A transcriptomic Analysis of the Activity and Mechanism of Action of a Ruthenium(II)-Based Antimicrobial That Induces Minimal Evolution of Pathogen Resistance

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Supplementary 1 – Chemistry methods

The ligand TPPHZ was synthesised through established procedure.[1]

S-1a. [Ru(3,4,7,8-tetramethyl-1,10-phenanthroline)$_2$Cl$_2$]$^{2+}$, [2]

$RuCl_3.3H_2O$ (1.14 g, 5.50 mmol), TMP (2.4 g, 10.16 mmol) and LiCl (1.47 g, 34.68 mmol) were heated for 8 h under reflux in DMF (19 mL). The reaction mixture was cooled to room temperature and acetone was added (100 mL). The solution was stored at 4 °C for 16 h forming a dark purple precipitate. The product was washed with water and ethanol and dried *in vacuo*. Mass = 2.07 g (3.21 mmol, 63.2 %) purple solid. MS m/z (%): 609.1 (62) [M −Cl]+, 637.1 (100) [M]+ 667.1 (44) [M + Na]+. Carbon monoxide displaced one of the chlorines.

$[M\text{-}3(PF_6)]^{3+}$. ^{1}H NMR (MeCN-d_6) δ (splitting integration): 2.1 (s, 48H), 7.8 (s, 4H), 7.9 (t, 8H), 8.2 (dd, 4H), 8.4 (s, 8H)

S-1b. [{Ru(TMP)$_2$}$_2$(tpphz)]$^{4+}$, [3]

$Ru(TMP)_2Cl_2]^{2+}$ (1.12 g, 1.73 mmol) and TPPHZ (0.260 g, 0.68 mmol) were added to a 1:1 solution of ethanol and water (80 mL). The solution was refluxed for 12 h under argon. After completion the reaction mixture was cooled to room temperature and stored at 4 °C for 16 h. The red solution was filtered, and ethanol removed by rotary evaporation. A saturating amount of NH_4PF_6 was added; this caused the formation of a dark red precipitate. The precipitate was collected *via* vacuum filtration, washed with water and recrystallised in acetonitrile by addition of diethyl ether. The product was dried *in vacuo* and purified on an alumina column, solvent system: 95 % MeCN, 3 % dH_2O and 2 % KNO3. Mass = 1.22 g (0.58 mmol, 85.7 % yield). MS; m/z (%): 911 (10) [M − 2(PF6)]2+, 559 (100) $[M\text{-}3(PF_6)]^{3+}$. ^{1}H NMR (MeCN-d_6) δ (splitting integration): 2.1 (s, 48H), 7.8 (s, 4H), 7.9 (t, 8H), 8.2 (dd, 4H), 8.4 (s, 8H), 9.9 (dd, 4H). ^{1}H NMR (Acetone-d6) δ (splitting integration): 2.1 (dt, 48H), 8.0 (m, 4H), 8.1 (s, 4H), 8.2 (s, 4H), 8.52 (d, 4H), 8.6 (s, 8 H), 10.1 (d, 4H). Elemental analysis [{Ru(3, 4, 7, 8-Tetramethyl-1,10-phenanthroline)$_2$}$_2$(tpphz)](PF6)$_4$·5.5H2O, $C88H87N14O5.5Ru_2P_4F_{24}$ Calculated: C; 47.93, H; 3.97, N: 8.89. Found C; 47.92, H; 3.83, N; 8.82. Accurate mass analysis: $C88H76N14[102Ru]_2^{4+}$ Calculated 383.1111. Found 383.1112**.**

S-1c. Anion metathesis

The hexafluorophosphate salt of each complex was dissolved in the minimum volume of acetone, and a saturated solution of tetrabutylammonium chloride in acetone added. The resultant precipitated chloride salt was collected by filtration, washed with cold acetone, and dried *in vacuo*.

Supplementary 2 – Supporting data

Table S-1 - Primer sequences designed for transcriptomic analysis. Annealing temperature and GC percentage stated for each sequence.

Gene		tm	gc%	Sequence
hcaT	Forward	59.4	47.62	cgctcggctatttcacatact
	Reverse	59.89	45.45	gacgctccagaaaggtagaaaa
idnT	Forward	60.61	45.45	agggattgctttactcctgctt
	Reverse	59.91	45.45	ttcgactgaggtgacgacttta
recN	Forward	58.77	40.91	acaactgaccatcagcaacttt
	Reverse	59.94	38.1	catgccgctatgaaaatcaat
recA	Forward	60.37	40.91	atggctatcgacgaaaacaaac
	Reverse	60.2	40.91	catgatggagcctttaccaaat
bhsA	Forward	59.43	36.36	aaaaacgtaaaaaccctcatcg
	Reverse	59.35	45	cgacttttttgttggccttct
ibpA	Forward	60.2	36.36	aactttgatttatccccgcttt
	Reverse	59.77	36.36	atagcaatgcggtaatggtttt
umuC	Forward	59.5	40.91	atgtttgccctctgtgatgtaa
	Reverse	59.99	40.91	caaccgtcattattcgatagca
yrbF	Forward	60.51	45.45	gtctgtggcgaatttagtcgat
	Reverse	59.85	50	agacggagtagcgtcgttttac
spy	Forward	59.98	50	ctgcactgtttgttgcctctac
	Reverse	60.37	47.62	gaccgaacttgcctttatggt
sdhA	Forward	59.88	45.45	ccagtcagagaatttgatgcag
	Reverse	60.51	57.14	cgggaagaccttagagagcag
ompF	Forward	61.2	54.55	gtgatcgtccctgctctgttag
	Reverse	59.97	45.45	ccttggagaaataatgcagacc

Table S-2 - Kinetic turbidimetric solubility plate readings – absorbance at 620 nm

Concentration µM	1^{4+} (1)			1^{4+} (2)			Nicardipine (1)			Nicardipine (2)		
0.2	0.130	0.123	0.149	0.132	0.125	0.152	0.435	0.504	0.540	0.499	0.531	0.514
2	0.039	0.054	0.061	0.053	0.045	0.040	0.171	0.195	0.186	0.195	0.192	0.175
4	0.012	0.012	0.012	0.012	0.011	0.013	0.002	0.009	0.018	0.004	0.021	0.009
20	0.005	0.007	0.006	0.006	0.006	0.006	0.000	0.000	0.000	0.000	0.000	0.000
40	0.001	0.002	0.002	0.002	0.002	0.001	0.000	0.000	0.000	0.000	0.000	0.000
100	0.000	0.001	0.001	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000
200	0.000	0.001	0.001	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Figure S-1 - Variation in colony morphology of *Escherichia coli* after exposure to 1^{4+}

Cultures exposed to 1^{4+} consistently show differing colony size and morphology when plated onto rich medium in the absence of the compound. Microscopy images were taken using a GX Microscope L2000A at 40x magnification. (A-C) Images of typical colonies of *Escherichia coli*. (D-E) Images of colonies of *Escherichia coli* after exposure to 1^{4+}.

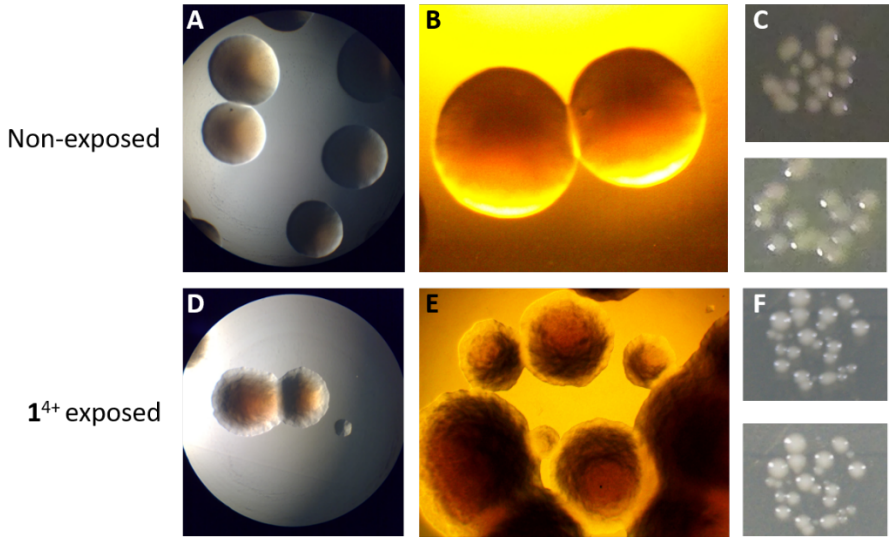

Figure S-2 – Minimal inhibitory and bactericidal concentrations for *Escherichia coli* BL21 and porin knockout mutants after exposure to 1^{4+}.

Strain	MIC (µM) ± SEM	MBC (µM) ± SEM
BL21 Gold (DE3)	0.91 ± 0.07	1.39 ± 0.14
ΔompA	1.39 ± 0.14	4.17 ± 0.89
ΔlamB	0.87 ± 0.07	1.74 ± 0.14
ΔompF	1.04 ± 0	1.48 ±0.07
ΔompC-ΔompF	1.04 ± 0	2.43 ± 0.38
ΔompC-ΔompF-ΔlamB	1.39 ± 0.14	2.60 ± 0.43

N = 3 ± SEM. Origin of strains[4].

Supplementary references

(1) Bolger, J.; Gourdon, A.; Ishow, E.; Launay, J.-P. Mononuclear and Binuclear Tetrapyrido [3, 2-a: 2', 3'-C: 3'', 2''-H: 2''', 3'''-"J] Phenazine (Tpphz) Ruthenium and Osmium Complexes. *Inorg. Chem.* **1996**, *35*, 2937−2944.

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