Electronic Supplementary Material (ESI) for Inorganic Chemistry Frontiers.

Electronic Supporting Information

Mitochondrial-Targeting Monofunctional Platinum(II)-Lonidamine Conjugates for Cancer Cells De-energization

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Experimental

Chemical Reagents and cell lines

All the reagents and solvents were of analytical grade and used as received without further purification. Cisplatin (cDDP) was purchased from Shandong Boyuan Pharmaceutical Co., Ltd. China. 1,10-Phenanthroline (Phen), 4,7-diphenyl-1,10-phenanthroline (DIP), N-(aminopropyl) imidazole, anhydrous sodium sulfate and silver nitrate were purchased from Sigma-Aldrich and used as received without further purification. Lonidamine was purchased from J&K Scientific. Other reagents were supplied by Energy Chemicals, China and used as received. Water was deionized and ultrafiltered by a Milli-Q apparatus (Millipore Corporation, China). Annexin V conjugated with fluorescein isothiocyanate (Annexin V-FITC), propidium iodide (PI) and H_2DCF-DA (2',7'-dichlorodihydrofluorescein diacetate) were purchased from Sigma-Aldrich. AxyPrep Blood gDNA MiniPrep kit was purchased from Corning (USA). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was purchased from Life Technologies, USA. Cellular ATP quantification assay kit were purchased from Promega (USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich. pBR322 plasmid DNA was purchased from ThermoFisher Scientific. Glucose, pyruvate, oligomycin, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), rotenone, antimycin A, succinate, and adenosine diphosphate (ADP) were purchased from Sigma. The antibodies used for western blotting was purchased from Beyotime Biotechnology, China. All the compounds tested were dissolved in DMSO just before the experiments, and the final concentration of DMSO was kept at 0.5% (v/v). Human lung cancer A549, human prostate cancer PC3, human ovarian cancer CaOV3, human breast cancer MCF7 and MDA-MB-231, and human mammary epithelial MCF10A cell lines were purchased from American type culture collection (ATCC).

Instrumentation

NMR spectra were recorded on a Bruker Avance 500 spectrometer (Germany). ESI-MS were recorded on a Thermo Finnigan LCQ DECA XP spectrometer (USA). Cell images were captured by a Zeiss LSM 710 Confocal. Flow cytometric analysis was done using a BD FACS Calibur™ flow cytometer (Becton Dickinson, USA). Microanalysis of elements (C, H, and N) was carried out using an Elemental Vario EL CHNS analyzer (Germany).

Methods

Synthesis of L

Ligand L was synthesized according to the procedure reported earlier.[1] Yield 68%. ^{1}H NMR (DMSO-d_6) δ (ppm) 1.95−2.02 (m, 2H), 3.27−3.31 (dd, 2H), 4.0−4.03 (t, 2H), 5.84 (s, 2H), 6.76−6.78 (d, 1H), 6.89 (s, 1H), 7.29−7.38 (m, 2H), 7.46−7.50 (t, 1H), 7.67−7.76 (m, 3H), 8.23−8.25 (d, 1H), 8.49−8.51 (t, 1H).

Synthesis of MPL-I

$AgNO_3$ (83 mg) was added to a solution of cDDP (150 mg) in 20 mL DMF, and the mixture was stirred at 45 °C for 16 h. The mixture was allowed to cool and the precipitated AgCl was removed by centrifugation at 9000 g for 10 min. Ligand L (203 mg, 0.47 mmol) in DMF (6 mL) was added to the supernatant and the reaction was left on stirring for 24 h at 35 °C. After that, the solvent was removed on a rotary evaporator and the product was re-dissolved in CH_2Cl_2 and precipitated with diethyl ether. Yield 76%. HPLC Purity >95%. ^{1}H NMR ($CDCl_3$) δ (ppm) 1.98 (s, 2H), 3.33 (s, 2H), 3.98 (s, 2H), 4.10−4.17 (d, 3H), 4.54 (s, 3H), 5.47 (s, 2H), 6.69−6.71 (d, 1H), 7.11−7.23 (m, 5H), 7.60−7.66 (d, 1H), 8.20−8.26 (dd, 2H). ^{13}C NMR (DMSO−d_6) (ppm) 30.39, 35.43, 45.28, 49.43, 110.21, 120.38, 121.95, 122.18, 122.57, 127.08, 127.71, 128.21, 128.95, 130.08, 132.73, 133.13, 133.36, 138.03, 138.91, 140.99, 161.84. ESI-MS (negative mode, m/z) observed (calculated): 692.41 (692.93).

Synthesis of MPL-II

$AgNO_3$ (22 mg) was added to a solution of [Pt(Phen)Cl_2] (60 mg) in 25 mL DMF, and the mixture was stirred at 45 °C for 16 h. The mixture was allowed to cool and the precipitated AgCl was removed by centrifugation at 9000 g for 10 min. Ligand L (56 mg, 0.13 mmol) in 2 mL DMF was added to the supernatant, and the reaction was left on stirring for 24 h at 35 °C. After that, the solvent was removed on a rotary evaporator and product was re-dissolved in CH_2Cl_2 and precipitated with diethyl ether. Yield 59%. HPLC Purity >95%. ^{1}H NMR (DMSO−d_6) δ (ppm) 2.11−2.19 (m, 2H), 3.39−3.46 (m, 2H), 4.29−4.33 (t, 2H), 5.77 (s, 2H), 6.65−6.69 (t, 1H), 7.22−7.26 (t, 1H), 7.33−7.44 (m, 2H), 7.48−7.52 (m, 1H), 7.60 (d, 1H), 7.64−7.71 (m, 1H), 7.77 (s, 1H), 8.05−8.08 (dd, 1H), 8.11−8.17 (m, 1H), 8.19−8.24 (m, 2H), 8.33 (s, 2H), 8.56−8.59 (t, 1H), 8.73 (s, 1H), 9.06−9.15 (dt, 2H), 9.59−9.61 (d, 2H). ^{13}C NMR (DMSO−d_6) (ppm) 30.61, 36.30, 46.58, 49.96, 110.65, 122.48, 122.69, 123.09, 126.61, 127.27, 127.58, 128.31, 128.57, 129.37, 130.38, 130.98, 131.14, 133.13, 133.60, 133.91, 138.66, 140.56, 141.24, 141.52, 147.15, 147.78, 149.77, 150.96, 162.42. ESI-MS (negative mode, m/z) observed (calculated): 838.59 (839.08).

Synthesis of MPL-III

$AgNO_3$ (45 mg) was added to a solution of [Pt(DIP)Cl_2] (150 mg) in 30 mL DMF, and the mixture was stirred at 45 °C for 16 h. The mixture was allowed to cool and the precipitated AgCl was removed by centrifugation at 9000 g for 10 min. Ligand L (107 mg, 0.25 mmol) in 2 mL DMF was added to the supernatant, and the reaction left on stirring for 24 h at 35 °C. After that, the solvent was removed on a rotary evaporator and the product was re-dissolved in CH_2Cl_2 and precipitated with diethyl ether. Yield 63%. HPLC Purity >95%. ^{1}H NMR (DMSO−d_6) δ (ppm) 2.15−2.18 (d, 2H), 3.41−3.43 (d, 2H), 4.34−4.35 (d, 2H), 5.76 (s, 2H), 6.65−6.67 (d, 1H), 7.15−7.22 (dd, 1H), 7.33−7.39 (dd, 2H), 7.54−7.56 (d, 1H), 7.60−7.64 (m, 2H), 7.69−7.76 (m, 10H), 8.07−8.08 (d, 1H), 8.12−8.22 (dd, 3H), 8.25−8.27 (d, 1H), 8.37−8.39 (d, 1H), 8.56−8.59 (t, 1H), 8.80 (s, 1H), 9.72−9.74 (d, 2H). ^{13}C NMR (DMSO−d_6) (ppm) 30.59, 36.34, 46.65, 49.98, 110.61, 122.36 , 122.63, 123.03, 126.55, 127.24, 127.56, 128.32, 128.71, 128.88, 129.36 , 129.80, 130.05, 130.63, 133.12, 133.62, 133.88, 135.36, 138.62, 140.69, 141.50, 147.66, 148.25, 149.31, 150.62, 151.59, 152.26, 162.40. ESI-MS (negative mode, m/z) observed (calculated): 838.59 (839.08).

HPLC Analysis

The HPLC characterization of **MPL-I**, **MPL-II** and **MPL-III** was performed on a reversed phase HPLC system using 250 × 4.5 mm ODS column. The HPLC profiles were recorded on UV detection at 254 nm.

Water (A) and methanol (B) was used as a mobile phase for a gradient elution at a flow rate of 1 mL min^{-1}. The HPLC elution program was as follows: 5% B (0 min) →100% B (linear increase in 20 min).

Intracellular Distribution

MDA-MB-231 cells (10^6) were treated with **MPL-I**, **MPL-II**, **MPL-III** or cDDP at a concentration of 10 μM for 24 h. The cells were washed twice with PBS, collected by trypsin and re-suspended in PBS (1 mL). Mitochondria isolation kit (Beyotime Institute of Biotechnology, China) was used to separate the nuclei, mitochondrion-free cytoplasm and mitochondria. Samples were prepared according to the procedure described previously.[2] The content of Pt was tested by ICP-MS and the control value was subtracted.

Cell Cycle analysis

MDA-MB-231 cells (10^6) were treated with **MPL-III** (5 or 10 μM) or cDDP (10 μM) for 24 h. The cells were then fixed in ice-cold ethanol (70%) for 6 h, treated with RNase A, stained with PI in PBS for 15 min. The samples were analyzed by flow cytometry.

Annexin V-FITC and propidium iodide (PI) staining

MDA-MB-231 cells (10^6) cells were treated with **MPL-III** (5 or 10 μM) or cDDP (10 μM) for 48 h. The cells were stained with Annexin V-FITC and PI, and the samples were detected by flow cytometry. Data were analysed by the Flowjo 7.6.1 software.

DNA Platination

MDA-MB-231 cells (10^6) were treated with cDDP (10 μM) or **MPL-III** (10 μM) for 24 h. NcDNA were extracted and purified by the commercial spin column quantification kits (AxyPrep Blood gDNA MiniPrep kit). The quantification and purity of DNA were measured by NanoDrop (MD2000, Gene Company Limited) according to the manufacturer's instructions. The Pt content was quantified by ICP-MS.

MDA-MB-231 cells (10^6) were treated with **MPL-III** and cDDP (10 μM) for 24 h. Mitochondria were isolated from cells by mitochondria isolation kit, and mtDNA was purified by TIANamp Genomic DNA kit. Quality and quantity of mtDNA were determined as reported previously.[3]

Cell-free DNA binding was examined by 1% agarose gel electrophoresis in TAE running buffer (40 mM Tris acetate/1 mM EDTA, pH 7.4) on pBR322 plasmid DNA for 2 h at 70 V. Different concentrations of **MPL-III** were incubated with pBR322 plasmid DNA (200 ng μL^{-1}, 10 μL) at 37 °C for 4 h in Tris-HCl buffer (50 mM Tris, 50 mM NaCl, pH 7.4).

Western Blot

The assays were performed according to similar procedures previously reported.[3] Briefly, MDA-MB-231 cells (10^6) were treated with **MPL-III** (5 or 10 μM) and cDDP (10 μM) for 24 h. The cells were collected and lysed by ice-cold RIPA lysis buffer supplemented with protease and phosphatase inhibitor. The isolation of the cytosolic and mitochondrial protein fractions was performed using a literature method.{Eymin, 1999 #263} Protein samples were separated on SDS-PAGE on 12% gel and transferred to a PVDF membrane. The membrane was incubated with primary monoclonal antibodies (cytochrome C and Cyclin D1) at 4 °C overnight. Subsequently, the membrane was incubated with peroxidase-labelled goat anti rabbit HRP secondary antibody for 2 h. Western blots were visualized by an enhanced chemiluminescence detection system.

Transmission Electron Microscopy

MDA-MB-231 cells were treated with 10 μM of **MPL-III** at 37 ºC for 24 h. The cells were collected and fixed for 6 h at 4 ºC in PBS (pH 7.4) containing 2.5% glutaraldehyde. The cells were then treated with osmium tetroxide, stained with uranyl acetate and lead citrate, and visualized under a transmission electron microscope (JEM 100 CX, JEOL, Tokyo, Japan).

Measurement of MMP

MDA-MB-231 cells (10^6) were treated with **MPL-III** (5 or 10 μM) and cDDP (10 μM) for 24 h. The cells were collected and the pellets were re-suspended in JC-1 staining solutions for 20 min. Fluorescence intensity change in MDA-MB-231 cells were measured using flow cytometry (FACS Calibur™, Becton Dickinson, NJ, USA). Data were analysed by FlowJo software (Tree Star, OR, USA).

Mitochondrial Bioenergetics

MDA-MB-231 cells were treated with **MPL-III** (10 μM) and CDDP (10 μM) for 24 h at 37 °C. The assay was performed according to the method previously reported.[4] The key parameters of mitochondrial function were assessed using the XF Cell Mito Stress Test Kit with the Seahorse XFe24 analyzer (Seahorse

Bioscience, Billerica, USA) by directly measuring the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) according to the manufacturer's instructions.

Intracellular ATP Level

Cellular ATP levels were measured using the Cell Titer-Glo® Luminescent Cell Viability assay kit (G7570, Promega, USA) according to the manufacturer's instructions. MDA-MB-231 cells were seeded in 96 well plates and treated with **MPL-III** (5 or 10 μM) and cDDP (10 μM) for 24 h. The Cell Titer-Glo® reagent was added and the plate was incubated for 10 min. The luminescence was recorded using a microplate reader (Infinite M200 Pro, Tacan, Switzerland).

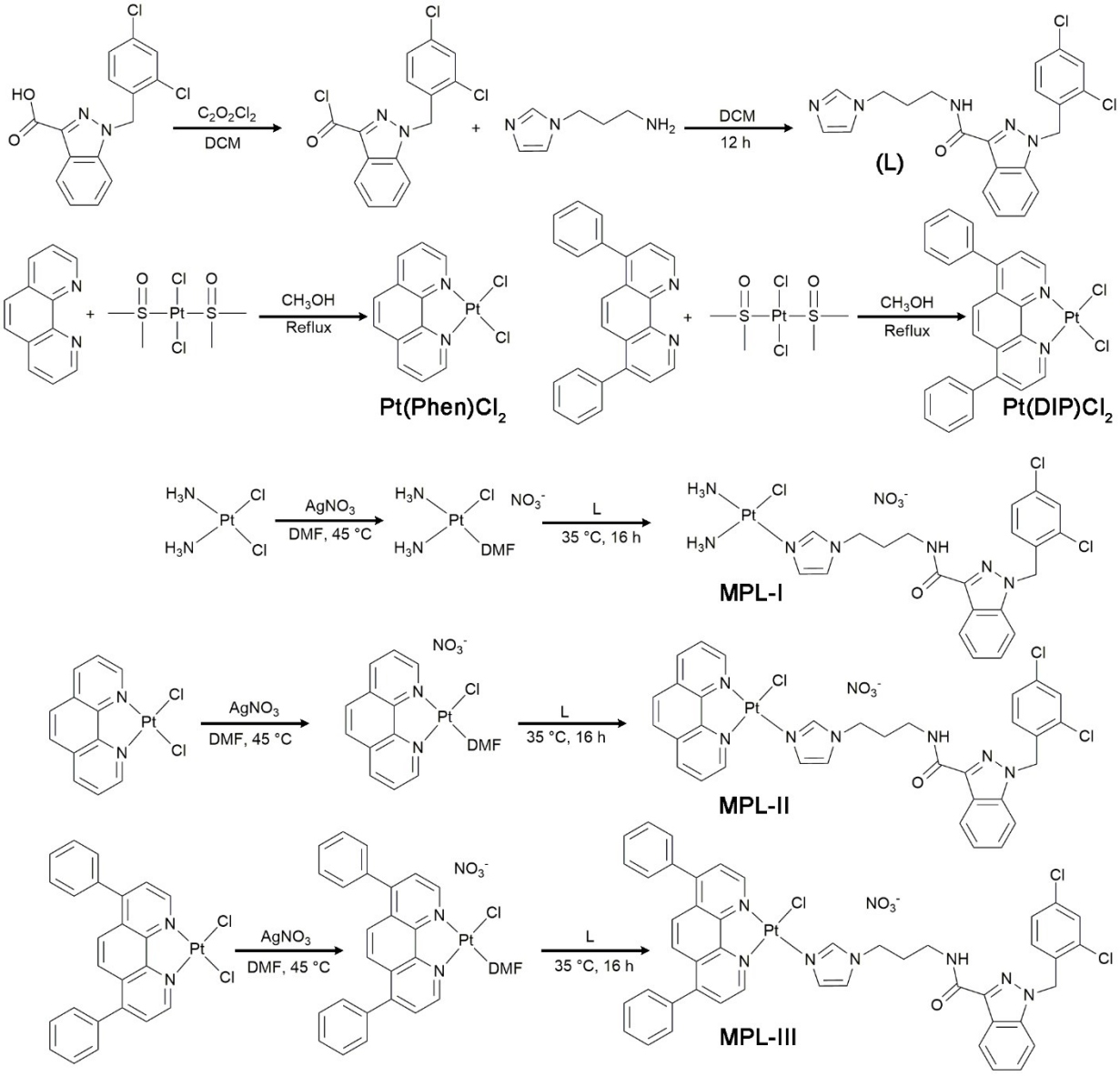
Cellular ROS Detection

MDA-MB-231 (10^6) were treated with **MPL-III** (5 or 10 μM) or cDDP (10 μM) for 24 h. The cells were then washed twice with PBS and re-suspended in serum-free medium. Cells were stained with H_2DCF-DA (10 μM) at 37°C for 20 min in the dark. The fluorescence intensity in MDA-MB-231 cells was measured by flow cytometry (FACS CaliburTM, Becton Dickinson, USA). λ_{ex} = 485 nm, λ_{em} = 520 nm.

RNA-Seq and Bioinformatics

MDA-MB-231 cells were treated with **MPL-III** (10 μM) for 24 h. Total RNA was purified using RNeasy mini kit (Qiagen, Hilden, Germany). RNA purity was checked using the kaiaoK5500®Spectrophotometer (Kaiao, Beijing, China). The integrity and concentration of RNA was assessed using the RNA Nano 6000 Assay Kit (Agilent Technologies, CA, USA). A total amount of 2 μg RNA per sample was used as input. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (#E7530L, NEB, USA). RNA concentration of library was measured using Qubit® RNA Assay Kit in Qubit® 3.0 to preliminary quantify and then dilute to 1 ng/μL. Insert size was assessed using the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA), and qualified insert size was accurate quantification using StepOnePlus™ Real-Time PCR System. The libraries were sequenced on an Illumina platform and 150 bp paired-end reads were generated. Sequencing reads were mapped to reference human genome sequence (NCBI 36.1 [hg19] assembly by TopHat (Version 2.0.6). Genes with false discovery rate (FDR) of < 0.05 and > 200 bp were considered as differentially expressed. Gene Set Enrichment Analysis (GSEA) was performed following the standard procedure (http://www.broadinstitute.org/gsea/doc/GSEAUserGuideFrame.html) as described by GSEA user guide.

Supplementary Scheme, Figures and Tables



Scheme S1. Synthetic routes of **MPL-I**, **MPL-II** and **MPL-III**.

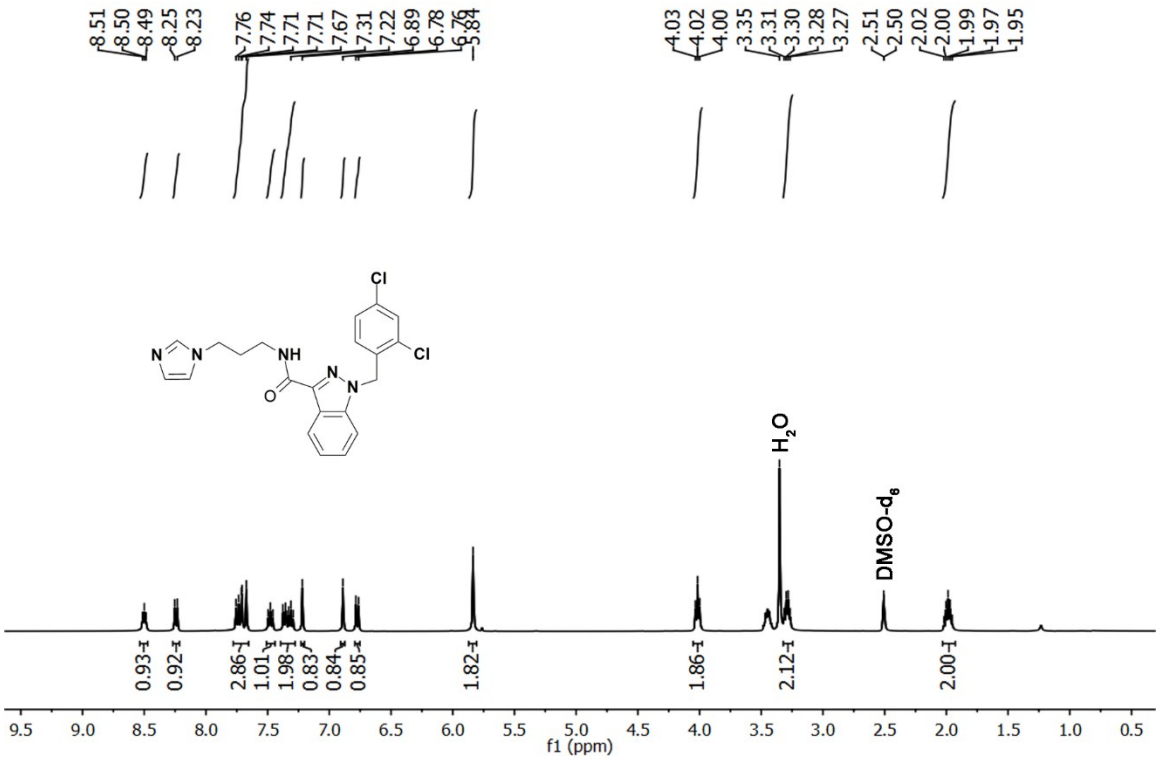


Fig. S1 ^{1}H NMR spectrum of **L** in DMSO-d_6.

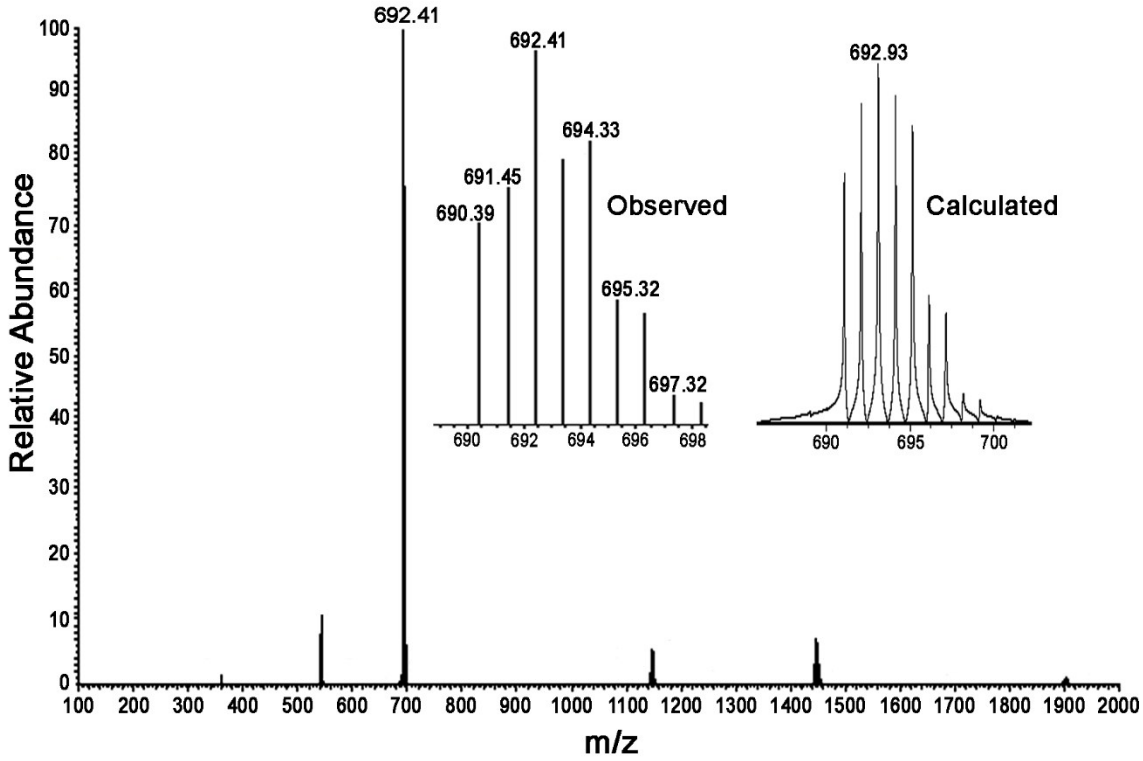


Fig. S2 ESI-MS spectrum of **MPL-I**.

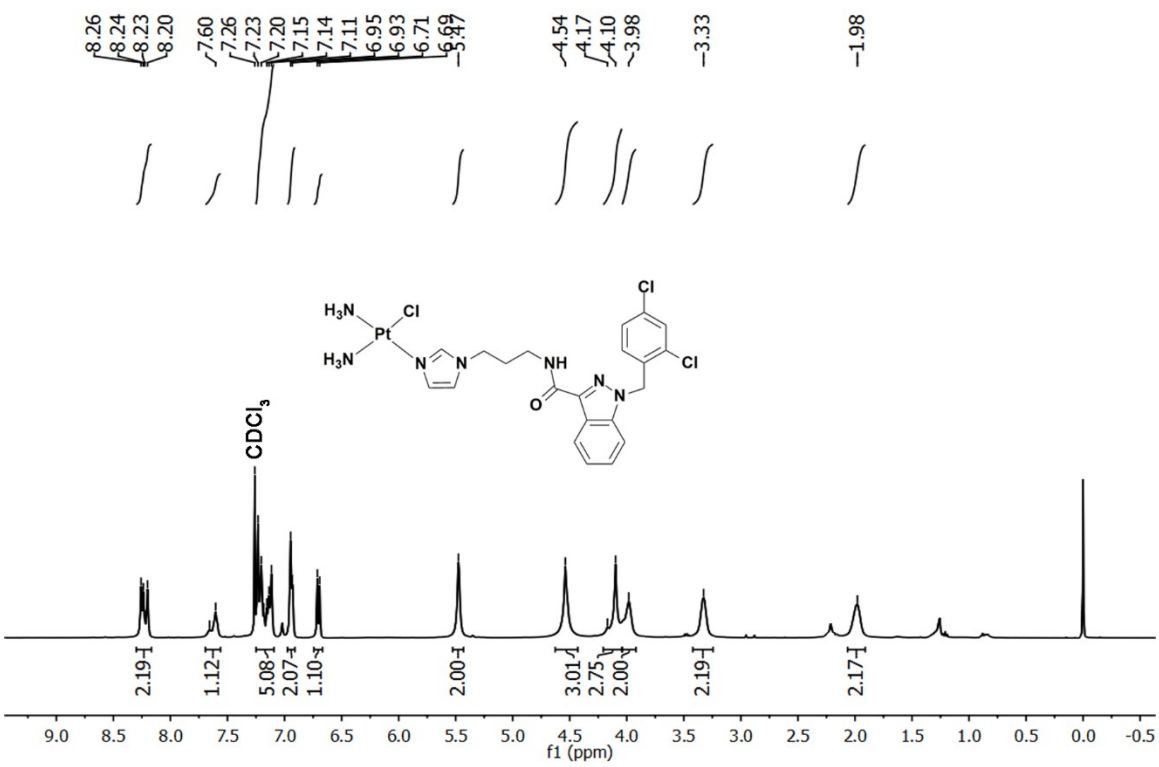


Fig. S3 ^{1}H NMR spectrum of **MPL-I** in $CDCl_3$.

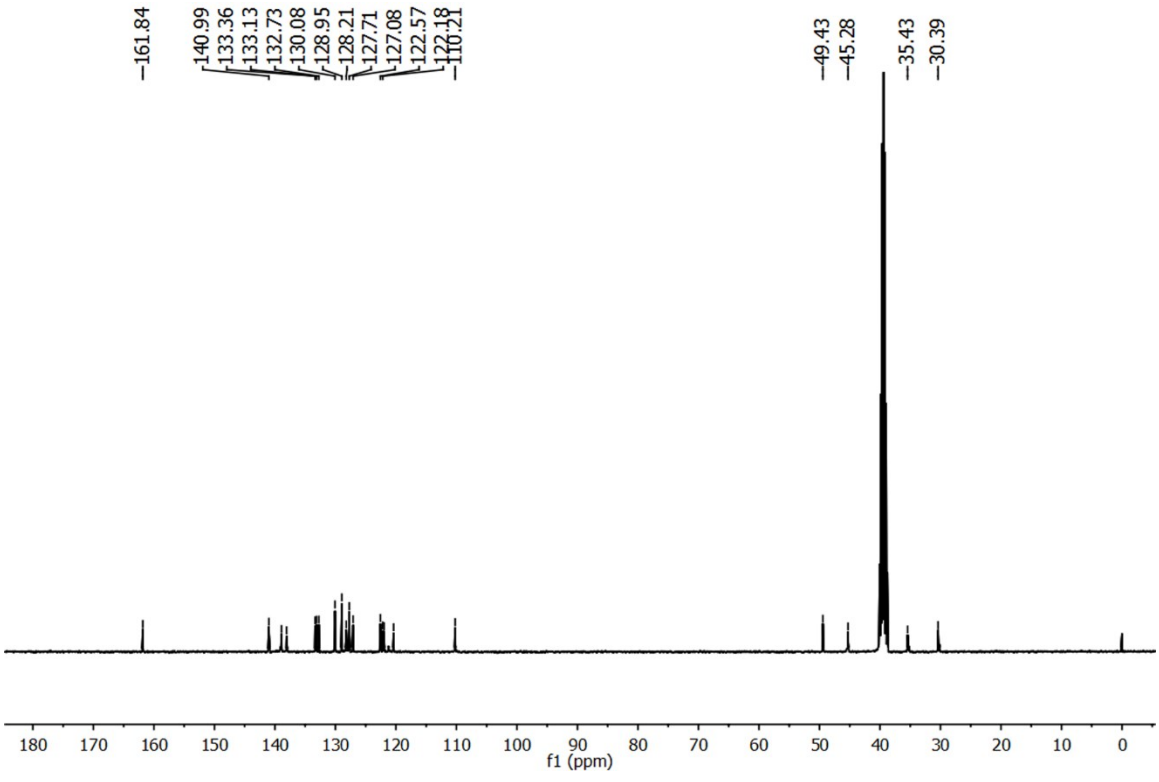


Fig. S4 ^{13}C NMR spectrum of **MPL-I** in DMSO-d_6.

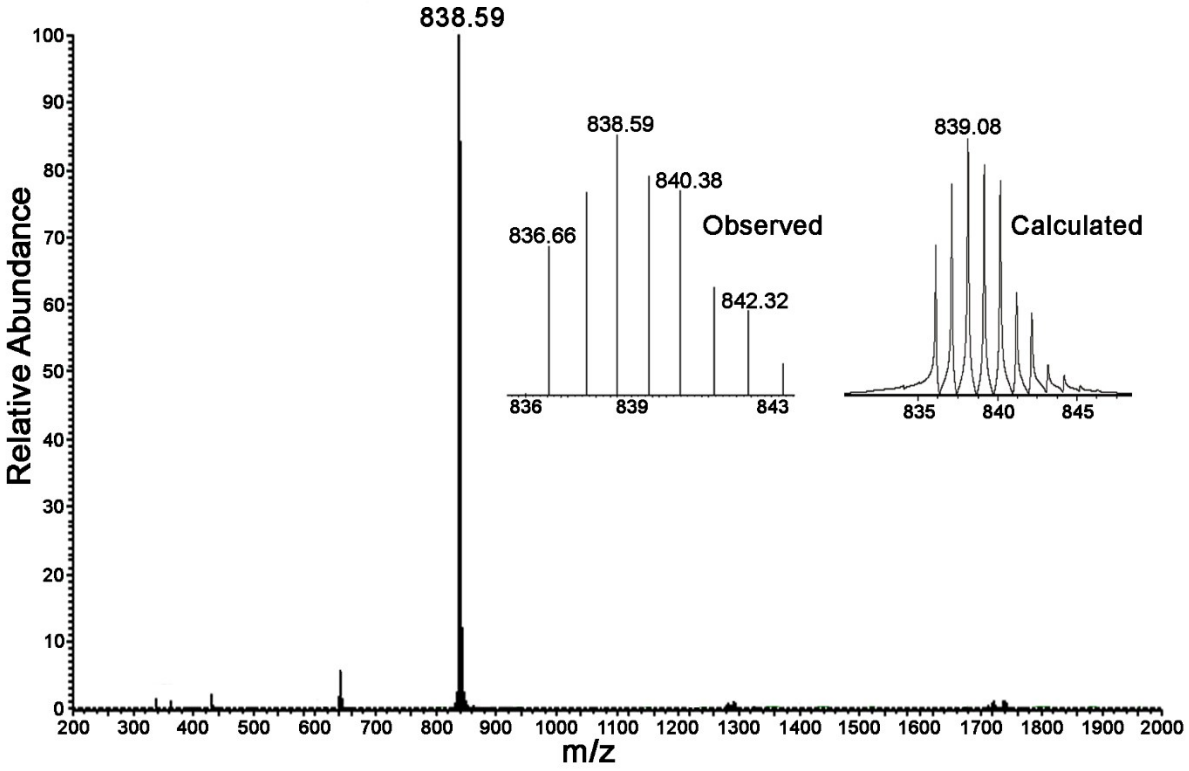


Fig. S5 ESI-MS spectrum of **MPL-II**.

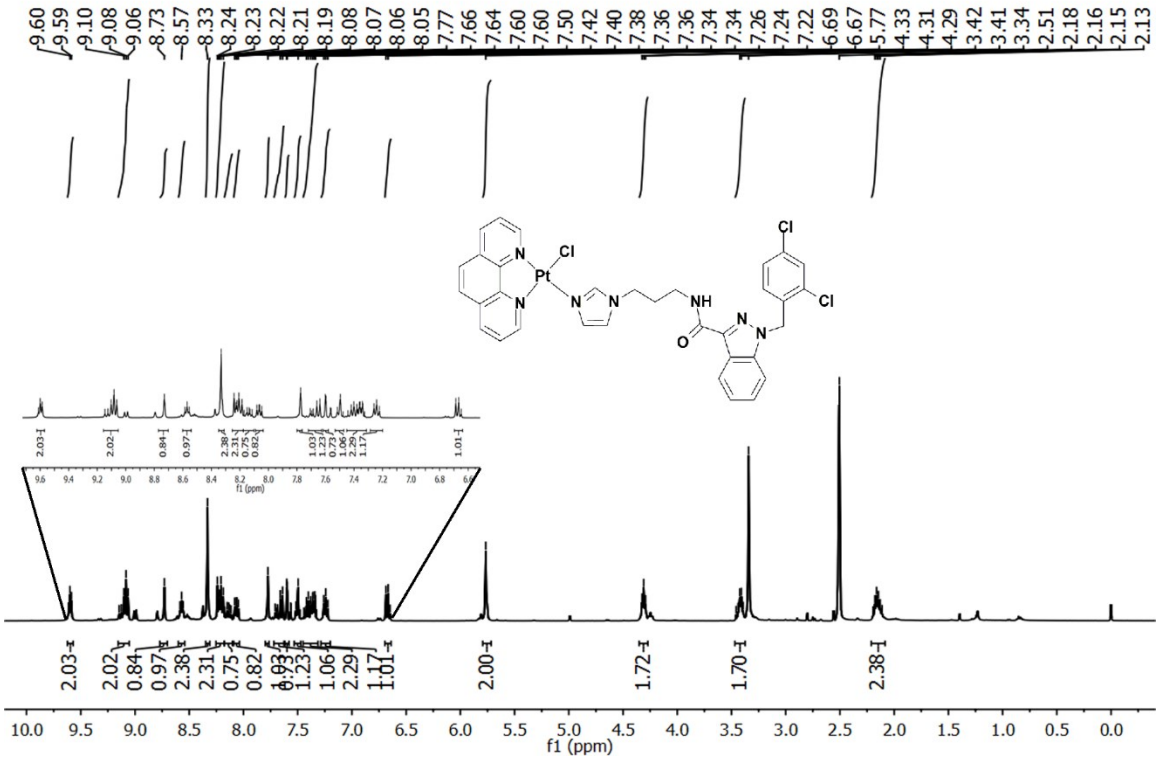


Fig. S6 ^{1}H NMR spectrum of **MPL-II** in DMSO-d_6.

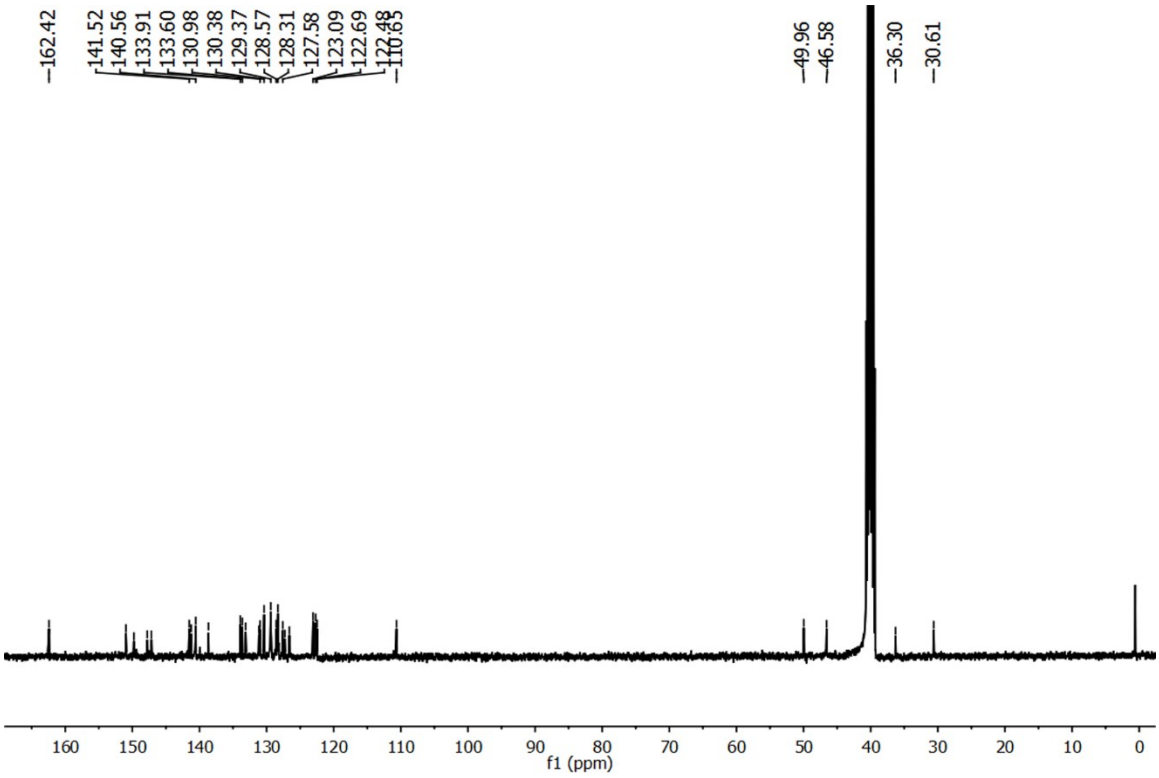


Fig. S7 ^{13}C NMR of **MPL-II** in DMSO-d_6

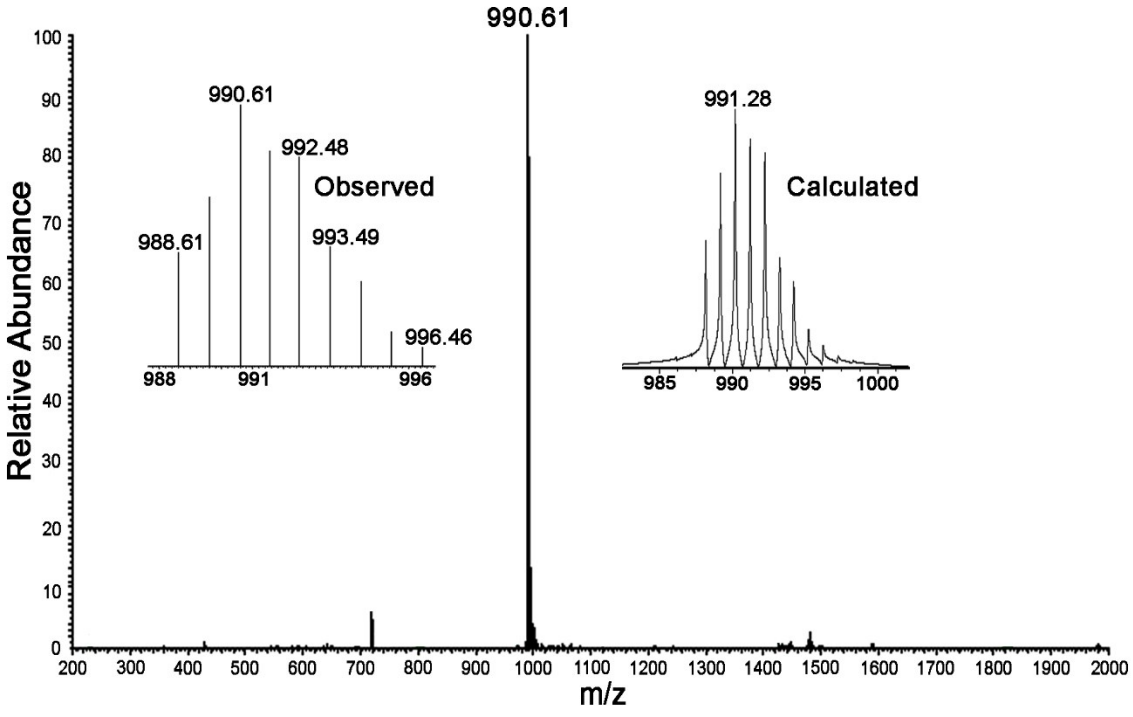


Fig. S8 ESI-MS Spectrum of **MPL-III**.

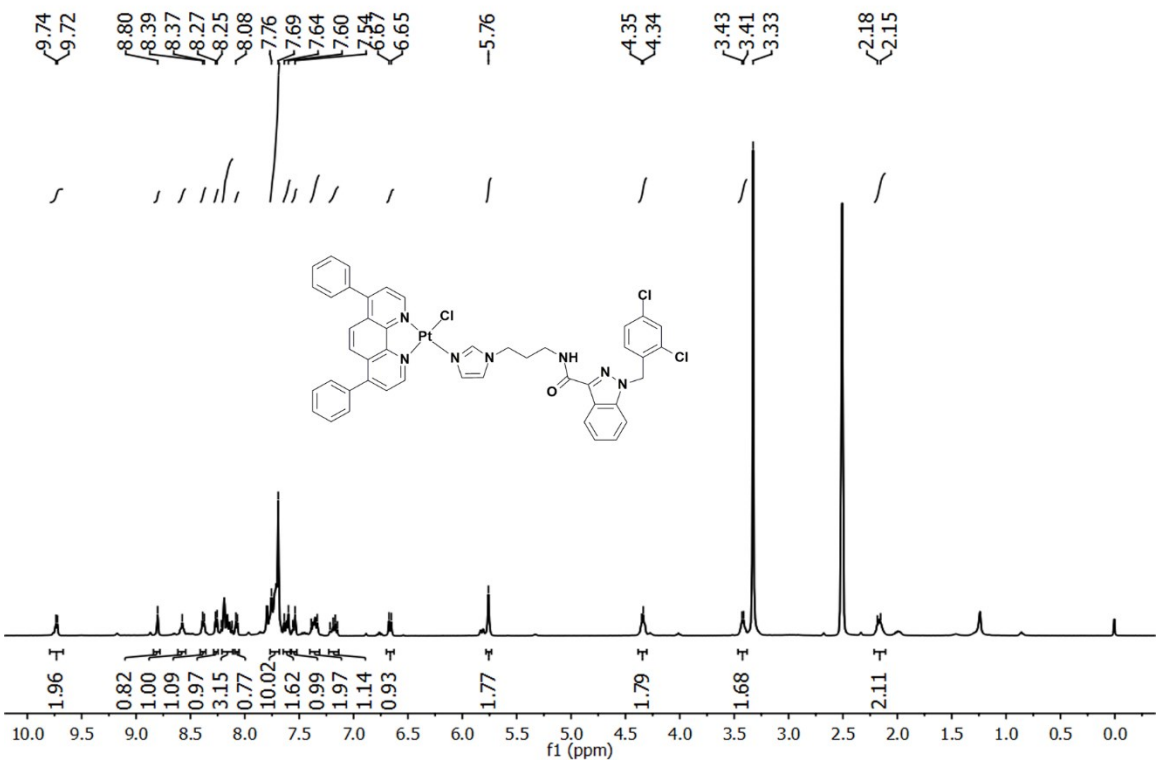


Fig. S9 ^{1}H NMR spectrum of **MPL-III** in DMSO-d6.

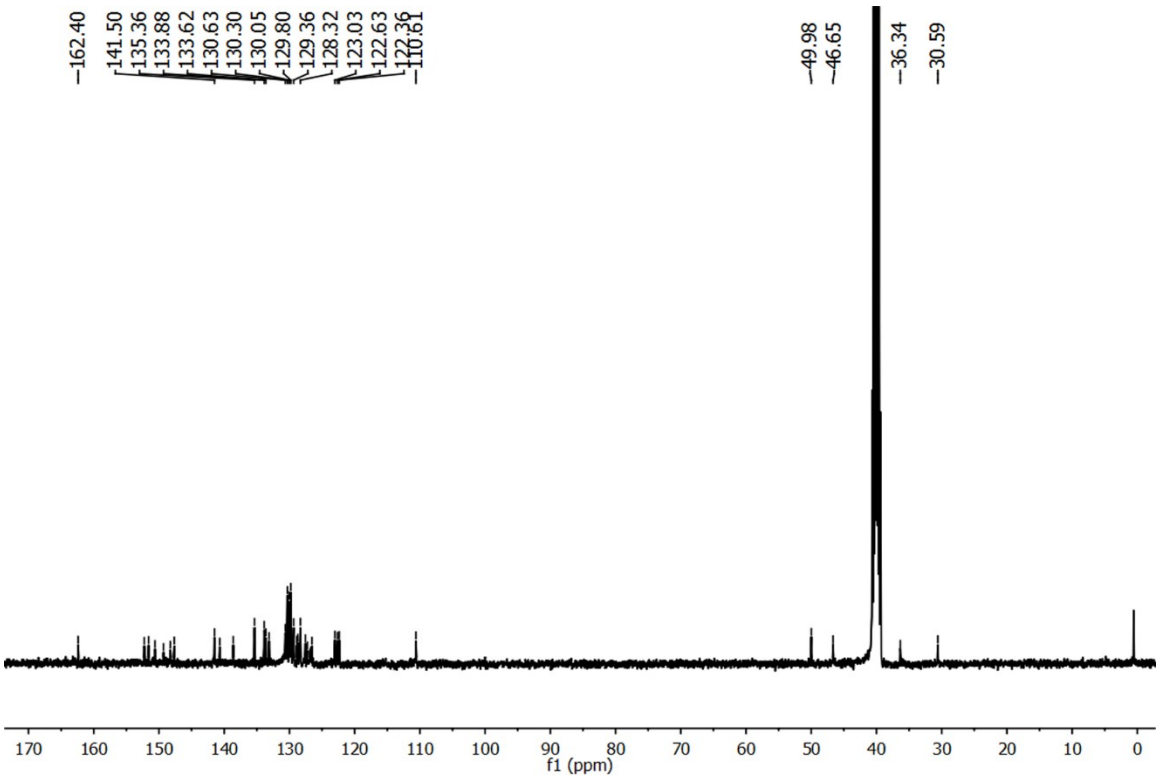


Fig. S10 ^{13}C NMR of **MPL-III** in DMSO-d_6.

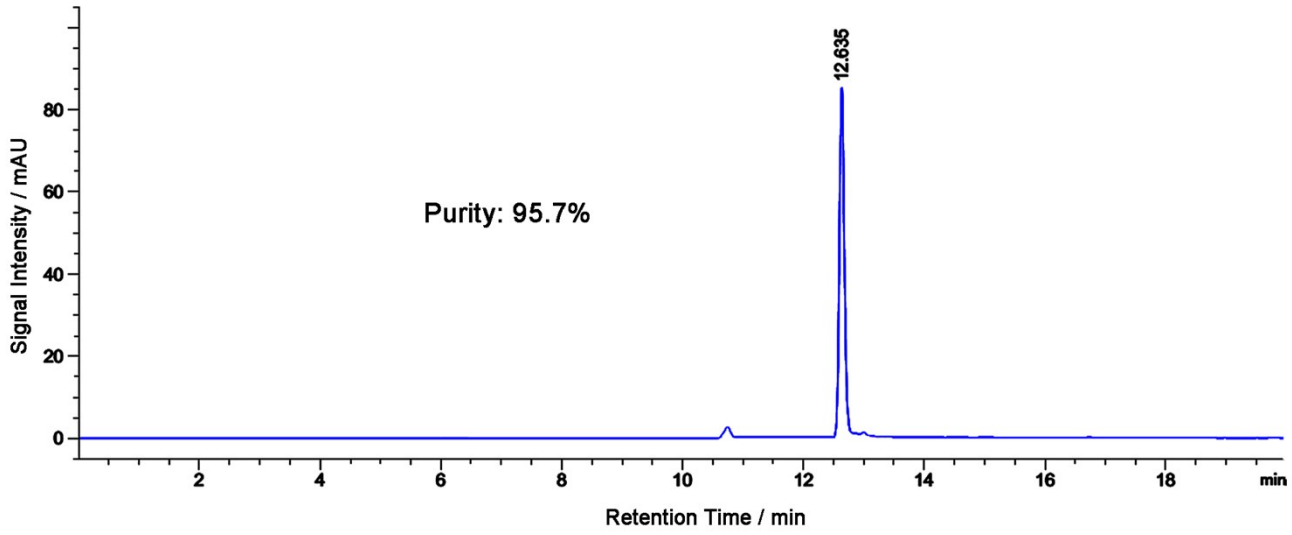


Fig. S11 HPLC chromatogram of **MPL-I**

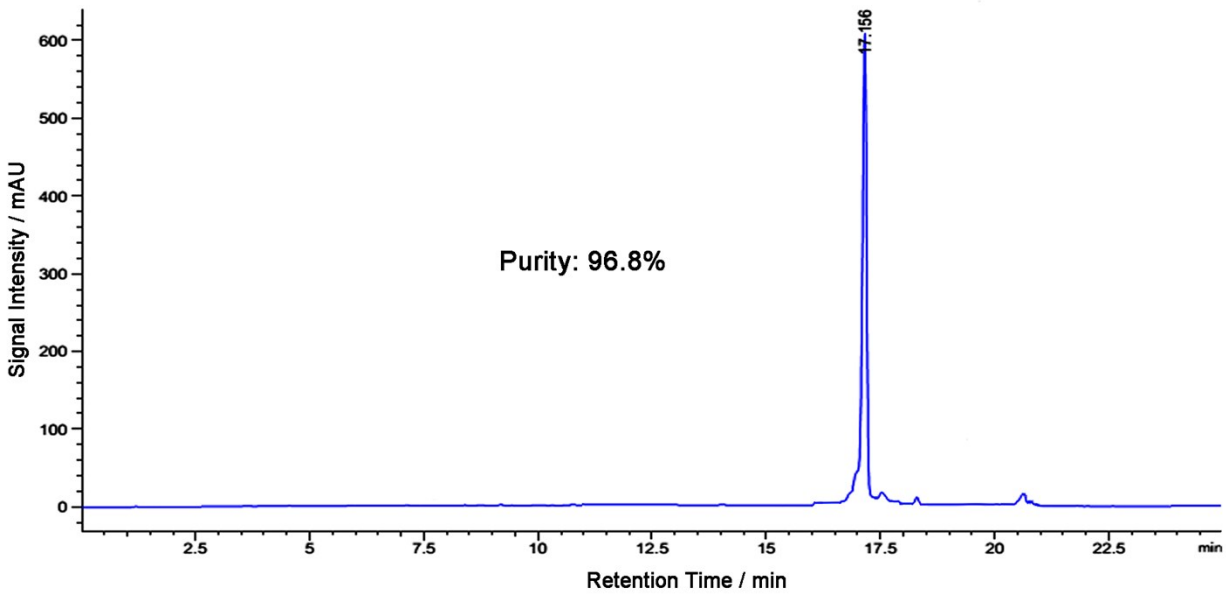


Fig. S12 HPLC chromatogram of **MPL-II**

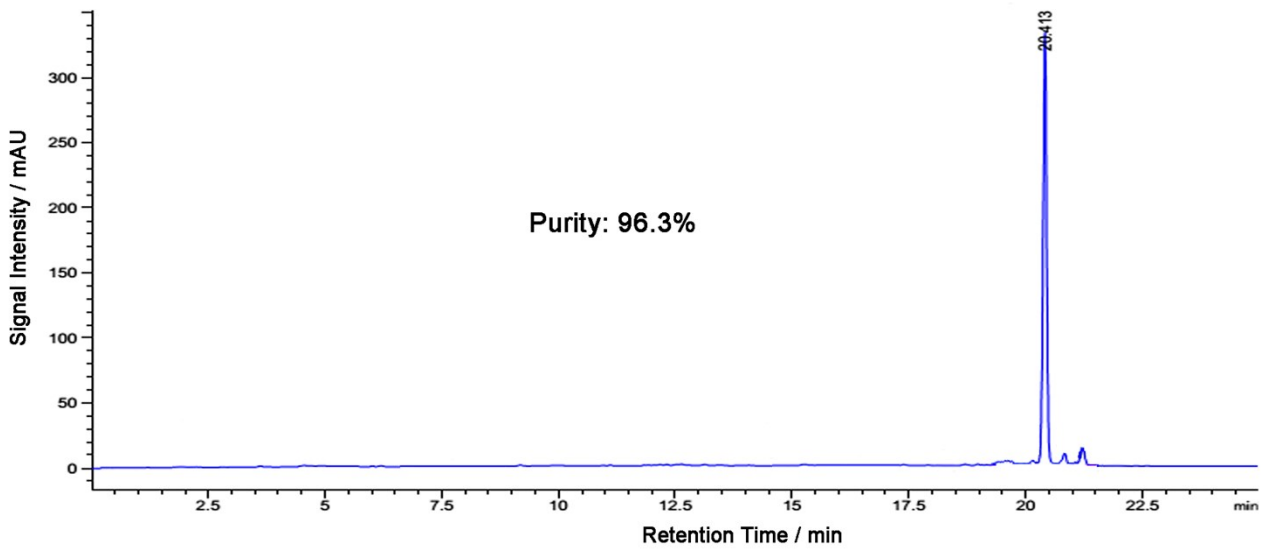


Fig. S13 HPLC chromatogram of **MPL-III**

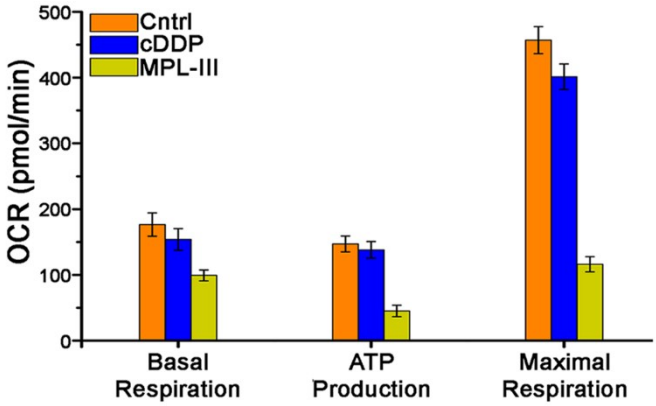


Fig. S14 Impact of cDDP and **MPL-III** on the key parameters of mitochondrial respiration calculated from Figure 3C. Cells were treated with cDDP (10 μM) or **MPL-III** (10 μM) for 24 h.

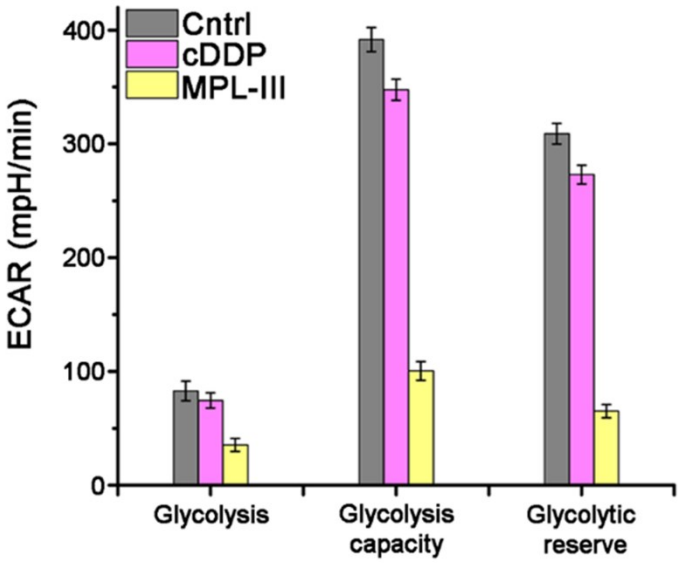


Fig. S15 Impact of cDDP and **MPL-III** on the key parameters of glycolysis calculated from Figure 3D. Cells were treated with cDDP (10 μM) or **MPL-III** (10 μM) for 24 h.

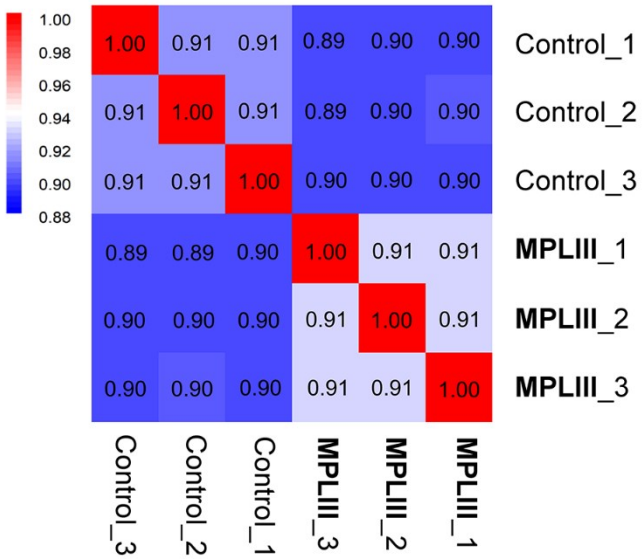


Fig. S16 Heat map diagram of Pearson correlation coefficient between the samples.

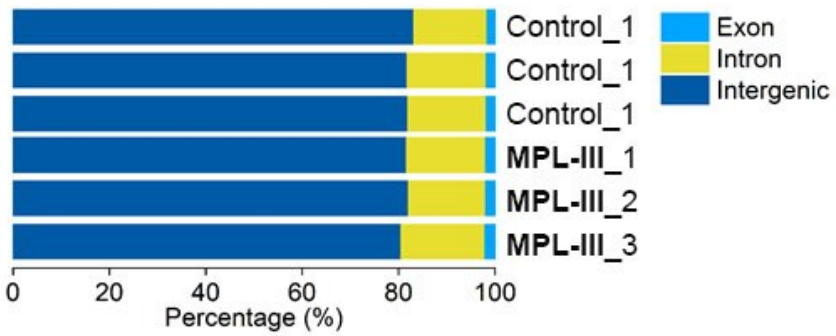


Fig. S17 The genomic location of mapped transcripts in **MPL-III** *vs* Control in MDA-MB-231 cells.

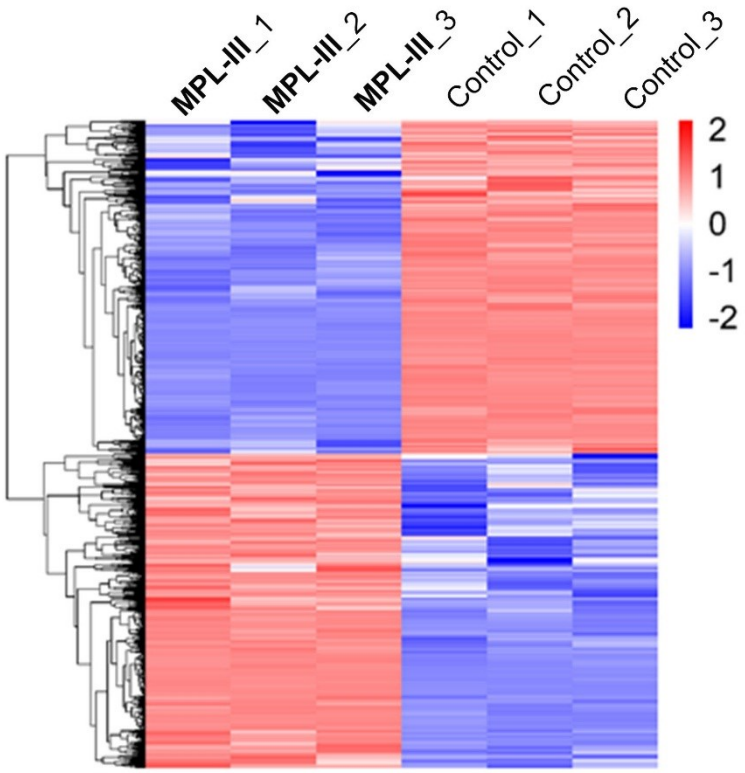


Fig. S18 Cluster analysis and Heatmap displays the overview of the differentially expressed genes induced by **MPL-III** treatment. Each column represents a sample, and each row represents a gene. Colors represent the expression level of the genes.

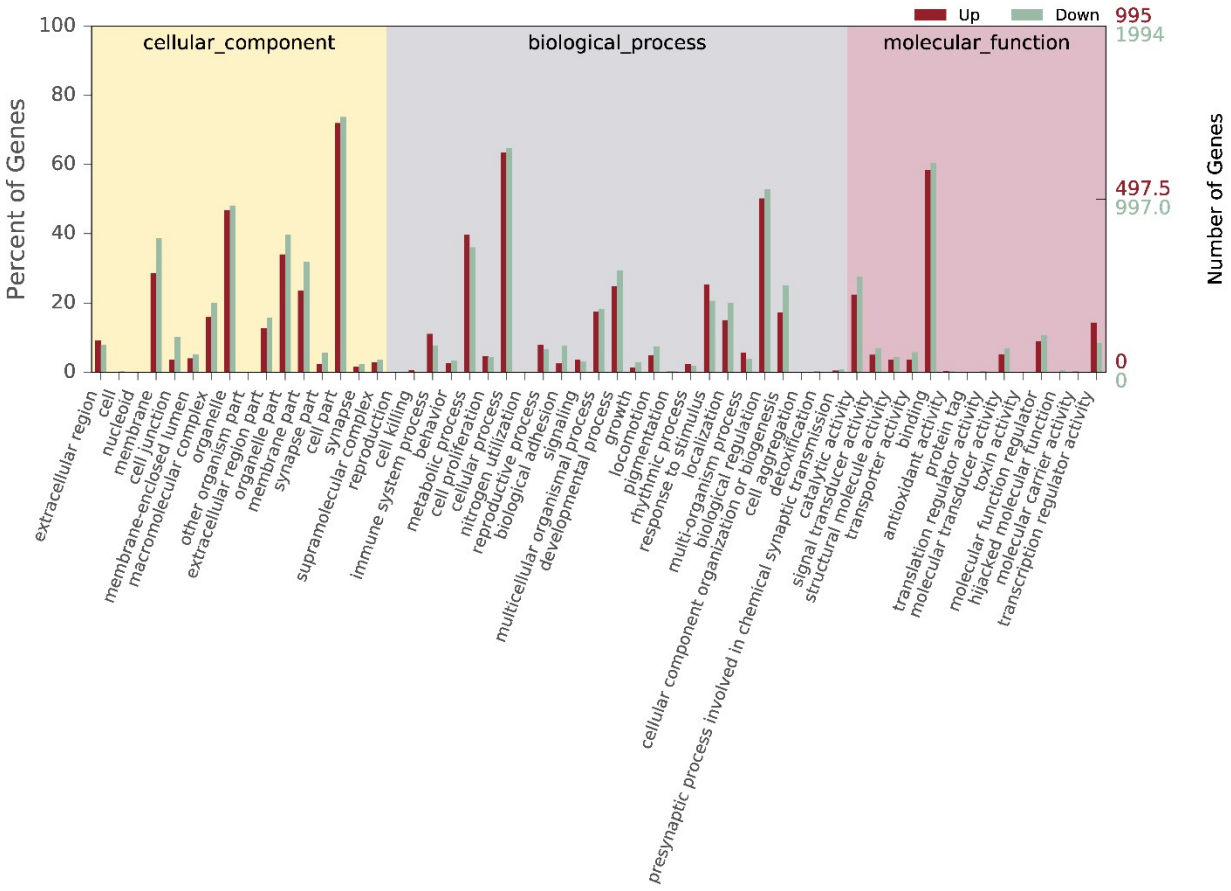


Fig. S19 Gene Ontology categorization of cellular component, biological process and molecular function for assembled unigenes of the transcriptome induced by **MPL-III** treatment.

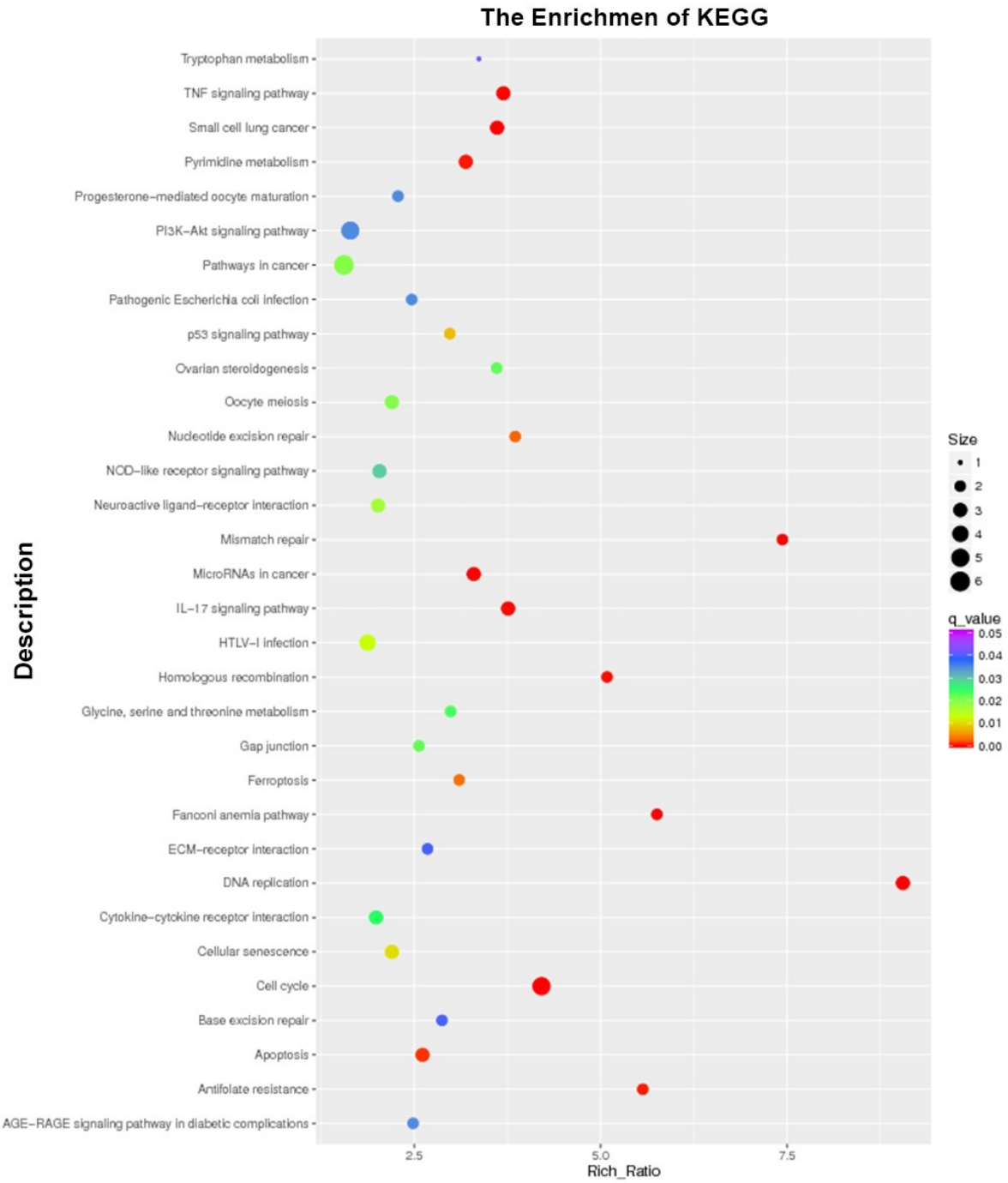


Fig. S20 Pathways mediated by **MPL-III** treatment identified by KEGG enrichment analysis (http://www.kegg.jp/kegg/kegg1.html).

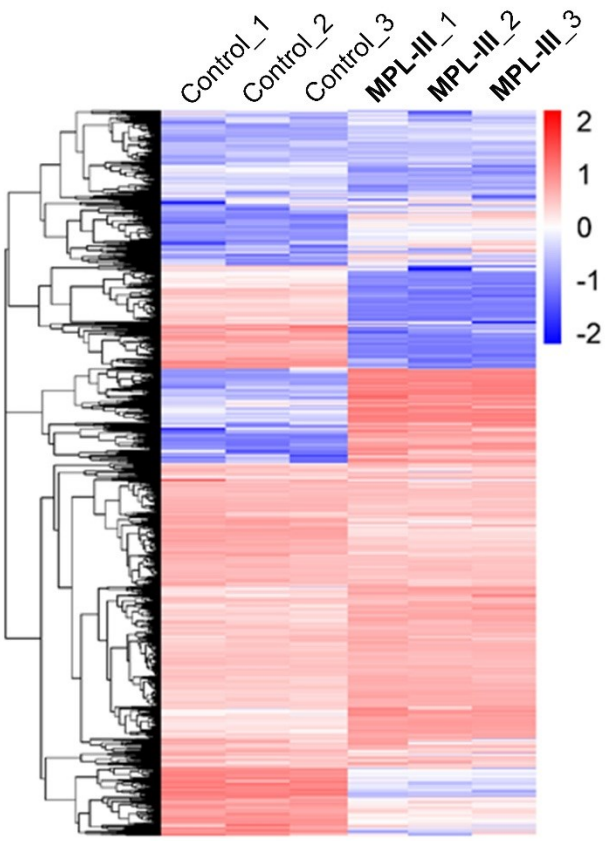


Fig. S21 Cluster analysis and Heatmap displays the overview of the differentially expressed transcriptional factor (TF) genes induced by **MPL-III** treatment. Each column represents a sample, and each row represents a gene. Colors represent the expression level of the genes.

Table S1. List of genes that are up-regulated or down-regulated upon treatment with **MPL-III**. These genes meet the selection threshold of fold change (FC) > 2 and $p < 0.05$.

Table S2. List of transcription factor (TF) genes that are up-regulated or down-regulated upon treatment with **MPL-III**. These genes meet the selection threshold of fold change (FC) > 2 and $p < 0.05$.

References

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