Trinuclear Rhenium(I)-based Metallocages as Anticancer Agent Towards Human Cervical Cancer Cells

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Figure S13. Evaluation of antimetastatic effects of **Re-C1** and **Re-C2** on HeLa cells using scratch assay, untreated cells kept as negative control and cisplatin used as a positive control, (Scale bar: 100 μm). (B) Percentage of scratch closure at 0,6 and 12 h of treatment.

Table S2. IC^{50} values of rhenium metallocycles/metallocages towards human cancer cells.

References

Experimental Section

General Data. $Re_2(CO)_{10}$, 1,3,5-tris(bromomethyl)benzene, 1,3,5-tris(bromomethyl)-2,4,6-triethylbenzene, 2,3-diaminonaphthalene, formic acid, and NaH (55-60%) in mineral oil were purchased and used as received. THF and toluene were procured from commercial sources and used after purification by following standard methods. Ligands L^{tbim} and $L^{Et\text{-}tnim}$ were prepared by reported methods.[S1] ATR-IR spectra were recorded on a Nicolet iS5 IR spectrophotometer. ^{1}H NMR spectra were recorded on a Bruker AVANCEIII 500 instrument. ESI-MS spectra were recorded on Bruker-maXis mass spectrometer. Single crystal X−ray data of crystals of **Re-C1** and **Re-C2** were collected on a Rigaku Oxford diffractometer (λ(Mo Kα) = 0.71073 Å). The structures were solved by direct methods using SHELXS-97 (Sheldrick 2008) and refined using the SHELXL-2018/3 program (within the WinGX program package).[S2-S4] Non-H atoms were refined anisotropically (Table S1). The structure has been deposited with the Cambridge Crystallographic Data Centre (CCDC 2265703 and 2265704).

Synthesis of *fac*-[{Re(CO)$_3$(nim)}$_3$L^{tbim}] (Re-C1)

A mixture of $Re_2(CO)_{10}$ (100.0 mg, 0.153 mmol), H-nim (51.6 mg, 0.307 mmol), L^{tbim} (47.8 mg, 0.102 mmol) and toluene (10 mL) was kept in a Teflon flask which was then placed in a steel container. It was kept in an oven maintained at 160 °C for 48 h and then cooled to 25 °C. Dark brown crystalline product of **Re-C1** was filtered and washed with excess hexane. Yield: 46 % (126.3 mg). ^{1}H NMR (500 MHz, d_6-DMSO, ppm): 10.23 (s, 3H,H^A, nim), 8.66 (d, J = 8.40 Hz, 3H, H^b, L^{tbim}), 8.06 (t, J = 7.84 Hz, 3H, H^e, L^{tbim}), 7.92 (d, J = 8.20 Hz, 3H, H^c, L^{tbim}), 7.85 (t, J = 7.8 Hz, 3H, H^d, L^{tbim}), 7.06 (m, 6H, H^C, nim), 6.81 (m, 6H, H^D, nim), 6.60 (s, 6H, H^B, nim), 5.53 (s, 3H, H^f, L^{tbim}), 5.20 (s, 3H, H^a, L^{tbim}), and 4.31 (s, 6H, –CH_2–, L^{tbim}). ESI (HRMS). Calcd for $C_{72}H_{46}N_{12}O_9Re_3$ [M + H]$^+$: *m/z* 1781.2155. Found: *m/z* 1781.2206. ATR-IR (cm^{-1}): 2012 (C≡O) and 1883 (C≡O).

Synthesis of *fac*-[{Re(CO)$_3$(nim)}$_3$L$^{Et\text{-}tnim}$] (Re-C2)

A mixture of $Re_2(CO)_{10}$ (100.0 mg, 0.153 mmol), H-nim (51.67 mg, 0.307 mmol), $L^{Et\text{-}tnim}$ (71.90 mg, 0.102 mmol) and toluene (10 mL) was kept in a Teflon flask which was then placed in a steel container. It was kept in an oven maintained at 160 °C for 48 h and then cooled to 25 °C. Brown crystalline product of **Re-C2** was filtered and washed with excess hexane. Yield: 33% (102.1 mg).^{1}H-NMR (500 MHz, d_6-DMSO, ppm): 10.36 (s, 3H, H^A, nim), 9.26 (s, 3H, H^b, $L^{Et\text{-}tnim}$), 8.65 (m, 3H, H^c, $L^{Et\text{-}tnim}$), 8.50 (s, 3H, H^g, $L^{Et\text{-}tnim}$), 8.31(m, 3H, H^f, $L^{Et\text{-}tnim}$), 7.79 (m, 6H, H^{d,e}, $L^{Et\text{-}tnim}$), 6.92 (m, 6H, H^C, nim), 6.70 (s, 6H, H^B, nim), 6.61 (m, 6H, H^D, nim), 5.38 (s, 3H, H^a, $L^{Et\text{-}tnim}$), 4.55 (s, 6H, –CH_2–, $L^{Et\text{-}tnim}$), 0.18 (t, J = 7.5 Hz, 9H, H^i, $L^{Et\text{-}tnim}$), and -0.24 (m, 6H, H^h, $L^{Et\text{-}tnim}$). ESI (HRMS). Calcd for $C_{90}H_{64}N_{12}O_9Re_3$ [M + H]$^+$: *m/z* 2015.3564. Found: *m/z* 2015.3449. ATR-IR (cm^{-1}): 2010 (C≡O) and 1879 (C≡O).

Biological Studies

Materials and Methods

High glucose Dulbecco's Modified Eagle Medium (DMEM), phosphate buffer (pH 7.0), trypsin−EDTA, Dimethyl sulfoxide (DMSO), and US origin fetal bovine serum (FBS) were acquired from HiMedia Chemicals, Mumbai, India. 0.2 μm syringe filters were purchased from Sartorius (Carrigtwohill, Ireland). Milli-Q water was taken from the Millipore, Billerica, MA system.

Cell lines and maintenance

Mouse Embryonic fibroblast L929 (P^{58}) and human cervical cancer cell line HeLa (P^{79}) were obtained from the National Center for Cell Science (NCCS), Pune, India. The cell lines were cultured in high glucose DMEM medium supplemented with 10% (v/v) FBS, 1% L-glutamine, and 100 U/streptomycin/penicillin and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. All the biological experiments were performed at a final concentration of 0.1% DMSO.

Cell viability on HeLa

Cell viability of **Re-C1** and **Re-C2** were studied in the Human Cervical Cancer cell line HeLa using the MTT assay. HeLa (6×10^3 cells/well) cells were seeded in 96-well plates with DMEM as the growth medium (100 μL/well). After 24 h, DMEM media was replaced with fresh media containing 0 μM, 5 μM, 10 μM, 20 μM and 30 μM of **Re-C1**, **Re-C2**, cisplatin separately and kept for an incubation period of 24 h. After 24 h, MTT solution (5 mg/mL) was added followed by 3 h of incubation. Consequently, the supernatant of the MTT solution was removed carefully, and formazan crystals formed were dissolved by adding DMSO. The absorbance of dissolved formazan crystals was measured at 570 nm using a microplate reader (BIORAD Microplate Reader).

Live dead assay

HeLa cells (6×10^3 cells/well) were seeded in 96-well plates and incubated for 24 h allowing complete adherence. Then the cells were treated with the respective IC_{50} values of **Re-C1**, **Re-C2** and cisplatin (positive Control). After treatment, the plate consisting of cells was kept in CO_2 incubator for 24 h. FDA (stain live cells with green fluorescence) and PI (stain dead cells with red fluorescence) were used to see the live and dead cell population. FDA and PI were used in 20 μM concentrations. An Olympus fluorescence microscope was used to capture the images of live and dead cells.

Crystal violet assay

The crystal violet assay gives a quantitative idea of the amount of cell death by staining the live cells which were attached to the bottom of the plates.[S5-S6] Dead cells were washed using 1XPBS. The crystal violet assay was used to quantify the cell proliferation in the presence of **Re-C1**, **Re-C2**, and cisplatin by measuring the absorbance of cells stained with crystal violet at regular intervals. The metallocages showed high antiproliferation activity, almost reducing the cell growth by more than 50% at 24 h compared to the untreated cells. Further increasing the time to 72 h leads to ~75% decrease in proliferation activity. HeLa cells (1×10^6 cells/well) were seeded in a 96-well plate to perform the

crystal violet assay. Cells were incubated for 24 h for complete adherence to the bottom of the well. On the next day, cells were treated with respective IC_{50} values of **Re-C1**, **Re-C2**, and cisplatin (positive control) and kept for further 24 h incubation. After that, cells were stained with crystal violet (0.5%) and kept for 30 mins in dark condition. Then the cells were gently washed with 1XPBS, and methanol to wash out the extra crystal violet stain, and the plates were kept for 1 h for complete drying. Absorbance was recorded using a plate reader at 570 nm.

Scratch assay

HeLa cells were seeded in a 24-well plate at a density 5x105 cells/well. After 24 h of incubation, the wounds were created in the cells using a micro tip. The cells were then treated with respective IC_{50} value of **Re-C1**, **Re-C2** and cisplatin. The effect on migration was analyzed under the microscope at 0, 6, 12, and 24 h. FDA/PI stain was also used to observe the live and dead cells after 24 h of incubation. The % of scratch closure at the respective time points was calculated compared to 't_0'.

Ethidium Bromide (EtBr) displacement assay

For this experiment, EtBr (2 nM) was pre-incubated with CT-DNA (100 μM of base pair equivalent to 0.064 mg/ml) for 1 h at room temperature. This preincubated CT-DNA, **Re-C1** (20 μM), **Re-C2** (20 μM), and cisplatin (25 μM) (positive control) were dissolved in 10 mM Tris-HCl pH 7.4 and 0.1% DMSO, respectively, and were incubated at room temperature for at least 30 mins. The concentrations of DMSO were kept constant. The main stock of **Re-C1**, **Re-C2**, cisplatin were made in 100% DMSO and diluted with Tris HCl upto 1% DMSO concentrations. Concentrations of DMSO in all samples were kept at a constant level. After 30 mins, incubation reading was measured using a fluorescence plate reader (excitation wavelength 480 nm and emission wavelength 530 - 650 nm).

Circular Dichroism (CD) in CT-DNA

Metallocages **Re-C1** (20 μM), **Re-C2** (20 μM) and cisplatin (25 μM) were incubated with CT-DNA (100 μM of base pair equivalent to 0.064 mg/ml) for 1 h at room temperature. CT-DNA stock was prepared in Tris-HCl (5 mM) buffer solution. CT-DNA without any incubation was used as a negative control and cisplatin incubated CT-DNA was used as a positive control. Measurements were recorded on a JASCO J-810 spectropolarimeter in a 0.2 cm cuvette, scanning from 210 to 300 nm, and the respective absorbance of the compounds and the buffer was subtracted from each curve. Three cycles were performed for each reading.

Immunofluorescence assay

HeLa cells (5×10^4 cells/well) were seeded on top of the sterile, clean cover glass in 12-well plates and incubated for 24 h for complete attachment. Next day, cells were washed with PBS and incubated with **Re-C1** (20 μM), **Re-C2** (20 μM) and cisplatin (25 μM) supplemented with fresh 2% FBS containing high glucose DMEM media. After incubation, cells were washed with PBS and fixed by using 4% PFA for 12 mins. The fixative solutions were discarded after 12 mins and washed with PBS to remove excess PFA solutions. Then cells were allowed for immunostaining, for which first the cells

were blocked with blocking buffer to avoid any unspecific binding, adding diluted primary antibody (γH2AX) and kept for 24 h incubation in dark condition at 4 °C. Next day, the cells were washed twice with PBS and incubated with diluted fluorochrome-conjugated secondary antibody for 2 h and coated with DAPI (1 µg/ml). Images were captured by Biorad fluorescent cell imager.

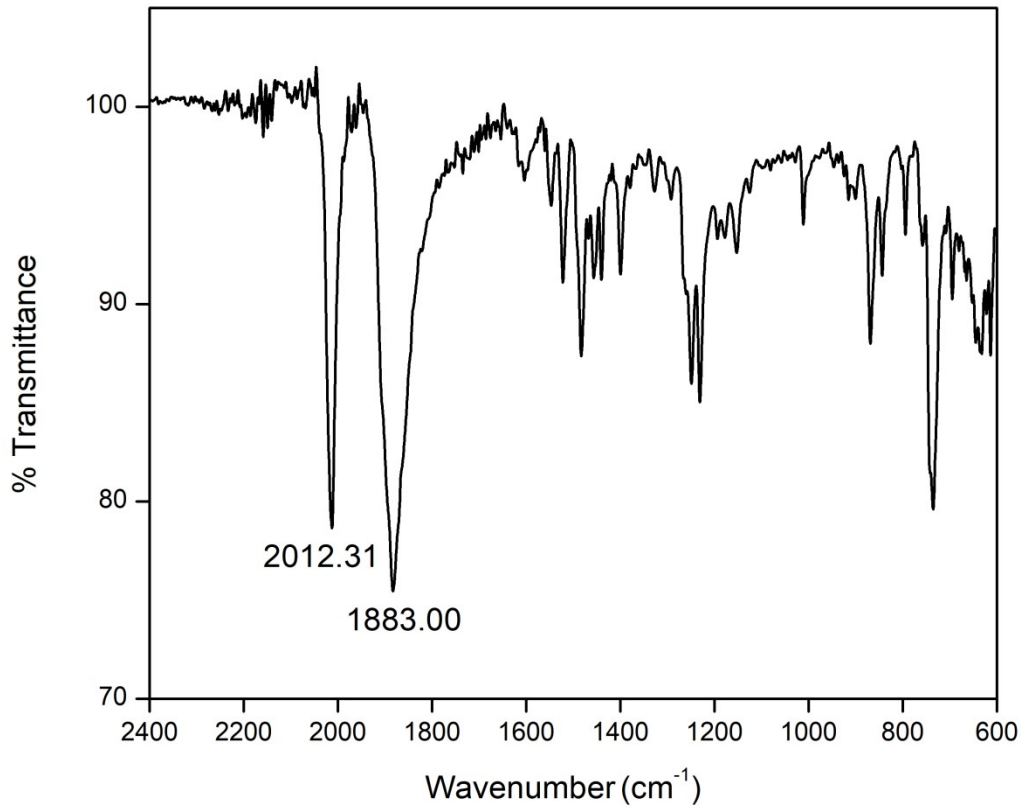

Figure S1. ATR-IR spectrum of **Re-C1**.

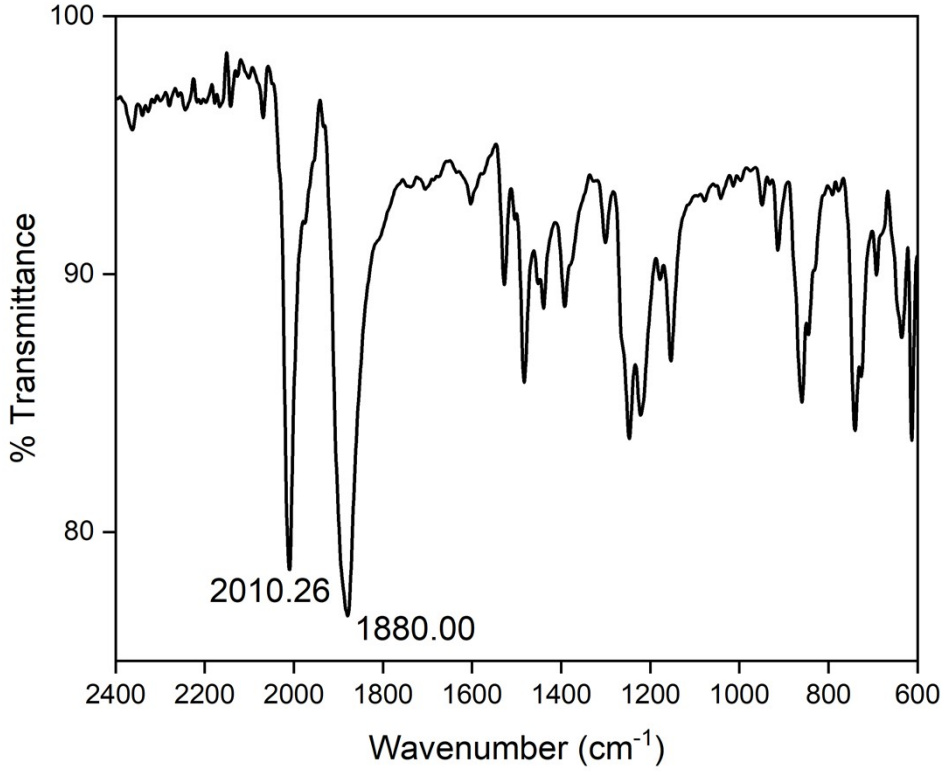

Figure S2. ATR-IR spectrum of **Re-C2**.

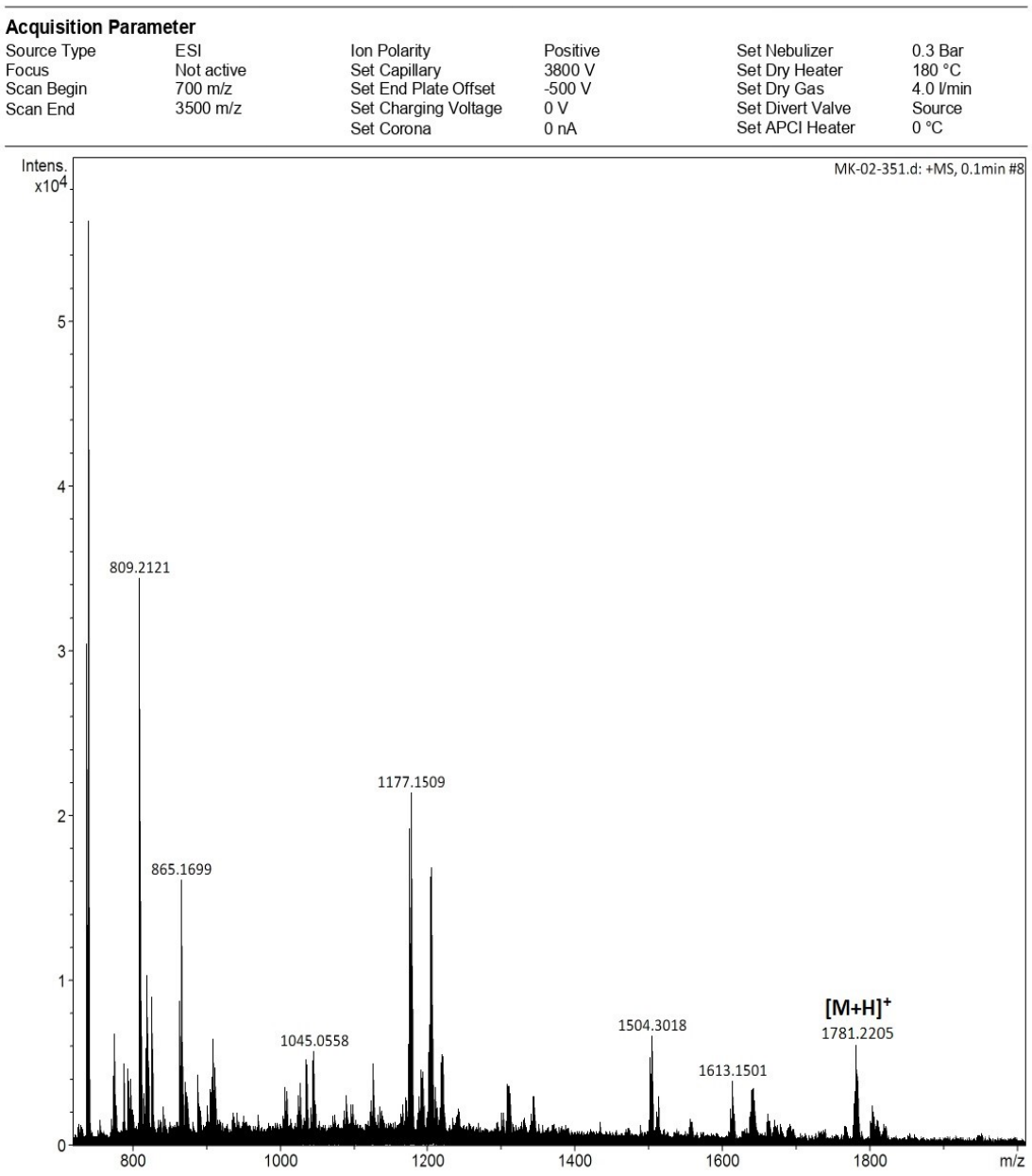

Figure S3. Experimental ESI mass spectrum of [**Re-C1** + H]$^+$ in positive ion mode.

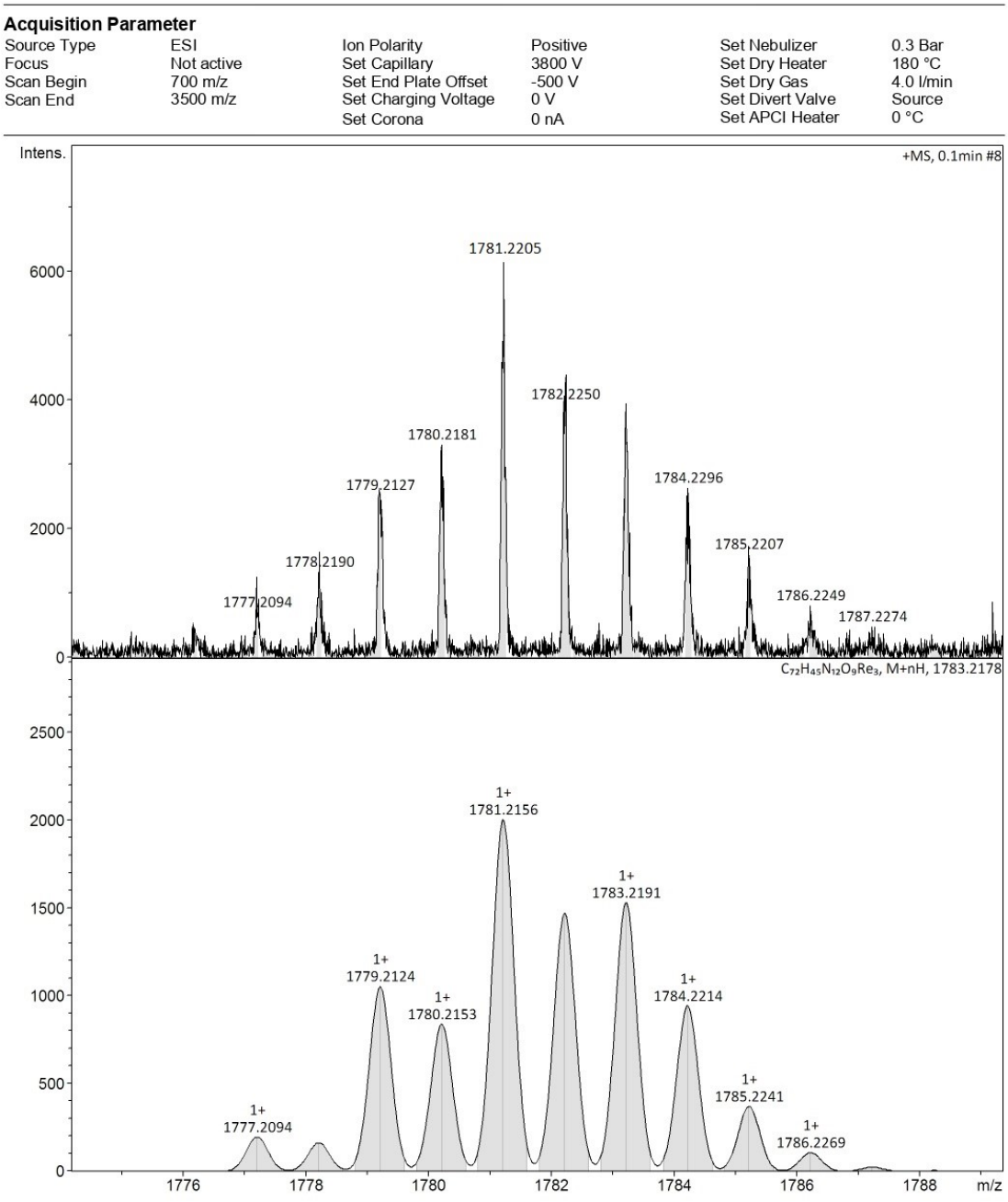

Figure S4. Experimental (Top) and calculated (Bottom) ESI mass spectra of [**Re-C1** + H]$^+$ in positive ion mode.

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.3 Bar
Focus	Not active	Set Capillary	3800 V	Set Dry Heater	180 °C
Scan Begin	200 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	3000 m/z	Set Charging Voltage	0 V	Set Divert Valve	Source
		Set Corona	0 nA	Set APCI Heater	0 °C

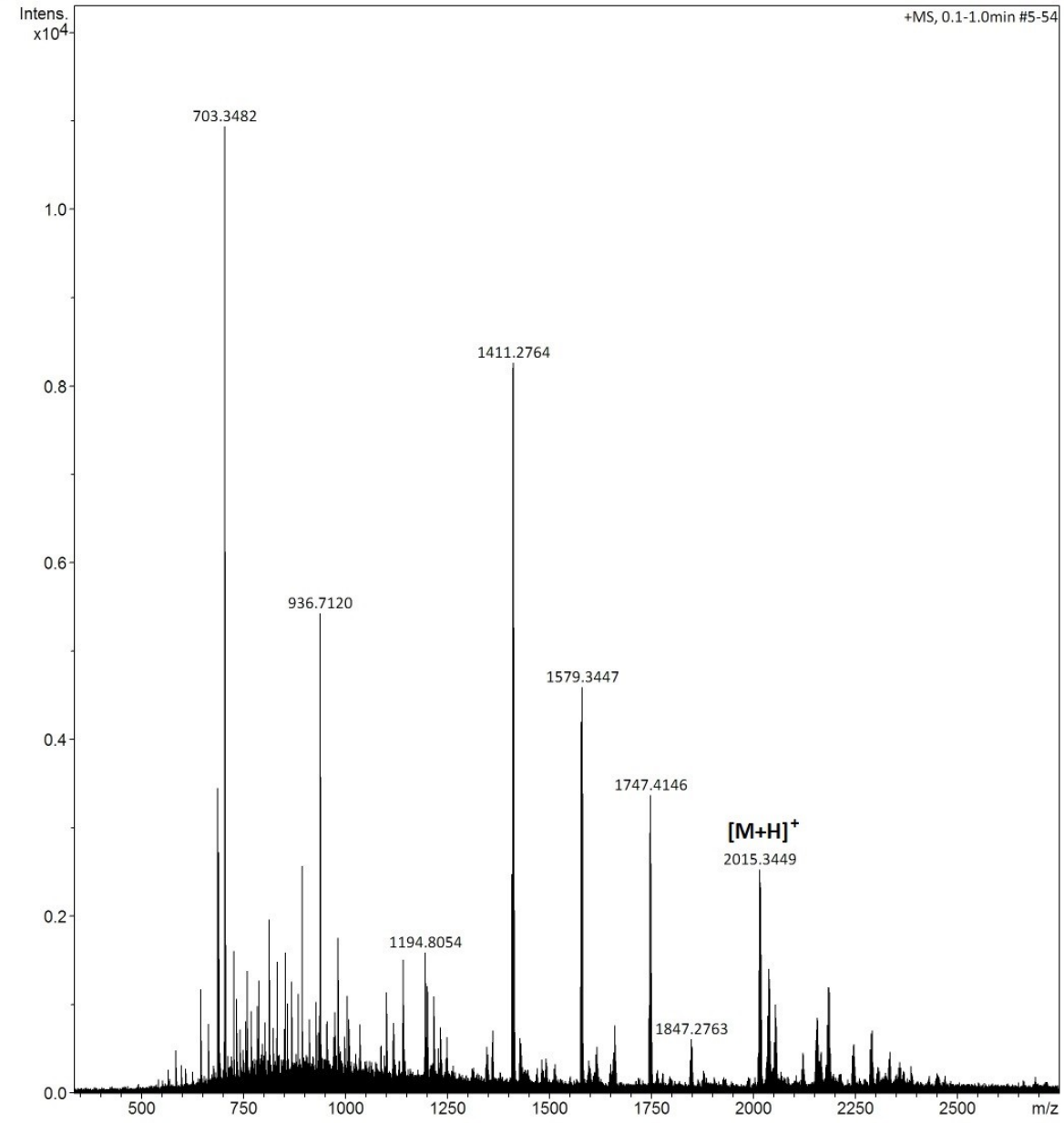

Figure S5. Experimental ESI mass spectrum of [**Re-C2** + H]$^+$ in positive ion mode.

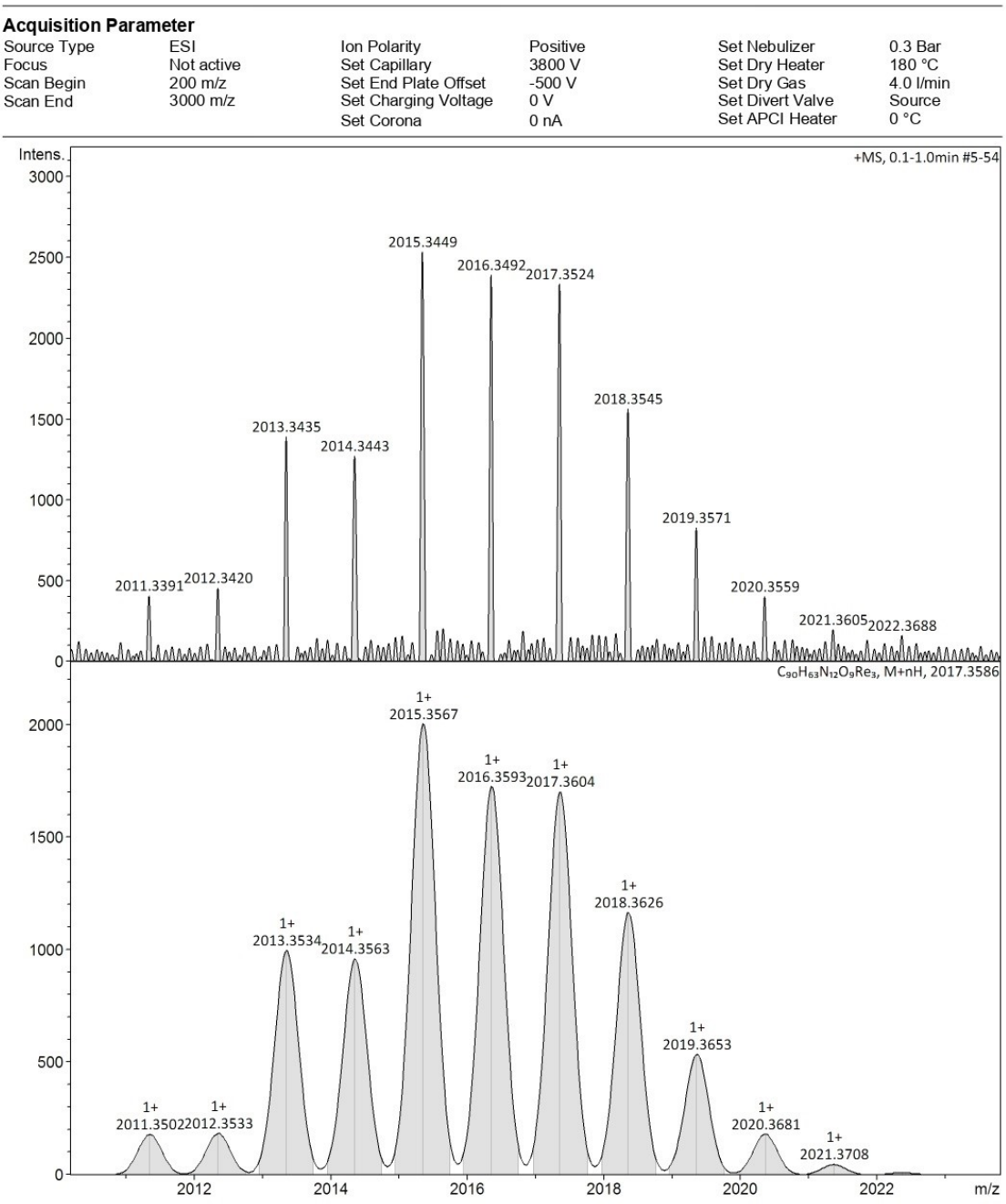

Figure S6. Experimental (Top) and calculated (Bottom) ESI mass spectra of [**Re-C2** + H]$^+$ in positive ion mode.

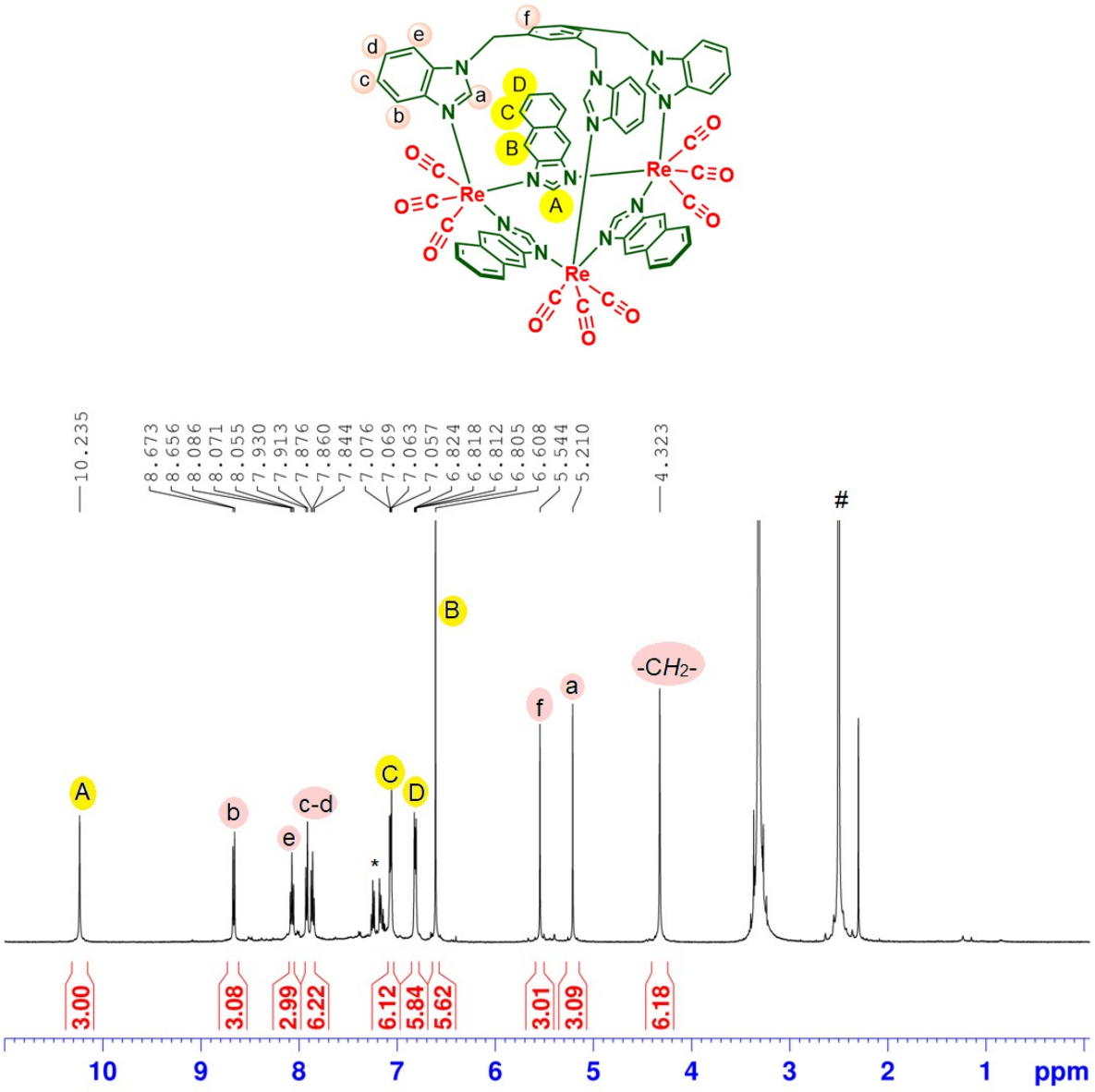

Figure S7. ^{1}H-NMR spectrum of **Re-C1** in DMSO-d_6 (∗ = toluene, # = DMSO-d_6).

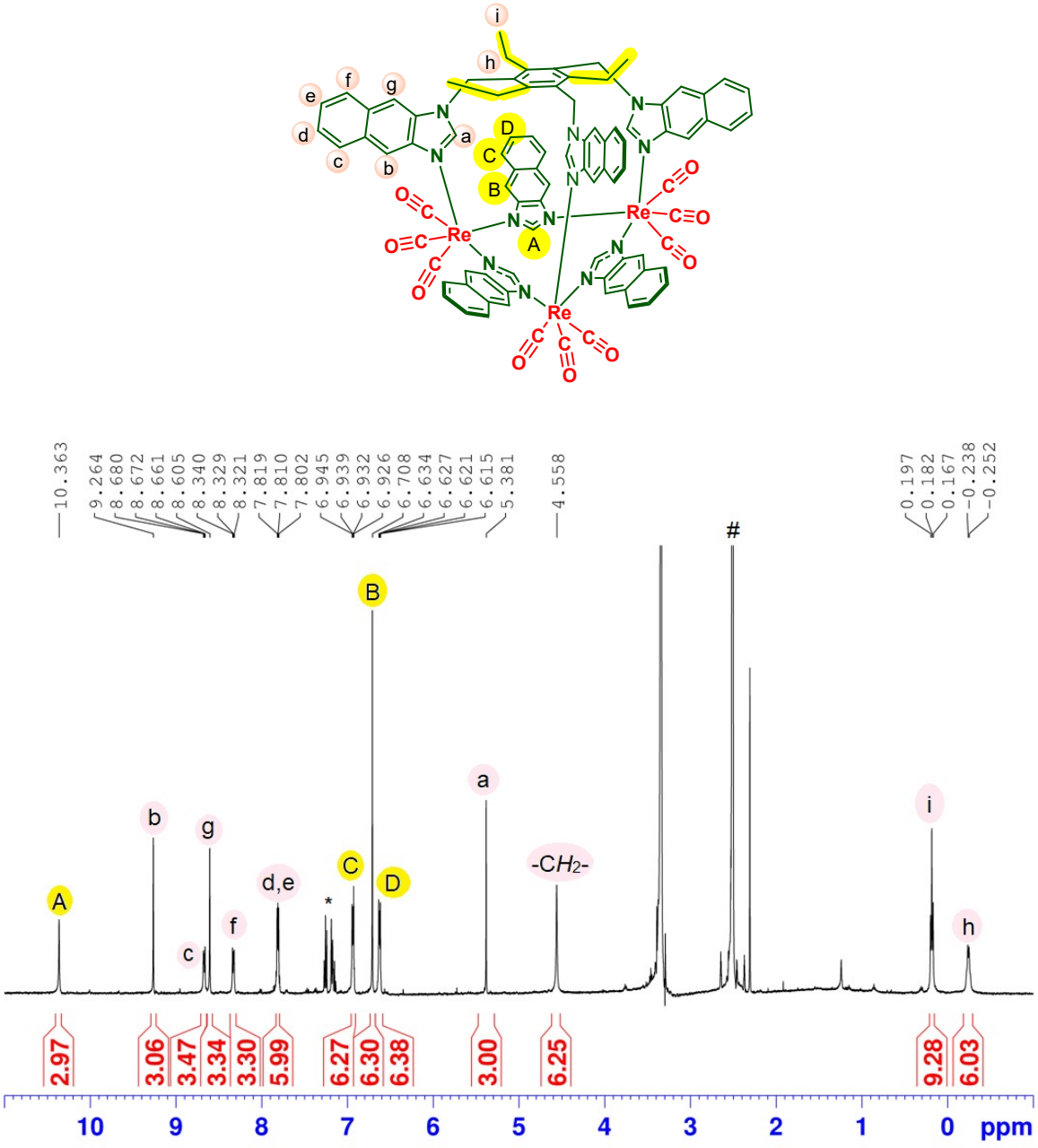

Figure S8. ^{1}H-NMR spectrum of **Re-C2** in DMSO-d_6 (∗ = toluene, # = DMSO-d_6).

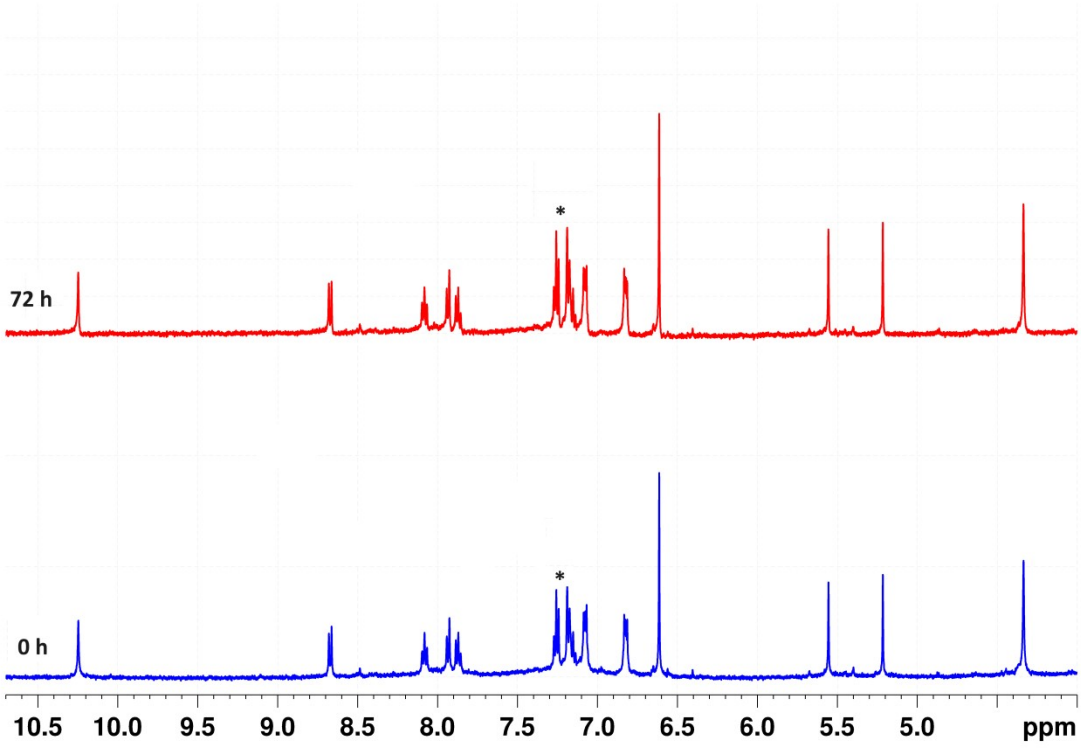

Figure S9. ^{1}H-NMR spectrum of **Re-C1** in DMSO-d_6 at 0 h and 72 h. (∗ = toluene).

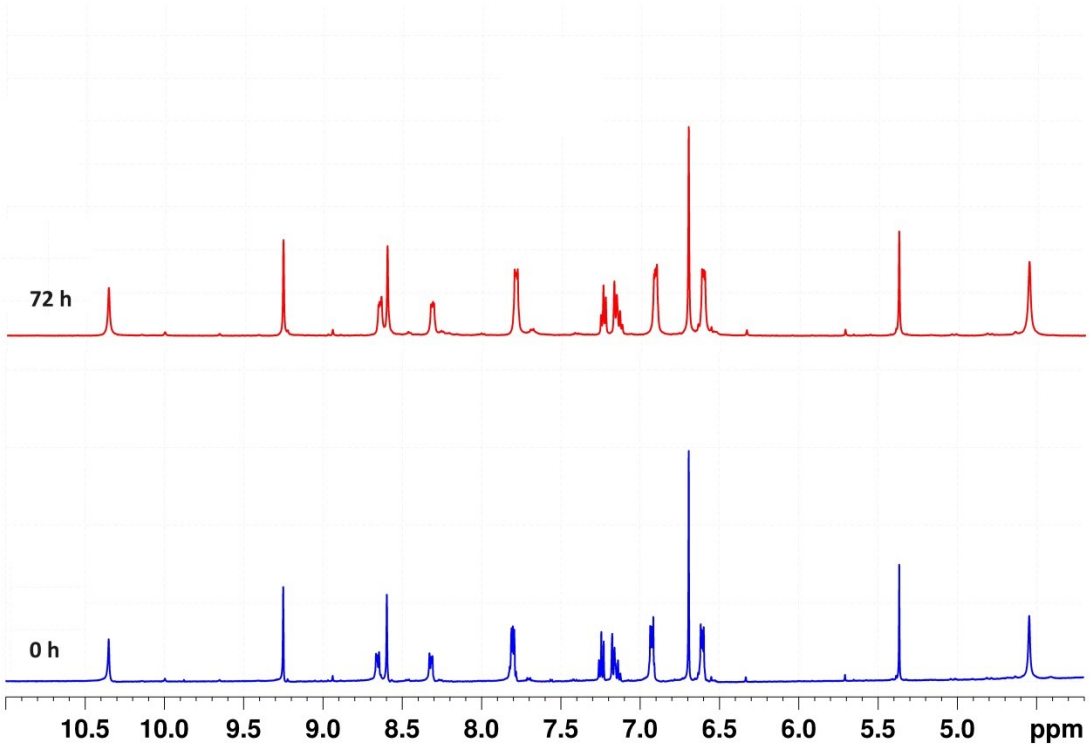

Figure S10. ^{1}H-NMR spectrum of **Re-C2** in DMSO-d_6 at 0 h and 72 h. (∗ = toluene).

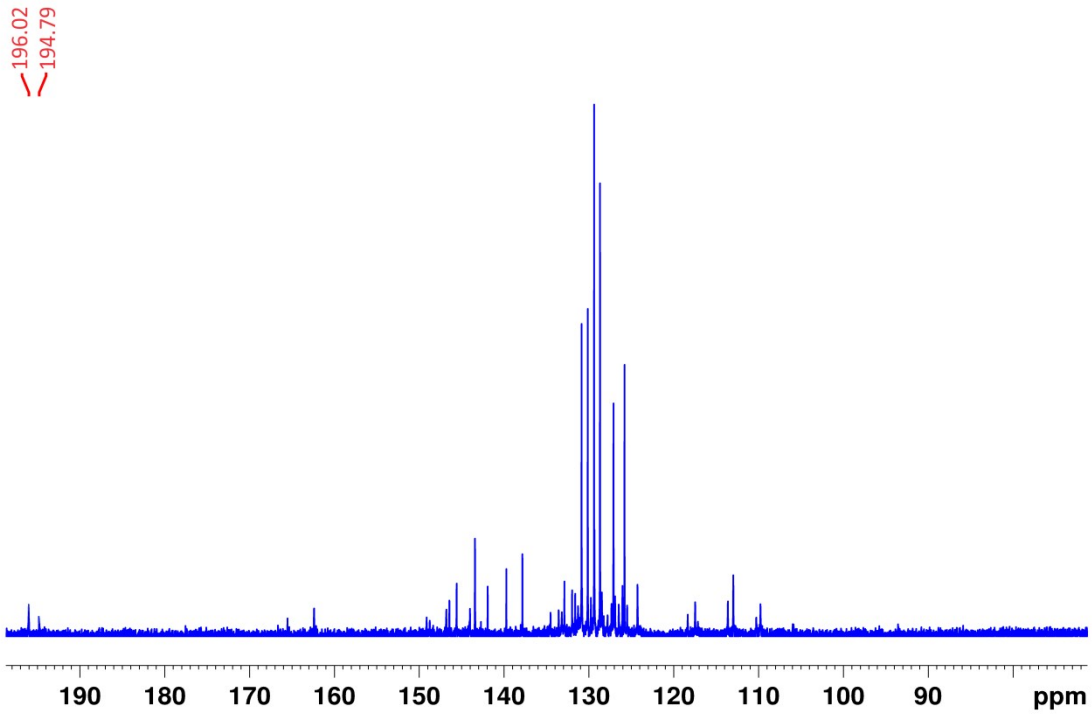

Figure S11. ^{13}C-NMR spectrum of **Re-C2** in DMSO-d_6.

	Re-C1	**Re-C2**
chemical formula	$(C_{72}H_{45}N_{12}O_9Re_3)_2(C_7H_8)_3$	$(C_{90}H_{63}N_{12}O_9Re_3)_2(C_7H_8)_3$
formula weight	3838.0	4306.64
crystal system	monoclinic	monoclinic
space group	$P2_1/c$	P $2_1/n$
a (Å)	24.1112(4)	18.1144(4)
b (Å)	15.4419(3)	19.0355(5)
c (Å)	20.2080(3)	25.3196(8)
α (deg)	90	90
β (deg)	93.724(1)	95.563(2)
γ (deg)	90	90
V (Å^3)	7508.0(2)	8689.5(4)
Z	2	2
T (K)	296	296
λ (Å)	0.71073	0.71073
D_{calc} (g cm^{-3})	1.698	1.646
μ (mm^{-1})	4.891	4.236
R_1 [I > 2σ(I)]	0.0494	0.0528
wR_2 (all data)	0.1255	0.1278
GooF	1.045	1.045
CCDC no.	2265703	2265704

Table S1. Crystallographic data and structure refinement for **Re-C1** and **Re-C2**.

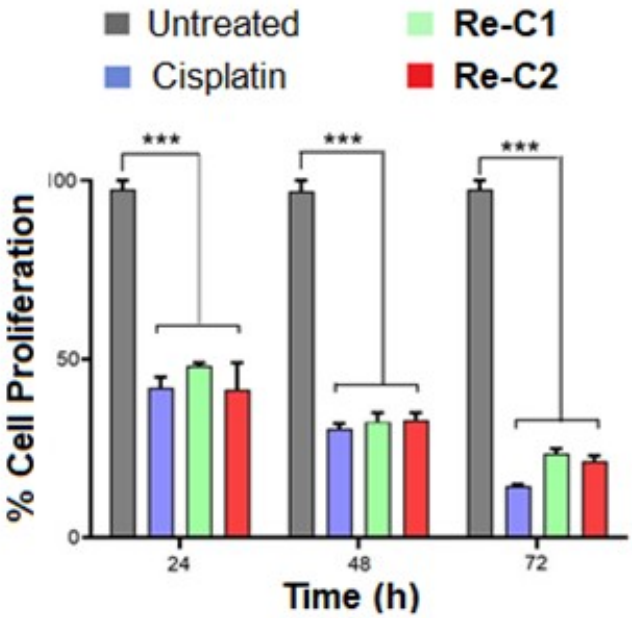

Figure S12. Evaluation of proliferation percentage of HeLa cells treated with **Re-C1**, **Re-C2** and cisplatin via crystal violet assay.

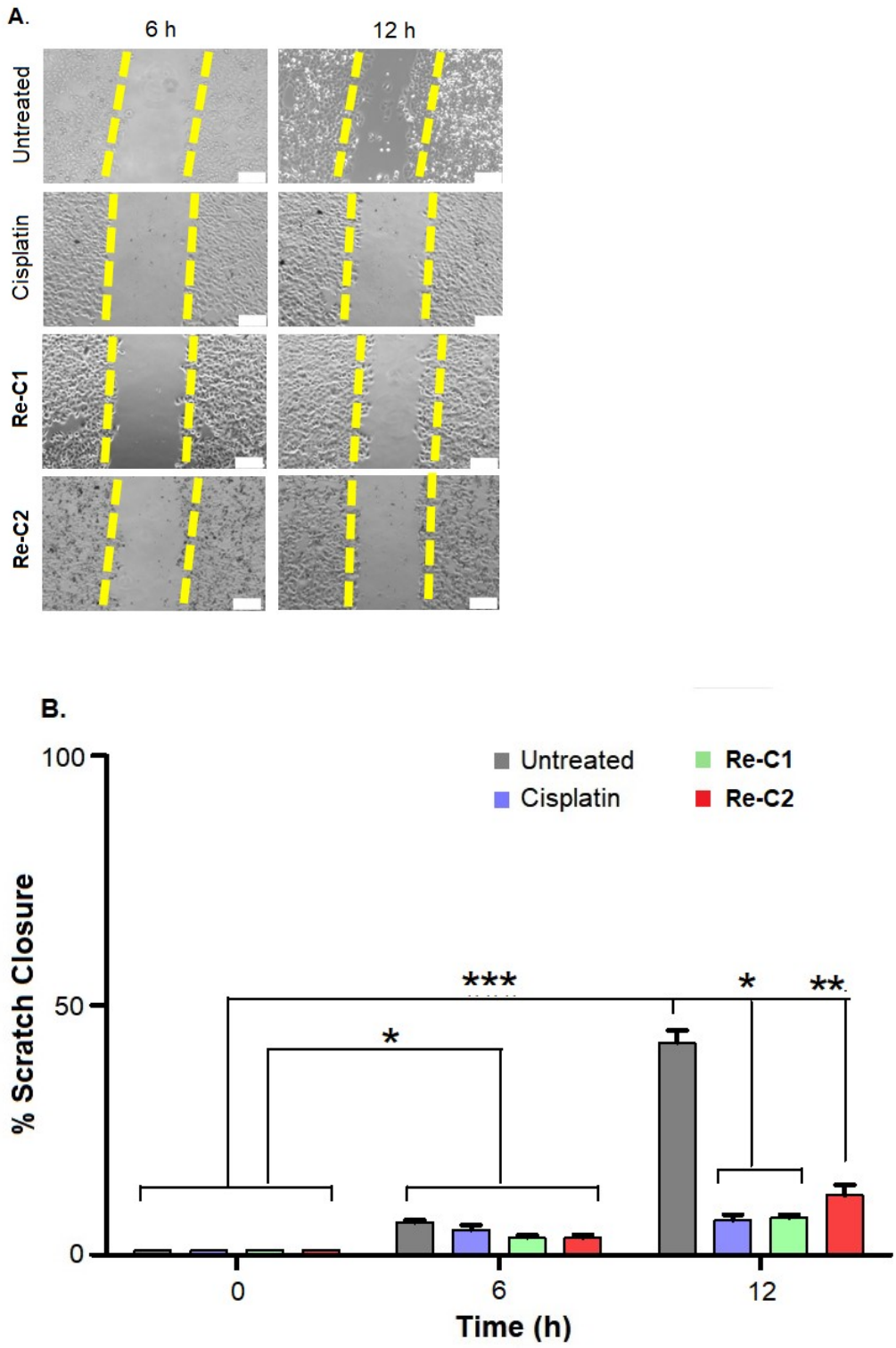

Figure S13. Evaluation of antimetastatic effects of **Re-C1** and **Re-C2** on HeLa cells using scratch assay, untreated cells kept as negative control and cisplatin used as a positive control, (Scale bar: 100 µm). (B) Percentage of scratch closure at 0,6 and 12 h of treatment.

Complex	Cell line (Type)	IC50 (μM)
fac-$[Re(CO)_3(\mu\text{-}N\text{-}L^1\text{-}N)Br]_2$[S7]	A549 (Lung cancer) HeLa (Cervical cancer) HCT-15 (Colon cancer) HepG2 (Liver cancer) K562 (Leukemia)	>100 23.4 ± 1.4 48.2 ± 3.4 30.1 ± 1.6 27.3 ± 1.3
fac-$[Re(CO)_3(\mu\text{-}N\text{-}L^2\text{-}N)Br]_2$[S7]	A549 (Lung cancer) HeLa (Cervical cancer) HCT-15 (Colon cancer) HepG2 (Liver cancer) K562 (Leukemia)	41.4 ± 2.2 63.4 ± 3.1 42.2 ± 3.1 23.3 ± 1.2 >100
fac-$[Re(CO)_3(\mu\text{-}N\text{-}L^3\text{-}N)Br]_2$[S7]	A549 (Lung cancer) HeLa (Cervical cancer) HCT-15 (Colon cancer) HepG2 (Liver cancer) K562 (Leukemia)	>100 22.8 ± 2.5 46.9 ± 3.1 29.4 ± 2.8 26.4 ± 1.9
fac-$[Re(CO)_3(\mu\text{-}N\text{-}L^4\text{-}N)Br]_2$[S7]	A549 (Lung cancer) HeLa (Cervical cancer) HCT-15 (Colon cancer) HepG2 (Liver cancer) K562 (Leukemia)	24.7 ± 2.9 21.2 ± 2.7 22.1 ± 2.3 27.3 ± 2.6 24.1 ± 1.7
fac-$[Re(CO)_3Br(\mu\text{-}L^5)]_2$[S8]	A549 (Lung cancer) MCF-7 (Breast cancer) HCT-15 (Colon cancer) HeLa (Cervical cancer) HepG2 (Liver cancer) K562 (Leukemia)	23.9 ± 4.7 >100 >100 51.4 ± 4.5 34.8 ± 5.6 60.6 ± 6.2
fac-$[Re(CO)_3Br(\mu\text{-}L^6)]_2$[S8]	HCT-15 (Colon cancer) HeLa (Cervical cancer) HepG2 (Liver cancer) K562 (Leukemia)	5.4±2.9 29.3±3.7 21.5±4.6 43.7±2.1
fac-$[Re(CO)_3Br(\mu\text{-}L^7)]_2$[S8]	A549 (Lung cancer) HCT-15 (Colon cancer) HeLa (Cervical cancer) HepG2 (Liver cancer) K562 (Leukemia)	12.3±3.8 20.9±5.7 20.8±4.5 8.9±3.5 8.3±3.4

fac-[$(CO)_3Re(\mu\text{-}L^8)(\mu\text{-}L^{10})Re(CO)_3$] [S9]	A549 (Lung cancer) HeLa (Cervical cancer) HCT-15 (Colon cancer)	95.72 ± 8.2 >100 20.80 ± 1.9
fac-[(CO)3$Re(\mu\text{-}L^9)(\mu\text{-}L^{10})Re(CO)_3$] [S9]	A549 (Lung cancer) HeLa (Cervical cancer)	56.15 ± 2.9 54.19 ± 2.4
fac-[$(CO)_3Re(\mu\text{-}L^8)(\mu\text{-}L^{11})Re(CO)_3$] [S9]	A549 (Lung cancer) HeLa (Cervical cancer)	88.49 ± 1.2 29.81 ± 2.5
fac-[$(CO)_3Re(\mu\text{-}L^9)(\mu\text{-}L^{11})Re(CO)_3$] [S9]	A549 (Lung cancer) HeLa (Cervical cancer)	65.23 ± 7.2 40.49 ± 1.9
fac-[$(CO)_3Re(\mu\text{-}L^8)(\mu\text{-}L^{12})Re(CO)_3$] [S9]	A549 (Lung cancer) HeLa (Cervical cancer) HCT-15 (Colon cancer)	29.65 ± 1.4 64.50 ± 1.9 30.53 ± 1.3
fac-[$(CO)_3Re(\mu\text{-}L^{14})_2Re(CO)_3(\mu\text{-}L^{17})$] [S10]	A549 (Lung cancer) HCT-15 (Colon cancer) HepG2 (Liver cancer)	>100 54.7 ± 3.2 8.57 ± 5.3
fac-[$(CO)_3Re(\mu\text{-}L^{15})_2Re(CO)_3(\mu\text{-}L^{17})$] [S10]	A549 (Lung cancer) HCT-15 (Colon cancer) HepG2 (Liver cancer)	89.2 ± 1.2 66.1 ± 9.8 61.7 ± 2.4
fac-[$(CO)_3Re(\mu\text{-}L^{16})_2Re(CO)_3(\mu\text{-}L^{17})$] [S10]	A549 (Lung cancer) HCT-15 (Colon cancer) HeLa (Cervical cancer) HepG2 (Liver cancer)	19.2 ± 4.5 24.8 ± 5.6 35.98 ± 4.6 8.46 ± 5.6
fac-[$(CO)_3Re(\mu\text{-}L^{13})_2Re(CO)_3(\mu\text{-}L^{18})$] [S10]	A549 (Lung cancer) HCT-15 (Colon cancer) HeLa (Cervical cancer)	74.5 ± 0.4 69.3 ± 0.4 86.89 ± 0.2
fac-[$(CO)_3Re(\mu\text{-}L^{15})_2Re(CO)_3(\mu\text{-}L^{18})$] [S10]	A549 (Lung cancer) HCT-15 (Colon cancer) HeLa (Cervical cancer)	89.8 ± 0.7 93.8 ± 0.4 91.3 ± 0.3
	A549 (Lung cancer)	73.9 ± 0.8

fac-[$(CO)_3Re(\mu$-$L^{16})_2Re(CO)_3(\mu$-$L^{18})$] [S10]	HCT-15 (Colon cancer) HeLa (Cervical cancer)	97.5 ± 0.9 97.2 ± 0.2
fac-[$Re(CO)_3Br(\mu$-$L^{17})]_2$[S11]	HCT-15 (Colon cancer) HepG2 (Liver cancer)	33.2 ± 2.7 14.2 ± 4.8
fac-[$Re(CO)_3Br(\mu$-$L^{19})]_2$[S11]	A549 (Lung cancer) MCF-7 (Breast cancer) HCT-15 (Colon cancer) HeLa (Cervical cancer) HepG2 (Liver cancer) K562 (Leukemia)	37.6 ± 7.2 89.5 ± 4.8 86.7 ± 3.6 12.4 ± 2.9 31.6 ± 4.5 29.5 ± 5.8
Re-C1	HeLa (Cervical cancer)	20 ± 5
Re-C2	HeLa (Cervical cancer)	20 ± 5

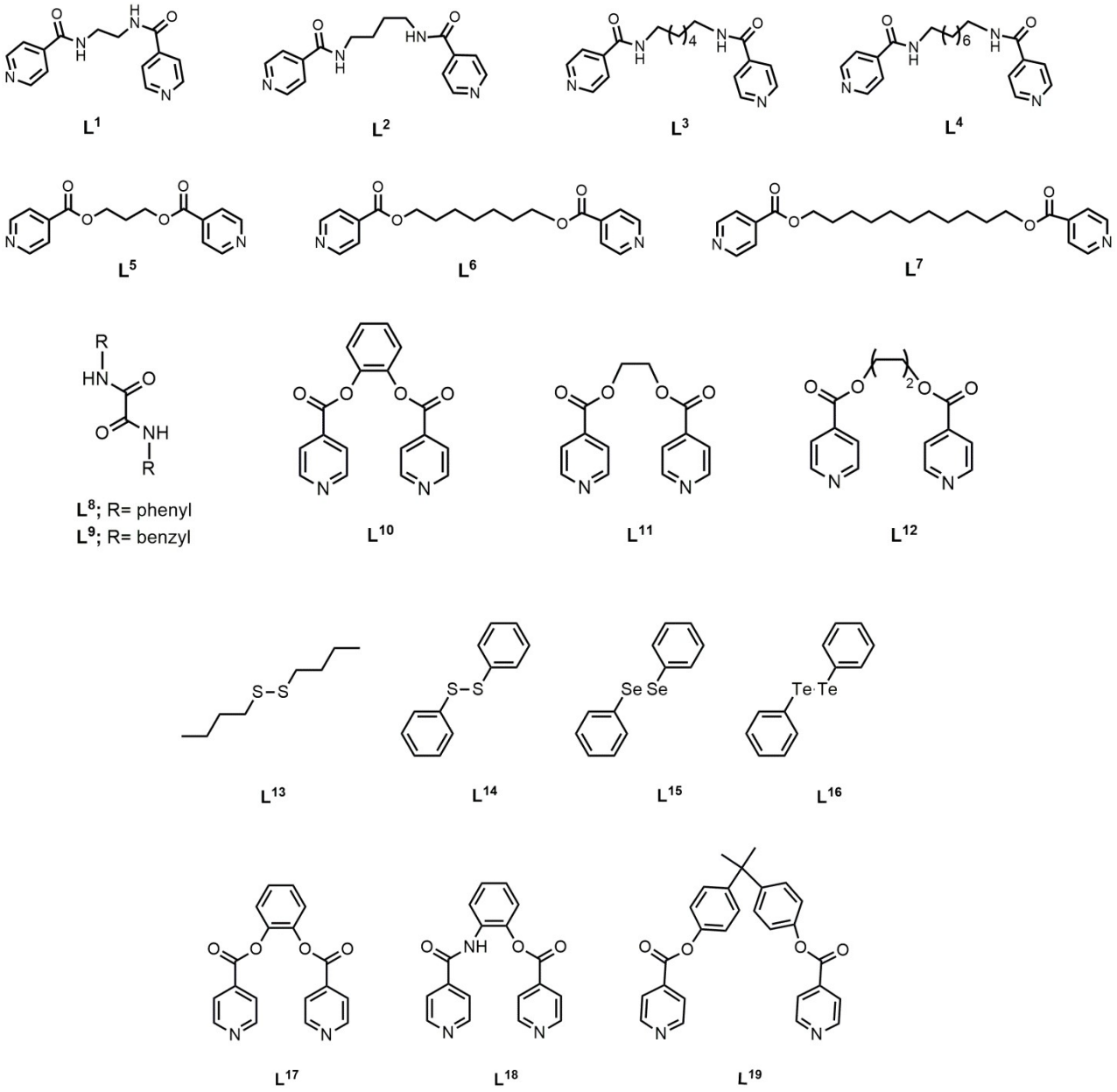

Table S2. IC^{50} values of rhenium metallocycles/metallocages towards human cancer cells.

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