SUPPORTING INFORMATION

to

Platinum(terpyridine) Complexes with *N*-Heterocyclic Carbene Co-Ligands: High Antiproliferative Activity and Low Toxicity *in vivo*

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Experimental

Materials and methods

All reagents were purchased from commercial suppliers and used without further purification. The terpyridine ligands 2,6-bis(2-pyridyl)-4[1*H*]-pyridone (**a**),[1] and 4'-phenyl-2,2':6',2''-terpyridine (**b**)[2] were prepared following literature methods. The pro-carbene 1,3-dimethylbenzimidazolium iodide was prepared following the procedure outlined by de Robillard and co-workers,[3] after which a counterion exchange was carried out using excess NH_4PF_6 in MeOH to prepare 1,3-dimethylbenzimidazolium hexafluorophosphate.

NMR spectra were recorded at 298.15 K on Bruker Avance AVIII 400 MHz NMR spectrometers [^{1}H (400.13 MHz); ^{13}C{^{1}H} (100.62 MHz); ^{31}P{^{1}H} (161.95 MHz) or ^{1}H (399.89 MHz); ^{13}C{^{1}H} (100.55 MHz); ^{31}P{^{1}H} (161.88 MHz)]. Mass spectra were recorded on a Bruker micrOTOF-Q II ESI-MS in positive ion mode. Elemental analyses were conducted using a vario El cube (Elementar Analysensysteme GmbH, Hanau, Germany).

Preparation of *cis*-[dichlorido(1,3-dimethylbenzimidazol-2-ylidene)(dimethylsulfoxide)platinum(II)] 1

cis-$[PtCl_2(DMSO)_2]$ (0.26 g, 0.60 mmol) and 1,3-dimethylbenzimidazolium hexafluorophosphate (0.18 g, 0.61 mmol) were heated at 90 °C in DMSO (1.50 mL) in the presence of $NaHCO_3$ (0.062 g, 0.74 mmol). After 24 h, DCM was added, and the organic layer was washed with water and dried over Na_2SO_4. The volume of the solvent was reduced to ~2 mL and diethyl ether was added to yield an off-white solid. Yield: 0.27 g (91%). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 7.74–7.81 (m, 2H, H5 & H8), 7.39–7.47 (m, 2H, H6 & H7), 4.16 (s, 6H, CH_3), 2.54 (s, 6H, $(CH_3)_2SO$). ^{13}C{^{1}H} NMR (100.6 MHz, DMSO-d_6): δ (ppm) = 154.4 (C2), 133.6 (C4 & C9), 123.8 (C6 & C7), 111.4 (C5 & C8), 40.4 ($CH_{3(DMSO)}$), 34.3 (CH_3). MS (ESI$^+$): *m/z* 454.0309 $[M - Cl]^+$ (calcd. 454.0314); 472.0405 $[M - Cl + H_2O]^+$ (calcd. 472.0420); 511.9908

[M + Na]$^+$ (calc. 511.9900). Elemental analysis calcd. (%) for $C_{11}H_{16}Cl_2N_2OSPt$ (490.31 g/mol): C 26.95, H 3.29, N 5.71; Found C 27.29, H 3.44, N 6.07.

Synthesis of [(terpyridine) (1,3-dimethylbenzimidazol-2-ylidene)platinum(II)] (hexafluorophosphate)$_n$ complexes

[Pt(**a'**)(NHC)]$PF_6 \cdot 2H_2O$ (**2a'**)

Compound **1** (0.14 g, 0.29 mmol) was dissolved in acetonitrile (5 mL) and added to a solution of 2,6-bis(2-pyridyl)-4[1*H*]-pyridone **a** (0.076 g, 0.30 mmol) in acetonitrile (10 mL), which was degassed under N_2 for 15 min. The reaction mixture was refluxed for 3 d, after which the solvent was removed and the yellow solid was washed with DCM to remove any unreacted starting material. Yield: 0.16 g (81%). ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ (ppm) = 8.57 (d, $^3J_{HH}$ = 7.9 Hz, 2H, H6 & H6"); 8.47 (t, $^3J_{HH}$ = 8.0 Hz, 2H, H4 & H4"); 8.15-8.25 (m, 4H, H3' & H5' & H3 & H3"); 7.89-7.97 (m, 2H, H11 & H14); 7.65 (t, $^3J_{HH}$ = 6.6 Hz, 2H, H5 & H5"); 7.52-7.59 (m, 2H, H12 & H13); 4.25 (s, 6H, CH_3). The chloride salt (0.13 g, 0.19 mmol) was dissolved in MeOH, followed by the addition of NH_4PF_6 (0.32 g, 1.97 mmol) and stirred at room temperature for 1 h. Water was added and the yellow solid collected by suction filtration, washed with water, MeOH (min. volume) and diethyl ether. Yield: 0.13 g (87%). ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ (ppm) = 8.42 (d, $^3J_{HH}$ = 8.0 Hz, 2H, H6 & H6"); 8.34 (t, $^3J_{HH}$ = 8.0 Hz, 2H, H4 & H4"); 8.10 (d, $^3J_{HH}$ = 5.6 Hz, 2H, H3 & H3"); 7.87-7.93 (m, 2H, H11 & H14); 7.46-7.57 (m, 4H, H5 & H5" & H12 & H13); 7.29 (s, 2H, H3' & H5'); 4.22 (s, 6H, CH_3). ^{13}C{^{1}H} NMR (100.6 MHz, DMSO-*d*$_6$): δ (ppm) = 177.6 (CC=O); 168.1 ($C_{carbene}$); 161.6 (C2 & C2"); 154.9 (C3 & C3"); 152.8 (C2' & C6'); 142.1 (C4 & C4"); 135.4 (C10 & C15); 128.8 (C5 & C5"); 124.7 (C6 & C6"); 124.3 (C12 & C13); 115.3 (C3' & C5'); 112.3 (C11 & C14); 35.0 (CH_3). ^{31}P{^{1}H} NMR (161.8 MHz, DMSO-*d*$_6$): –144.2 (sept.). MS (ESI+): m/z 589.1305 [M – PF_6]$^+$ (calcd. 589.1312); 295.0710 [M + H – Cl – PF_6]$^{2+}$ (calcd. 295.0691). Elemental analysis calcd. (%) for $[C_{24}H_{20}N_5OPt]PF_6 \cdot 2H_2O$ (770.52 g/mol): C 37.41, H 3.14, N 9.09; Found C 37.61, H 3.14, N 9.16.

[Pt(**b**)(NHC)]$(PF_6)_2$ (**2b**)

Compound **1** (0.12 g, 0.25 mmol) was dissolved in acetonitrile (10 mL) and degassed for 15 min before 4'-phenyl-2,2':6',2''-terpyridine **b** (0.074 g, 0.24 mmol) was added. The reaction mixture was refluxed for 3 d, after which the solvent was removed and the solid suspended in MeOH. NH_4PF_6 (0.40 g, 2.46 mmol) was added and the reaction mixture allowed to stir at room temperature for 1 h. After the addition of water, the yellow solid was collected and washed with water, DCM (min. volume) and diethyl ether. Yield: 0.15 g (64%). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 9.19 (s, 2H, H3' & H5'); 8.97 (d, $^3J_{HH}$ = 8.3 Hz, 2H, H3 & H3"); 8.58 (t, $^3J_{HH}$ = 7.8 Hz, 2H, H4 & H4"); 8.20–8.34 (m, 4H, H6 & H6" & H2* & H5*); 7.91–7.98 (m, 2H, H11 & H14); 7.68–7.79 (m, 5H, H5 & H5" & H4* & H3* & H5*); 7.55–7.62 (m, 2H, H12 & H13); 4.26 (s, 6H, CH_3). ^{13}C{^{1}H} NMR (100.6 MHz, DMSO-d_6): δ (ppm) = 163.7 ($C_{carbene}$); 158.5 (C2 & C2"); 155.4 (C6 & C6"); 154.5 (C2' & C6'); 154.0 (C4'); 142.7 (C4 & C4"); 135.0 (C10 & C15); 134.9 (C1*); 131.7 (C4*); 130.1 (C3* & C5*); 129.5 (C5 & C5"); 128.1 (C2* & C6*); 126.4 (C3 & C3"); 124.1 (C12 & C13); 121.5 (C3' & C5'); 112.0 (C11 & C14); 34.7 (CH_3). ^{31}P{^{1}H} NMR (161.8 MHz, DMSO-d_6): –144.1 (sept.). MS (ESI+): m/z 325.0873 $[M - 2PF_6]^{2+}$ (calcd. 325.0875). Elemental analysis calcd. (%) for $[C_{30}H_{25}N_5Pt](PF_6)_2$ (940.56 g/mol): C 38.31, H 2.68, N 7.45; Found C 38.23, H 2.70, N 7.38.

Conversion of 2a' to 2a

Upon the addition of 1 equivalent of HCl to **2a'**, the carbonyl oxygen atom was protonated and this was reflected in the ^{1}H NMR spectrum (400 MHz, DMSO-d_6): δ (ppm) = 8.61 (d, $^3J_{HH}$ = 8.1 Hz, 2H, H6 & H6"); 8.47 (t, $^3J_{HH}$ = 7.7 Hz, 2H, H4 & H4"); 8.19 (d, $^3J_{HH}$ = 5.6 Hz, 2H, H3 & H3"); 8.11 (s, 2H, H3' & H5'); 7.89–7.95 (m, 2H, H11 & H14); 7.62–7.68 (m, 2H, H5 & H5"); 7.53–7.59 (m, 2H, H12 & H13); 4.23 (s, 6H, CH_3).

Sulforhodamine B cytotoxicity assay

HCT116, SW480 and NCI-H460 cells were supplied by ATCC, while SiHa cells were from Dr. David Cowan, Ontario Cancer Institute, Canada. The cells were grown in αMEM (Life Technologies) supplemented with 5% fetal calf serum (Moregate Biotech) at 37 °C in a humidified incubator with 5% CO_2. The cells were seeded at 750 (HCT116, NCI-H460), 4000 (SiHa) or 5000 (SW480) cells per well in 96-well plates

and left to settle for 24 h. The compounds were added to the plates in a series of 3-fold dilutions, containing a maximum of 0.5% DMSO at the highest concentration. The assay was terminated after 72 h by addition of 10% trichloroacetic acid (Merck Millipore) at 4 °C for 1 h. The cells were stained with 0.4% sulforhodamine B (Sigma-Aldrich) in 1% acetic acid for 30 min in the dark at room temperature and then washed with 1% acetic acid to remove unbound dye. The stain was dissolved in unbuffered Tris base (10 mM; Serva) for 30 min on a plate shaker in the dark and quantified on a BioTek EL808 microplate reader at an absorbance wavelength of 490 nm with 450 nm as the reference wavelength to determine the percentage of cell growth inhibition by determining the absorbance of each sample relative to a negative (no inhibitor) and a no-growth control (day 0). The IC_{50} values were calculated with SigmaPlot 12.5 using a three-parameter logistic sigmoidal dose–response curve between the calculated growth inhibition and the compound concentration. The presented IC_{50} values are the mean of at least 3 independent experiments, where 10 concentrations were tested in duplicate for each compound.

Cellular accumulation experiments

For the cell accumulation studies, HCT116 cells (4×10^5/well) were seeded into 6-well plates and allowed to settle for 24 h. The compounds (1 µM) were added for 24 h at 37 °C and under 5% CO_2 atmosphere before the medium was removed and the wells were washed twice with 1 mL of ice-cold PBS buffer. The cells were lysed with 500 µL of HNO_3 (68 %, Suprapure, Merck) for 1 h, after which 400 µL of the lysate was transferred to a 15 mL-falcon tube and diluted to a final volume of 8 mL with H_2O (18.2 MΩ, Millipore). Rh was added as the internal standard at a final concentration of 3 µg/L.

The samples were analysed using an Agilent 7700 ICP-MS with an ASX-500 autosampler (CETAC Technologies) in a Serie SuSi laminar flow hood (SPECTEC). The instrument was equipped with a MicroMist nebuliser and a Scott double pass spray chamber. The carrier gas flow rate was 1.10 L/min, the plasma gas flow rate was 15.0 L/min, RF Power was set to 1550 W, and the sample depth was 6.0 mm. The instrument was tuned for ^{7}Li, ^{89}Y and ^{205}Tl. When analysing cell samples, a blank and a 3 µg/L calibration standard were measured for every 12 samples as quality controls. The monitored isotopes were ^{194}Pt, ^{195}Pt and ^{103}Rh.

Pt calibration standards were prepared from a 1000 ± 5 µg/mL Pt standard solution (Inorganic Ventures), diluted with a matrix-matched solution with regard to HNO_3 and Rh concentration, producing calibration standards with concentrations of 0, 0.2, 0.5, 1, 3 and 5 µg/L.

LOD and LOQ. Calibration curves for Pt were constructed in concentrations ranging from 0–5 µg/L. The limit of detection (LOD) was defined as 3 times the random error in the y-direction ($S_{y/x}$) of the calibration curve divided by the slope of the calibration curve. The limit of quantification (LOQ) was defined as 10 times the random error in the y-direction ($S_{y/x}$) of the calibration curve divided by the slope of the calibration curve.

In vivo toxicity using zebrafish

Wildtype (strain AB) zebrafish (*Danio rerio*) embryos were used to determine the *in vivo* toxicity of **2b** compared to cisplatin and vehicle control (1% v/v DMSO in E3 medium). At 5 hours post-fertilisation (hpf), 15 healthy embryos per treatment group were treated with either DMSO, **2b** or cisplatin at a range of concentrations (0.5 µM, 1 µM, 4 µM, 8 µM, 16 µM and 32 µM) in E3 medium, and maintained at 28.5 °C in the dark. Survival was monitored using a stereomicroscope at 1, 2, 3, 4, and 5 days post-fertilisation (dpf) and dead embryos were removed. Embryos with an absent heartbeat were considered dead. At 5 dpf, embryos were examined for deformities, including delayed hatching/development, spinal deformities, yolk sac and pericardial oedema, and impaired swimming ability. Three independent replicates were performed.

All zebrafish work was approved by the University of Auckland Animal Ethics Committee (AEC22563). Zebrafish experiments and husbandry complied with the good practice guides set by the New Zealand National Ethics Advisory Committee.

DNA gel electrophoresis

pUC19 (2686 base pairs, Takara Bio) was transformed into DH5α cells, and positive transformants were selected on Ampicillin lysogeny broth (LB)-Agar plates. Colonies were grown in LB and the plasmid was isolated using a NucleoSpin Plasmid (mini-prep) isolation kit (Machery-Nagel) and quantified by UV-vis spectroscopy at 260 nm with a N60 NanoPhotometer (Implen) to be 135 nM (236 ng/µL). The incubated samples consisted of pUC19 (50 nM) with cisplatin or **2b** at r_b values of 0–2 (Pt : nucleotide ratio). The samples were incubated at 37 °C and mixed at 300 rpm for 18

h in the dark. To identify the type of interaction the plasmid may undergo with the compounds, linearised pUC19 plasmid was run side-by-side with the treated samples (data not shown). The 0.8% agarose gel was cast (Mini Sub-Cell GT Gel Caster, Bio-Rad) and a 30 lane comb (Bio-Rad) with agarose (1.50 mg, Fisher Scientific) and Tris-acetate (40 mM)-ethylenediaminetetraacetic acid (1 mM) buffer (TAE; 200 mL). A second set of gels was cast in the same manner except EtBr (ethidium bromide, Bio-rad, 20 µL) was added prior to polymerisation. The gel was placed into the buffer gel tank (wide Mini-Sub Cell GT Cell, Bio-Rad) along with running buffer (TAE). Controls and samples were then loaded and run at 60 V for ~2.5 h. After the electrophoresis, the gel with EtBr embedded was immediately imaged (Bio-Rad Gel Doc XR+). The gel containing no EtBr was stained in 0.5 µg/mL EtBr solution for 15 min and then de-stained in deionised water for 15 min as per the manufacturer's instructions. The freshly de-stained gel was imaged as the other gel.

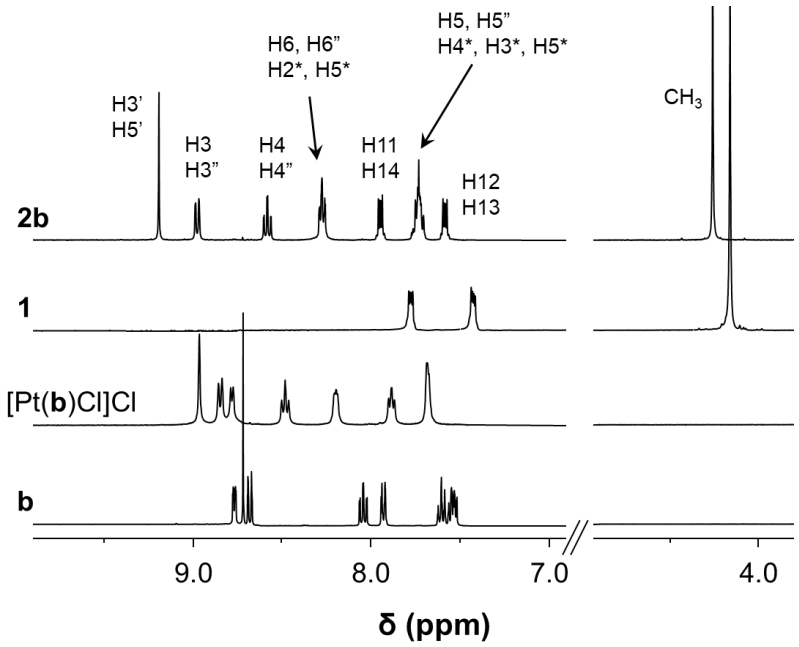

Figure S1. Comparison of the ^{1}H NMR spectra of **b**, [Pt(**b**)Cl]Cl, **1** and **2b** recorded in DMSO-d_6.

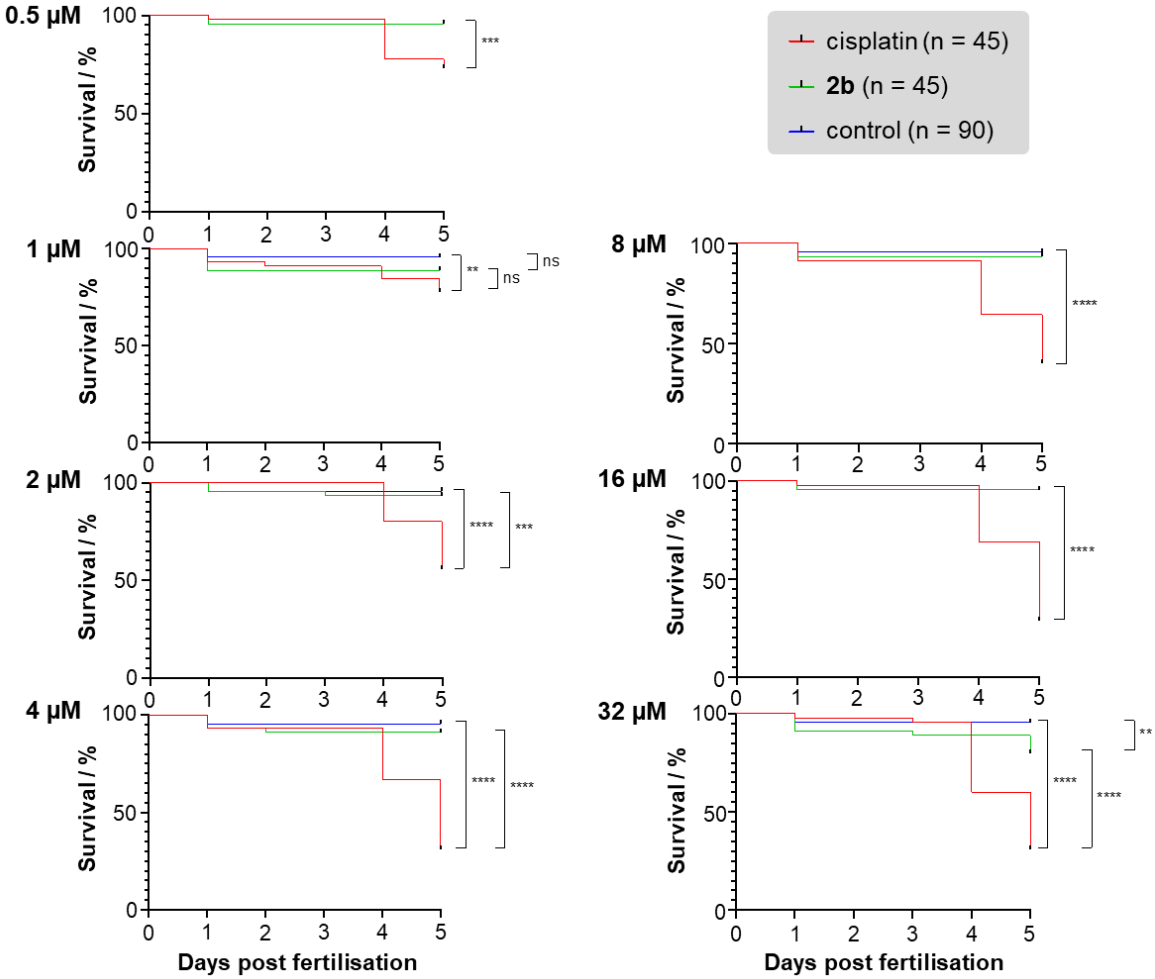

Figure S2. Kaplan-Meier Curves tracking the survival of zebrafish over three replicate experiments (n = 15 embryos per replicate; control: n = 90) when treated at 5 hours post-fertilisation with cisplatin or **2b** compared to control (1% v/v DMSO in E3 medium). Significance calculated by log-rank test: ns ($p > 0.05$), * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), and **** ($p \leq 0.0001$).

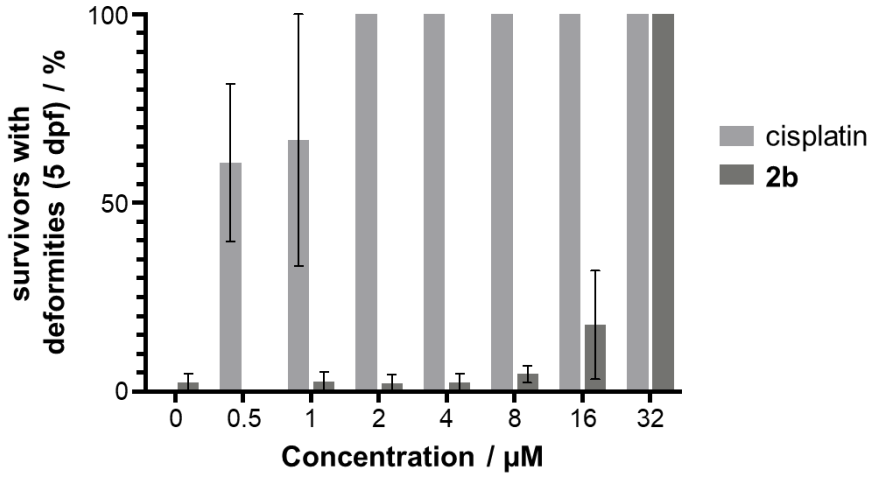

Figure S3. Zebrafish with deformities 5 days post-fertilisation (5 dpf) after treatment with cisplatin or **2b**. Delayed hatching, affected movement, oedemas, abnormal eye size, scoliosis, heart abnormalities, and reduced size were considered as deformities. Data is collected from three independent experiments, with error bars indicating the standard error about the mean.

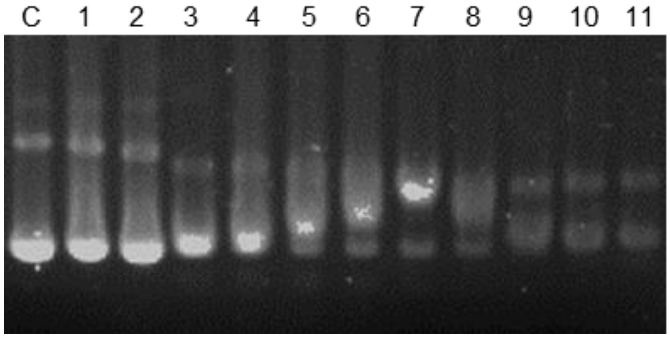

Figure S4. Agarose gel of pUC19 plasmid DNA after incubation with cisplatin for 18 h at 37 °C in the dark. The gel was stained with EtBr post-electrophoresis. Molar ratio of platinum per nucleotide (r_b) in each lane: C (control) – 0; 1 – 0.005; 2 – 0.010; 3 – 0.025; 4 – 0.050; 5 – 0.075; 6 – 0.10; 7 – 0.15; 8 – 0.25; 9 – 0.50; 10 – 0.75; 11 – 1.00. The upper bands are the open-circular plasmid and the bottom bands are the supercoiled forms.

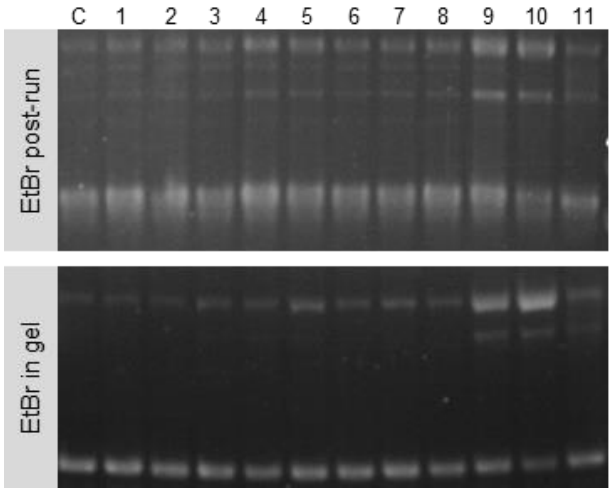

Figure S5. Agarose gel of pUC19 plasmid DNA after incubation with **b** for 18 h at 37 °C in the dark. The gel was run with EtBr or stained with EtBr post-electrophoresis. Molar ratio of platinum per nucleotide (r_b) in each lane: C (control) – 0; 1 – 0.005; 2 – 0.010; 3 – 0.025; 4 – 0.050; 5 – 0.075; 6 – 0.10; 7 – 0.15; 8 – 0.25; 9 – 0.50; 10 – 0.75; 11 – 1.00. The upper bands are the open-circular plasmid and the bottom bands are the supercoiled forms.

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2. Benavides, P. A.; Matias, T. A.; Arak, K., *Dalton Trans.* **2017,** *46*, 15567-15572.
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