Biguanide Irdium(III) Complexes with Potent Antimicrobial Activity

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Supporting Information

Synthesis of Chelating Ligands L6-L10:

TolSul-Big-Tol (L6). The ligand was synthesized according to a published literature with following modifications:[1] A solution of 1-(o-tolyl)biguanide (360 mg, 1.88 mmol) in dichloromethane (150 mL) was placed in a round bottom flask. A solution of toluenesulfonyl chloride (300 mg, 1.58 mmol) in dichloromethane (50 mL) was added slowly via a dropping funnel, and the mixture stirred vigorously for 12 h. Solvent was removed on a rotary evaporator to give a white solid which was purified on a chromatography column (MeOH/DCM, 7/93(v/v)). Yield = 294 mg (54 %). ^{1}H NMR (400 MHz, MeOD-d$_4$): δ_H 2.23 (s, 3H), 2.40 (s, 3H), 7.19-7.23 (m, 3H), 7.28 (d, J = 6.8 Hz, 1H), 7.32 (d, J = 8.0 Hz, 2H), 7.76 (d, J = 8.2 Hz, 2H). ESI-MS: *Calc* for $[C_{16}H_{19}N_5O_2S + Na]^+$ 368.1 m/z, found: 368.1 m/z.

4-(BrCH$_2$)-PhSul-Big-Tol (L7). This ligand was obtained following the above method using 400 mg of 1-(o-tolyl)biguanide and 470 mg of 4-(bromomethyl)benzene sulfonyl chloride. Yield = 252 mg (34%). ^{1}H NMR (400 MHz, DMSO-d$_6$): δ_H 2.15 (s, 3H), 4.73 (s, 2H), 7.17 (d, J = 7.3 Hz, 2H), 7.24 (d, J = 7.0 Hz, 2H), 7.59 (d, J = 8.2 Hz, 2H), 7.80 (d, J = 8.4 Hz, 2H). ESI-MS: *Calc* for $[C_{16}H_{18}BrN_5O_2S + H]^+$ 446.0 *m/z*, found: 446.2 *m/z*.

4-F-PhSul-Big-Tol (L8). This ligand was obtained following the above method using 400 mg of 1-(o-tolyl)biguanide and 340 mg of 4-fluoro-benzenesulfonyl chloride. Yield = 390 mg (64%). ^{1}H NMR (400 MHz, MeOD-d$_4$): δ_H 2.24 (s, 3H), 7.19-7.26 (m, 5H), 7.28 (d, J = 6.6 Hz, 1H), 7.91-7.94 (m, 4H). ESI-MS: *Calc* for $[C_{15}H_{16}FN_5O_2S + Na]^+$ 372.0 *m/z*, found: 371.9 *m/z*.

4-NO$_2$-PhSul-Big-Tol (L9). This ligand was obtained following the above method using 360 mg of 1-(o-tolyl)biguanide and 300 mg of 4-nitro-benzenesulfonyl chloride. Yield = 240 mg (47%). ^{1}H NMR (400 MHz, DMSO-d$_6$): δ_H 2.17 (s, 3H), 7.01 (t, J = 6.8 Hz, 1H), 7.26 (dd, J = 7.6 Hz, 22 Hz, 3H), 8.05 (d, J = 8.4 Hz, 2H), 8.34 (d, J = 3.4 Hz, 2H). ESI-MS: *Calc* for $[C_{15}H_{16}N_6O_4S + H]^+$ 377.1 *m/z*, found: 376.9 *m/z*.

Dan-Big-Tol (L10). This ligand was obtained following the above method using 354 mg of 1-(o-tolyl)biguanide and 300 mg of dansyl chloride. Yield = 251 mg (53%). ^{1}H NMR (400 MHz, MeOD-d$_4$): δ_H 2.05 (s, 3H), 2.86 (s, 6H), 7.11-7.16 (m, 2H), 7.20-7.26 (m, 3H), 7.33 (t, J = 7.9 Hz, 1H), 7.54 (t, J = 8.3 Hz, 1H), 8.23 (d, J = 6.6 Hz, 1H), 8.41 (d, J = 8.7 Hz, 1H), 8.49 (d, J = 8.5 Hz, 1H). ESI-MS: *Calc* for $[C_{21}H_{24}N_6O_2S + H]^+$ 425.1 *m/z*, found: 425.0 *m/z*.

Summary of Protocols for Experiments Carried out by CO-ADD[2]

Preparation of Samples and Antibiotic Standards

Colistin and vancomycin were used as positive bacterial inhibitor standards for Gram-negative and Gram-positive bacteria, respectively. Fluconazole was used as a positive fungal inhibitor standard for *C. albicans* and *C. neoformans*. The antibiotics were provided in 4 concentrations, with 2 above and 2 below its MIC value, and plated into the first 8 wells of column 23 of the 384-well NBS plates. The quality control (QC) of the assays was determined by the antimicrobial controls and the Z'-factor (using positive and negative controls). Each plate was deemed to fulfil the quality criteria (pass QC), if the Z'-factor was above 0.4, and the antimicrobial standards showed full range of activity, with full growth inhibition at their highest concentration, and no growth inhibition at their lowest concentration.

Antibacterial Assays

All bacteria were cultured in Cation-adjusted Mueller Hinton Broth (CAMHB) at 37 °C overnight. A sample of each culture was then diluted 40-fold in fresh broth and incubated at 37 °C for 1.5 - 3 h. The resultant mid-log phase cultures were diluted (CFU/mL measured by OD600), then added to each well of the compound containing plates, giving a cell density of 5 $\times 10^5$ CFU/mL and a total volume of 50 μL. All the plates were covered and incubated at 37 °C for 18 h without shaking. Inhibition of bacterial growth was determined measuring absorbance at 600 nm (OD_{600}), using a Tecan M1000 Pro monochromator plate reader. The percentage of growth inhibition was calculated for each well, using the negative control (media only) and positive control (bacteria without inhibitors) on the same plate as references. The significance of the inhibition values was determined by modified Z-scores, calculated using the median and MAD of the samples (no controls) on the same plate. Samples with inhibition value above 80% and Z-Score above 2.5 for either replicate (n = 2 on different plates) were classed as actives. Samples with inhibition values between 50 - 80% and Z-Score above 2.5 for either replicate (n = 2 on different plates) were classed as partial actives.

Antifungal Assays

Fungi strains were cultured for 3 days on Yeast Extract-Peptone Dextrose (YPD) agar at 30 °C. A yeast suspension of 1×10^6 to 5×10^6 CFU/mL (as determined by OD_{530}) was prepared

from five colonies. The suspension was subsequently diluted and added to each well of the compound-containing plates giving a final cell density of fungi suspension of 2.5×10^3 CFU/mL and a total volume of 50 μL. All plates were covered and incubated at 35 °C for 24 h without shaking. Growth inhibition of *C. albicans* was determined measuring absorbance at 530 nm (OD_{530}), while the growth inhibition of *C. neoformans* was determined measuring the difference in absorbance between 600 and 570 nm ($OD_{600-570}$), after the addition of resazurin (0.001% final concentration) and incubation at 35 °C for additional 2 h. The absorbance was measured using a Biotek Synergy HTX plate reader. The percentage of growth inhibition was calculated for each well, using the negative control (media only) and positive control (fungi without inhibitors) on the same plate. The significance of the inhibition values was determined by modified Z-scores, calculated using the median and MAD of the samples (no controls) on the same plate. Samples with inhibition value above 80% and Z-Score above 2.5 for either replicate (n = 2 on different plates) were classed as actives. Samples with inhibition values between 50 - 80% and Z-Score above 2.5 for either replicate (n = 2 on different plates) were classed as partial actives.

Cytotoxicity Assays

Growth inhibitions of *HEK-293* and *RBC* cells were determined measuring fluorescence at ex: 530/10 nm and em: 590/10 nm (F560/590), after the addition of resazurin (25 μg/mL final concentration) and incubation at 37 °C and 5% CO_2, for additional 3 h. The fluorescence was measured using a Tecan M1000 Pro monochromator plate reader. The percentage of growth inhibition was calculated for each well, using the Negative Control (media only) and Positive Control (cell culture without inhibitors) on the same plate.

CC_{50} (Concentration at 50% Cytotoxicity against *HEK-293*) were calculated by curve fitting the inhibition values vs. log (concentration) using Sigmoidal dose-response function, with variable values for bottom, top and slope. The curve fitting is implemented using Pipeline Pilot's dose-response component (giving similar results to similar tools such as GraphPad's Prism and IDBS's XlFit). Any value with > indicates a sample with no activity (low DMax value) or samples with CC_{50} values above the maximum tested concentration (higher DMax value).

HC_{50} (Concentration at 50% haemolytic activity, human red blood cells) were calculated by curve fitting the inhibition values vs. log (concentration) using Sigmoidal dose-response

function, with variable values for bottom, top and slope. The curve fitting is implemented using Pipeline Pilot's dose-response component (giving similar results to similar tools such as GraphPad's Prism and IDBS's XlFit).

Cytopathic Effect. *HaCat* keratinocyte cells were cultured in freshly prepared growth media (including 10 mL of Dulbecco's Modified Eagle's medium (DMEM) supplemented with 3.7 g/L sodium bicarbonate, and 10% heat inactivated foetal bovine serum. *HaCaT* cells were incubated overnight in 96-well plates at a concentration of 1×10^4 cells per well, to allow cell adhesion. Cells were treated with concentration range of 0-256 µg mL^{-1} for complexes **4-7**, **11**, **12** and **14** in 200 µL of growth medium at 37 ºC with 5% CO_2. The cytopathic effect was checked every hour under microscopy.

Table S1. Crystallographic Data for Complexes 1 and 4

	1	4
Formula	$C_{14}H_{26}Cl_2IrN_5$	$C_{29}H_{32}ClIrN_5$
FW	527.50	683.64
Temp (K)	150(2)	150(2)
Crystal system	monoclinic	triclinic
Space group	$P2_1/c$	P-1
a (Å)	13.09904(18)	9.80400(17)
b (Å)	7.96973(11)	15.8991(2)
c (Å)	17.6195(2)	18.4457(3)
α (°)	90	82.7271(13)
β (°)	92.2718(14)	86.7456(14)
γ (°)	90	72.2390(14)
Volume ($Å^3$)	1837.95(4)	2715.64(8)
Z	4	4
Dcalc(mg/cm^3)	1.906	1.672
$\mu(mm^{-1})$	7.559	5.043
F(000)	1024.0	1350.0
Crystal size (mm^3)	$0.35 \times 0.35 \times 0.08$ orange block	$0.5 \times 0.13 \times 0.038$ brown block
Reflections measured	29267	83915
Indep reflection	6136	18356
R1 [I>2σ(I)]	0.0258	0.0330
wR2 (all data)	0.0903	0.0751
CCDC deposit no	1846267	1846268

Table S2. Hydrogen Bond Lengths (Å) and Angles (º) for Complex 1

D-H	H...A	D...A	<(DHA)	
0.88	2.56	3.344(3)	148.8	N4-H4...Cl2_$1
0.88	2.35	3.189(4)	158.9	N6-H6B...Cl2_$1
0.88	2.58	3.361(3)	148.0	N5-H5...Cl2
0.88	2.46	3.268(4)	152.4	N6-H6A...Cl2

Table S3. Hydrogen Bond Lengths (Å) and Angles (º) for Complex 4

D-H	H...A	D...A	<(DHA)	
0.88	2.43	3.273(3)	161.8	N107-H10A_b...Cl1
0.88	2.54	3.375(3)	157.8	N108-H108...Cl1
0.88	2.00	2.838(9)	157.9	N110-H11A...O01_$1b
0.88	2.49	3.294(4)	152.7	N110-H11B...Cl2_$2
0.88	2.41	3.243(3)	158.4	N207-H207...Cl1
0.88	2.46	3.303(3)	161.2	N208-H208...Cl1
0.88	2.39	3.218(3)	157.1	N210-H21A...Cl2

Table S4. Antimicrobial Activity of Ir^{III} Complexes 1-14 as MICs (µg/mL; µM in brackets), and HPLC retention times

Comp.	MRSA[a]	E. coli	K. pneumoniae	P. aeruginosa	A. baumanii	C. albicans	C. neoformans	R-T (min)[c]
1	32 (59.7)	>32 (>59.7)	>32 (>59.7)	>32 (>59.7)	>32 (>59.7)	>32 (>59.7)	>32 (>59.7)	13.0±1.4
2	16 (27.2)	>32 (>54.3)	>32 (>54.3)	>32 (>54.3)	>32 (>54.3)	>32 (>54.3)	>32 (>54.3)	14.4±0.6
3	4 (6.3)	16 (25)	>32 (>50)	>32 (>50)	32 (>50)	4 (6.3)	4 (6.3)	17.8±0.3
4	1 (1.5)	4 (5.8)	32 (46.6)	32 (46.6)	4 (5.8)	1 (1.5)	0.25 (0.4)	25.2±1.1
5	1 (1.4)	8 (11.2)	>32 (>44.8)	>32 (>44.8)	4 (5.6)	1 (1.4)	0.25 (0.4)	23.4±0.3
6	1 (1.3)	4 (5.4)	16 (21.6)	32 (43.2)	4 (5.4)	1 (1.3)	0.25 (0.3)	22.6±0.3
7	1 (1.4)	4 (5.5)	32 (44)	>32 (>44)	4 (5.5)	1 (1.4)	0.5 (0.7)	22.5±0.3
8	1 (1.2)	8 (9.3)	8 (9.3)	>32 (>37.2)	4 (4.7)	1 (1.2)	0.5 (0.6)	20.93±0.02
9	1 (1.1)	8 (8.8)	16 (17.6)	>32 (>35)	16 (17.6)	1 (1.1)	0.5 (0.6)	20.95±0.02
10	1 (1.2)	>32 (>38)	>32 (>38)	>32 (>38)	>32 (>38)	2 (2.4)	1 (1.2)	21.44±0.07
11	1 (1.1)	>32 (>35)	>32 (>35)	>32 (>35)	>32 (>35)	2 (2.2)	2 (2.2)	32.2±0.1
12	1 (1.2)	>32 (>38)	>32 (>38)	>32 (>38)	>32 (>38)	2 (2.4)	2 (2.4)	36.93±0.08
13	1 (1.2)	>32 (>38)	>32 (>38)	>32 (>38)	>32 (>38)	2 (2.4)	1 (1.2)	34.89±0.02
14	1 (1.1)	>32 (>35)	>32 (>35)	>32 (>35)	>32 (>35)	2 (2.2)	1 (1.1)	32.42±0.01
Col[b]	>32 (>27.7)	0.125 (0.11)	0.125 (0.11)	0.25 (0.22)	0.25 (0.22)	>32 (>27.7)	32 (27.7)	n. d.
Van	1 (0.7)	>32 (>22)	>32 (>22)	>32 (>22)	>32 (>22)	>32 (>22)	>32 (>22)	n. d.
Flu	>32 (>104)	>32 (>104)	>32 (>104)	>32 (>104)	>32 (>104)	0.125 (0.41)	8 (26)	n. d.

[a]Bacterial strains: MRSA: ATCC 43300; *E. coli*, ATCC 25922; MDR *K. pneumoniae*, ATCC 700603; *P. aeruginosa*, ATCC 27853; *A. baumannii*, ATCC 19606. Fungus strains: *C. albicans*, ATCC 90028; *C. neoformans*, ATCC 208821. [b]Model antibiotics: colistin (Col) and vancomycin (Van) as antibacterial agents; fluconazole (Flu) as antifungal agent; [c]R-T: retention time by RP-HPLC.

Table S5. Antibacterial Activity (MICs, µg/mL) of Complexes 4-10 against *S. aureus* and *S. pyogenes* under Anaerobic and Aerobic Conditions

Complex	*S. aureus*		*S. pyogenes*	
	anaerobic	aerobic	anaerobic	aerobic
4	2	2	0.5	0.25
5	2	2	0.5	0.25
6	2	2	0.5	0.125
7	2	1	0.5	0.125
8	2	0.5	1	0.25
9	2	0.5	1	0.25
10	2	0.5	1	0.25

Table S6. Selectivity Factors (SF) for Complexes 4-14 against *HEK-293* (CC_{50}/MBC) Cells and *RBC* (HC_{50}/MBC in brackets) versus Bactericidal Activity against Gram-positive bacteria (MBC) from Table S5

Complex	*S. aureus*	*B. subtilis*	*S. pyogenes*	*S. epidermidis*	*E. faecalis*
			SF : CC_{50}/MBC (HC_{50}/MBC)		
4	>16 (3)	>64 (12)	>32 (6)	>64 (12)	>4 (0.8)
5	>16 (6)	>64 (24)	>128 (42)	>64 (24)	>4 (1.5)
6	9 (10)	9 (11)	34 (42)	69 (84)	17 (0.7)
7	>32 (7)	>64 (29)	>256 (118)	>64 (29)	>8 (4)
8	24 (7)	24 (7)	95 (27)	24 (7)	0.7 (0.2)
9	4 (2)	17 (8)	68 (29)	34 (17)	1.1 (5)
10	n.d.	>2 (0.9)	>128 (57)	n.d.	n.d.
11	n.d.	>2 (>2)	>16 (>16)	n.d.	n.d.
12	n.d.	>1 (>1)	>4 (>4)	n.d.	n.d.
13	n.d.	>2 (>1)	>4 (3)	n.d.	n.d.
14	n.d.	>1 (>1)	>8 (>8)	n.d.	n.d.

Table S7. Dependence of Antibacterial Activity of Complexes 4-10 as MICs (µg/mL) on Time of Storage of Solutions of Complexes in CAMH Broth Medium at Various Temperatures (18 to +42 ºC) from 1-21 Days

Complex	Day 1			Day 4			Day 8	Day 21
	-18 ºC	18 ºC	42 ºC	-18 ºC	18 ºC	42 ºC	42 ºC	42 ºC
4	2	2	2	2	1	2	2	4
5	2	2	2	2	1	4	1	4
6	2	2	2	2	1	4	8	4
7	2	2	1	2	2	2	2	4
8	4	2	1	1	4	2	4	16
9	2	2	1	2	1	1	2	8
10	2	1	1	2	2	4	8	32

Table S8. *S. aureus* Biofilm Disruption (log cell number) by Complexes 4-9 at Various Concentrations (2-100 µg/mL)

Complex	Complex Concentration (µg/mL)							Negative Control
	100	50	30	20	10	5	2	
	log (cell number)							
4	3.33±0.35	3.73±0.05	7.40±0.02	8.10±0.19	8.84±0.06	8.92±0.08	9.07±0.11	
5	3.40±0.17	3.97±0.07	7.35±0.04	7.99±0.08	8.59±0.11	8.81±0.29	8.86±0.09	
6	3.91±0.12	4.09±0.09	7.44±0.03	8.16±0.11	8.89±0.11	8.88±0.03	8.96±0.10	
7	3.61±0.39	4.05±0.02	7.12±0.07	8.03±0.12	8.78±0.15	8.77±0.21	8.80±0.04	9.13±0.11
8	3.89±0.21	4.98±0.09	7.42±0.11	8.14±0.14	8.76±0.15	9.17±0.11	8.93±0.08	
9	3.85±0.14	6.11±0.07	7.86±0.09	8.22±0.09	8.74±0.24	8.99±0.09	8.90±0.05	

Table S9. Results of ANOVA for the Difference in Numbers of Bacteria after Treatment with Various Concentrations of Complexes and for Negative Controls (see Figure 4).[3]

	Df	Sum Sq	Mean Sq	F value	Pr (>F)
complex	5	23.9	4.8	44.10	<2e-16 ***
concentration	6	2779.6	463.3	4281.14	<2e-16 ***
complex: concentration	30	54.5	1.8	16.79	<2e-16 ***
residuals	84	9.1	0.1		

There are statistically significant effects of complex and concentration numbers of bacteria, and the effect of concentration differs between the complexes (Significant interaction).

Table S10. MBC/MIC Ratio of for Complexes 1-14 against Gram-positive Bacteria.

Complex	*S. aureus*	*B. subtilis*	*S. pyogenes*	*S. epidermidis*	*E. faecalis*
1	n.d.	n.d.	4	4	n.d.
2	2	2	2	2	4
3	1	1	1	4	4
4	1	1	4	2	8
5	1	1	1	1	8
6	1	2	4	1	32
7	2	2	1	1	4
8	2	2	1	2	64
9	8	4	1	1	16
10	>64	32	1	>128	n.d.
11	>64	32	2	>64	n.d.
12	>64	32	8	>128	n.d.
13	>64	32	16	>128	n.d.
14	>64	64	4	>128	n.d.

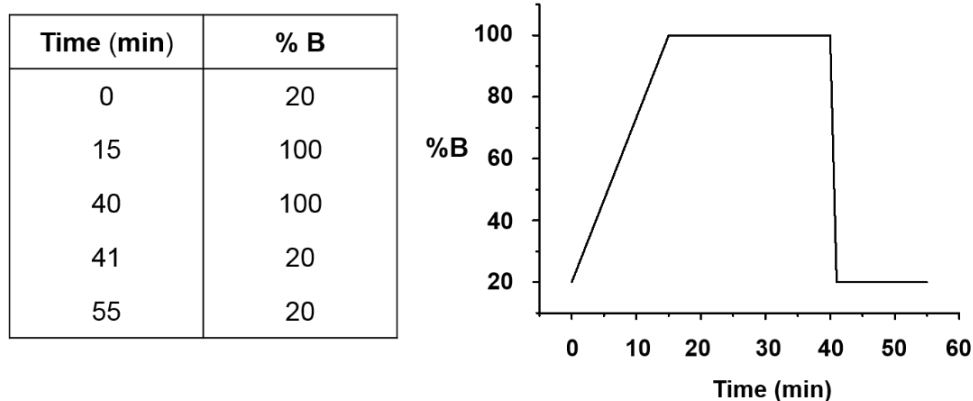| Time (min)	% B
0	20
15	100
40	100
41	20
55	20

Figure S1. Elution conditions for determination of relative hydrophobicity measurements by RP-HPLC. Solvent A: H_2O 50 mM NaCl; Solvent B: H_2O/CH_3CN 1:1 50 mM NaCl.
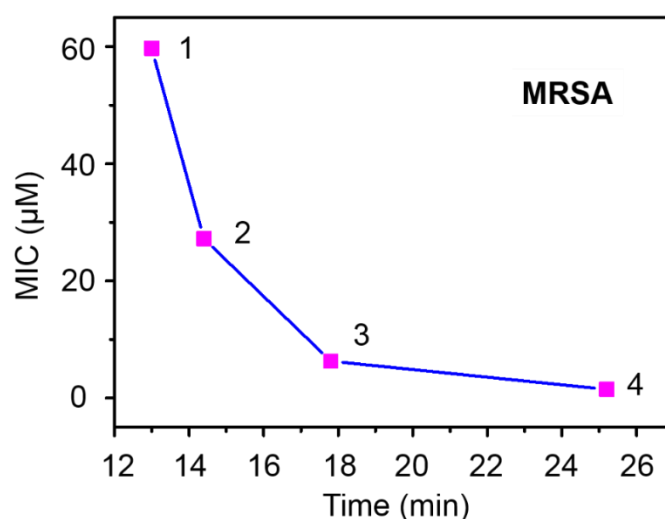

Figure S2. Plot of retention time (min) versus MICs (µM) for complexes **1-4** against MRSA. It is evident that the antibacterial activity of complexes increases with increase of the RP-HPLC retention times. The hydrophobicity of the substituent on the Cp^X ring increases from Me to Ph to biphenyl in complexes **1**, **2** and **3**, respectively, and complex **4** also has the biphenyl substituent (Chart 2).
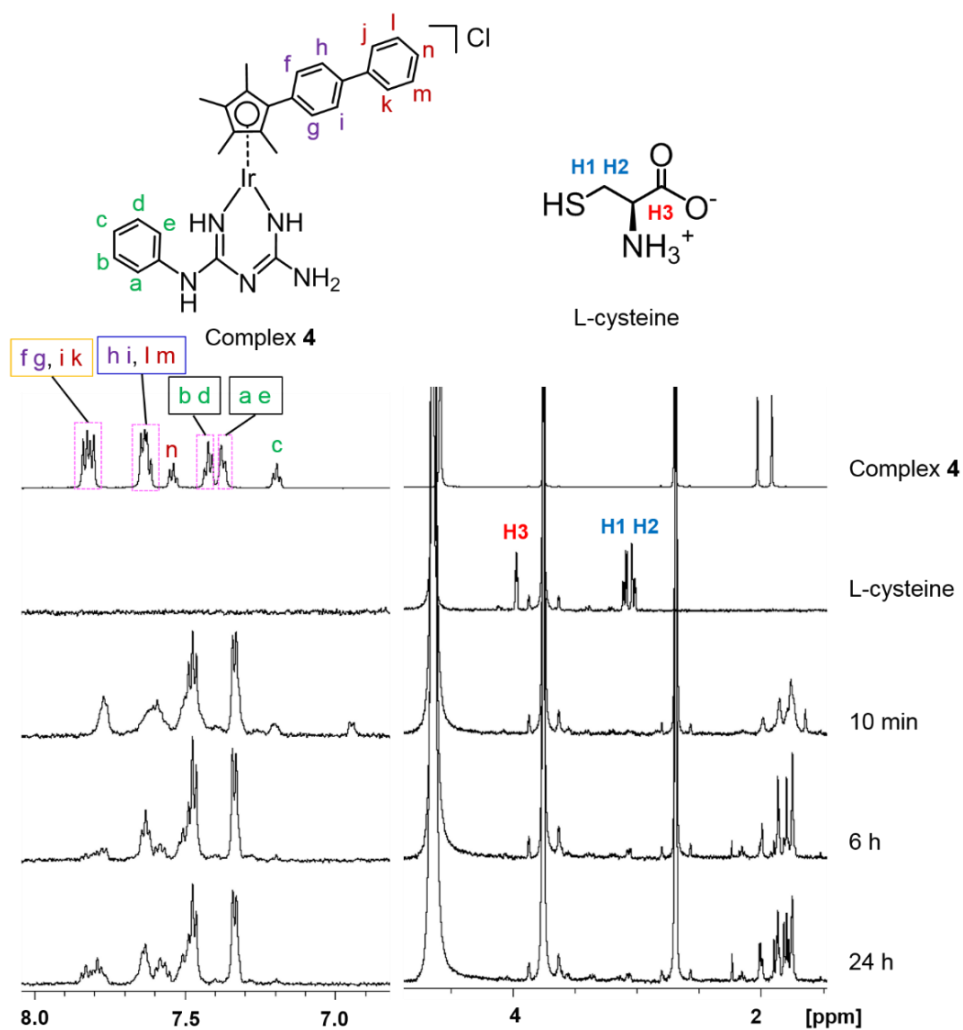
Figure S3. Reaction of complex **4** (2 mM in 20% DMSO-d_6/80% D_2O) with L-cysteine (1 mol equiv) monitored by ^1H NMR at 37 °C, pH* 7.1.
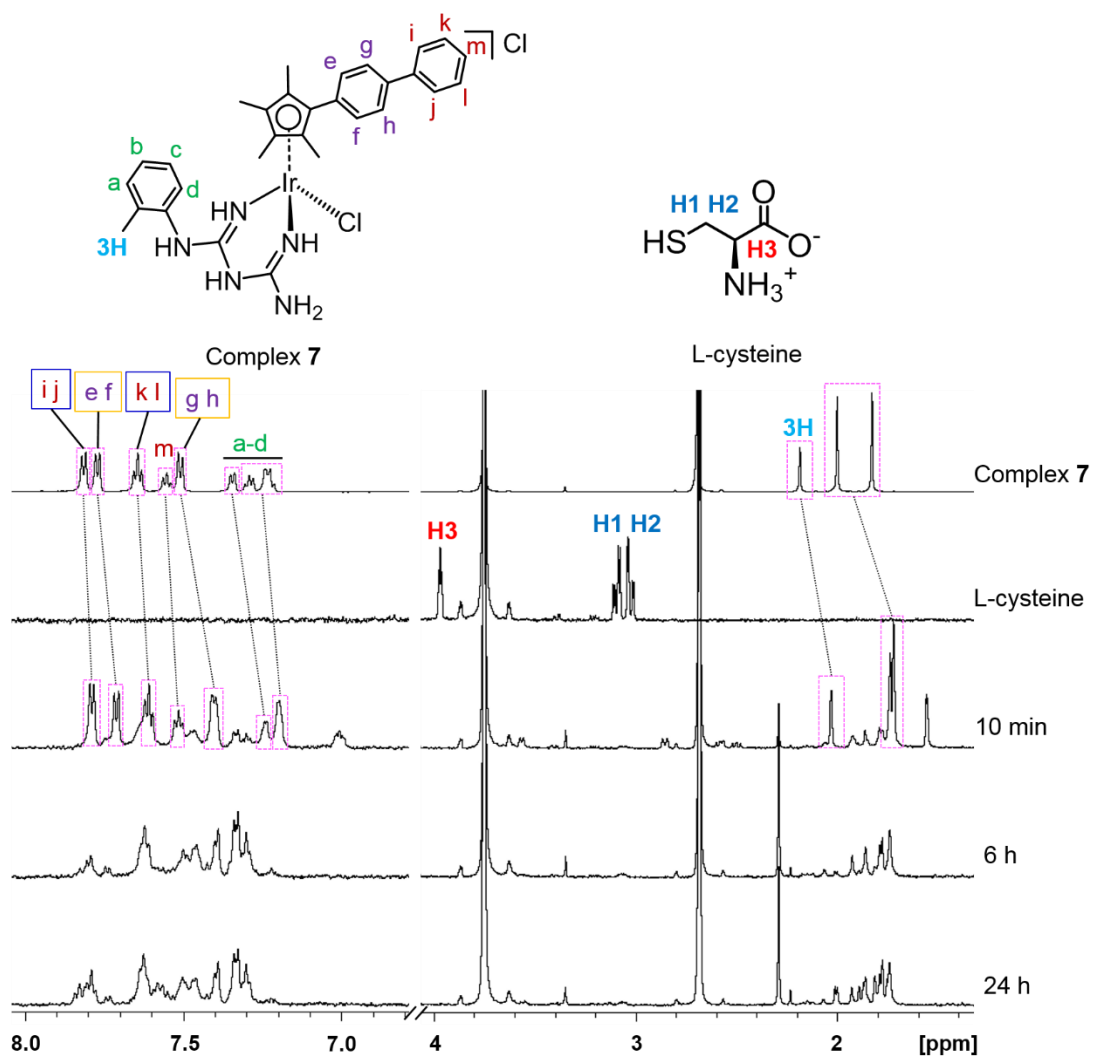
Figure S4. Reaction of complex **7** (2 mM in 20% DMSO-d_6/80% D_2O) with L-cysteine (1 mol equiv) monitored by ^1H NMR at 37 °C, pH* 7.1.
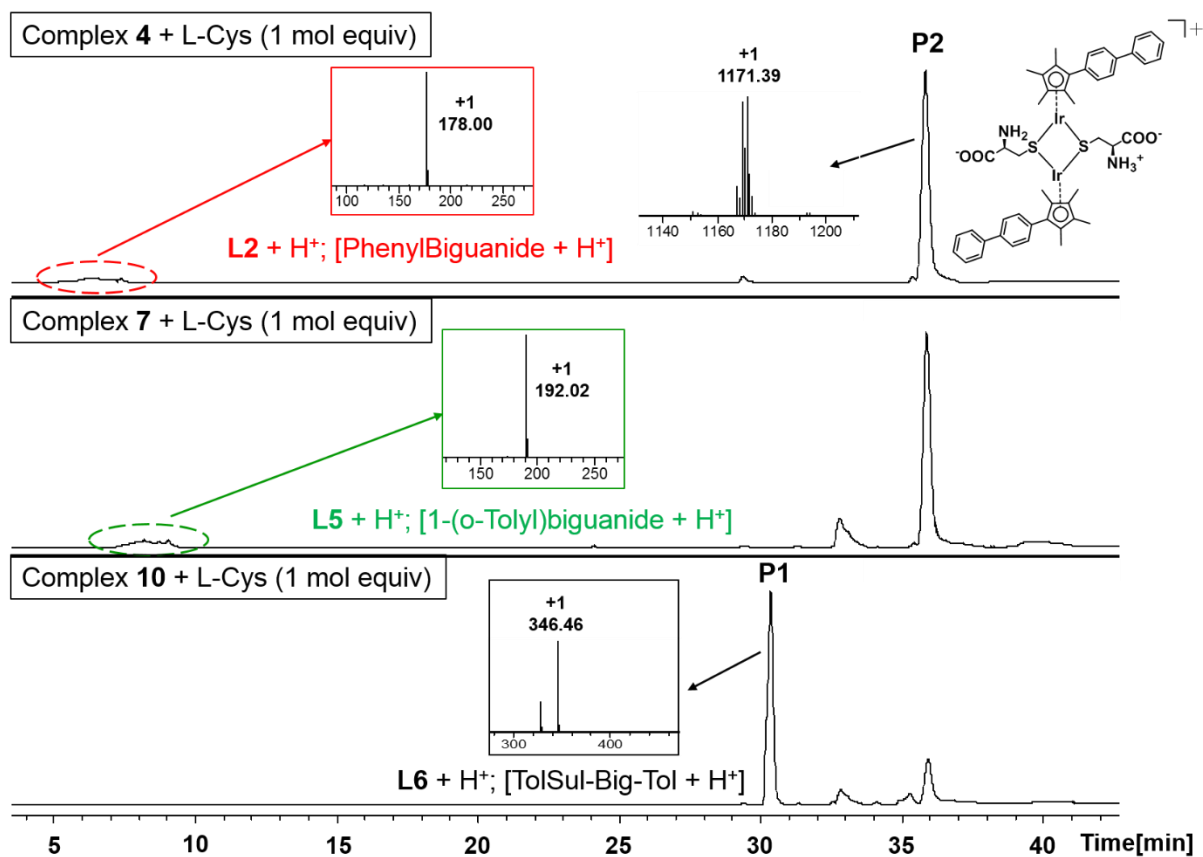
Figure S5. Reaction of complexes **4**, **7** and **10** (2 mM, MeOH/H$_2$O, 2:3 (v/v)) with L-cysteine (2 mM, H$_2$O, L-Cys) monitored by LC-MS after 24 h incubation at 37 °C, pH 7.1 ± 0.1. The dinuclear bridged complex [(Cp^{Xbiph})Ir(L-Cys)]$_2^{2+}$ (**P2**, detected by MS as [(Cp^{Xbiph})Ir(L-Cys)-H]$_2^+$) is the major product after 24 h with concomitant release of free biguanide ligand.

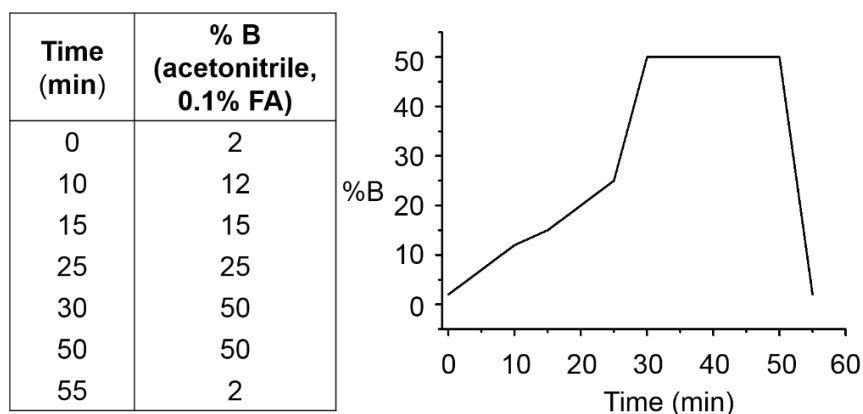| Time (min)	% B (acetonitrile, 0.1% FA)
0	2
10	12
15	15
25	25
30	50
50	50
55	2

Figure S6. LC-MS gradients for identification of [(Cp^{Xbiph})Ir(L-Cys)]$_2^{2+}$ from the reaction of complexes **4**, **7** and **10** with L-Cys using H$_2$O with 0.1% formic acid (v/v, FA) (Solvent A) and CH$_3$CN with 0.1% FA (v/v) (solvent B) as eluents. Column type: ZORBAX Eclipse XDB-C18, 9.4 × 250 mm, 5 µm.
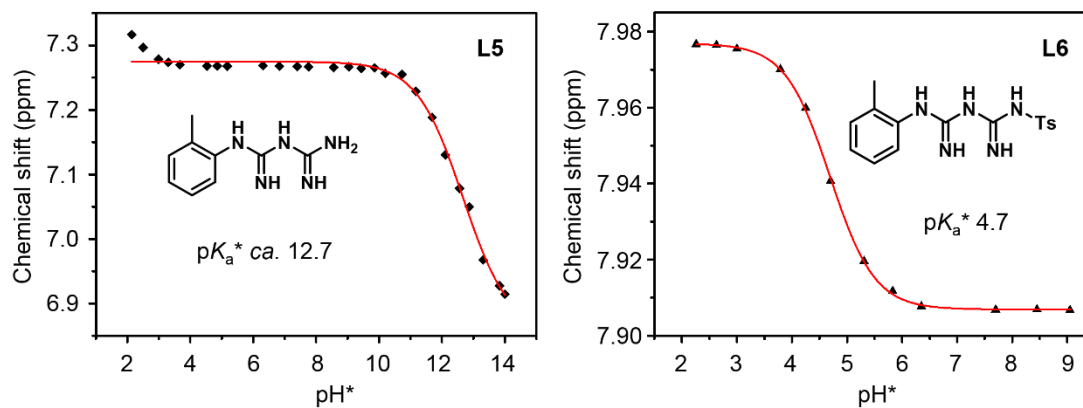
Figure S7. Dependence of chemical shift of low field ^{1}H NMR resonances (tolyl proton) of the ligands **L5** (1-(o-tolyl)Big) and **L6** (TolSul-Big-Tol) in 20% MeOD-d$_4$ /80% D$_2$O on pH* over the range 2-14. The solid lines are the best fits corresponding to pK_a* values of above 12 and 4.7 ± 0.1 for **L5** and **L6**, respectively.

References:

(1) Soldevila-Barreda, J. J.; Bruijnincx, P. C. A.; Habtemariam, A.; Clarkson, G. J.; Deeth, R. J.; Sadler, P. J. Improved catalytic activity of ruthenium−arene complexes in the reduction of NAD$^+$. *Organometallics* **2012**, *31*, 5958−5967.

(2) Blaskovich, M. A. T.; Zuegg, J.; Elliott, A. G.; Cooper, M. A. Helping chemists discover new antibiotics. *ACS Infect. Dis.* **2015**, *1*, 285−287.

(3) R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing. **2016** Vienna, Austria. URL https://www.R-project.org/.