the percentage of acetonitrile increased over a 40 min gradient (2 to 40% over 40 mins). The peaks were quantified with Empower software (**Figure S2**).

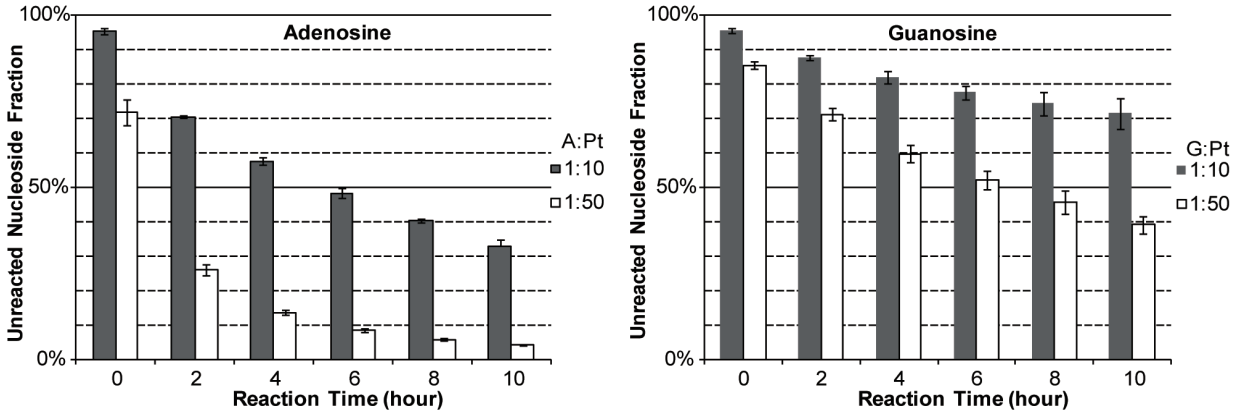

Figure S2. HPLC (C18) analysis of reactions between excess monoaquated Oplatin with single nucleosides (1:10 or 1:50 ratio of nucleoside: platinum) (unreacted adenosine (left panel); unreacted guanosine (right panel)).

References:

1. C. J. Ziegler, K. E. Sandman, C. H. Liang and S. J. Lippard, *J Biol Inorg Chem*, 1999, **4**, 402-411.

2. L. Dalla Via, O. Gia, S. M. Magno, A. Dolmella, D. Marton and V. Di Noto, *Inorg Chim Acta*, 2006, **359**, 4197-4206.

3. G. Spedding, *Ribosomes and Protein Synthesis: A Practical Approach"*, Oxford University Press, 1990.

4. D. Moazed, S. Stern and H. F. Noller, *J Mol Biol*, 1986, **187**, 399-416.

5. M. Hägerlöf, P. Papsai, C. S. Chow and S. K. Elmroth, *J Biol Inorg Chem*, 2006, **11**, 974-990.

6. R. L. Lundblad and F. Macdonald, *Handbook of Biochemistry and Molecular Biology*, CRC Press, Boca Raton, 2010.