Electronic Supplementary Material (ESI) for Metallomics.

Electronic Supplementary Information (ESI)

Pharmaco-genomic Investigations of Organo-iridium Anticancer Complexes Reveal Novel Mechanism of Action

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Supplementary Material

Methods

Roswell Park Memorial Institute (RPMI-1640) medium, as well as foetal bovine serum, L-glutamine, penicillin/streptomycin mixture, trypsin, trypsin/EDTA, phosphate buffered saline (PBS) were purchased from PAA Laboratories GmbH. HPLC grade ethanol, β-mercaptoethanol, PI (>94%), Annexin V-FITC Apoptosis Detection Kit and RNAse A were obtained from Sigma Aldrich. For RNA sequencing, cell shredders and mini-prep kits were purchased from Qiagen.

Cell maintenance

The A2780, human ovarian carcinoma cell line was obtained from the European Collection of Cell Cultures (ECACC). Cells were grown in RPMI-1640 medium supplemented with 10% (v/v) foetal calf serum, 1% (v/v) 2 mM glutamine and 1% (v/v) penicillin (10 k units/mL)/streptomycin (10 mg/mL). All cells were maintained in 75 mL culture flasks at 310 K with 5% CO_2 humidified atmosphere. Cells were grown as adherent monolayers and split when 80-90% confluent, using 0.25% trypsin.

Screening in the Sanger cell panel

Briefly, cells were seeded in 96 well plates at ca.15% confluency and left to incubate for 24 h at 310 K, 5% CO_2, 95% air and 100% relative humidity. For adherent cell lines, cells were treated with nine concentrations of each compound (2-fold dilution series over 256-fold concentration range) and returned to the incubator for 72 h. Cells were then fixed with 4% formaldehyde for 30 min and stained with 1 μM Syto60 for 1 h. Quantitation of fluorescent signal intensity was performed using a plate reader at excitation/emission wavelengths of 630/695 nm. For suspension cell lines, cells were treated with compound immediately following plating, and returned to the incubator for 72 h. Cells were stained with 55 μg/mL Resazurin, prepared in glutathione-free medium, for 4 h. Quantitation of fluorescent signal intensity was performed using a plate reader at excitation/emission wavelengths of 535/595 nm. MANOVA analysis was performed by the Sanger Bioinformatics Institute. All Figures presented here were reconstructed using the R statistical programme.

RNA sequencing

Experimental

A2780 cells were seeded in P100 Petri dishes at 3 x 10^6 cells per plate in 10 mL RPMI-1640 medium. Plates were incubated for 24 h at 310 K, 5% CO_2, 95% air and 100% relative humidity. Stock solutions of each compound and of the vehicle control were prepared in 5% (v/v) DMSO, 10% (v/v) saline, and 85% (v/v) RPMI-1640 medium. Cells were exposed to complex **2** at a final concentration of 400 nM. The final DMSO concentration for all cell samples did not exceed 0.05% v/v. After compound addition, cells were incubated for a further 4, 12, 24 and 48 h. Medium was aspirated from cells and cells were washed twice with PBS before trypsinising and collection. To each sample, 600 µL RLT lysis buffer (Qiagen) was added and the samples vortexed. Lysate was pipetted directly into QIAshredder spin columns (Qiagen) and centrifuged. Lysate was transfered to gDNA eliminator spin columns (RNeasy plus mini kit, Qiagen) and centrifuged. Columns were discarded and 600 uL of 70% ethanol was added to each sample flow-through. Samples were transfered into RNeasy spin columns (RNeasy plus mini kit, Qiagen) and centrifuged. Column-bound RNA samples were washed with RW1 and RPE buffer (RNeasy plus mini kit, Qiagen) before RNA collection in 70 µL RNAse-free water. Samples were stored at 193 K for no more than 2 months. Samples were diluted 1:10 fold in RNAse-free water and run on a NanoDrop 1000 spectrophotometer machine and the absorbance at 230, 260 and 280 nm recorded to calculate the 260/230 and 260/280 ratios. Samples with A260/230 >2.0 and A260/280 >1.9 were passed. The concentration of RNA in each solution was also estimated using the NanoDrop and was verified using a 2100 Agilent Bioanalyzer and an RNA 6000 Nano Kit (Agilent), and the Qubit assay (Life Technologies). All samples had a RNA integrity number (RIN) > 9.50. A minimum of 1 µg RNA for each sample was transferred to Oxford Genomics Centre (Wellcome Trust Centre for Human Genetics) in a total of 30 µL RNAse-free water in skirted 96 well plates.

Reverse phase protein microarrays (RRPA)

4 x 10^5 A2780 cells were seeded per well in 6-well plates, with samples in duplicate. Cells were pre-incubated in drug-free media for 48 h at 310 K in a

5% CO_2 humidified atmosphere. After this, cells were treated at 150 nM and 450 nM of complex **2** for 4, 24, 48 and 72 h. Control samples were treated with medium containing 0.1% DMSO. Following exposure, drug-containing medium was removed, and cells were washed twice with PBS and lysed with CLB1 buffer (Zeptosens-Bayer) according to manufacturer's instructions. Cell lysates were normalised to a uniform protein concentration of 2 mg/mL with CLB1 buffer (Zeptosens-Bayer) prior to preparing a final 4-fold concentration series of; 0.2; 0.15; 0.1 and 0.75 mg/mL in spotting buffer CSBL1 (Zeptosens-Bayer). The diluted concentration series of each sample was printed onto hydrophobic Zeptosens protein microarray chips (ZeptoChip™, Zeptosens-Bayer) under environmentally controlled conditions (constant 50% humidity at 287 K) using a non-contact printer (Nanoplotter 2.1e, GeSiM). A single 400 pL droplet of each lysate concentration was deposited onto the Zeptosens chip. A reference grid of Alexa Fluor 647 conjugated BSA was spotted onto each sub-array, each sample concentration series was spotted in between reference columns. After array printing, the arrays were blocked with an aerosol of BSA solution using a custom designed nebuliser device (ZeptoFOG™, Zeptosen-Bayer) for 1.5 h to prevent non-specific antibody binding. The protein array chips were subsequently washed in double deionised water (DDW) and dried prior to performing a dual antibody immunoassay comprising of a 24 h incubation of primary antibodies followed by 2.5 h incubation with secondary Alexa Fluor 647 conjugated antibody detection reagent (anti-rabbit or anti-mouse 647 Fab, Invitrogen). Following secondary antibody incubation and a final wash step in BSA solution, the immunostained arrays were imaged using the ZeptoREADER instrument (Zeptosens-Bayer). For each-sub-array, five separate images were acquired using different exposure times ranging from 0.5-10 s. Microarray images representing the longest exposure without saturation of fluorescent signal detection were automatically selected for analysis using the ZeptoView™ 3.1 software. A weighted linear fit through the 4-fold concentration series was used to calculate the relative fluorescence intensity (RFI) value for each sample replicate. Local normalisation of sample signal to the reference BSA grid was used to compensate for any intra- or inter-array/chip variation. RFI values were further normalised to a house keeping protein and to the negative

control, to provide the final RFI to represent the relative abundance of total, phosphorylated and cleaved proteins in compound-treated samples relative to the DMSO control for each time point.

Table S1. Summary statistics for RNA sequencing experiment.

Description	Yield Mb Q20	% Mapped	Avg. Quality score
4 h control	975.855	98.45	36.7
4 h control	901.135	98.6	36.65
4 h control	701.445	98.9	36.8
4 h drug	1016.67	99.1	36.7
4 h drug	640.475	98.75	36.85
4 h drug	901.065	98.1	36.65
12 h control	968.815	98.65	36.65
12 h control	1208.1	97	36.25
12 h control	658.935	98.75	37.25
12 h drug	710.245	97.8	37.1
12 h drug	651.6	98.75	37.3
12 h drug	728.13	98.9	37.2
24 h control	591.46	98.9	37.3
24 h control	763.42	98.25	37.2
24 h control	663.875	98.6	37.2
24 h drug	794.815	98.2	37.2
24 h drug	754.52	98.65	36.95
24 h drug	864.57	97.5	36.85
48 h control	876.415	98.15	36.95
48 h control	1003.08	97.95	37
48 h control	853.78	98.25	37
48 h drug	894.37	95.55	36.8
48 h drug	811.94	97.3	36.9
48 h drug	1026.57	97.6	36.8

Table S2. Pathway analysis showing the top five most-mapped processes for DEGs with -1.0 < LogFC > 1.0 and FDR < 0.05 after exposure to **2**. IPA has identified pathways of interest, with associated significance *p*- and z-values.

Time point (h)	Upstream regulator	p-value	Activation z-score	Predicted activity
4	TNF	3.25×10^{-15}	3.676	Activated
	LY294002	2.67×10^{-14}	-2.413	Inhibited
	PDGF BB	2.15×10^{-13}	4.766	Activated
	TGFβ1	2.62×10^{-13}	3.063	Activated
	ERK	1.31×10^{-12}	4.221	Activated
12	TNF	1.48×10^{-14}	3.238	Activated
	UO126	2.19×10^{-11}	-2.470	Inhibited
	LY294002	2.34×10^{-11}	-2.466	Inhibited
	beta-estradiol	3.29×10^{-11}	-0.400	-
	TREM1	6.67×10^{-11}	1.771	Activated
24	TGFβ1	1.30×10^{-24}	2.206	Activated
	TNF	3.94×10^{-22}	6.101	Activated
	LPS	7.13×10^{-22}	7.223	Activated
	PDGF BB	1.33×10^{-21}	5.287	Activated
	beta-estradiol	3.42×10^{-21}	2.230	Activated
48	TNF	4.93×10^{-12}	3.646	Activated
	IL1β	7.74×10^{-09}	3.343	Activated
	LPS	1.87×10^{-09}	2.211	Activated
	ESR1	3.17×10^{-09}	0.282	-
	TGFβ1	3.48×10^{-09}	1.717	Activated

Table S3. Generation of total ROS and superoxide by complex **2** in A2780 ovarian carcinoma cells exposed to IC_{50} concentrations. Values obtained from triplicate experiments. Determination of statistical significance by two-sample independent Welch t-test assuming unequal variance: $p \leq 0.05$ *, $p \leq 0.01$ **, $p \leq 0.001$ ***, $p \leq 0.0001$ ****.

	High Superoxide	**High ROS and Superoxide**	**High ROS**	**Low ROS and Superoxide**
	FL1-FL2+	**FL1+FL2+**	**FL1+FL2-**	**FL1-FL2-**
	Q1	**Q2**	**Q3**	**Q4**
Neg CTL	0.17 ± 0.07	0	0	99.82 ± 0.07
Complex 2	0.1 ± 0.1	84 ± 1 ****	16 ± 1 ****	0.3 ± 0.5

Table S4. List of publicly-released compounds tested by the Sanger Institute in the cell line screen

681640
(5Z)-7-Oxozeaenol
17-AAG
A-443654
A-770041
ABT-263
ABT-869
ABT-888
AC220
AG-014699
AICAR
AKT inhibitor VIII
AMG-706
AP-24534
AR-42
AS601245
AS605240/KIN001-173
AT-7519
ATRA
AUY922
AV-951
AX11492
Axitinib
AZ628
AZD-0530
AZD-2281
AZD2281
AZD6244
AZD6244
AZD6482
AZD6482
AZD7762
AZD8055
BAY 61-3606
Bexarotene
BI-2536
BIBW2992
Bicalutamide
BIRB 0796
BIX02189
Bleomycin
BMN-673
BMS-345541
BMS-509744
BMS-536924
BMS-708163
BMS-754807
Bortezomib
Bosutinib
Bryostatin 1
BX-795
CAL-101
Camptothecin
CAY10603
CCT007093
CCT018159
CEP-701
Cetuximab
CGP-082996
CGP-60474
CH5424802
CHIR-99021
CHIR-99021
CI-1040
Cisplatin
CMK
CP466722
CP724714
CUDC-101
CX-5461
Cyclopamine
Cytarabine
Dasatinib
DMOG
Docetaxel
Doxorubicin
EHT 1864
EKB-569
Elesclomol
Embelin
Epothilone B
Erlotinib
Etoposide
EX-527
FH535
FK866
FR-180204
FTI-277
GDC-0449
GDC0941
GDC0941
Gefitinib
Gemcitabine
Genentech Cpd 10
GNF-2
GSK-1904529A
GSK-650394
GSK1070916
GSK1120212
GSK2118436
GSK2126458
GSK269962A
GSK429286A
GSK690693
GW 441756
GW843682X
HG-5-113-01
HG-5-88-01
HG-6-64-1 (KIN001-206)
I-BET
Imatinib
INCB-18424
IPA-3
JNJ-26854165
JNK Inhibitor VIII
JNK-9L
JQ1
JQ12
JW-7-24-1
JW-7-52-1
KIN001-055
KIN001-102
KIN001-135
KIN001-167/ZSTK474
KIN001-175/BX-912
KIN001-201/TAK-715
KIN001-236
KIN001-242/FMK
KIN001-244
KIN001-260
KIN001-266
KIN001-270
KU-55933
Lapatinib
LAQ824
Lenalidomide
LFM-A13
LY317615
Masitinib
Methotrexate
MG-132
Midostaurin
Mitomycin C
MK-2206
MLN4924
MP470
MPS-1-IN-1
MS-275
NG-25
Nilotinib
NPK76-II-72-1
NSC-207895
NSC-87877
NU-7441
Nutlin-3a

NVP-BEZ235
NVP-BHG712
NVP-TAE684
Obatoclax Mesylate
OSI-027
OSI-906
OSI-930
OSU-03012
PAC-1
Paclitaxel
Parthenolide
Pazopanib
PD-0325901
PD-0332991
PD-173074
PF-02341066
PF-4708671
PF-562271
PHA-665752
PHA-793887
PI-103
PIK-93
piperlongumine
PLX4720
PLX4720 (for rescreen control)
"PXD101, Belinostat"
Pyrimethamine
QL-VIII-58
QL-X-138
QL-XI-92
QL-XII-47
QL-XII-61
QS11
Rapamycin
RDEA119
RDEA119
RO-3306
Roscovitine
rTRAIL
S-Trityl-L-cysteine
Salubrinal
SB 216763
SB-505124
SB-715992
SB52334
SB590885
Shikonin
SL 0101-1
SN-38
SNX-2112
Sorafenib
STF-62247
Sunitinib
T0901317
Tamoxifen
Temozolomide
Temsirolimus
TG101348
TGX221
Thapsigargin
THZ-2-102-1
THZ-2-49
Tipifarnib
TL-1-85
TL-2-105
TPCA-1
Tubastatin A
TW 37
UNC0638
UNC0638
Vinblastine
Vinorelbine
VNLG/124
Vorinostat
VX-11e
VX-680
VX-702
WH-4-023
WZ-1-84
WZ3105
XAV 939
XL-184
XL-880
XMD11-85h
XMD13-2
XMD14-99
XMD15-27
XMD8-85
XMD8-92
Y-39983
YK 4-279
YM155
YM201636
Z-LLNle-CHO
ZG-10
"Zibotentan, ZD4054"
ZM-447439

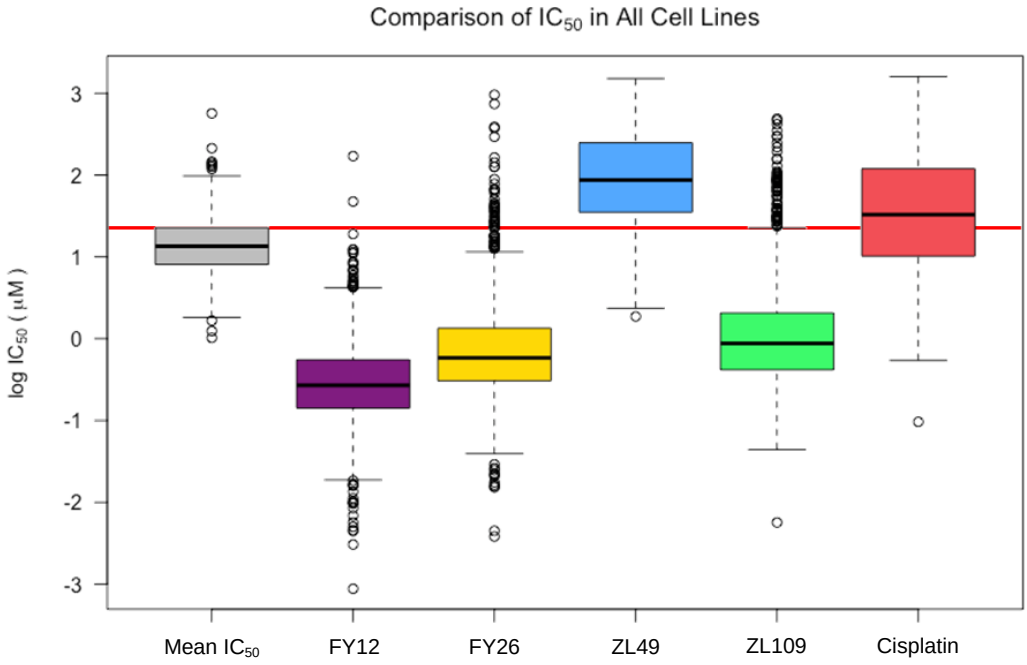

Figure S1. Box and whisker plot showing the distribution of log IC_{50} values for complexes **1** (ZL49) (blue), **2** (ZL109) (green) and CDDP (red) in all cell lines as well as the distribution of the mean log IC_{50} values for 202 drugs in the screen (grey). Whiskers extend to whichever is the lower value of the upper/lower quartile +1.5x the interquartile range, or the maximum/minimum y value, respectively. Cell lines which are less sensitive to **2** are highlighted in a red box. Data for osmium complexes **3** (FY26) and **4** (FY12) are also shown for comparison. For structures see Figure 1.

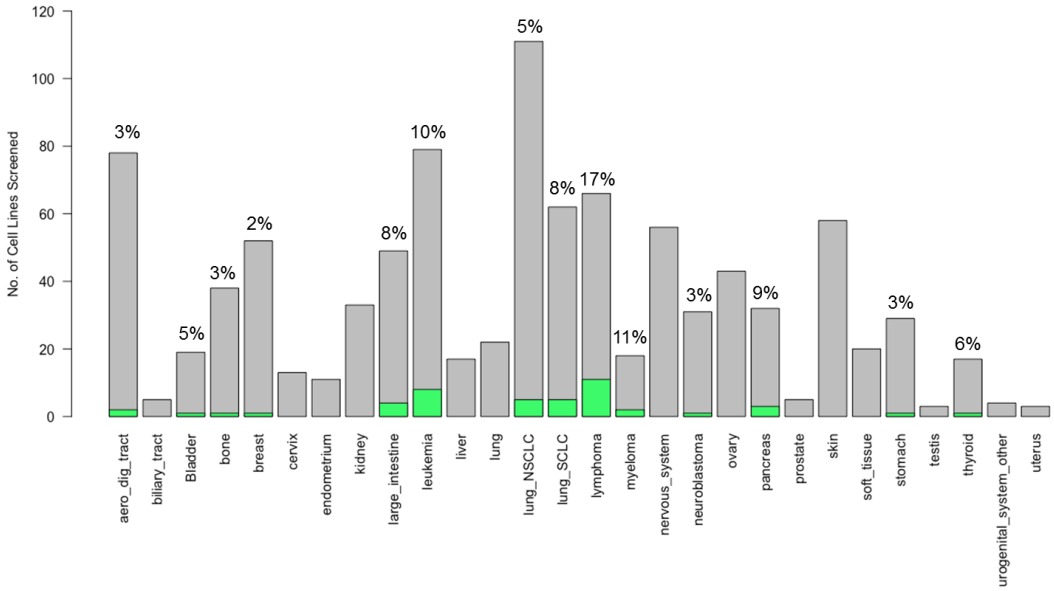

Figure S2. Bar plot of the number of cell lines of each tissue type screened against organo-iridium complex **2.** Cell lines significantly insensitive to **2** highlighted in green with the corresponding % of total cell lines of that type. Tissue groups where no percentage is given contained no cell lines resistant to **2**.

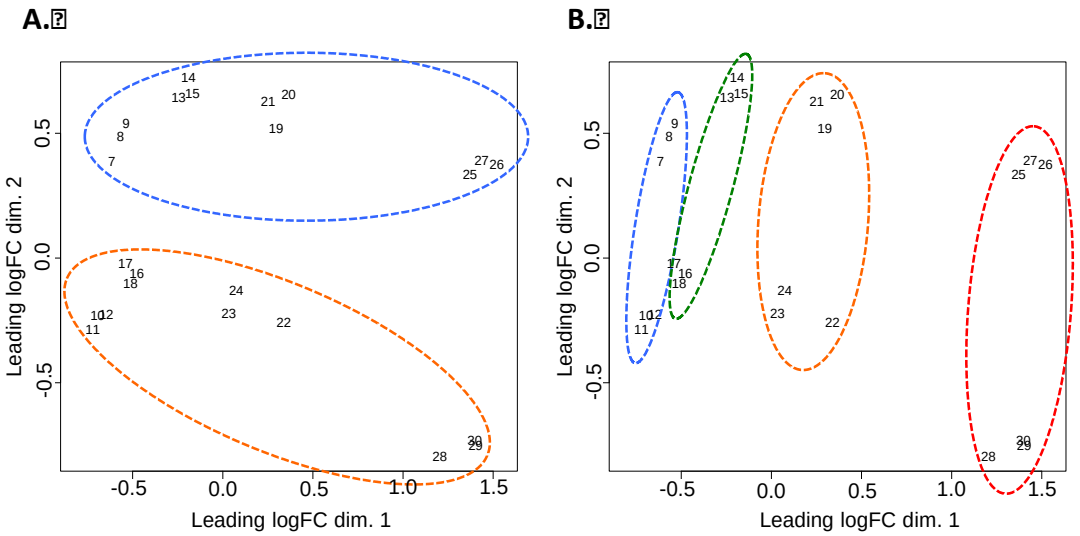

Figure S3. Multidimensional scaling (MDS) plots for RNA sequencing data. **(A)** Samples grouped as control (blue) and **2**-exposed (orange), demonstrating a differential drug-induced response. **(B)** Grouping of samples across the time series, with 4 h control and **2**-exposed samples in blue, 12 h in green, 24 h in orange and 48 h in red The contrasting behavior of the 48 h datasets compared to earlier time points is evident.

Figure S3 shows natural separation of the samples into clusters, and good agreement between the triplicate measurements. The biggest source of variation is by time point, the second by exposure-status, i.e. whether they are exposed as a control or to a compound.

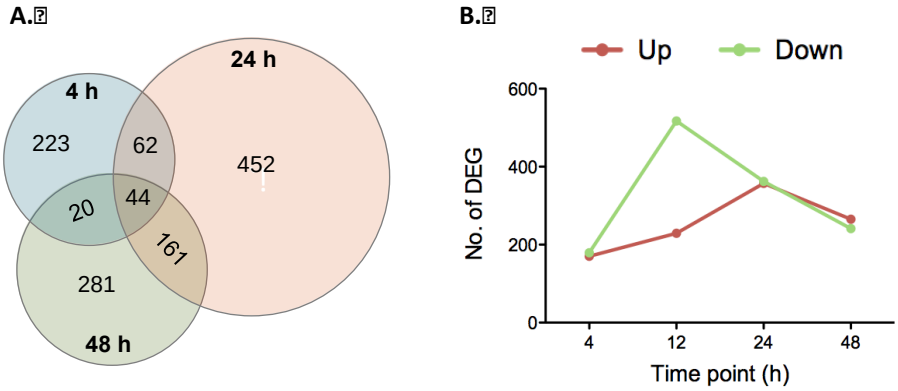

Figure S4. (A) Venn diagram showing the number of differentially-expressed genes at 4, 24 and 48 h after exposure to **2**. Only those genes with -1.0 < LogFC > 1.0 and FDR < 0.05 are included. **(B)** Graph showing the number of up- (red) and down-regulated (green) genes at each time point. Only those genes with -1.0 < LogFC > 1.0 and FDR < 0.05 are included.

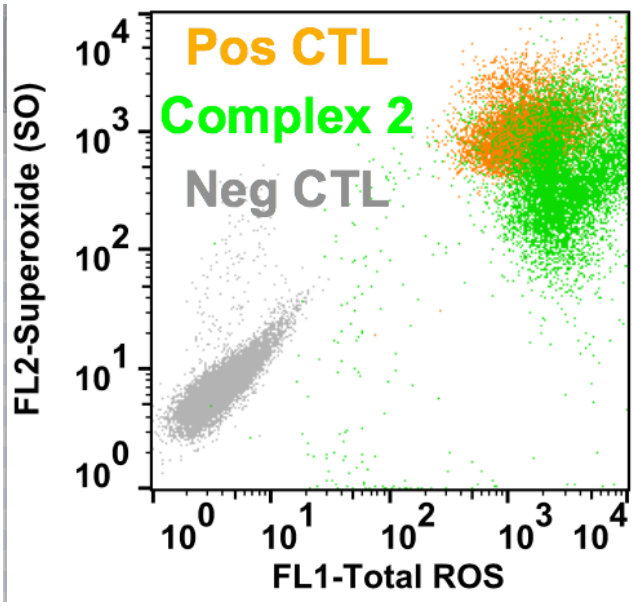

Figure S5. Generation of reactive oxygen species (ROS) and superoxide (SO) analysis by flow cytometry of A2780 ovarian carcinoma cells exposed to complex **2** for 24 h at IC_{50} concentration at 310 K. Cells stained with orange/green fluorescent reagents. Pyocyanin was the positive control (orange).

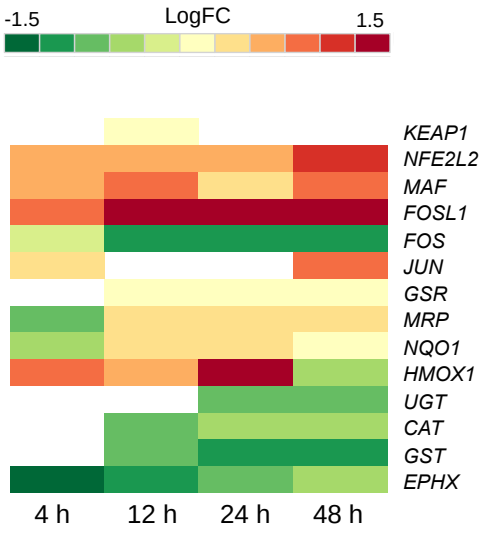

Figure S6. Heat map of DEGs In the oxidative stress response pathway in response to **FY26** (complex **3**) published previously.[1] Only DEGs with FDR < 0.10 are included.

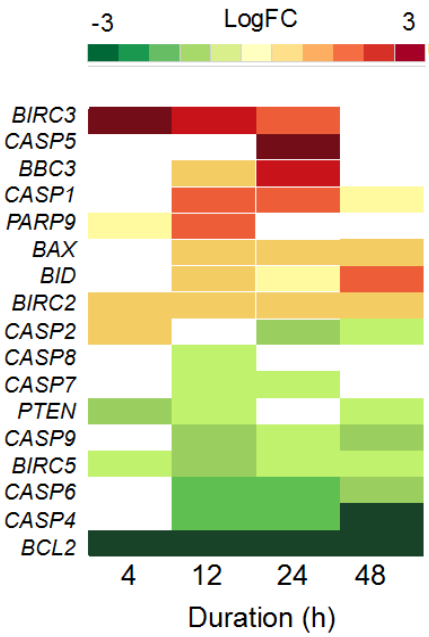

Figure S7. Heat map of DEGs for **ZL109** (complex **2**) in the apoptotic pathway. Only DEGs with FDR < 0.10 are included.

Reference

1 J. M. Hearn, I. Romero-Canelón, B. Qamar, Z. Liu, I. Hands-Portman and P. J. Sadler, Organometallic iridium(III) anticancer complexes with new mechanisms of action: NCI-60 screening, mitochondrial targeting, and apoptosis, *ACS Chem. Biol.*, 2013, **8**, 1335–1343.