



UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Phosphorescent heteroleptic iridium(III)
complexes: applications for therapy and
photodynamic therapy of cancer

Complejos heterolépticos fosforescentes de
iridio(III): aplicaciones para terapia y fototerapia
del cáncer

Dña. Gloria Vigueras Bautista
2021

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iridio(III): aplicaciones para terapia y fototerapia
del cáncer

Memoria presentada por **Dña. Gloria Viguera Bautista** para optar al
grado de Doctor en Química con mención internacional por la
Universidad de Murcia

Fdo: Gloria Viguera Bautista

Debido a que la Tesis ha sido redactada en inglés, se incluye un resumen en castellano, con una extensión de más de 2000 palabras, encuadernado como parte de la Tesis, en cumplimiento de Artículo 18 del Reglamento de Doctorado de la Universidad de Murcia (Redacción de la Tesis).



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La presentación de la Tesis Doctoral titulada “Complejos heterolépticos fosforescentes de iridio(III): aplicaciones para terapia y fototerapia del cáncer”, realizada por D^a. Gloria Vigueras Bautista, bajo nuestra inmediata dirección y supervisión, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

En Murcia, a 2 de julio de 2021

Fdo. José Ruiz López

Fdo. Natalia Cutillas Aulló

(firmado al margen con certificados electrónicos reconocidos)

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Este trabajo pone punto final a una importante etapa de mi vida. He disfrutado mucho aprendiendo del iridio y de sus excelentes propiedades, con su luz y brillo propios, y me alegra haber colaborado aunque sea un poquito en la lucha de esta enfermedad que está día a día más arraigada en nuestra sociedad.

Quiero agradecer a todos los que me han acompañado durante este camino y me han ayudado en cada problema que ha surgido. Gracias por cada frase de apoyo, por escucharme, por vuestros consejos, por preocuparos, por las risas, por vuestros ánimos. También gracias a aquellos que pasaron por el camino, y que aunque no se quedaron, dejaron pequeñas huellas que duran hoy día. Gracias a los que inspiraron mi carrera investigadora.

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He aprendido muchísimo bajo vuestra dirección, gracias.

“La vida no es fácil para ninguno de nosotros. Debemos tener perseverancia y sobre todo, confianza en nosotros mismos. Debemos creer que estamos dotados para algo y que esto debe ser alcanzado”

-Marie Curie

A mi yaya

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Abbreviations

A	Absorption
ACQ	Aggregation-caused quenching
APC	Antigen presenting cell
ATP	Adenosine triphosphate
bpy	2,2'-bipyridine
br	broad
BSA	Bovine Serum Albumin
calcd	calculated
CCDC	Cambridge Crystallographic Data Center
CDDP	cisplatin
Cg	ring centroid
CGC	Caenorhabditis Genetic Center
CI	Cell Index
COSY	correlation spectroscopy
Cp*	pentamethylcyclopentadienyl
CRT	calreticulin
CSC	cancer stem cell
CT	charge transfer
cys	cysteine
d	doublet
DAMPs	Damage-associated molecular patterns
DBB	<i>o</i> -dibenzoylbenzene
DC	dendritic cell
DCF	2'-7'-dichlorofluorescein
DCFH-DA	dichloro-dihydro-fluorescein diacetate
dd	doublet of doublets
DFT	Density Functional Theo

DHE	Dihydroethidium
DIC	differential interference contrast
DIPEA	<i>N,N</i> -diisopropylamine
DLS	dynamic light scattering
DMEM	Dulbecco's modified Eagle médium
DMF	<i>N,N</i> -dimethylformamide
DMSO	dimethylsulfoxide
DOX	doxorubicin
DPBF	1,3-diphenylisobenzofuran
dppn	benzo[<i>i</i>]dipyrido[3,2- <i>a</i> :2,3- <i>c</i>]phenazine
dppz	dipyrido[3,2- <i>a</i> :2,3- <i>c</i>]phenazine
dppz-izdo	dipyrido[3,2- <i>a</i> :2,3- <i>c</i>]phenazine-10,11-imidazolone-2
dpq	dipyrido[3,2- <i>f</i> :2,3- <i>h</i>]quinoxaline
EBSS	Earle's balanced salt solution
Ebselen	2-phenyl-1,2-benzisoselenazol-3(2H)-one
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Em	emission
EPR	enhanced permeability and retention
EtOH	ethanol
ER	Endoplasmic reticulum
ESI MS	Electrospray Ionization Mass Spectrometry
F	Fluorescence
FAAS	Flame Atomic Absorption Spectrometry
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FITC	fluorescein isothiocyante
fw	water fraction

Glu	glutamic acid
Gly	glycine
GSH	glutathion
HATU	1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5 b]- -pyridinium 3-oxide hexafluorophosphate
His	histidine
HMGB1	High mobility group protein B1
HPD	hematoporphyrin derivate
HPLC	High Pressure Liquid Chromatography
HSA	human serum albumin
HSP90	Heat shock protein 90
HSQC	Heteronuclear Single Quantum Correlation
IC	internal conversion
ICD	Immunogenic Cell Death
ICP	Inductively Coupled Plasma
IFN γ	Interferon-gamma
ILCT	Intra-Ligand Charge Transfer
IPTG	Isopropyl β -D-1-thiogalactopyranoside
ISC	intersystem crossing
LC	Ligand-centered
LED	Light Emitting Diode
LFSE	Ligand-Field Stabilization Energy
LLCT	Ligand-to-Ligand Charge Transfer
LMCT	Ligand-to-Metal Charge Transfer
m	multiplet
MC	Metal-centered
Me	Methyl
MeI	Methyl iodide
MeOH	Methanol

MDR	Multidrug Resistance
MLCT	Metal-to-Ligand Charge Transfer
MRI	Magnetic resonance imaging
MS	mass
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
m/z	mass to charge ratio
NAD ⁺	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide hydride
NGM	Nematode Growth Medium
NIR	Near-Infrared
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
NP	nanoparticle
OLED	Organic Light-Emitting Diode
P	Phosphorescence
PACT	photoactivated chemotherapy
PBS	phosphate buffer saline
pbz	2-phenylbenzimidazole
PCC	Pearson's correlation coefficient
PDT	photodynamic therapy
PEG	polyethyleneglycol
Ph	phenyl
Phen	1,10-phenanthroline
PI	Phototoxic Index
ppm	parts per million
PS	photosensitizer
q	quadruplet
ROS	Reactive Oxygen Species
RP-HPLC	Reverse Phase High Pressure Liquid Chromatography
RPMI	Roswell Park Memorial Institute

RT	room temperature
RTCA	Real-Time Cell Analyzer
s	singlet
SAR	Structure-Activity Relationship
SD	Standard Deviation
SF	Selectivity Factor
SOSG	Singlet Oxygen Sensor Green
SSTR	Somatostatin Receptor
t	triplet
TCRPs	Time-dependent cell-response profiles
td	triplet of doublets
TEM	Transmission Electron Microscopy
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
TMRE	tetramethylrhodamine ethyl ester
TOF MS	Time-of-flight mass spectrometry
tR	Retention time
UV	ultraviolet
Vis	Visible
vol	volume
VR	vibrational relaxation
VTP	vascular-targeted PDT

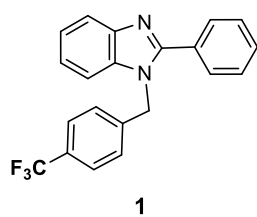
Cell lines

518A2	Human melanoma
A2780	Human ovarian carcinoma
A2780cisR	Human ovarian carcinoma with acquired resistance to cisplatin
A375	Human melanoma
BGM	Healthy kidney cell line from African green monkey
CHO	Normal Chinese hamster ovarian cells
HeLa	Human cervical carcinoma
MCF-7	Human breast cancer cells
MCF10-A	Human breast normal cells

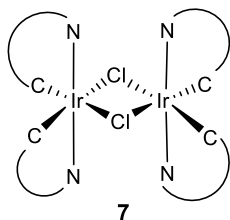
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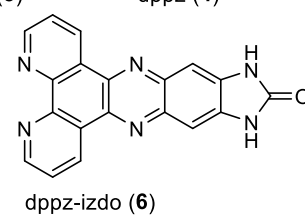
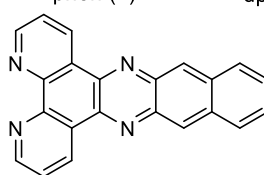
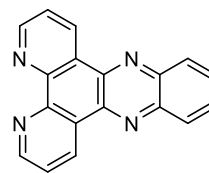
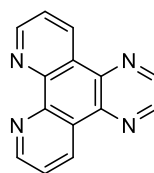
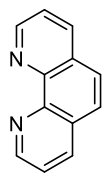
HC^N Pro-ligand



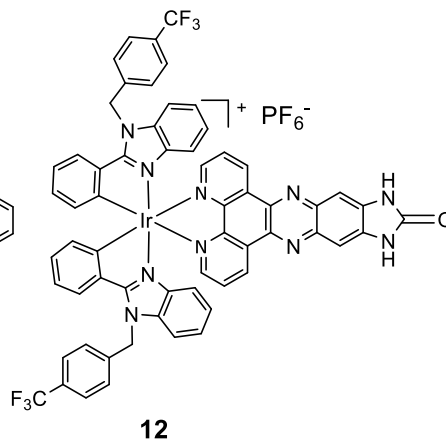
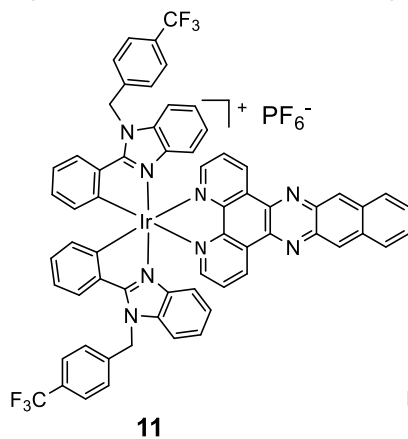
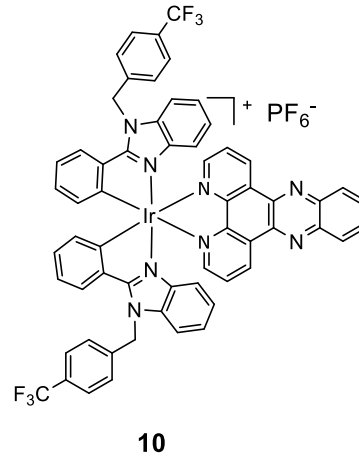
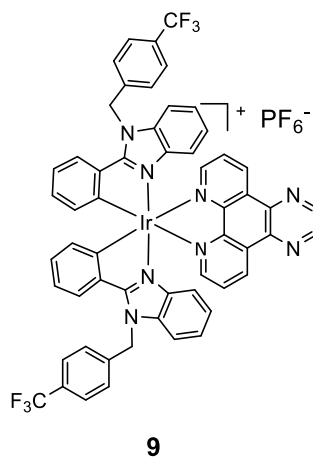
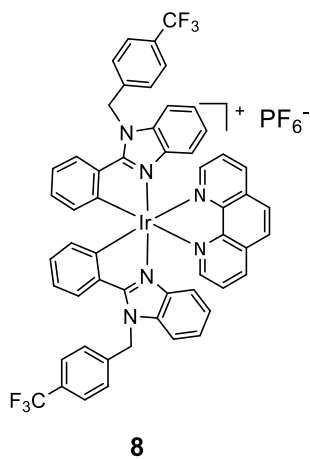
Dimer



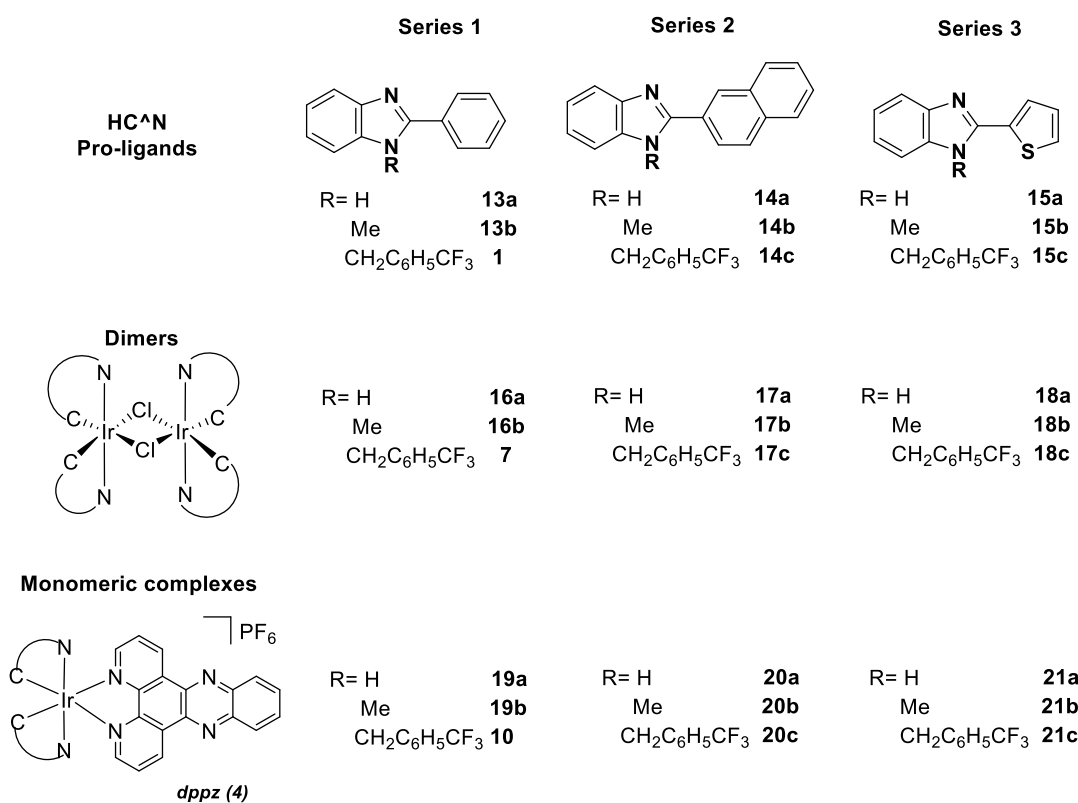
N^N Ligands



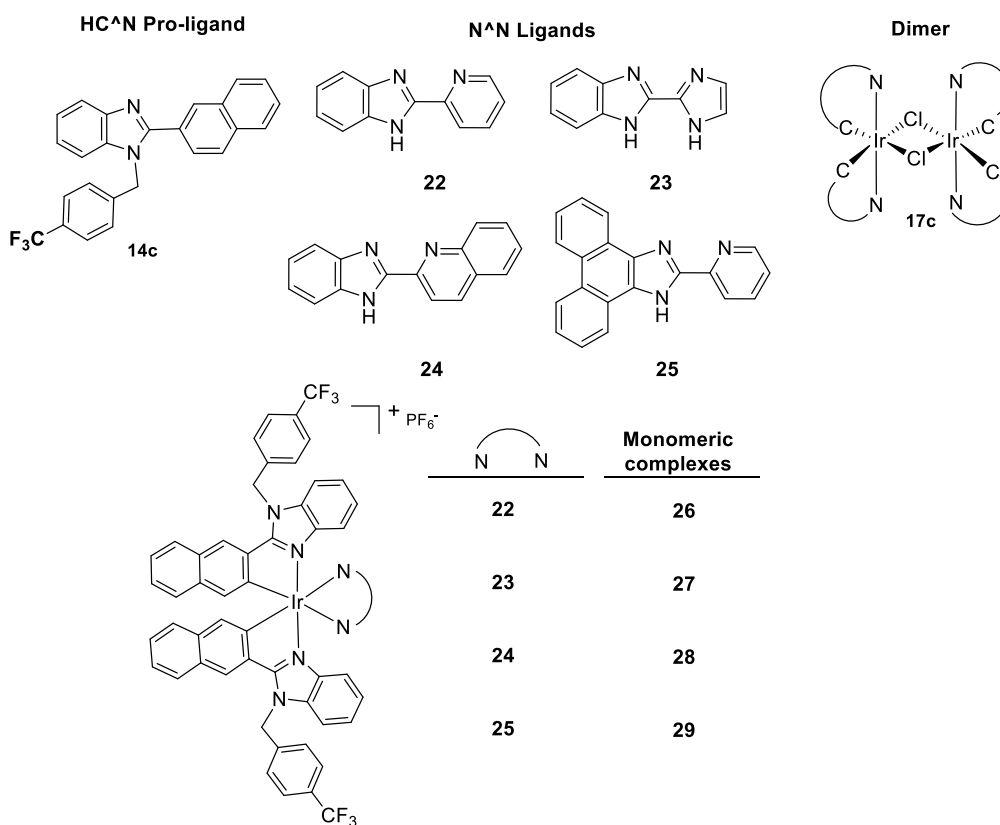
Monomeric complexes



Chapter 3.

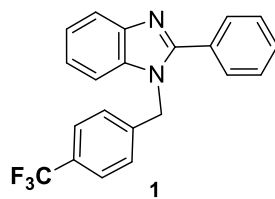


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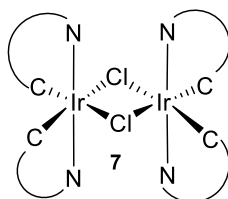


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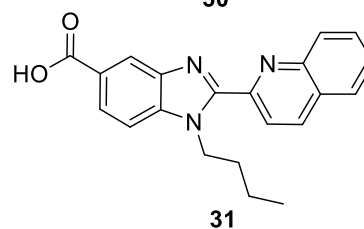
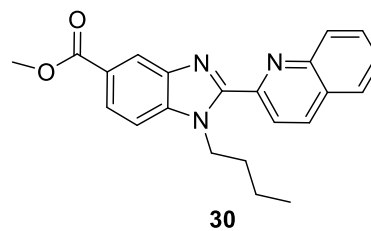
HC^N pro-ligand



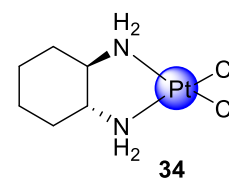
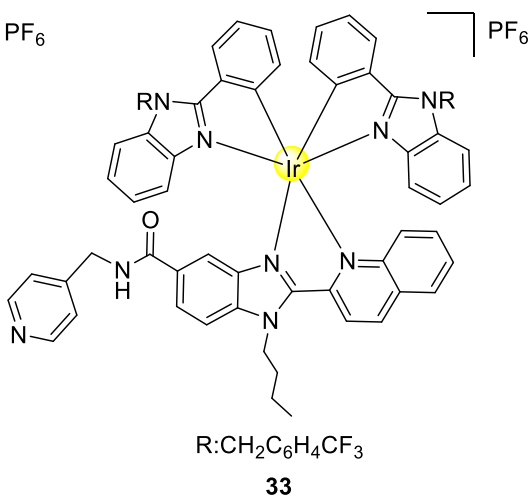
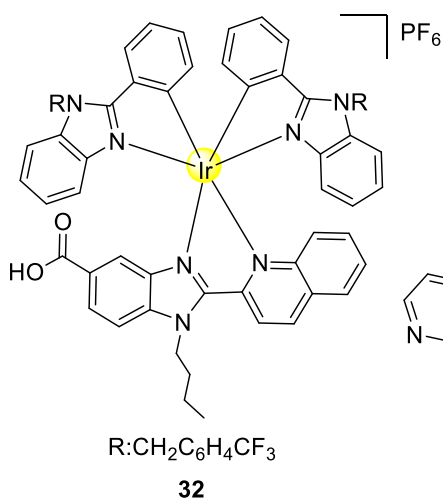
Ir(III) dimer



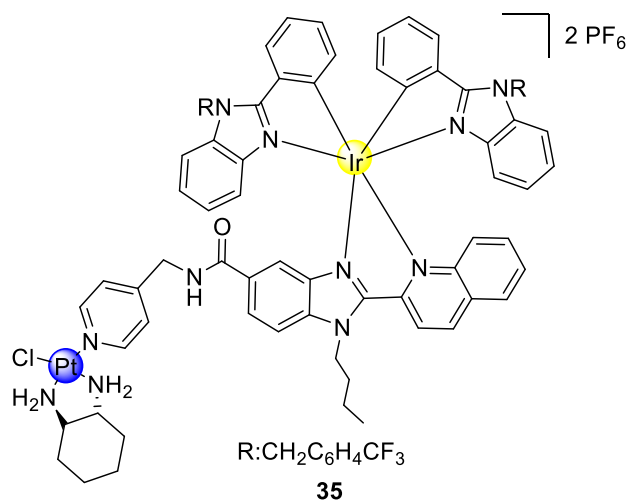
N^N Ligands



Monomeric complexes

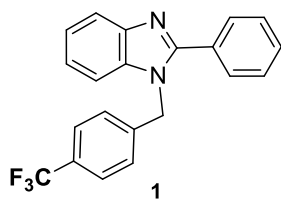


Heterobimetallic complex

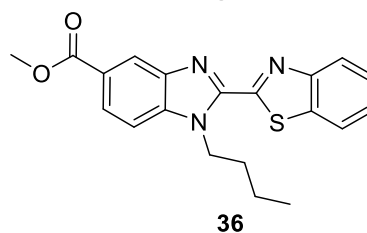


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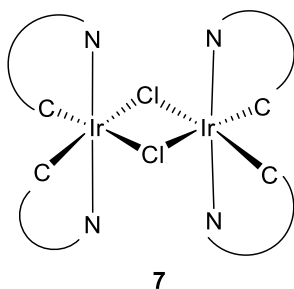
HC[^]N Pro-ligand



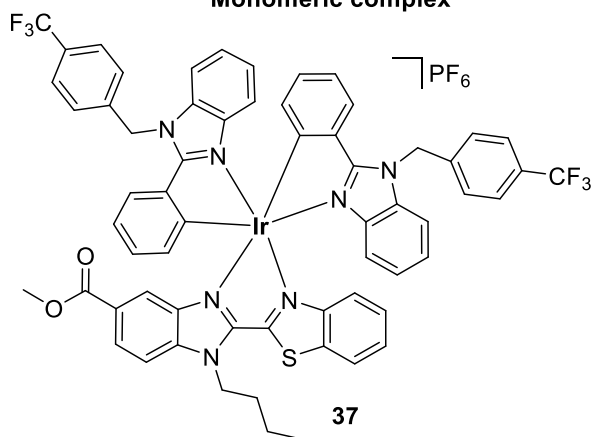
N[^]N Ligand



Dimer

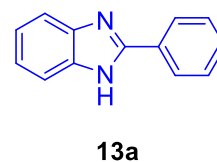
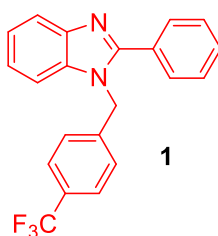


Monomeric complex

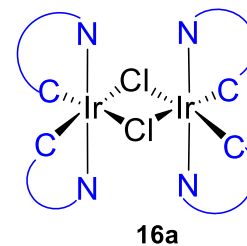
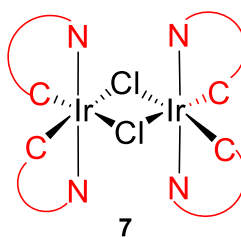


Chapter 7.

HC[^]N Pro-ligands

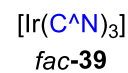
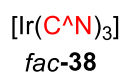


Dimers

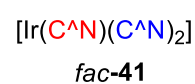
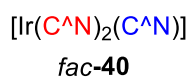


Monomeric complexes

Homoleptic



Heteroleptic



Chapter 1

Introduction and Objectives

1 1A 1A	2 2A 2A	3 3A 3A	4 4A 4A	5 5A 5A	6 6A 6A	7 7A 7A	8 8A 8A	9 9A 9A	10 10A 10A	11 11A 11A	12 12A 12A	13 13A 13A	14 14A 14A	15 15A 15A	16 16A 16A	17 17A 17A	18 18A 18A
1 H Hydrogen 1.008	2 He Helium 4.003																
3 Li Lithium 6.941	4 Be Beryllium 9.012																
11 Na Sodium 22.990	12 Mg Magnesium 24.305																
19 K Potassium 39.098	20 Ca Calcium 40.078	21 Sc Scandium 44.956	22 Ti Titanium 47.867	23 V Vanadium 50.942	24 Cr Chromium 51.996	25 Mn Manganese 54.938	26 Fe Iron 55.845	27 Co Cobalt 58.933	28 Ni Nickel 58.693	29 Cu Copper 63.546	30 Zn Zinc 65.38	31 Ga Gallium 69.723	32 Ge Germanium 72.631	33 As Arsenic 74.922	34 Se Selenium 78.971		
37 Rb Rubidium 85.468	38 Sr Strontium 87.62	39 Y Yttrium 88.906	40 Zr Zirconium 91.224	41 Nb Niobium 92.906	42 Mo Molybdenum 95.95	43 Tc Technetium 98.907	44 Ru Ruthenium 101.07	45 Rh Rhodium 102.906	46 Pd Palladium 106.42	47 Ag Silver 107.868	48 Cd Cadmium 112.414	49 In Indium 114.818	50 Sn Tin 118.711	51 Sb Antimony 121.760	52 Te Tellurium 127.6		
55 Cs Cesium 132.905	56 Ba Barium 137.328	57-71 Lanthanide Series	72 Hf Hafnium 178.49	73 Ta Tantalum 180.948	74 W Tungsten 183.84	75 Re Rhenium 186.207	76 Os Osmium 190.23	77 Ir Iridium 192.217	78 Pt Platinum 195.085	79 Au Gold 196.967	80 Hg Mercury 200.592	81 Tl Thallium 204.383	82 Pb Lead 207.2	83 Bi Bismuth 208.980	84 Po Polonium [209]		
87 Fr Francium 223.020	88 Ra Radium 226.025	89-103 Actinide Series	104 Rf Rutherfordium [261]	105 Db Dubnium [262]	106 Sg Seaborgium [266]	107 Bh Bohrium [264]	108 Hs Hassium [269]	109 Mt Meitnerium [278]	110 Ds Darmstadtium [281]	111 Rg Roentgenium [289]	112 Cn Copernicium [285]	113 Nh Nihonium [286]	114 Fl Flerovium [289]	115 Mc Moscovium [288]	116 Lv Livermorium [293]		
		57 La Lanthanum 138.905	58 Ce Cerium 140.116	59 Pr Praseodymium 140.908	60 Nd Neodymium 144.243	61 Pm Promethium 144.913	62 Sm Samarium 150.36	63 Eu Europium 151.964	64 Gd Gadolinium 157.25	65 Tb Terbium 158.925	66 Dy Dysprosium 162.500	67 Ho Holmium 164.930	68 Er Erbium 167.259	69 Tm Thulium 168.934	70 Yb Ytterbium 173.055	71 Lu Lutetium 174.967	
		89 Ac Actinium 227.038	90 Th Thorium 232.038	91 Pa Protactinium 231.036	92 U Uranium 238.029	93 Np Neptunium 237.048	94 Pu Plutonium 244.064	95 Am Americium 243.061	96 Cm Curium 247.070	97 Bk Berkelium 247.070	98 Cf Californium 251.080	99 Es Einsteinium [254]	100 Fm Fermium 257.095	101 Md Mendelevium [261]	102 No Nobelium [265]	103 Lr Lawrencium [262]	

Catalysis

Anti-cancer

PDT

Ir

Imaging

Therapeutics

Luminescence

1.1. CANCER AND PLATINUM METALLODRUGS

Cancer is currently the second leading cause of death in developed countries despite the huge advancement in drug research and technology for cancer treatment during the past decades. As reported by the World Health Organization ([WHO](#)), cancer was responsible for approximately 9.6 million deaths in 2018. Globally, about 1 in 6 deaths is due to this illness. With global cancer morbidity increasing, the development of new cancer treatments is crucial.

The conventional anticancer treatments (**Figure 1.1**) rely mainly on surgery, chemotherapy, and radiation therapy. Other therapies (immunotherapy,¹ targeted therapy^{2,3} or photodynamic therapy (PDT⁴, *vide infra*)) have aroused great interest in recent years. However, some of these therapies may be insufficient to address a problem of such magnitude. For example, only localized cancers can be treated by surgery, and tumors such as the pancreas and hepatocellular carcinoma, cannot be removed by this surgical procedure. On the other hand, radiation therapy carries the risk of inducing secondary neoplasms due to high-energy radiation used. In immunotherapy of cancer, a key obstacle is the immunosuppressive environment of the growing tumor.⁵

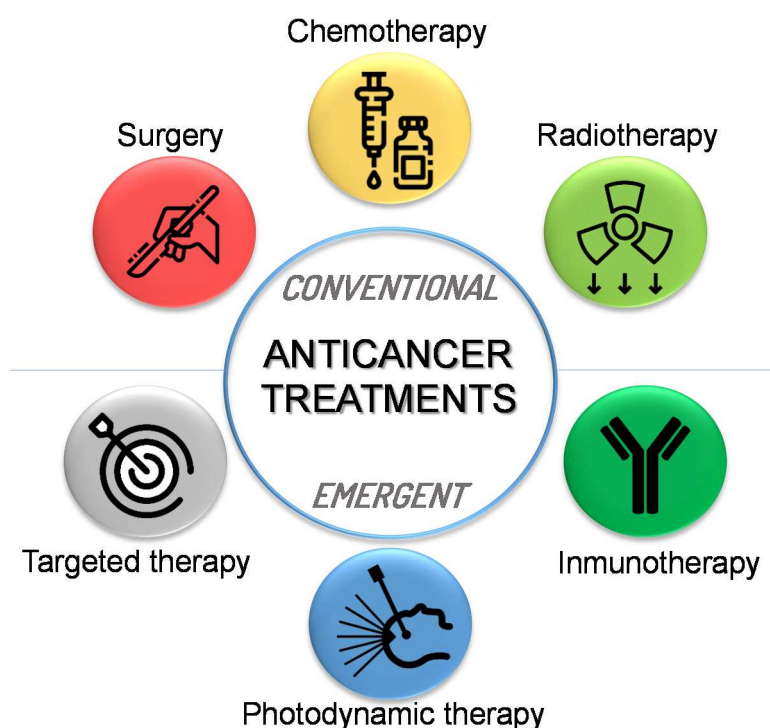
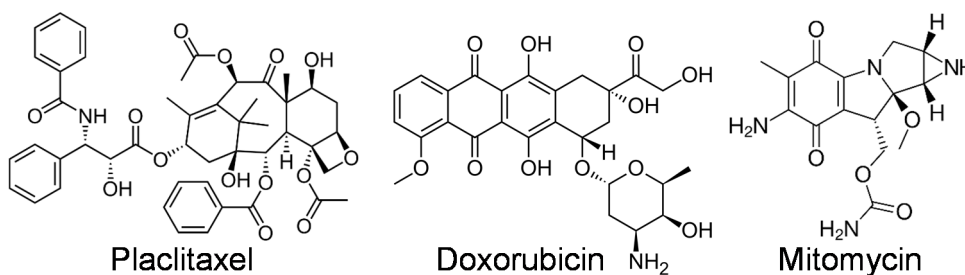


Figure 1.1. Types of anti-cancer treatment.

Chemotherapy, meanwhile, attempts to cause the cancer cells' death to arrest the tumor's progression through the use of drugs (organic or inorganic), usually attacking cellular DNA or metabolic sites essential for cell replication. Ideally, these anticancer drugs should interfere with cellular processes that only malignant cells possess. Unfortunately, most clinically approved anticancer agents do not specifically recognize tumor cells; instead, they affect all kinds of cells (normal and neoplastic).⁶ In addition, cancer-related multidrug resistance (MDR) can directly lead to chemotherapy failure.^{7,8} Luckily, there is a continuous effort to develop of new chemotherapeutic drugs that allow overcoming these drawbacks.

Most of the drugs used in the clinic are organic molecules⁹ (Paclitaxel, Doxorubicin, Mitomycin, etc.; **Figure 1.2A**). However, the serendipitous discovery of the biological activity of cisplatin (*cis*-diamminedichloridoplatinum(II), **Figure 1.2B**)¹⁰ and its subsequent approval as a therapeutic drug in 1978 has transformed cancer treatment over the last 50 years. Several platinum complexes with a similar structure (carboplatin and oxaliplatin; **Figure 1.2B**) have also been approved by the FDA (U.S. Food and Drug Administration) and are used worldwide in cancer therapy. Nowadays, these three platinum-containing drugs are utilized to treat of almost 50% of cancer patients going through chemotherapy,¹¹ employed alone or in combination with other agents to treat various solid tumors.

A. Organic-based drugs in clinical use



B. Platinum metallodrugs in clinical use

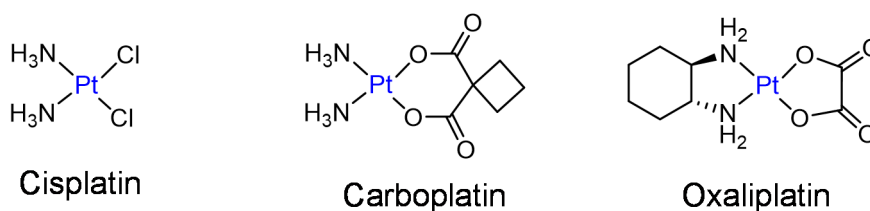


Figure 1.2. A) Some organic FDA-approved anti-cancer drugs B) FDA-approved platinum-containing drugs

The mechanism of action of cisplatin (CDDP) has been extensively studied through the years. Surprisingly, only 1% of intracellular CDDP forms covalent bonds with nuclear DNA lead to cell death. Briefly, CDDP is injected intravenously since oral administration would produce its hydrolysis in the stomach and, also, due to its low solubility in the aqueous medium. Once in the bloodstream ($[\text{Cl}^-] = 100 \text{ mM}$), part of the complex can react with some proteins in a non-selective way, especially with those with thiol groups.¹² This is excreted through the kidneys, where it causes nephrotoxicity. The remaining drug enters cells both by passive diffusion and by active transport. The intracellular Cl^- ion concentration is not high enough (4-10 mM) to avoid hydrolysis of the complex (**Figure 1.3**), forming the cationic species $[\text{PtCl}(\text{NH}_3)_2(\text{OH}_2)]^+$ and $[\text{Pt}(\text{NH}_3)_2(\text{OH}_2)_2]^{2+}$, whose positive charge prevents it from leaving the cell again. Since platinum(II) is a soft acid, it preferably interacts with the nitrogen atoms of the nucleobases. The most accessible position is the N7 of the guanine. The different adducts formed, among them 1,2-d(GpG) (60-65%) and 1,2-d(ApG) (20-55%), modify the conformation of the double helix making it more unstable. Specifically, the majority 1,2-d adduct (GpG) causes unwinding of the double helix and folding towards the major groove. On the other hand, they enable the binding of specific nuclear proteins through the minor groove, as occurs, fundamentally, with proteins of the high mobility group (High-mobility group box protein 1, HMGB1) that “mask” the CDDP/DNA adducts, preventing the lesion repair from the nucleotide excision repair machinery.¹³ CDDP can also interact with other nucleophiles such as RNA molecules, the N- and S-donor moieties of biomolecules (e.g., glutathione),¹⁴ and proteins, disrupting essential cellular processes.¹⁵⁻¹⁷ The interaction between CDDP and RNA can impact cisplatin bioactivity, influencing its toxicity and interfering in processes such as protein translation.¹⁸ In addition, the union of CDPP to chaperonage proteins such as HSP90 can inhibit its protein-folding assistance function.¹⁵ Furthermore, CDDP can induce cellular damage by generating reactive oxygen species (ROS), producing mitochondrial dysfunction through mtDNA adducts, and cause electron transport chain imbalance. All these processes ultimately lead to cell death through apoptosis.¹⁹⁻²¹

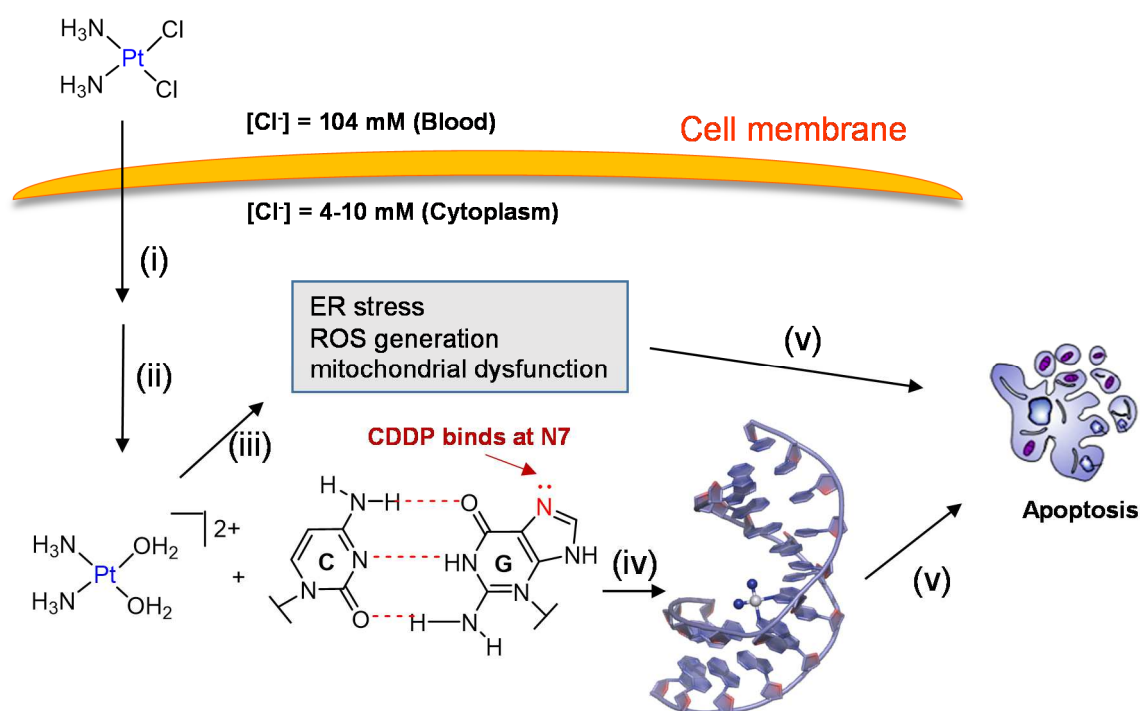


Figure 1.3. Mechanism of action of cisplatin: (i) Cellular uptake, (ii) aquation/ activation, (iii) some of the alternative under-reported effects of cisplatin, (iv) DNA binding and (v) activation of cell death signalling pathways and promotion of apoptosis. Adapted from reference.²²

The structures of carboplatin and oxaliplatin have been modified to increase the stability of the platinum compounds before their ultimate target, the nuclear DNA. Thus, the half-life of the aquation reaction, which is approximately 2 h for the generation of $\text{cis-}[\text{PtCl}(\text{NH}_3)_2(\text{OH}_2)]^+$, drastically decreases in these complexes over weeks to months.²³ As a consequence, carboplatin has lower toxicity and equivalent efficacy than cisplatin, so it has become the drug selected for those tumor types.²⁴ Instead, oxaliplatin is effective against colorectal cancer²⁵ and contains the diaminocyclohexane (DACH) ligand that correlates with a lack of cross-resistance with CDDP.²⁶ Recently, Lippard *et al.* have demonstrated that oxaliplatin has a different mechanism of action to cisplatin and carboplatin. This platinum complex of third-generation induces ribosome biogenesis stress and does not cause DNA damage, as was previously believed.²⁷

Unfortunately, despite their success and history, small platinum agents suffer many limitations: poor stability, lack of tumor selectivity, high toxicity,²⁸ and multiple mechanisms of resistance (inherent and acquired).²⁹ Continued efforts to improve upon platinum anticancer agents have been studied (**Figure 1.4**): *Trans*-platinum(II) complexes,³⁰ monofunctional Pt(II) complexes,³¹ platinum compounds that bind non-covalently to DNA,³² among others. In addition, Pt(IV) complexes³³ have aroused great interest because of the inertness of the low-spin d⁶ Pt(IV) center to ligand substitution

reactions, which minimizes side reactions with biomolecules other than DNA. Thus, they are considered pro-drugs until the Pt(IV) reduction to Pt(II) active species inside the cell. However, there are not new drugs of all of these classes used in the clinic since the FDA approval of oxaliplatin in 2002.³⁴ All this has supported the development of new drug candidates based on other metals.

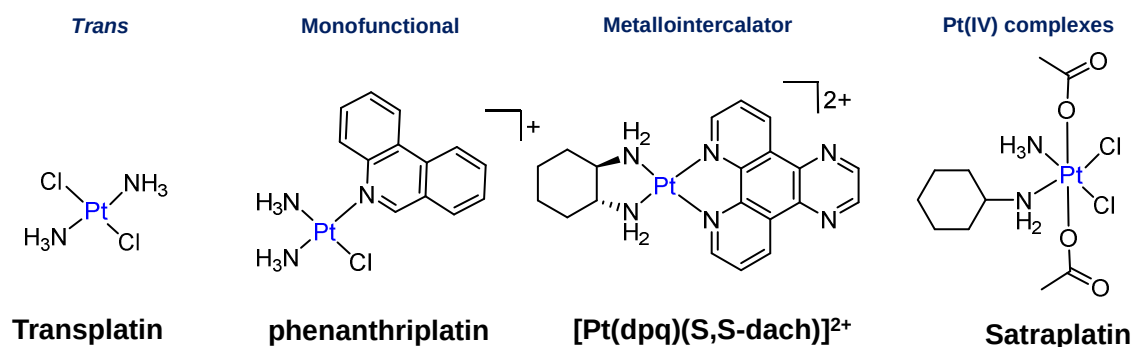


Figure 1.4. Example of chemical structures of non-conventional platinum anti-cancer complexes.

1.2. ALTERNATIVE METALLODRUGS: OVERCOMING THE DRAWBACKS

Compare to organic-based drugs, organometallic complexes are by far more versatile due to possessing a wide range of coordination numbers, geometries, and oxidation states^{35,36} (**Figure 1.5A**) that allow fine-tuning the biological activities of the metallodrugs. These features increase the interest as pharmaceuticals for the use of antitumor drugs and diagnostic agents.³⁷ Gold,^{38,39} ruthenium,^{40,41} iridium,^{42,43} rhodium,^{44,45} osmium,^{41,46} rhenium,^{47,48} and other so-called noble elements have been highly researched in recent years. Organometallic agents combine the advantages of classic inorganic systems with the high stability of an organic scaffold. In addition, they are relatively lipophilic and can be synthesized using a wide variety of organic ligands allowing the introduction of stereospecificity with access to a large number of structural possibilities. The strong metal-carbon (M-C) bond provides an increased metal complex stability against ligand exchange reactions and biological reduction. Moreover, the properties of the cyclometalated compounds can be easily modified by tuning both the ancillary or the anionic cyclometalated ligands, thus improving their pharmacokinetics and cytotoxicity.⁴⁹

After Pt(II)-based drugs, ruthenium complexes (both classical and organometallic) are among the most studied therapeutic agents. Thus, the classical Ru(III) compound known as NAMI-A (**Figure 1.5B**) was the first compound of this metal to enter phases of clinical trials. However, recently these studies have been suspended because of the lack of a specific target.⁵⁰ On the other hand, the Ru(III) complex known as BOLD-100 (formerly IT-139

and KP1339; **Figure 1.5B**) is currently in the clinical trial phase I/II with up-and-coming prospects.^{51–53} In biological conditions, two oxidation states are stable for ruthenium, Ru(II) and Ru(III), where the latter is less reactive. Among the advantages, Ru-based drugs include (a) an octahedral geometry, which allows for a broader range of reaction mechanisms involving the metal center, (b) several accessible oxidation states within physiological conditions, and (c) the ability of Ru to mimic Fe, which binds to transferrin proteins,⁵⁴ whose receptors are overexpressed mainly on the surface of carcinogenic cells.

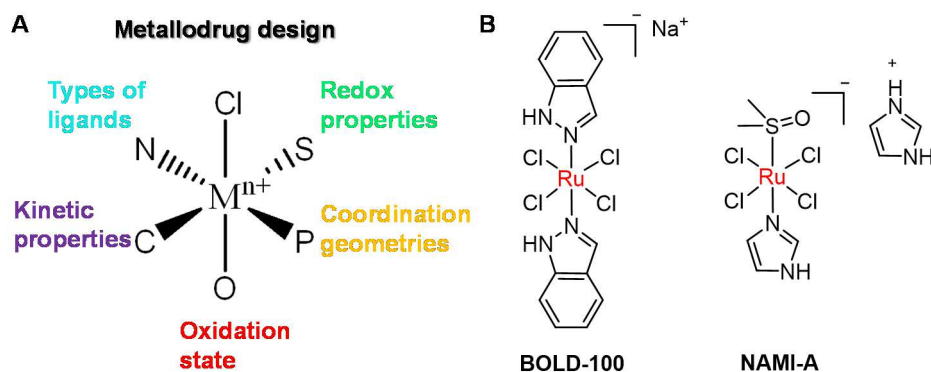


Figure 1.5. A) Advantages in metallodrug design. B) Structures of the classical Ru(III) octahedral complexes **BOLD-100** and **NAMI-A**.

The history of anti-cancer Ru chemistry shows three basic classes of active compounds: Ru(III) compounds (e.g., **NAMI-A**, **Figure 1.5B**), Ru(II)-arene complexes of the type $[(\eta^6\text{-arene})\text{RuL}_1\text{L}_2\text{Cl}]$ (e.g., **RAPTA-C**) or $[(\eta^6\text{-arene})\text{Ru}(\text{C}^{\wedge}\text{N})\text{Cl}]$ (**Figure 1.6A**), and tris-heteroleptic polypyridyl complexes (**Figure 1.6B**). These three groups of active compounds have different mechanisms of action. For example, some arene compounds can interact with DNA, whereas others can inhibit protein translation.⁵⁵ Tris-heteroleptic polypyridyl complexes can include a cyclometalating ligand to activate their cytotoxicity,^{56,57} while complexes of the type $[\text{Ru}(\text{N}^{\wedge}\text{N})_3]^{2+}$ are actively being studied as photosensitizers (PSs) in PDT.⁵⁸

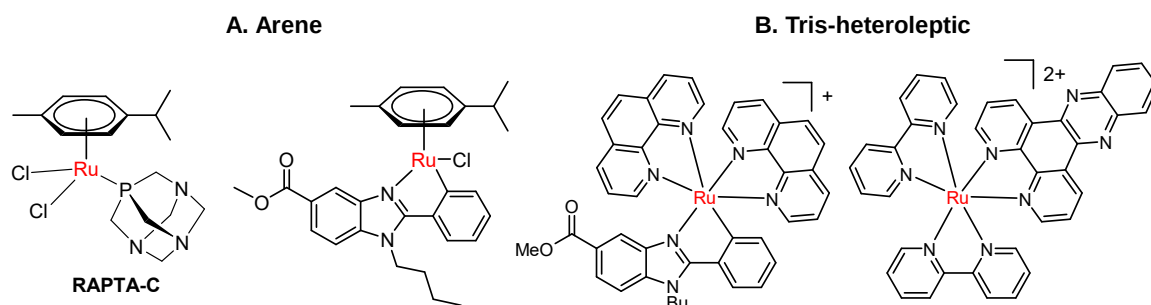


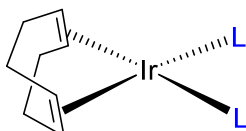
Figure 1.6. Example of chemical structures of two families of Ru(II) complexes: A) Arene complexes of the type $[(\eta^6\text{-arene})\text{RuL}_1\text{L}_2\text{Cl}]$ ⁵⁹ and $[(\eta^6\text{-arene})\text{Ru}(\text{C}^{\wedge}\text{N})\text{Cl}]$,⁶⁰ B) Tris-heteroleptic complexes of general formula $[\text{Ru}(\text{N}^{\wedge}\text{N})_2(\text{C}^{\wedge}\text{N})]^{+}$ ⁶¹ and $[\text{Ru}(\text{N}^{\wedge}\text{N})_3]^{2+}$.⁶²

1.3. IRIIDIUM BASED-THERAPEUTICS. PHOTOPHYSICAL PROPERTIES AND ANTINCANCER ACTIVITY

Iridium is a third-row transition metal, a member of the platinum group of “precious metals.” Discovered in 1803 by the English chemist Tennant, as an impurity in platinum, iridium is undoubtedly the rarest element and the most corrosion-resistant metal in the earth's crust, possessing excellent chemical and physical properties.

The iridium chemistry is fascinating and diverse: a wide range of oxidation states (−1 to +9), three coordination numbers (4, 5, and 6), and varied coordination geometries. The most common oxidation states of iridium are +1 and +3. The cation Ir(I) is a d^8 electron system and forms tetra-coordinate or pentacoordinate complexes whose molecular geometry is square planar or trigonal bipyramidal, respectively. In the oxidation state +3, iridium is a $5d^6$ center, and its compounds are hexacoordinate and octahedral.⁶³ It is interesting to know that in the 70s, the anti-cancer complexes based on iridium were centered on d^8 square-planar 1,5-cyclooctadiene Ir(I) compounds (**Figure 1.7A**), owing to their structural similarity to those of Pt(II). However, nowadays, the development of therapeutic agents is based on Ir(III) complexes (**Figure 1.7B**). This oxidation state is more stable than Ir(I), with a higher coordination number (6 versus 4), which can give great structural diversity due to the extensive range of ligands.

A. Ir(I) anti-cancer compounds



B. Ir(III) anti-cancer compounds

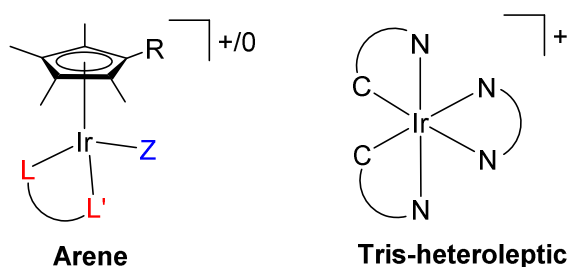


Figure 1.7. Coordination geometries for A) Ir(I) and B) Ir(III) therapeutic compounds.

In organometallics, Ir(III) complexes have shown an incredible range of properties that make them appropriate for many applications, extending from catalysis to optoelectronics. The most significant functions of Ir(III) compounds are their use as catalysts in different reactions⁶⁴ (metathesis of alkanes, hydrogenation, water oxidation, etc), and their use in

electronic devices, such as luminescent chemosensors⁶⁵ or photoelectronic sensors. The articles associated with these applications have been growing exponentially until 2016 but have decreased slightly in the last three years. In contrast, the use of iridium complexes in therapy is increasing, although it is still in its beginning (**Figure 1.8**). Unlike Ru(II) complexes, isoelectronic Ir(III) compounds were not considered potentially useful as cytotoxic due to the inertness of the metal center. However, the development of new iridium compounds with biomedical purposes has increased because they can modulate their reactivity from kinetically inert species to other labile species with the proper choice of auxiliary ligands.⁶⁶ Furthermore, Ir(III) complexes show several advantages regarding platinum and ruthenium compounds that support their therapeutic potential: easier synthesis and purification, air and moisture stability,⁶⁶ and impressive tunability of their optoelectronic properties.^{63,67}

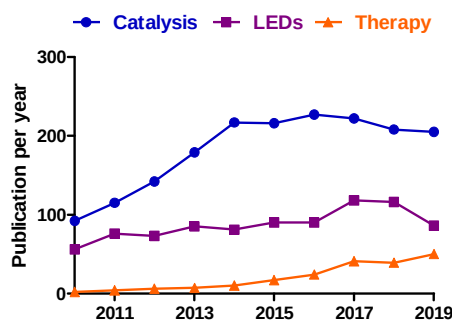


Figure 1.8. Published articles between 2010 and 2019 on iridium compounds applications (Web of Science, core collection, 2020-April).

Ir(III) anti-cancer agents can be divided mainly into two types of complexes: arene complexes and tris-chelate complexes (**Figure 1.7B**).

Arene complexes, also known as ‘half-sandwich’ Ir(III) complexes, have proven their potential chemotherapy activity towards various types of cancer.^{68,69} These pseudo-octahedral iridium complexes of the type $[(\eta^5\text{-Cp}^x)\text{Ir}(\text{L}^{\wedge}\text{L}')\text{Z}]^{0/+}$ (**Figure 1.7B**), where Cp^x is pentamethylcyclopentadienyl (Cp^*) or extended Cp^* , $\text{L}^{\wedge}\text{L}'$ is a chelating $\text{C}^{\wedge}\text{N}$ or $\text{N}^{\wedge}\text{N}$ ligand and Z a neutral pyridyl ligand or an anionic chloride, have been widely investigated by Sadler and co-workers.^{69,70} The ligands usually rule these complexes' electronic and physiological properties so that an intelligent design can lead to a wide range of biological activities. For example, the anti-cancer activity of cyclopentadienyl organoiridium(III) compounds can be activated by changing a neutral $\text{N}^{\wedge}\text{N}$ -chelating ligand to a negatively charged $\text{C}^{\wedge}\text{N}$ analog. Also, the rapid ligand exchange reactions in these complexes (on the order of few seconds) are connected to their antitumor activities. In addition, these Ir compounds can act as catalytic drugs or possess redox mechanisms of action.^{69,71}

Interestingly, some of these compounds can inhibit biologically relevant enzymes,^{72,73} and some cyclometalated benzimidazole Ir(III) complexes have shown dual cytotoxic and antiangiogenesis activities.^{60,74}

On the other hand, octahedral cyclometalated iridium complexes have aroused great interest since, in addition to presenting antitumor properties, they possess an intrinsic luminescence, so they can be used simultaneously in therapy and diagnosis. These octahedral complexes can be mono-, bis-, and tris- cyclometalated, which broadens the range of potentially available structures, which can be ionic or neutral.

In this thesis, Ir(III) complexes of the type $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{N}^{\wedge}\text{N})]^+$ and $[\text{Ir}(\text{C}^{\wedge}\text{N})_3]$ have been developed fundamentally, so we will focus on the properties of these compounds.

Both types of cyclometalated Ir(III) complexes are commonly synthesized through two steps: first with the formation of the dichlorido-bridged dinuclear Ir(III) complex $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\mu\text{-Cl})_2]_2$ from the reaction between the cyclometalating ligand ($\text{HC}^{\wedge}\text{N}$) and $\text{IrCl}_3 \cdot x\text{H}_2\text{O}$, and then through the reaction between the dimer and a third ligand, to obtain the bis- or tris-cyclometalated monomers (**Figure 1.9**). Bis-cyclometalated Ir(III) compounds are generally obtained as racemic mixtures of the Δ and Λ enantiomers,⁷⁵ although, the separation of these enantiomers has rarely occurred.^{76–78} While, for tris-cyclometalated complexes, two isomers are possible, facial (*fac*) and meridional (*mer*),⁷⁹ being the latest easily isomerized to the more stable *fac* isomer using heat or light.⁸⁰ Cyclometalated Ir(III) complexes can be categorized as heteroleptic when different ligands are integrated to the iridium center (e.g., bis-cyclometalated complexes) or homoleptic when three identical bidentate ligands are involved in the complex.

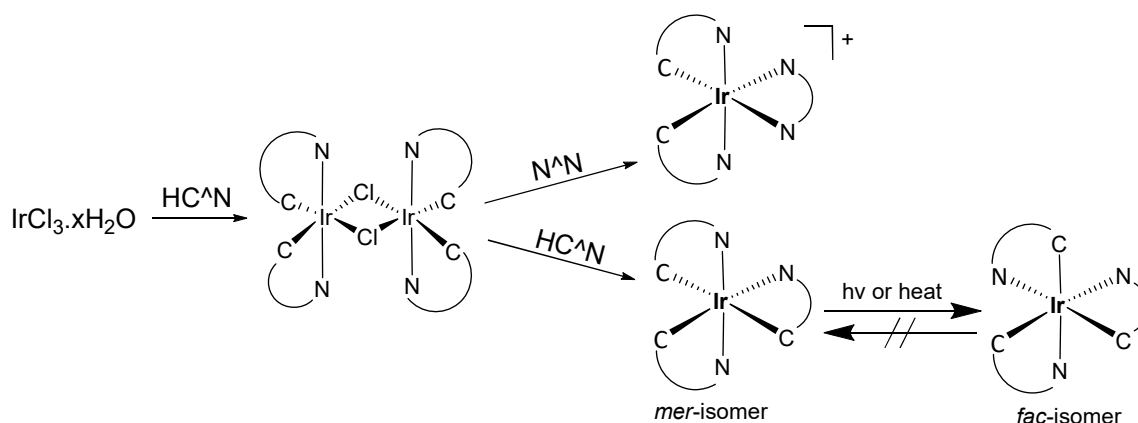


Figure 1.9. General synthetic scheme for bis- and tris- cyclometalated Ir(III) complexes.

1.3.1. Photophysical properties of octahedral Ir(III) complexes

Before introducing the properties of these complexes, it is advisable to review the Jablonski diagram (**Figure 1.10A**).^{81,82} After light absorption (A), a molecule is usually excited from the ground singlet state (S_0) to the excited singlet state S_n . The excitation process occurs so rapidly (on the order of 10^{-15} s) that no rearrangement of the nuclear framework occurs during this process (Frank-Condon principle). In the excited state, a molecule relaxes through internal conversion (IC) to higher vibrational levels of the first excited singlet state, S_1 , within 10^{-11} - 10^{-14} s. Then, via vibrational relaxation (VR), the molecule quickly relaxes (10^{-12} s) to the lowest vibrational level of S_1 (Kasha's rule) by dissipating energy for collisions between molecules. The spacing of the vibrational energy levels of the excited states is similar to that of the ground state. As a result, the vibrational structures seen in the emission and absorption spectra are comparable. If the radiative depopulation occurs from this S_1 state, the spontaneous emission is known as fluorescence (F). If the spin of an excited electron is reversed by intersystem crossing (ISC), leaving the molecule in the first excited triplet state, T_1 , the emission is termed phosphorescence (P). This emission is usually shifted to longer wavelengths (lower energy) than fluorescence. Because the S_1/T_3 transition is also spin forbidden, triplet state lifetimes are very long after fluorescence (microsecond to millisecond time scale).

The chemical nature of cyclometalated Ir(III) complexes can be readily tuned by chemical variations of the coordinated and auxiliary ligands, allowing fine-tuning of their photoluminescent characteristics. This family of complexes possesses excellent photophysical properties⁶³ that overcome the disadvantages of classic organic luminophores:⁸³

- ✓ High photostability, which avoids photobleaching in long-term excitation
- ✓ Efficient tuning of the emission color with slight modifications in their structures
- ✓ Large stoke shifts (**Figure 1.10B**) allow easily distinguishing emission overexcitation and eliminating self-quenching processes.
- ✓ Long luminescent lifetimes (τ)
- ✓ High phosphorescence quantum yields (Φ_P)

These favorable characteristics are essentially ascribed to the strong spin-orbit coupling (SOC) induced by the 5d orbitals of Ir^{3+} ion ($\xi_{\text{Ir}} = 4430 \text{ cm}^{-1}$).^{84,85}

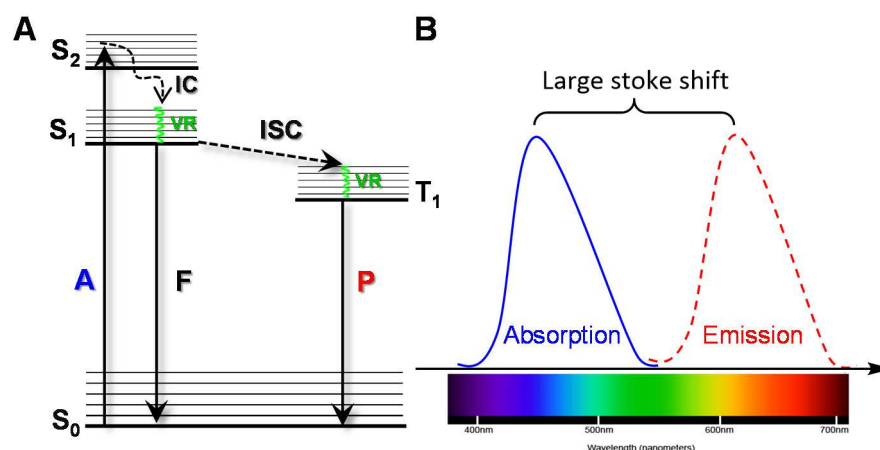


Figure 1.10. A) Jablonski diagram with the electronic levels and possible transitions between singlet (S) and triplet (T) states of a luminescent molecule. A: absorption, F: fluorescence, P: phosphorescence, IC: internal conversion, VR: vibrational relaxation, ISC: intersystem crossing. B) Example of large stoke shift of a molecule.

In these d^6 complexes, the degenerated d-orbitals are destabilized and divided by a certain energy Δ_0 (**Figure 1. 11A**) in an octahedral ligand field. This energy between the three orbitals t_{2g} (d_{xy} , d_{xz} , d_{yz}) and the two orbitals e_g (d_{z^2} , $d_{x^2-y^2}$) is determined by the metal oxidation state, the atomic number, and the field strength of the ligand (whose order establishes the spectrochemical series).⁸⁶ It is additionally possible to portray the molecular orbitals (MOs) of the metal complex and the transitions between it and the ligands, using the same ligand field theory. According to the localization and nature of these orbitals, we can distinguish different electronic transitions (**Figure 1. 11B**): metal-centered (MC), ligand-centered (LC), and charge transfer (CT), the latter occurring from metal-to-ligand (MLCT), ligand-to-metal (LMCT) or ligand-to-ligand (LLCT). Furthermore, the strong SOC of the Ir(III) center enables efficient intersystem crossing (ISC) from singlet to the forbidden triplet excited states $^3\text{MLCT}$, ^3LC , and $^3\text{LLCT}$.^{86,87} Also, an increase in the ligand-field stabilization energy (LFSE) in these complexes, the higher ionic charge of the metal, and the larger size of the d orbitals result in a less thermally accessible MC^3 non-emissive state, unlike their Fe(II) and Ru(II) d^6 analogs.^{88,89}

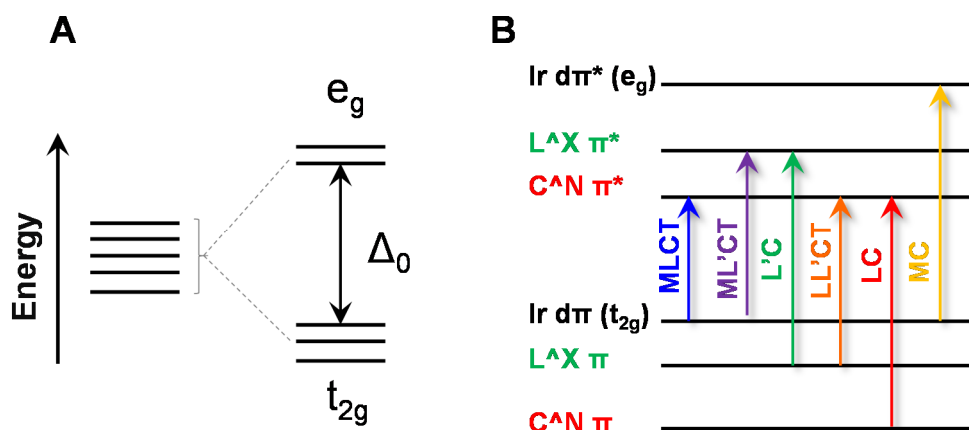


Figure 1. 11. A) Orbital diagram of a d^6 complex in an octahedral ligand field. B) Diagram of the electronic energy levels for a generic $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{L}^{\wedge}\text{X})]$. LC: ligand-centered, MC: metal-centered, MLCT: metal-to-ligand charge transfer, LLCT: ligand-to-ligand charge transfer. Adapted from reference.⁶³

We can obtain a greater understanding of the electronic properties of cyclometalated Ir(III) complexes taking advantage of the good predictive power of density functional theory (DFT) calculations. Using $[\text{Ir}(\text{ppy})_2(\text{bpy})]^+$ (ppy: 2-phenylpyridine; bpy: 2,2'-bipyridine) as an “archetype” complex, it is possible to illustrate some general properties. The highest occupied molecular orbital (HOMO) generally has contributions from an Ir d -orbital and also from the π -system of the aryl rings of the $\text{C}^{\wedge}\text{N}$ ligands. In contrast, the lowest unoccupied molecular orbital (LUMO) has primarily π^* -character localized on the ancillary $\text{N}^{\wedge}\text{N}$ ligand (**Figure 1.12**).^{90,91} More expensive spin unrestricted DFT optimizations on the lowest triplet state of the complex confirmed that the lowest energy emitting state is expected to originate from triplet metal-to-ligand charge transfer ($^3\text{MLCT}$) states admixed with ligand-to-ligand charge transfer (ancillary-based, $^3\text{LL}'\text{CT}$) or ligand centered (^3LC) state character through the population of higher-lying vacant $\text{C}^{\wedge}\text{N}$ -based orbitals.^{89,92} A less commonly encountered situation occurs when strong electron donor groups are substituents on the diimine ligand, and an intra-ligand charge transfer (ILCT) arises.⁹² Interestingly, the nature of emission can be classified by the band shape and luminescence lifetimes. Thus, structured bands showing vibronic progressions result from $^3\text{MLCT}/^3\text{LC}$ states, while broad featureless bands are observed for $^3\text{MLCT}/^3\text{LL}'\text{CT}$ states.⁹¹ In addition, emission lifetimes on the order of hundreds of nanoseconds are typical of $^3\text{MLCT}$ states, while lifetimes for states with an analogous emission intensity but more marked ^3LC character can reach a few tens of microseconds.⁸⁹

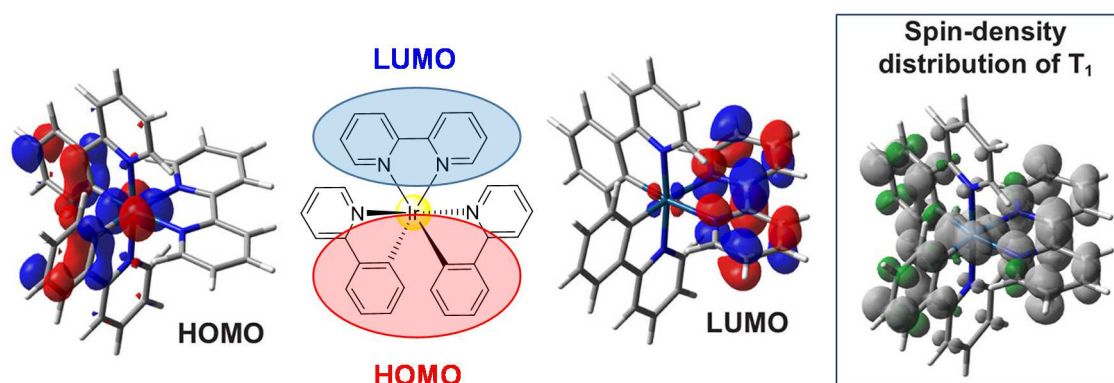


Figure 1.12. DFT predictions of the electron density distribution on the HOMO and LUMO. HOMO: localized on the metal center and the phenyl rings of the cyclometalating ligands. LUMO: localized on the N[^]N ligand. Spin-density distribution of the triplet excited state. Adapted from reference.⁹³

The aforementioned indicates that a rational modification of the ligands around the Ir center can tune the key parameters to achieve new complexes for many applications. These parameters include the emission color, photoluminescence quantum yield (Φ), and the excited-state lifetimes (τ). Therefore, bis-cyclometalated complexes show tunable emission color over a wide range of the visible spectrum and even part of the near-infrared (NIR) region through different ligands since the energy levels of the N[^]N and C[^]N units can be tuned independently of one another.⁹⁴ For example, the placement of electron-donating groups on the ancillary ligand leads to an overall destabilization of the lowest unoccupied molecular orbital (LUMO) and a resulting blue shift in the emission spectrum.⁹⁵ Thus, we can get complexes with an intense blue emission (suitable for OLED applications^{96,97}) and complexes with a red-NIR emission (650–950 nm), which is optimal for use as bioimaging agents, combining virtues of good tissue penetration and low autofluorescence in this range.⁹⁸

The phosphorescent emission quantum yield, Φ , is defined as the ratio between the number of photons emitted and the number of photons absorbed. To change this value, some modifications can be carried out. For example, the quantum yield of the “archetype” complex $[\text{Ir}(\text{ppy})_2(\text{bpy})]^+$ is reported to be just 9%, yet other derivatives are found to possess much higher quantum yields depending upon emission wavelength and steric effects.^{99,100} Hence, a broad range of phosphorescent quantum yields, as well as incredible color versatility and different excited-state lifetimes, has been achieved with 2-phenylpyridine-based iridium(III) complexes with simple modifications in the ligands (**Figure 1.13**).^{85,101} For all this, bis-cyclometalated Ir(III) compounds have been widely studied as cellular imaging reagents and biomolecular probes.^{102,103}

On the other hand, the neutral tris-cyclometalated complexes $[\text{Ir}(\text{C}^{\wedge}\text{N})_3]$ often exhibit higher quantum yields than the cationic bis-cyclometalated complexes analogs.⁶³ However, its application in living systems is limited due to its lack of charge, which makes it difficult to enter the cell, and therefore, modifications must be made to solubilize them.^{104,105}

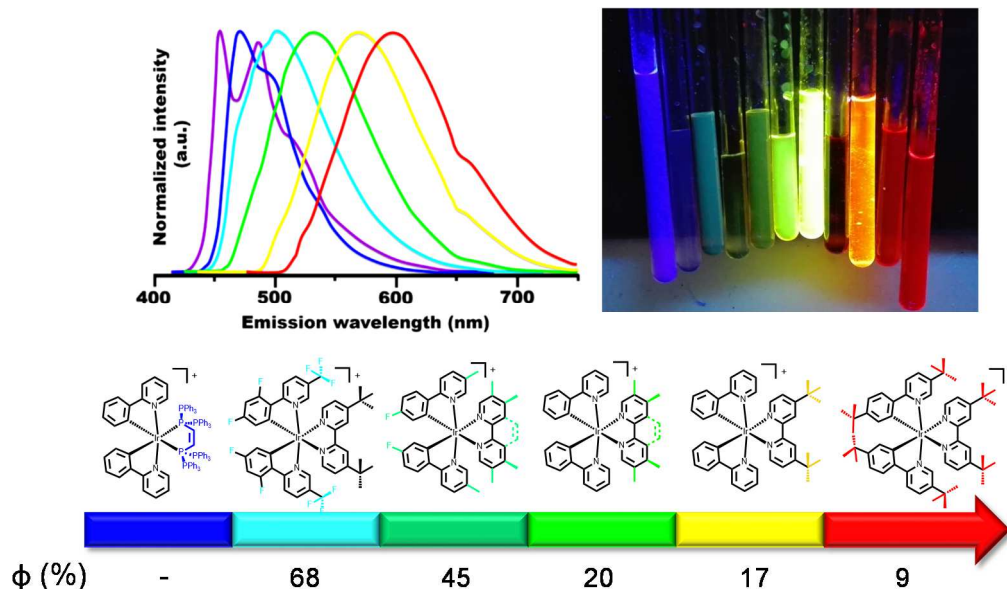


Figure 1.13. Emission quantum yields of cationic luminescent iridium(III) complexes in acetonitrile involving 2-phenylpyridine as a cyclometalated ligand, in the order of increasing emission wavelength (blue to red). Adapted from references.^{85,88}

1.3.2. Cyclometalated Ir(III) anti-cancer theranostic agents

In addition to the role of luminophore, bis-cyclometalated Ir(III) complexes also have a great potential to act as organometallic anti-cancer agents.^{106,107} The combination of both features has allowed the development of new anticancer theranostic agents, which offer both therapeutic promise and potential for diagnosis¹⁰⁸ (**Figure 1.14**).

The successful design of cyclometalated iridium(III) theranostic agents with particular biological properties is still relatively difficult to rationalize and usually relies on lipophilicity, the complex's intrinsic charge, and its aqueous solubility. However, given all the research done so far, the strategies to design Ir(III) complexes to fight cancer can be broadly divided into three main types: organelle-targeted agents, protein-targeted inhibitors, and photosensitizers in PDT (*vide infra*).

For the design of the first ones, we don't have a pattern to follow because there is no clear relationship between the localization inside the cell and the structure of compounds.¹⁰⁶ Sometimes, with a simple change in the structure or the charge, the complexes can be directed to one organelle or another (e.g., **Figure 1.15A**). However, in some cases, it is

possible to target more specifically with organelle-targeting moieties, which are divided into small molecules and peptides. Thus, small molecules such as morpholine¹⁰⁹ and triphenylphosphine (TPP)¹¹⁰ or peptides as octaarginine¹¹¹ can be used to target lysosomes, mitochondria, or nucleus, respectively (see **Figure 1.15B**).

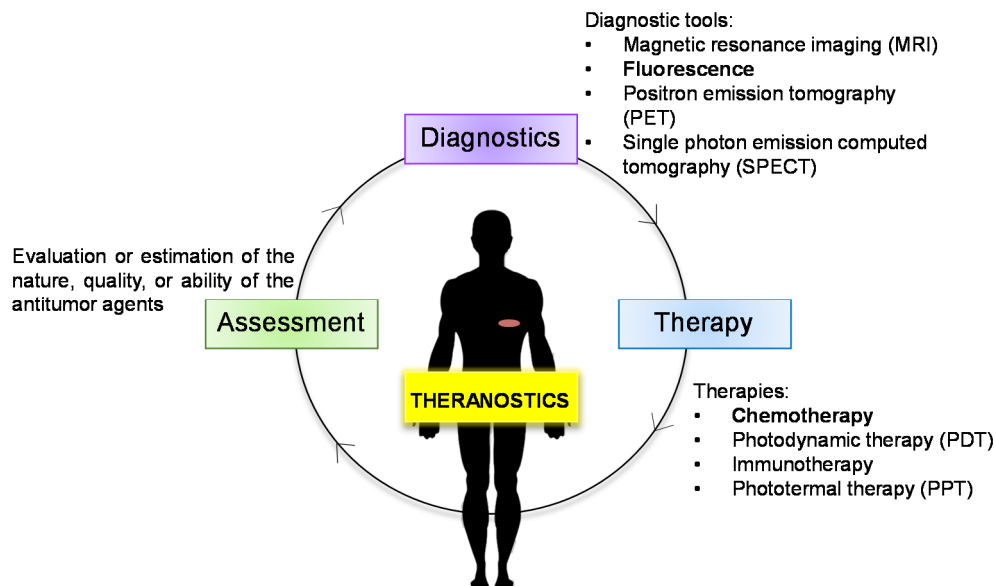


Figure 1.14. Representation of theranostics: a combination of therapy and diagnostics.

On the other hand, the complexes can be bioconjugated to biological or targeting vectors (normally proteins) to induce a specific biological behavior.¹¹² For example, one approach is based on ligands whose receptors are overexpressed in cancer cells compared to healthy cells. Accordingly, a complex conjugated to octreotide-based peptides can target tumor cells overexpressing somatostatin subtype-2 receptors (SSTR2)¹¹³ (**Figure 1.16A**). Another Ir-peptide conjugate with an integrin targeting motif RGD can selectively target angiogenic cancer cells because $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are implicated in tumor angiogenesis¹¹⁴ (**Figure 1.16B**).

The complexes can also be designed to act as an enzyme or protein-protein interaction inhibitors. For instance, in developing organometallic Ir(III) compounds as effective and specific enzyme inhibitors, Meggers and co-workers have pioneered.¹¹⁵ In this regard, our research group synthesized an iridium complex with thiosemicarbazide N[^]S chelate ligand (**Figure 1.17A**),¹¹⁶ inhibiting cathepsin B (involved in invasion and migration of many tumors).

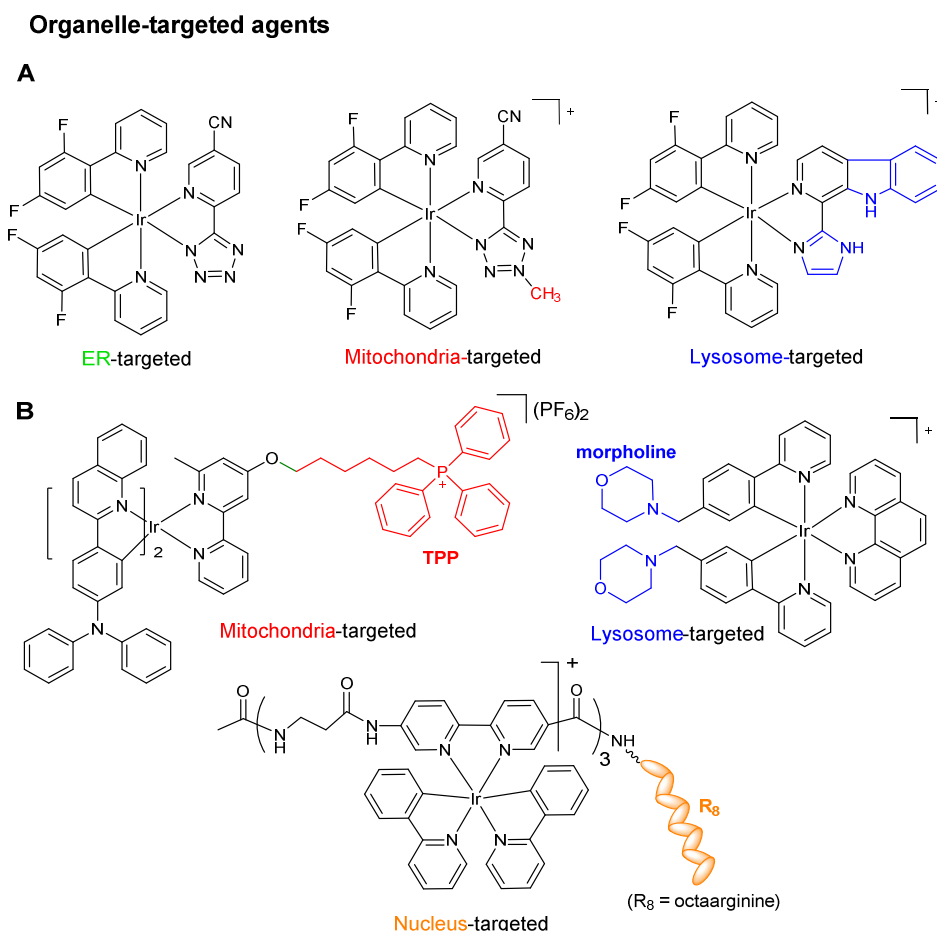


Figure 1.15. A) Changes in the structure of some Ir(III) complexes targeted different organelles: ER, Mitochondria¹¹⁷ and Lysosome¹¹⁸ B) Ir(III) complexes specifically target to mitochondria,¹¹⁰ lysosome¹⁰⁹ or nucleus.¹¹¹

Protein-protein interactions (PPIs) represent a pivotal role in the functions of the cell and, therefore, constitute promising targets for drug discovery. Ma and Leung, innovators in the field, published a very interesting related review in 2014.⁶⁶ PPIs disorders are concerned with oncogenesis because these interactions regulate the receptor tyrosine kinases to induce oncogenic signals to allow cancer progression. For example, anomalous activation of Ras/Raf/mitogen-activated protein kinase (MEK)/extracellular-signal-regulated kinase (ERK) signaling pathway improves tumor initiation, evolution, and metastasis and consequently, occurs frequently in the most recurrent metastasizing tumor, renal cell carcinoma.¹⁰⁶ In this sense, in 2017, Ma and Leung designed an Ir(III) compound (**Figure 1.17B**) with the diimine ligand 4,4'-dinonyl-2,2'-bipyridine,¹¹⁹ which inhibited the H-Ras/Raf-1 interaction and its downstream pathways.

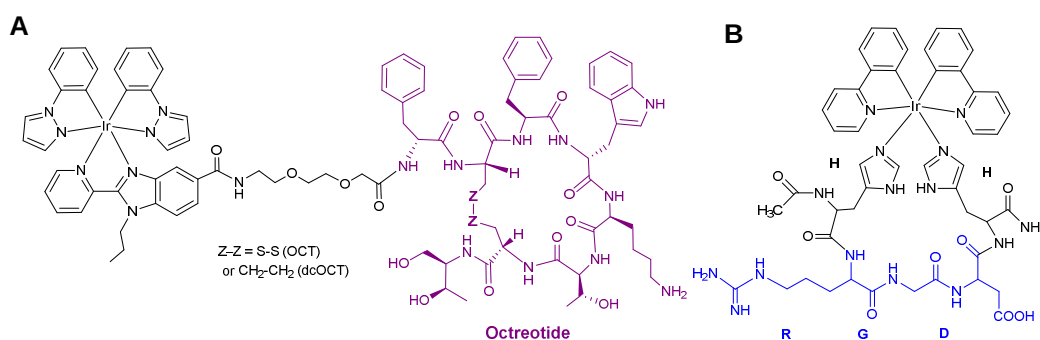


Figure 1.16. A) Somatostatin-targeted Ir(III) complex¹¹³ and B) Integrins-targeted Ir(III) complex.¹¹⁴

On the other hand, there is an increased number of publications based on Ir(III) photosensitizers to fight cancer (see Section 1.4).

Despite everything, a rationalization between the activity and structure of the compounds seems difficult to achieve. The experimental conditions used by the research groups worldwide usually are very different, with variations occurring on the biological protocol, the cell lines used, incubation time, and the compound concentration. These features frequently lead to results that are not just dependent on the chemical nature and characteristics of the metal complex. Nevertheless, the field has rapidly increased in the past decades.

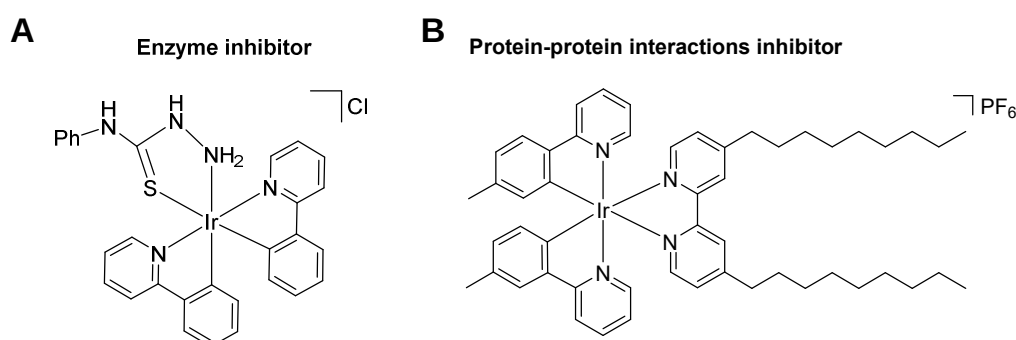


Figure 1.17. Examples of A) an enzyme inhibitor¹¹⁶ and B) protein–protein interactions inhibitor.¹¹⁹

1.4. PHOTOTHERAPEUTIC TRANSITION METAL COMPLEXES: PDT AND PACT

The transition metal complexes allow, with the tunability of their excited states, designing drugs with the desired photobiological mechanisms that are not possible with organic compounds. The modes of light-mediated activation of these antiproliferative drugs can be generally divided into two main categories: (1) photosensitization reactions that involve reactive oxygen species (photodynamic therapy, PDT) and (2) photocleavage reactions of the metal-ligand bond (photoactivated chemotherapy, PACT).

1.4.1. Photodynamic anti-cancer therapy

Light has been used for hundreds of years in therapy for different ailments. Egyptian, Indian and Chinese ancestors used light to treat various diseases, including psoriasis, vitiligo, or even skin cancer.⁴ At the end of the 19th century, Niels Finsen began contemporary investigations into phototherapy.¹²⁰ Finsen treated patients with lupus vulgaris using concentrated beams of UV light, and thanks to this revolutionary approach to treating diseases, he was awarded a Nobel Prize in Physiology and Medicine in 1903. Thus, the discovery in the 1960s of hematoporphyrin derivative (isolated from hemoglobin) as an effective antitumor drug activated with light marked the start of modern photodynamic anti-cancer therapy (**Figure 1.18**).

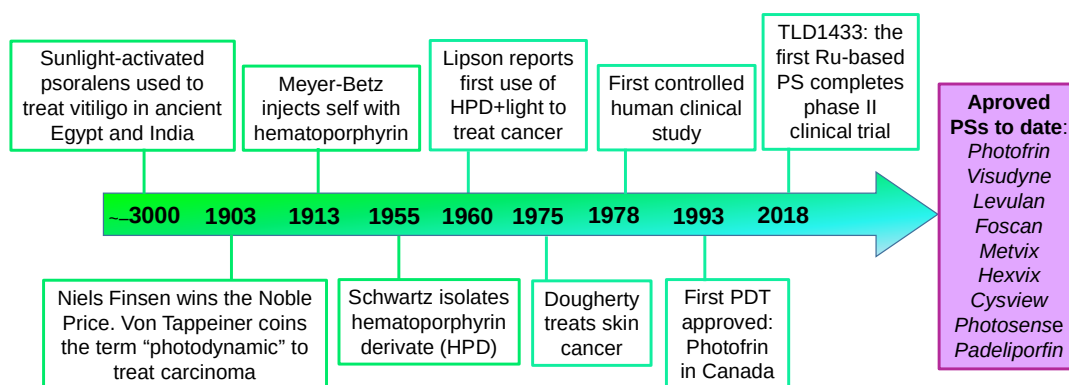


Figure 1.18. Timeline of the historical development of PDT. PS = photosensitizer. Adapted from reference.⁵⁸

In general terms, we can define PDT as an approved non-invasive medical procedure that uses light to activate a drug over a flexible time in a precise space. It is based on the production of toxic singlet oxygen ($^1\text{O}_2$) and/or reactive oxygen species (ROS) through the combination of three non-toxic components: the drug, also called photosensitizer (PS), molecular oxygen ($^3\text{O}_2$), and light. After light irradiation at a wavelength in its range of absorption, the PS is excited from the ground singlet state (S_0) to the excited singlet state (S_1) and then undergo intersystem crossing (ISC) to reach the first excited triplet state (T_1). T_1 triggers the photodynamic therapy through two paths (type I and type II) based on the different photochemical reaction processes (**Figure 1.19**). Type I photoprocess consists of electron or hydrogen transfer to the around biological substrates, generating free radicals that can react with $^3\text{O}_2$ to form ROS (e.g., hydroxyl radicals, peroxides or superoxide anions). Instead, type II mainly involves energy transfer from the photosensitizer to ground-state $^3\text{O}_2$ to yield singlet oxygen ($^1\text{O}_2$). The photo-generated ROS and $^1\text{O}_2$ during PDT induce oxidative stress interrupting normal cell functions that finally drive tumor cell death.

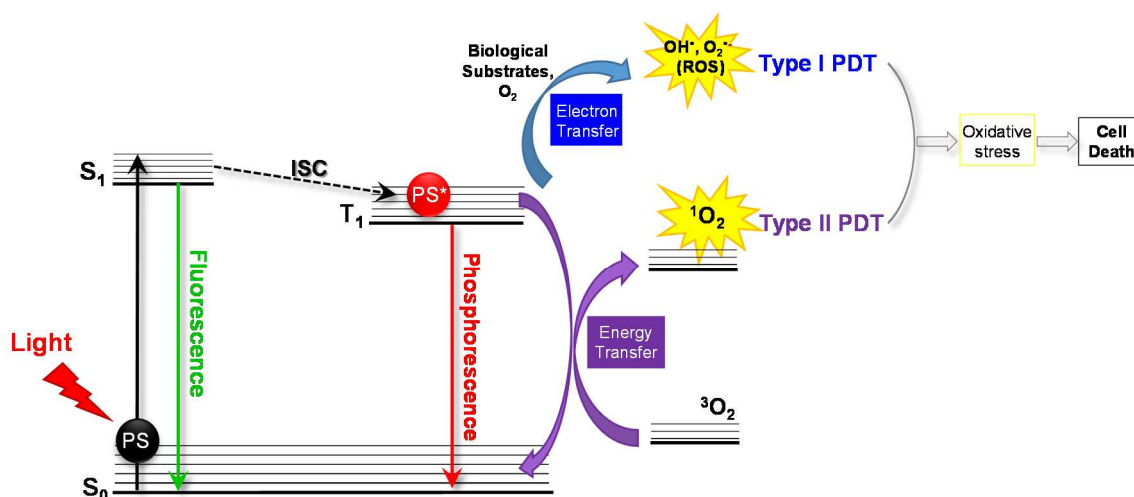


Figure 1.19. Jablonski diagram showing type I and type II PDT photoreactions.

Both mechanisms have complete dependence on molecular oxygen;⁵⁸ thus, immediate oxygen consumption during the therapy could worsen the intrinsic tumor's hypoxic condition. However, in the type I mechanism, some reactive oxygen species could be less oxygen-dependent. For instance, superoxide anion radical ($O_2^{\bullet-}$) disproportionation reaction or Haber–Weiss/Fenton reaction would compensate for this oxygen consumption.^{121,122}

To be applicable in the clinic, a PS should meet some requirements. On the one hand, the PS should be non-toxic in the dark, exhibiting strong phototoxicity. This fact is depicted by the phototoxic index (PI), calculated as the ratio of PS toxicity in the dark and upon light irradiation ($PI = [IC_{50}]_{\text{dark}}/[IC_{50}]_{\text{light}}$, being IC_{50} the concentration of drug required for 50% of cancer cells inhibition). On the other hand, the PS should localize mainly in tumor cells and preferably have great absorption in the “phototherapeutic window” (650–850 nm) to minimize both autofluorescence and light toxicity and maximize tissue penetration of the light.¹²³ Undoubtedly, for deep-seated or thicker solid tumors, tissue-penetrating near-infrared light is necessary. Nevertheless, if PDT is applied topically (e.g., skin cancer), there is no reason to sacrifice PDT potency for deeper penetration through the human tissues.⁵⁸

Current FDA-approved photosensitizers for PDT treatment are mostly porphyrin-based compounds¹²⁴ (phthalocyanines, bacteriochlorins, chlorins, and similar structures). Nonetheless, their use in the clinic is restricted by the low quantum yield of singlet oxygen generation, the short lifetime of their excited state, and rapid photodegradation (photobleaching).¹²⁵ These facts, in addition to their significant side effects, have encouraged the design of new classes of PSs. One of the strategies is the use of metals.

required for applying this strategy are associated with a high cost and are currently not present in clinics.¹³⁰ Nevertheless, a new technique named “interstitial PDT” allows irradiating with light internal organs such as the pancreas, the brain, or even the lungs through the use of optical fibers that are directly introduced into the tumor.¹³¹

In the last year, there has been an increase in the publication of Ir(III)-based PSs in high-impact index journals,^{132–136} which shows great interest in developing these kinds of drugs. Some of these complexes (with high PI even under hypoxia) are shown in **Figure 1.21**.

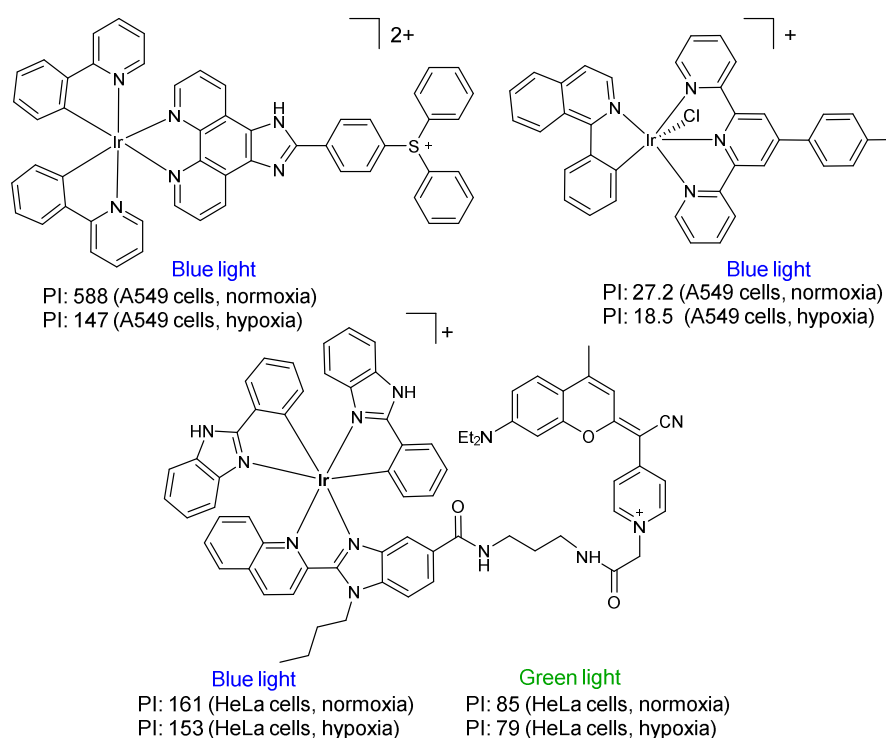


Figure 1.21. Examples of Ir(III)-based PSs with their PI in normoxia and hypoxia.^{132–134}

In the same way as for iridium theranostic agents, each research group uses different conditions (light potency (W/cm^2), determination of singlet oxygen quantum yields, cell lines, etc.) and different light sources. Thus, it's difficult to compare the activity from one PS and another because there are no generalized protocols.

1.4.2. Photoactivated chemotherapeutic (PACT)

Inorganic photoactivated chemotherapy (PACT) is an approach where a protected bioactive caged compound (M–L, **Figure 1.22A**) is triggered and released by light irradiation. Cleavage of the M–L bond can occur via photoreduction, photosubstitution, or radical mechanisms, and either the fragment M or L, or both, may have biological

A

<Caged>
No effect

Active molecules

B

DNA

P450 Enzyme

Ru(II) complexes have been the most studied metal compounds as photoactivated chemotherapeutic agents.^{131,137,138} The photochemical reaction can happen at any step during the decay to the ground state, and is therefore highly connected to the excited state configurations, the nature and energy of close states, and the accessibility of the dynamic process.¹³⁹ Thus, Ru(II) compounds can populate triplet metal-centered (³MC)¹³⁸ which have a strong dissociative character, leading to ligand substitution. An example of this type of complexes is shown in **Figure 1.22B**: a ruthenium complex containing P450 inhibitors for dual DNA damage and enzyme inhibition.

In contrast, the mixed $^3\text{MLCT}$ and ^3LC states of iridium complexes turn them into highly photostable drugs, and their use in PACT continues undeveloped.¹⁰⁶ Thus, only two examples of photoactivated chemotherapeutic Ir(III) compounds have been described. Lo *et al.* designed¹⁴⁰ a photoactivatable iridium agent (**Figure 1.23A**) similar to the Ru(II) compound previously reported by Gasser,¹⁴¹ using the photocaging technology to avoid the Ir(III) complex activation before irradiation. The active iridium compound $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{bpy-CH}_2\text{NH}_2)]$ ($\text{bpy-CH}_2\text{NH}_2$ = 4-aminomethyl-4'-methyl-2,2'-bipyridine) was modified with a PEG chain using a nitrobenzyl photolabile protecting group (PPG). In addition, Mao and

co-workers synthesized three Ir(III) complexes (**Figure 1.23B**)¹⁴² with two five-membered heterocyclic ligands, mainly because of the coordination of these monodentate ligands to the metal is more unstable as compared with bidentate ligands. After blue light irradiation (425 nm), the monodentate heterocyclic ligands were dissociated from the metal center, showing a high light-initiated anti-cancer therapeutic effectiveness.

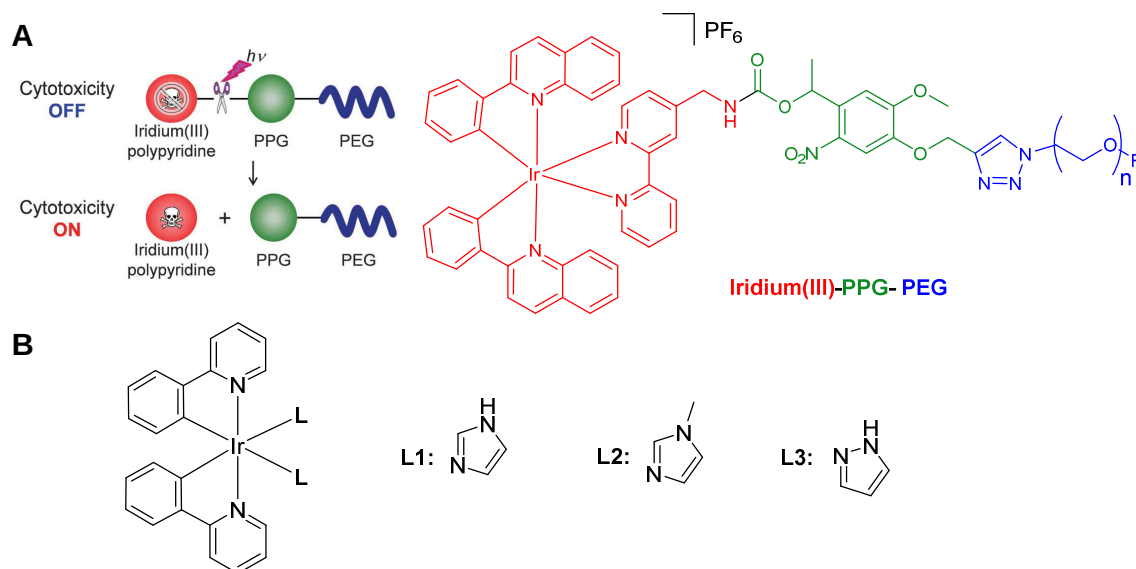


Figure 1.23. A) Photorelease of the PEG pendant from the iridium(III)-PPG-PEG complex.¹⁴⁰ B) Chemical structures of complexes designed by Mao.¹⁴²

1.5. OBJECTIVES

This thesis is outlined in the context of the synthesis and characterization of new octahedral Ir(III) complexes and the study of their potential as anti-cancer drugs in the therapy and phototherapy of cancer. The work is structured in eight chapters, including a general introduction (chapter 1) and final conclusions (chapter 8). Chapters 2, 3, and 4 will focus on a structure-activity relationship (SAR) study of cationic Ir(III) complexes, where we will introduce modifications in both the N[^]N and the C[^]N ligands to study the effect on their photodynamic activity. In the following chapters (5, 6, and 7), we will elucidate the synergistic effect of using two metals in the same molecule, the advantage of operating *in vivo* models in the investigation of a new drug, and the need to use nanoparticles to increase the accumulation and biocompatibility of neutral iridium complexes respectively. This research field is multidisciplinary, and consequently, the results obtained from both *in vitro* and *in vivo* biological studies, and the preparation of nanoparticles loaded with some selected iridium complexes, have required the collaboration with other research groups

with expertise in these fields: Prof. Viktor Brabec (Institute of Biophysics of Brno, Czech Republic), Dr. Fernando Gandía-Herrero (Biochemist and Molecular Biology A Department, University of Murcia) and Dr. Vicente Marchán (Organic Chemistry Department, University of Barcelona).

The aims of each chapter are:

1. Synthesis and characterization of bis-cyclometalated Ir(III) complexes bearing different polypyridyl N[^]N ligands with π -extended conjugation to investigate their photophysical properties and cytotoxic potential in dark conditions and after irradiation with blue light.
2. Modification of cyclometalating ligand in a series of Ir(III) complexes of the type [Ir(C[^]N)₂(dppz)][PF₆] to tune the photophysical properties, lipophilicity, cellular uptake efficacy, and phototherapy activity even under normoxia and hypoxia.
3. Development of novel bis-cyclometalated Ir(III) complexes modifying the N[^]N ligand based on benzimidazole backbone containing NH groups to study their activity against melanoma cancer cells under blue light irradiation and deepen into its mechanism of action.
4. Design and synthesize of a new dinuclear Ir(III)-Pt(II) complex to overcome resistance to drugs such as cisplatin with a synergistic effect of two metal centers and incorporate features like traceability.
5. Synthesis of a new Ir(III) complex to investigate its potential as an anti-cancer drug *in vivo* in a recently developed *Caenorhabditis Elegans* tumoral model.
6. Synthesis and characterization of four neutral tris-cyclometalated Ir(III) complexes and their vehiculation using polymeric nanocapsules to improve their biocompatibility and cellular uptake and study their potential activity against multidrug-resistant HeLa cancer cells.

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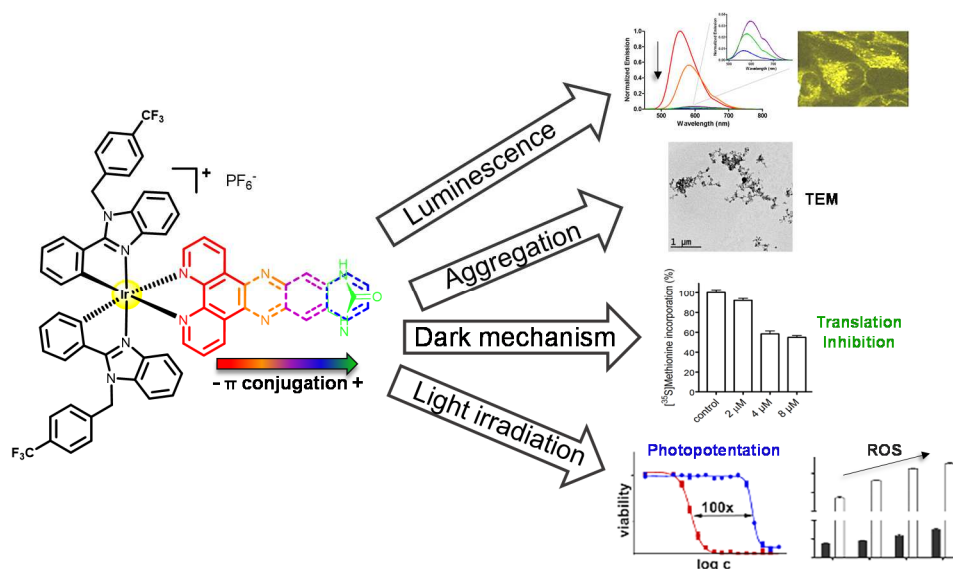
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Chapter 2

Effect of polypyridyl ligands on the antitumor activity of phosphorescent Ir(III) complexes under dark and light conditions



The biological experiments included in this chapter were carried out by Dr. Viktor Brabec (Institute of Biophysics, Czech Academy of Sciences, Brno, Czech Republic).

2.1. INTRODUCTION

As commented in Chapter 1, the antiproliferative activity of cisplatin arises from the ability of this complex to generate covalent adducts with nuclear DNA, which ultimately triggers cell death.¹ There are many metallodrugs with this traditional mode of action,² but the side effects observed by the patients undergoing chemotherapy with this kind of drugs are due, among other things, to the lack of selectivity of this covalent binding. Therefore, new strategies have been developed for the discovery of novel metallodrugs with different mechanisms of action as targeting cellular organelles.³

The endoplasmic reticulum (ER), which has an important role in the synthesis of protein, lipid, and hormones,⁴⁻⁶ is not well explored as the target for anticancer agents compared to the other subcellular targets.⁴ Most metal-based drugs directed at this organelle cause ER stress, leading to subsequent cell death.^{7,8} However, obtaining metal-based translation inhibitor drugs is virtually non-existent, only some complexes are known.⁹⁻¹¹ Our research group has developed some series of Ru(II) complexes that interfered with protein synthesis with a markedly higher potency than conventional inhibitors of DNA translation.^{9,12} More recently, Ruiz, Gandía-Herrero *et al.* have reported new half-sandwich osmium(II) complexes that reduced protein translation *in vitro* and were able to reduce *in vivo* tumor growth using the animal model *C. elegans*.¹³ Furthermore, the antitumor platinum complex oxaliplatin can induce ribosome biogenesis stress causing cancer cell death and not by eliciting a DNA-damage response, as believed up to recently.¹⁴

As mentioned previously, bis-cyclometalated iridium(III) complexes have attracted much attention as possible PDT agents due to their convenient tunable photophysical properties.¹⁵ Ir(III) complexes exhibit significantly larger Stokes shifts than do organic PDT agents; this greatly reduces the interference between excitation and emission. Remarkably, photophysical properties such as absorption wavelength, quantum yields, and emission lifetimes can be tuned by modifying the cyclometalated ligand and/or ancillary ligand to optimize PDT requirements. Moreover, Ir(III) compounds also show high photochemical stability, in contrast to the fast photodegradation (photobleaching) of porphyrin derivatives.¹⁶

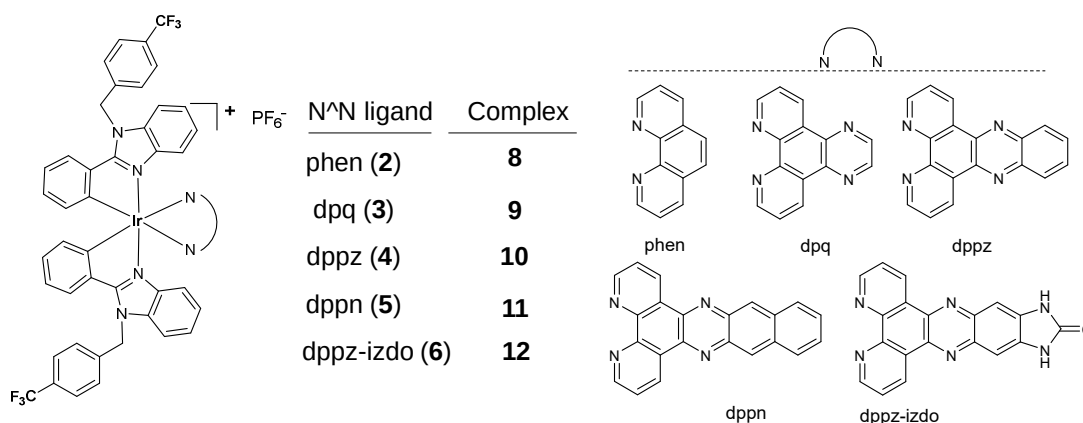
In this chapter, we describe a series of phosphorescent organometallic Ir(III) complexes (**8-12**) of the type $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{N}^{\wedge}\text{N})][\text{PF}_6]$ (**Scheme 2.1**), in which the cyclometalating ligand (**1**) 2-phenyl-1-[4-(trifluoromethyl)benzyl]-1H-benzo[d]imidazol remains as the $\text{C}^{\wedge}\text{N}$ backbone and the diimine ligand is varied $\{\text{N}^{\wedge}\text{N}=\text{phen}$ (**2**), dpq (**3**), dppz (**4**), dppn (**5**), and

dppz-izdo (**6**); phen=1,10-phenanthroline; dpq=dipyrido[3,2-d:2',3'-f]quinoxaline; dppz=dipyrido[3,2-a:2',3'-c]phenazine; dppn=benzo[i]dipyrido[3,2-a:2',3'-c]phenazine; dppz-izdo=dipyrido[3,2-a:2',3'-c]phenazine-10,11-imidazolone} with the aim to fine-tune the photophysical and antitumor properties of these kind of interesting drugs.¹⁷ Particularly, benzimidazole is a widely used pharmacophore,¹⁸ and the choice of the trifluoromethylbenzyl group on the nitrogen atom of the benzimidazole provides a higher lipophilicity of the ligand. The *in vitro* antiproliferative activities of **8–12** were studied against various human cancer cell lines both under dark and irradiation conditions. The complexes preferentially targeted the endoplasmic reticulum and the mechanism of action of **8** and **12** in the dark, which included the induction of apoptosis, also involved inhibition of protein translation. In addition, their activity increased upon blue light irradiation with a large increase (~10–30-fold) of the intracellular ROS level.

2.2. RESULTS AND DISCUSSION

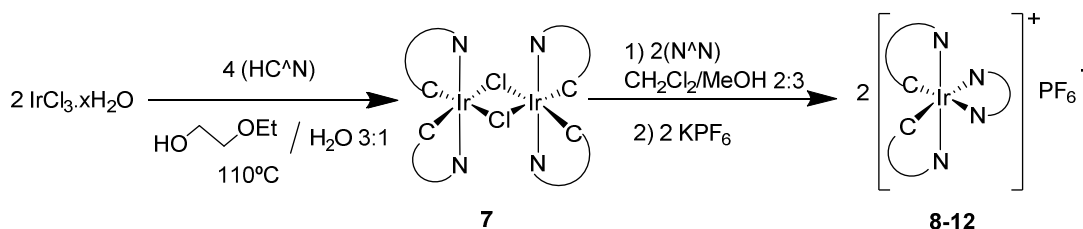
2.2.1. Synthesis and characterization

Five new luminescent iridium(III) complexes **8–12** (**Scheme 2.1**) (C[^]N = 2-phenyl-1-[4-(trifluoromethyl)benzyl]-1H-benzo[d]imidazol-κN,C; N[^]N = phen **8**, dpq **9**, dppz **10**, dppn **11** and dppz-izdo **12**) were synthesized and characterized to investigate the effect of the degree of the π conjugation of the polypyridyl ligands on the photophysical properties and their biological activity. The HC[^]N pro-ligand (**1**) 2-phenyl-1-[4-(trifluoromethyl)benzyl]-1H-benzo[d]imidazol-κN,C was synthesized as previously reported by us¹⁹ (**Scheme E2.1** in the Experimental Section). The N[^]N ligand 1,10-phenanthroline (**2**) is commercial. The quinoxaline and phenazine N[^]N ligands (dpq (**3**), dppz (**4**), dppn (**5**), and dppz-izdo (**6**)) were synthesized through Schiff-base condensation of 1,10-phenanthroline-5,6-dione (phendio) with ethylenediamine, o-phenylenediamine, 2,3-diaminonaphthalene or 5,6-diaminobenzimidazolone-2 (**Scheme E2.2** in the Experimental Section) as previously reported.^{20,21}



Scheme 2.1. Structures of new iridium(III) complexes **8–12**.

The complexes **8–12** were obtained in good yields (65–86%) in the form of racemic mixtures (Λ and Δ enantiomers), by reaction of the chloro-bridged Ir(III) dimer **7** with the corresponding N^N ligand in a 1:2 molar ratio (**Scheme 2.2**). All compounds were characterized by ^1H and ^{13}C NMR spectroscopy (**Figures S2.1–S2.7**) and positive-ion HR ESI-MS (**Figure S2.8**), and they were shown to be at least 96% pure by both elemental analysis and RP-HPLC in DMSO (**Figure S2.9**). The NMR spectra of new complexes exhibited one set of peaks for the two equivalent cyclometalating ligands due to the symmetric nature of the coordination environment around the metal. The positive-ion ESI mass spectra showed the $[\text{M}-\text{PF}_6]^+$ signals with the expected isotopic distribution pattern.



Scheme 2.2. Synthetic route for the preparation of the new Ir(III) complexes **8–12**.

2.2.2. Structures determination

Single crystals suitable for X-ray diffraction analysis were obtained by slow diffusion of hexane into saturated solutions of **9** and **10** in dichloromethane and 1,2-dichloroethane, respectively. Their molecular structures with hydrogen-bonding schemes are shown in **Figure 2.1**. Crystallographic data are given in **Tables S2.1–S2.6**, and selected bond lengths and angles are listed in **Table S2.7**. The iridium atoms have a pseudo-octahedral coordination geometry, and the largest deviation is represented by the bite angle (from 77.6 to 77.988) of the ancillary ligand. In the C^N ligands, the two Ir-C bonds are in a mutual cis disposition and their high trans influence results in Ir-N bond lengths that are slightly shorter than in the N^N ligands. These findings are commonly observed for reported bis-

cyclometalated Ir(III) complexes.²² The iridium complex cation of **9** crystallized with one PF₆ anion and approximately 1.75 methylene chloride solvent molecules. The positive complex cation of **10** crystallized with one PF₆ anion and approximately 2.375 1,2-dichloroethane and one hexane solvent molecules per formula unit, exhibiting disorder of the solvent molecules within the solvent-occupied voids between the complex molecules. Besides the important cation-anion Coulomb interactions, the packing in the structures of **9** and **10** is organized (according to an analysis by PLATON²³) by intermolecular C-H...F²⁴⁻²⁷ (Tables S2.8, and S2.11), C-H...Cl contacts (only for **3**), C-H... π interactions^{28,29} (Scheme S2.1, Tables S2.9, and S2.12, Figures S2.11 and S1.14), and π - π interactions^{30,31} (Scheme S2.2, Tables S2.10, and S2.13, Figure S2.12). The π - π interactions between the dipyridophenazine ligands of **10** lead to the pairwise arrangement of two symmetry-related molecules (Figure 2.1C).

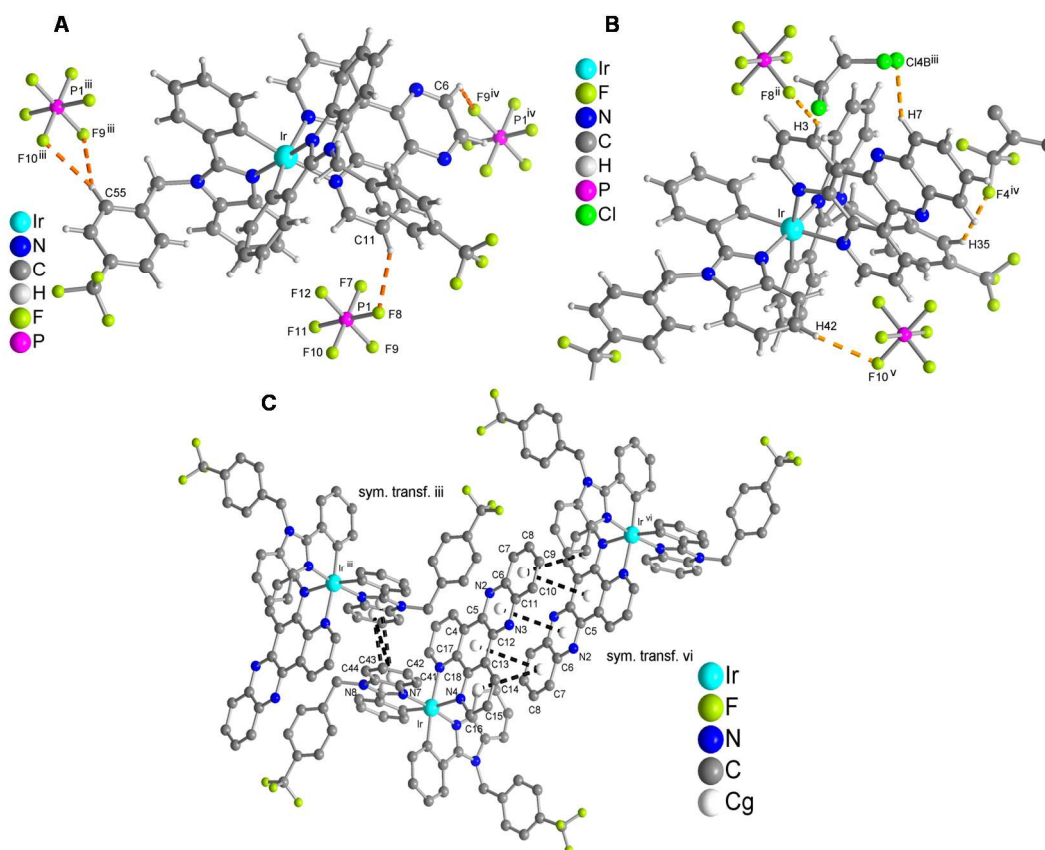


Figure 2.1. Molecular structures and part of hydrogen bonding schemes A) for complex **9**, showing the Ir(III) complex cation and three surrounding PF₆ anions in the crystal structure (CH₂Cl₂ solvent molecules of crystallization were omitted for clarity). B) for complex **10**, showing the Ir(III) complex cation and two surrounding PF₆ anions, a trifluoromethylphenyl fragment from an adjacent cation, and a C₂H₄Cl₂ solvent molecule in the crystal structure. C) π - π interactions in the packing of **10** in the crystal, indicated as dashed black lines. Details of these π - π interactions, including the symmetry transformations are given in Table S2.13 (Cg = ring centroid).

2.2.3. Photophysical properties

The UV/Vis absorption and emission spectra were recorded for **8–12** in CH₃CN, Tris buffer (1% DMSO), and water (1% DMSO) at room temperature (**Table 2.1**, **Figures S2.15–S2.17**). The UV/Vis spectra of complexes are characterized by multiple bands (**Figure 2.2A** for **8–12** in water (1% DMSO) and **Figure S2.16**).

Table 2.1. UV/Vis absorption bands, extinction coefficients and emission wavelengths for **8–12**^a

Compound	Solvent	λ_{abs} , nm ($\epsilon/10^4 \text{ M}^{-1} \text{ cm}^{-1}$)	$\lambda_{\text{exc}}/\lambda_{\text{em}}$, nm
8	ACN	251 (3.18), 299 (2.22), 307 (2.31), 380 (0.79)	381/565
	H ₂ O ^b	235 (4.76), 297 (3.24), 381 (1.18)	381/557
	TrisHCl ^b	238 (4.02), 274 (3.50), 309 (3.10), 379 (1.39)	381/555
9	ACN	260 (5.64), 291 (3.37), 314 (3.47), 378 (0.98)	381/581
	H ₂ O ^b	254 (6.03), 296 (3.95), 381 (1.13)	381/582
	TrisHCl ^b	259 (5.71), 300 (4.13), 383 (1.32)	381/581
10	ACN	242 (4.57), 280 (7.19), 362 (2.04), 382 (1.32)	380/615
	H ₂ O ^b	245 (5.41), 281 (6.18), 380 (1.78)	380/596
	TrisHCl ^b	249 (3.98), 280 (4.41), 364 (2.07), 384 (1.94)	380/605
11	ACN	241 (6.11), 326 (8.10), 395 (1.51), 421 (1.05)	380/575
	H ₂ O ^b	242 (6.47), 310 (5.65), 380 (1.52)	380/566
	TrisHCl ^b	248 (3.58), 324 (3.32), 400 (1.42)	380/566
12	ACN	235 (6.15), 307 (6.03), 413 (3.09), 505 (0.36)	405/582
	H ₂ O ^b	232 (9.41), 308 (5.62), 415 (2.30)	405/580
	TrisHCl ^b	239 (5.02), 311 (4.41), 412 (2.28)	405/585

^a[Ir complex] = 10 μM in aerated solvent. ^b 1% DMSO

All complexes display intense and structured absorption bands at approximately 230–350 nm. The magnitudes of their molar extinction coefficients in this region ($\sim 4\text{--}6 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$, **Table 2.1**) are consistent with spin-allowed ligand-centered (¹LC) $\pi\text{--}\pi^*$ transitions arising from cyclometalated (C^N) and diimine (N^N) ligands.³² The less

intense absorbance bands and shoulders at $\lambda > 350$ nm can be mainly attributable to mixed singlet and triplet metal-to-ligand charge-transfer (spin-allowed singlet-to-singlet $^1\text{MLCT}$ and spin-forbidden singlet-to-triplet $^3\text{MLCT}$) and ligand-to-ligand charge-transfer (LLCT) transitions.^{33–35} The intensities of these absorption bands gradually increased from complex **8** to complex **12** when the π -conjugation was increased on the diimine ligands, as happened in other Ir(III) polypyridyl complexes previously described.³² Upon excitation at $\lambda = 380$ nm (for **8–11**) or $\lambda = 405$ nm (for **12**), they exhibit yellow to orange emission (**Figure 2.2B** and **S2.17**) slightly sensitive to the nature of the solvent (**Table 2.1**). The emission lifetimes of **8–10** and **12** in water are shown in **Table 2.2** and they are consistent with excited states of triplet parentage.³⁶ The lifetime of **11** could not be measured because the intensity in the decay kinetics curves was very low and matched with the background (prompt in **Figure S2.18**).

Table 2.2. Excitation wavelengths (λ_{exc}), emission wavelengths (λ_{em}), emission lifetimes (τ_{em}), and quantum yields (Φ_{em}) of **8–12** in water (1% DMSO).

Compound	λ_{exc} , nm ^a	λ_{em} , nm	τ_{em} , ns ^b	Φ_{em} , % ^d
8	381	557	54 (12%) 310 (88%)	22.3
9	381	582	55 (9%) 290 (91%)	10.5
10	380	595	34 (22%) 170 (78%)	1.5
11	380	566	— ^c	<1
12	405	580	22 (25%) 190 (75%)	<1

^a λ_{exc} maxima. ^b Emission lifetimes measured in aerated solutions. ^c Too weak to be measured. ^d Emission quantum yields measured in deaerated solution

A decrease in the emission intensity (**Figure 2.2B**) and emission quantum yields (**Table 2.2**) is observed in water upon increasing the length of the polypyridyl ligand. As the emission spectra remain the same in the presence or absence of oxygen (**Figure 2.2C**), the decrease in the emission intensity is attributed to the luminescence aggregation-caused quenching (ACQ) effect.^{37–39}

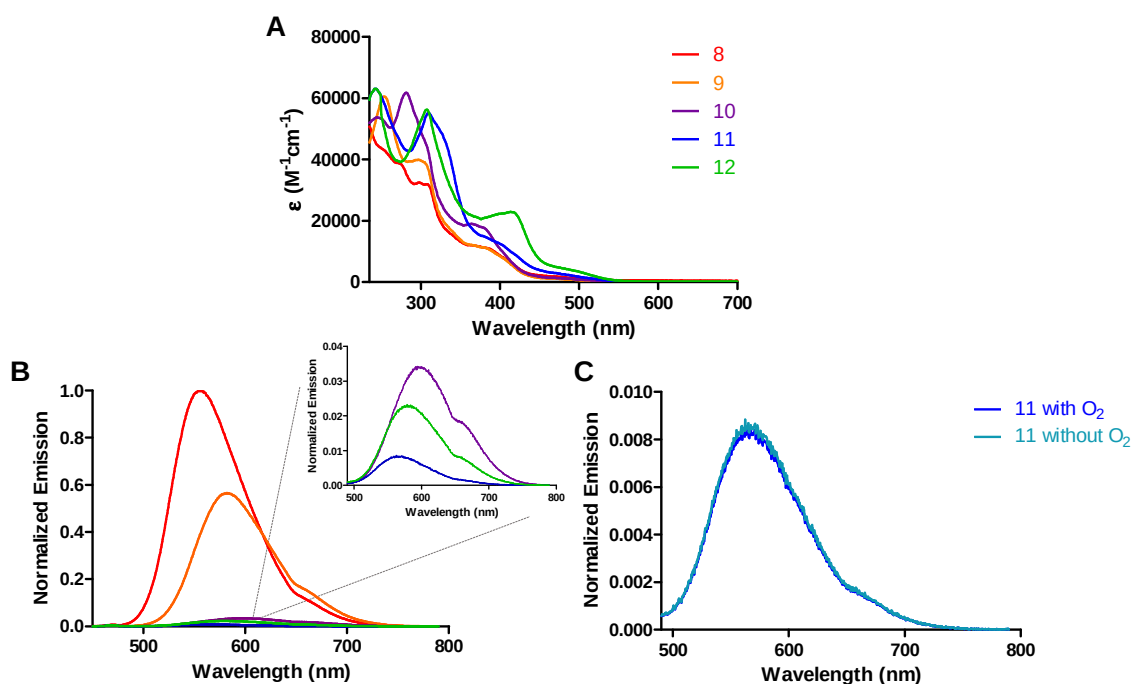


Figure 2.2. A) UV/Vis absorption and B) emission spectra of complexes **8-12** in water at room temperature. $\lambda_{\text{exc}} = 405$ nm. C) Emission spectra of **11** in aerated and deaerated water solution. $\lambda_{\text{exc}} = 405$ nm. In all measures, the concentration was 10 μM and the final content of DMSO was 1%.

To study the aggregation for the new compounds, ^1H -NMR spectroscopy was used. The aromatic proton resonances of the N^N ligand shown an upfield shift in the spectrum of **11** with increasing the concentration (**Figure 2.3A**) and at different compositions of mixtures of $[\text{D}_6]\text{DMSO}$ and D_2O (**Figure 2.3B** for **11** and **Figure S2.19** for **12**). This can be attributed to favorable π - π stacking, which is well known to lead to the formation of dimers or higher-order aggregates in solution.^{40,41} In fact, the proton signals become poorly resolved when the percentage of water increases from 0 to 30% (for **11**, **Figure 2.3B**) and from 0% to 50% (for **12**, **Figure S2.19**). In both cases, an increase in the water content up to 70% lead to the virtual disappearance of the resonances in the ^1H -NMR spectra. This behavior is not observed for complexes with a lower degree of π conjugation of the polypyridyl ligand (**Figure S2.20** for complex **8**). Spherical and elongated aggregates in water were observed by transmission electron microscopy (TEM) for **11** (**Figure 2.4A** and **S2.21**), and **12** (**Figure 2.4B**) respectively verifying the occurrence of aggregation.

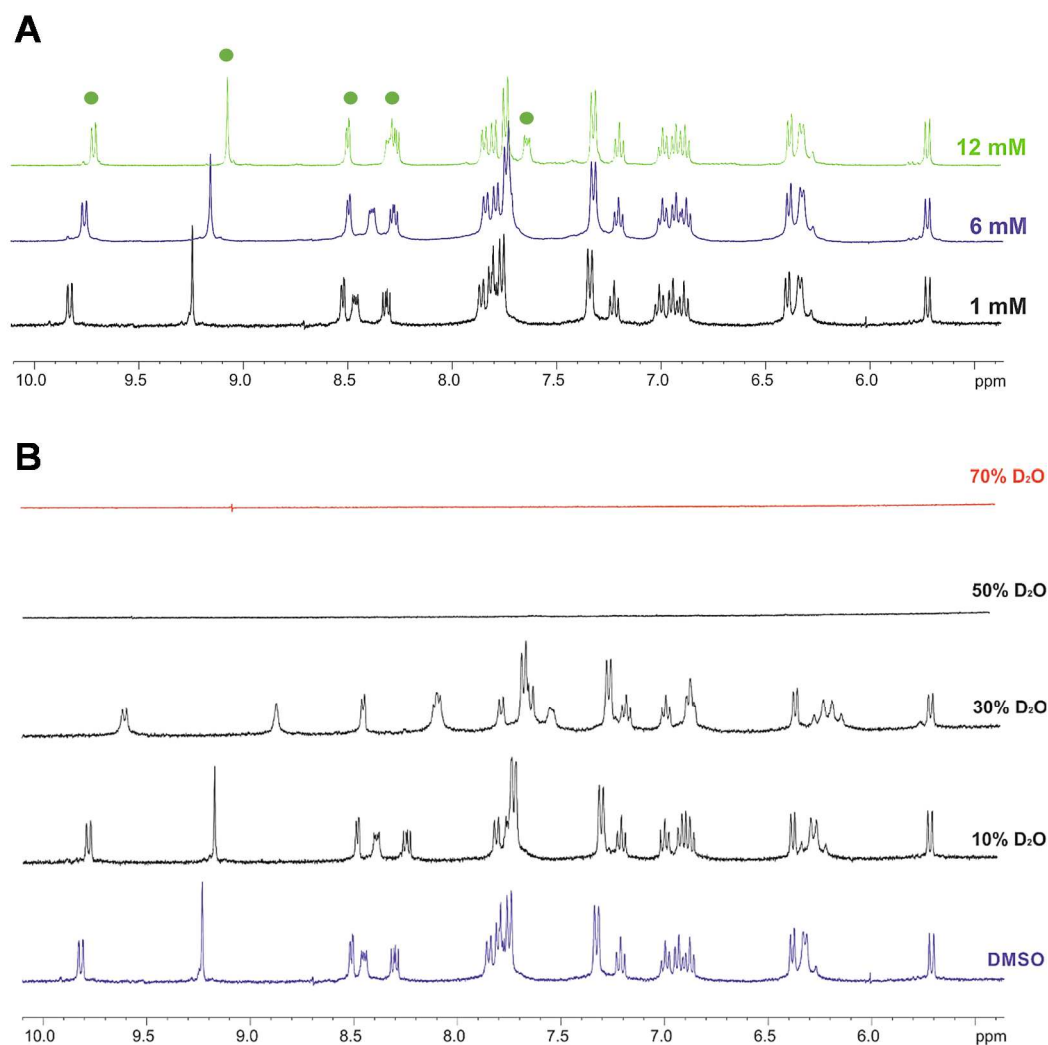


Figure 2.3. A) **11** at different concentration in $[D_6]$ DMSO (400 MHz, 25 °C). Some proton signals ($N^{\wedge}N$ ligand) show an upfield shift when the concentration increases (green dots). B) 1H NMR traces of **11** at different composition of D_2O in $[D_6]$ DMSO (1×10^{-3} M, 400 MHz, 25 °C).

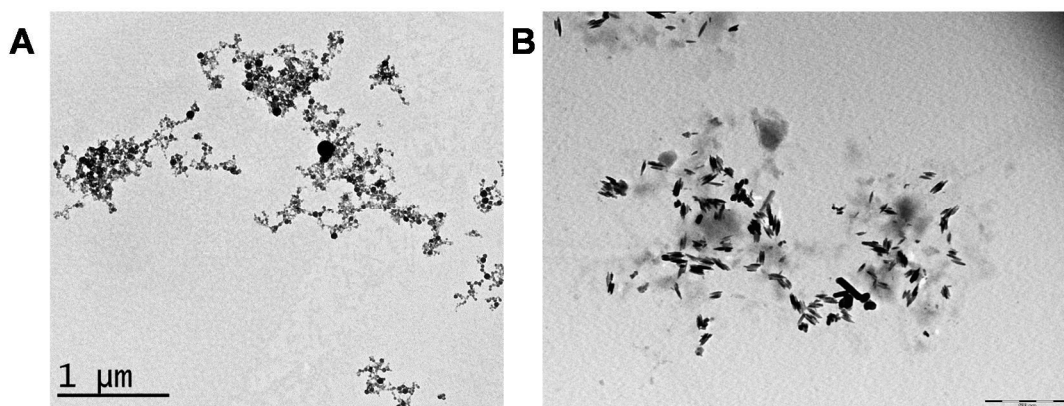


Figure 2.4. TEM images of the aggregates prepared A) for **11** and B) for **12** in water:DMSO (99:1) ($10 \mu M$).

2.2.4. Stability and photostability studies

Before the biological study, it is important to know the solution stability of the new compounds. Since the complexes are not soluble in water, we need to dissolve them in a minimal amount of DMSO to solubilize them. Thus, the stability was studied first in DMSO by ^1H -NMR, and no free ligand was observed after 24 h at room temperature (**Figure 2.5A**). The stability of complexes was also confirmed after 24 h in RPMI culture medium by HPLC (**Figure 2.5B** and **Figure S2.22**).

It is also important to check the photostability of the complexes to ensure that they are not degraded by the action of light. The photostability in water (1% DMSO) was confirmed for **10** by UV/Vis, not observing changes in the spectra before and after light irradiation at 420 nm for 15 min (**Figure 2.5C**).

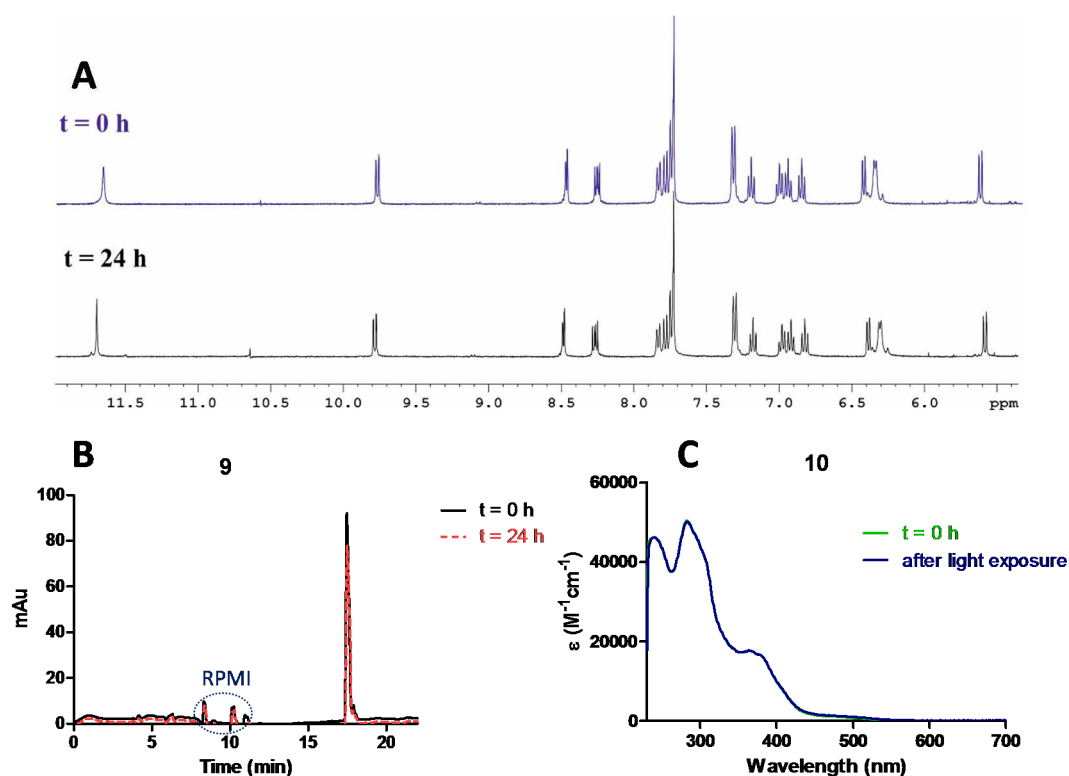


Figure 2.5. A) ^1H NMR spectra of **12** measured after dissolving immediately in $[\text{D}_6]\text{DMSO}$ (top) and after 24 h (bottom) at RT. B) HPLC chromatogram of complex **9** in RPMI culture medium (5 % DMSO) at $t = 0$ h (—) and $t = 24$ h (---) incubation. C) UV/Vis spectra of complex **10** before ($t = 0$ h) and after light exposure.

2.2.5. Cytotoxicity studies towards human cancer cells

The antiproliferative activity of complexes **8-12** were evaluated *in vitro* against several human tumor cell lines: ovarian A2780, cisplatin-resistant ovarian A2780cisR, cervix HeLa, and breast MCF-7 cancer cells. The standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to obtain their cytotoxicity values.

Table 2.3. Cytotoxicity [IC_{50}^a mean values (μM)] obtained for complexes **8-12** and cisplatin.^b

	A2780	A2780cisR	RF ^c	HeLa	MCF-7	MCF-10A	SF ^d
Cisplatin	5.4 ± 0.4	15.5 ± 0.4	2.9	14.0 ± 0.9	16.6 ± 0.7	20 ± 2	1.2
8	0.63 ± 0.03	0.38 ± 0.09	0.6	1.8 ± 0.1	0.7 ± 0.1	2.6 ± 0.3	3.7
9	1.1 ± 0.1	0.37 ± 0.09	0.3	0.9 ± 0.2	0.5 ± 0.1	3.2 ± 0.2	6.4
10	2.9 ± 0.6	2.80 ± 0.01	0.9	6.1 ± 0.3	6.3 ± 0.8	9.9 ± 0.8	1.6
11	20 ± 3	20 ± 2	1.0	> 25 μM	23 ± 2	40 ± 4	1.7
12	4.7 ± 0.6	3.4 ± 0.5	0.7	23 ± 3	11 ± 3	41 ± 3	3.7

^a Concentration of drug which exhibited 50% cell viability ^bThe results are expressed as mean values ± SD of three independent experiments, each of them made in quadruplicate. ^c Resistance factor (RF) is defined as IC_{50} (resistant cells)/ IC_{50} (sensitive cells). ^d Selectivity Factor (SF) is defined as IC_{50} (healthy MCF-10A cells)/ IC_{50} (cancer MCF-7 cells).

Cisplatin was also evaluated under the same experimental conditions for comparative purposes. As indicated in **Table 2.3**, the new Ir(III) complexes were toxic in all cancer cell lines tested and their antitumor potency decreased in the order **8~9>10>12>11**, when the π conjugation of the polypyridyl ligand and the lipophilicity of the complex (determined by the retention times in RP-HPLC analyses; **Table 2.4** and **Figure S2.9**) increase. All iridium compounds could overcome acquired resistance to cisplatin and were at least as cytotoxic in cisplatin-resistant A2780cisR cells as in the wild type A2780 line. The resistance factor, defined as the ratio of the inhibitory concentration (IC_{50}) values in resistant (A2780cisR) and cisplatin-sensitive parent cells (A2780), was in the range of 0.34 to 1.02 for the complexes, whereas it was markedly higher for cisplatin (2.89). Moreover, all complexes (except **11**) were also more toxic than cisplatin in breast MCF-7 cells, which are naturally resistant towards cisplatin. These results indicate that the mechanism of action of the complexes should be different from that of clinically used cisplatin and its direct derivatives, which allows the Ir(III) compounds to overcome the resistance mechanisms operating in the case of conventional platinum drugs. Remarkably, **8-12** showed less cytotoxic in noncancerous breast cells, MCF-10A, and exhibited higher selectivity for breast cancer cells, MCF-7, than cisplatin (**Table 2.3**).

2.2.6. Cellular accumulation

The biological activity of metallodrugs is closely dependent on their ability to cross cell membranes and enter the cell. Therefore, the intracellular content of Ir was determined in human HeLa cancer cells treated with equimolar (10 μ M) concentrations of complexes **8**–**12** for 5 and 24 h. The iridium (or platinum in the case of cisplatin) contents in the samples were determined by inductively coupled plasma mass spectrometry (ICP-MS). The viability of these cells was approximately 85–97% and no marked increase in dead cells was observed in comparison with the control. Both live and dead cells were harvested and included in the experiment.

The amount of iridium that accumulated in cells after 5 or 24 h of treatment increased in the order **8**<**9**<**10**~**11**~**12** (Table 2.4). Although most lipophilic complexes **10** and **11** showed the highest accumulation, the correlation does not work for **12**. The effect of the N^N ligand on drug lipophilicity was studied by comparing their RP-HPLC retention times (t_R) on a C18 column (Table 2.4 and Figure S2.9). This is considered a reliable method to estimate the relative hydrophobicities, as the RP-HPLC behavior of a compound depends on its hydrophobic interactions with the nonpolar stationary phase: more lipophilic complexes should have larger retention times.^{42–45}

This correlation suggests a role of passive diffusion in the cellular uptake of these compounds (except for **12**). Interestingly, most active **8** showed the lowest accumulation in cells, whereas much less active **11** and **12** showed an accumulation almost one order of magnitude higher. Thus, the cytotoxicities of the new Ir(III) complexes do not seem to reflect their ability to accumulate in cells.

Table 2.4. Retention times (t_R) by RP-HPLC, cellular accumulation of cisplatin and complexes **8**–**12** in HeLa cells^a

	t_R (min)	5h	24h
cisplatin	–	22.7 $\pm$ 0.7	110 $\pm$ 10
8	17.1	34 $\pm$ 4	61.0 $\pm$ 0.9
9	17.4	105 $\pm$ 11	219 $\pm$ 9
10	18.5	305 $\pm$ 5	536 $\pm$ 12
11	19.1	275 $\pm$ 11	461 $\pm$ 55
12	17.2	246.0 $\pm$ 13	406 $\pm$ 12

^aCellular uptake of the investigated metal compounds (10 μ M) into HeLa cells after 5 or 24 h. Each value in the table is in pmol Pt or Ir/10⁶ cells. The results are expressed as the mean $\pm$ SD of three independent experiments.

2.2.7. Mechanism of action studies

The profiles of time-dependent cell-response curves have been shown to be a method of partially identify the mechanism of biological action of small molecules.^{46,47} Thus, we used impedance-based time-dependent cell-response profiling as the predictive method for the mechanism of action of complexes. The time-dependent cell-response profiles (TCRPs) of **8** and **12** are characterized by an initial increase in the cell index (CI) above the level of the CI for the control, followed by a peak with a width that is inversely proportional to the concentration of the investigated compound applied to the cells (shown in **Figure 2.6** for HeLa and A2780 cells treated with **8**). The slope of the CI decline is directly proportional to the treated concentration.

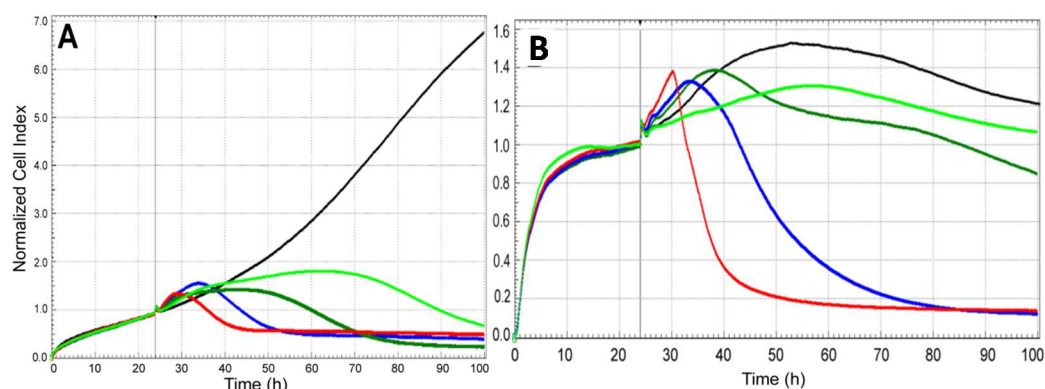


Figure 2.6. The TCRPs of A) A2780 and B) HeLa cells treated with increasing concentrations of **8**. Curves: untreated control (black line), **8** at the concentration of 1 μ M (light green), 3 μ M (dark green), 10 μ M (blue) and 20 μ M (red). Data were normalized to the time of the treatment and expressed as the normalized cell index. Curve profiles of **12** were found to be the same as for **8** at approximately 5-times higher concentrations. Thus, only results for **8** are shown.

The acquired profiles (**Figure 2.6**) were compared to those of a library of compounds that were clustered into groups based on the similarities of their mechanism of action. The TCRPs of **8** and **12** were the same as those of the group of compounds affecting protein synthesis. In addition, a complete match with anisomycin was found.⁴⁶ This model compound inhibits peptidyl transferase, which is an integral part of the large subunit of ribosomes and catalyzes peptide-bond formation in the elongation step of translation during protein biosynthesis.^{48,49} Altogether, the obtained TCRPs for the investigated compounds are consistent with the view and support the hypothesis that a possible mechanism of action of the complexes involves inhibition of protein translation.

To support this hypothesis, the incorporation of [³⁵S]methionine was detected as a measure of translation activity of the cells in the presence of the new compounds. In these experiments, we intentionally used low concentrations of **8** or **12** and a short incubation

time (1 h) to secure cell viability to detect mainly the upstream effects of the Ir(III) complexes. HeLa cells were further incubated with [^{35}S]-Met for 2 h to allow their incorporation into the newly synthesized proteins. As shown **Figure 2.7**, complex **8** significantly decreased protein synthesis.

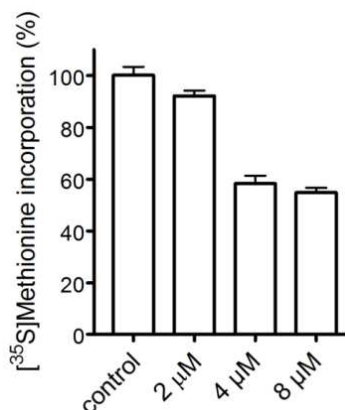


Figure 2.7. Effect of **8** on translation in HeLa cells as a measure of incorporation of ^{35}S -methionine. The radioactivities of control, untreated cells were taken as 100 %. Cells were treated with **8** at indicated concentrations for 1 h. Bars represent mean $\pm$ SD from two independent measurements.

2.2.8. Cellular localization and distribution

Fluorescence confocal microscopy was used to find out the cellular distribution of the new complexes. In this study, two representative compounds were selected: **8**, which shows a high quantum yield value in cell-free media, and **12**, despite having a luminescence aggregation-caused quenching (ACQ) effect in water solutions.

As indicated in **Figure 2.8A**, the luminescence from both complexes was localized preferentially out of the cell nucleus, and the majority of the luminescence was associated with the cytoplasm. Based on the results of real-time cell electronic sensing (**Figure 2.6**) and the fact that DNA is a very unlikely target for the complexes, we also performed a co-localization study, which may suggest the intracellular target organelle.

The luminescence signal coming from Ir(III) complexes almost completely matched the luminescence coming from the endoplasmic reticulum (ER)-Tracker marked organelles (**Figure 2.8**). Co-localization of **8** and **12** with the ER was also analyzed by using Pearson's correlation coefficient (PCC).⁵⁰ The calculated PCCs were 0.43 ± 0.03 and 0.60 ± 0.02 for **8** and **12**, respectively, and confirmed a relatively high level of co-localization with the ER. These results underscore the ability of **8** and **12** to target the ER preferentially.

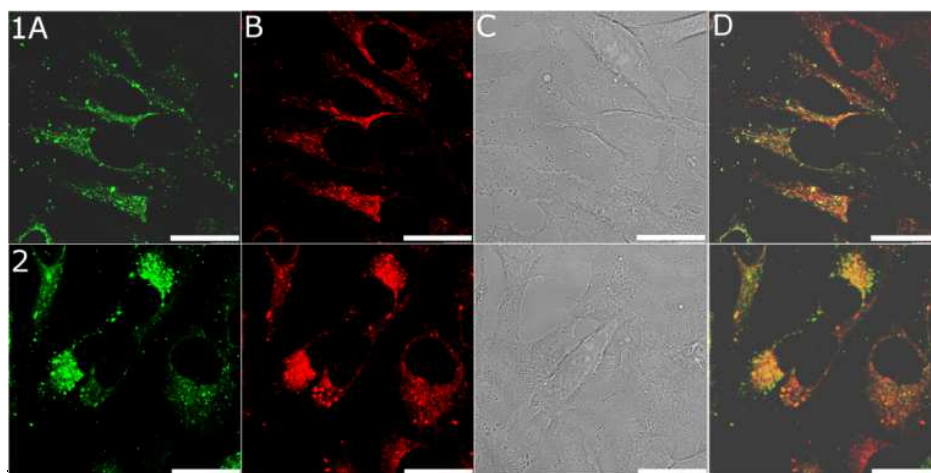


Figure 2.8. Colocalization studies of **8** and **12** in HeLa cells. HeLa cells were treated with 5 μ M of **8** (upper images) and **12** (bottom images) for 2 h and co-stained with the ER tracker. Channels: A) luminescence signal from the tested compounds, B) ER-tracker fluorescence, C) bright field, D) overlay of the luminescence channels. Samples were sequentially scanned on a confocal laser scanning microscope, and the scale bars are 25 μ m.

2.2.9. Cell-death detection studies

Many metal-based complexes kill cancer cells by triggering cellular processes leading to apoptosis.^{51–53} However, cytotoxic compounds may also kill cells through non-apoptotic cell-death pathways such as necrosis.^{54–56} To distinguish whether the cells treated with the Ir(III) complexes underwent apoptosis or necrosis, DNA fragmentation induced by various concentrations of **8** and **12** was quantified by a specific ELISA colorimetric kit. This analysis allows relative amounts of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) to be measured after they are produced during the apoptotic process or if these fragments are released from necrotic cells. These results in **Figure 2.9** demonstrate that treatment of cancer cells with both complexes led primarily to apoptotic events.

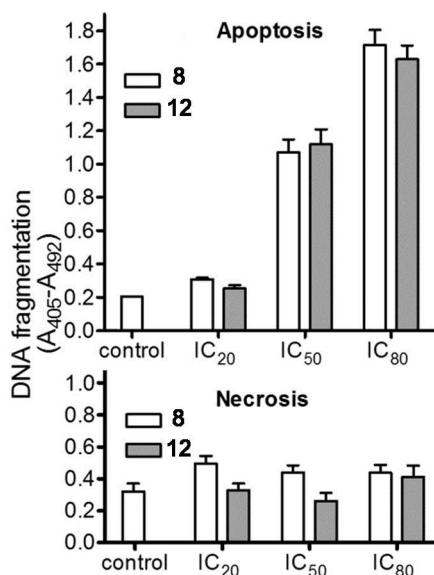


Figure 2.9. Detection of apoptosis and necrosis in HeLa cells, treated with equitoxic (IC₂₀, IC₅₀, and IC₈₀) concentrations of **8** and **12** for 24 h. Data show mean $\pm$ SD from two independent experiments, each of them made in duplicate.

2.2.10. Photo-toxicity

We also studied the effect of visible light on the antitumor properties of the new Ir(III) compounds. HeLa cells were treated with **8-12** in Earle's balanced salt solution (EBSS) for 1 h in the dark to give the compounds time to enter the cells, and subsequently, the cells were irradiated with blue light ($\lambda_{\text{max}}=420$ nm) for 1 h. After irradiation, the cells were washed and incubated in complete Dulbecco's modified Eagle medium (DMEM) (without Ir complex) for another 70 h (recovery time). Then, the viability of the cells was assessed by the MTT test, as described in the Experimental Section.

Table 2.5. Phototoxicity [IC₅₀ mean values (μM)] of the new Ir(III) complexes in HeLa cells^a

	Dark	420 nm	PI ^b
8	11.5 $\pm$ 0.2	0.044 $\pm$ 0.006	261
9	10.36 $\pm$ 0.06	0.103 $\pm$ 0.005	100
10	> 100	0.866 $\pm$ 0.009	> 115
11	> 50	0.58 $\pm$ 0.01	> 86
12	> 50	0.35 $\pm$ 0.01	> 143

^aThe results are expressed as mean values $\pm$ SD of three independent experiments, each of them made in quadruplicate. ^b PI (phototoxic index) is defined as the ratio of the toxic effect in the dark and upon light irradiation; PI = [IC₅₀]_{dark} / [IC₅₀]_{420nm}.

As observed in **Table 2.5**, the cytotoxic effects of all complexes in HeLa cells were significantly potentiated by irradiation with visible light. The phototoxicities of **8-12** were elevated by more than two orders of magnitude compared to the samples in the dark (the phototoxic indexes fall in the range of >86-261).

2.2.11. Determination of intracellular reactive oxygen species (ROS)

It was already shown that upon irradiation Ir(III) complexes could significantly increase intracellular reactive oxygen species (ROS) levels.^{57–59} Therefore, intracellular ROS were quantified to determine the oxidative stress in cells treated with **8** or **12** in the dark and after irradiation. A method developed by Robinson et al.^{60,61} was used. This method was designed to provide a highly sensitive, quantifiable, real-time assessment of ROS production.⁴⁷ To evaluate ROS formation, HeLa cells untreated or treated with **8** or **12** in the dark or under irradiation conditions ($\lambda_{\text{max}}=420$ nm) were incubated with 10 μM 2',7'-dichloro-dihydro-fluorescein diacetate (DCFH-DA) for 30 min in the dark. DCFH-DA, after cleavage by cellular esterases, is oxidized by ROS to yield 2'-7'-dichlorofluorescein (DCF), a fluorescent compound of which fluorescence was measured. The data demonstrating the generation of ROS in HeLa cells induced by various concentrations of **8** or **12** are showed in **Figure 2.10**.

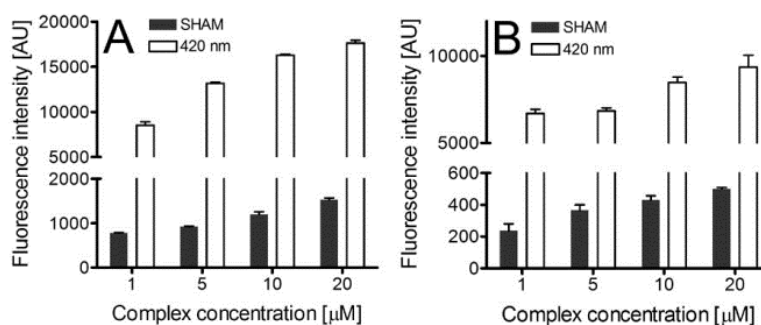


Figure 2.10. ROS generation in HeLa cells induced by various concentrations of A) **8** or B) **12**. The values shown represent the fluorescence intensity in AU obtained for various concentrations of the Ir(III) complexes from which the luminescence intensity obtained for the untreated control cells was subtracted. The fluorescence intensities yielded by the untreated cells in the dark or under irradiation conditions were 333 ± 57 AU or 438 ± 57 AU, respectively. Data represent mean $\pm$ SD from three independent measurements.

Treatment of HeLa cells with **8** in the dark resulted in a concentration-dependent increase in fluorescence (**Figure 2.10A**, black bars), which indicated the production of ROS. Importantly, the fluorescence was markedly (11–14-fold) increased if the treated cells were irradiated (**Figure 2.10A**, white bars). This implies that the level of intracellular ROS induced by **8** could be markedly elevated by irradiation with blue light. Complex **12** showed qualitatively similar effects (i.e., 18–28-fold increase in intracellular ROS level upon irradiation, **Figure 2.10B**), but altogether, it was less effective in inducing ROS than **8**, which likely reflects its lower cytotoxicity under both dark and light conditions.

2.3. CONCLUSIONS

We have explored the influence of the degree of the π conjugation of the polypyridyl ligands on the photophysical properties and the biological activity of five Ir(III) complexes of the type $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{N}^{\wedge}\text{N})][\text{PF}_6]$ with 2-phenyl-1-[4-(trifluoromethyl)benzyl]-1H-benzo[d]imidazole cyclometalating ligand as the $\text{C}^{\wedge}\text{N}$ backbone.

The complexes showed different photophysical properties depending on the length and degree of π conjugation of the $\text{N}^{\wedge}\text{N}$ ligand; the largest (dppn and dppz-izdo) exhibited a luminescence aggregation-caused quenching effect in cell-free media. Their antitumoral study reveal that less lipophilic complexes **8** and **9** (with the lowest level of cellular accumulation) exhibited the highest toxicities in A2780, HeLa, and MCF-7 cancer cells, and they were markedly more efficient than clinically used cisplatin. In contrast, the cytotoxicities of more lipophilic **11** and **12** were considerably diminished (IC_{50} values were ~4–55-fold higher). It is noteworthy that the investigated agents displayed the capability to overcome acquired and inherent resistance to cisplatin (in A2780cisR and MCF-7 cells, respectively) and were more selective for breast cancer cells than healthy breast cells. In addition, the cytotoxicity of the Ir(III) complexes did not reflect their cellular accumulation and that they preferentially targeted the endoplasmic reticulum. The time-dependent cell response profiles and estimations of the incorporation of $[\text{}^{35}\text{S}]$ methionine as a measure of translation activity of the cells, suggested that a possible mechanism of action involved inhibition of protein translation. Moreover, **8** and **12** induced apoptosis similarly to other antitumor metal-based drugs. Our examinations also indicated that the toxic effect of all complexes in HeLa cells was significantly elevated (by more than two orders of magnitude) by irradiation with visible light ($\lambda = 420 \text{ nm}$). This photopotential was attributed to a marked increase (~10–30-fold) in intracellular reactive oxygen species. To our knowledge, the Ir(III) compounds investigated in this study represent the first iridium complexes that induce cancer cell death by inhibiting cell translation, whose activity could be markedly photo-enhanced.

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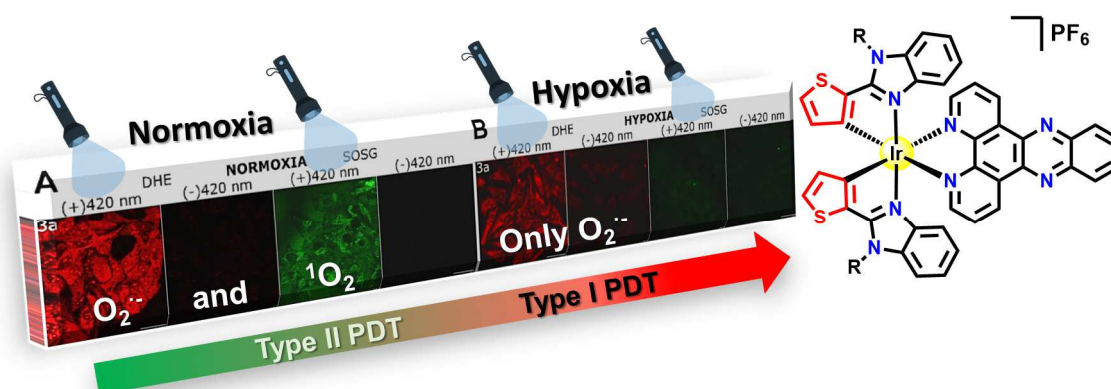
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Chapter 3

Modifying the cyclometalated ligand in
dipyridophenazine Ir(III) complexes to
study their photodynamic activity in
normoxia and hypoxia



The biological experiments included in this chapter were carried out by Dr. Viktor Brabec (Institute of Biophysics, Czech Academy of Sciences, Brno, Czech Republic).

3.1. INTRODUCTION

As an emerging treatment modality, photodynamic therapy (PDT) shows several advantages: temporal and spatial control of the irradiation in the target site, minimal invasiveness, low general organ toxicity, and lack of initiating resistance.^{1–3} Currently, most reported PDT systems operate via a type II mechanism, in which PSs transfer their excited-state energy to the surrounding oxygen for producing singlet oxygen. However, the native microenvironment in solid tumors is hypoxic (0.02–2% O₂) due to their rapid growth and insufficient oxygen supply (**Figure 3.1A**).^{4,5} Besides, quick oxygen consumption during the treatment further disturbs the hypoxic condition and limits the therapeutic effect. As a result, a hypoxic environment seems to be a significant restriction of PDT, and resolving this Achilles' heel arouses general interests.^{6,7}

Among other strategies, type I PDT appears to be one way to overcome hypoxia limitation.^{8–11} The different reactive oxygen species (ROS; hydroxyl radicals, superoxide anions, hydrogen peroxide, or peroxy radicals) do not depend on a single mechanism of action, making it difficult for tumor cells to develop resistance PDT.⁹ In addition, some of these ROS, such as superoxide radicals, could be less oxygen-dependent with disproportionation reactions or Fenton/Haber–Weiss reactions (**Figure 3.1B**) that would compensate for the oxygen consumption.^{12–14}

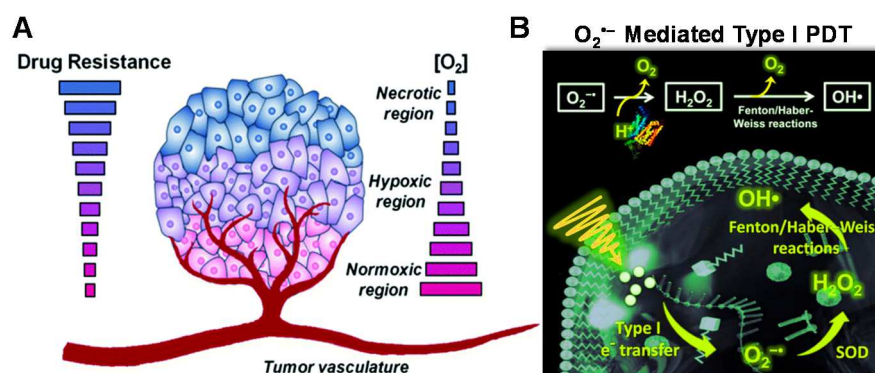


Figure 3.1. A) Hypoxic tumor microenvironment and associated resistance.¹⁵ B) Compensation of oxygen consumption by superoxide radical disproportionation reaction.¹⁴

Superoxide radicals (O₂^{•-}) have been identified as the main oxidant for cancer treatment and as an adjuvant to synergize the chemotherapy.^{16,17} An excess of O₂^{•-} in the cells can react with proteins, lipids, and DNA, irreversibly damaging cellular components and resulting in tumor cell death.¹⁸ Recently, Li *et al.* have confirmed the intracellular generation of superoxide anion radicals of benzophenothiazines-derived agents, after near-infrared light irradiation even under hypoxia.^{12,19} On the other hand, a superoxide anions

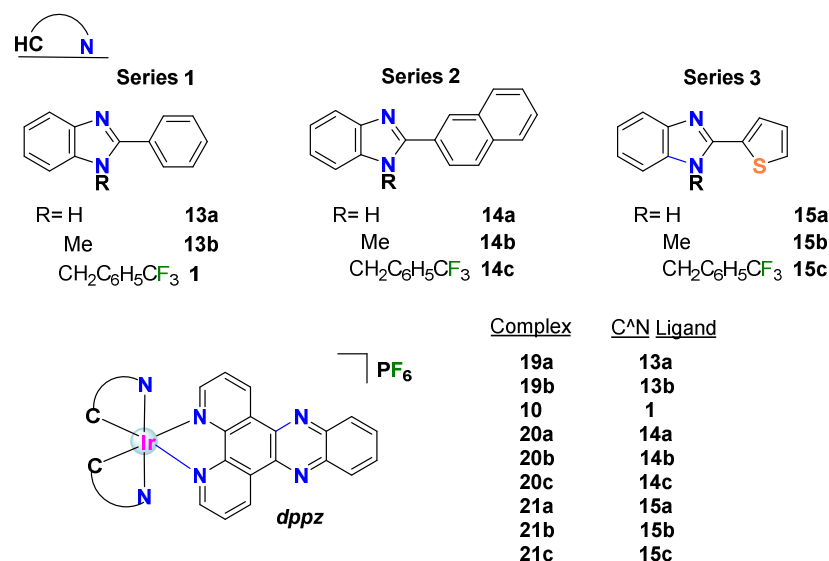
photogenerator based on a cyclometalated iridium compound conjugated to coumarin has shown promising PDT activity towards hypoxic tumors.²⁰

Herein we report the design and synthesis of three new series of octahedral Ir(III) complexes of the general formula $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{dppz})][\text{PF}_6]$ (dppz = dipyrido[3,2-*a*:2',3'-*c*]phenazine), which include some modifications on the $\text{C}^{\wedge}\text{N}$ ligands such as the incorporation of a sulfur heteroatom or a larger π conjugation to tune the photophysical properties, lipophilicity, cellular uptake efficacy, and phototherapy activity even under hypoxia.²¹ The new compounds have been investigated in human cervical cancer HeLa cells both in the dark and after irradiation by blue light under normoxic and hypoxic conditions. We demonstrate that in HeLa cells, the new complexes can switch from Type II to Type I photochemical mechanism, and therefore, they can be active in all areas of a solid tumor, adapting their mechanism of action according to the oxygen concentration. This fact supposes overcoming the Achilles' heel of PDT, related to its limited applicability to treat hypoxic tumor cells and thus resistance to PDT.

3.2. RESULTS AND DISCUSSION

3.2.1. Synthesis and characterization

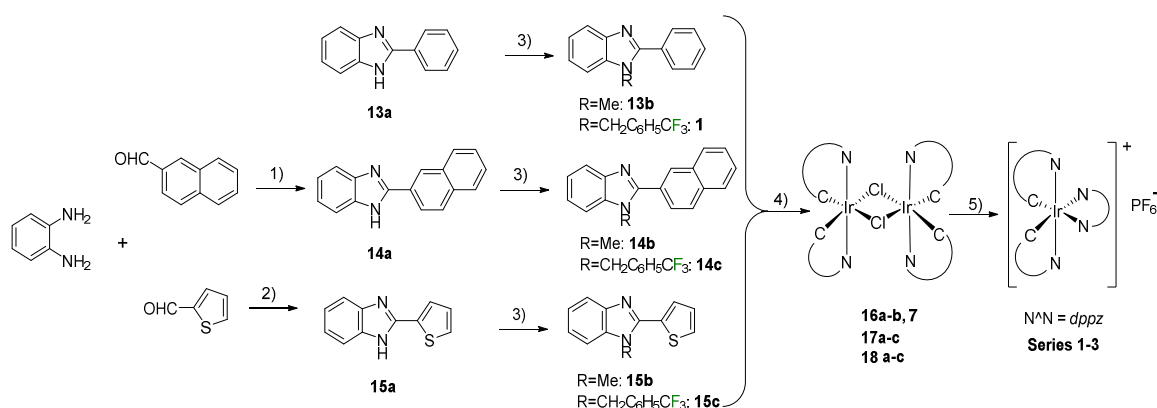
Three series of luminescent octahedral Ir(III) complexes of the type $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{dppz})][\text{PF}_6]$ (**Scheme 3.1**) were synthesized to study the effect on their antitumor photodynamic activity by modifying the cyclometalating ligand.



Scheme 3.1. Structures of the pro-ligands $\text{HC}^{\wedge}\text{N}$ and new iridium compounds **19a-b**, **20a-c** and **21a-c**, showing labels and abbreviations used throughout the whole text.

Consistently, nine different C[^]N ligands based on the benzimidazole core were designed and incorporated into the metal, six of them containing 2-phenyl (**13a**, **13b**, and **1**) or 2-naphthalen-2-yl (**14a-c**) groups and three others containing a sulfur heteroatom (**15a-c**). In addition, hydrogen, methyl and 4-(trifluoromethyl) benzyl substituents (a, b, and c compounds, respectively) were included in the imidazole unit. The dppz ligand was chosen as the N[^]N ligand due to its interesting PDT activity exhibited by the previously reported complex **10** (Chapter 2).²² The new pro-ligands were synthesized by slight modifications of protocols reported in the literature (**Scheme 3.2**, and **Schemes E2.3-E2.6** in the Experimental Section).²³⁻²⁵

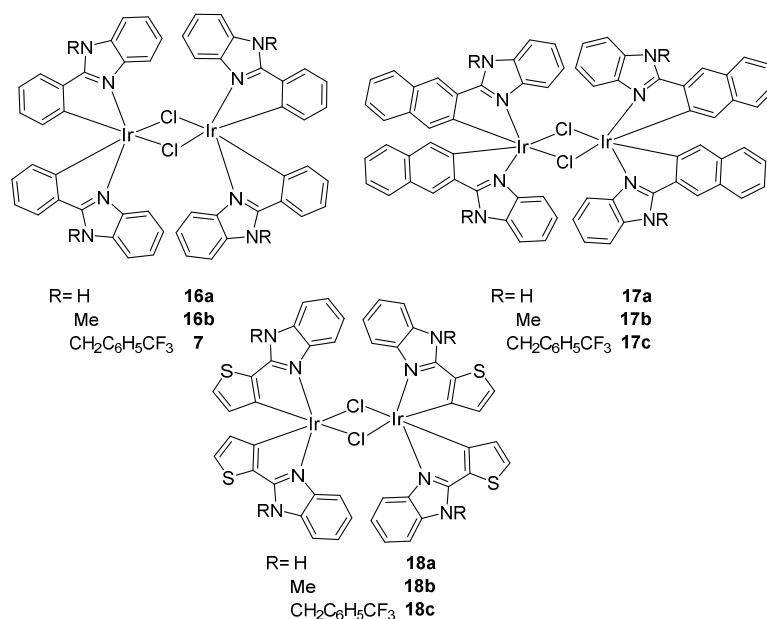
The new monomeric Ir(III) complexes were synthesized in two stages as previously described in Chapter 2. First, dimeric iridium(III) precursors [Ir(C[^]N)₂(μ-Cl)]₂ **16a-b**, **17a-c**, **18a-c** (**Scheme 3.2** and **Scheme 3.3**) were obtained by reacting IrCl₃·H₂O and the different HC[^]N pro-ligands. Then, the corresponding dimer was reacted with dppz in a 1:2 molar ratio yielding orange to red monomers **19a-21c**.



Scheme 3.2. Synthetic route for pro-ligands and new Ir(III) compounds. Reaction conditions: (1) DMF, 80°C, 5 days; (2) NaHSO₃, H₂O, reflux, 20 min; (3) MeI or CH₂C₆H₄CF₃Br, Cs₂CO₃, CH₃CN, r.t., 24h; (4) IrCl₃·H₂O, 2-ethoxyethanol/H₂O, reflux, 24h; (5) dppz, CH₂Cl₂/MeOH (2:3), reflux; then KPF₆, r.t.

The complexes were characterized by ¹H, ¹³C NMR spectroscopy (**Figures S3.1-S3.11**), and positive-ion ESI-HRMS (**Figures S3.12-S3.14**). Due to the symmetric nature of the coordination environment around the iridium center, the NMR spectra of new compounds exhibit one set of peaks for the two equivalent C[^]N ligands (**Figure 3.2**). The positive-ion ESI mass spectra clearly showed the molecular peaks corresponding to [M – PF₆]⁺. On the other hand, complexes were shown to be at least 96% pure by both elemental

analysis and reverse-phase RP-HPLC (**Figure S3.15**). In addition, the order of expected lipophilicity was verified using RP-HPLC, i.e., $H < Me < CH_2C_6H_4CF_3$ -p (**Figure S3.15**).



Scheme 3.3. Chemical structures of Ir(III) dimers.

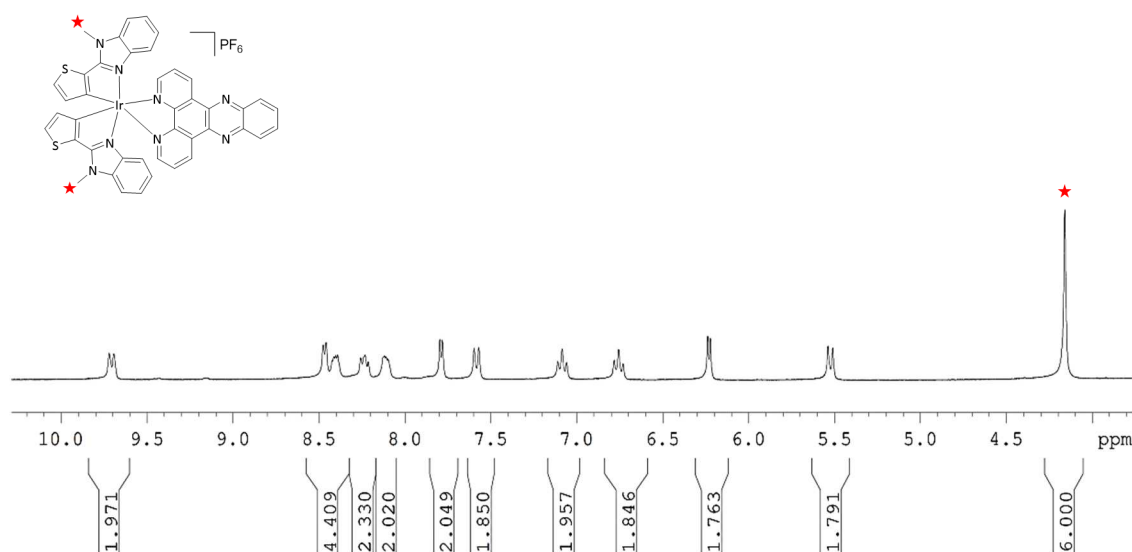


Figure 3.2. 1H NMR in $[D_6]DMSO$ for **21b**. The peak of the CH_3 groups integrates by 6 due to the equivalence of both C N ligands.

3.2.2. Structures determination

The crystal structure of **10** has been previously reported in Chapter 2.²² Single crystals of **20b**·3 $C_2H_4Cl_2$ ·0.33 hexane suitable for X-ray diffraction analysis were obtained by slow diffusion of hexane into a saturated solution of **1,2**- $C_2H_4Cl_2$. The structure of **20b** is shown in **Figure 3.3A** and **S3.16**. Crystallographic data are given in **Tables S3.1–S3.3**.

The Ir atom has a distorted octahedral coordination geometry with the expected cis-C,C, and trans-N,N mutual disposition for the C[^]N ligands. The Ir–C_{C[^]N} and Ir–N_{C[^]N} bond distances lie within the expected ranges for the C[^]N ligands, that is, very close to 2.0 Å.²² The Ir–N bond lengths for the dppz ancillary ligand (2.130 (4) and 2.131 (4) Å) are significantly longer due to the strong trans influence attributed to the C atoms of the C[^]N ligands.^{22,26} The iridium cation of **20b** crystallized with one PF₆ anion and, as stated above, about three 1,2-C₂H₄Cl₂ and about third hexane solvent molecules per formula unit. Apart from the important cation-anion Coulomb interactions, the packing in the structure of **20b** is organized (according to an analysis by PLATON²⁷ by intermolecular C–H···F (Table S3.4),^{28–31} C–H··· π interactions (Table S3.5 and Scheme S3.1),^{32,33} and π – π interactions (Table S3.6 and Scheme S3.2).^{34,35} The π – π interactions between the dppz ligands of **20b** are shown in Figure 3.3B.

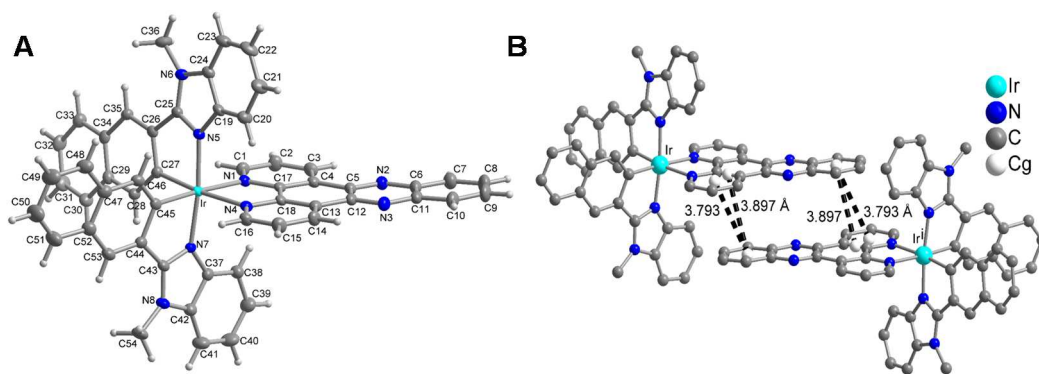


Figure 3.3. A) Molecular structure within the crystal of **20b** showing only the iridium cation (50% thermal ellipsoids), and full atom numbering. Selected bond lengths (Å) and angles (deg) for **20b**: Ir–N1 = 2.130 (4), Ir–N4 = 2.131 (4), Ir–N5 = 2.038 (4), Ir–N7 = 2.043 (4), Ir–C27 = 2.023 (5), Ir–C45 = 2.019 (5), N1–Ir–N4 = 77.49 (15), C27–Ir–N5 = 79.66 (17), C45–Ir–N7 = 79.87 (17). B) π – π interactions in the packing of **20b**, indicated as dashed black lines.

3.2.3. Photophysical properties

The UV/Vis absorption spectra of the new complexes were recorded in both water (1% DMSO) and acetonitrile at room temperature (Figure 3.4, Figures S3.17–S3.19 and Table S3.7). All complexes exhibit intense and structured absorption bands in the ultraviolet region below 350 nm that can be assigned to spin-allowed ligand-centered (¹LC) π – π^* transitions within the N[^]N and C[^]N ligands (Figure 3.4). The less intense absorption bands and shoulders recorded at $\lambda > 350$ nm could be attributed to spin-allowed metal to ligand (¹MLCT), spin forbidden singlet-to-triplet (³MLCT) charge-transfer, and ligand-to-ligand charge-transfer (LLCT) transitions.^{36–38}

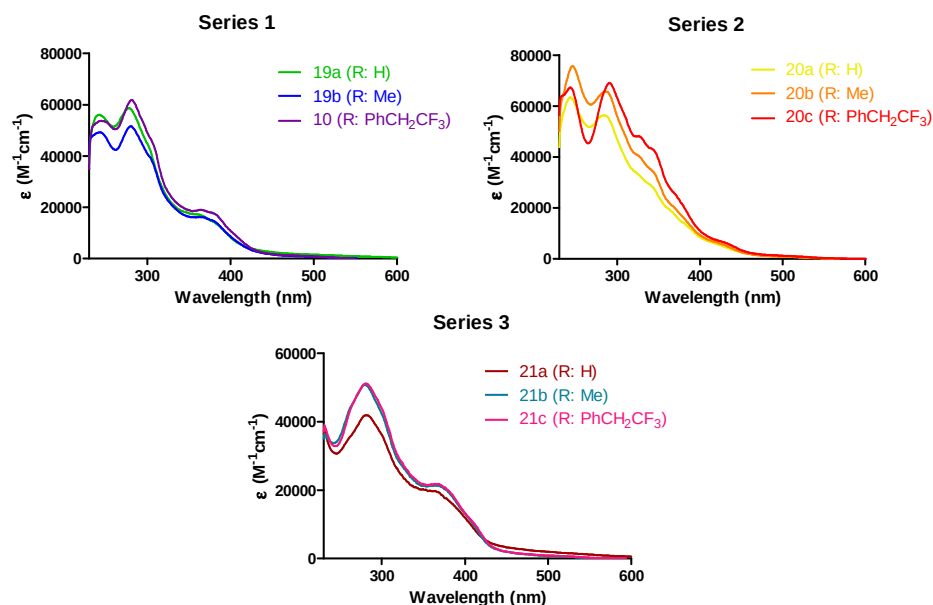


Figure 3.4. UV/Vis absorption spectra of series 1, 2 and 3 in water (1% DMSO).

Apart from the above-mentioned features common to all the complexes, some differences were found in the absorption spectra of these derivatives depending on the C^N ligand. In particular, complexes **20a**, **20b** and **20c** with 2-naphthalen-2-yl C^N ligand displayed the higher intense bands in the 250–350 nm range ($40000 < \epsilon < 75000 \text{ M}^{-1}\cdot\text{cm}^{-1}$) (**Figure 3.4**), whereas complexes **21a**, **21b** and **21c** bearing the C^N ligand with the sulfur heteroatom, showed the higher molar extinction coefficient above 420 nm ($\epsilon > 20000 \text{ M}^{-1}\cdot\text{cm}^{-1}$) (**Figure 3.5**). Noteworthy, high molar absorption coefficient values observed at the visible region are suitable for visible-light-driven applications.³⁹

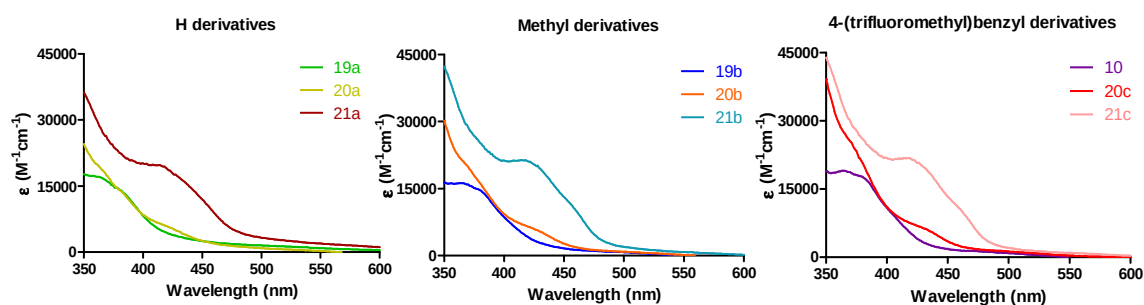


Figure 3.5. Less intense absorption bands and shoulders in UV/Vis spectra of complexes.

The emission spectra of complexes were measured in water (1% DMSO) at 25 °C (**Figure 3.6**, **S3.20**, and **Table 3.1**). Upon excitation at λ_{exc} maxima, the new iridium compounds exhibit orange to red phosphorescence emissions (**Table 3.1**) in water. These emission wavelengths (λ_{em}) were higher for the methyl derivatives within each series that might be due to the electron-donating nature of the CH_3 groups in **19b**, **20b** and **21b**.⁴⁰

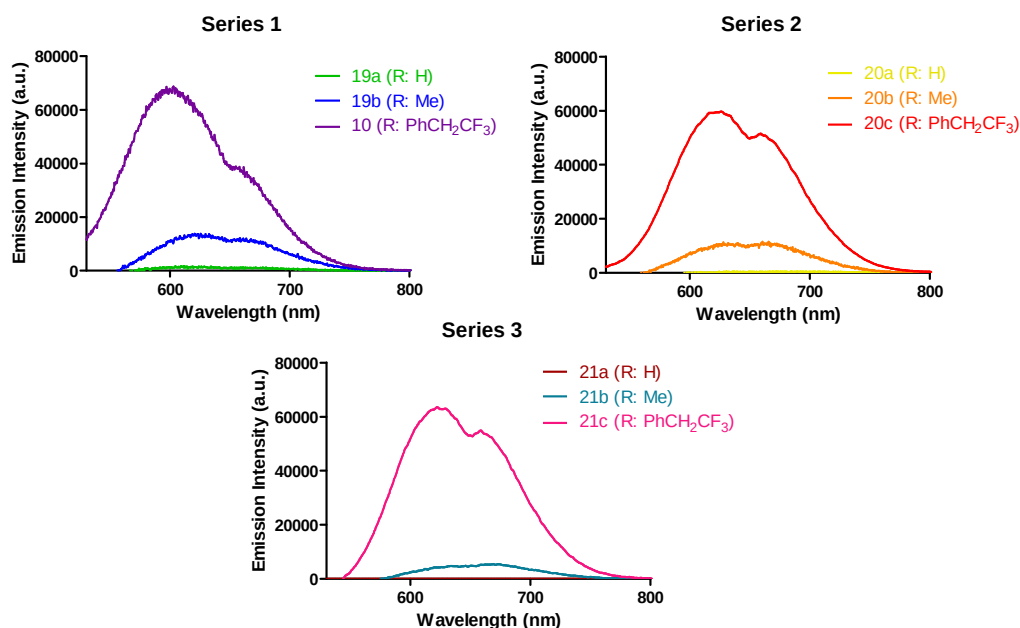


Figure 3.6. Emission spectra of series 1, 2, and 3 in water (1% DMSO), 10 μM . $\lambda_{\text{exc}} = 420 \text{ nm}$.

The emission lifetimes of complexes in water show a bi-exponential decay with a short (18–46 ns) and a long (170 – 364 ns) component (**Table 3.1** and **Figures S3.21–23**) which supports the triplet nature of the emission state.⁴¹ The quantum yields in the deaerated water solution are below 1.5% for all compounds (**Table 3.1**). This weak emission is common in some other Ir(III) complexes containing dppz,^{42,43} and could be explained by the increase of the non-radiative process due to the interaction of the solvent with non-chelating nitrogen atoms of the dppz ligand.⁴³ Moreover, the decrease in the emission intensity could also be attributed to the luminescence aggregation-caused quenching (ACQ) effect, explained in Chapter 2.²² To test the extent of aggregation for the new compounds, ¹H NMR spectroscopy was used. Thus, an upfield shift of some aromatic peaks of the dppz ligand was observed in the spectrum of **19b** when the concentration increases (**Figure S3.24**), as well as at different compositions of mixtures of [D₆]DMSO and D₂O (**Figure S3.25**). This effect can be attributed to favorable π – π stacking (as observed in the crystal structure of **20b** in **Figure 3.3B**). In addition, the emission spectra is non-oxygen dependent (**Figure S3.26**) as reported before for other Ir(III) polypyridyl complexes with π -expansive ligands.^{22,44}

Table 3.1. Excitation (λ_{exc}), emission (λ_{em}) wavelengths, emission lifetimes (τ_{em}), and quantum yields (Φ_{em}) of Ir(III) complexes in water (1% DMSO).

Compound	λ_{exc} , nm ^a	λ_{em} , nm	τ_{em} , ns ^b	Φ_{em} , % ^b
19a	385	612	46 (4%) 308 (96%)	<1
19b	381	625	35 (19%) 282 (81%)	<1
10	380	602	34 (22%) 170 (78%)	1.5
20a	385	660	39 (6%) 364 (94%)	<1
20b	400	665	18.3 (4%) 292 (96%)	<1
20c	380	626	33 (26%) 214 (74%)	1.0
21a	380	— ^c	— ^c	— ^c
21b	390	667	35 (6%) 323 (94%)	<1
21c	385	624	22 (15%) 244 (85%)	1.1

^a λ_{exc} maxima. ^b Emission lifetimes and quantum yields measured in deaerated solution, respectively. ^c Too weak to be measured.

Furthermore, there is a significant influence of the substituent on the imidazole units on the emission intensity (**Figure 3.6** and **S3.20**). The complexes with the 4-(trifluoromethyl) benzyl substituent exhibit the highest emission intensity of the three series, whereas complexes **19a**, **20a**, and **21a** (H derivatives) suffer important emission quenching, probably by H-bonding interactions. Despite that, these complexes have pH-sensitive emissions (**Figure S3.27**). To exclude another explanation of the observed pH-dependent luminescence, we also tested the luminescence of **19b** at different pH values (**Figure 3.7**). Whereas for complex **19a** (R = H) the luminescence at pH 3.3 was 16-fold of that observed at pH 7.4, the increase for **19b** (R = Me) was only 1.2-fold. This implies that the increase in luminescence is mainly due to the protonation/deprotonation processes of the benzimidazolyl-NH,⁴⁵ increasing the emission intensity at acidic pH.

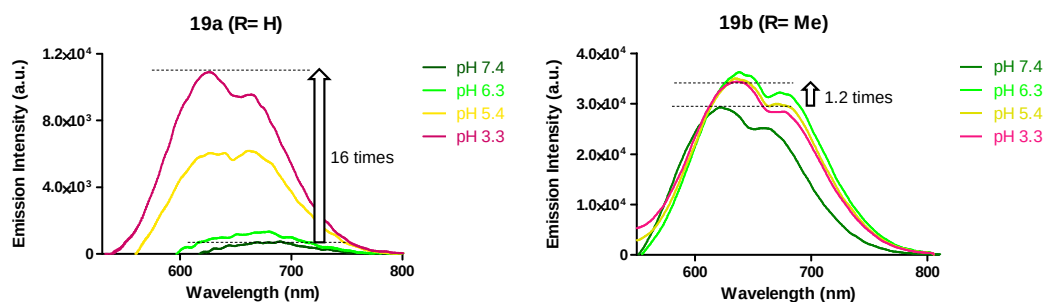


Figure 3.7. pH dependence on the luminescence for **19a** and **19b** in buffer/DMSO (99:1).

3.2.4. Stability and photostability studies

The stability in DMSO and Roswell Park Memorial Institute (RPMI) culture medium of **19b**, **20a**, and **21c** at 24 h was checked by RP-HPLC. As shown in **Figure 3.8**, the peak of each compound does not change after 24h.

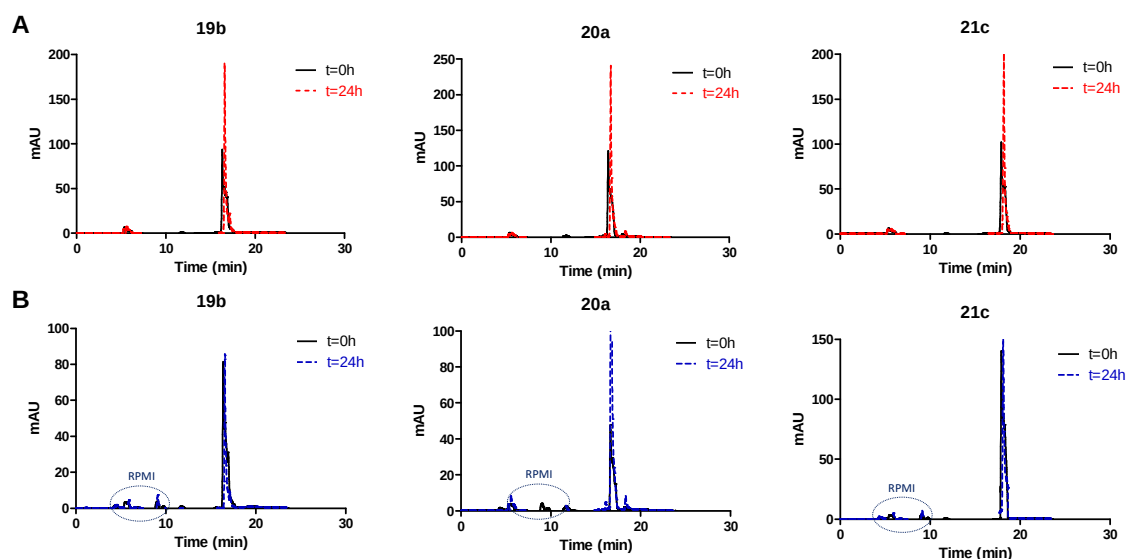


Figure 3.8. HPLC chromatograms of complexes **19b**, **20a**, and **21c** in A) DMSO at $t=0$ h (—) and $t=24$ h (---) incubation and B) RPMI culture medium (contain 5% DMSO) at $t=0$ h (—) and $t=24$ h (---) incubation.

The photostability in water (1% DMSO) was checked for **21a–c** by absorption spectroscopy under irradiation at 450 nm for 1 h. The UV/Vis spectra remained the same before and after light exposure (**Figure 3.9**).

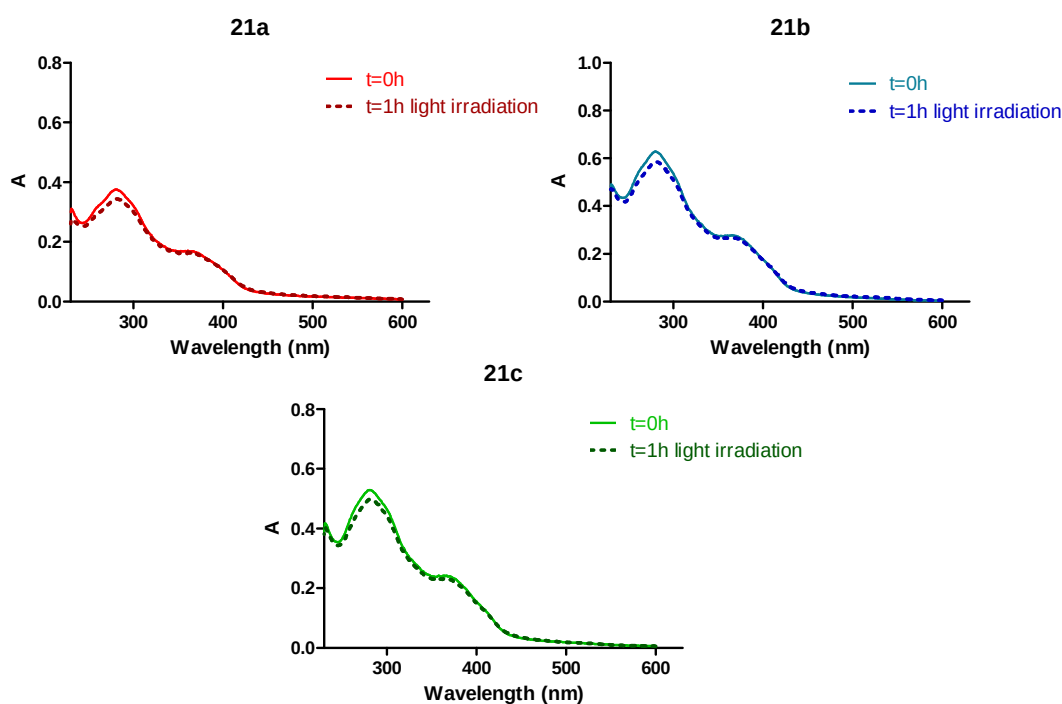


Figure 3.9. Photostability of complexes **21a–c** in water under 450 nm at $t=0$ h and after 1h of irradiation.

3.2.5. Photo-toxicity studies towards human cancer cells

The antitumor activity of new complexes and complex **10**²² for comparative purposes was examined in the dark and after irradiation by visible light (1 h, $\lambda_{\text{max}} = 420 \text{ nm}$, $77 \pm 3 \text{ W m}^{-2}$) in human cervical cancer HeLa cells.

As shown in **Table 3.2**, some of the complexes display a significant antiproliferative effect in the dark. Within the three series, the 1-methyl benzimidazole complexes (**19b**, **20b**, and **21b**) were the most active. In addition, the antitumor potency of all compounds was increased by irradiation with blue light, being 2–115 times more active. Particularly, the 4-(trifluoromethyl) benzyl derivatives (**10**, **20c**, and **21c**) have low toxicity in the dark and therefore exhibit a high phototoxicity index, which makes them promising candidates for PDT.

Furthermore, the antitumor effect of all complexes was also tested in hypoxia (2% O₂). Remarkably, the compounds were also significantly active under hypoxic conditions (**Table 3.2**), being the activity of the compounds of series 2 and 3 similar to that determined in normoxia (21% O₂). Therefore, the results obtained under hypoxia suggest that the new complexes may represent candidate compounds for the treatment of hypoxic tumors.

Table 3.2. IC₅₀ values (μM) obtained for HeLa cells treated with the investigated Ir complexes in the dark or after irradiation by visible light (1 h, $\lambda_{\text{max}} = 420 \text{ nm}$, $77 \pm 3 \text{ Wm}^{-2}$) determined by the MTT assay^a

	Normoxia			Hypoxia		
	Dark	420 nm	PI ^b	Dark	420 nm	PI ^b
19a	21 $\pm$ 2	0.7 $\pm$ 0.1	30	> 50	2.5 $\pm$ 0.4	> 20
19b	2.57 $\pm$ 0.03	0.48 $\pm$ 0.02	5.3	9.5 $\pm$ 1.0	1.3 $\pm$ 0.1	7.3
10	> 100	0.866 $\pm$ 0.009	> 115	> 100	2.7 $\pm$ 0.3	> 37
20a	> 50	2.64 $\pm$ 0.04	> 19	> 50	2.3 $\pm$ 0.2	> 22
20b	3.5 $\pm$ 0.2	0.21 $\pm$ 0.02	17	4.4 $\pm$ 0.5	0.85 $\pm$ 0.19	> 5.2
20c	> 50	1.26 $\pm$ 0.04	> 40	> 50	1.15 $\pm$ 0.13	> 43
21a	2.9 $\pm$ 0.2	1.4 $\pm$ 0.2	2.1	3.1 $\pm$ 0.5	2.2 $\pm$ 0.3	1.4
21b	1.1 $\pm$ 0.3	0.016 $\pm$ 0.006	69	0.97 $\pm$ 0.1	0.02 $\pm$ 0.01	42
21c	13 $\pm$ 1	0.64 $\pm$ 0.09	20	15 $\pm$ 4	0.71 $\pm$ 0.06	21

^a The data are expressed as mean values $\pm$ SD of three independent experiments, each of them made in quadruplicate. ^b PI (phototoxic index) is defined as the ratio of the toxic effect in the dark and upon light irradiation; $\text{PI} = [\text{IC}_{50}]_{\text{dark}} / [\text{IC}_{50}]_{420\text{nm}}$.

3.2.6. Cellular accumulation

Intracellular accumulation and cellular uptake of metallodrugs is an important step in performing their biological actions. Thus, we determined the amount of Ir or Pt from the investigated complexes and cisplatin accumulated in the HeLa cells after their incubation

at their equimolar concentrations (10 μM). As shown in **Table 3.3**, the highest iridium cellular content was observed for methyl derivatives (**19b**, **20b**, and **21b**), which nicely correlated with their highest toxic effects (in the dark and after irradiation).

Table 3.3. Accumulation of Ir or Pt from new compounds or cisplatin in HeLa cells^a

	ng Ir or Pt/10 ⁶ cells	pmol Ir or Pt/10 ⁶ cells
19a	87 $\pm$ 7	455 $\pm$ 35
19b	352 $\pm$ 25	1832 $\pm$ 131
10	59 $\pm$ 4	307 $\pm$ 20
20a	26.5 $\pm$ 0.9	138 $\pm$ 5
20b	157 $\pm$ 24	817 $\pm$ 125
20c	63 $\pm$ 11	327 $\pm$ 56
21a	82 $\pm$ 9	425 $\pm$ 49
21b	321 $\pm$ 17	1672 $\pm$ 89
21c	119 $\pm$ 5	621 $\pm$ 27
Cisplatin	4.6 $\pm$ 0.5	24 $\pm$ 3

^a Cells were treated with the investigated Ir(III) compounds and cisplatin (10 μM) and incubated for 5 h at 37 °C. Cellular complex accumulation was expressed in ng of iridium or platinum per 10⁶ cells, or pmol of Ir or Pt per 10⁶ cells. Results are the mean $\pm$ SDs from three independent experiments.

3.2.7. Cellular localization and distribution

A co-localization study was performed to determine the intracellular distribution of Ir(III) complexes by confocal laser-scanning microscope. For these studies, the 4-(trifluoromethyl)benzyl derivatives (**10**, **20c**, and **21c**) were selected because they showed the highest quantum yields in water. In contrast, the internal fluorescence of other series (**19a–21a** and **19b–21b**) was low so that the signal was under the detection limit.

HeLa cells were treated with 5 μM of **10**, **20c**, and **21c** for 18 h and subsequently, cells were co-incubated with the ER-TrackerTM. As shown in **Figure 3.10** (panels 2 and 4), the luminescence from complexes **10** and **20c** matched very well to the signal of organelles stained by the ER-Tracker. Furthermore, Pearson's correlation coefficient (PCC) was used to quantify the co-localization of iridium compounds with the ER. The PCCs above the threshold were 0.61 and 0.68 for **10** and **20c**, respectively (**Table 3.4**), indicating that both complexes highly co-localized with the ER.

Table 3.4. PCCs calculated for complexes **10**, **20c**, and **21c**.^a

Compound	Mitochondria	Endoplasmic reticulum
10	0.41 $\pm$ 0.06	0.61 $\pm$ 0.04
20c	0.44 $\pm$ 0.03	0.68 $\pm$ 0.05
21c	0.62 $\pm$ 0.06	0.33 $\pm$ 0.04

^a Complexes were investigated for their co-localization in the mitochondria and ER of HeLa cells. Results are the means from three independent experiments with at least eight pictures for individual data set.

Interestingly, the calculated PCC for **21c** was significantly lower than those found for **10** and **20c** (PCC of 0.33, **Table 3.4**) suggesting inferior co-localization of **21c** with the ER. Next, the co-localization of complexes with mitochondria was tested as well. As indicated in **Figure 3.10** (panels 5 and 6) and **Table 3.4**, the fluorescence signal coming from **21c** co-localized with the fluorescence from the Mito-Tracker much better than with that from the ER-Tracker. The PCC calculated for mitochondrial localization was 0.62, which is almost double that the coefficient found for ER. Therefore, **21c** has a preferential localization in mitochondria. In comparison, **10** and **20c** target ER preferentially.

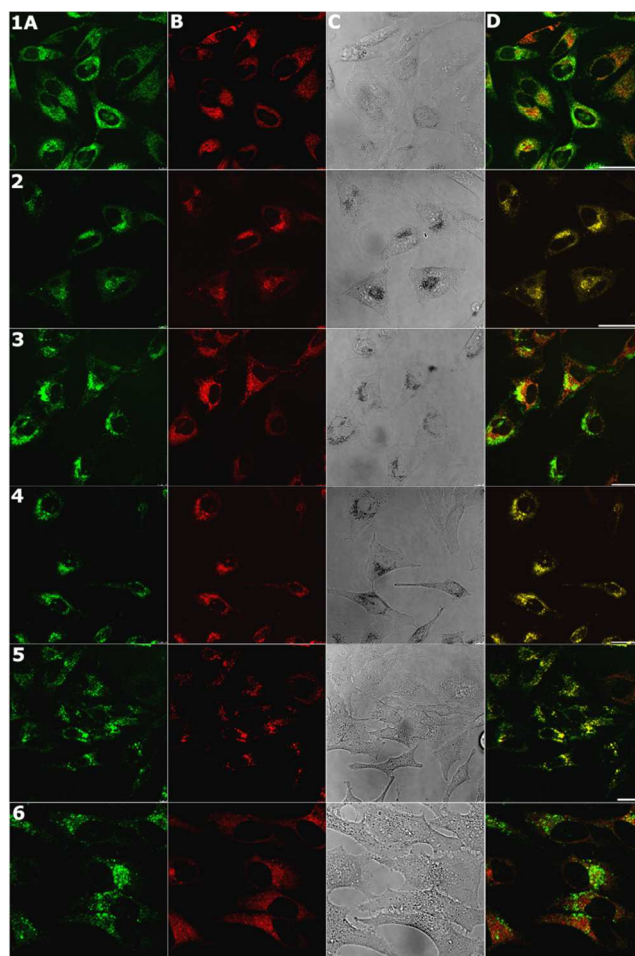


Figure 3.10. Co-localization of compounds in HeLa cells. Cells were treated with 5 μM **10** (panels 1, 2); **20c** (panels 3, 4); or **21c** (panels 5, 6) and co-stained with MitoTrackerTM (panels 1, 3, and 5) or ER-TrackerTM (panels 2, 4, 6). Channels: A) luminescence coming from complexes, B) ER- or Mito-Tracker fluorescence, C) bright field, D) overlay of the fluorescence channels. Scale bars represent 30 μm .

3.2.8. Effect on the mitochondrial membrane potential

The great localization of **21c** in mitochondria prompted us to examine the effect of the iridium compounds in the early stage of their action on this bio-energetically essential cellular organelle. For this purpose, a method based on the measurement of the

mitochondrial membrane potential by tetramethylrhodamine ethyl ester (TMRE), was used.⁴⁶ TMRE is a cell-permeant dye, easily accumulating in active mitochondria. Depolarized or damaged mitochondria cannot accumulate TMRE, and thus the cells with inactive mitochondria exhibit lower fluorescence.

HeLa cells were treated with **10**, **20c**, and **21c** or cisplatin (10 μ M) for 5 h and then analyzed by flow cytometry. As observed in **Figure 3.11** and **S3.28**, complex **21c** was the only compound of the 4-(trifluoromethyl)benzyl derivatives that significantly sequestered the mitochondrial uptake of TMRE, which correlates with the results of the co-localization studies by confocal microscopy (**Figure 3.10**).

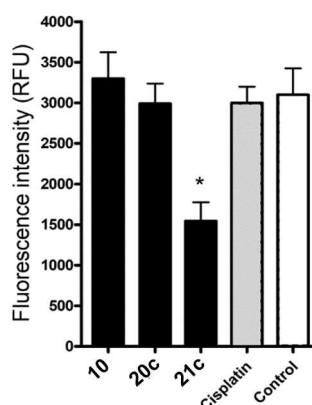


Figure 3.11. Mitochondrial membrane potential. HeLa cells were treated with the complexes (10 μ M) for 5 h, subsequently stained with TMRE, and analyzed by using flow cytometry. Data are expressed as the mean fluorescence intensities calculated from two independent experiments; error bars are the standard deviations. *Star at the top of the bar denotes a significant difference from the untreated control calculated by using Student's t-test ($p < 0.01$).

3.2.9. Determination of intracellular reactive oxygen species (ROS)

Mitochondrial damage is accompanied by oxidative stress and an increase of the intracellular ROS level. Moreover, a large number of iridium complexes was already shown to behave as photosensitizers^{22,47–49} by increasing the intracellular ROS if they are irradiated. Thus, the ability of **21a**, **21b** and **21c** to affect the ROS level was investigated in HeLa cells after their treatment in the dark or under irradiation ($\lambda_{\text{max}} = 420$ nm). HeLa cells untreated or pre-treated with complexes of series 3 were incubated with 2',7'-dichlorodihydro-fluorescein diacetate (DCFH-DA).

As indicated in **Figure 3.12**, HeLa cells treated with complexes **21a**, **21b**, and **21c** in the dark resulted, after subsequent incubation with DCFH-DA, in a concentration-dependent increase in fluorescence as compared to the control untreated cells (**Figure 3.12A** and **C–E**). That fact showed the production of ROS in cells even in the dark, whereas the treatment with compounds **21a** and **21c** in the dark hardly increased the intracellular

ROS as compared to the untreated cells. Furthermore, the increase caused by **21b** was markedly more effective, which correlates with its highest antitumor activity in the dark. The fluorescence was significantly enhanced due to irradiation of the cells by blue light before the addition of DCFH-DA (see **Figure 3.12B** and white bars in **Figure 3.12C–E**). These results can be interpreted to mean that the generation of intracellular ROS by complexes of series 3 inside cells was considerably potentiated after irradiation. The ROS level in irradiated cells treated with **21a** was lower than that in the cells treated with the other two complexes **21b** and **21c**, which likely reflects its lower phototoxic index (**Table 3.2**).

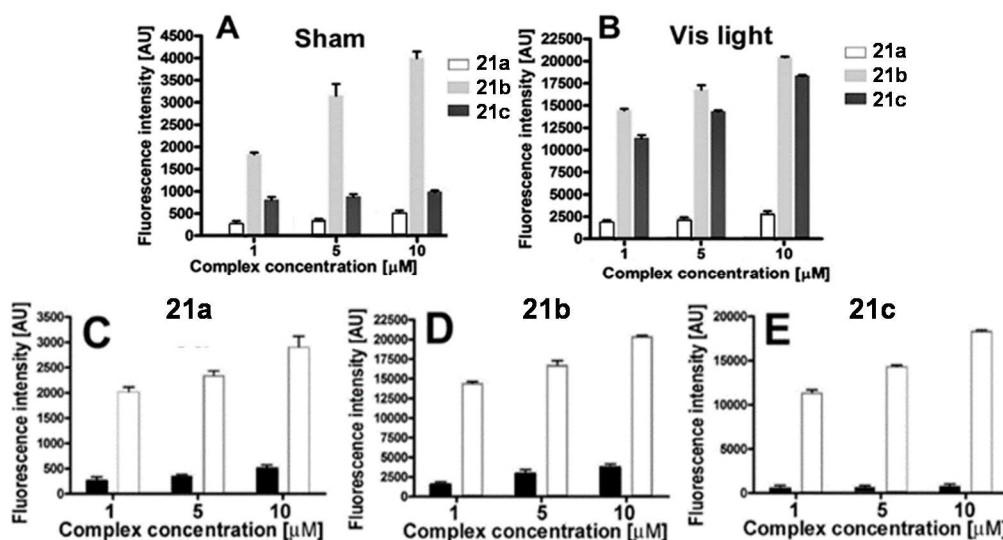


Figure 3.12. ROS generation of **21a**, **21b**, and **21c** in the dark or after irradiation (1 h) in HeLa cells. Top panels: Comparison of effects of **21a–c** A) in the dark or B) after irradiation. Bottom panels: Comparison of ROS induction in the dark (black columns) and under irradiation (empty columns) for complexes C) **21a**, D) **21b**, and E) **21c**. Data represent means $\pm$ SD of three independent experiments.

To find out the specific ROS produced in cancer cells treated with the new compounds, a set of ROS inhibitors or scavengers was employed. Cells were pre-treated with the selective ROS scavengers for 1 h at 37 °C and immediately after this pre-treatment, the cells were treated with the investigated complexes (10 μ M) for 2 h (1 h of incubation in the dark followed by 1 h under blue light irradiation). The cells were then collected and stained using a fluorogenic ROS sensor (CellRox® Oxidative stress Deep red reagent) and fluorescence intensity was analyzed by flow cytometry. In its reduced state, CellROX™ is non-fluorescent; however, upon oxidation by ROS, this reagent exhibits bright near-infrared fluorescence that serves as a direct measurement of the ROS levels in live cells. With an excitation/emission maxima of 640/665 nm, the fluorescence of the oxidized CellROX™ Deep Red reagent does not interfere with the luminescence of the new compounds.

The effects of various ROS scavengers on the intracellular level of ROS after the irradiation are summarized in **Figure 3.13** and **S3.29**. Since the fluorescence signal was not inhibited in the presence of hydroxyl radical ($\text{OH}^\bullet$) and hydrogen peroxide (H_2O_2) scavengers such as mannitol and sodium pyruvate, respectively, even at high concentrations (50 and 10 mM, respectively), both the hydroxyl radicals and hydrogen peroxide are unlikely to be responsible for oxidative stress. In contrast, the presence of the cell-permeable superoxide scavenger Tiron resulted in the decrease of fluorescence in the cell treated with **21a–c**, indicating that superoxide anions ($\text{O}_2^{\bullet-}$) are likely candidates responsible for oxidative stress induced by irradiation. To test the possibility that the photopotential of **21a–c** also involves the formation of singlet oxygen, the fluorescence was measured in the presence of sodium azide, which is one of the most effective singlet-oxygen quenchers.⁵⁰

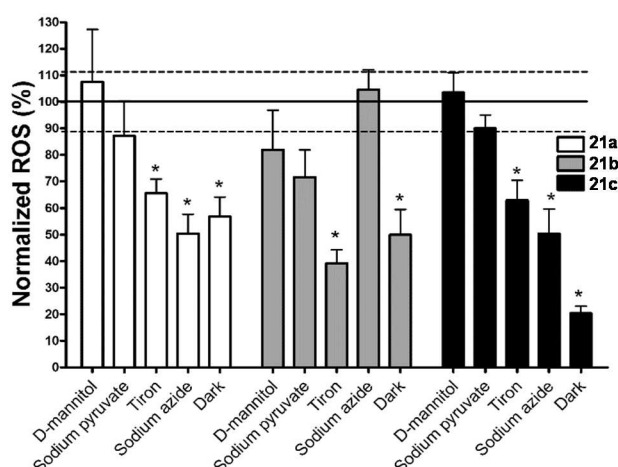


Figure 3.13. Determination of specific ROS generated by **21a–c**. Cells were pre-incubated for 1 h with specific ROS scavengers: D-mannitol (50 mM), sodium azide (5 mM), sodium pyruvate (10 mM), and tiron (5 mM). Cells were treated with **21a–c** (10 μM), incubated for 1 h in the dark followed by irradiation (1 h). Scavengers remained throughout the experiment. The fluorescence signals from positive controls (irradiated cells treated with **21a–c** without scavengers) were set to 100% and are indicated as the full horizontal line with SD (dashed lines). Stars at the top of the bars indicate a significant difference from the positive control with $p \leq 0.01$ calculated by using analysis of variance (ANOVA) and non-parametric student's t-test; error bars represent the SDs.

As shown in **Figure 3.13**, **S3.29**, and **S3.30**, oxidation of the reporter reagent was inhibited in the presence of sodium azide if the cells were treated with **21a** and **21c**, but not **21b**. The conclusion that the photopotential of **21a** and **21c** also involves the formation of singlet oxygen is consistent with the results of the experiments performed in a cell-free medium using 1,3-diphenylisobenzofuran (DPBF; **Figure E3.1** in Experimental Section)^{10,51} demonstrating that under blue light irradiation, **21a** and **21c** produced singlet-oxygen (**Figure 3.14**). The effect of the individual scavengers on cells was checked by flow cytometry and cells were analyzed for flow cell debris and granularity as indicators of the

cell culture condition (**Figure S3.30**). No significant effect on cells was observed under the conditions of the experiment.

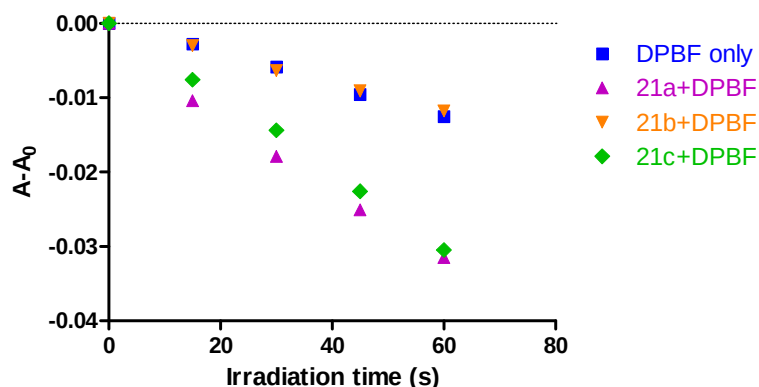


Figure 3.14. Rate of decay of DPBF sensitized by complexes **21a-c** in PBS solution (1 % DMSO) as shown by the decrease in the absorbance at 418 nm.

Given the strong photopotentialization of cytotoxicity of new complexes under both normoxic and hypoxic conditions (**Table 3.2**), the ROS generation was also studied under hypoxic conditions by standard dihydroethidium (DHE) and singlet oxygen sensor green (SOSG) staining assays.¹⁹ For comparative purposes, the same procedures were applied to the cells cultivated under normoxic conditions. As indicated in **Figure 3.15**, in the normoxic environment (21% O₂), bright red fluorescence related to the oxidation of DHE by O₂^{•-} was observed by confocal microscopy with the signal markedly increased after exposure to 420 nm light, in agreement with the ROS studies described above (**Figure 3.12** and **Figure 3.13**). Importantly, remarkable DHE fluorescence was also detected in HeLa cells in a hypoxic environment, indicating the photoactivated generation of superoxide radicals even at a low O₂ concentration. In contrast, a slight, but notable, green fluorescence of singlet oxygen sensor green (SOSG) after irradiation of cells treated with **21a** and **21c** under normoxic, but not in hypoxic conditions, indicating the formation of singlet oxygen mainly in normoxia, but not under hypoxia. These results correspond with the photopotentialization of **21a-c** also in hypoxia indicating that under both normal and low oxygen concentrations, the formation of superoxide radicals is mainly responsible for the cytotoxic effect of that complexes, and the biological effect of these radicals predominates over the singlet oxygen.

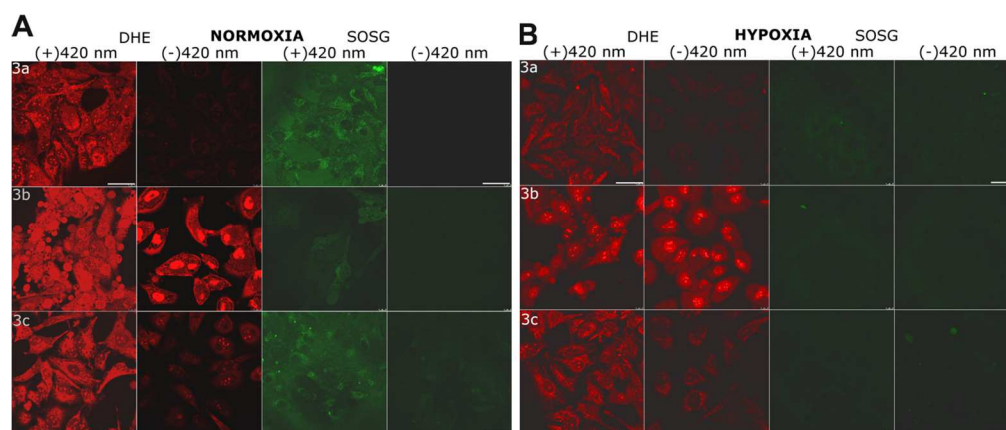


Figure 3.15. ROS detection in HeLa cells treated with **21a-c** under A) normoxic (21% O₂) or B) hypoxic (2% O₂) conditions by confocal microscopy using DHE and SOSG as the O₂^{•-} and ¹O₂ fluorescence indicator, respectively. Cells were treated with complexes (10 μM) for 1 h followed by 1 h of irradiation in photoreactor [(+)420 nm] or kept in the dark [(-)420 nm]. Scale bar represents 40 μm.

3.2.10. Cell-death detection studies

We investigated the progression of the HeLa cells death treated with all complexes, including cell fragmentation and generation of apoptotic bodies using a previously published flow cytometry-based protocol.⁵² Notably, compounds **19b**, **20b**, and **21b** were markedly more effective in killing the cells than **19a-21a** and **10**, **20a-b** (Figure 3.16). These observations nicely correlate with the potency of complexes to affect their proliferative activity and viability (Table 3.2). The number of early apoptotic cells largely increased after the irradiation of cells treated with compounds of series 2 and 3. Interestingly, a substantial increase of apoptotic bodies was detected after the treatment with the methyl derivatives (**19b**, **20b**, and **21b**). Moreover, as a consequence of the irradiation of the HeLa cells pre-treated with all compounds, fractions of cells were also undergoing necrotic processes. These results show that under irradiation by visible light, the complexes are phototoxic and induce cell death via both, apoptotic and necrotic pathways. Although apoptosis is the preferred pathway for cancer cell death because is an orderly and programmed process, it has been observed for several tumors that they are resistant to this kind of cell death.^{53,54} Therefore, the photoactivatable Ir(III) complexes of this study, which are capable of killing cells through both apoptosis and necrosis, are highly sought as very promising candidates for chemotherapeutics acting in human tumors resistant to existing antitumor drugs.

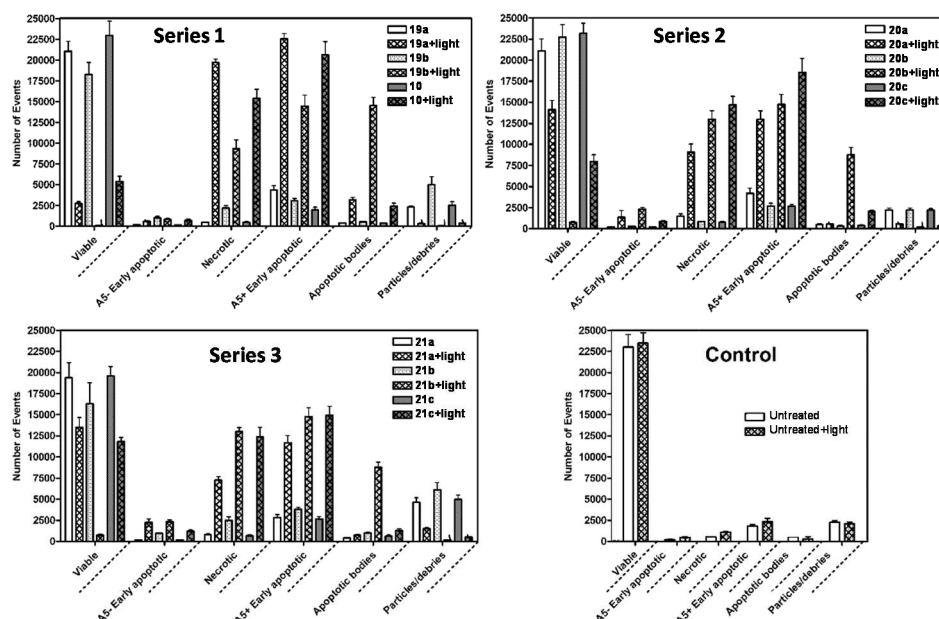


Figure 3.16. Cell death detection. HeLa cells were treated with all complexes (10 μ M) for 2 h [1 h of pre-incubation followed by 1 h of irradiation with light (420 nm) (double-hatched bars) or kept in the dark (empty bars)]. Cells were stained with annexin-V-FITC and TO-PRO-3. Samples were analyzed by flow cytometry. The seven-stage electronic gating strategy was used to identify cell populations at various stages of cell death: viable cells, annexin-V^(negative) (A5⁻) early apoptotic cells, necrotic cells, annexin-V^(positive) (A5⁺) early apoptotic cells, apoptotic bodies, and A5⁻ particles/debris. Data are expressed as the means of detected events from two independent experiments $\pm$ SDs. Acquisition of 30000 events was used for each sample.

3.3. CONCLUSIONS

Nine octahedral biscyclometalated Ir(III) complexes bearing C^N ligands based on 2-phenyl-, 2-(naphthalen-2-yl)- or 2-(thiophen-2-yl)-benzimidazole scaffolds (series 1, 2, and 3, respectively) have been synthesized. The substituent in the imidazole unit of the C^N ligand plays a fundamental role in the photophysical properties of the complexes and their antiproliferative activity in human cervical cancer HeLa cells under normoxic and hypoxic conditions. The methyl derivatives (**19b**, **20b**, and **21b**) were the most active in the dark while complexes with the group CH₂C₆H₄CF₃-p (**10**, **20c**, and **21c**) exhibit the lower toxicity in the dark and have a high PI. On the other hand, the substitution in position 2 of the benzimidazole seems to be directly related to the location in the cell: the sulfur-containing complex **21c** mostly localized in mitochondria, whereas the 2-aryl-benzimidazole complexes targeted ER. The ROS production investigated by DHE and SOSG staining assays showed that the generation of superoxide radicals competed with singlet oxygen production in cells in normoxia, but became dominant under hypoxia. The importance of the switch from Type II to Type I photochemical mechanism by the new compounds is that they can be active in all areas of a solid tumor, adapting their mechanism of action according to the oxygen concentration, thereby overcoming the Achilles' heel of PDT.

3.4. REFERENCES

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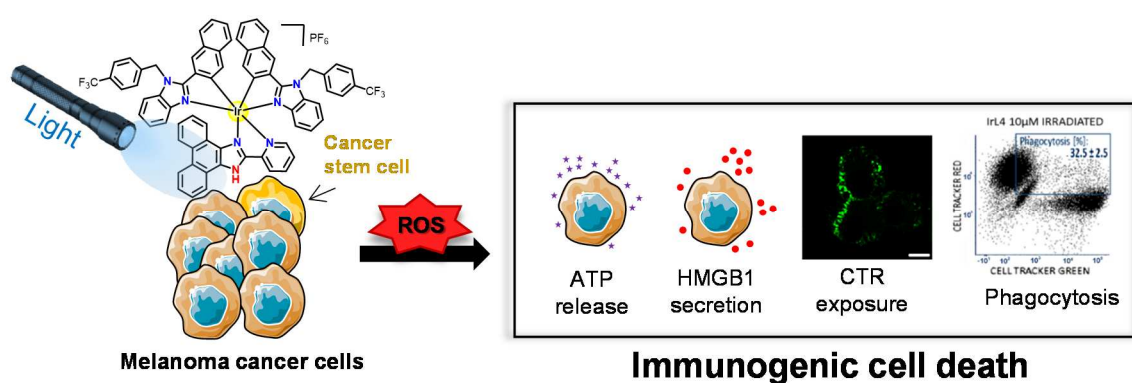
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Chapter 4

Photoactivated Ir(III) complexes that target cancer stem cells and induce immunogenic cell death in melanoma cells



The biological experiments included in this chapter were carried out by Dr. Viktor Brabec (Institute of Biophysics, Czech Academy of Sciences, Brno, Czech Republic).

4.1. INTRODUCTION

Cancer recurrence and metastasis have been strongly linked to the existence of cancer stem cells (CSCs).¹ These cells are responsible for self-renewing and give rise to other, more differentiated cells that form the bulk of the tumor.² There is hard evidence of CSCs in solid cancers, such as breast, brain, colon, pancreas, and melanoma.^{3,4} CSCs can overcome chemotherapy, radiotherapy, and immune attack, which trigger tumor relapse. This unsuccessful recognition of CSCs by the immune system is because of their ability of antigen-editing and maintenance of only limited antigen presentation.⁵ Current research suggests immunotherapeutic strategies that target CSCs may improve the efficacy of cancer treatment, using in combination with traditional cytotoxic therapies.⁶ Thus, developing new immuno-chemotherapeutic agents capable of reducing tumor mass by cytotoxic mechanisms and removing residual CSCs by immunological activation simultaneously⁷ holds hope for improving cancer patients' survival and life quality, including those in the metastatic stage of the disease.

One of the approaches by which chemotherapeutics can induce a tumor-targeting immune response is through immunogenic cell death (ICD). This rare immunostimulatory form of cell death is a chain process characterized by the release into the extracellular space of damage-associated molecular patterns (DAMPs) that are recognized by antigen-presenting cells (APCs) and activate dendritic cells (DCs) can lead to the subsequent development and activation of tumor-specific effector T cells and memory T cells⁸ (**Figure 4.1**). Molecularly, these signals or DAMPs, also known as biochemical hallmarks, are mainly: i) the surface exposure of the chaperone calreticulin (CRT) during the early apoptotic stage of the cell as a result of stress on the endoplasmic reticulum (ER) induced by the drug, acting as an 'eat me' signal against DCs, ii) extracellular secretion of adenosine triphosphate (ATP) during the late apoptotic stage, acting as a 'find me' signal and recruiting DCs, and iii) passive release of high mobility group protein B1 (HMGB1) during the final necrotic stage of the cell, constituting the last danger signal recognized by DCs before activating of T lymphocytes.⁹ In cooperation, these factors initiate gradual processes of dendritic cell recruitment, phagocytosis, antigen processing, maturation, and antigen presentation to T cells. Finally, the cascade process produces cytotoxic cytokines like IFN- γ involving $\gamma\delta$ T cells and cytotoxic CD8⁺ T lymphocytes, resulting in the killing of target cells.⁸ The absence of any of this three hallmarks suppresses the activation of ICD.

Of the clinically approved anti-cancer platinum(II) drugs (cisplatin, carboplatin, and oxaliplatin), only oxaliplatin can induce ICD.¹⁰ Despite exciting advances in the field of bio-inorganic chemistry regarding the design of new anticancer metal drugs (based on platinum, iridium, ruthenium, etc),¹¹ their immuno-modulatory features have not been addressed systematically so far.

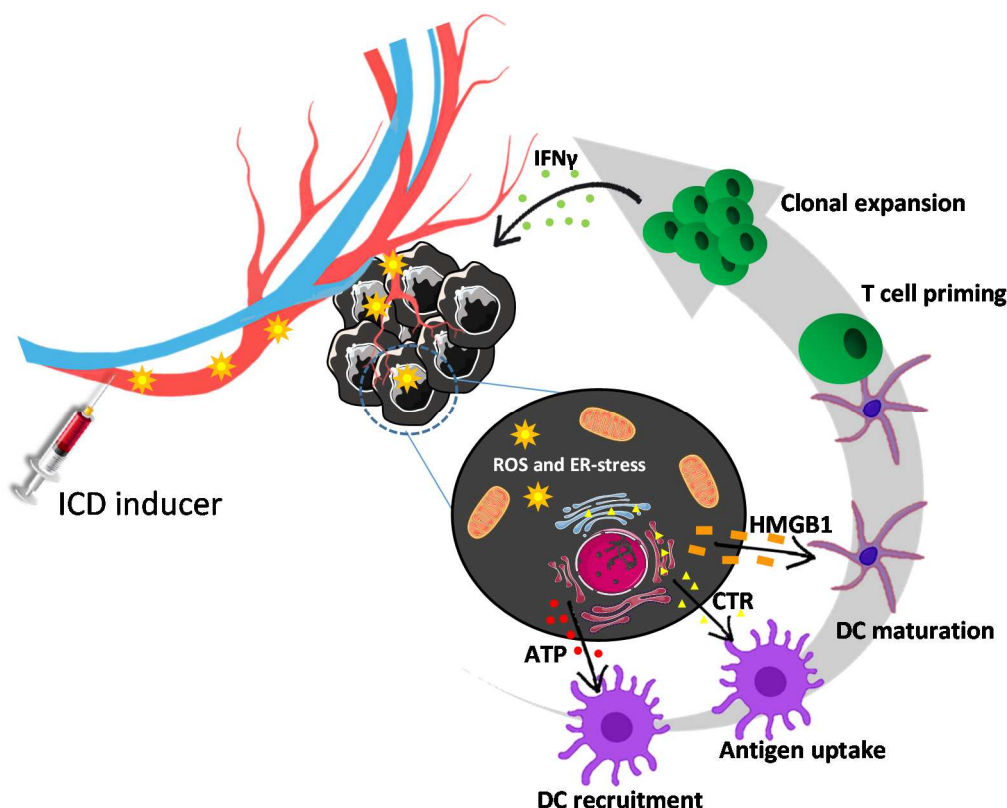


Figure 4.1. Molecular hallmarks of immunogenic cell death (ICD). ATP, adenosine triphosphate CTR, calreticulin; HMGB1, high-mobility group box 1; DC, dendritic cell; IFN, interferon.

In the last years, it has been shown that photodynamic therapy (PDT) can produce ICD in cancer cells.^{12–16} As commented before, PDT is an antitumor approach using the activation of a drug by light, directly destroying local tumor cells due to the generation of toxic singlet oxygen and/or reactive oxygen species (ROS). Recently, some studies have demonstrated that PDT can exhibit immunomodulatory functions apart from its direct tumor-destroying capability. This phototherapy can stimulate the innate and adaptive immune responses, killing untreated primary or metastatic tumor cells and promoting anti-cancer immunity to avoid the tumor repeat.¹⁷ The induction of ROS-based ER stress is vital for the danger signaling pathways activation.^{18,19} Consequently, this makes each photosensitizer with a high production of ROS a promising drug for ICD induction.

On the other hand, metastatic malignant melanoma stays quite possibly the most dreaded skin cancer around the planet. To date, PDT has not exhibited high efficiency

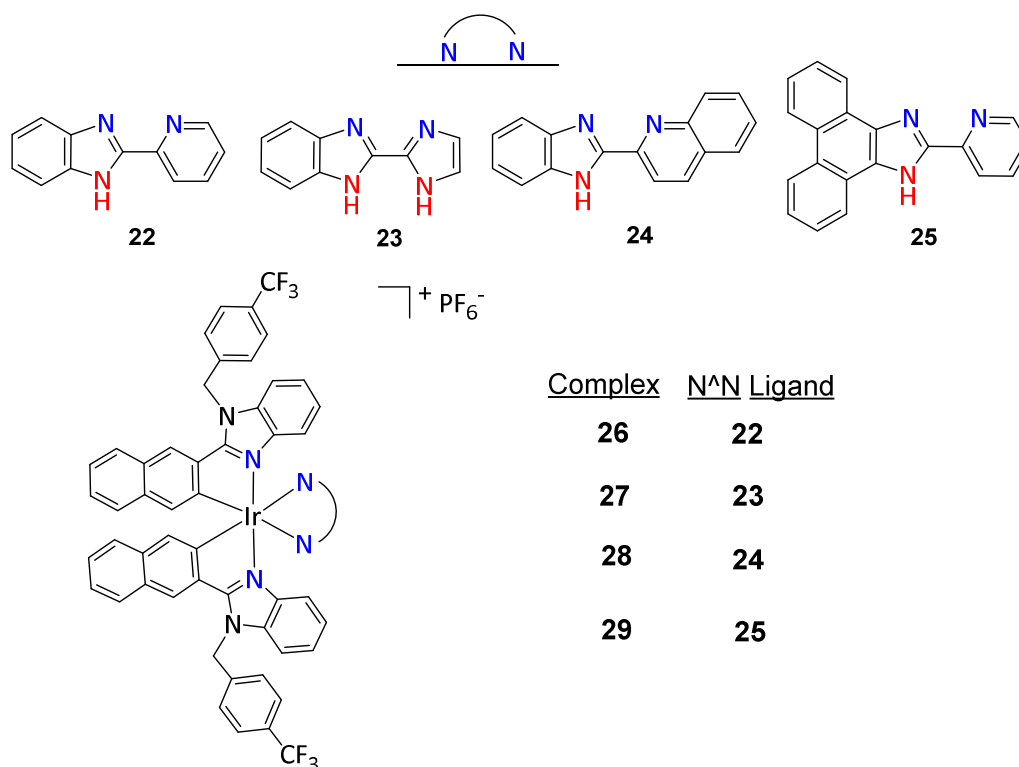
against melanoma, primarily because of the high dark toxicity of the most recognized photosensitizers (PSs), melanin pigmentation, and anti-oxidant defense mechanisms in melanoma cells.²⁰ However, since melanoma is a limited cutaneous tumor and the skin is not difficult to illuminate with light, PDT is as yet quite possibly the most encouraging helpful method.²¹ Furthermore, using PSs that are not active in the dark and display a high ROS generation under light irradiation can increase the success of this phototherapeutic modality against melanoma. In this sense, Ir(III) complexes have attracted much attention as possible PDT agents due to their highly populated triplet excited states for ROS production and their easy structural modification that allows precise tuning of their photophysical and photobiological properties.^{22,23} Additionally, these Ir(III) PSs are usually excited by short-wavelength UV/Vis light (generally in the blue region), limiting their potential application for deep-seated tumors but providing adequate PDT potency for superficial treatments as melanoma.²⁴

Herein, we report the design and synthesis of new four phosphorescent Ir(III) complexes and their potency towards melanoma and HeLa cancer cells after blue light irradiation. In addition, we show that the 2-(pyridin-2-yl)-1H-phenanthro[9,10-d]imidazole-bearing complex, **29**, can evoke ICD in melanoma cells under photoirradiation. Moreover, **29** also exhibits a promising photoactivity in three-dimensional *in vitro* models that mimic features of the *in vivo* environment, and it is selective toward CSCs-like melanoma cancer cells only after blue light irradiation. To the best of our knowledge, this is the first iridium complex that selectively targets CSCs and induces ICD hallmarks in melanoma by light irradiation.

4.2. RESULTS AND DISCUSSION

4.2.1. Synthesis and characterization

A series of four luminescent and cationic Ir(III) complexes of the type $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{N}^{\wedge}\text{N})][\text{PF}_6]$ (**Scheme 4. 1**) were synthesized to investigate the effect of modifying the ancillary ligand on their phototherapy activity.



Scheme 4. 1. Structures of new iridium complexes **26-29**.

The HC^N pro-ligand was chosen because it provided low dark toxicity and one of the highest phototoxicity indexes in the complexes of Chapter 3. The different N^N ligands (**22-25**) were based on a common 1*H*-benzo[*d*]imidazole backbone containing NH groups in their structure. According to literature, these N^N ligands with NH groups seems to have a crucial role in the activity of the PS because they provided very low toxicity of the complexes in the dark.²⁵⁻²⁷ **23** and **24** were prepared by condensation reactions between *o*-phenylenediamine and the corresponding aldehydes using an adaptation method reported in the literature²⁸ (**Schemes E2.7** and **E2.8** in Experimental Section). **25** was synthesized by the reaction between picolinaldehyde and phenanthrene-9,10-dione using an adaptation method reported in the literature²⁹ (**Scheme E2.9** in Experimental Section). The new complexes were synthesized similarly to previously reported methods.³⁰⁻³² First, the cyclometalated Ir(III) dimer was obtained by reacting iridium(III) chloride hydrate and HC^N pro-ligand. Then, the dimer **17c** (described in Chapter 3) was reacted with the corresponding N^N ligand (**22-25**) in a 1:2 molar ratio yielding the yellow to orange monomers **26-29**. The investigated complexes were characterized by ¹H and ¹³C NMR spectroscopy (**Figures S4.1-6**), and positive-ion ESI-HRMS (**Figure S4.7-8**). The NMR spectra of the new complexes exhibit one set of peaks for each of the two inequivalent C^N ligands due to the asymmetric nature of the coordination environment around the iridium

center. The positive-ion ESI mass spectra displayed the expected molecular ion ($[M-PF_6]^+$) with the expected isotopic distribution pattern (**Figure S4.8**). Complexes were shown to be at least 96 % pure by both reverse-phase RP-HPLC in DMSO (**Figure S4.9**) and elemental analysis.

4.2.2. Photophysical properties

The UV/Vis absorption spectra and the luminescence behaviors of the complexes were measured in water (1% DMSO) and acetonitrile at room temperature (**Figure 4.2** and **S4.10-11**). All complexes demonstrate strong absorbance in the range 230-330 nm ($\epsilon \sim 100000-70000 \text{ M}^{-1}\text{cm}^{-1}$) owing to $\pi \rightarrow \pi^*$ electronic transitions located on the C[^]N and N[^]N ligands. The less intense broad bands at 350-400 nm ($\epsilon \sim 30000-12000 \text{ M}^{-1}\text{cm}^{-1}$) as well as the bands in the visible range ($> 400 \text{ nm}$) could be attributed to spin-allowed metal to ligand ($^1\text{MLCT}$), spin forbidden singlet-to-triplet ($^3\text{MLCT}$) charge-transfer and ligand-to-ligand charge-transfer ($^{1,3}\text{LLCT}$) transitions.³³⁻³⁵

Upon excitation at 405 nm, **26-29** exhibit yellow to orange phosphorescent emissions in water (**Figure 4.2** and **Table 4.1**), showing **28** and **29** a red-shift in their emission bands probably due to the increased π -conjugation of the N[^]N ligand.^{36,37} It has been reported that the lowest unoccupied molecular orbital (LUMO) is based on benzimidazole for the complex with a similar structure.³⁸ Thus, the addition of a phenyl ring on N[^]N ligands stabilizes LUMO and leads to the decline in the energy gap, which induces the red-shift in the emission maximum of these complexes. The emission quantum yields in the deoxygenated water (**Table 4.1**) were lower for **26** and **27** ($<1\%$) and **29** showed the highest quantum yield (7.3%).

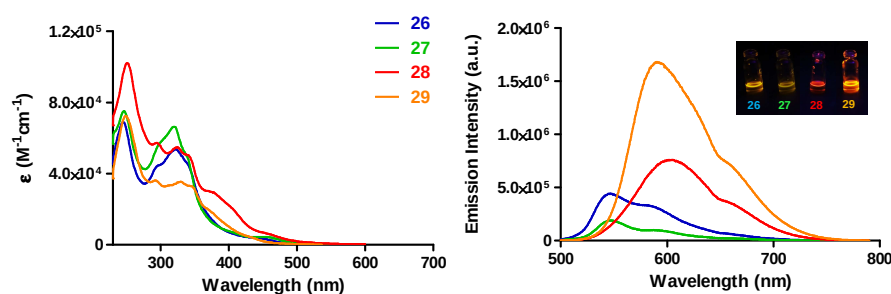


Figure 4.2. UV/Vis and emission spectra of **26-29** in water (1% DMSO). $\lambda_{\text{exc}} = 405 \text{ nm}$.

The excited-state lifetimes in water for these complexes at room temperature show a bi-exponential decay with a short (21 – 62 ns) and a long (173 – 237 ns) component (**Table 4.1**). The bi-exponential intensity decays are shown in **Figure S4.12** and they are consistent with triplet excited states, which is in line with the other reported Ir(III) complexes.³⁹⁻⁴¹

Table 4.1. Excitation (λ_{exc}), emission (λ_{em}) wavelengths, emission lifetimes (τ_{em}), and quantum yields (Φ_{em}) of **26-29** in water (1% DMSO).

Compound	λ_{exc} , nm ^a	λ_{em} , nm	τ_{em} , ns ^b	Φ_{em} , % ^b
26	370	547	21 (20%), 237 (80%)	<1
27	350	549	— ^c	<1
28	390	604	38 (36%), 173 (64%)	1.0
29	380	595	62 (15%), 227 (85%)	7.3

^a λ_{exc} maxima. ^b Emission lifetimes and quantum yields measured in deaerated solution. ^c Too weak to be measured.

To study the influence of the NH group of the N[^]N ligand in the luminescence of the new complexes, the emission intensity of these compounds was studied in a mixture of water and DMSO with different water fractions and at different pHs. As shown in **Figure 4.3**, the emission intensity reached a maximum when the water content of the **26**, **27**, and **28** mixtures increased up to 30%. When the percentage increased to 99%, these mixtures exhibited almost no emission (suffer important emission quenching). Remarkably, **29** exhibited a very similar emission intensity in all water fractions. Among these complexes, only complex **27** showed a significant pH-sensitive emission (**Figure S4.13**).

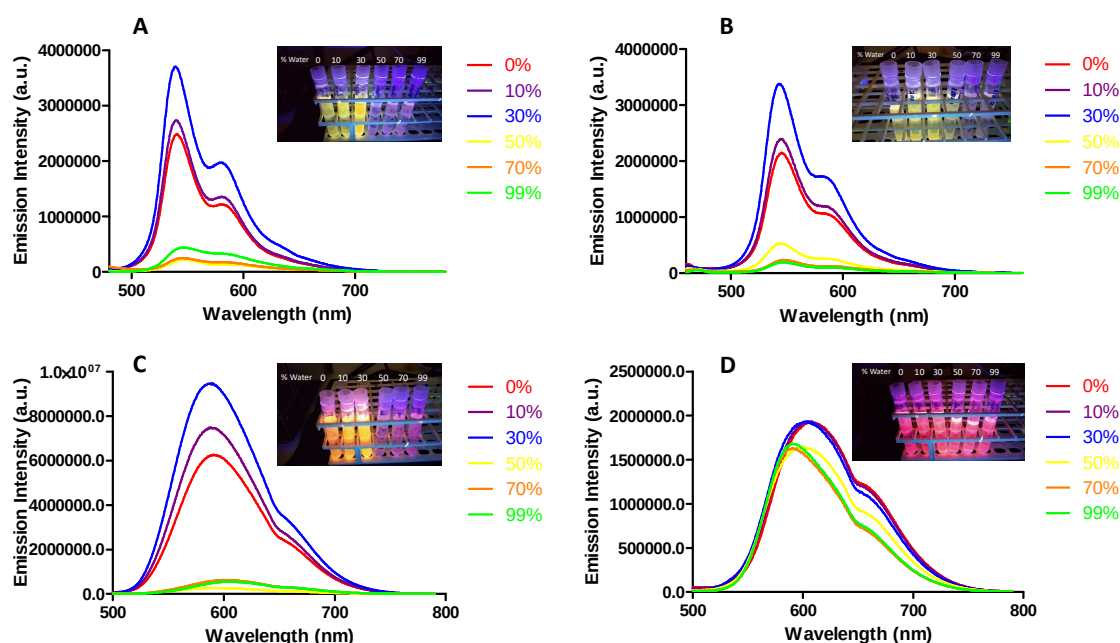


Figure 4.3. Emission spectra of A) **26**, B) **27**, C) **28**, D) **29** in DMSO/water mixtures with different water fractions (fw). The excitation wavelength was 405 nm.

4.2.3. Stability and photostability studies

The stability of the complexes in solution was ascertained by NMR and UV/Vis techniques. The stability of **26-29** in DMSO at room temperature was checked by NMR, no free ligands were detected after 7 days (**Figure 4.4A**). The stability was also checked in Roswell Park Memorial Institute (RPMI) culture medium by UV/Vis (**Figure 4.4B** and **S4.14**).

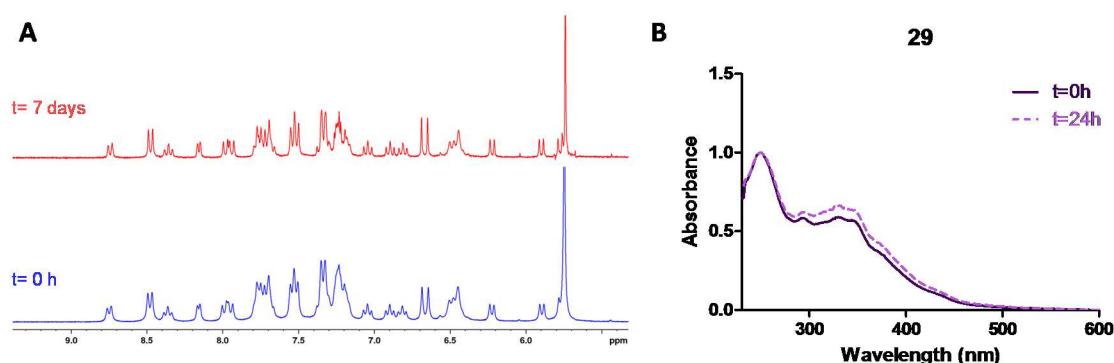


Figure 4.4. A) ^1H NMR spectra of **26** measured after dissolving immediately in $[\text{D}_6]\text{DMSO}$ (bottom) and after 7 days (top) at RT. B) UV/Vis spectra of complex **29** before ($t = 0$ h) and after 24 h in RPMI culture medium.

The photostability in water (1% DMSO) was tested for complexes **26-29** by UV/Vis spectroscopy after irradiation with blue light ($\lambda = 465$ nm, 8.4 mW/cm 2) for 1 h. The absorption spectra remained the same before and after light exposure (**Figure 4.5**).

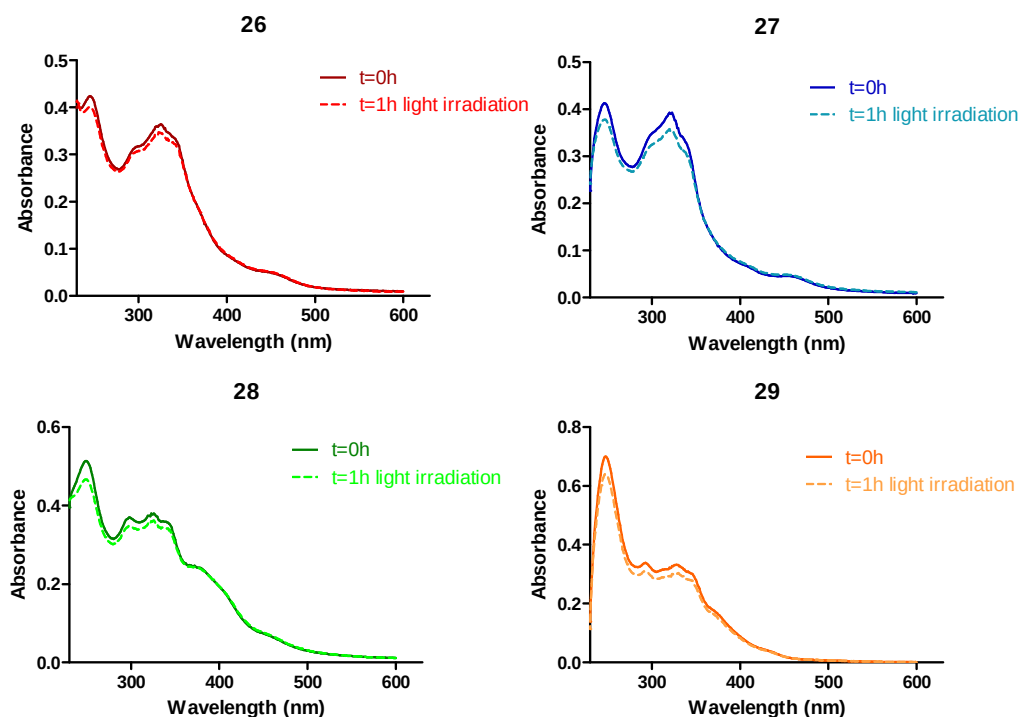


Figure 4.5. Photostability of complexes **26-27** in water under 465 nm at $t = 0$ h and after 1 h of irradiation.

4.2.4. Photo-oxidation of NADH by the new Ir(III) complexes in cell-free media

Ir(III) complexes have been effectively utilized as photocatalysts in numerous organic transformations.⁴² More specifically, they have also been used in photooxidation reactions, in which they are efficient photosensitizers due to their ability to produce superoxide anion radicals ($\text{O}_2^{\cdot-}$) or singlet oxygen ($^1\text{O}_2$).⁴³ Therefore, before the biological assays, we studied if the new complexes could photocatalytically oxidize NADH. This coenzyme is an essential molecule in living cells, which participates in maintaining the intracellular redox balance, and has recently been studied as a photooxidation test.^{44,45} The photocatalytic oxidation of NADH (100 μM) by **26-29** (5 μM) in PBS (0.5 % DMSO) was observed by UV/Vis spectroscopy (**Figure 4.6** and **S4.17**). While the UV/Vis spectrum of NADH remained unchanged in the dark and in the presence of light for 30 min (**Figure 4.6A** and **S4.15**) and the spectrum did not change in the presence of complexes in the dark neither (**Figure 4.6B** and **S4.16**), under blue light (465 nm, 8.4 mW/cm²) in air, the absorbance of NADH at 339 nm decreased gradually with all complexes (**Figure S4.17**). Surprisingly, **29** was able to completely oxidize NADH to NAD^+ after 30 min of irradiation (**Figure 4.6C**) and showed the highest NADH oxidation turnover number (TON = 16.4 after 30 min) with a turnover frequency (TOF) of 32.9 h⁻¹ (**Table 4.2**). In addition, this TON was greater than the (non-photoactivated) Ir(III) half-sandwich catalyst [(η^5 -Cp^xbiph)Ir(phpy)py]PF₆ (TON = 7.6 after 20 h) previously reported.⁴⁶

Table 4.2. TON and TOF of the new Ir(III) compounds. TON is defined as the number of moles of NADH that the Ir compound can convert in 30 min. TOF is calculated from the concentration of oxidized NADH (calculated by the difference of concentration of NADH) after 30 min divided by the concentration of complexes.

Compound	TON	TOF ($\times 10^4$) (s ⁻¹)	TOF (h ⁻¹)
26	10.1	55.9	20.1
27	4.2	23.2	8.3
28	9.9	55.5	20.0
29	16.4	91.3	32.9

When the photocatalytic reaction is carried out in hypoxic conditions (after bubbling nitrogen for 30 min), complex **29** still maintains a certain ability to oxidize NADH (**Figure 4.6D**).

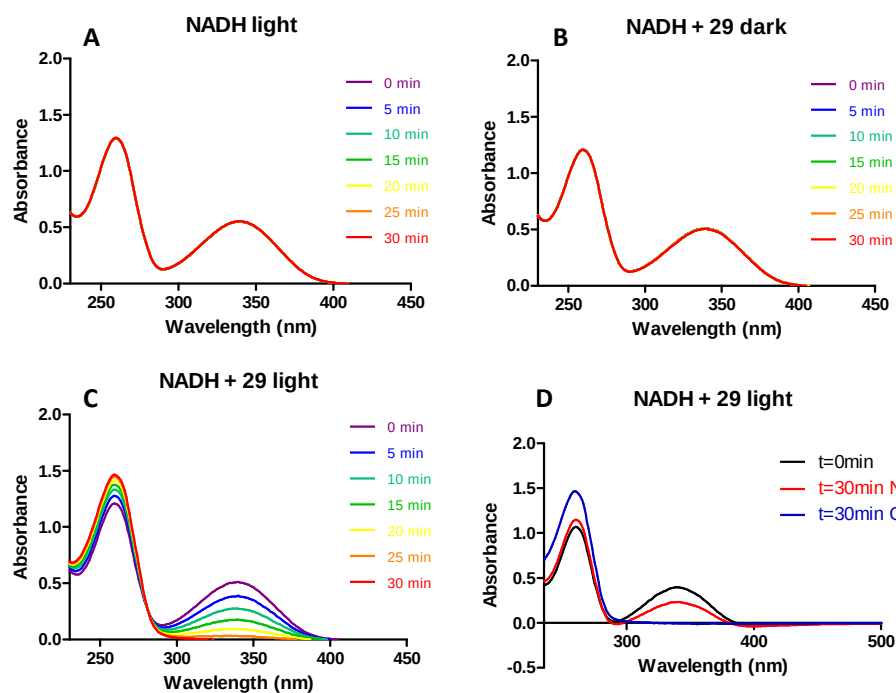


Figure 4.6. UV/Vis spectra for the photocatalytic oxidation of NADH (100 μM) by **29** (5 μM) in PBS at RT. A) NADH under 465 nm blue light irradiation. B) NADH in presence of complex **29** in the dark for 30 min. C) NADH in presence of complex **29** under continuous 465 nm light irradiation. D) NADH in presence of **29** at $t=0$ h (—), $t=30$ min irradiation with O_2 (—) and $t=30$ min irradiation under N_2 atmosphere (—).

4.2.5. Log P by HPLC-based method

The partition coefficient $\log P$ is an important factor for cellular uptake and anti-cancer activity, which provides a measure of hydrophobicity. Recently, Keppler *et al.*⁴⁷ have designed a new HPLC-based method that allows transforming the chromatographic lipophilicity parameter ϕ_0 in $\log P$ (see Experimental section and **Figures S4.18-19**). ϕ_0 is defined as the percentage of the organic phase in the eluent at which the partition of an analyte between the mobile and stationary phase is equal. This chromatographic lipophilicity parameter ranges between 0–100, and higher values indicate a stronger lipophilic character of the analyte. ϕ_0 and $\log P$ values for Ir complexes are shown in **Table 4.3**.

Table 4.3. Chromatographic lipophilicity parameter ϕ_0 and $\log P$ values of new iridium complexes calculated with the HPLC-based method validated by Keppler *et al.*

Compound	ϕ_0	$\log P$ (HPLC) ^a
26	84.0	3.4 ± 0.01
27	84.8	3.5 ± 0.01
28	88.0	4.0 ± 0.01
29	89.3	4.2 ± 0.1

^aResults are the mean $\pm$ SDs from two independent experiments.

4.2.6. Photo-toxicity studies towards human cancer cells

The phototoxic activity of **26-29** was determined against human skin melanoma cells (A375 and 518.A2) and human cervix adenocarcinoma cells (HeLa) in the dark and after irradiation (**Table 4.4, 4.5** and **4.6**). These cell lines were selected because skin and cervical cancer belong to the cancer types predisposed to photodynamic therapy and the tumors are easily accessible for therapy by irradiation. The tested compounds were added to the cells in EBSS and incubated for 1 h in the dark. Afterward, the cells were irradiated for 1 h ($\lambda_{\text{max}} = 420 \text{ nm}$, $7.7 \pm 3 \text{ mW/m}^2$) or left in the dark. Subsequently, EBSS was removed, and cells were returned to the incubator in the complete, Ir-complex free, DMEM medium. The metabolic activity of the cells was determined 72 h after irradiation using standard MTT assay. To assess the effect of oxygen, the experiments were performed in normoxia (21% O₂, 5% CO₂) or under hypoxic conditions (2% O₂, 5% CO₂).

Table 4.4. IC₅₀ values (μM) obtained for A375 melanoma cells treated with new complexes in the dark or after irradiation by visible light (1 h, $\lambda_{\text{max}} = 420 \text{ nm}$, $7.7 \pm 3 \text{ mW/m}^2$) determined by the MTT assay in normoxic or hypoxic conditions.^a

	Normoxia				Hypoxia		
	A375	Dark	420 nm	PI ^b	Dark	420 nm	PI ^b
26		>300	9.7 ± 2.5	>30	>300	18.3 ± 0.5	>16
27		>300	>50		>300	>50	
28		>300	8.1 ± 2.1	>37	>300	25.0 ± 2.8	>12
29		>300	3.2 ± 0.7	>100	>300	6.9 ± 0.4	>43

^aThe data are expressed as mean values $\pm$ SD of three independent experiments, each of them made in triplicate. ^bPI (phototoxic index) is defined as the ratio of the toxic effect in the dark and upon light irradiation; $\text{PI} = [\text{IC}_{50}]_{\text{dark}} / [\text{IC}_{50}]_{420\text{nm}}$.

Table 4.5. IC₅₀ values (μM) obtained for 518.A2 melanoma cells treated with new complexes in the dark or after irradiation by visible light (1 h, $\lambda_{\text{max}} = 420 \text{ nm}$, $7.7 \pm 3 \text{ mW/m}^2$) determined by the MTT assay in normoxic or hypoxic conditions.^a

	Normoxia				Hypoxia		
	518.A2	Dark	420 nm	PI ^b	Dark	420 nm	PI ^b
26		>300	15 ± 2	>20	>300	>50	>17
27		>300	>50		>300	>50	
28		>300	14 ± 2	>21	>300	48 ± 3	>17
29		>300	3.3 ± 0.2	>90	>300	7 ± 1	>43

^aThe data are expressed as mean values $\pm$ SD of three independent experiments, each of them made in triplicate. ^bPI (phototoxic index) is defined as the ratio of the toxic effect in the dark and upon light irradiation; $\text{PI} = [\text{IC}_{50}]_{\text{dark}} / [\text{IC}_{50}]_{420\text{nm}}$.

Table 4.6. IC₅₀ values (μM) obtained for HeLa cells treated with new complexes in the dark or after irradiation by visible light (1 h, λ_{max} = 420 nm, 7.7 ± 3 mW/m²) determined by the MTT assay in normoxic or hypoxic conditions.^a

	Normoxia				Hypoxia		
	HeLa	Dark	420 nm	PI ^b	Dark	420 nm	PI ^b
26		>300	13.4 ± 1.9	>22	>300	17.3 ± 3.2	>17
27		>300	>50		>300	>50	
28		>300	9.7 ± 0.9	>31	>300	17.9 ± 4.9	>17
29		>300	4.5 ± 0.6	>66	>300	6.1 ± 2.0	>49

^aThe data are expressed as mean values ± SD of three independent experiments, each of them made in triplicate. ^bPI (phototoxic index) is defined as the ratio of the toxic effect in the dark and upon light irradiation; PI = [IC₅₀]_{dark} / [IC₅₀]_{420nm}.

All tested complexes have no effect on cellular viability and proliferation in the dark, even at very high (300 μM) concentrations. However, when irradiated, they show a significant effect on melanoma and cervical carcinoma cell lines in both normoxia and hypoxia. Remarkably, complex **29** was the most active, demonstrating the highest phototoxicity index in the two cell lines.

The data obtained by measurement of the metabolization of MTT could be affected by the possible impact of tested compounds on mitochondrial metabolism. Therefore, the above-described experiments focused on the Ir-complexes' antiproliferative activity have also been performed using an assay based on neutral red uptake into cellular lysosomes. As shown in **Table S4.2**, the neutral red assay confirmed the same trend in the biological activity of all tested compounds in the dark and after irradiation, as found by MTT, being complex **29** the most effective.

4.2.7. Cellular accumulation

To evaluate the effect of cellular uptake and accumulation on the biological activity of new complexes, the intracellular content of Ir in A375 cells was determined by ICP-MS after the cells were treated with tested compounds at their equimolar concentrations (5 μM).

The data collected in **Table 4.7** indicate that the most active complex **29** markedly accumulates more effectively as compared with **26**, **27**, and **28** (possibly due to the elevated uptake through cellular membrane caused by the greater lipophilicity of ligand **25**). Complex **29** also showed the highest log P value (**Table 4.3**).

Table 4.7. Accumulation of Ir from new Ir(III) complexes in A375 cancer cells.^a

pmol Ir /10 ⁶ cells	
26	14± 1
27	9 ± 4
28	14 ± 3
29	92 ± 2

^a Results are the mean ± SDs from two independent experiments.

Therefore, the highest photoactivity of **29** can be likely associated with its increased cellular uptake and accumulation. Interestingly, **27**, which was inactive towards cancer cells, accumulated to a comparable extent as more active complexes **26** and **28**. This observation indicates that factors other than the reduced intracellular transport are responsible for the biological ineffectiveness of **27**.

4.2.8. Determination of intracellular reactive oxygen species (ROS)

As shown in previous chapters, the phototoxicity of iridium complexes is associated with their ability to arouse ROS generation inside cancer cells. Thus, the intracellular levels of ROS were quantified in A375 cells by using the CellROX® deep red assay. In this assay, the fluorescence intensity at 660 nm ($\lambda_{\text{exc}} = 640$ nm) was determined as a measure of ROS concentration. The tested complexes do not contribute to the final fluorescence signal by the excitation wavelength used.

As demonstrated in **Figure 4.7**, none of the new compounds cause ROS production in the dark. When irradiated, the intracellular ROS level was significantly elevated for cells treated with **26**, **28**, and **29**, being **29** markedly more effective than the **26** and **28**. However, irradiation by blue light does not affect ROS production in cells treated with complex **27**. The results of this experiment correlate with the data on phototoxicity; the complexes showing significant phototoxicity (PI > 22-100) demonstrated the photoinduced increase of intracellular ROS, whereas non-toxic **27** was unable to modulate ROS level after irradiation. The superior effect of **29** might be connected with its high cellular accumulation and its good performance as a photocatalyst agent of NADH.

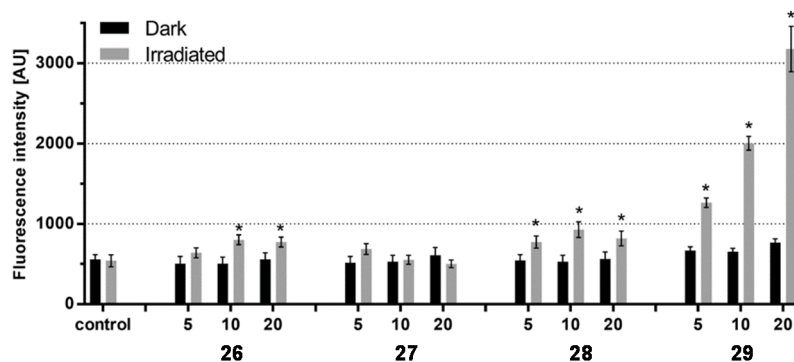


Figure 4.7. ROS generation in A375 cells induced by increasing concentrations (5, 10, 20 μM) of **26**-**29**. Data represent means $\pm$ SD of two measurements. Stars (*) at the top of the bars show a significant difference from the control (irradiated) with $p \leq 0.01$. Statistical significance was calculated using the Students' t-test.

The results described above clearly show that **29** possesses the best biological properties: the highest PI, cellular uptake, and photoinduced ROS generation. Moreover, **29** showed the strongest luminescence in cell-free media (the highest quantum yield), which, together with the high uptake, enables its localization in cells by confocal microscopy. Based on that, complex **29** was selected for further experiments focused on a detailed mechanism of its biological action.

4.2.9. Cellular localization and distribution

Intracellular localization of complex **29** were studied to understand the mechanism underlying the photoactivity of this compound. For this purpose, confocal microscopy was employed, as **29** favorably exhibits a high emission at $\lambda_{\text{exc}} = 405 \text{ nm}$.

As shown in **Figure 4.8**, the luminescence signal from **29** was localized mainly out of the cell nucleus, with most of the signal associated with the cytoplasm (Panels 1A and 2A). To find out the intracellular target organelle, a co-localization study was also performed. The phosphorescence signal coming from **29** was compared to those from Mito-Tracker (panels 1A-C) or ER-Tracker (panels 2A-C). As indicated, the signal from **29** matched the fluorescence coming from Mito-Tracker marked organelles better than that coming from ER-Tracker. Pearson's correlation coefficients (PCCs) were found to be 0.63 ± 0.10 and 0.35 ± 0.09 for mitochondria and ER, respectively. The calculated PCCs confirmed a preferential accumulation of **29** in mitochondria. However, a positive value of PCC calculated for co-localization with the signal coming from the endoplasmic reticulum suggests that **29** can be partially associated also with ER.

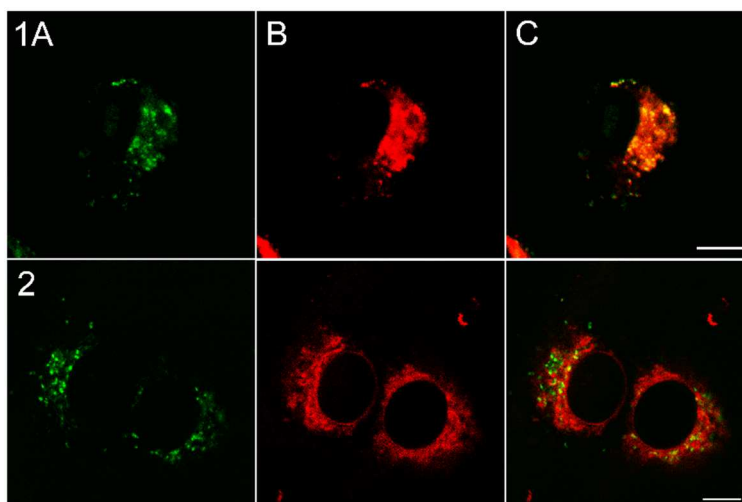


Figure 4.8. Co-localization of **29** in A375 melanoma cells. Cells were treated with **29** (5 μ M) and co-stained with Mito-TrackerTM (panels 1) or ER-TrackerTM (panels 2). Channels: (1A, 2A) phosphorescence coming from **29**, (1B) Mito-Tracker fluorescence, (2B) ER-tracker fluorescence, (1C, 2C) overlay of the channels. Scale bars represent 10 μ m.

4.2.10. Induction of immunogenic cell death in melanoma cells

Some conventional antitumor drugs (including oxaliplatin or doxorubicin) can also induce ICD, enhancing their anti-cancer efficiency. As commented before, it has been shown that ICD-associated immunogenicity can be evoked through ROS, and it is frequently associated with ER stress.⁴⁸ Recently, mitochondria have emerged as organelles with key roles in the immune system. It is hypothesized that the alteration of mitochondria in cancer cells could be an important target for developing an efficient ICD inducer for use in cancer immunotherapy.⁴⁹ Thus, it was reasonable to assume that the photoactivated complex **29**, which generates ROS and accumulates in mitochondria and partially also in ER, could induce ICD. Therefore, biochemical hallmarks such as active secretion of ATP, extracellular secretion of nuclear HMGB1, translocation of calreticulin from ER, and immunogenic phagocytosis, were studied to prove whether **29** induces ICD *in vitro* under light irradiation.

a. Analysis of the extracellular release of ATP and HMGB1

Extracellular ATP and HMGB1 released from A375 melanoma cells were detected by using ATP bioluminescence assay kit CLSII and a colorimetric HMGB1 ELISA kit respectively after the cells were treated with various concentrations of **29** (5, 10, and 20 μ M) for 1 h in the dark and subsequently irradiated (420 nm, 7.7 ± 3 mW/m²). As for the positive controls, doxorubicin and oxaliplatin were also included in the experiment.

The results demonstrated the ability of **29** to provoke ATP (**Figure 4.9A**) and HMGB1 (**Figure 4.9B**) secretion after the cells were irradiated. This effect was concentration-dependent. In contrast, a very low extracellular release of nuclear HMGB1 and ATP (insignificantly different from the untreated cells) was detected if the melanoma cells were treated with **29** in the dark.

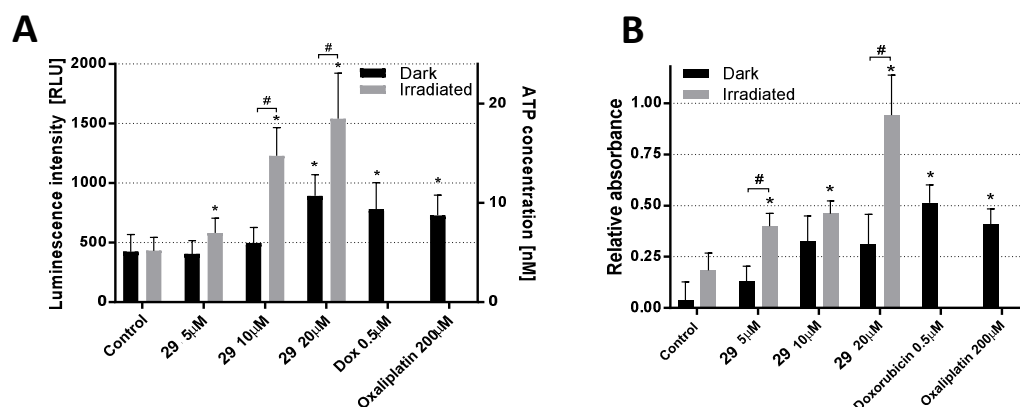


Figure 4.9. Extracellular A) ATP or B) HMGB1 levels in A375 cells. Cells were treated with **29** and positive control ICD agents: doxorubicin and oxaliplatin at the concentrations indicated in the figure. The drug treatment period was 2 h (1 h of incubation in the dark followed by 1 h irradiation with 420 nm or kept in the dark) with 22 h of recovery time in a drug-free medium. Symbols at the top of the bars (*, #) denote a significant difference from untreated control (*) or between irradiated and non-irradiated samples (#). Statistical significance was calculated by using the Students't-test with the $p \leq 0.01$. Three independent experiments were done with quadruplicate for each sample set.

b. Detection of calreticulin (CRT) exposure

The effect of **29** on the exposure of calreticulin in melanoma cells was analyzed by using flow cytometry after the cells were stained with anti-CRT-alexa fluor 488 conjugate. Before analysis, the cells were co-stained with propidium iodide. Propidium iodide-negative cells were gated out to eliminate possible false-positive results due to the non-specific accumulation of anti-CRT antibodies through the compromised cell membranes of dying cells. Single cells (1×10^4 cells) were analyzed and plotted on the histogram with a bi-exponential scale (**Figure 4.10** and **S4.20**, **Table S4.3**).

Well-known ICD inducers doxorubicin and oxaliplatin (both used as a positive control) caused a relatively extensive induction of CRT exposure. Similarly, the amount of ecto-CRT (calreticulin translocated from ER to the outer surface of the cell membrane) observed on the surface of cells treated with **29** increased significantly in a concentration-dependent manner when the cells were irradiated. However, no effect on CRT exposure was observed for cells treated with **29** in the dark.

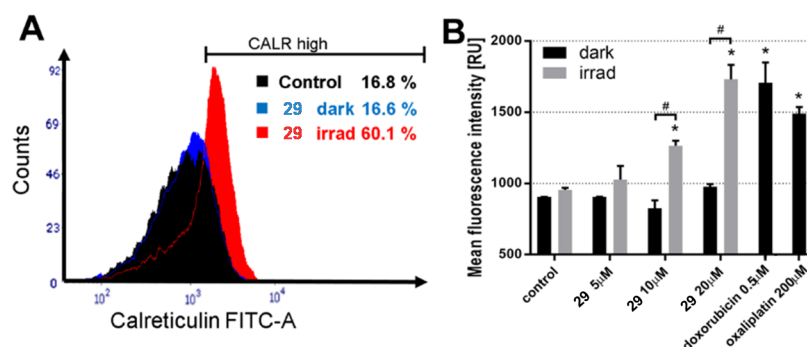


Figure 4.10. Flow cytometric analysis of ecto-CRT exposure after the treatment of A375 cells with tested compounds. A) Representative histograms of cell samples treated with 20 μ M **29** in dark or under irradiation. B) Analysis of samples for overall mean fluorescence. Stars (*) at the top of the bars indicate a statistically significant difference from untreated control ($p \leq 0.05$) or (#) the statistically significant difference between irradiated and non-irradiated samples ($p \leq 0.05$). The bars represent the mean $\pm$ SD from two independent experiments.

To confirm data obtained by flow cytometry, CRT exposure was also pictured by confocal microscopy. The cells treated with **29** in the dark or under irradiation were fixed with 4% formaldehyde and stained with anti-CRT-alexa fluor 488 conjugate. Samples were visualized on a confocal microscope, and the representative images are shown in **Figure 4.11** and **S4.21**. As indicated, the fluorescence signal of anti-CRT-alexa fluor 488 antibodies is observed on the cell membrane of the cells treated with **29**, but only after the cells were irradiated (**Figure 4.11**, bottom panels). Qualitatively identical results were obtained when the cells were treated with oxaliplatin or doxorubicin in the dark (**Figure S4.21**). In contrast, the cells treated with **29** in the dark yielded no fluorescence signal indicating a lack of CRT exposure (**Figure 4.11**, top panels), similarly to untreated control cells (**Figure S4.21**). Thus, results of the confocal microscopy experiments are consistent with those found by flow cytometric analysis (**Figure 4.10**), demonstrating that **29** encourages exposure of CRT to the cell surface only when the complex is photoactivated.

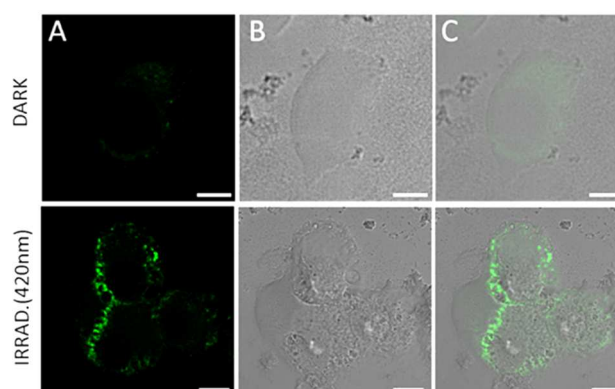


Figure 4.11. Immunofluorescence analysis of ecto-CRT exposure by confocal microscopy. A375 cells were treated with **29** (20 μ M) for 1 h pre-incubation, irradiated with blue light (bottom panels) or in the dark (top panels) followed by 22 h of recovery in a cell-free medium. Channels: A) Fluorescence signal from anti-CRT-Alexa fluor 488 conjugate. B) Bright field. C) Merge of the fluorescence and bright field channels. Scale bars represent 10 μ m.

c. *In vitro* phagocytosis assay

Another important step of ICD is an engulfment of dying cancer cells by macrophages that facilitates immuno-stimulatory clearance of cancer cell corpses.⁵⁰ A stimulation of immunogenic phagocytosis is one of the most important biochemical hallmarks of ICD.⁵¹ For these purposes, the *in vitro* phagocytosis assay was applied to identify possible ICD inducers.^{52,53} Therefore, we employ this assay to evaluate the ability of **29** to provoke phagocytosis of cancer cells by macrophages.

Briefly, murine cancer cells CT26 (treated with **29**, doxorubicin, or untreated) were co-incubated with J774.A1 murine macrophages for 2.5 h. To distinguish these two cell populations and facilitate detection, cancer cells were pre-stained green, whereas the macrophages were stained red. The samples were then analyzed by flow cytometry for the red and green fluorescence. The cells showing signals in both channels (double-stained) indicated phagocytosis. Representative flow cytometric densitograms for cells treated with **29** (10 μ M) in the dark or under irradiation are shown in **Figure 4.12A**. The data were quantitatively evaluated, and the percentage of phagocytosis was calculated as the number of double-stained macrophages to the total number of macrophages x100.

The results show that irradiation of cells treated with **29** significantly potentiated phagocytoses of tumor cells. In contrast, cells treated with **29** in the dark or non-treated control were phagocytized very reluctantly: 4.3 ± 1.6 or 12.6 ± 3.8 % for untreated control or **29** in the dark, respectively, vs. $32.5 \pm 2.5\%$ for irradiated **29** (see also **Figure S4.22**). Interestingly, the level of phagocytosis induced by 10 μ M **29** after irradiation was almost double that induced by the same concentration of doxorubicin, a well-known inducer of ICD and phagocytosis (**Figure 4.12B** and **Figure S4.22**).⁵⁴ Oxaliplatin, although capable of the effective induction of ICD,¹⁰ was unable to promote phagocytosis in agreement with previously published data.⁵³ Thus, the results suggest that **29**, when irradiated, stimulates phagocytosis with high efficiency, in contrast to clinically used Pt-based drugs.

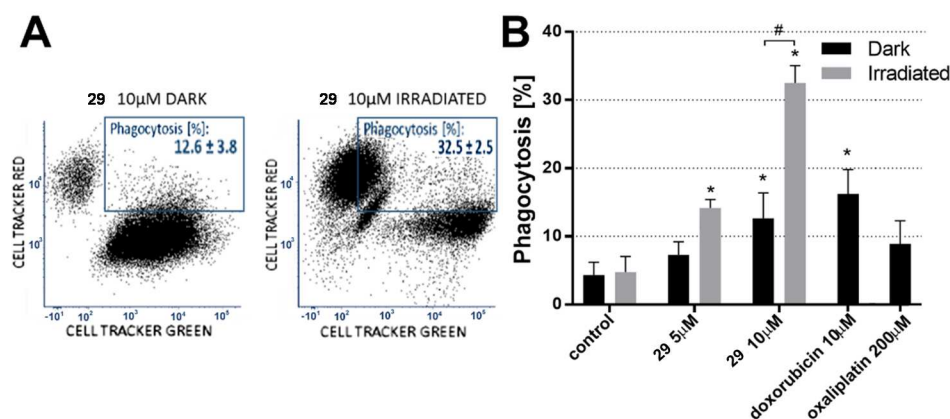


Figure 4.12. Phagocytosis assay. A) Representative flow cytometry density plots showing the phagocytosis after the treatment with **29** (10 μ M) in the dark or upon irradiation. B) Quantitative analysis of phagocytosis by flow cytometry. Symbols at the top of the bars (*, #) denote a significant difference from untreated control (*) or between irradiated and non-irradiated samples (#). Statistical significance was calculated using the Students' t-test with the $p \leq 0.01$. The experiment was performed in duplicate.

4.2.11. Activity against cancer stem cells

In an assortment of tumors, including melanoma, a small subset of stem-like cells (cancer stem cells, CSCs) was identified.⁵⁵ Although there is still no consensus on the CSCs classification criteria,⁵⁵ the expression of cell surface markers is commonly used to isolate and enrich CSCs from different tumors. This expression of CSC surface markers is tissue type-specific and in melanoma, stem cell-like subpopulation expressing CD20 marker has been described.⁵⁶

Therefore, CD20 expressing melanoma cells (A375CD20+) was tested to study the ability of **29** to inhibit the growth of these CSCs-like melanoma cells. The sensitivity of A375CD20+ cells was compared with CD20 non-expressing (A375CD20-). Additionally, we tested the effect of **29** in 3D cell culture, which mimics the tumor microenvironment, including intercellular interactions, oxygen gradient, and drug penetration more closely than conventional 2D cell cultures.^{57,58} A CSCs-selective agent abamectin was included in the experiment as a control.⁵⁹

The A375CD20+ and A375CD20- cells were cultured as single-cell suspensions in serum-free media to form melanospheres. 96 h old spheroids were then treated with **29** for 1 h in the dark and subsequently irradiated (420 nm, 1 h) or kept in the dark. After that, the drug-containing medium was removed, and melanospheres were incubated for a further 72 h for recovery. The viability of cells was then tested by using CellTiter-Glo® 3D Cell Viability Assay.

Complex **29** was found to be selective toward CD20+ cells under light irradiation (**Table 4.8**). Interestingly, **29** was approximately as effective as abamectin in the dark, with the advantage that selectivity of **29** (IC_{50} A375CD20- / IC_{50} A375CD20+ ratio) was markedly higher.

Table 4.8. IC_{50} values^a (μ M) for the tested compounds in human melanospheres determined using CellTiter-Glo® 3D Cell Viability Assay.

A375 sorted	CD20+		CD20-	
	Dark	Light	Dark	Light
29	>20	7.4 ± 1.1	>20	>20
Abamectin	6.5 ± 0.7	8.0 ± 0.5	11.4 ± 0.3	11.0

^a IC_{50} values were determined on 96-h old spheroids treated with the investigated compounds for 1 h followed by irradiation/dark for 1 h. After the treatment, the spheroids were washed out and cultured in a drug-free medium for 72 h. The data represent the mean $\pm$ SD from 3 measurements.

Late investigations have discovered that, in contrast to bulk breast cancer cells in which ROS levels are elevated due to their higher metabolism rate, CSCs possess low levels of ROS.⁶⁰ The adaptation of CSCs to survive in ROS-deficient conditions implies high sensitivity of CSCs towards ROS elevation by exogenous chemicals.⁶¹ Our data (**Figure 4.7**) revealed that **29**, after irradiation, markedly increases the level of ROS in melanoma cells. Therefore, the CSC-specificity observed for **29** could arise from its ability to activate the generation of this reactive oxygen species by blue light.

To verify this hypothesis, the penetration and distribution of complex **29** in A375 spheroids formed from both unsorted and CD20+ or CD20- sorted cells have been tested using confocal microscopy. As indicated in **Figure 4.13** and **Figure 4.14**, the short-time (2 h) incubation with **29** resulted in surface-distribution of complex **29**, while an effective penetration of **29** into spheroids was observed after a longer incubation. After 24 h, the luminescence signal of **29** was apparent in cells localized near the spheroid centrum. It is also obvious that the penetration is comparable for both CD20+ and CD20- cell spheroids. Thus, the selectivity of **29** for CSC-like CD20+ cells (**Table 4.8**) cannot be attributed to the increased or selective uptake. Thus, these results further support the view that the ability of **29** to generate intracellular ROS after irradiation could, at least partly, explain its CSC-selectivity.

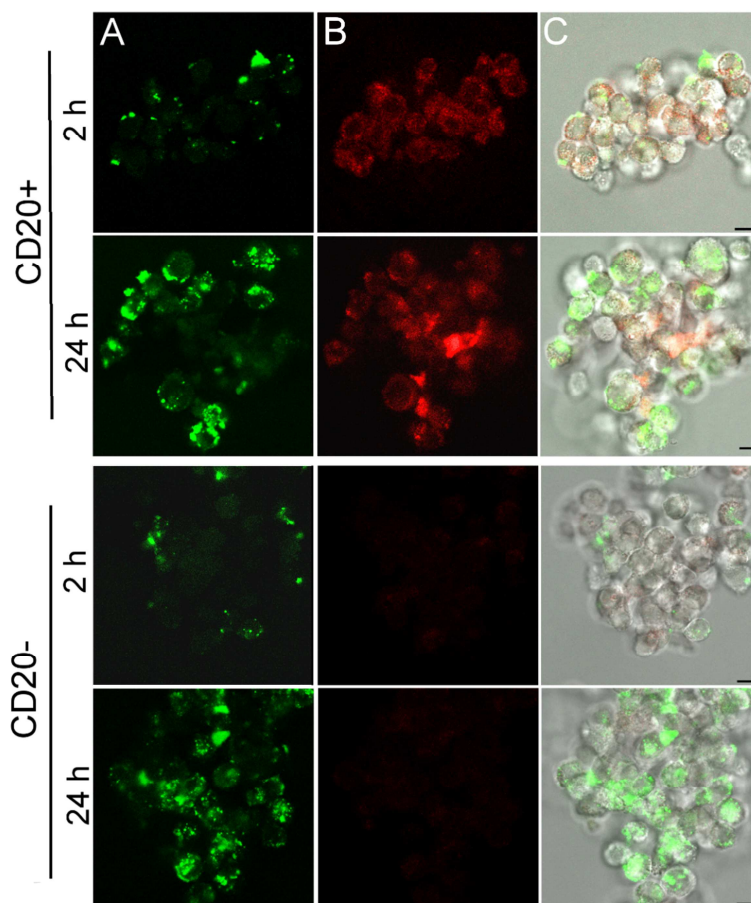


Figure 4.13. Penetration of **29** into 3D spheroids generated from A375CD20+ and A375CD20- cells. Cell spheroids (diameter: 40 – 60 μm) were treated with **29** (10 μM) and incubated for 2 h or 24 h in the dark. Spheroids were subsequently fixed and stained with APC anti-human CD20 antibody. Channels: Panels A, phosphorescence from **29**. Panels B, CD20 antibody. Panels C, overlay of the luminescence channels and bright field. Scale bar represents 10 μm .

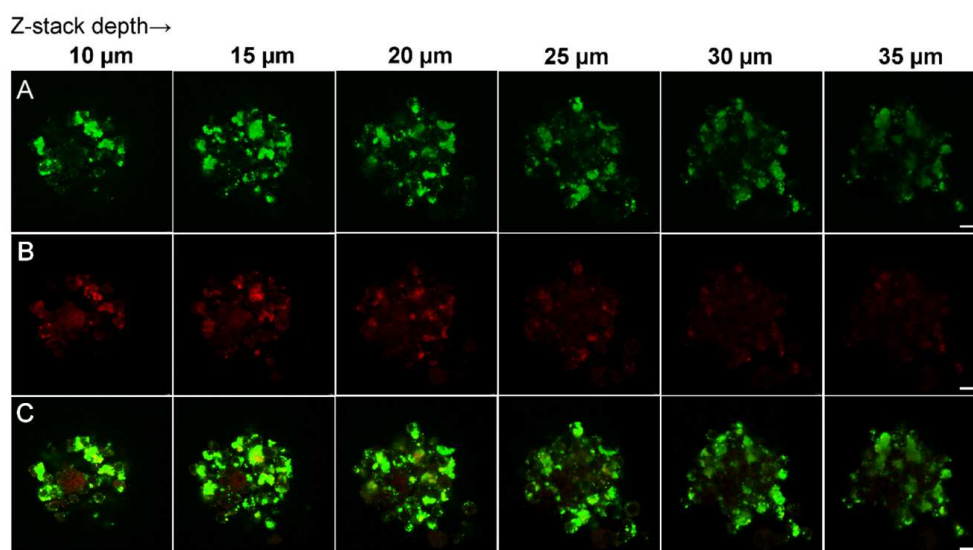


Figure 4.14. Penetration of **29** to 3D spheroid generated from unsorted A375 cells. Spheroids (diameter: 80 μm) were treated with **29** (10 μM) and incubated in the dark for 24 h. After washing, cells were co-stained with APC anti-human CD20 antibody. Channels: Panels A, phosphorescence from **29**. Panels B, CD20 antibody. Panels C, overlay of the luminescence channels. Confocal z-stacks were acquired in defined steps (10-35 μm). Scale bar represents 20 μm .

4.3. CONCLUSIONS

A rational design and efficient synthesis of four phosphorescent and photoactivated Ir(III) complexes is described. The choice of the auxiliary ligands was based on a common 1H-benzo[d]imidazole backbone containing one or two NH groups in their structure, which usually provide low cytotoxicity to their correspondent complexes in the dark according to literature, a crucial condition to be met for the design of ideal photosensitizers. In a cell-free medium, **26-29** were able to photo-catalytically oxidize NADH under blue light irradiation, with a very high TOF. The new complexes exhibited very low toxicity in the dark even at high concentrations (300 μ M) against human skin melanoma cells (A375 and 518.A2) and human cervix adenocarcinoma cells (HeLa). They showed high phototoxicity by blue light irradiation under normoxic and hypoxic conditions, being **29** the most active with the highest phototoxicity indexes (46-100). This complex also displayed the highest accumulation in cancer cells and the most elevated photoinduced ROS generation. On the other hand, **29** showed preferential localization in the mitochondria (PCCs = 0.63), although the co-localization study with ER-Tracker suggests that this complex can also be partially associated with this organelle.

In addition, the data introduced in this chapter indicate that **29** induces immunogenic cell death in melanoma to the same extent as known ICD inducers (oxaliplatin and doxorubicin), but only if the cells are irradiated by light. On the other hand, **29** showed a potent ability to inhibit the growth of CSCs-like melanoma cells after light irradiation, being approximately as effective as the CSCs-specific drug abamectin in the dark, but with a selectivity markedly higher toward the CSCs.

As far as we know, **29** is the first iridium complex to induce ICD in melanoma cells and target selectively CSCs by blue light irradiation. Therefore, these results represent a significant scientific advance in the field of cancer chemotherapeutic agents with an alternative mechanism of action, developing promising CSCs-directed candidates for immunostimulation phototherapy, which could suppress side effects with a selective activation with light and fights against acquired resistance and metastasis of cancers such as melanoma.

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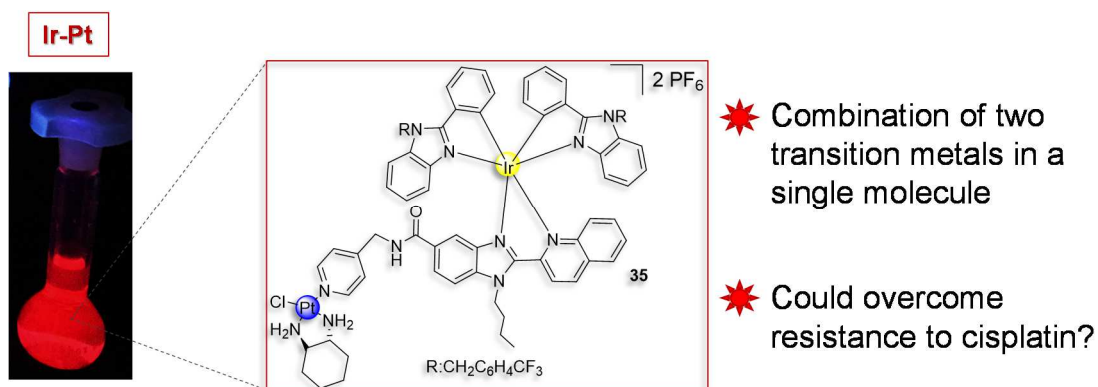
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Chapter 5

Synthesis and characterization of a new heterobimetallic Ir(III)-Pt(II) complex that overcomes cisplatin resistance



The biological experiments included in this chapter were carried out by Enrique Ortega and Pezhman Ashooh (Departamento de Química Inorgánica, Universidad de Murcia).

5.1. INTRODUCTION

The discovery of the biological activity of cisplatin and its analogs marked the beginning of developing antitumor drugs based on metals. As commented before, cisplatin is still one of the most extensively used drugs today, but its efficacy is limited by its low selectivity for cancer cells and acquired or intrinsic resistance of tumor cells to the drug. Cisplatin resistance mechanisms^{1,2} can be divided into three stages: (1) the pre-target stage, based on cisplatin accumulation inside the cell, where the intracellular content of the drug is reduced by decreasing of compound uptake and increasing efflux, (2) the on-target stage, based upon the inhibition of cisplatin-DNA adducts, triggering by DNA repair mechanisms,³ (3) the post-target stage, involving the cell death or apoptosis by DNA damage, which is controlled by some anti-apoptotic proteins. Different approaches to overcoming this resistance have been studied: nanomaterials for the drug delivery,³ Pt(IV) compounds,⁴ and hetero-nuclear complexes.⁵⁻⁷

The combination of two or more transition metals by coordination chemistry can improve the antitumor potency of metallodrugs. In addition, these heterobimetallic or multi-metallic complexes may induce additive or even synergistic effects using two or more metals in a single molecule.⁷⁻¹⁰ In contrast to the investigation of mononuclear anti-cancer complexes, dinuclear metal compounds have been less well studied. Nevertheless, these dinuclear species can show extraordinary properties and capacities due to tuning the bridging ligands, the two metal centers, or the cyclometalating ligands.¹¹ The development of a new dinuclear complex can be complicated at times, posing the following challenges: low production yields due to complicated synthetic processes,^{12,13} poor optical properties with low luminescence quantum yields produced by the considerable contribution of non-radiative excited states from the bridging ligands,^{14,15} relative large molecular weights which can decrease their solubility in biological media, etc. Nonetheless, significant progress has been made with dinuclear complexes in biological applications in the last years.¹⁶⁻¹⁹

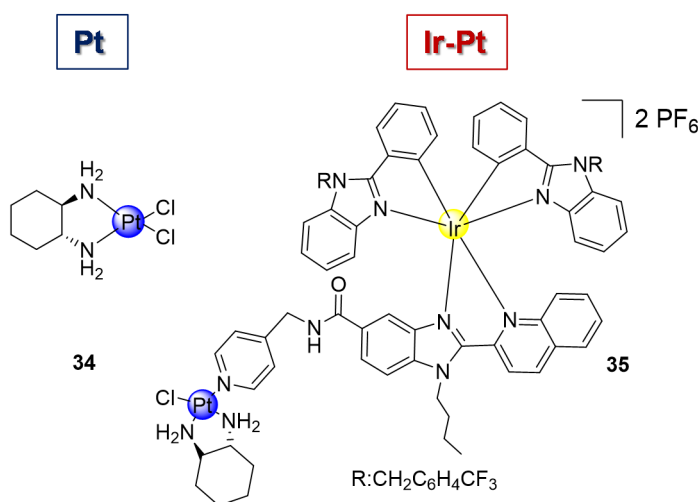
Given the importance of cellular tracking for developing new and more effective metal drugs, proper selection of different metallic fragments would make it possible to increase the therapeutic activity with a synergistic effect and incorporate key features like traceability.²⁰ Cyclometalated Ir(III) compounds have been demonstrated to be excellent candidates for their use as emissive tags of therapeutic drugs.^{21,22} Thus, inspired by the need to overcome the disadvantages of current platinum drugs and in the excellent photophysical

properties of bis-cyclometalated Ir(III) complexes, we design and synthesis the new dinuclear Ir(III)-Pt(II) complex **35** (**Scheme 5.1**) of general formula $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{N}^{\wedge}\text{N})(\mu\text{-NHCH}_2\text{py})\text{Pt}(\text{DACH})\text{Cl}]^{2+}$ (where $\text{C}^{\wedge}\text{N}$ = 2-phenyl-1-[4-(trifluoromethyl)benzyl]-1H-benzo[d]imidazol-kN,C; $\text{N}^{\wedge}\text{N}$ = an in-house ligand²³ modified with 4-(aminomethyl)pyridine; DACH = 1R, 2R)-(-)-diaminocyclohexane) to investigate its potential as an anti-cancer drug. This heterobimetallic complex showed an intense red phosphorescent in water granted by the Ir(III) moiety. The antiproliferative study revealed the ability of **35** to kill cisplatin-resistant cervical and ovarian cancer cells. In addition, the dinuclear complex exhibited less toxicity than cisplatin towards healthy kidney cells. Further studies on cell death induction revealed that, in contrast to cisplatin, which induced apoptosis in A2780 cells, **35** was causing necrosis rather than apoptosis after 24 h.

5.2. RESULTS AND DISCUSSION

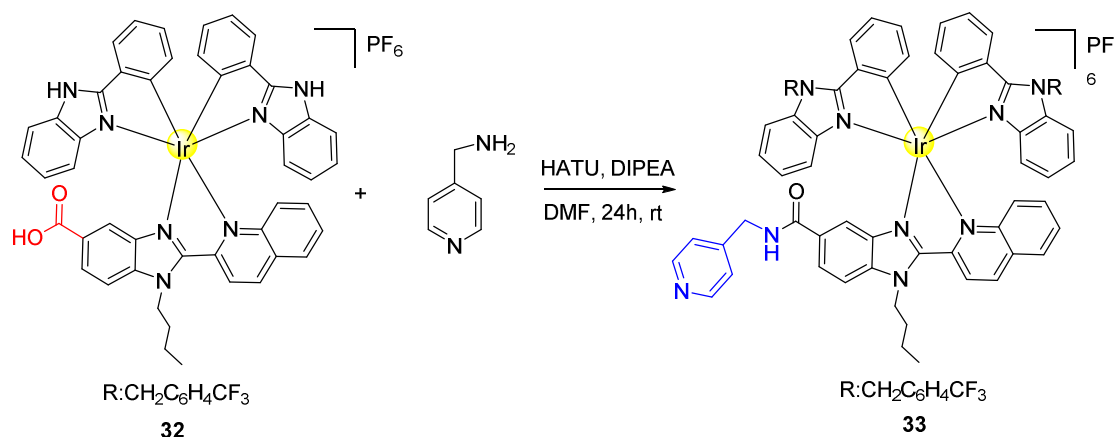
5.2.1. Synthesis and characterization

A novel red luminescent heterobimetallic Ir(III)-Pt(II) complex **35** (**Scheme 5.1**) was designed and synthesized. The platinum complex **34** was used in this study with comparative purposes.



Scheme 5.1. Structures of heterobimetallic Ir(III)-Pt(II) complex **35** and the platinum complex **34** used with comparative purposes.

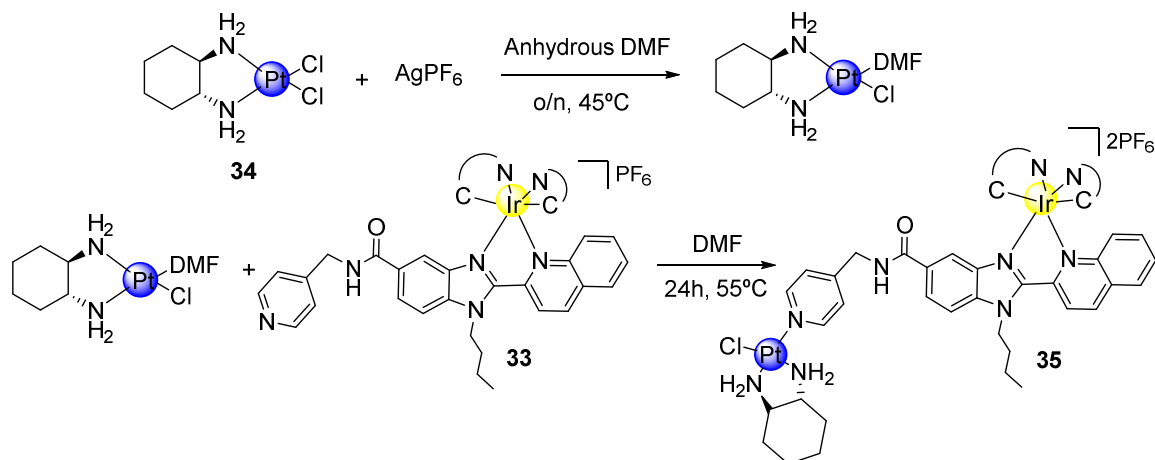
First, the Ir(III) complex **32** (**Scheme 5.2**) was obtained with an acid group in the $\text{N}^{\wedge}\text{N}$ ligand to synthesize the Ir complex **33** functionalized with 4-(aminomethyl)pyridine, a linker which was used for binding to Pt(II) complex. This 4-(aminomethyl)pyridine-based linker has already been used successfully in the synthesis of other dinuclear complexes.^{24,25}



Scheme 5.2. Synthesis of Ir(III) complex **33** with the 4-(aminomethyl)pyridine-based linker.

As a Pt(II) complex, cisplatin was used first, but the synthesis with **33** resulted in a mixture of two major compounds (**Figure 5.1A**): the desired one and the other resulting from the substitution of an ammonia ligand for the pyridine. That fact was observed by ESI-MS (**Figure 5.1B**) and thin-layer chromatography (TLC). However, the compounds were tough to separate by column chromatography due to their similar R_f . Therefore, we decided to choose the Pt(II) complex **34** (**Scheme 5.1**), which possesses the chelated diamine ligand (1R, 2R)-(-)-diaminocyclohexane (DACH). This diamine is used to synthesize oxaliplatin and correlates with a lack of cross-resistance of oxaliplatin with cisplatin.²⁶

The dinuclear complex **35** was obtained in a good yield (72%) using a modification of a method of literature²⁷ (**Scheme 5.3**). First, by reaction of the platinum complex **34** and AgPF_6 in anhydrous DMF overnight at 45 °C, and then adding a solution of **33** in DMF dropwise over the previous platinum complex solution and stirring for 24 h in the dark at 55 °C.



Scheme 5.3. Synthesis of heterodinuclear complex **35**.

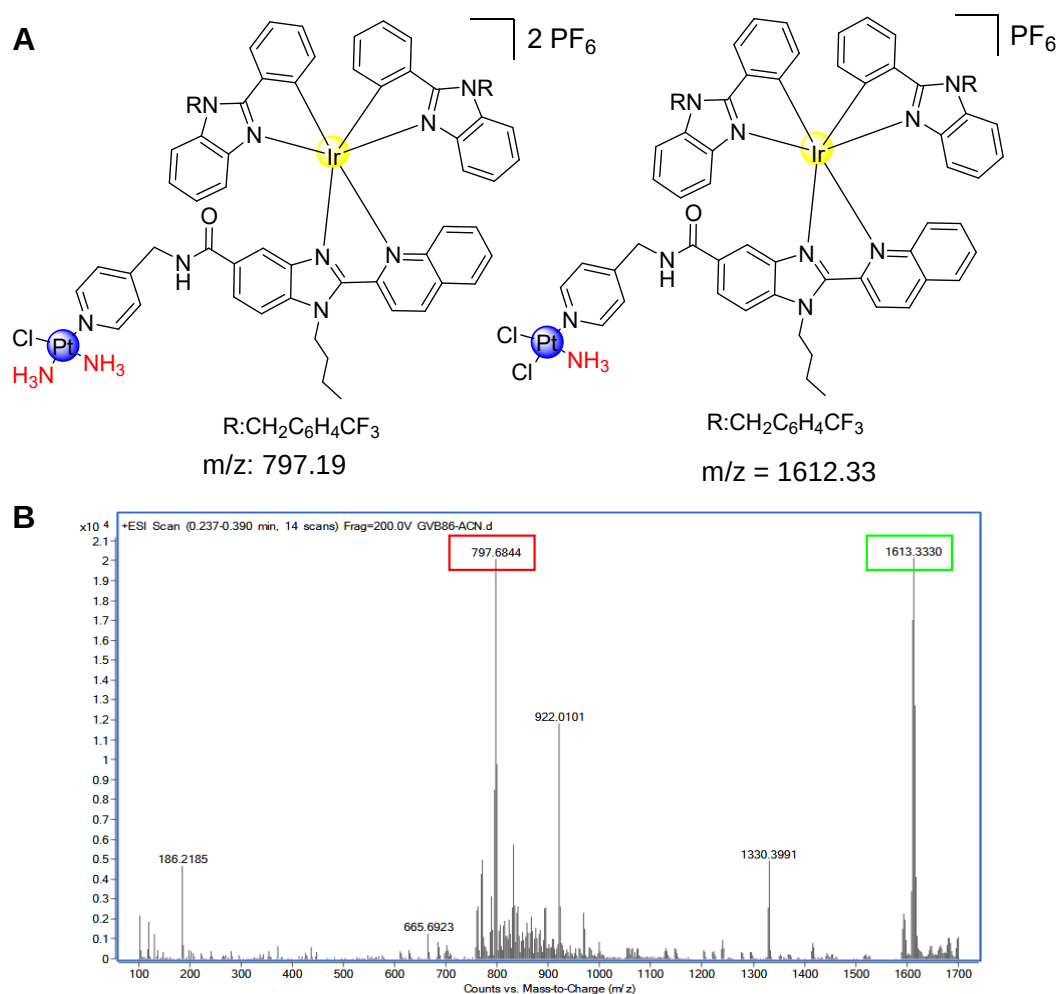


Figure 5.1. A) Major products in the reaction of **33** with cisplatin. B) MS spectra of the mixture.

All complexes were characterized by ¹H and ¹³C NMR spectroscopy (**Figures S5.2-S5.8**), positive-ion HR ESI-MS (**Figure S5.9-S5.11**), and elemental analysis. The ¹H-NMR spectrum of **33** exhibits new signals between 9-8 ppm, a singlet around 6.6 ppm and a multiplet around 4 ppm that confirm the functionalization of complex **32** with 4-(aminomethyl)pyridine (**Figures S5.12**).

The formation of the new heterodinuclear complex **35** was checked by NMR (**Figures S5.6-S5.8**) and confirmed with ESI-MS, where the MS spectra clearly showed the molecular peaks corresponding to m/2 ratio (**Figure 5.2**).

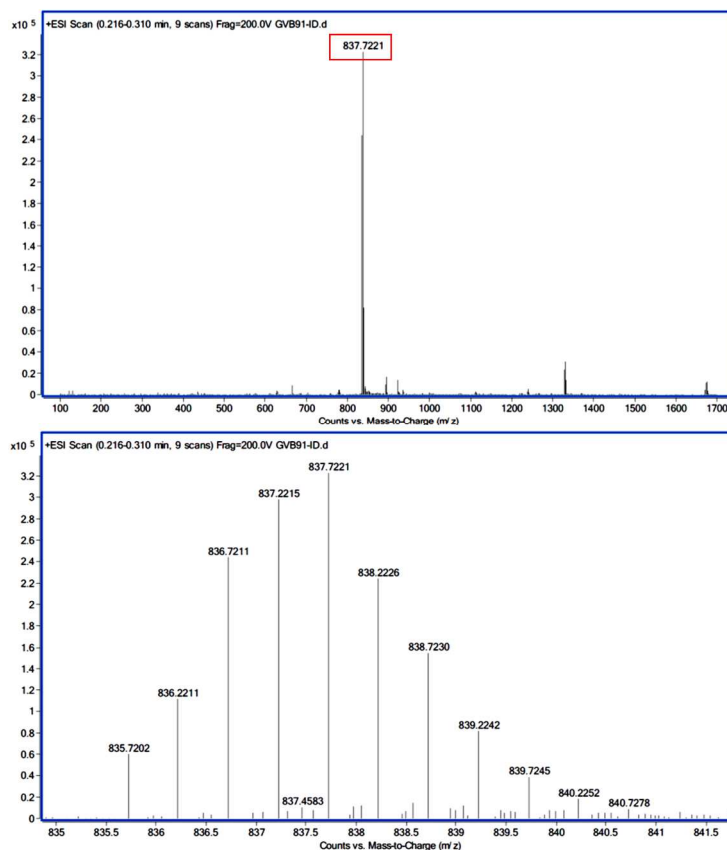


Figure 5.2. MS spectra of Ir-Pt complex **35** in DMF.

5.2.2. Stability studies

The free chlorine in the molecule caused us to study the solution stability of the dinuclear complex previous to the luminescence and biological studies.

As **35** is not soluble in water, its stability was tested first in DMSO by ^1H NMR spectroscopy at room temperature. As observed in **Figure S5.13**, after 24 hours, new signals appear on the spectrum.

Then, the stability of **35** was studied in DMF, which is another solvent compatible with the biological medium in small percentages. No changes were detected in the NMR spectrum after 72h (**Figure 5.3**). The Ir-Pt complex was also stable for at least 24 hours in water containing 1% DMF at room temperature, as indicated by the unchanged MS spectra of the compound (**Figure 5.4** and **Figure S5.11**). Therefore, subsequent studies were carried out at DMF and DMF:water mixtures.

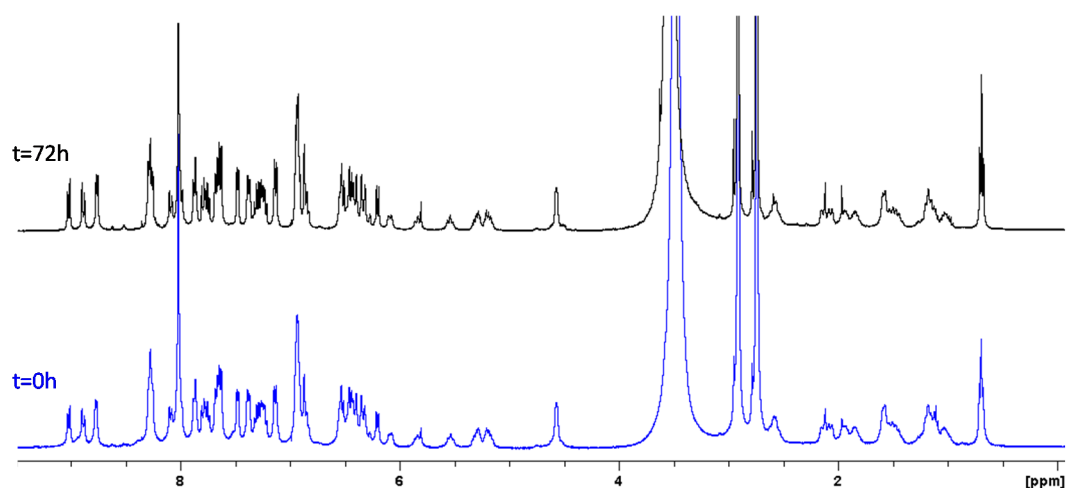


Figure 5.3. ^1H NMR spectra of **35** measured after dissolving immediately in $[\text{D}_7]\text{DMF}$ (bottom) and after 72 hours (top) at RT.

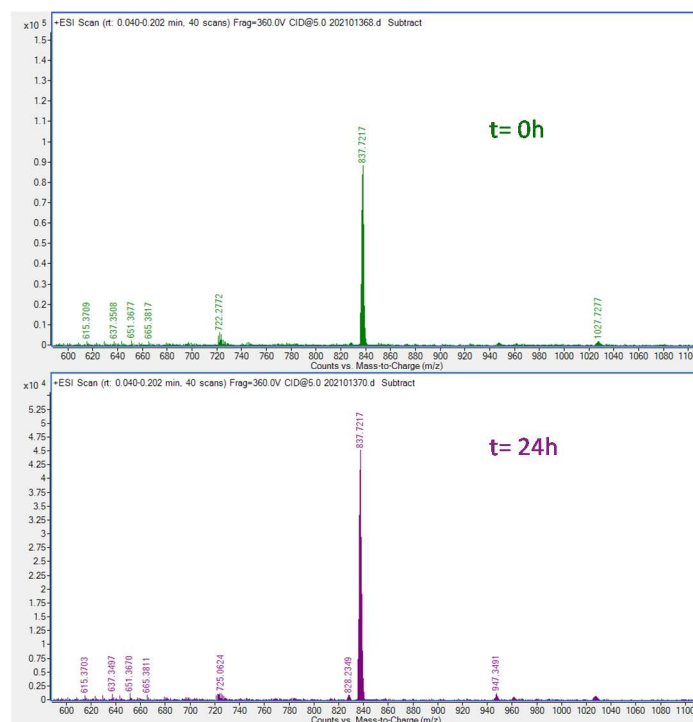


Figure 5.4. MS spectra of Ir-Pt complex **35** at $t=0$ h and 24 h in water (1% DMF).

Physiological pH values (pH 5.0–8.0) did not substantially affect the stability of the heterodinuclear complex either (**Figure 5.5**). The stability was also studied in water using different chloride concentrations: 0 mM, 4 mM (intracellular) and 100 mM Cl^- (bloodstream). As observed in **Figure 5.6**, the UV/Vis spectrum of heterodinuclear complex **35** remains the same at 0, 1, 5 and 24 h, confirming its stability against hydrolysis.

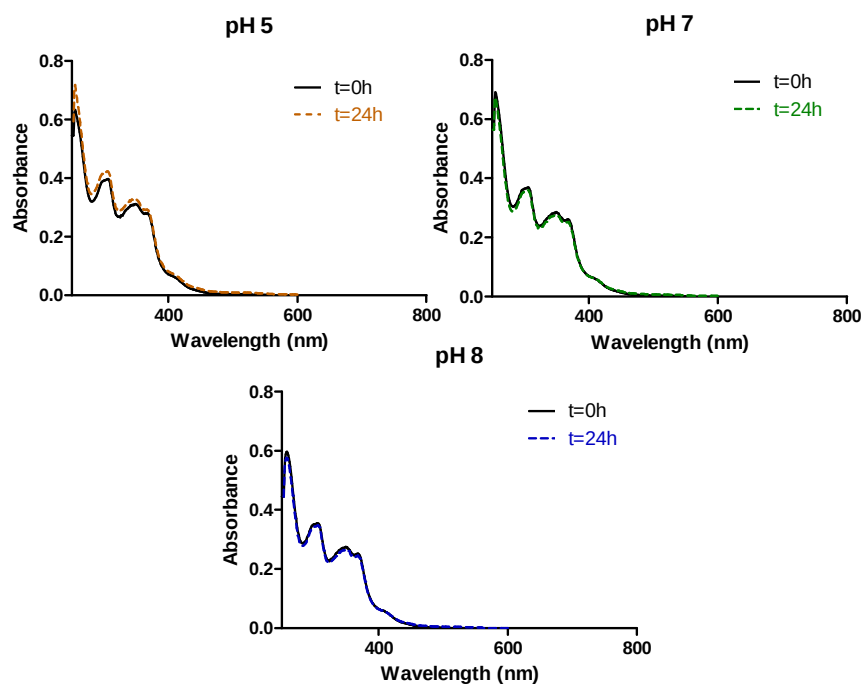


Figure 5.5. UV/Vis absorption spectra of 35 (10 μ M, water/DMF, 50:50) measured at 0 h and 24 hours in aqueous solution at different pH values. The pH was adjusted with 0.1M NaOH and HCl (10%) solutions.

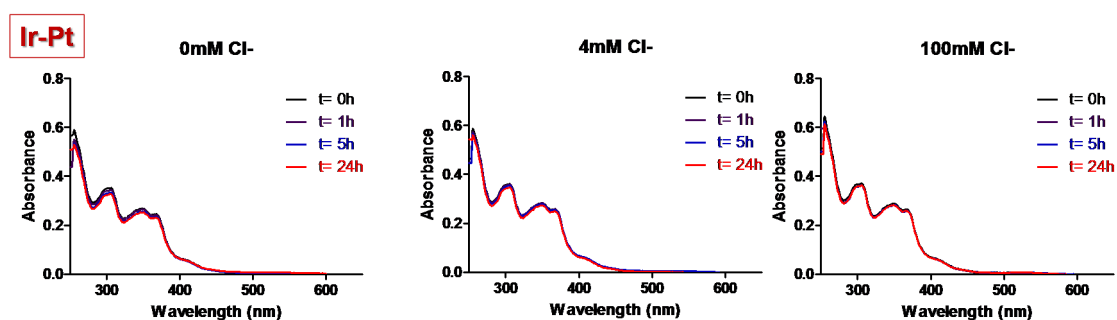


Figure 5.6. UV/Vis spectra of complex 35 in water:DMF (50:50, v:v) with different chloride concentrations (0, 4 and 100 mM) at 0, 1, 5 and 24 h.

Finally, the stability in RPMI culture medium was verified by UV/Vis, not observing changes after 48h (**Figure 5.7**).

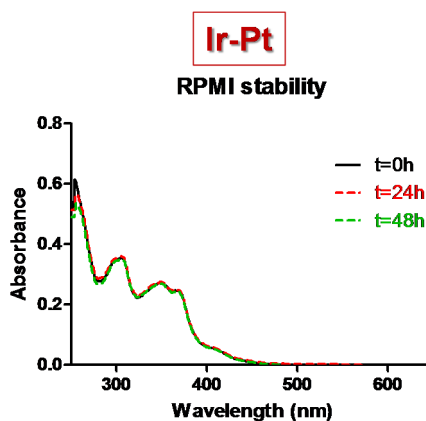


Figure 5.7. UV/Vis spectra of complex 35 (Ir-Pt) in RPMI:DMF (50:50, v:v) at 0, 24 and 48 h.

5.2.3. Photophysical properties

The photophysical properties of Ir-Pt complex **35** were investigated in DMF and water (1% DMF) solution (**Table 5.1**). The UV/Vis absorption spectra (**Figure 5.8A**) showed the typical absorption bands of cyclometalated Ir(III) complexes. Thus, intense bands between 230 and 350 nm can be assigned to ligand-centered (LC) transitions within the C[^]N and N[^]N ligands of Ir(III) moiety. Bands at $\lambda > 350$ nm as well as the bands in the visible range (> 400 nm) could be attributed to spin-allowed metal to ligand (¹MLCT), spin forbidden singlet-to-triplet (³MLCT) charge-transfer and ligand-to-ligand charge-transfer (^{1,3}LLCT) transitions.²⁸ Upon excitation at λ_{exc} maxima, the heterodinuclear complex exhibits an intense red (632-662 nm) phosphorescence emission in both solvents (**Figure 5.8B**). The luminescence quantum yields were found to be similar ($\Phi_{\text{em,DMF}} = 12.1\%$, $\Phi_{\text{em,water}} = 8.8\%$). The complex was found to have excited state lifetimes in the nanosecond range (**Table 5.1**, **Figure S5.14**) which is in the same range as other published phosphorescent Ir(III) complexes.^{29–31}

Table 5.1. Excitation (λ_{exc}), emission (λ_{em}) wavelengths, emission lifetimes (τ_{em}), and quantum yields (Φ_{em}) of **35** in DMF and water.

Compound	Solvent	λ_{exc} , nm ^a	λ_{em} , nm	τ_{em} , ns ^b	Φ_{em} , % ^b
Ir-Pt complex 35	DMF	370	662	558 (100%)	12.1
	Water ^c	368	632	70 (84%)	8.8
				25 (16%)	

^a λ_{exc} maxima. ^b Degassed by bubbling 20 min under argon. ^c 1% DMF

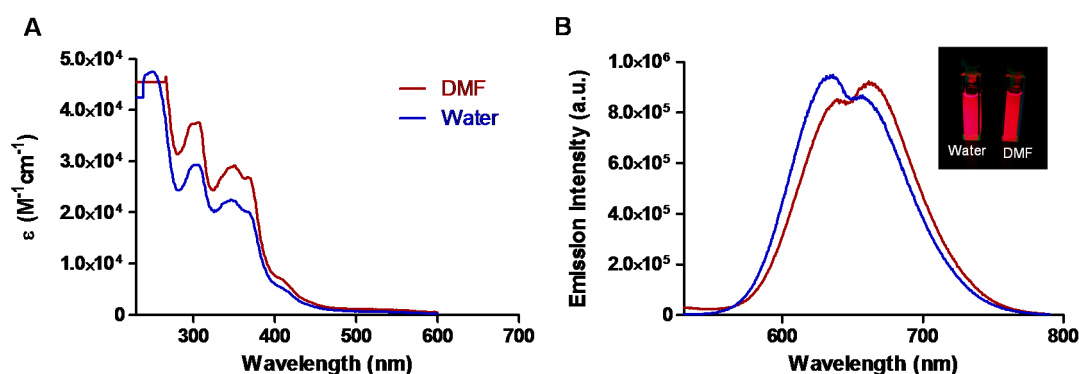


Figure 5.8. A) UV/Vis and B) Emission spectra of **35** in DMF and water (1% DMF). $\lambda_{\text{exc}} = 405$ nm.

5.2.4. Interaction with biomolecules

The reactivity of the heterodinuclear complex **35** with relevant biomolecules was studied by UV/Vis. Glutathione and L-histidine (**Figure 5.9A**) were used as models for amino acid residues on proteins. Glutathione is a thiol-containing tripeptide (γ -L-Glu-L-Cys-Gly, GSH) present in most cells with an intracellular concentration (10 mM) substantially higher than the extracellular levels (2 mM).³² Intracellular interactions between cisplatin and glutathione have previously been found to be deactivated, and cisplatin-resistant tumor cells often contain elevated levels of glutathione.³³ However, there are no definitive conclusions in the expression of thiol-containing proteins and resistance to cisplatin.³⁴ Interestingly, the strong affinity of sulfur for platinum complexes has led to the coadministration of platinum drugs with glutathione as a chemoprotectant to prevent the toxic side-effects of Pt-S adducts in proteins.³⁵ On the other hand, L-histidine is a highly versatile amino acid that plays key roles in the activity of many proteins and peptides, acting as a hydrogen bond donor or acceptor, an aromatic residue, or as a coordinating ligand.³⁶ Histidine (His) is also important biologically, being the precursor for histamine, which is involved in many physiological processes. Gupta *et al.* have recently reported a cyclometalated trinuclear Ir(III)/Pt(II) complex as a new protein-binding agent based on specific interactions with the amino acid histidine.³⁷ Thus, the histidine residues from proteins reversibly coordinate with the Pt center replacing the chloride ion of the trinuclear complex.

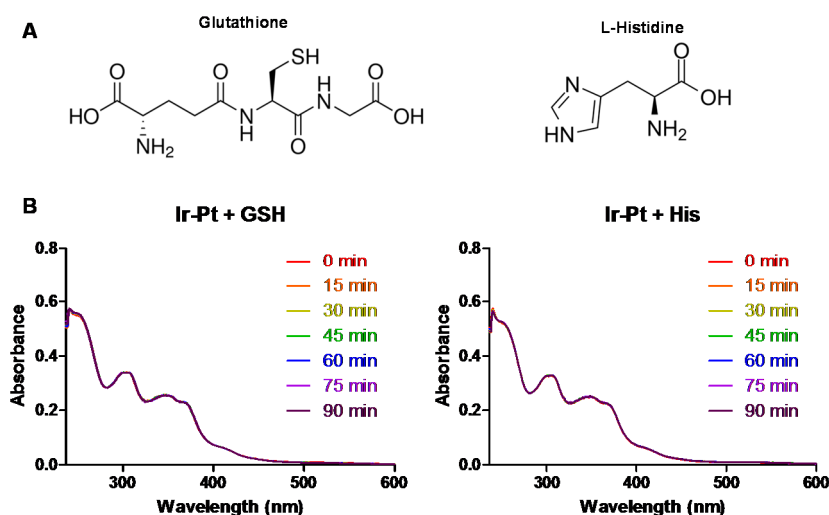


Figure 5.9. A) Chemical structures of glutathione and L-histidine. B) UV/Vis spectra of **35** (10 μ M) in water (1% DMF) after adding 10 mM of glutathione and histidine.

As observed in **Figure 5.9B**, the UV/Vis spectra of **35** remain unchanged in the presence of 10 mM of glutathione and histidine for 90 min. This fact tests the stability of the chlorine ligand in the heterobimetallic complex against substitution reactions.

5.2.5. Cytotoxicity study towards human cancer cells

To probe whether the new heterobimetallic Ir(III)–Pt(II) complex had the potential as an anti-cancer agent and any ability to counteract the cisplatin resistance, we tested its cytotoxicity against A2780 cells and cisplatin-resistant A2780cisR and HeLa cell lines. The Pt complex **34** was studied with comparative purposes. As observed in **Table 5.2**, the Ir–Pt complex **35** showed high cytotoxicity in the tested cell lines, with IC₅₀ values ranging from 3.6 to 7.6 μM. In addition, Ir–Pt displayed approximately 14-fold higher potency than cisplatin in A2780cisR cells and 5-fold higher potency than cisplatin in HeLa cells, indicating that **35** has better anticancer potential toward cisplatin-resistant cancer cells. Furthermore, **35** exhibited a higher selectivity towards tumor cells than **34** and cisplatin, as shown in the study in non-tumorigenic renal BGM (African green monkey kidney) cells.

Table 5.2. IC₅₀ values [μM] of complexes **34**, **35** and cisplatin after 48 h.

	A2780	A2780cisR (RF ^b)	HeLa	BGM	SF ^c
34 (Pt)	12.6 ± 0.9	13.7 ± 2.4 (1.1)	>100	>100	>8
35 (Ir-Pt)	3.6 ± 0.2	3.6 ± 0.3 (1.0)	7.6 ± 0.9	>100	>28
Cisplatin	2.0 ± 0.6	50 ± 0.3 (25)	33 ± 4.0	16.5 ± 0.16	8.3

^a The data are expressed as mean values ± SD of two independent experiments, each of them made in quadruplicate. ^bResistance factor: IC₅₀ A2780cis/IC₅₀ A2780; ^cselectivity factor: IC₅₀ BGM /IC₅₀ A2780

5.2.6. Metal accumulation in isolated DNA

Several metal-based drugs,^{38,39} including cisplatin, have demonstrated their capability to bind and damage nuclear DNA, and therefore, DNA can be considered one of their principal target. Thus, metal accumulation in isolated DNA in HeLa cells was measured by ICP-MS to investigate if the dinuclear complex **35** could bind DNA. As observed in **Table 5.3**, the amount of Ir and Pt bound to DNA in the heterodinuclear complex was very small compared to cisplatin, so DNA does not appear to be its main target. However, finding the same Ir and Pt accumulation in **35** clearly indicates that the complex is not broken inside the cell.

Table 5.3. ICP-MS analysis to determine metal accumulation in isolated DNA in HeLa cells after 2 h treatment with 5 μ M of Ir-Pt complex **35** and cisplatin.

	pg metal/ μ g DNA	
	Ir-DNA	Pt-DNA
35	11.8 ± 0.4	14.9 ± 1.7
Cisplatin	0.9 ± 0.1	84.6 ± 5.2

^a Cells were treated with **35** and cisplatin (5 μ M) and incubated for 2 h at 37 °C. Complex accumulation was expressed in pg of iridium or platinum per μ g DNA. Results are the mean $\pm$ SDs from two independent experiments.

5.2.7. Cell-death studies and cell cycle analysis

In order to elucidate the mode of cell death induced by heterodinuclear complex **35**, dual Annexin V-FLUOS/Propidium Iodide (PI) staining assays were performed to discriminate apoptotic and necrotic cell death induction. Compared to cisplatin and **34** treatment, which showed positive staining for Annexin V due to apoptosis, A2780 cells treated with the concentration of IC_{50} and $2 \times IC_{50}$ (2.5 and 5 μ M respectively) of **35** revealed a PI-positive population after 24 h (**Figure 5.10**).

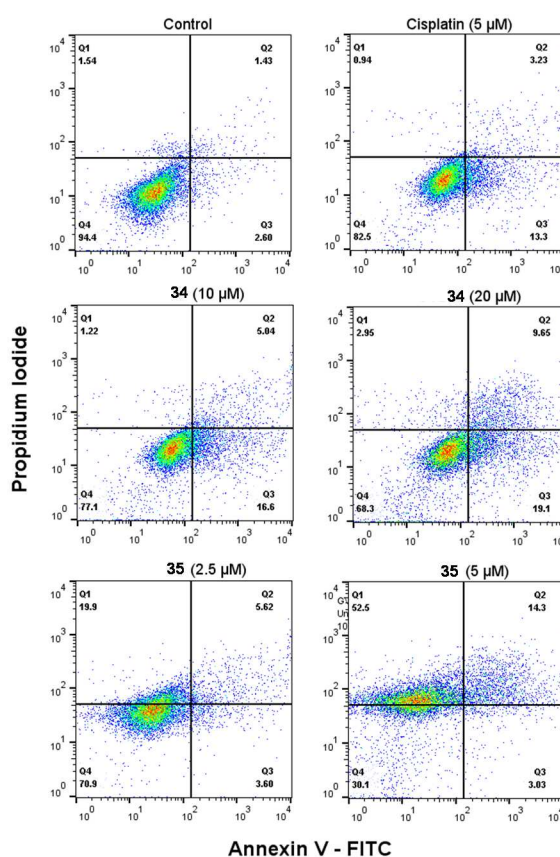


Figure 5.10. Apoptosis assay of A2780 cells incubated with **34** and **35** at the concentration of IC_{50} and $2 \times IC_{50}$ for 24h.

This induction of necrotic cell death could be one of those responsible for overcoming resistance to cisplatin during the post-target stage.⁵

In addition, to investigate the mechanism of action underpinning the Ir(III)-Pt(II) complex effects, the cell cycle progression of cancer cells was studied. The impact of **35** on cell cycle distribution was examined in A2780 cancer cells after 24 h. Fluorescence-activated cell sorting (FACS) analysis showed that complex **35** not induced an effect in the cell cycle in contrast to cisplatin and complex **34**, which induce G2/M and S phase arrest (Figure 5.11).

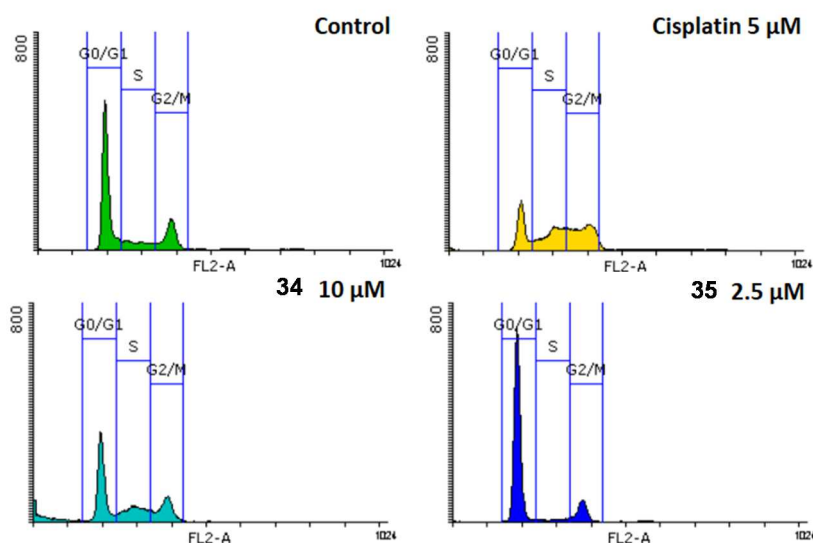


Figure 5.11. Cell cycle analysis in A2780 cells incubated with **34**, **35** and cisplatin at indicated concentrations (IC_{50}) for 24h.

New studies will be carried out to investigate further the mechanism of action of this promising Ir(III)-Pt(II) antitumor agent.

5.3. CONCLUSIONS

A new dinuclear Ir(III)-Pt(II) complex was designed and synthesized. This heterobimetallic complex showed an intense red phosphorescence in water granted by the Ir(III) moiety. In addition, the stability studies in solution and the interaction with biomolecules showed the resistance of the chloride ligand to substitution and hydrolysis reactions. The antitumoral study revealed the ability of **35** to kill cisplatin-resistant cervical and ovarian cancer cells. In addition, the dinuclear complex exhibited no effect in non-tumorigenic normal renal cells, showing its better selectivity towards cancer cells than cisplatin.

Further studies on cell death induction revealed that, in contrast to cisplatin, which induced apoptosis in A2780 cells, complex **35** was causing necrosis rather than apoptosis. In addition, the dinuclear complex appears not to affect the cell cycle in contrast to the platinum precursor and cisplatin, which induce G2/M and S phase arrest. All of the above indicates that the Ir(III)-Pt(II) heterodinuclear complex could have a different mechanism of action than cisplatin.

To get more conclusions, we need a deeper biological study of the dinuclear complex. Even so, this preliminary study reveals a promising antitumor agent that could overcome the cisplatin resistance and represents a further step in the development of new heterodinuclear Ir–Pt complexes with dual antitumor and luminescent properties, which is still a developing area.

5.4. REFERENCES

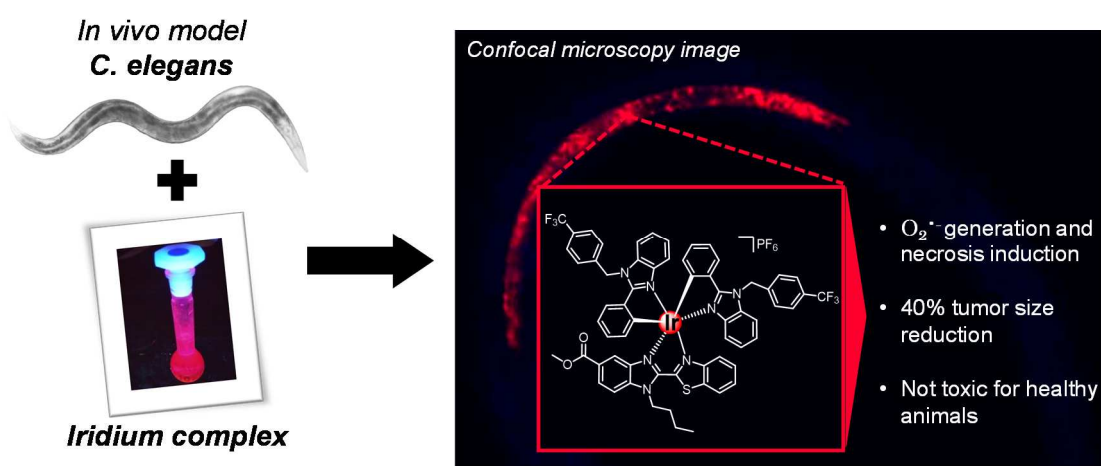
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Chapter 6

A red phosphorescent iridium(III) complex as a promising antitumor agent: *in vivo* studies in *C. elegans*



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6.1. INTRODUCTION

The excellent photophysical properties of Ir(III) complexes commented in Chapter 1 have triggered a growing interest in developing these complexes as biological probes and visualizing reagents.¹ That fact has allowed deeper mechanism of action studies inside the cells or even inside transparent organisms as zebrafish. Thus, *in vivo* studies using zebrafish as an animal model have evidenced the application of Ir(III) compounds as visualizing reagents^{2–4} (**Figure 6.1**).

To advance in the research of new anti-cancer drugs is important to demonstrate the measurable effects of the drug against this human disease. For this purpose, the preclinical oncology models have extended significantly in the last decades. Such models allow the demonstration of *in vivo* effectiveness, along with the confirmation of the mechanism of action and the main target of the drug candidate.⁵

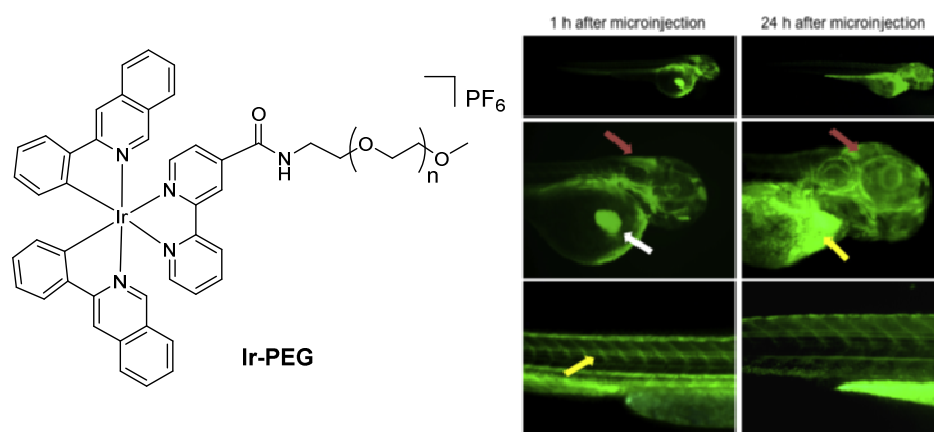


Figure 6.1. *In vivo* distribution of Ir-PEG complex in zebrafish larva.²

Despite its simplicity, the nematode worm *Caenorhabditis elegans* (*C. elegans*) has been widely studied as an animal model in biochemical, pharmaceutical, and medical research, mainly in the characterization of novel drug targets.^{6,7} This animal model has a whole organism (with digestive, reproductive, and nervous systems), and their transparency also enables the visualization of fluorescently labeled tissues in mutant strains. Additionally, most human disease genes and disease pathways are present in *C. elegans*, as confirmed by comparing the human and *C. elegans* genomes.⁸

The application of mammals for *in vivo* studies is common. However, testing first in *C. elegans* supposes a decrease in the use of mammals in research, compliant with the 3R principles (Reduction, Refinement, and Replacement) in animal testing.⁹ Thus, the *Caenorhabditis elegans* tumoral model has been recently developed.¹⁰ The tumor in *C.*

elegans is induced using RNAi technology: the knockdown of the *gld-1* gene prevents the germ cells from exiting from mitosis and consequently induces cell proliferation through the gonad, forming a germline tumor lethal to the animal (**Figure 6.2**).^{11,12} Once cancer has been generated, this tumoral strain of *C. elegans* can be used to investigate the effects of the drug *in vivo*.

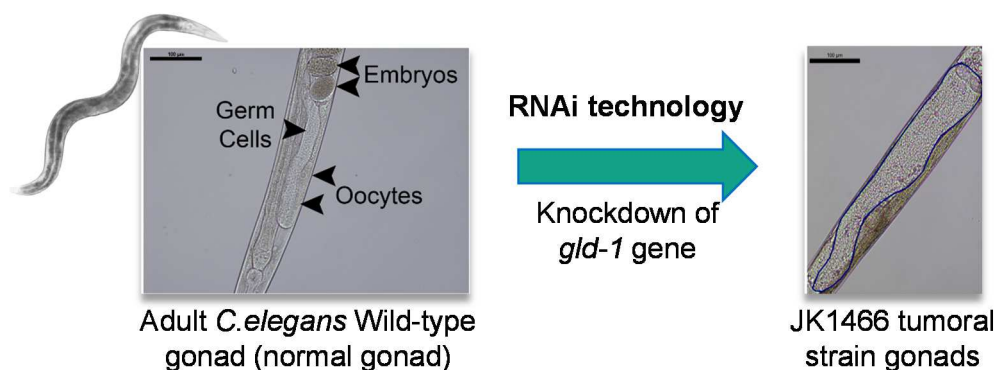


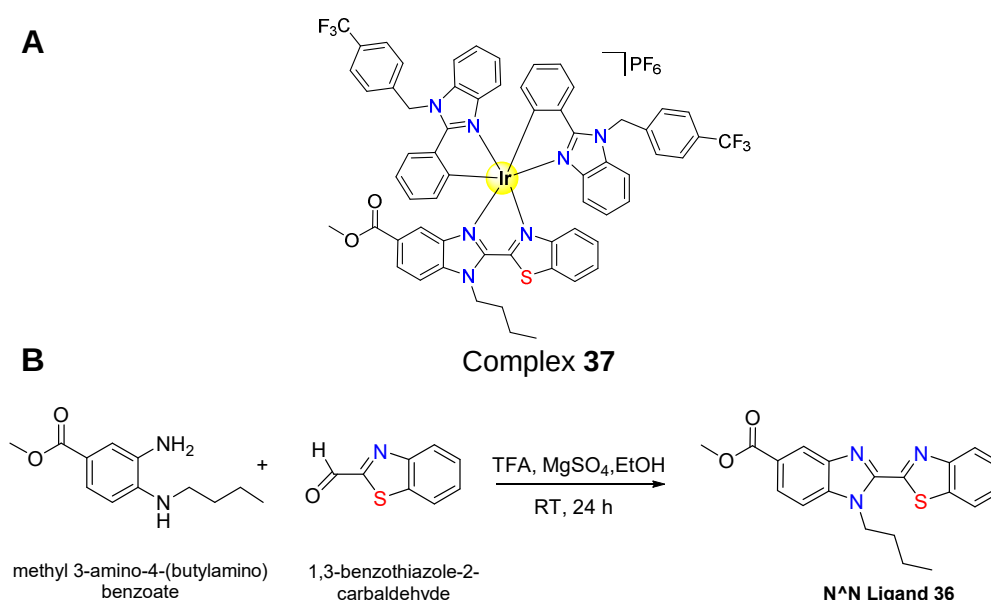
Figure 6.2. RNAi technology to form a germline tumor in the animal gonad.

Herein we report the synthesis and characterization of a new red phosphorescent iridium complex $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{N}^{\wedge}\text{N})][\text{PF}_6]$ (**37**) (where $\text{C}^{\wedge}\text{N}$ = 2-phenyl-1-[4-(trifluoromethyl)benzyl]-1H-benzo[d]imidazol-kN,C and $\text{N}^{\wedge}\text{N}$ = methyl 2-(benzo[d]thiazol-2-yl)-1-butyl-1H-benzo[d]imidazole-5-carboxylate) in order to investigate its potential as anti-cancer drug *in vivo*. The pro-ligand **1** was selected as the $\text{C}^{\wedge}\text{N}$ ligand due to the excellent photophysical properties it provides the final complexes in previous chapters. As $\text{N}^{\wedge}\text{N}$ ligand was selected a new 2-benzothiazol-yl benzimidazole core with an ester group as a handy tool for further functionalization and a butyl group for N-substitution in order to modulate its lipophilic properties. On the other hand, the choice of a sulfur heteroatom in this ligand has been made to tune the emission color and the cellular uptake efficacy of the iridium complex. The antitumoral capacities of **37** were tested *in vitro* in ovarian cell lines and *in vivo* in a recently developed *Caenorhabditis elegans* tumoral model. The use of an animal model in this work have allowed a study in depth of the underlying mechanism of action of the new iridium complex *in vivo*.

6.2. RESULTS AND DISCUSSION

6.2.1. Synthesis and characterization

A new red luminescent iridium complex (**37**) of the type $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{N}^{\wedge}\text{N})][\text{PF}_6]$ (**Scheme 6.1A**) was designed and synthesized. The $\text{HC}^{\wedge}\text{N}$ proligand (**1**) was synthesized as previously reported.¹³ The new $\text{N}^{\wedge}\text{N}$ ligand methyl 2-(benzo[d]thiazol-2-yl)-1-butyl-1H-benzo[d]imidazole-5-carboxylate (**36**) was synthesized by condensation of the intermediate methyl 3-amino-4-(butylamino)benzoate,¹⁴ with 1,3-benzothiazole-2-carbaldehyde in ethanol with a catalytic amount of trifluoroacetic acid at room temperature for 24 h (**Scheme 6.1B**). Complex **37** was obtained in a good yield (72%) by reaction of the cyclometalated iridium(III) dimer **7** with the $\text{N}^{\wedge}\text{N}$ ligand in a 1:2 molar ratio. The new ligand and the Ir(III) complex were characterized by ^1H and ^{13}C NMR spectroscopy (**Figures S6.1-6.5**) and positive-ion HR ESI-MS (**Figures S6.6-6.7**), showing the $[\text{M}-\text{PF}_6]^+$ signals with the expected isotopic distribution pattern. The ^1H -NMR spectrum of the new complex exhibits one set of peaks for each of the two inequivalent $\text{C}^{\wedge}\text{N}$ ligands due to the asymmetric nature of the coordination environment around the metal. The corresponding signals of the butyl group of the $\text{N}^{\wedge}\text{N}$ ligand appears between 5.02 and 0.69 ppm (**Figure S6.4**). Complex **37** was shown to be at least 96 % pure by both reverse-phase RP-HPLC in DMSO (**Figure S6.8**) and elemental analysis.



Scheme 6.1. A) Structure of new iridium complex **37**. B) Synthesis of the new $\text{N}^{\wedge}\text{N}$ ligand.

6.2.2. Photophysical properties

The UV/Vis absorption and emission spectra of **37** were recorded in both acetonitrile and water (1% DMSO) at room temperature (**Figure 6.3**). The new complex displays intense absorption bands at $\lambda < 350$ nm that can be assigned to ligand-centered (LC) transitions within the C[^]N and N[^]N ligands. Bands at $\lambda > 350$ nm and the lowest energetic absorption wavelength could be associated with ligand-to-ligand charge transfer (LLCT) and metal to ligand charge transfer (MLCT) phenomena.¹⁵ Recent theoretical calculations based on a similar Ir(III) complex, suggest that the parent Ir(pbz)₂(N[^]N) complex (pbz: 2-phenylbenzimidazole) adopts a push–pull architecture, where the metallic center and the pbz ligand play the role of donor, and the N[^]N moiety acts as the acceptor.¹⁵ This fact supposes that the decoration of ligands triggered an enhancement in the charge transfer and a shift in the absorption to larger wavelengths. Upon excitation at λ_{exc} maxima, **37** exhibits an intense red (~ 665 nm) phosphorescence emission in both solvents (**Figure 6.3** and **Figures S6.9**).

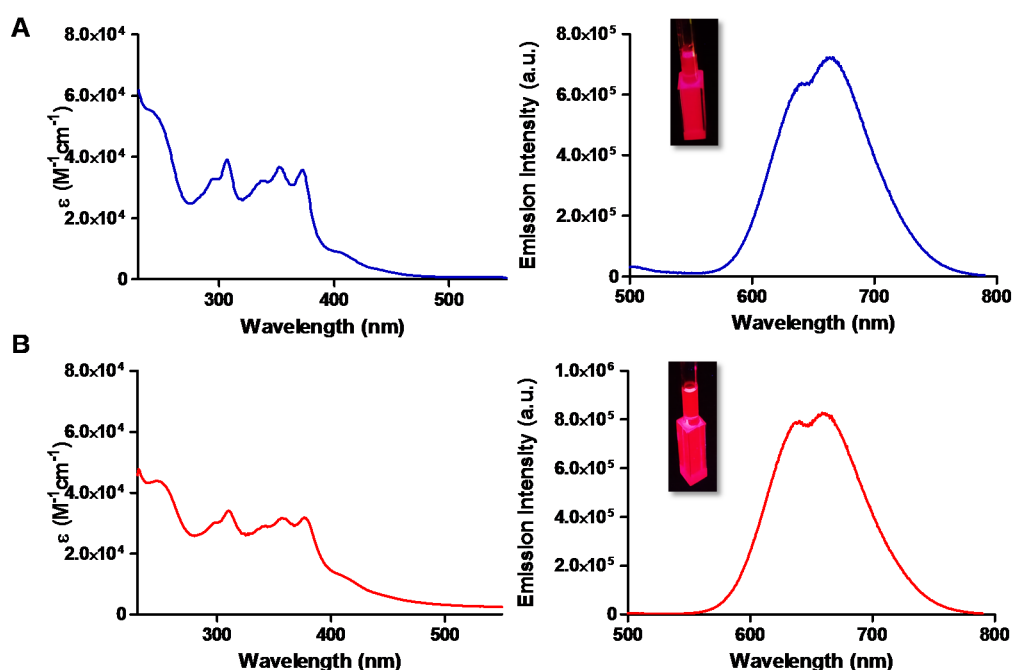


Figure 6.3. UV/Vis and emission spectra of **37** in A) acetonitrile and B) water (1% DMSO). $\lambda_{\text{exc}} = 405$ nm.

The lifetimes of the new Ir(III) complex in acetonitrile and water (**Table 6.1**) were of hundreds of nanoseconds, indicating the phosphorescence nature of the emission.^{16–18} The study of the emission quantum yield in aerated and deaerated solutions showed no influence of the oxygen in the emission in water, but that didn't occur in acetonitrile. On the other hand, in aerated solution, the quantum yield in water is higher than in acetonitrile (**Table**

6.1). Therefore, the change in the emission intensity was studied in a mixture of water and DMSO with different water fractions. As shown in **Figure 6.4**, the emission intensity increases 3 times when the water content mixtures increased up from 75% to 95%, which is suitable for further confocal microscopy in the *in vivo* study.

Table 6.1. Excitation (λ_{exc}), emission (λ_{em}) wavelengths, emission lifetimes (τ_{em}), and quantum yields (Φ_{em}) of **37** in acetonitrile and water (1% DMSO).

Solvent	λ_{exc} , nm ^a	λ_{em} , nm	aerated solution		deaerated solution ^b	
			τ_{em} , ns	Φ_{em} , %	τ_{em} , ns	Φ_{em} , %
Acetonitrile	372	665	223 (100%)	3.9	393 (96%)	6.9
					744 (4%)	
Water (1% DMSO)	376	661	181 (13%)	6.0	370 (89%)	6.1
			413 (87%)		1237 (11%)	

^[a] λ_{exc} maxima. ^[b] Degassed by bubbling 20 min under argon

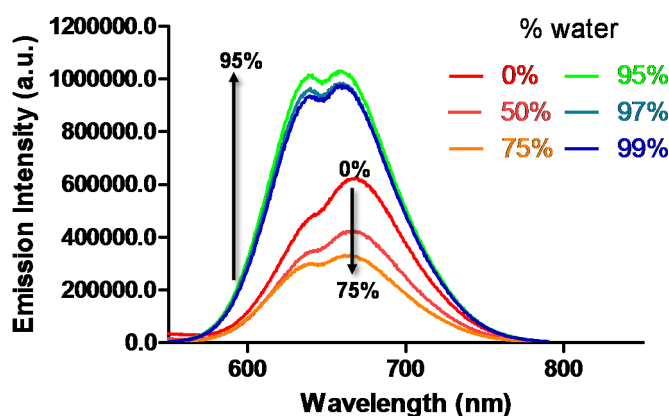


Figure 6.4. Emission spectra of **37** in DMSO/water mixtures with different water fractions (fw). The excitation wavelength was 405 nm.

6.2.3. Stability studies

Before the *in vitro* and *in vivo* studies, it is very important to know the solution stability of the new compound. The stability of the complex in DMSO was studied by ¹H NMR spectroscopy, and no free ligand was detected after 5 days at room temperature (**Figure 6.5**).

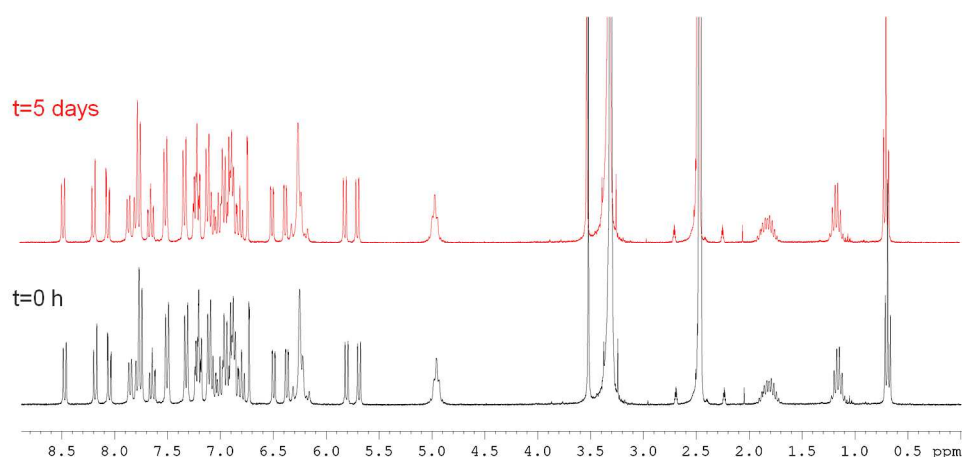


Figure 6.5. ^1H NMR spectra of **37** measured after dissolving immediately in $[\text{D}_6]\text{DMSO}$ (bottom) and after 5 days (top) at RT.

The stability of **37** was also confirmed after 24 h in Roswell Park Memorial Institute (RPMI) culture medium by UV/Vis upon dissolving the compound in 1% DMSO (**Figure 6.6**).

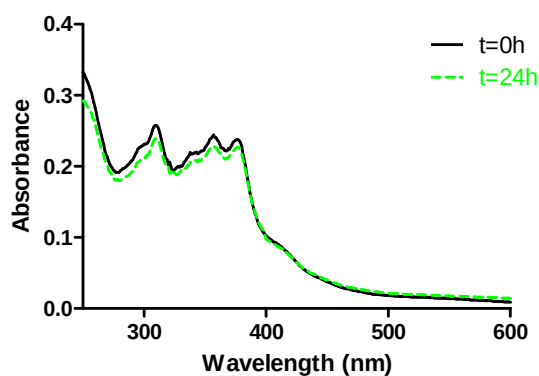


Figure 6.6. UV/Vis spectra of **37** in RPMI medium (1% DMSO) measured at $t=0\text{h}$ and after 24h.

C. elegans has an intestinal pH oscillating between 3.5 and 6.¹⁹ To ensure the permanence of the iridium complex inside the worms, we also performed the stability at different pH. As shown in **Figure 6.7**, the UV/Vis spectrum of **37** remains the same in the pH range of 7.4 to 3.3.

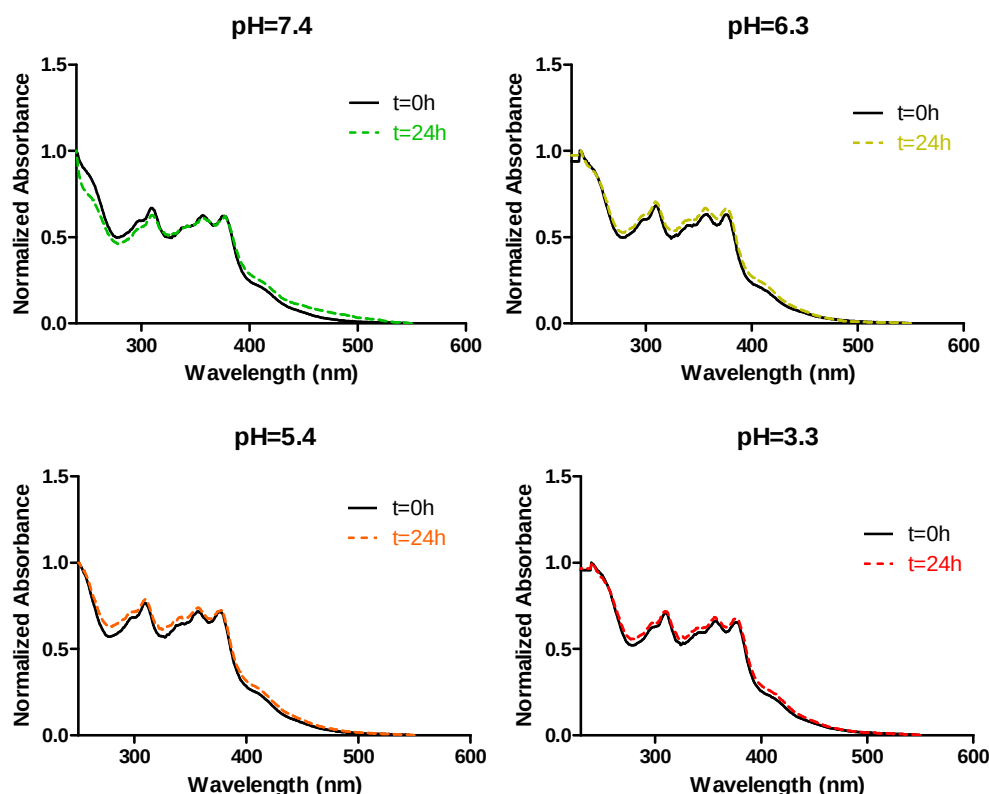


Figure 6.7. Effect of pH on Ir(III) complex stability in disodium hydrogen phosphate/citric acid buffer solutions (pH 3.3, 5.4, 6.3 and 7.4) with 10% DMSO.

In vitro study

6.2.4. Cytotoxicity studies towards human cancer cells

The cytotoxicity of complex **37** was tested against human ovarian carcinoma cells (A2780), cisplatin-resistant A2780 cells (A2780cisR), and normal ovarian cells (CHO) using the standard MTT assay. With an IC_{50} value of 0.17 μ M, the Ir(III) complex showed a much higher antiproliferative effect than the common clinical drug cisplatin against ovarian cancer cell lines (**Table 6.2**). Remarkably, the iridium complex also exhibited high cytotoxicity against A2780cisR cells with a resistance factor below 1. In addition, under the same experimental conditions, **37** achieved 12-fold higher cytotoxicity against ovarian cancer cells than against normal ovarian cells. This highly selective factor *in vitro* suggested that **37** could act preferentially in tumor cells in the model animal *C. elegans*.

Table 6.2. IC₅₀ values [μ M] of complex and cisplatin after 48 h.

	A2780	A2780cisR (RF ^a)	CHO	SF ^b
37	0.17 $\pm$ 0.02	0.168 $\pm$ 0.007 (0.98)	2.0 $\pm$ 0.4	11.8
Cisplatin	2.3 $\pm$ 0.2	22 $\pm$ 4 (9.56)	7.8 $\pm$ 0.8	3.4

^aResistance factor: IC₅₀ A2780cis/IC₅₀ A2780; ^bselectivity factor: IC₅₀ CHO /IC₅₀ A2780

6.2.5. Cellular localization and distribution

The localization within cancer cells was investigated by confocal microscopy owing to the intrinsic phosphorescence of complex **37**. Co-staining with mitochondria-specific probe MitoTracker Green FM was performed in A2780 cells (**Figure 6.8**), where a partial overlapping pattern between **37** and MitoTracker was observed after 2 h treatment (**Figure 6.8D**), displaying a Pearson's correlation coefficient of 0.56 (**Figure S6.10**). Moreover, the luminescence of complex **37** was also observed in the cytoplasmic membrane. This distinctive localization pattern reminded us that of a previously published similar Ir(III) complex accumulated in the actin cortex,¹³ suggests that **37** might be able to target the actin cortex as well.

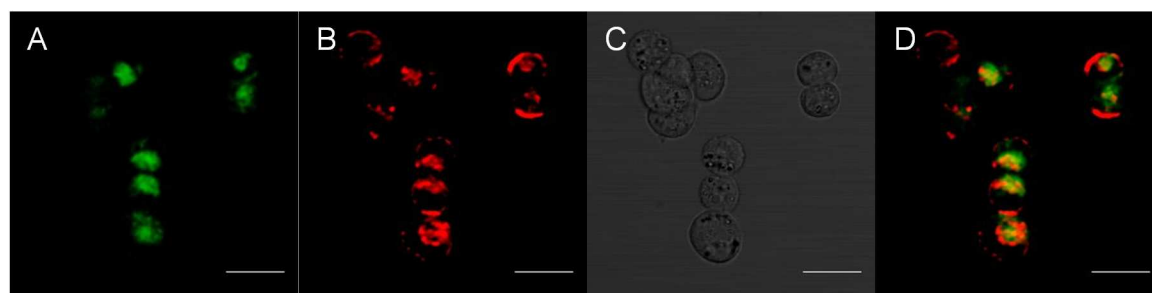


Figure 6.8. Co-localization study of **37** in A2780 cells. A2780 cells were treated with 5 μ M of **37** for 2 h and co-stained with the MitoTracker Green^{FM} (0.1 μ M). Channels: A) Mito-Tracker fluorescence, B) luminescence signal from Ir compound, C) bright field, D) overlay of the luminescence channels. Scale bar = 10 μ m.

6.2.6. Cell-death induction

Necrosis is an acute form of cell death due to energetics failure that produces a loss of nuclear and plasma membrane integrities. Although necrosis has been long considered an unregulated process, recent evidence has demonstrated that necrosis can also be a regulated form of cell death.²⁰ Dual Annexin V-FLUOS/Propidium Iodide (PI) staining assays were performed to discriminate apoptotic and necrotic cell death induction. Compared to cisplatin treatment, which showed positive staining for Annexin V due to apoptosis being induced, A2780 cells treated with 2 μ M of **37** revealed a PI-positive population after 24 h

(**Figure 6.9**). Indeed, the increment in complex **37** concentration (5 μM) in the treatment induced a more significant population of PI-positive cells. Thus, the study revealed that, in contrast to cisplatin, **37** was causing necrosis rather than apoptosis after 24 h.

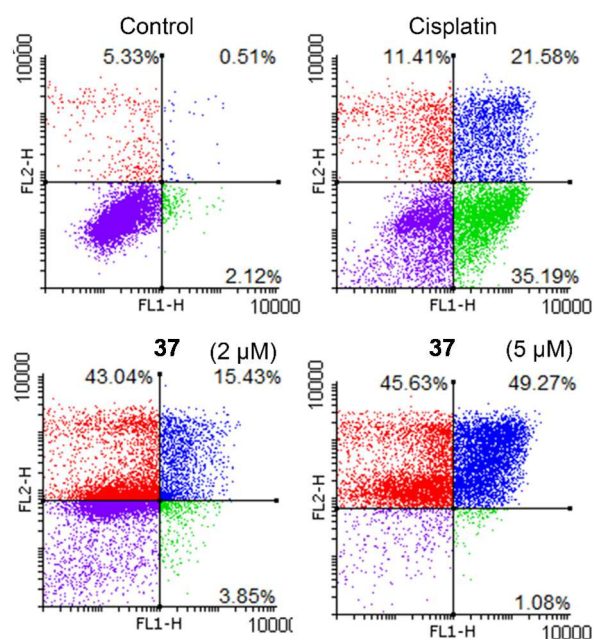


Figure 6.9. Dual Annexin-FLUOS (FL1-H)/Propidium iodide (FL2-H) flow cytometric analysis of A2780 untreated cells, cisplatin (2 μM) treated cells, cells treated with **37** at 2 and 5 μM . Cisplatin was used as a positive control for apoptosis induction.

6.2.7. Superoxide anion production

Since elevated ROS, and particularly high superoxide anion levels, can trigger necrotic cell death,^{21,22} we decided to measure the amount of ROS inside A2780 cells upon exposure to Ir(III) complex. Flow cytometry analysis of A2780 cells using DCFH-DA showed that ROS levels in **37**-exposed cells were comparable to those present in untreated cells (**Figure S6.11**). However, the use of specific fluorescent superoxide anion probe dihydroethidium (DHE) revealed a significant rise of red fluorescence in ovarian cancer cells after 2 h treatment with **37** at 5 μM (**Figure 6.10**). Furthermore, the DHE fluorescence was reduced in the well-known ROS scavenger N-acetylcysteine (NAC) presence. Moreover, complex **21b** (Chapter 3) was used as a positive control for superoxide generation.

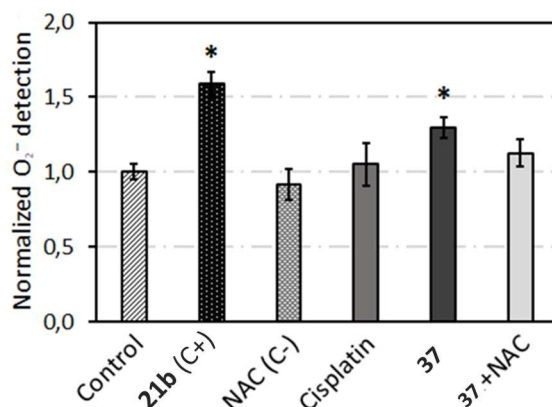


Figure 6.10. Normalized levels of superoxide production in A2780 cells after treatments with **37** (5 μ M) or cisplatin (5 μ M) measured by flow cytometry. N-acetylcysteine (NAC) at 2 mM was used as a negative control, whereas **21b** (5 μ M) was used as a positive control for superoxide generation. Data expressed as Mean RFI $\pm$ SD from two independent experiments (n=2 replicates). *p < 0.05 unpaired t-test.

In vivo study

6.2.8. Assimilation of Ir(III) complex by the model animal *C. elegans*

The bright phosphorescence of the compound allowed the detection *in vivo* and its quantification inside *Caenorhabditis elegans* worms, as showed in **Figure 6.11**. The images showed that the animals could assimilate the compound supplied along with the *E. coli*, and it was detected in the intestinal lumen (**Figure 6.11B**). As expected, the assimilation was related to the complex concentration provided, showing a dose-dependent behavior (**Figure 6.11C**). *C. elegans* have gustatory and olfactory receptors to detect food, danger, or other worms. The responses to chemosensory stimuli produce different patterns in the animals as changes in feeding, motility, avoidance of the compound or dauer arrest.²³ Thus, assimilation of the complex suggests that the worms were not repelled by it as happens with some other compounds like heavy metals, detergents, bitter alkaloids, and some organic molecules.^{24,25}

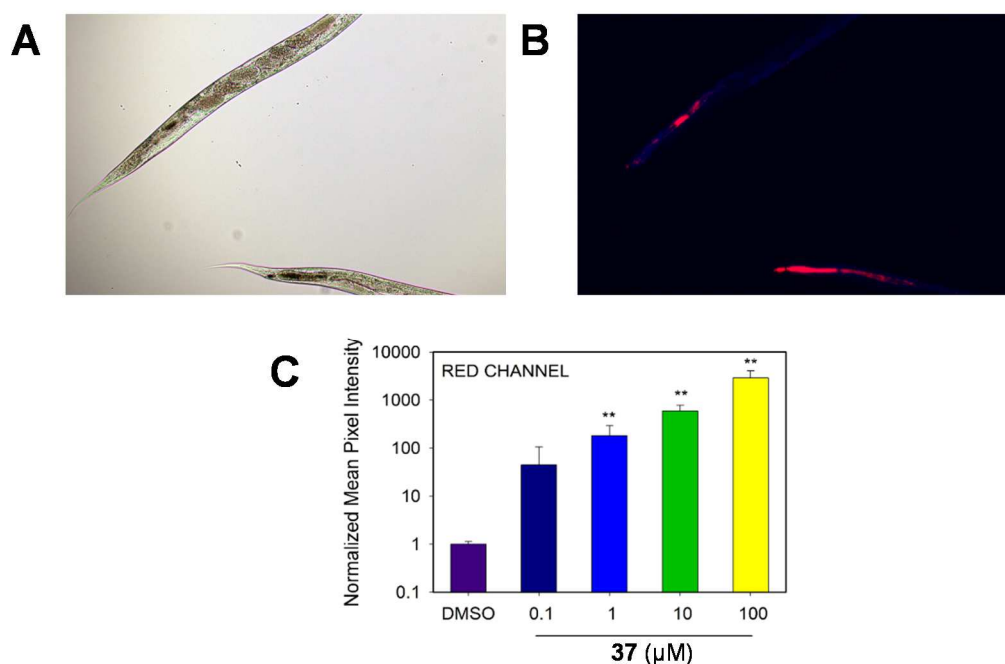


Figure 6.11. Accumulation of **37** *in vivo*. (A-C) Representative images of N2 animals exposed to the iridium complex **37**. A) Brightfield at 100 μM. B) Luminescence image at 100 μM. C) Quantification of the fluorescence intensity inside the animals, data is represented as mean ± S.D, n > 15, two independent experiments were performed, **significantly at $p \leq 0.05$ by ANOVA test. Scale bars 200 μm.

6.2.9. Antitumoral potential of the iridium complex *in vivo*

The chemotherapeutic effects of **37** were evaluated using the mutant strain JK1466; these animals have suppressed the *gld-1* gene involved in the oocyte fate determination. *gld-1* is necessary for the regulation of mitosis and meiosis entry in the germ line cells. Without it, germ cells are arrested in the mitosis phase and proliferate, forming large tumors in the proximal gonad.²⁶ Gonads of normal young adults showed oocytes and embryos in the proximal zone and germinal cells in the distal zone, as displayed in **Figure 6.12A**. In contrast, worms of the tumoral strain showed proliferative germ cells in all the gonad regions and the absence of oocytes and embryos (**Figure 6.12B**). In addition, the acridine orange stain allowed the visualization of the individual cells in the gonad and their morphology, showing that oocytes and embryos are larger than germinal cells. The tumoral strain JK1466 has bigger and swollen tumoral gonads (**Figure 6.12B**). However, the size and the swell were reduced when the animals were treated with the iridium complex **37** (**Figure 6.12C**).

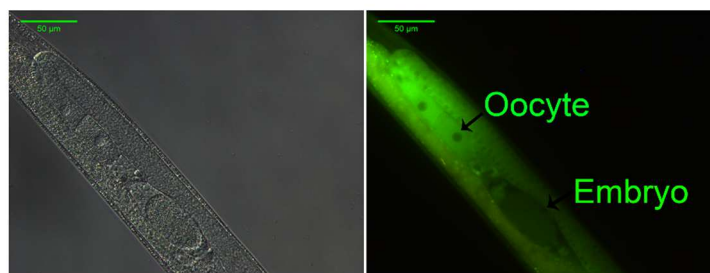
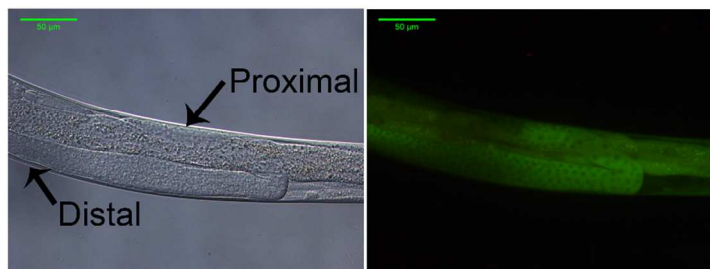
A Wild-type gonad**B JK1466 tumor gonad****C JK1466 tumor gonad + Ir complex**

Figure 6.12. A) Wild-type gonad, B) control JK1466 tumor gonad and C) tumor gonads of JK1466 animals treated with 100 μM of **37** visualized with differential interference contrast (DIC) and stained with acridine orange.

Measurements of the tumor area were performed using different concentrations of **37** (0.1, 1, 10 and 100 μM). Cisplatin was used as a control. The results are shown in **Figure 6.13** and **Table S6.1**. The higher concentration tested (100 μM) reduced the tumor growth by 41.03 % ($p < 0.0001$), while cisplatin reduced the tumor size by 42.49 % ($p < 0.0001$), showing the efficacy of this novel iridium complex as an antitumoral agent.

Moreover, the *C. elegans* tumoral strain JK1466 and the wild-type strain were used to estimate the chronicle exposure toxicity of the **37**. Previous studies on *C. elegans* treated with iridium(III) complexes reported their use for biosensing and bioimaging *in vivo*. However, the studies did not evaluate the possible toxicity effects of the compounds in the worms.^{27–29} Furthermore, toxicity studies of other metal complexes such as ruthenium ones reported a 20% decrease in the survival of *C. elegans* treated with 100 μM of these complexes for 24 h.^{30,31}

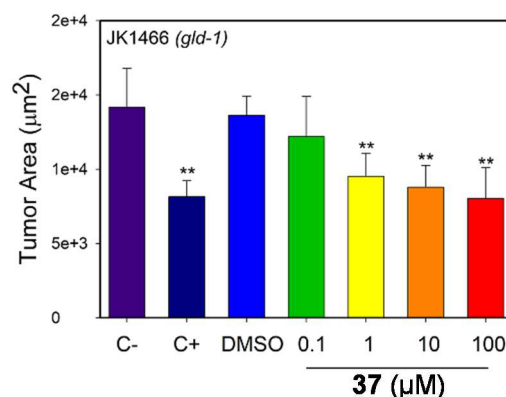


Figure 6.13. Tumor size evaluation data is represented as mean $\pm$ S.D, $n > 10$, two independent experiments were performed, **significantly at $p \leq 0.05$ by ANOVA test. Scale bars 50 μm .

The results obtained showed low toxicity for the wild-type animals treated with 100 μM of the complex as their mean lifespan was unaltered (**Figure S6.12A**), and a significant increase was observed in the mean lifespan of the tumoral animals (**Figure S6.12B**), suggesting that the tumor reduction was improving the health status of the animals.

6.2.10. ROS accumulation in *C. elegans*

Reactive oxygen species in *C. elegans* was measured by using the fluorescent probe DCFH-DA. The probe detects peroxy, hydroxyl, or alkoxy radicals and some nitrogen species, being an assay for generalized oxidative stress rather than a detection method for specific species.³² The treatment with cisplatin increased the total ROS levels 4.1-fold ($p \leq 0.00001$) compared with the control, while the ROS levels produced by **37** were not significantly different to control levels (**Figure S6.13**). Since the DCFH-DA probe shows the general redox state of the worms but cannot detect superoxide anions,³³ dihydroethidium (DHE) was used as a fluorescent probe (**Figure 6.14**) in order to check if the same could be happening in full animals. As a positive control, the widely studied superoxide generator paraquat (methyl viologen) was used.³⁴

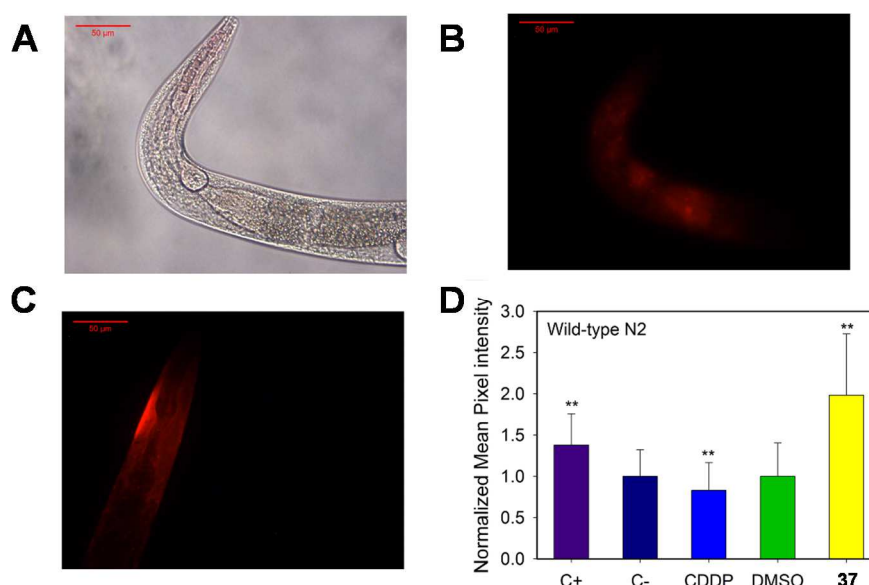


Figure 6.14. *In vivo* effects of **37** in superoxide generation. (A-C) Representative images of wild-type *C. elegans* stained with DHE to detected superoxide anions. A) Positive control (200 μ M paraquat) worm in brightfield. B) The same animal visualized under fluorescence visualization. C) Worm treated with 100 μ M iridium complex under fluorescence visualization. D) Quantification of superoxide levels presented as mean $\pm$ SD, $n > 10$, two independent experiments were performed, **significantly at $p \leq 0.05$ by ANOVA test.

Consistently with the results in cells, **37** treatment increased the superoxide levels inside the worms, in contrast to cisplatin (**Figure 6.14D**). Furthermore, the iridium complex generated more superoxide anions than paraquat at the same concentration. Remarkably, the treatment with the Ir(III) complex did not affect the mean lifespan of the wild-type animals and increased the lifespan of the tumoral strain, consistent with previous results that reported an increase of superoxide levels produced by paraquat did not reduce the lifespan of wild-type animals.³³

6.2.11. Morphology of *C. elegans* tumoral cells

Necrotic cells are morphologically different from apoptotic cells, as they form swelled, enlarged corpses, while apoptotic cells are smaller in size, more compact, and have a button-like form.³⁵ The morphology of the tumoral cells in the animal gonads was studied using acridine orange. Under normal conditions, the stain does not penetrate cell nuclei. However, when the cell nuclear membrane is compromised –as happens in apoptotic and necrotic death– the acridine orange binds to the DNA and the compound's fluorescence is increased, showing cell corpses much more fluorescent than normal cells (**Figure 6.15**). While the gonad cells of the animals treated with cisplatin showed small corpses similar to apoptosis corpses (**Figure 6.15A**), the gonad cells treated with the iridium complex were swollen and enlarged, properties associated with necrosis rather than to apoptosis (**Figure**

6.15B). These results indicate that the iridium complex is likely to reduce tumor growth by activating cell death by necrosis, consistent with the result in cells and in contrast to cisplatin, which promotes cell death by apoptosis.

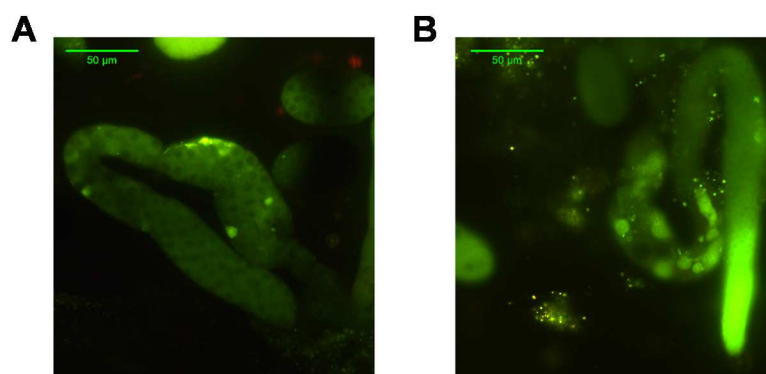


Figure 6.15. Representative images of extracted gonads of tumoral animals stained with acridine orange and treated with A) 100 μ M of cisplatin or B) 100 μ M of **37**. Scale bars 50 μ m.

6.3. CONCLUSIONS

A new inert Ir(III) complex of the type $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{N}^{\wedge}\text{N})][\text{PF}_6]$ has been synthesized and characterized to investigate its potential as an antitumor drug *in vivo*. Complex **37**, containing a new 2-benzothiazol-yl benzimidazole core with an ester group as $\text{N}^{\wedge}\text{N}$ ligand, showed an intense red luminescence in water, which is suitable to visualize the complex inside the cell or *in vivo*. The potential anti-cancer effects were evaluated using cell lines and a recently developed *C. elegans* tumoral model, in which the tumor reduction and the survival of treated specimens can be measured. Complex **37** reduced the tumor size as much as cisplatin in the *C. elegans* tumoral model, being less toxic for the animals. In the ovarian cancer cell lines, the IC_{50} was much lower than cisplatin, while the selectivity factor of the compound was higher.

Interestingly, **37** induces necrosis rather than apoptosis in A2780 cells and localizes both in cytoplasmic membrane and mitochondria (Pearson coefficient = 0.56 for the latter) of cancer cells as shown by confocal microscopy. Furthermore, the measurements of superoxide anions showed increased levels both in ovarian cell lines and in *C. elegans*, suggesting that these radicals play a crucial role in the mechanism of action of **37** that leads to cell death by necrosis, a non-apoptotic cell death that is another efficient way to overcome cisplatin resistance during the post-target stage. The aforementioned indicates that the antitumor mechanism of action of the complex evaluated *in vitro* corresponds pretty well too *in vivo* study, and therefore shows the antitumor action prevalence of the drug when moving from two-dimensional *in vitro* models to the *in vivo* environment.

6.4. REFERENCES

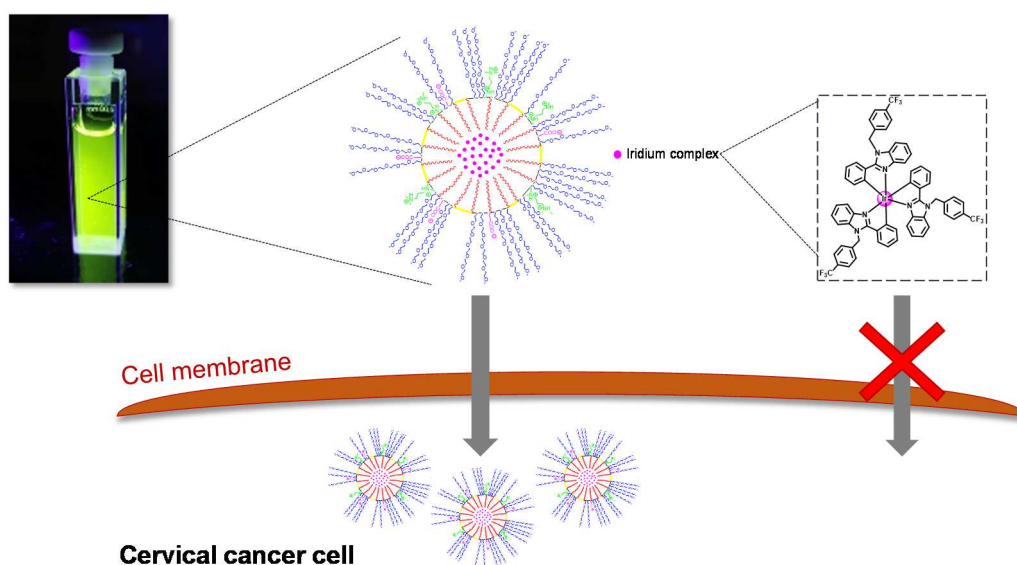
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Chapter 7

Vehiculation of highly luminescent neutral Ir(III) complexes to improve their biocompatibility and activity against HeLa cells



The biological experiments included in this chapter were carried out by Enrique Ortega Forte (Departamento de Química Inorgánica, Universidad de Murcia). The preparation and characterization of nanoparticles were carried out by Joaquin Bonelli Blasco (Universidad de Barcelona, Nanobiotechnological Polymers Division, Ecopol Tech).

7.1. INTRODUCTION

So far, the most studied phosphorescent materials are neutral Ir(III) complexes.^{1,2} This is because they possess excellent properties: stability of the octahedral geometry, triplet emission lifetimes of microseconds that ensure high efficiency in the fabricated organic light-emitting diodes (OLEDs), high emission quantum yields, and tunable emission color.¹ These phosphorescent neutral Ir(III) compounds can be divided into two main categories: bis- and tris-cyclometalated.

Tris-cyclometalated iridium(III) complexes show geometric *fac-mer* isomerism (**Figure 7.1**). Of the two possible isomers, the *fac*-isomer is thermodynamically favored, while the *mer*-isomer is kinetically favored. The lower thermodynamic stability of the meridional isomer is mainly because two carbon atoms (with a high *trans* effect) of the C^N ligands are mutually in a *trans* arrangement.³ This has caused that in therapeutic applications, the *fac* isomer is mainly used although, generally, it is obtained with a lower yield. The reaction temperature, time, and type of the cyclometalated ligand affect the synthesis processes' facial/meridional product ratio. Thus, the *mer*-isomer can be obtained by performing the reactions at lower temperatures (inhibiting the *fac*-isomers formation), often supported by admixing of base (e.g., triethylamine).⁴ On the other hand, the *fac*-isomer synthesis requires more tough conditions: ligand excess and higher temperatures (e.g., refluxing in 2-ethoxyethanol or glycerol). The meridional isomer can be efficiently transformed to the facial at high temperatures or irradiating with light.⁵

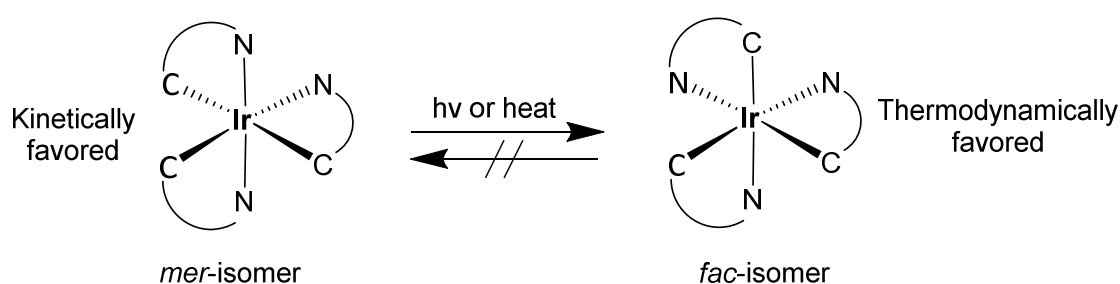


Figure 7.1. Facial and meridional isomers of the tris-cyclometalated iridium complexes.

Recently, the OLEDs application of phosphorescent benzimidazole-based cyclometalated iridium compounds has been studied.⁶ Benzimidazole-type cyclometalating ligands are essential components to prepare highly efficient Ir(III) emissive phosphors because of their excellent thermal stability, excellent electron mobility, and flexible modification. However, as commented in Chapter 1, applying these highly luminescent neutral complexes in living systems is limited due to its lack of charge, making it difficult to enter the cell. Thus, the complex structures can be modified with groups that increase water solubility,^{7,8} cationic peptides,^{9,10} or pH-sensitive groups.^{11,12} Furthermore, nanocarriers that can transport the complexes inside the cells are another emergent strategy.¹³

Nanomedicine is a developing method for treating different diseases where sub-micron particles (e.g., nanoparticles) are employed in medicine.^{14,15} Recent advances in nanotechnology have opened up unprecedented possibilities for the efficient delivery and protection of drugs with the development of several types of drug-loaded nanocarriers (**Figure 7.2A**), such as liposomes, carbon and inorganic nanomaterials, and polymeric particles.¹⁶ Recently, nanotechnology has been very necessary to obtain vaccines against the SARS-CoV-2 virus. For instance, lipid nanoparticles are a vital component of the new Pfizer and Moderna mRNA COVID-19 vaccines, playing a pivotal role in transporting and protecting the mRNA effectively to the right place in cells.^{17,18}

In cancer therapy, the characteristics of nanoparticles (NPs), including their small sizes, high surface area, favorable drug release profiles, water solubility, and targeting modifications, can allow them to reach target tumor tissue better and release drugs in a stable and controlled manner. In addition, NPs can protect the payloads from degradation and premature release and accumulate in leaky vasculature while sparing healthy cells thanks to the enhanced permeability and retention (EPR) effect (**Figure 7.2B**). This effect exploits the atypical vascular architecture, the leaky blood vessels, and a damaged lymphatic drainage system of tumor tissue,¹⁹ resulting in the efficient retention in the tumor interstitium. Accordingly, incorporating the drug into nanoparticles improves biodistribution, solubility, pharmacokinetics, and toxicity, reducing the adverse side effects observed with conventional treatments.²⁰ Thus, Doxil® (liposomal doxorubicin) is the first liposome nanomedicine approved for the Food and Drug Administration (FDA) that exhibits passive tumor targeting features and has fewer side effects comparing free doxorubicin (DOX).²¹

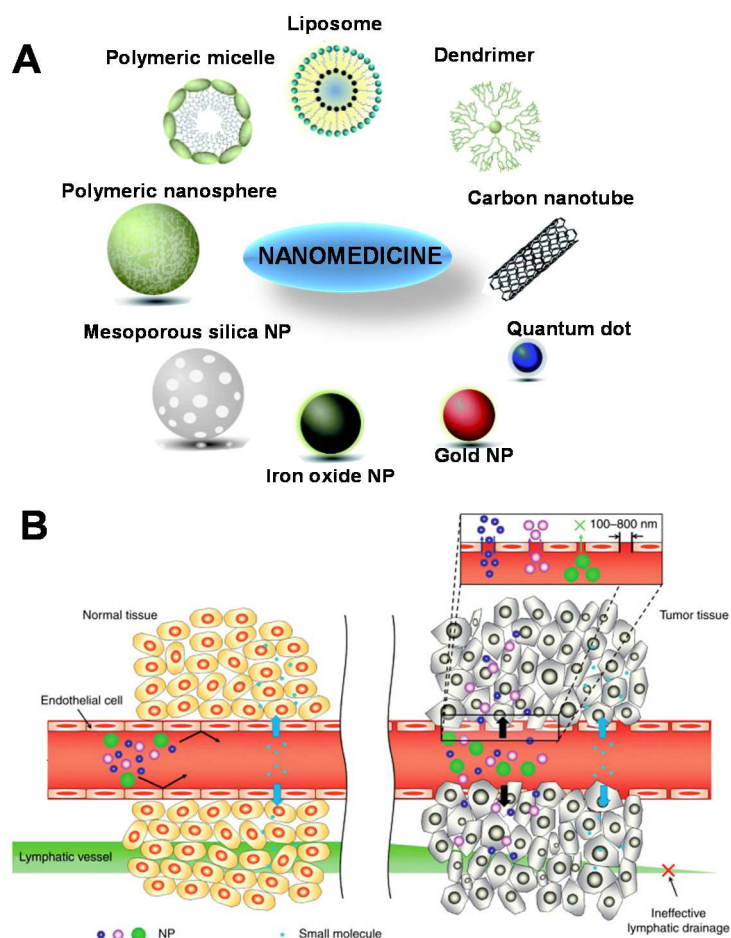


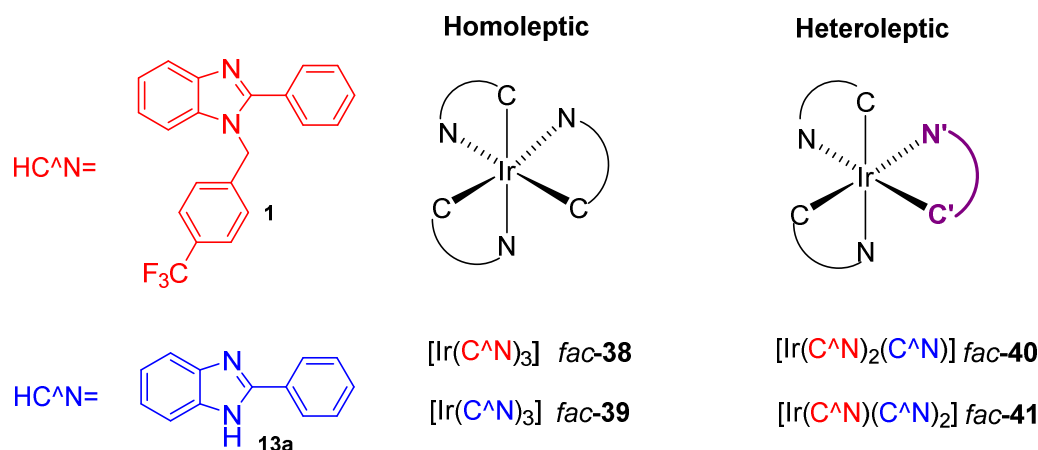
Figure 7.2. A) Organic and inorganic nanomaterials used in medicine. B) Schematic EPR effect showing differential uptake of NPs and small molecules based on their size across normal and tumor tissues. Adapted from reference.²²

Herein we report the synthesis of four new neutral tris-cyclometalated Ir(III) complexes: two homoleptic $[\text{Ir}(\text{C}^{\wedge}\text{N})_3]$ and two heteroleptic $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{C}'^{\wedge}\text{N}')]_2$. They exhibited high emission intensity and quantum yields in organic mediums. However, they could not enter the cell due to its lack of charge (which also limited its solubility in water), as demonstrated by confocal microscopy. Thus, two of them were encapsulated using polyurethane-polyurea hybrid nanocapsules to improve their biocompatibility and cellular uptake and study their potential activity against multidrug-resistant HeLa cancer cells. Incorporating neutral Ir(III) complexes into nanoparticles offers an exciting improvement in delivery, targeting, and cellular uptake for a safer and more successful therapy in the future.

7.2. RESULTS AND DISCUSSION

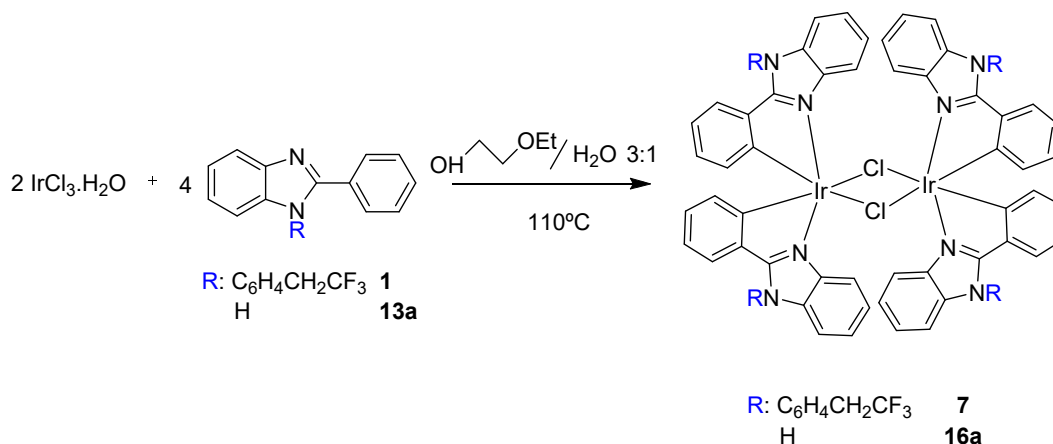
7.2.1. Synthesis and characterization

A series of four luminescent, tris-cyclometalated Ir(III) complexes of the type $[\text{Ir}(\text{C}^{\wedge}\text{N})_3]$ and $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{C}^{\wedge}\text{N}^{\prime})]$ (**Scheme 7.1**) were synthesized and characterized.



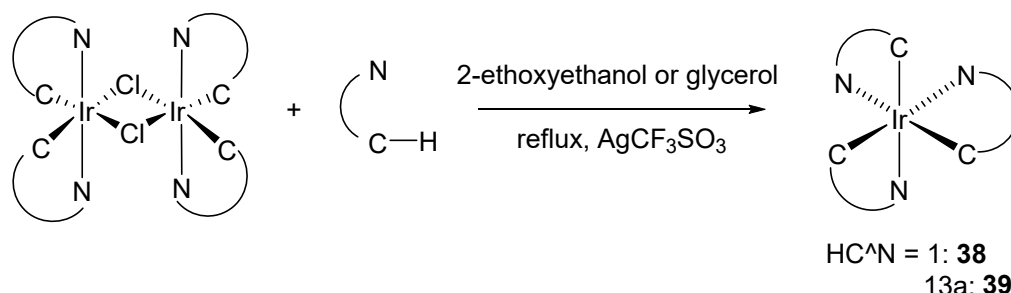
Scheme 7.1. Structures of new tris-cyclometalated iridium complexes.

The $\text{HC}^{\wedge}\text{N}$ pro-ligands have in common the benzimidazole core that has been demonstrated to be an extensively used pharmacophore^{23–25} and has given good results in previous chapters of this thesis. 2-phenyl-1H-benzo [d]imidazole (**13a**) is commercial and 2-phenyl-1-[4-(trifluoromethyl)benzyl]-1H-benzo[d]imidazole (**1**) was synthesized according to literature.²⁶ The new neutral iridium(III) complexes (two homoleptic and two heteroleptic) were synthesized in the thermodynamically favored *fac*-configuration. The first step for all of them was the synthesis of the dimer by reaction of iridium(III) chloride hydrate with the corresponding $\text{HC}^{\wedge}\text{N}$ pro-ligand (**Scheme 7.2**).



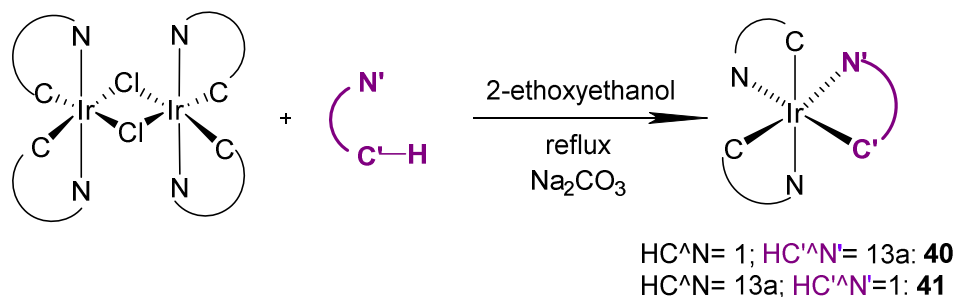
Scheme 7.2. Synthesis of starting dimeric iridium complexes (**7** and **16a** previously reported in Chapters 2 and 3, respectively).

Homoleptic complexes were synthesized by reaction of dimer complex (**7** or **16a**) with an excess of ligand, using a high boiling point solvent (2-ethoxyethanol for complex **38**²⁷ and glycerol for complex **39**⁷) and silver triflate to extract the chlorides (**Scheme 7.3**).



Scheme 7.3. Synthesis of new homoleptic Ir(III) complexes.

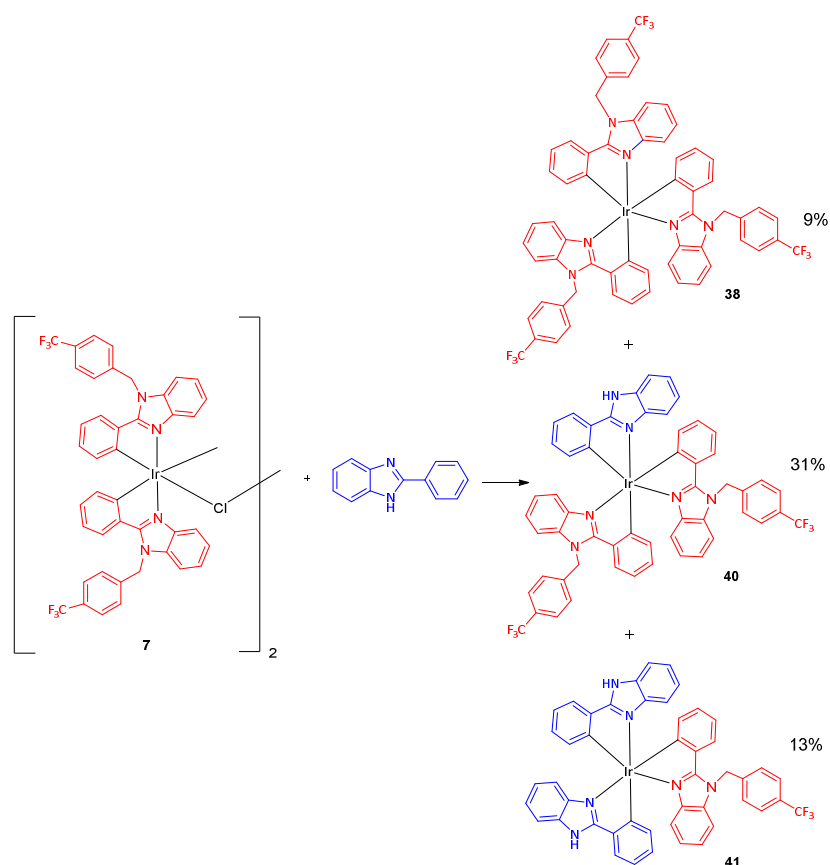
Heteroleptic complexes were synthesized in reflux of 2-ethoxyethanol for 3 hours using sodium carbonate (**Scheme 7.4**).²⁸ The product of the reaction was a mixture of the three possible neutral compounds: [Ir(C^NN)₃], [Ir(C^NN)₂(C'^NN')] and [Ir(C^NN)(C'^NN')₂], whose proportion depends on the starting dimer (**Scheme 7.5** and **Scheme 7.6**). Similar behavior as previously described.²⁹



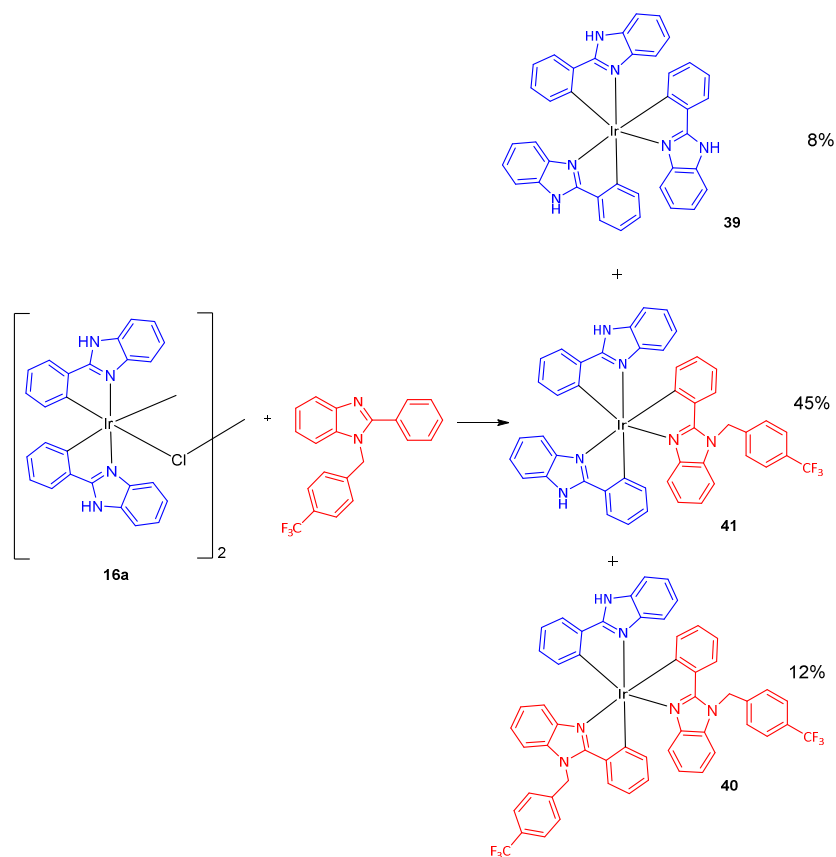
Scheme 7.4. Synthesis of new heteroleptic Ir(III) complexes.

The purification of heteroleptic complexes was carried out using silica column chromatography and dichloromethane as eluent, taking advantage of the differences in the retention times (R_f) of the complexes with a different number of NH groups. As a result, the complex **40** (**Scheme 7.5**) was eluted in second place (31%), preceded by the complex **38** (9%), and finally, the most retained product was the complex **41** (13%).

In the synthesis of complex **41**, that complex was the main product with a yield of 45% (**Scheme 7.6**). The compound with more NH groups and more retained was the homoleptic complex **39** (8%), and the first product was the complex **40** (12%).



Scheme 7.5. The mixture of the three neutral compounds obtained in the synthesis of **40**.



Scheme 7.6. The mixture of the three neutral compounds obtained in the synthesis of **41**.

All investigated complexes were characterized by ^1H and ^{13}C NMR spectroscopy (**Figures S7.1-S7.4**) and positive-ion ESI-HRMS (**Figure S7.5**). The *fac-mer* configuration of the complexes can be assigned using ^1H -NMR. In *fac*-tris-cyclometalated homoleptic complexes, the three C $\wedge$ N ligands surrounding the central atom are magnetically equivalent due to the C_3 symmetry of the compounds. This fact makes the total number of resonances equal to the number of resonances of a single C $\wedge$ N ligand.³⁰ The signals in the ^1H -NMR spectrum of **39** (**Figure 7.3**) correspond to eight aromatic protons of three equivalent **13a** ligands. The same occurs to **38** (**Figure S7.1**).

Recently, *fac* isomerism has been assigned to heteroleptic Ir(III) complexes with phenylpyridine ligands based on the synthesis temperature ($\sim 135\text{ }^\circ\text{C}$) and the simplicity of their ^1H -NMR spectra.³¹ In our case, given the impossibility of obtaining valid monocrystals for X-ray diffraction, the *fac* configuration was assigned for heteroleptic complexes **40** and **41** based on their ^1H -NMR spectra and due to they were synthesised at 136°C . In all cases, the positive-ion ESI mass spectra exhibit the expected molecular ion (**Figure S7.5**).

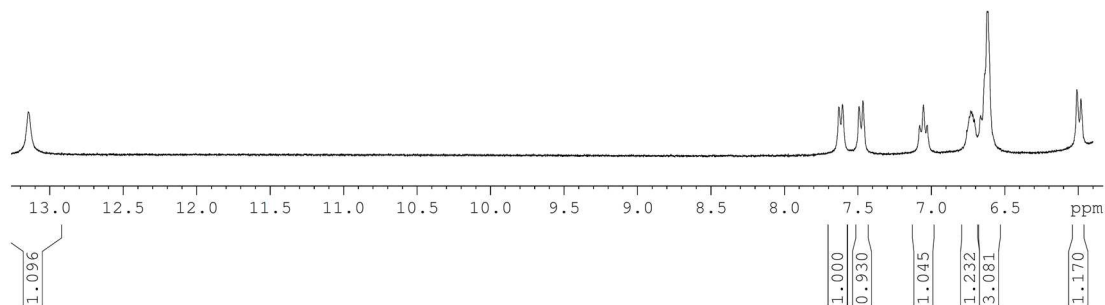


Figure 7.3. The ^1H -NMR spectrum of **39** in $[\text{D}_6]\text{DMSO}$.

The complexes with the highest number of NH groups (**39** and **41**) showed more significant solubility in DMSO, while **38** and **40** exhibited greater solubility in less polar solvents (e.g., dichloromethane). In any case, none displayed great solubility in DMSO:water mixtures, probably due to its neutral nature. Therefore, the solution stability study was carried out in DMSO. All complexes were shown to be stable in DMSO after 3 days by ^1H -NMR (**Figure 7.4, S7.6-S7.8**).

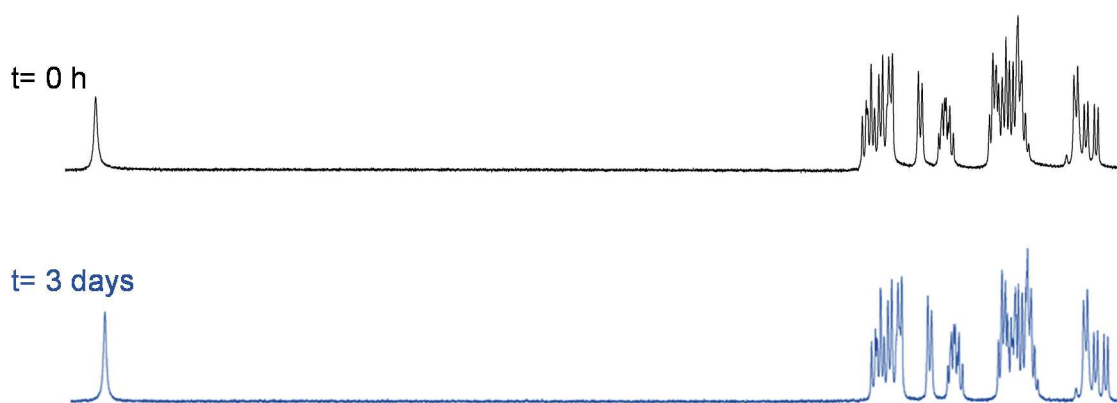


Figure 7.4. ^1H NMR spectra of **41** measured after dissolving immediately in $[\text{D}_6]\text{DMSO}$ (top) and after three days (bottom) at RT.

7.2.2. Photophysical properties

The UV/Vis absorption spectra of complexes (**Figure 7.5**) were recorded in CH_2Cl_2 and DMSO solutions (10 μM). It was only impossible to measure the luminescent properties of **39** in dichloromethane due to its insolubility in this solvent. All complexes exhibit characteristic absorptions of the tris-cyclometalated iridium complexes,^{32–35} with very similar spectra. Between 240 and 330 nm, it can be observed intense bands (molar extinction coefficients of the order of 10^4 M cm^{-1}) assigned to the permitted transitions centered on the ligands ($^1\pi\text{-}\pi^*$). On the other hand, the broad bands of lower energy between 330 and 440 nm and the rest of weak absorptions between 440–520 nm are assigned to $^1\text{MLCT}$ and $^3\text{MLCT}$ transitions.

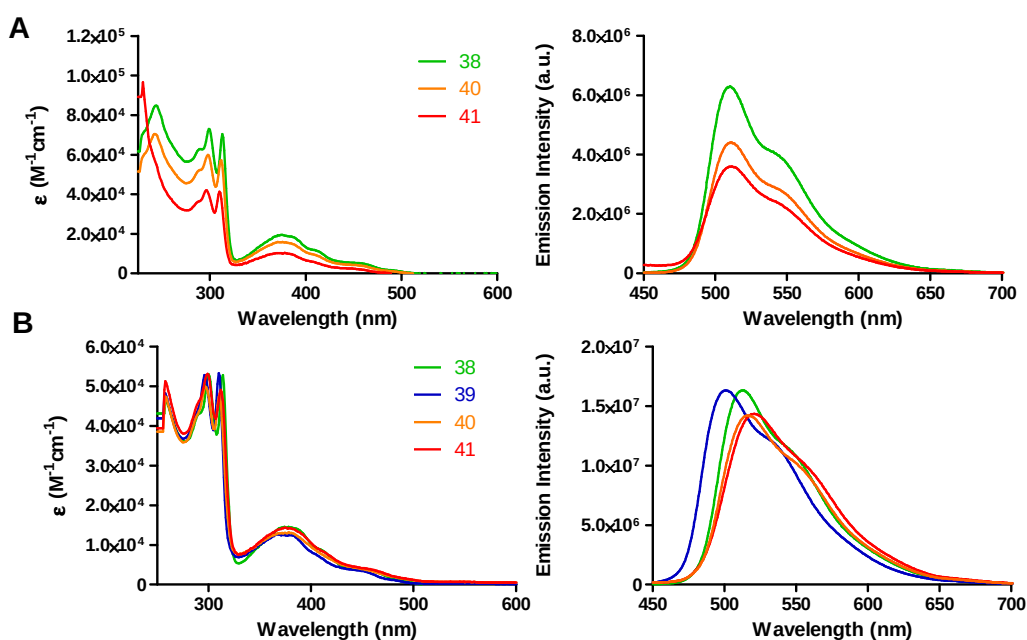


Figure 7.5. UV/Vis and emission spectra of **38–41** in A) aerated CH_2Cl_2 , $\lambda_{\text{exc}} = 380 \text{ nm}$ and B) aerated DMSO, $\lambda_{\text{exc}} = 405 \text{ nm}$.

The photophysical properties are further summarized in **Table 7.1**. Upon excitation at the absorption wavelength maxima, the complexes exhibit very similar green phosphorescent emissions in both solvents. However, the heteroleptic complexes (**40** and **41**) show slight redshifts relative to the homoleptic. On the other hand, **38-41** exhibited high emission quantum yields (Φ). As shown in **Table 7.1**, the Φ in degassed solution were in the range of 65-81%, while in air-equilibrated solutions, the quantum yields decreased over 3%. On the other hand, their luminescence lifetimes go from nanoseconds in aerated solution to μ s in degassed solution. These long-lived excited states (**Figure S7.9**) compare well with the respective values for other phosphorescent Ir(III) compounds, which supports the triplet nature of the emission state.^{32,33}

Table 7.1. Excitation (λ_{exc}), emission (λ_{em}) wavelengths, emission lifetimes (τ_{em}), and quantum yields (Φ_{em}) of complexes in dichloromethane and DMSO

Compound	Solvent	λ_{exc} , nm ^a	λ_{em} , nm	Aerated		Degassed	
				τ_{em} , ns	Φ_{em}	τ_{em} , ns ^b	Φ_{em} ^b
38	CH ₂ Cl ₂	375	509	35.6 (7%)	0.026	764 (53%)	0.784
				47.9 (93%)		1295 (47%)	
				171 (99.7%)		1230 (100%)	
	DMSO	380	512	47.8 (0.3%)	0.086		0.810
39	CH ₂ Cl ₂	--	--	--	--	--	--
				128 (26%)	0.085	1181 (100%)	0.678
				153 (74%)			
	DMSO	390	500				
40	CH ₂ Cl ₂	380	511	46.7 (98%)	0.025	596 (37%)	0.655
				139 (2%)		777 (63%)	
				171 (100%)		1286 (100%)	
	DMSO	385	517		0.094		0.799
41	CH ₂ Cl ₂	375	510	58 (96%)	0.031	716 (40%)	0.705
				98 (4%)		1053 (60%)	
				180 (38%)		1233 (100%)	
	DMSO	400	520	153 (62%)	0.104		0.742

^a λ_{exc} maxima. ^b 20 min under argon.

7.2.3. Preliminary biological study

The cytotoxicity of complex **38** was evaluated against human ovarian and cervical tumor cell lines (A2780, cisplatin-resistant A2780cisR and multidrug-resistant HeLa³⁶) and against the BGM healthy cell line (green primate kidney) by MTT-based assay. For comparison purposes, the cytotoxicity of cisplatin was also evaluated under the same experimental conditions. **Table 7.2** shows the IC₅₀ values obtained and indicates that complex **38** is not toxic against any of the lines tested. These results may be a consequence of the zero interference of the complex in cellular functions, the impossibility of crossing the cell membrane due to the lack of charge or a sum of both effects.

Table 7.2. Cytotoxicity [IC₅₀ mean values (μM)] obtained for **38** and cisplatin.^a

	A2780	A2780CisR	HeLa	BGM
38	>80	>80	>100	>80
Cisplatin	1.45 ± 0.16	71.99 ± 0.54	33 ± 4.01	16.62 ± 0.16

^a The results are expressed as mean values ± SD of three independent experiments, each of them made in quadruplicate.

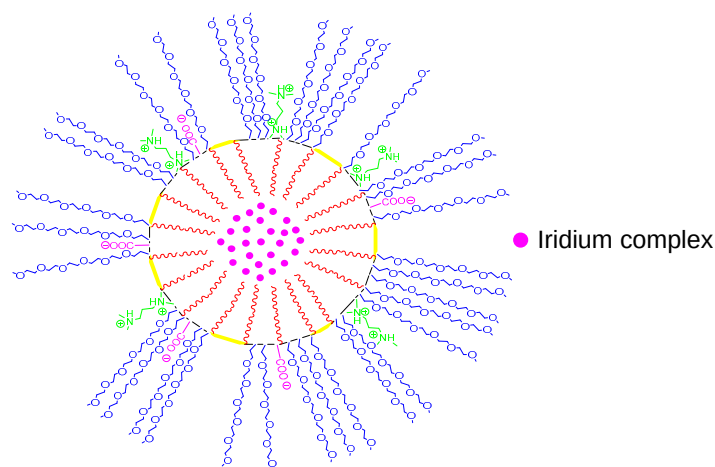
Thus, the cellular location of the complex was studied by confocal microscopy in A2780 cells. As shown in **Figure 7.6**, the iridium compound seems to be not able to penetrate the cell. These facts motivated encapsulation of complexes **38** and **40** (one homoleptic and another heteroleptic) due to their excellent photophysical properties and to investigate their possible biological activity.



Figure 7.6. Confocal microscopy studies of A2780 cells treated with **38**. A) Luminescence channel; B) bright-field channel; C) merge of the luminescence and bright field channels.

7.2.4. Encapsulation of 38 and 40

The encapsulation of neutral Ir complexes was performed by Joaquin Bonelli Blasco (University of Barcelona, Nanobiotechnological Polymers Division, Ecopol Tech). The preparation of Ir(III) complex-loaded nanocapsules (**Scheme 7.7**) involved two main processes: (1) the preparation of an amphiphilic polyurethane/polyurea amino terminal-reactive prepolymer and (2) the iridium complex nanoencapsulation. The physical encapsulation of complexes relying on non-covalent interactions between the drug and the polymer matrix.



Scheme 7.7. Schematic representation of the structure of a metallodrug-loaded nanocapsule, showing the orientation of the hydrophilic and hydrophobic tails of the prepolymer.

The encapsulation reaction was controlled by Infrared (IR) spectroscopy, and the nanocapsules obtained were characterized by dynamic light scattering (DLS). The particle size distribution of the Ir(III) complex-loaded nanoparticles (**NPs-38** and **NPs-40**) was measured by DLS and compared with non-loaded NPs. As observed in **Table S7.1**, the size was approximately 18 nm for **NPs-38/40**, which was slightly lower than non-loaded NPs (22 nm), with a very low standard deviation. The Z-potential of the nanocapsules (**Figure S7.10**) was determined at different pH values (6.5, 7.0, and 7.5). The Ir(III) complex-loaded nanoparticles are slightly anionic under physiological conditions (pH = 7.4), mainly those containing the heteroleptic complex **40**, but become cationic at low pH values. The above allows a prolonged circulation through the bloodstream and a slow accumulation in the tumor tissues where the environment is acidic (pH = 5.8-6-9). The nanoparticles, therefore, become cationic there, increasing their internalization capacity through association with negatively charged cell receptors.

The amount of **38** and **40** inside the NPs was determined by ICP-MS analysis (**Table S7.2**). The encapsulation efficiency was relatively high in both cases ($\sim 40\%$), considering that no external surfactants were required. As a result, the following loading of both iridium compounds was obtained: $130\ \mu\text{M}$ in **NPs-38** and $206\ \mu\text{M}$ in **NPs-40**.

7.2.5. Photophysical study of nanoparticles loaded with complexes

The UV/Vis absorption and emission spectra were recorded in water solutions for the nanocapsules (**Figure 7.7**). The lifetimes and quantum yields for NPs are summarized in **Table 7.3**. As observed in **Figure 7.7**, both absorption spectrum and emission bands of **NPs-38** and **NPs-40** resemble that registered with **38** and **40** in CH_2Cl_2 (**Figure 7.5**). In addition, the luminescence lifetimes increased from ~ 70 - $90\ \text{ns}$ in air-equilibrated solutions to $\sim 1.5\ \mu\text{s}$ in degassed solutions, as it happened with the free complexes in CH_2Cl_2 (**Table 7.1**, **Figure S7.11**). This fact is indicative of the hydrophobic environment generated by the nanoparticles.

On the other hand, the nanocapsules (**NPs-38** and **NPs-40**) exhibited high emission quantum yield in water (**Table 7.3**). Unlike free complexes, which were easily quenched by oxygen, the oxygen quenching of the emission was less efficient when the complexes were encapsulated in the nanoparticles. While the emission quantum yields of **38** and **40** in an air-equilibrated solvent ($\Phi_{\text{air}} \approx 3\%$) have a 30-fold reduction in luminescence features concerning degassed solutions ($\Phi \approx 78\%$), **NPs-38** and **NPs-40** showed a less difference between aerated ($\Phi_{\text{air}} \approx 20\%$) and degassed ($\Phi \approx 40\%$) solutions. Interestingly, the nanocapsules displayed quantum yields of 12-20% in aerated aqueous solution, which indicated that these nanostructures can be used in the presence of dissolved oxygen and still exhibit a high quantum yield.

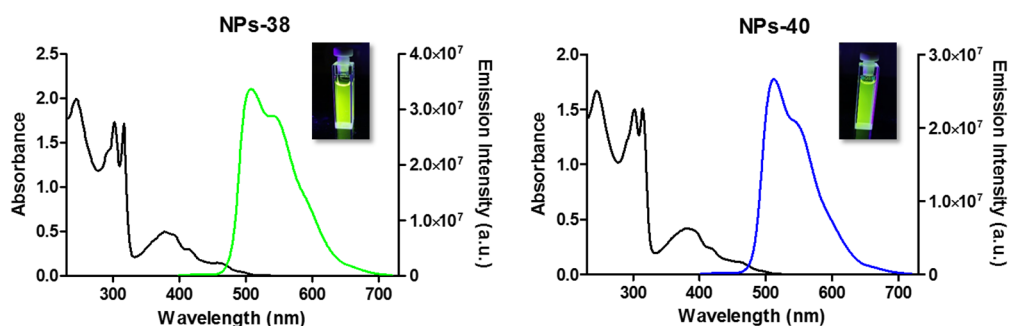


Figure 7.7. UV-visible and emission spectra in aerated water solutions of A) **NPs-38** ($\lambda_{\text{exc}} = 378\ \text{nm}$) and B) **NPs-40** ($\lambda_{\text{exc}} = 382\ \text{nm}$).

Table 7.3. Excitation (λ_{exc}), emission (λ_{em}) wavelengths, emission lifetimes (τ_{em}), and quantum yields (Φ_{em}) of **NPs-38** and **NPs-40** in water.

Compound	Solvent	λ_{exc} , nm ^a	λ_{em} , nm	aerated		degassed	
				τ_{em} , ns ^b	Φ_{em} ^b	τ_{em} , ns ^c	Φ_{em} ^c
NPs-38	Water	378	507	22.3	0.197	417	0.411
				(8%)		(6%)	
				75.6 (92%)		1623 (94%)	
NPs-40	Water	382	511	19.5	0.124	282	0.198
				(21%)		(6%)	
				95.1 (79%)		1537 (94%)	

^[a] λ_{exc} maxima. ^[b] air equilibrated solution ^[c] deaerated solution (20 min under argon).

7.2.6. Cellular accumulation

To find out if the nanoparticles were able to enter the cell, their cellular uptake and accumulation in HeLa cells were determined by ICP-MS after the cells were treated with the tested compounds or nanocapsule agents.

The data collected in **Table 7.4** indicate that the nanoparticles (**NPs-38** and **NPs-40**) accumulate more effectively (6 to 21– fold higher) than free complexes **38** and **40**, confirming the vehiculation of the complexes inside the cell.

Table 7.4. Intracellular iridium content measured by ICP-MS after 30 min treatment.^a

ng Ir/10 ⁶ cells	
Control	0.81 ± 0.2
38	24.96 ± 1.05
NPs-38	532.84 ± 35.89
40	60.30 ± 2.04
NPs-40	356.94 ± 26.44

^a Results are the mean ± SDs from two independent experiments.

7.2.7. Cytotoxicity studies of the nanoparticles

The cytotoxicity of the nanoparticles loaded with iridium complexes (**NPs-38** and **NPs-40**) was evaluated against cervical cancer cells (HeLa) and non-tumorigenic normal renal cells (BGM) using MTT-based assays. The obtained IC₅₀ values, expressed as a function of the concentration of complex inside of nanoparticles, are shown in **Table 7.5**. Treatment with **NPs-38** and **NPs-40** resulted in high antitumor activity against cancer cells, while non-loaded NPs did not affect the cells (**Table S7.3**). These results indicated that the

cytotoxic action of the Ir(III) complexes greatly improved upon encapsulation. Furthermore, as observed in **Table 7.5**, the treatment with **NPs-38** and **NPs-40** affected the viability of HeLa cells after 24 and 48 h. On the other hand, an absence of cytotoxicity was observed in BGM healthy cells, thus indicating the ability of NPs-loaded agents to eliminate cancer cells over normal dividing cells preferentially.

Table 7.5. Cytotoxicity [IC_{50} mean values (μM)] obtained for nanoparticles and cisplatin in HeLa cells,^a

	HeLa			BGM
	2 h	24 h	48 h	24h
NPs-38^b	22.48 $\pm$ 1.14	2.89 $\pm$ 0.20	1.77 $\pm$ 0.06	>86
NPs-40^b	47.60 $\pm$ 6.35	3.56 $\pm$ 0.19	2.92 $\pm$ 0.13	>95
Cisplatin	-	-	34.08 $\pm$ 4.10	16.80 $\pm$ 0.17

^aThe results are expressed as mean values $\pm$ SD of two independent experiments; ^b IC_{50} mean values obtained as a function of the amount of Ir complex inside of nanoparticles.

7.3. CONCLUSIONS

Four neutral tris-cyclometalated Ir(III) complexes (two homoleptic and two heteroleptic) were synthesized and characterized. All complexes exhibited very high green phosphorescent emission in both dichloromethane and DMSO solvents. However, the iridium complexes could not penetrate the cell due to their lack of charge, as shown by confocal microscopy. Therefore, two of them were encapsulated using polyurethane-polyurea hybrid nanocapsules to improve their biocompatibility and cellular accumulation. The cellular uptake of Ir(III) compounds determined by ICP-MS showed that the iridium content inside cancer cells treated with **NPs-38** and **NPs-40** was 6 to 21– fold higher than those treated with **38** and **40**, thus suggesting that treatment with Ir-loaded NPs allowed Ir compounds to enter cells rapidly.

Furthermore, the antiproliferative study demonstrated the effective cytotoxic action of **NPs-38** and **NPs-40** against multidrug-resistant HeLa cancer cells. In addition, NPs-loaded agents showed low cytotoxicity in the non-tumorigenic kidney cells BGM, indicating the ability of **NPs-38** and **NPs-40** to eliminate cancer cells over normal cells preferentially. Thus, this incorporation of neutral Ir(III) complexes into nanoparticles could offer an exciting improvement in delivery, targeting, and cellular uptake for a safer and more successful cervical cancer therapy.

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Chapter 8

Conclusions

1. A structure-activity relationship (SAR) study for *in vitro* antiproliferative activity of a new series of cationic iridium complexes of the type $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{N}^{\wedge}\text{N})][\text{PF}_6]$, in which the cyclometalating ligand 2-phenyl-1-[4-(trifluoromethyl)benzyl]-1H-benzo[d]imidazole remains as the $\text{C}^{\wedge}\text{N}$ backbone and the diimine ligand is varied $\{\text{N}^{\wedge}\text{N}=\text{phen, dpq, dppz, dppn, and dppz-izdo}\}$, demonstrates that the auxiliary $\text{N}^{\wedge}\text{N}$ ligand affects both the photophysical properties and the antiproliferative activity of the complexes. The photophysical properties are modified depending on the length and degree of π conjugation of the $\text{N}^{\wedge}\text{N}$ ligand; the complexes with dppn and dppz-izdo exhibit a luminescence ACQ effect in water. Their cytotoxicity under dark conditions decreases as the length of the $\text{N}^{\wedge}\text{N}$ ligand increases, but an enhancement of the activity of these complexes is observed under blue light irradiation with a marked increase in intracellular reactive oxygen species (ROS). In addition, they preferentially target the endoplasmic reticulum and their possible mechanism of action in the dark involve inhibition of protein translation.
2. A second SAR study was performed by modifying the $\text{C}^{\wedge}\text{N}$ ligand based on 2-phenyl-, 2-(naphthalen-2-yl)- or 2-(thiophen-2-yl)-benzimidazole scaffolds in complexes of the general formula $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{dppz})][\text{PF}_6]$, demonstrates the influence of this ligand in their photodynamic activity. The complexes with the substituent 4-(trifluoromethyl) benzyl in the imidazole unit of the $\text{C}^{\wedge}\text{N}$ ligand show the highest emission intensity of the three series, whereas H derivatives suffer important emission quenching. The substituent in the imidazole unit also plays a fundamental role in their antiproliferative activity in human cervical cancer HeLa cells under normoxia and hypoxia. Thus, the methyl derivatives were the most active in the dark while complexes with the group $\text{CH}_2\text{C}_6\text{H}_4\text{CF}_3$ -p exhibit the lower toxicity in the dark and have a high phototoxicity index. On the other hand, the substitution in position 2 of the benzimidazole seems to be directly related to the location in the cell: the sulfur-containing complexes mostly localize in mitochondria, whereas the 2-aryl-benzimidazole complexes targeted endoplasmic reticulum. The ROS production studied by DHE and SOSG staining assays with sulfur-containing complexes showed that the generation of superoxide radicals competed with singlet oxygen production in cells in normoxia, but became dominant under hypoxia, showing the switch from Type II to Type I photochemical mechanism.

3. The introduction of a benzimidazole scaffold with NH groups in the auxiliary ligand of $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{N}^{\wedge}\text{N})][\text{PF}_6]$ complexes results in very low cytotoxicity to their correspondent complexes in the dark, a crucial condition to be met for the design of ideal photosensitizers. The new complexes show high phototoxicity by blue light irradiation under normoxic and hypoxic conditions in human skin melanoma cells (A375 and 518.A2) and human cervix adenocarcinoma cells (HeLa), being **29** the most active with the highest phototoxicity indexes. This complex also displays the highest accumulation in cancer cells and the most elevated photoinduced ROS generation. On the other hand, **29** is localized in the mitochondria (PCCs = 0.63), although the co-localization study with ER-Tracker suggests that this complex can also be partially associated with this organelle. In addition, **29** induces immunogenic cell death in melanoma cells to the same extent as known ICD inducers (oxaliplatin and doxorubicin), but only if the cells are irradiated by light. Complex **29** shows a potent ability to inhibit the growth of CSCs-like melanoma cells after light irradiation, with markedly high selectivity toward the CSCs.
4. The combination of two transition metals in a single molecule in the dinuclear Ir(III)-Pt(II) complex of general formula $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{N}^{\wedge}\text{N})(\mu\text{-NHCH}_2\text{py})\text{Pt}(\text{DACH})\text{Cl}]^{2+}$ reveals the promising activity of this new complex to overcome resistance to drugs such as cisplatin. This heterobimetallic complex displays an intense red phosphorescence in water granted by the Ir(III) moiety. The antitumoral study shows the ability of the Ir(III)-Pt(II) complex to kill cisplatin-resistant cervical and ovarian cancer cells. In addition, the dinuclear complex exhibits no effect in non-tumorigenic normal renal cells, showing its better selectivity towards cancer cells than cisplatin. The heterodinuclear complex causes necrosis rather than apoptosis. In addition, the new compound appears not to affect the cell cycle in contrast to the platinum precursor and cisplatin which induce G2/M and S phase arrest. More studies are needed to fully investigate its mechanism of action.

5. A new bis-cyclometalated iridium complex has been synthesized to study the antitumor action prevalence of a drug when moving from two-dimensional *in vitro* models to the *in vivo* environment. This new inert iridium(III) compound containing a new 2-benzothiazol-yl benzimidazole core with an ester group as N^N ligand, shows an intense red luminescence in water. The potential effects are evaluated using cell lines and a recently developed *C. elegans* tumoral model. The new complex reduces the tumor size as much as cisplatin in the *C. elegans* tumoral model while being less toxic for the animals. In the ovarian cancer cell lines, the cytotoxicity is much lower than cisplatin, while the selectivity factor of the compound is higher. Interestingly, the iridium compound induces necrosis rather than apoptosis in A2780 cells. The measurements of superoxide anions show increased levels both in ovarian cell lines and in *C. elegans* suggesting that the superoxide anions play an important role in the mechanism of action. Thus, the antitumor mechanism of action of the complex evaluated *in vitro* corresponds pretty well too *in vivo* study.
6. The design of highly luminescent neutral Ir(III) complexes of the type $[\text{Ir}(\text{C}^{\wedge}\text{N})_3]$ and $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{C}'^{\wedge}\text{N}')]]$ proves the influence of their neutral nature in the biological study. These iridium complexes are not able to penetrate the cell due to their lack of charge, as shown by confocal microscopy. Therefore, two of them were encapsulated using polyurethane-polyurea hybrid nanocapsules to improve their biocompatibility and cellular accumulation. The cellular uptake shows that the iridium content inside cancer cells treated with Ir-loaded nanoparticles is 6 to 21-fold higher than those treated with free complexes. The antiproliferative study demonstrates the effective cytotoxic action of nanoparticles against multidrug-resistant HeLa cancer cells. In addition, these agents show low cytotoxicity in the non-tumorigenic kidney cells BGM, indicating the ability of the Ir-loaded nanoparticles to preferentially eliminate cancer cells over normal cells. This incorporation of neutral Ir(III) complexes into nanoparticles offer an exciting improvement in delivery, targeting, and cellular uptake for a more safer and successful therapy of cervical cancer.

Experimental Section

E1. INSTRUMENTAL AND SPECTROSCOPIC TECHNIQUES

E1.1. General Materials

The iridium chloride ($\text{IrCl}_3 \cdot \text{H}_2\text{O}$) was obtained from Johnson Matthey. The commercial cyclometalating pro-ligand 2-phenyl-1H-benzo [d]imidazole (**13a**) and the N^N ligand 2-(pyridin-2-yl)-1H-benzo[d]imidazole (**22**) was obtained from Sigma-Aldrich (Madrid, Spain) and used without further purification. Deuterated solvents were purchased from Euriso-top.

E1.2. Elemental analyses

The C, H, N and S analyses were performed with a Carlo Erba model EA 1108 microanalyzer.

E1.3. Direct insertion probe–mass spectrometry (MS-DIP)

Mass spectra were recorded on an Agilent Q-TOF 6550 hybrid spectrometer with JetStream electrospray + i-Funnel ionization source. Mass spectrometry data were acquired in the positive ionization mode. The isotopic distribution of the heaviest set of signals matched very closely to that calculated for the formulation of the complex cation in every case.

E1.4. RP-HPLC/MS spectra

The purity and stability of some iridium compounds were analyzed using an RP-HPLC/MS TOF 6220 equipped with a double binary pump (model G1312A), degasser, autosampler (model G1329A), diode array detector (model G1315D), and mass detector in series Agilent Technologies 1200. Chromatographic analyses were carried out on a Brisa C18 column (150 mm × 4.6 mm, 5 μm particle size). The mobile phase was a mixture of (A) $\text{H}_2\text{O}/\text{HCOOH}$ 0.1% and (B) acetonitrile/ HCOOH 0.1%. The flow rate was 0.6 mL min^{-1} in a linear gradient starting (see **Table E1.1** for the gradient used). Chromatograms were recorded at 280 nm. The HPLC system was controlled by ChemStation software (MASS HUNTER.). The mass detector was an ion trap spectrometer equipped with a dual-source electrospray-APCI. Mass spectrometry data were acquired in the positive ionization mode. The full scan mass covered the range from m/z 100 up to m/z 1000. Samples were dissolved in acetonitrile, DMSO or DMF.

Table E1.1 HPLC method

Time (min)	0.1% acid formic in H ₂ O	0.1% acid formic in CH ₃ CN
0-14	90	10
14-20	10	90
20-25	90	10

E1.5. Nuclear Magnetic Resonance spectra

¹H NMR and ¹³C NMR spectra were recorded with a Bruker AC 300E, Bruker AV 400, or Bruker AV 600 NMR spectrometer. Chemical shifts are cited relative to SiMe₄ (¹H and ¹³C, external).

E1.6. UV/Vis absorption spectroscopy

UV/Vis spectroscopy was carried out on a PerkinElmer Lambda 750 S spectrometer with operating software.

E1.7. Emission spectroscopy, quantum yields and lifetime measurements

Emission spectra were obtained with a Horiba Jobin Yvon Fluorolog 3–22 modular spectrofluorometer with a 450 W xenon lamp. Measurements were performed in a right angle configuration using 10 mm quartz fluorescence cells for solutions at 298 K. Emission lifetimes (τ) were measured using an IBH FluoroHub TCSPC controller and a NanoLED pulse diode excitation source ($\tau < 10\mu\text{s}$); the estimated uncertainty is $\pm 10\%$ or better. Emission quantum yields (Φ) were measured using a Hamamatsu C11347 Absolute PL Quantum Yield Spectrometer; the estimated uncertainty is $\pm 5\%$ or better. For measurements in deaerated conditions, solutions of the samples were previously degassed by bubbling argon for 30 min.

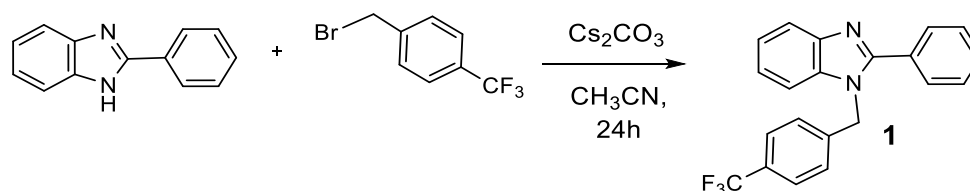
E1.8. Transmission electron microscopy

TEM experiments were performed on a PHILIPS TECNAI 12 transmission electron and JEOL 1011 transmission electron microscopes. The samples were prepared by dropping a few drops of water solutions (1% DMSO) of complexes **11** and **12** onto a carbon-coated nickel grid. Slow evaporation of solvents in the air for 10 min was allowed before placing the samples into the sample chamber of the instrument.

E2. SYNTHESIS PROCEDURES

E2.1. Synthesis of ligands

Preparation of HC^N proligand **1**

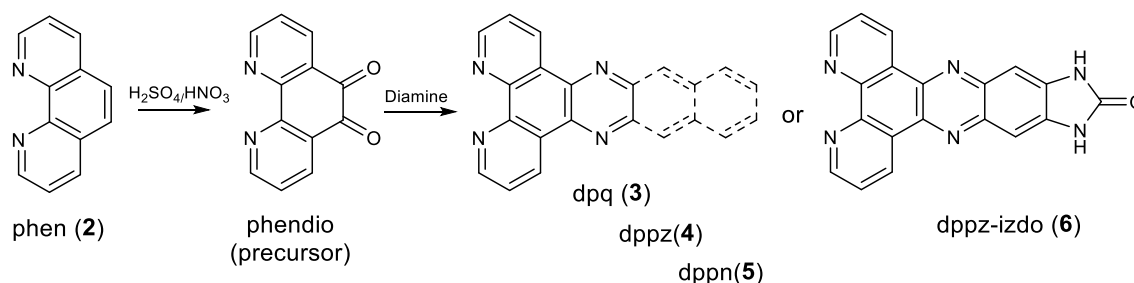


Scheme E2.1 Synthesis of HC^N proligand **1**.

The N-benylation was performed as previously reported.¹ A suspension of 2-phenylbenzimidazol (**1** mmol), cesium carbonate (1.5 mmol), and the 1-(bromomethyl)-4-(trifluoromethyl)benzene (1.05 mmol) in acetonitrile (1 mL/ 0.1 mmol benzimidazole) was stirred at RT for 24 h under nitrogen atmosphere. The progress of the reaction was monitored by TLC. After completion of the reaction, the mixture was concentrated in vacuo. The residue was suspended in a mixture of CH_2Cl_2 and sat. NaHCO_3 (vol % 50:50, 10 mL/0.1 mmol benzimidazole). The aqueous layer was extracted with CH_2Cl_2 (2 x 10 mL/0.1 mmol benzimidazole). The combined organic layers were dried over Mg_2SO_4 , filtered and the solvent was removed under reduced pressure.

2-phenyl-1-[4-(trifluoromethyl)benzyl]-1H-benzo[d]imidazole (**1**). White solid. Isolated yield: 92%. Reported.¹

Preparation of N^N ligands **3-6**



Scheme E2.2. Synthesis of polypyridyl N^N ligands **3-6**.

The precursor **phendio** and ligands **3-5** were synthesized using an adaptation method of reference.²

Preparation of precursor 1,10-phenanthroline-5,6-dione (phendio).

1,10-Phenanthroline (**2**) (10 mmol) and potassium bromide (15 mmol) were thoroughly mixed and slowly added to an ice-cold mixture of H₂SO₄ (20 mL) and HNO₃ (10 mL). The solution was refluxed for 3 h at 100°C, then cooled to room temperature, poured onto crushed ice (~200 mL), and neutralized with an aqueous NaOH solution (40 g per 200 mL) to a pH between 4 and 5, yielding a yellow solution. The solution was extracted with CH₂Cl₂ (in 4×100mL portions). The organic phase was combined and dried with anhydrous MgSO₄ and then filtered before being evaporated to dryness. Bright yellow solid was obtained. Isolated yield: 82%. Reported.²

*Dipyrido[3,2-*f*:2,3-*h*]quinoxaline (3, dpq).*

To a solution of phendio (1 mmol) in water (20 mL) was added ethylenediamine (5 mmol), and the resultant suspension was refluxed for 12 h at 60°C. The resulting product was washed with water (10 mL) and a minimum volume of diethyl ether. Isolated yield: 83%. Reported.²

*Dipyrido[3,2-*a*:2,3-*c*]phenazine (4, dppz).*

A methanolic solution (10 mL) of 1,2-phenylenediamine (1 mmol) was refluxed until it was dissolved. A warm ethanolic solution of phendio (1 mmol) was prepared (10 mL), added over the methanolic solution, and refluxed with constant stirring for 3 h. The resulting solution was vacuum-filtered and recrystallized from EtOH, producing gray filaments. Isolated Yield: 56%. Reported.²

*Benzo[*I*]dipyrido[3,2-*a*:2,3-*c*]phenazine (5, dppn).*

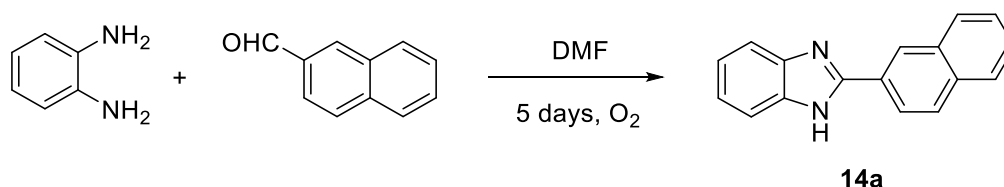
To a warm solution of phendio (1 mmol) in EtOH (32 mL) was added 2,3-diaminonaphthalene (1 mmol), and the resulting suspension was refluxed for 3 h, during which time an orange precipitate formed. The solid was vacuum-filtered, washed with cold EtOH, and allowed to dry. Isolated yield: 76%. Reported.²

*Dipyrido[3,2-*a*:2,3-*c*]phenazine-10,11-imidazolone-2 (6, dppz-idzo).*

The ligand **6** was synthesized using an adaptation method of reference.³ Phendio (1 mmol) was dissolved in 35 mL of EtOH with stirring. 5,6-diaminobenzimidazolone-2 (1 mmol) was added to this solution and the mixture was refluxed for 12 h at 78°C. The yellow

precipitate formed during that time was collected by filtration and dried. Isolated yield: 84%. Reported.³

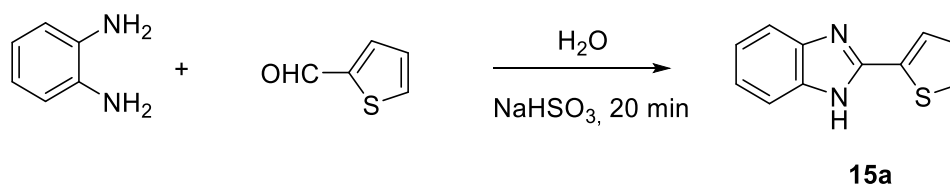
Preparation of 2-(naphthalen-2-yl)-1H-benzo[d]imidazole (14a):



Scheme E2.3. Synthesis of HC[^]N pro-ligand **14a**.

Pro-ligand **14a** was synthesized using an adaptation method of reference.⁴ 2-Naphthaldehyde (7.77 mmol) and o-phenylenediamine (7.4 mmol) were dissolved in 2 ml of DMF. The mixture was stirred at 80°C for 5 days. Then, the reaction mixture was suspended in 30 mL of CH₂Cl₂ and was extracted with water (5 x 60 mL). The organic layer was dried over MgSO₄, filtered and the solvent was removed under reduced pressure. The residue was stirred with diethyl ether for 3 h, and the solid was filtered. Brown solid. Isolated yield: 40%. Reported.⁴

Preparation of 2-(thiophen-2-yl)-1H-benzo[d]imidazole (15a):

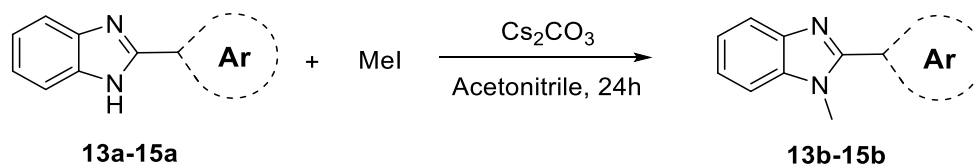


Scheme E2.4. Synthesis of HC[^]N proligand **15a**.

Pro-ligand **15a** was synthesized with a slight modification of the method of literature.⁵ Thiophene-2-carbaldehyde (1.0 mmol) and NaHSO₃ (11.0 eq, 1.14 g) was dissolved in H₂O (4.0 mL). When the mixture reached refluxing temperature, o-phenylenediamine (1.0 mmol) was added. The resulting mixture was stirred for 30 min. Then, the solid was filtered, washed with water (2 x 4 mL), and air-dried.

Pale yellow solid. Isolated yield: 78%. Reported.⁶

Preparation of pro-ligands **13b**, **14b**, and **15b**:



Scheme E2.5. Synthesis of HC^N pro-ligands **13b**, **14b**, and **15b**.

The N-methylation was performed with a modification of the method of literature.¹

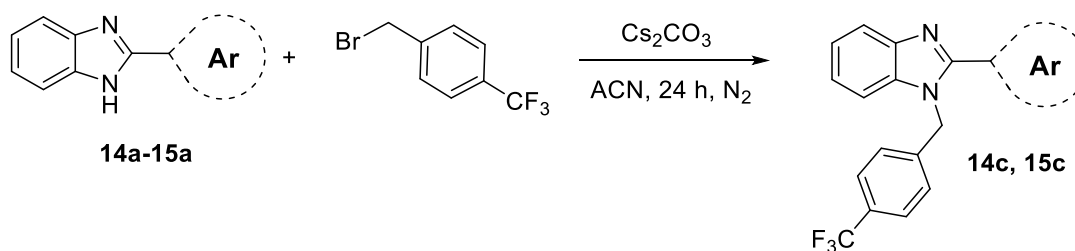
A suspension of **13a**, **14a**, or **15a** (1 mmol), cesium carbonate (1.5 mmol), and the methyl iodide (1.8 mmol) in acetonitrile (1 mL/ 0.1 mmol benzimidazole) was stirred at RT for 24 h under nitrogen atmosphere. The progress of the reaction was monitored by TLC. After completion of the reaction, the mixture was concentrated in vacuo. The residue was suspended in a mixture of CH₂Cl₂ and sat. NaHCO₃ (vol % 50:50, 10 mL/0.1 mmol **13a-15a**). The aqueous layer was extracted with CH₂Cl₂ (2 x 10 mL/0.1 mmol **13a-15a**). The combined organic layers were dried over MgSO₄, filtered and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel using ethyl acetate/hexane (2:3) as eluent.

1-methyl-2-phenyl-1H-benzo[d]imidazole (**13b**). Brown solid. Isolated yield: 74%. Reported.⁷

1-methyl-2-(naphthalen-2-yl)-1H-benzo[d]imidazole (**14b**). Brown solid. Isolated yield: 51%. Reported.⁸

1-methyl-2-(thiophen-2-yl)-1H-benzo[d]imidazole (**15b**). Brown solid. Isolated yield: 70%. Reported.⁹

Preparation of pro-ligands **14c** and **15c**:



Scheme E2.6. Synthesis of HC^N pro-ligands **14c**, and **15c**.

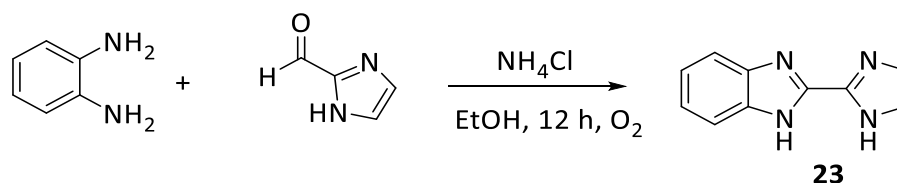
The N-benzylation was performed as previously described for pro-ligand **1**.

2-(naphthalen-2-yl)-1-(4-(trifluoromethyl)benzyl)-1H-benzo[d]imidazole (**14c**). Pale brown solid. Isolated yield: 80%. ¹H-NMR (400 MHz, CDCl₃): δ 8.16 (s, 1H), 7.97 (d, J=

8.2 Hz, 2H), 7.92 (d, J = 8.2 Hz, 1H), 7.81-7.78 (m, 2H), 7.66 (d, J = 8.2 Hz, 2H), 7.61-7.54 (m, 2H), 7.41-7.38 (m, 1H), 7.33-7.23 (m, 4H), 5.60 (s, 2H). m/z = 403.1426 $[M+H]^+$.

2-(thiophen-2-yl)-1-(4-(trifluoromethyl)benzyl)-1H-benzo[d]imidazole (**15c**). White solid. Isolated yield: 87%. $^1\text{H-NMR}$ (300 MHz, CDCl_3): 7.86 (dd, J = 0.6, 8.0 Hz, 1H), 7.59 (d, J = 8.2 Hz, 2H), 7.49-7.47 (m, 1H), 7.34-7.19 (m, 6H), 7.16-7.06 (m, 1H), 5.64 (s, 2H). m/z = 359.081 $[M+H]^+$.

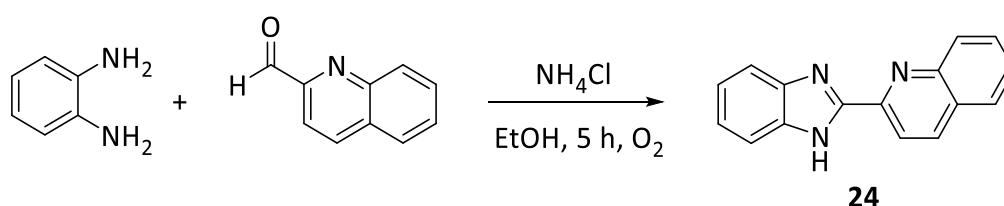
Preparation of 2-(1H-imidazol-2-yl)-1H-benzo[d]imidazole (23):



Scheme E2.7. Synthesis of the N[^]N ligand **23**.

Ligand **23** was synthesized using an adaptation method of reference.¹⁰ A suspension of o-phenylenediamine (1.6 mmol), 1H-imidazole-2-carbaldehyde (1.6 mmol), and ammonium chloride (1.4 mmol) in 5 mL of EtOH, was stirred at 80 °C for 12 h. After completion of the reaction, the mixture was then poured into ice-cold water and the precipitated solid was filtered and washed with water thoroughly. White solid. Isolated yield: 48%. Reported.¹¹

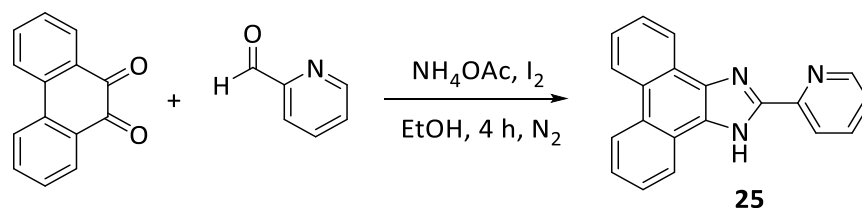
Preparation of 2-(1H-benzo[d]imidazol-2-yl)quinolone (24):



Scheme E2.8. Synthesis of N[^]N ligand **24**.

Ligand **24** was also synthesized using an adaptation method of reference.¹⁰ A suspension of o-phenylenediamine (1.6 mmol), 2-quinolinecarboxyaldehyde (1.6 mmol), and ammonium chloride (1.4 mmol) in 5 mL of EtOH, was stirred at 80 °C for 5 h. After completion of the reaction, the mixture was then poured into ice-cold water and the product was precipitated as an orange solid. The solid was filtered, washed with water thoroughly, and purified by column chromatography on silica gel with a mixture of $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (95:5). Pale yellow solid. Isolated yield: 35%. Reported.¹⁰

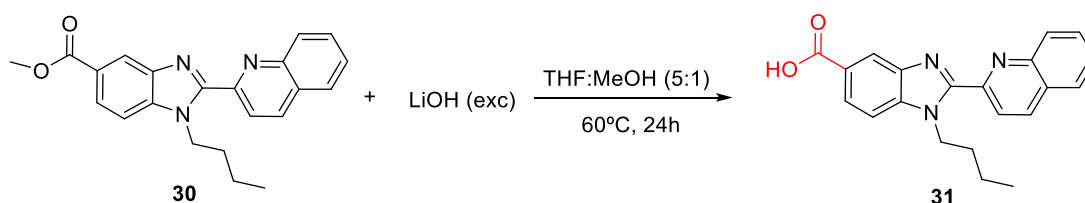
Preparation of 2-(pyridin-2-yl)-1H-phenanthro[9,10-d]imidazole (25):



Scheme E2.9. Synthesis of N[^]N ligand **25**.

Ligand **25** was synthesized using an adaptation method of reference.¹² A suspension of picolinaldehyde (1.0 mmol), phenanthrene-9,10-dione (1.0 mmol), NH₄OAc (3.0 mmol) and I₂ (0.05 mmol) in 15 mL of EtOH was stirred at 80 °C for 4 h under nitrogen atmosphere. The progress of the reaction was monitored by TLC. After completion of the reaction, the mixture was diluted with water (having a small amount of ammonium sulfate) and the pale yellow precipitate was filtered and washed with water. The product was purified by column chromatography on silica gel with a mixture of CH₂Cl₂/MeOH (95:5). White solid. Isolated yield: 56%. Reported.¹³

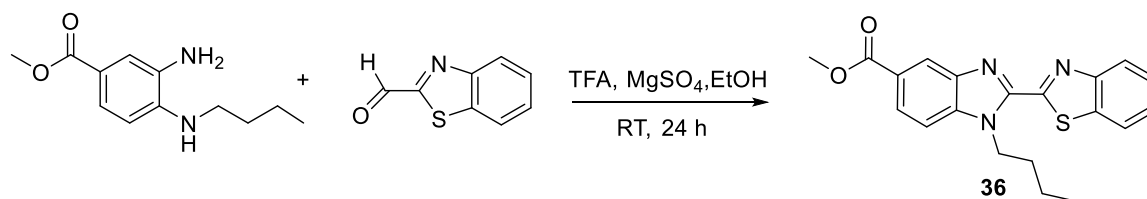
Preparation of 1-butyl-2-(quinolin-2-yl)-1H-benzo[d]imidazole-5-carboxylic acid (31):



Scheme E2.10. Synthesis of the N[^]N ligand **31** with the carboxylic group.

The precursor methyl 1-butyl-2-(quinolin-2-yl)-1H-benzo[d]imidazole-5-carboxylate, **30**, was synthesized as previously reported.¹ **31** was synthesized by hydrolysis of **30** using a method described by us.¹⁴ **30** (0.446 mmol) was dissolved in 10 mL of THF/MeOH (5:1). A saturated solution of LiOH was added slowly with constant stirring until pH = 8-9. The reaction mixture was stirred at 60 °C for 24 h, and the progress of the reaction was monitored by TLC. After completing the reaction, the solvent was removed under reduced pressure, and the resulting residue was suspended in a mixture of water and diethyl ether. The aqueous layer was separated and concentrated using a rotary evaporator to remove any traces of diethyl ether. Next, HCl 10% was carefully added dropwise until pH = 5-6. The precipitated solid was filtered and washed with water. White solid. Isolated yield: 75%; Reported.¹⁴

Preparation of methyl 2-(benzo[d]thiazol-2-yl)-1-butyl-1H-benzo[d]imidazole-5-carboxylate (**36**):



Scheme E2.11. Synthesis of the N[^]N ligand **36**.

The N[^]N ligand **36** was synthesized with a method adapted from literature.¹ Methyl 3-amino-4-(butylamino)benzoate (1.12 mmol) and 1,3-benzothiazole-2-carbaldehyde (1.12 mmol) were dissolved in ethanol (7 mL) in a round-bottom flask. Then, trifluoroacetic acid (0.11 mmol) and magnesium sulfate (5.56 mmol) were added and the reaction was stirred at RT for 24 h. The mixture was filtered to remove magnesium sulfate and the filtrate was concentrated and dissolved in CH₂Cl₂. Dichloromethane was extracted with water (2 × 10 mL) and brine (10 mL), dried on MgSO₄, and concentrated under reduced pressure. The brown solid was washed with hexane. Isolated yield: 32%. ¹H-NMR (400 MHz, CDCl₃): δ 8.60 (d, J=0.8 Hz, 1H), 8.11-8.10 (m, 2H), 8.01-7.99 (m, 1H), 7.56-7.53 (m, 1H), 7.51-7.48 (m, 2H), 4.98 (t, J=7.6 Hz, 2H), 3.98 (s, 3H), 1.97-1.93 (m, 2H), 1.51-1.44 (m, 2H), 1.03 (t, J= 7.2 Hz, 3H). ¹³C NMR (100 MHz, CDCl₃): δ 168.37, 160.11, 155.07, 147.63, 143.41, 140.74, 136.50, 127.53, 127.41, 126.62, 126.35, 125.04, 124.07, 122.80, 111.10, 53.21, 46.38, 33.08, 21.08, 14.76. ESI-MS (pos. ion mode, CH₃CN): m/z = 366.1278 [M+H]⁺. Anal. Calcd. for C₂₀H₁₉N₃O₂S: C, 65.73; H, 5.24; N, 11.50; S, 8.77. Found: C, 65.5; H, 5.0; N, 11.3; S, 8.8 (%).

E2.2. Synthesis of cationic Ir(III) complexes

The cationic monomeric iridium complexes corresponding to Chapters 2-6 were synthesized in two steps using the method previously described for these types of compounds.^{1,15,16}

Step 1. Synthesis of dimeric complexes [(Ir(μ-Cl)(C[^]N)₂]₂:

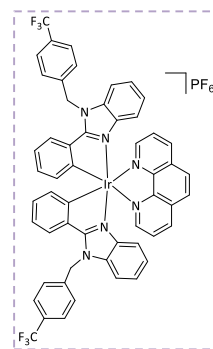
HC[^]N pro-ligands (2.2 mmol) and iridium(III) chloride (1 mmol) were suspended in 2-ethoxyethanol/deionized H₂O (3:1) in a Schlenk flask. The mixture was stirred at 110 °C for 24 h under a nitrogen atmosphere. The reaction was cooled to RT, and the solid obtained

was collected by filtration. The product was washed with water and cold EtOH and used in the next step without further purification.

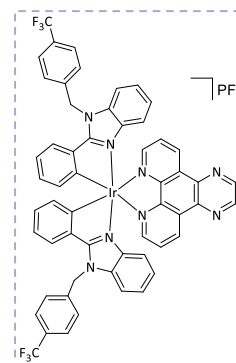
Step 2. Synthesis of monomeric complexes $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{N}^{\wedge}\text{N})][\text{PF}_6]$:

Cyclometalated Ir(III) dimers (1.0 mmol) and the respective $\text{N}^{\wedge}\text{N}$ ligands (2.1 mmol) were dissolved in $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (2:3, v/v) in a Schlenk flask. The mixture was stirred at 58°C for 24 h under a nitrogen atmosphere. After cooling the reaction to RT, an excess amount of KPF_6 (2.5 mmol) was added, and the mixture was stirred for 30 min. The solvent was removed under reduced pressure, and the resultant solid was washed with water. The product was recrystallized from CH_2Cl_2 and ether. The complex **21a** was further purified by column chromatography on neutral alumina with a mixture of $\text{CH}_2\text{Cl}_2/\text{ACN}$ (8:2). Complex **28** was purified by column chromatography on neutral alumina with a mixture of $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (95:5).

Complex 8. Yellow solid. Isolated yield: 65%. ^1H -NMR (400 MHz, $[\text{D}_6]$ DMSO): δ 8.95 (dd, J = 8.4, 1.2 Hz, 2H), 8.43 (dd, J = 5.2, 1.2 Hz, 2H), 8.37 (s, 2H), 8.20-8.01 (m, 2H), 7.82-7.72 (m, 8H), 7.29 (d, J = 8.0 Hz, 4H), 7.17 (t, J = 8.4 Hz, 2H), 7.01-6.90 (m, 4H), 6.78 (t, J = 7.6 Hz, 2H), 6.40 (dd, J = 7.6, 1.2 Hz, 2H), 6.29 (br s, NCH_2Ar , 4H), 5.31 (d, J = 8.4 Hz, 2H). ^{13}C -NMR (50 MHz, DMSO- d_6): δ 164.18, 153.10, 152.72, 148.60, 142.19, 140.31, 139.63, 136.54, 134.82, 134.38, 131.91, 129.86, 129.63, 128.35, 128.03, 127.31, 127.08, 125.70, 125.11, 123.75, 114.14, 113.30, 48.26; ESI-MS (pos. ion mode, DMSO): m/z = 1075.2492 $[\text{M}-\text{PF}_6]^+$. Anal. Calcd. for $\text{C}_{54}\text{H}_{36}\text{F}_{12}\text{IrN}_6\text{P}$: C, 53.16; H, 2.97; N, 6.89. Found: C, 53.04; H, 2.91; N, 6.81 (%).

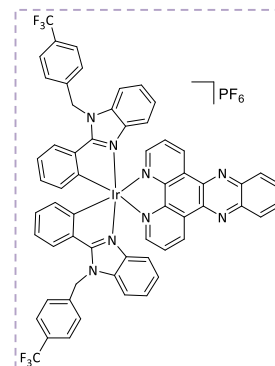


Complex 9. Bright orange solid. Isolated yield: 83%. ^1H -NMR (300 MHz, CD_3CN): δ 9.70 (dd, J = 8.4, 1.5 Hz, 2H), 9.21 (s, 2H), 8.60 (dd, J = 5.1, 3.6 Hz, 2H), 8.07 (dd, J = 8.1, 5.1 Hz, 2H), 7.72 (d, J = 7.2 Hz, 2H), 7.67 (d, J = 8.1 Hz, 4H), 7.50 (d, J = 8.4 Hz, 2H), 7.31 (d, J = 8.1 Hz, 4H), 7.18 (td, J = 7.2, 0.9 Hz, 2H), 6.98 (td, J = 7.5, 1.5 Hz, 2H), 6.90 (td, J = 7.5, 1.2 Hz, 2H), 6.79 (td, J = 8.4, 1.2 Hz, 2H), 6.53 (dd, J = 7.5, 0.9 Hz, 2H), 6.10 (br s, NCH_2Ar , 4H), 5.62 (d, J = 8.1 Hz, 2H). ^{13}C -NMR (75.4 MHz, CD_3CN): δ 164.35, 154.21, 152.38, 150.32, 147.74, 141.23, 140.67, 139.82, 136.42, 136.04, 134.86, 134.48, 131.55, 131.01, 128.78, 127.73, 126.94, 126.69, 125.52,



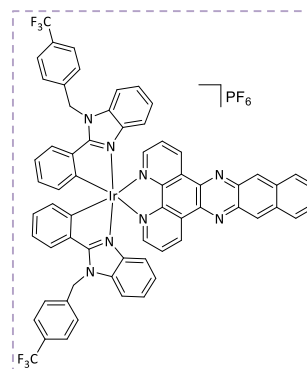
124.90, 123.62, 114.96, 112.04, 48.49. ESI-MS (pos ion mode, CH₃CN): m/z = 1127.2638 ([M-PF₆])⁺. Anal. Calcd. for C₅₆H₃₆F₁₂IrN₈P: C, 52.87; H, 2.85; N, 8.81. Found: C, 52.65; H, 2.65; N, 8.47 (%).

Complex 10. Orangish brown solid. Isolated yield: 79%. ¹H-NMR (400 MHz, CD₃CN): δ 9.81 (dd, J = 8.4, 1.2 Hz, 2H), 8.60 (dd, J = 5.2, 1.6 Hz, 2H), 8.46 (dd, J = 6.8, 3.6 Hz, 2H), 8.12 (dd, J = 6.8, 3.6 Hz, 2H), 8.07 (dd, J = 8.0, 2.8 Hz, 2H), 7.73 (d, J = 7.6 Hz, 2H), 7.68 (d, J = 8.0 Hz, 4H), 7.51 (d, J = 8.0 Hz, 2H), 7.33 (d, J = 8.4 Hz, 4H), 7.18 (t, J = 8 Hz, 2H), 6.99 (t, J = 6.8 Hz, 2H), 6.91 (t, J = 7.2 Hz, 2H), 6.82 (t, J = 8 Hz, 2H), 6.53 (d, J = 7.2 Hz, 2H), 6.12



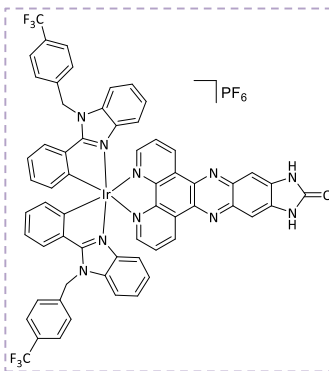
(br s, NCH₂Ar, 4H), 5.73 (d, J = 8.0 Hz, 2H). ¹³C-NMR (100 MHz, CD₃CN): δ 164.28, 154.16, 152.27, 151.36, 143.60, 141.12, 140.79, 139.76, 136.36, 136.07, 134.75, 134.35, 133.34, 131.54, 131.48, 130.45, 129.01, 127.64, 126.83, 126.61, 125.48, 124.83, 123.55, 114.97, 111.97, 48.41. ESI-MS (pos ion mode, CH₃CN): m/z = 1177.2761 ([M-PF₆])⁺. Anal. Calcd. for C₆₀H₃₈F₁₂IrN₈P: C, 54.50; H, 2.90; N, 8.47. Found: C, 54.19; H, 2.84; N, 8.23 (%).

Complex 11. Reddish solid. Isolated yield: 86%. ¹H-NMR (400 MHz, CD₃CN): δ 9.70 (dd, J = 8.0, 1.2 Hz, 2H), 8.97 (s, 2H), 8.58 (dd, J = 5.2, 1.2 Hz, 2H), 8.25-8.22 (m, 2H), 8.03 (dd, J = 8, 5.2 Hz, 2H), 7.75 (d, J = 8.0 Hz, 2H), 7.69 (d, J = 8.0 Hz, 4H), 7.64-7.62 (m, 2H), 7.53 (d, J = 8.0 Hz, 2H), 7.34 (d, J = 8.0 Hz, 4H), 7.20 (t, J = 7.6 Hz, 2H), 7.01 (t, J = 6.8 Hz, 2H), 6.92-6.88 (m, 4H), 6.54 (d, J = 7.6, 2H), 6.14 (br s, NCH₂Ar, 4H), 5.83 (d,

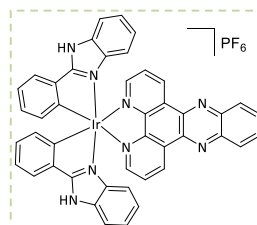


J = 8.0 Hz, 2H). ¹³C-NMR (75.4 MHz, CD₃CN): δ 164.42, 154.32, 152.36, 151.94, 141.50, 141.24, 139.91, 139.31, 136.50, 136.15, 135.92, 134.82, 134.46, 131.86, 131.63, 130.70, 129.41, 129.26, 129.02, 128.88, 127.77, 126.96, 126.76, 125.68, 125.01, 123.69, 115.18, 112.14, 48.53. ESI-MS (pos ion mode, CH₃CN): m/z = 1227.2871 ([M-PF₆])⁺. Anal. Calcd. for C₆₄H₄₀F₁₂IrN₈P: C, 56.02; H, 2.94; N, 8.17. Found: C, 55.86; H, 2.93; N, 7.91 (%).

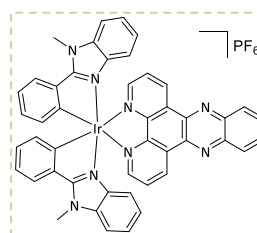
Complex 12. Orange solid. Isolated yield: 83%. ^1H -NMR (400 MHz, CD_3CN): δ 9.75 (d, J = 8.4 Hz, 2H), 8.54 (dd, J = 4.8, 1.2 Hz, 2H), 8.04 (dd, J = 8.24, 5.16 Hz, 2H), 7.75 (s, 2H), 7.73 (d, J = 8.0 Hz, 2H), 7.67 (d, J = 8.0 Hz, 4H), 7.53 (d, J = 8 Hz, 2H), 7.31 (d, J = 8.0 Hz, 4H), 7.17 (t, J = 8.0 Hz, 2H), 6.98 (t, J = 7.6 Hz, 2H), 6.89 (t, J = 7.6 Hz, 2H), 6.80 (t, J = 8.0 Hz, 2H), 6.51 (d, J = 7.6 Hz, 2H), 6.13 (br s, NCH_2Ar , 4H), 5.70 (d, J = 8.4 Hz, 2H). ^{13}C -NMR (75.4 MHz, CD_3CN): δ 164.44, 156.11, 153.48, 152.62, 150.63, 141.60, 141.23, 139.90, 137.96, 136.47, 135.49, 134.88, 134.46, 131.72, 131.58, 128.66, 127.77, 126.98, 126.93, 126.70, 125.57, 124.95, 123.74, 115.06, 112.10, 105.09, 48.53. ESI-MS (pos ion mode, CH_3CN): m/z = 1233.2884 ($[\text{M}-\text{PF}_6]^+$). Anal. Calcd. for $\text{C}_{61}\text{H}_{38}\text{F}_{12}\text{IrN}_{10}\text{OP}$: C, 53.16; H, 2.78; N, 10.16. Found: C, 52.98; H, 2.73; N, 9.90 (%).



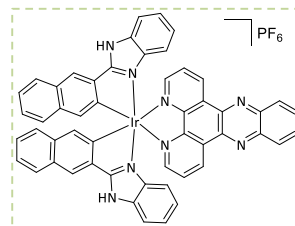
Complex 19a. Orange solid. Isolated yield: 83%. ^1H -NMR (300 MHz, $[\text{D}_6]\text{DMSO}$): δ 13.94 (br s, 2H), 9.76 (d, J = 8.4 Hz, 2H), 8.58 (d, J = 4.8 Hz, 2H), 8.48–8.45 (m, 2H), 8.26 (dd, J = 6, 13.2 Hz, 2H), 8.17–8.14 (m, 2H), 7.92 (d, J = 7.5 Hz, 2H), 7.54 (d, J = 7.8 Hz, 2H), 7.11–7.07 (m, 4H), 6.89 (t, J = 7.2 Hz, 2H), 6.75 (t, J = 8.1 Hz, 2H), 6.30 (d, J = 7.8 Hz, 2H), 5.55 (d, J = 8.4 Hz, 2H). ^{13}C -NMR (75.4 MHz, $[\text{D}_6]\text{DMSO}$): δ 164.17, 152.911, 150.26, 149.33, 142.02, 139.70, 139.24, 135.00, 134.24, 133.16, 132.63, 132.49, 130.48, 129.99, 129.33, 128.32, 124.39, 123.49, 123.11, 122.27, 113.01. ESI-MS (pos. ion mode, CH_3CN): m/z = 861.2045 $[\text{M} - \text{PF}_6]^+$. Anal. calcd for $\text{C}_{44}\text{H}_{28}\text{F}_6\text{IrN}_8\text{P}$: C, 52.54; H, 2.81; N, 11.14. Found: C, 52.60; H, 2.79; N, 11.0 (%).



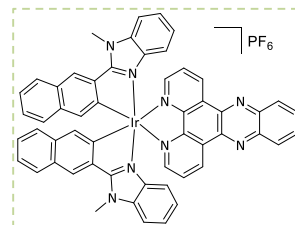
Complex 19b. Orange solid. Isolated yield: 68%. ^1H -NMR (300 MHz, $[\text{D}_6]\text{DMSO}$): δ 9.75 (d, J = 8.1 Hz, 2H), 8.50–8.44 (m, 4H), 8.25–8.21 (m, 2H), 8.17–8.09 (m, 4H), 7.75 (d, J = 8.4 Hz, 2H), 7.19–7.09 (m, 4H), 6.89 (t, J = 7.5 Hz, 2H), 6.77 (t, J = 7.5 Hz, 2H), 6.32 (d, J = 7.5 Hz, 2H), 5.53 (d, J = 8.4 Hz, 2H), 4.36 (s, 6H). ^{13}C -NMR (75.4 MHz, $[\text{D}_6]\text{DMSO}$): 164.36, 154.26, 152.38, 151.49, 143.49, 141.07, 139.79, 136.78, 136.51, 135.67, 134.39, 134.02, 131.75, 131.41, 130.79, 129.83, 127.46, 125.37, 124.46, 123.80, 114.54, 113.29, 33.68. ESI-MS (pos. ion mode, CH_3CN): m/z = 889.2393 $[\text{M} - \text{PF}_6]^+$. Anal. calcd for $\text{C}_{46}\text{H}_{32}\text{F}_6\text{IrN}_8\text{P}$: C, 54.43; H, 3.12; N, 10.84. Found: C, 53.44; H, 3.15; N, 10.73 (%).



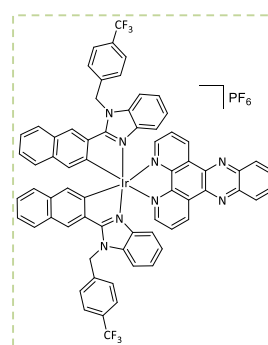
Complex 20a. Reddish solid. Isolated yield: 76%. $^1\text{H-NMR}$ (300 MHz, $[\text{D}_6]\text{DMSO}$): δ 14.24 (s, 2H), 9.77 (dd, $J = 1.5, 8.4$ Hz, 2H), 8.67 (dd, $J = 1.2, 5.1$ Hz, 2H), 8.49–8.43 (m, 4H), 8.19–8.11 (m, 4H), 7.81 (d, $J = 7.5$ Hz, 2H), 7.64 (d, $J = 8.1$ Hz, 2H), 7.28–7.13 (m, 8H), 6.78 (t, $J = 7.5$ Hz, 2H), 6.67 (s, 2H), 5.58 (d, $J = 6.67$ Hz, 2H). $^{13}\text{C-NMR}$ (75.4 MHz, $[\text{D}_6]\text{DMSO}$): δ 164.57, 154.44, 151.71, 143.99, 143.40, 141.07, 140.83, 136.43, 136.09, 135.72, 135.03, 133.87, 131.34, 131.09, 131.04, 130.71, 129.99, 129.79, 128.47, 127.49, 125.31, 125.11, 125.06, 124.92, 114.71. ESI-MS (pos. ion mode, CH_3CN): $m/z = 961.2406$ $[\text{M} - \text{PF}_6]^+$. Anal. calcd for $\text{C}_{52}\text{H}_{32}\text{F}_6\text{IrN}_8\text{P}$: C, 56.47; H, 2.92; N, 10.13. Found: C, 56.30; H, 2.89; N, 10.0 (%).



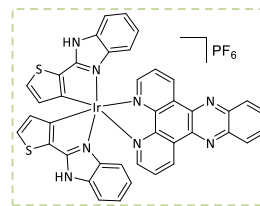
Complex 20b. Red solid. Isolated yield: 90%. $^1\text{H-NMR}$ (300 MHz, $[\text{D}_6]\text{DMSO}$): δ 9.76 (dd, $J = 1.8, 8.4$ Hz, 2H), 8.72 (s, 2H), 8.59 (dd, $J = 1.2, 5.4$ Hz, 2H), 8.48–8.45 (m, 2H), 8.19–8.14 (m, 4H), 7.99 (d, $J = 9.3$ Hz, 2H), 7.86 (d, $J = 8.4$ Hz, 2H), 7.33–7.16 (m, 8H), 6.80 (t, $J = 8.1$ Hz, 2H), 6.68 (s, 2H), 5.58 (d, $J = 8.4$ Hz, 2H), 4.54 (s, 6H). $^{13}\text{C-NMR}$ (75.4 MHz, $[\text{D}_6]\text{DMSO}$): 163.31, 154.42, 151.61, 145.07, 143.53, 141.09, 139.99, 137.19, 136.55, 135.72, 135.51, 134.03, 131.39, 131.33, 131.28, 130.79, 129.94, 128.84, 127.35, 127.13, 125.57, 125.28, 124.91, 114.93, 113.55, 34.06. ESI-MS (pos. ion mode, CH_3CN): $m/z = 989.2722$ $[\text{M} - \text{PF}_6]^+$. Anal. calcd for $\text{C}_{54}\text{H}_{36}\text{F}_6\text{IrN}_8\text{P}$: C, 57.19; H, 3.20; N, 9.88. Found: C, 57.10; H, 3.19; N, 9.80 (%).



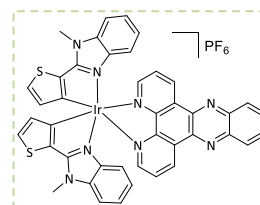
Complex 20c. Orangish brown solid. Isolated yield: 88%. $^1\text{H-NMR}$ (300 MHz, $[\text{D}_6]\text{DMSO}$): δ 9.78 (d, $J = 7.2$ Hz, 2H), 8.64 (d, $J = 4.9$ Hz, 2H), 8.49–8.47 (m, 4H), 8.23–8.15 (m, 4H), 7.92 (d, $J = 8.3$ Hz, 2H), 7.77–7.75 (m, 2H), 7.65 (d, $J = 8.3$ Hz, 4H), 7.49 (d, $J = 8.1$ Hz, 4H), 7.29–7.24 (m, 6H), 7.18–7.15 (m, 2H), 6.85 (t, $J = 7.7$ Hz, 2H), 6.59 (s, 2H), 6.45 (brs, NCH_2Ar , 4H), 5.62 (d, $J = 8.3$ Hz, 2H). $^{13}\text{C-NMR}$ (75.4 MHz, $[\text{D}_6]\text{DMSO}$): 163.02, 154.57, 151.69, 145.34, 143.56, 142.52, 141.26, 139.99, 137.23, 136.85, 135.70, 134.63, 134.07, 131.62, 131.13, 130.83, 130.67, 130.04, 129.61, 129.08, 128.52, 127.36, 127.15, 126.93, 126.29, 125.78, 125.45, 123.55, 115.32, 113.63, 48.45. ESI-MS (pos. ion mode, CH_3CN): $m/z = 1277.3030$ $[\text{M} - \text{PF}_6]^+$. Anal. calcd for $\text{C}_{68}\text{H}_{42}\text{F}_{12}\text{IrN}_8\text{P}$: C, 57.42; H, 2.98; N, 7.88. Found: C, 57.40; H, 2.96; N, 7.88 (%).



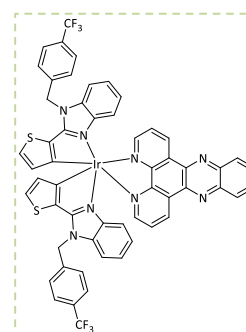
Complex 21a. Red solid. Isolated yield: 20%. ^1H -NMR (400 MHz, $[\text{D}_6]\text{DMSO}$): δ 13.77 (br s, 2H), 9.76 (d, J = 8.2 Hz, 2H), 8.53 (dd, J = 0.9, 5.2 Hz, 2H), 8.47 (dd, J = 3.4, 6.5 Hz, 2H), 8.28–8.25 (m, 2H), 8.15 (dd, J = 3.3, 6.4 Hz, 2H), 7.69 (d, J = 4.6 Hz, 2H), 7.44 (d, J = 7.9 Hz, 2H), 7.04 (t, J = 7.4 Hz, 2H), 6.73 (t, J = 7.4 Hz, 2H), 6.17 (d, J = 4.6 Hz, 2H), 5.54 (d, J = 8.1 Hz, 2H). ^{13}C -NMR (150 MHz, $[\text{D}_6]\text{DMSO}$): 171.41, 160.82, 153.29, 150.34, 142.15, 140.03, 139.70, 132.52, 130.68, 129.37, 128.30, 123.55, 123.25, 123.21, 122.18, 120.38, 115.57, 112.66, 111.94, 110.77. ESI-MS (pos. ion mode, CH_3CN): m/z = 873. 1150 $[\text{M} - \text{PF}_6]^+$. Anal. calcd for $\text{C}_{40}\text{H}_{24}\text{F}_6\text{IrN}_8\text{PS}_2$: C, 47.20; H, 2.38; N, 11.01; S, 6.30. Found: C, 47.18; H, 2.37; N, 11.01; S, 6.31 (%).



Complex 21b. Red solid. Isolated yield: 91%. ^1H -NMR (300 MHz, $[\text{D}_6]\text{DMSO}$): δ 9.70 (d, J = 8.1 Hz, 2H), 8.47 (d, J = 4.8 Hz, 2H), 8.42–8.39 (m, 2H), 8.25–8.21 (m, 2H), 8.11–8.09 (m, 2H), 7.79 (d, J = 4.5 Hz, 2H), 7.58 (d, J = 8.1 Hz, 2H), 7.08 (t, J = 7.5 Hz, 2H), 6.75 (t, J = 7.6 Hz, 2H), 6.23 (d, J = 4.6 Hz, 2H), 5.52 (d, J = 8.2 Hz, 2H), 4.16 (s, 6H). ^{13}C -NMR (100 MHz, $[\text{D}_6]\text{DMSO}$): 161.28, 154.87, 153.37, 150.15, 141.97, 139.55, 135.13, 134.27, 132.64, 132.52, 131.60, 129.95, 129.30, 128.33, 123.66, 122.56, 122.09, 112.01, 111.51, 31.69. ESI-MS (pos. Ion mode, CH_3CN): m/z = 901.1563 $[\text{M} - \text{PF}_6]^+$. Anal. calcd for $\text{C}_{42}\text{H}_{28}\text{F}_6\text{IrN}_8\text{PS}_2$: C, 48.23; H, 2.70; N, 10.71; S, 6.13. Found: C, 48.21; H, 2.70; N, 10.72; S, 6.12 (%).



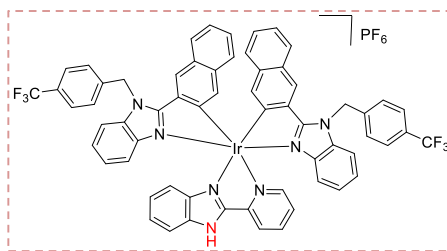
Complex 21c. Orange solid. Isolated yield: 55%. ^1H -NMR (300 MHz, $[\text{D}_6]\text{DMSO}$): δ 9.79 (d, J = 8.2 Hz, 2H), 8.49–8.44 (m, 4H), 8.32–8.28 (m, 2H), 8.16–8.28 (m, 2H), 7.79 (d, J = 4.7 Hz, 2H), 7.71 (d, J = 8.3 Hz, 4H), 7.60 (d, J = 8.3 Hz, 2H), 7.32 (d, J = 8.1 Hz, 4H), 7.07 (t, J = 7.8 Hz, 2H), 6.79 (t, J = 8.0 Hz, 2H), 6.26 (d, J = 4.6 Hz, 2H), 6.02–6.01 (m, 4H), 5.57 (d, J = 8.2 Hz, 2H). ^{13}C -NMR (75.4 MHz, $[\text{D}_6]\text{DMSO}$): 162.90, 157.45, 154.70, 151.71, 143.49, 141.98, 141.18, 141.06, 136.94, 135.24, 135.12, 134.06, 132.72, 131.63, 130.85, 129.93, 128.51, 127.42, 125.71, 124.20, 123.66, 113.86, 113.15, 49.18. ESI-MS (pos. ion mode, CH_3CN): m/z = 1189.1963 $[\text{M} - \text{PF}_6]^+$. Anal. calcd for $\text{C}_{56}\text{H}_{34}\text{F}_{12}\text{IrN}_8\text{PS}_2$: C, 50.41; H, 2.57; N, 8.40; S, 4.81. Found: C, 50.42; H, 2.58; N, 8.42 S, 4.81 (%).



Complex 26. Orange solid. Isolated yield: 80%. ^1H -

NMR (400 MHz, $[\text{D}_6]\text{DMSO}$): δ 15.00 (br s, 1H), 8.68 (d, J = 8 Hz, 1H), 8.47 (d, J = 11.2 Hz, 2H), 8.35 (t, J = 7.8 Hz, 1H), 8.15 (d, J = 4.8 Hz, 1H), 7.96 (q, J = 8.4 Hz, 2H), 7.76-7.69 (m, 6H), 7.52 (t, J = 8.2 Hz, 4H),

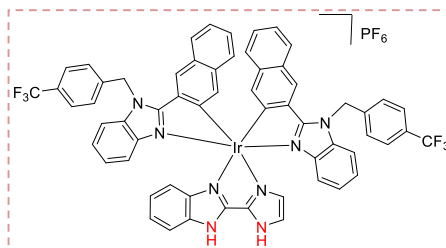
7.36-7.29 (m, 5H), 7.24-7.17 (m, 6H), 7.04 (t, J = 7.8 Hz, 1H), 6.89 (t, J = 7.8 Hz, 1H), 6.81 (t, J = 7.4 Hz, 1H), 6.66 (d, J = 16.4 Hz, 2H), 6.54-6.39 (m, , NCH_2Ar , 4H), 6.22 (d, J = 8.4 Hz, 1H), 5.89 (d, J = 8.4 Hz, 1H), 5.77 (d, J = 8.4 Hz, 1H). ^{13}C -NMR (75.4 MHz, $[\text{D}_6]\text{DMSO}$): δ 161.65, 154.46, 151.43, 148.98, 145.69, 141.49, 141.37, 141.20, 141.09, 139.97, 138.74, 138.55, 135.77, 135.71, 134.24, 133.91, 133.73, 133.48, 129.59, 129.44, 129.30, 129.18, 128.56, 128.40, 128.14, 127.98, 127.50, 127.08, 126.86, 125.89, 125.74, 125.31, 124.57, 124.28, 124.19, 124.07, 123.69, 123.63, 123.56, 116.51, 114.59, 114.05, 113.55, 112.27, 112.09, 46.96, 46.80. ESI-MS (pos. ion mode, CH_3CN): m/z = 1190.3053 $[\text{M} - \text{PF}_6]^+$. Anal. Calcd. for $\text{C}_{62}\text{H}_{41}\text{F}_{12}\text{IrN}_7\text{P}$: C, 55.77; H, 3.10; N, 7.34. Found: C, 55.81; H, 3.12; N, 7.32 (%).



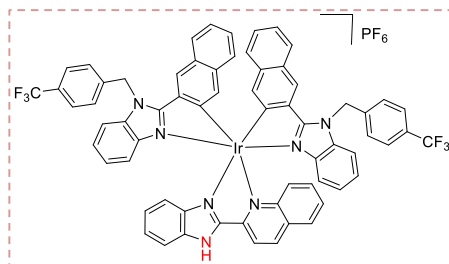
Complex 27. Yellow solid. Isolated yield: 72%. ^1H -

NMR (600 MHz, $[\text{D}_6]\text{DMSO}$): δ 14.03 (br s, 1H), 8.46 (s, 1H), 8.40 (s, 1H), 7.97 (d, J = 8.3 Hz, 1H), 7.91 (d, 8.3 Hz, 1H), 7.76-7.74 (m, 1H), 7.71 (d, J = 8.3 Hz, 4H), 7.65 (br s, 1H), 7.51-7.48 (m, 4H), 7.33-

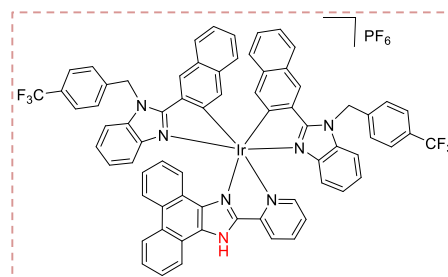
7.30 (m, 4H), 7.23-7.09 (m, 8H), 6.89 (t, J = 7.8 Hz, 1H), 6.76 (s, 2H), 6.71 (s, 1H), 6.66 (s, 1H), 6.53-6.43 (m, NCH_2Ar , 4H), 6.14 (d, J = 8.3 Hz, 1H), 6.02 (t, J = 8.5 Hz, 2H). ^{13}C -NMR (100 MHz, $[\text{D}_6]\text{DMSO}$): δ 162.00, 161.81, 141.35, 141.25, 138.89, 138.78, 135.73, 135.65, 134.54, 133.95, 133.77, 129.84, 129.37, 129.22, 129.01, 128.81, 128.42, 128.24, 128.04, 127.38, 127.27, 127.05, 126.84, 125.91, 125.71, 125.19, 125.10, 125.04, 124.85, 124.78, 124.43, 124.33, 124.10, 123.33, 123.05, 122.98, 115.94, 114.33, 114.22, 111.98, 46.89, 46.75. ESI-MS (pos ion mode, CH_3CN): m/z = 1179.2865 $[\text{M} - \text{PF}_6]^+$. Anal. Calcd. for $\text{C}_{60}\text{H}_{40}\text{F}_{12}\text{IrN}_8\text{P}$: C, 54.42; H, 3.04; N, 8.46. Found: C, 54.45; H, 3.02; N, 8.47 (%).



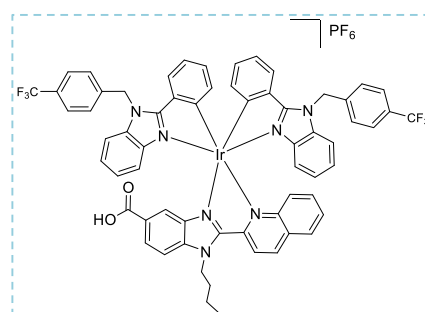
Complex 28. Orange solid. Isolated yield: 40%. ^1H -NMR (400 MHz, $[\text{D}_6]\text{DMSO}$): δ 8.62 (q, J = 9.3 Hz, 2H), 8.42 (d, J = 9.3 Hz, 2H), 8.20 (d, J = 8.7 Hz, 1H), 7.98 (d, J = 7.5 Hz, 1H), 7.90 (d, J = 8.4 Hz, 1H), 7.82 (d, J = 8.4 Hz, 1H), 7.76-7.74 (m, 1H), 7.71-7.68 (m, 1H), 7.61-7.57 (m, 5H), 7.45 (d, J = 8.1 Hz, 2H), 7.37-7.34 (m, 3H), 7.27-7.18 (m, 8H), 6.88-6.73 (m, 5H), 6.58 (s, 1H), 6.45-6.38 (m, NCH_2Ar , 4H), 6.28 (t, J = 7.7 Hz, 1H), 6.06 (d, J = 8.1 Hz, 1H), 5.75 (d, J = 8.1 Hz, 1H), 5.54 (d, J = 8.1 Hz, 1H). ^{13}C -NMR (75.4 MHz, $[\text{D}_6]\text{DMSO}$): δ 163.89, 163.48, 162.89, 158.65, 149.69, 147.07, 142.68, 142.21, 140.76, 140.51, 137.06, 136.97, 135.86, 135.72, 135.50, 135.11, 131.41, 130.62, 130.23, 130.08, 129.81, 129.48, 129.31, 128.63, 128.47, 128.37, 128.12, 127.20, 126.68, 126.30, 125.39, 125.23, 124.74, 124.49, 122.19, 121.36, 120.55, 116.75, 116.57, 116.02, 113.31, 112.86, 48.27. ESI-MS (pos ion mode, CH_3CN): m/z = 1240.3169 ($[\text{M} - \text{PF}_6]^+$). Anal. Calcd. for $\text{C}_{66}\text{H}_{43}\text{F}_{12}\text{IrN}_7\text{P}$: C, 57.22; H, 3.13; N, 7.08. Found: C, 57.24; H, 3.12; N, 7.10 (%).



Complex 29. Orangish brown solid. Isolated yield: 68%. ^1H -NMR (300 MHz, 343 K, $[\text{D}_6]\text{DMSO}$): δ 8.92-8.89 (m, 2H), 8.75 (d, J = 8.4 Hz, 1H), 8.57-8.46 (m, 3H), 8.38 (s, 1H), 8.11 (d, J = 5.4 Hz, 1H), 7.93 (d, J = 8.1 Hz, 1H), 7.91-7.78 (m, 6H), 7.74-7.72 (m, 2H), 7.64-7.58 (m, 4H), 7.35-7.13 (m, 7H), 7.22-7.04 (m, 6H), 6.88 (t, J = 7.7 Hz, 1H), 6.74 (d, J = 7.5 Hz, 2H), 6.61 (s, 1H), 6.54-6.45 (m, NCH_2Ar , 2H), 6.37-6.29 (m, NCH_2Ar , 2H), 5.79-5.75 (m, 2H). ^{13}C -NMR (75.4 MHz, 343 K, $[\text{D}_6]\text{DMSO}$): δ 165.24, 161.39, 160.59, 150.92, 145.98, 144.55, 141.20, 140.46, 140.01, 138.58, 138.05, 136.17, 135.37, 133.95, 133.86, 133.44, 132.53, 132.30, 130.18, 129.75, 129.58, 129.44, 128.78, 128.15, 127.32, 126.87, 126.67, 126.25, 126.06, 125.97, 125.66, 125.54, 125.34, 125.12, 124.95, 124.57, 124.44, 124.24, 123.93, 123.79, 123.44, 122.85, 121.83, 120.92, 118.81, 114.27, 113.06, 111.88, 111.74, 47.03, 46.65. ESI-MS (pos ion mode, CH_3CN): m/z = 1291.3182 ($[\text{M} + \text{H} - \text{PF}_6]^+$). Anal. Calcd. for $\text{C}_{70}\text{H}_{45}\text{F}_{12}\text{IrN}_7\text{P}$: C, 58.58; H, 3.16; N, 6.83. Found: C, 58.67; H, 3.17; N, 6.81 (%).



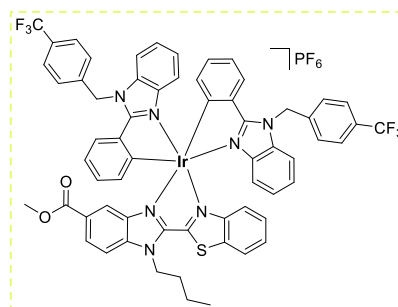
Complex 32. Red solid. Isolated yield: 72%. ^1H -NMR (400 MHz, $[\text{D}_6]\text{DMSO}$): δ 8.90 (d, J = 8.7 Hz, 1H), 8.67 (d, J = 8.7 Hz, 1H), 8.20 (d, J = 8.4 Hz, 1H), 8.09-8.01 (m,



3H), 7.82 (d, $J = 8.1$ Hz, 1H), 7.75 (d, $J = 7.9$ Hz, 1H), 7.70-7.67 (m, 2H), 7.63 (t, $J = 7.7$ Hz, 1H), 7.55 (d, $J = 8.2$ Hz, 2H), 7.45 (d, $J = 8.2$ Hz, 2H), 7.19 (t, $J = 8.0$ Hz, 2H), 7.10-7.08 (m, 3H), 7.01-6.99 (m, 2H), 6.96-6.91 (m, 2H), 6.88-6.82 (m, 5H), 6.42 (d, $J = 7.6$ Hz, 1H), 6.33-6.31 (m, 2H), 6.28-6.11 (m, 4H), 6.03 (d, $J = 8.2$ Hz, 1H), 5.14-4.93 (m, 2H), 1.73-1.54 (m, 2H), 0.90-0.70 (m, 2H), 0.52 (t, $J = 7.2$ Hz, 3H). ^{13}C -NMR (150 MHz, $[\text{D}_6]\text{DMSO}$): δ 166.37, 162.85, 162.10, 156.03, 154.21, 149.78, 148.15, 145.01, 141.57, 140.86, 140.73, 139.12, 138.99, 138.42, 138.34, 134.92, 134.85, 133.32, 132.47, 131.42, 130.53, 130.31, 129.32, 129.02, 128.37, 128.13, 126.64, 126.47, 126.03, 125.81, 125.54, 125.37, 125.28, 124.38, 123.87, 123.11, 122.67, 122.59, 121.96, 120.83, 120.10, 113.85, 113.67, 112.95, 111.89, 111.77, 46.74, 46.66, 45.92, 31.67, 18.76, 13.26. ESI-MS (pos. ion mode, DMSO): $m/z = 1240.3264$ $[\text{M} - \text{PF}_6]^+$. Anal. Calcd. for $\text{C}_{63}\text{H}_{47}\text{F}_{12}\text{IrN}_7\text{O}_2\text{P}$: C, 54.62; H, 3.42; N, 7.08. Found: C, 54.70; H, 3.42; N, 6.98; (%).

Complex 37. Red solid. Isolated yield: 73%. ^1H -NMR

(300 MHz, $[\text{D}_6]\text{DMSO}$): δ 8.47 (d, $J = 7.9$ Hz, 1H), 8.18 (d, $J = 8.8$ Hz, 1H), 8.05 (dd, $J = 8.8, 1.5$ Hz, 1H), 7.85 (d, $J = 8.0$ Hz, 1H), 7.79-7.73 (m, 3H), 7.67-7.61 (m, 1H), 7.50 (d, $J = 8.2$ Hz, 2H), 7.32 (d, $J = 8.3$ Hz, 2H), 7.23-7.18 (m, 3H), 7.13-7.06 (m, 3H), 7.05-6.76 (m, 8H), 6.73 (d, $J = 1.0$ Hz, 1H), 6.49 (dd, $J = 7.6, 1.2$ Hz, 1H), 6.37 (dd, $J = 7.7, 0.9$ Hz, 1H), 6.31-6.15 (m, 4H), 5.80 (d, $J = 8.2$ Hz, 1H), 5.69 (d, $J = 8.2$ Hz, 1H), 5.02-4.90 (m, 2H), 3.52 (s, 3H), 1.90-1.72 (m, 2H), 1.21-1.09 (m, 2H), 0.69 (t, $J = 7.2$ Hz, 3H). ^{13}C -NMR (150 MHz, $[\text{D}_6]\text{DMSO}$): δ 166.24, 164.27, 163.74, 158.83, 152.68, 151.96, 151.04, 147.32, 142.21, 140.57, 139.77, 139.56, 139.32, 136.49, 136.42, 135.61, 135.26, 135.11, 134.78, 134.34, 131.70, 131.58, 130.03, 129.83, 129.35, 128.25, 128.05, 127.95, 127.63, 127.29, 127.07, 126.73, 126.02, 125.75, 125.23, 124.07, 123.75, 123.03, 121.35, 114.89, 114.76, 114.61, 113.33, 113.20, 52.92, 48.08, 47.77, 33.37, 20.72, 14.81. ESI-MS (pos. ion mode, DMSO): $m/z = 1260.3054$ $[\text{M} - \text{PF}_6]^+$. Anal. Calcd. for $\text{C}_{62}\text{H}_{47}\text{F}_{12}\text{IrN}_7\text{O}_2\text{PS}$: C, 52.99; H, 3.37; N, 6.89; S, 2.28. Found: C, 53.01; H, 3.35; N, 6.87; S, 2.29 (%).



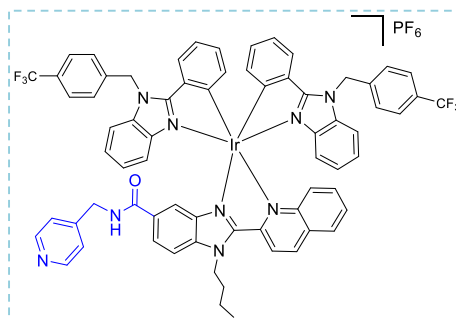
E2.3. Synthesis of the heterobimetallic Ir(III)-Pt(II) complex

The synthesis of the heterodinuclear complex of Chapter 5 was carried out, first synthesizing the iridium complex with the linker (complex **33**) and then by reacting it with the platinum complex **34**.

Preparation of complex **33**:

Ir complex **32** (1.3 mol equiv.) was coupled onto 4-(aminomethyl)pyridine (1.0 mol equiv.) with the coupling reagent HATU (1.2 mol equiv.) in anhydrous DMF in the presence of DIPEA (5 mol equiv.) for 24 h under nitrogen atmosphere at RT. Then, water was added, and the red solid was filtered and washed with water, cold MeOH, and diethylether.

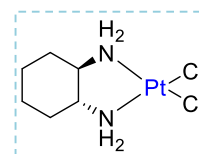
Red solid. Isolated yield: 90%. ^1H -NMR (300 MHz, $[\text{D}_6]\text{DMSO}$): δ 8.89 (d, J = 8.8 Hz, 1H), 8.67 (d, J = 8.8 Hz, 1H), 8.44 (d, J = 4.2 Hz, 2H), 8.29 (d, J = 5.6 Hz, 1H), 8.16 (d, J = 8.8 Hz, 2H), 8.03-7.91 (m, 2H), 7.76-7.66 (m, 4H), 7.60 (t, J = 7.0 Hz, 1H), 7.53-7.50 (m, 4H), 7.22-7.13 (m, 6H), 7.08-7.03 (m, 1H), 6.92-



6.76 (m, 8H), 6.63 (br s, 1H), 6.34-6.28 (m, 3H), 6.22-6.07 (m, 4H), 5.93 (d, J = 8.2 Hz, 1H), 5.15-4.97 (m, 2H), 4.41-4.27 (m, 2H), 1.70-1.57 (m, 2H), 0.91-0.72 (m, 2H), 0.50 (t, J = 7.1 Hz, 3H). ^{13}C - NMR (150 MHz, $[\text{D}_6]\text{DMSO}$): 167.26, 164.15, 163.66, 163.52, 157.11, 155.57, 151.14, 150.76, 149.49, 149.39, 146.67, 142.84, 142.09, 142.04, 140.46, 139.83, 139.59, 139.56, 136.23, 136.18, 134.77, 134.68, 134.21, 133.58, 132.89, 132.64, 131.84, 131.59, 130.58, 130.29, 129.83, 129.73, 129.50, 129.36, 128.09, 127.71, 127.26, 127.05, 126.82, 126.66, 126.09, 125.67, 125.23, 125.09, 124.01, 123.99, 123.47, 123.30, 122.04, 119.06, 115.32, 114.89, 114.29, 113.18, 113.07, 48.20, 47.96, 47.27, 43.07, 33.08, 20.16, 14.60. ESI-MS (pos. ion mode, DMSO): m/z = 1330.3912 $[\text{M} - \text{PF}_6]^+$. Anal. Calcd. for $\text{C}_{69}\text{H}_{53}\text{F}_{12}\text{IrN}_9\text{OP}$: C, 56.17; H, 3.62; N, 8.54. Found: C, 56.20; H, 3.63; N, 8.60 (%).

Preparation of platinum complex **34**:

The dichloro[(1R,2R)-*trans*-diaminocyclohexane]platinum(II) complex **34** was prepared according to standard literature procedures.^{17–19}

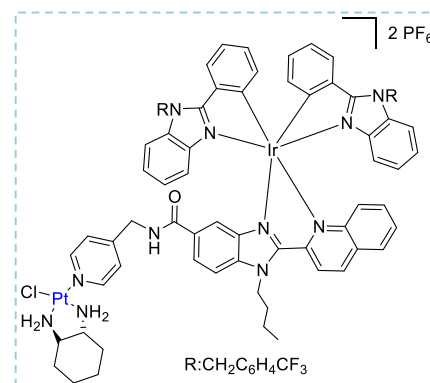


$\text{K}_2[\text{PtCl}_4]$ (7.23 mmol) was dissolved in 40 mL water. (1R, 2R)-(-)-diaminocyclohexane (7.23 mmol) was dissolved in 15 mL water, added to the dark red solution, and the reaction mixture was stirred at room temperature for 5 h in the dark. The yellow precipitate formed was filtered off and washed three times with water, EtOH, and diethyl ether. The product was dried five days over P_2O_5 under reduced pressure. Yellow solid. Isolated yield: 85%. Reported.¹⁷

Preparation of heterobimetallic complex **35**:

The heterobimetallic complex **35** was synthesized with a modification of a method of literature.²⁰ Pt complex **34** (0.039 mmol) and AgPF₆ (0.037 mmol) were stirred in anhydrous DMF (1 mL) overnight in the dark at 45 °C. The suspension was filtered with celite to remove AgCl. Then, an Ir complex **33** (0.031 mmol) solution in DMF (1 mL) was dropped into the previous platinum solution and stirred for 24 h in the dark at 55 °C. The DMF was removed, and the residue was treated with diethyl ether in constant stirring for 30 min. The precipitate obtained was filtered and dried under vacuum. Intense red solid.

Isolated yield: 81%. ¹H-NMR (400 MHz, [D₇]DMF): δ 9.03 (d, J = 8.8 Hz, 1H), 8.91 (d, J = 8.8 Hz, 1H), 8.77 (d, J = 6.5 Hz, 2H), 8.34-8.27 (m, 4H), 8.10 (t, J = 8.8 Hz, 1H), 8.03-8.00 (m, 1H), 7.90-7.86 (m, 2H), 7.81 (d, J = 8.6 Hz, 1H), 7.76 (t, J = 7.7 Hz, 1H), 7.69 (dd, J = 8.6, 1.2 Hz, 1H), 7.65 (d, J = 8.4 Hz, 2H), 7.50 (d, J = 5.3 Hz, 2H), 7.41-7.38 (m, 2H), 7.34-7.22 (m, 3H), 7.14



(d, J = 8.1 Hz, 2H), 6.97-6.92 (m, 4H), 6.88-6.84 (m, 2H), 6.56-6.52 (m, 2H), 6.47-6.29 (m, 5H), 6.22 (d, J = 8.0 Hz, 1H), 6.12 (d, J = 9.2 Hz, 1H), 5.85-5.80 (m, 1H), 5.63-5.54 (m, 1H), 5.35-5.16 (m, 2H), 4.64-4.53 (m, 2H), 2.66-2.51 (m, 2H), 2.16-2.07 (m, 2H), 2.00-1.80 (m, 2H), 1.60-1.43 (m, 5H), 1.28 (br s, 2H), 1.22-1.10 (m, 2H), 1.07-0.83 (m, 2H), 0.70 (t, J = 7.3 Hz, 3H). ¹³C- NMR (150 MHz, [D₇]DMF): δ 167.59, 164.38, 163.61, 157.35, 155.50, 153.56, 153.34, 151.22, 149.62, 146.84, 142.51, 141.96, 140.65, 140.01, 139.77, 139.72, 136.29, 136.17, 135.04, 134.79, 134.26, 133.52, 132.51, 131.48, 131.22, 130.52, 130.39, 129.99, 128.11, 127.67, 127.10, 126.85, 126.70, 126.62, 125.94, 125.37, 125.07, 124.84, 123.92, 123.73, 123.00, 121.98, 119.07, 115.72, 115.09, 113.94, 112.60, 64.29, 63.93, 63.45, 48.14, 47.89, 47.22, 42.96, 33.26, 33.11, 32.86, 32.66, 25.39, 25.26, 25.09, 20.25, 14.02. ESI-MS (pos. ion mode, DMF): m/z = 837.7221 [M - PF₆]²⁺. Anal. Calcd. for C₇₅H₆₇ClF₁₈IrN₁₁OP₂Pt: C, 45.84; H, 3.44; N, 7.84. Found: C, 45.70; H, 3.48; N, 7.77 (%). ¹⁹⁵Pt NMR (600 MHz, [D₇]DMF): δ = -2460 (s, 1Pt).

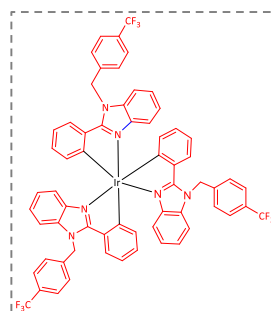
E2.4. Synthesis of neutral tris-cyclometalated Ir(III) complexes

The neutral complexes of Chapter 7 were synthesized in two stages. Firstly, through the formation of the dimer, as explained in section E2.2, and then through the synthesis of the monomer as indicated below:

Preparation of the homoleptic complex **38**:

Complex **38** was synthesized using an adaptation method of reference.²¹ The Ir(III) dimer **7** (0.081 mmol), 2-phenyl-1-[4-(trifluoromethyl)benzyl]-1H-benzo[d]imidazole (0.59 mmol), and silver triflate (0.16 mmol) in 2-ethoxyethanol (2 mL) was heated at 95 °C overnight under nitrogen atmosphere. The solvent was concentrated under reduced pressure, and the product was re-suspended in dichloromethane. The suspension was filtered with celite to remove the white AgCl precipitate, and the filtrate was solvent-stripped to dryness. Then, the obtained solid was washed with ethyl acetate to obtain the bright yellow product.

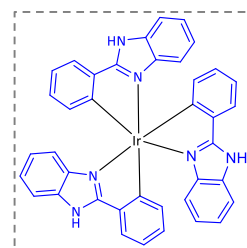
Bright yellow solid. Isolated yield: 65%. ¹H-NMR (300 MHz, [D₂] Methylene Chloride): δ 7.55 (d, J = 8.1 Hz, 2H), 7.46-7.44 (m, 1H), 7.34-7.18 (m, 4H), 6.87 (t, J = 7.2 Hz, 1H), 6.73-6.71 (m, 3H), 6.50 (d, J = 8.1 Hz, 1H), 6.07-5.86 (m, NCH₂Ar, 2H). ¹³C-NMR (75.4 MHz, [D₂] Methylene Chloride): δ 163.73, 142.89, 140.99, 138.44, 136.93, 135.85, 131.09, 130.66, 130.51, 127.37, 127.01, 126.96, 125.28, 124.29, 123.84, 120.71, 117.11, 110.76, 48.81. ESI-MS (pos. ion mode, CH₂Cl₂): m/z = 1247.3001 [M+H]⁺. Anal. Calcd. for C₆₃H₄₂F₁₂IrN₆: C, 60.72; H, 3.40; N, 6.74. Found: C, 60.66; H, 3.41; N, 6.62 (%).



Preparation of the homoleptic complex **39**:

Complex **39** was synthesized using an adaptation method of reference.²² The Ir(III) dimer **16a** (1 mmol), 2-phenyl-1H-benzo[d]imidazole (10 mmol), and silver triflate (2 mmol) were all suspended in 10 mL of ethylene glycol. Under a nitrogen atmosphere, the mixture was heated at 180 °C for 5 h. The reaction product was cooled to room temperature and poured into 100 mL of water after 5 h. To this solution, CH₂Cl₂ was added and extracted using a separatory funnel. Upon solvent evaporation from the organic layer, the crude product was purified by column chromatography on silica gel with a mixture of CH₂Cl₂/ACN (8:2).

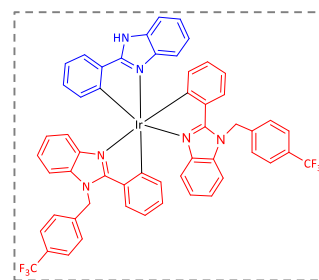
Green solid. Isolated yield: 27%. ¹H-NMR (300 MHz, [D₆]DMSO): δ 13.15 (s, 1H), 7.62 (d, J = 7.2 Hz, 1H), 7.48 (d, J = 7.8 Hz, 1H), 7.05 (t, J = 14.7 Hz, 1H), 6.73-6.66 (m, 1H), 6.62 (s, 3H), 6.00 (d, J = 8.1 Hz, 1H). ¹³C-NMR (75.4 MHz, [D₆]DMSO): δ 164.57, 161.84, 144.06, 138.10, 136.77, 134.98, 130.48, 124.61, 123.60, 120.28, 116.28, 113.23, 31.87. ESI-MS (pos ion mode, DMSO): m/z = 772.1926. Anal. Calcd. for C₃₉H₂₇IrN₆: C, 60.68; H, 3.53; N, 10.89. Found: C, 60.56; H, 3.49; N, 10.91 (%).



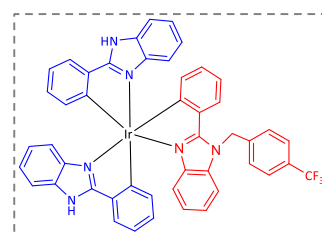
Preparation of the heteroleptic complexes 40 y 41:

The heteroleptic complexes were synthesized using an adaptation method of reference.²³ The iridium(III) dimers **7** or **16a** (1 mmol), the corresponding 2-phenylbenzimidazole-based ligands (10 mmol), and Na₂CO₃ (2 mmol) were all suspended in 2 mL of 2-ethoxyethanol. The mixture was refluxed for 3 h and the solid was filtered after cooling in the fridge for 2 hours. Finally, the crude was purified by a chromatography column on silica gel with dichloromethane as eluent.

Complex 40. Yellow solid. Isolated yield: 31%. ¹H-NMR (300 MHz, [D₂] Methylene Chloride): δ 9.59 (s, 1H), 7.56-7.45 (m, 8H), 7.35-7.19 (m, 6H), 7.24-7.14 (m, 3H), 6.95-6.73 (m, 12H), 6.46 (d, J=7.8 Hz, 1H), 6.40 (d, J=8.37 Hz, 1H), 6.32 (d, J=7.5 Hz, 1H), 6.07 (br s, 1H), 6.01 (br s, 1H), 5.93 (d, J=5.82 Hz, 1H), 5.71 (d, J=6.21 Hz, 1H). ¹³C-NMR (75.4 MHz, [D₂] Methylene Chloride): δ 174.54, 164.07, 163.92, 163.71, 143.47, 142.91, 142.77, 141.01, 138.51, 138.32, 137.82, 136.83, 135.68, 135.47, 134.10, 134.04, 131.08, 130.69, 130.55, 127.38, 127.01, 126.96, 125.42, 125.39, 124.48, 124.28, 123.91, 123.75, 123.66, 120.48, 120.42, 120.30, 117.09, 116.60, 112.60, 110.74, 110.62, 48.80. ESI-MS (pos. ion mode, DMSO): m/z = 1089.2669 [M+H]⁺. Anal. Calcd. for C₅₅H₃₇F₆IrN₆. C, 60.71; H, 3.43; N, 7.72. Found: C, 60.82; H, 3.43; N, 7.60 (%).



Complex 41. Green yellowish solid. Isolated yield: 45%. ¹H-NMR (300 MHz, [D₆]DMSO): δ 13.22 (s, 2H), 7.72 (d, J= 8.4 Hz, 1H) 7.68-7.64 (m, 2H), 7.59 (d, J= 8.0 Hz, 2H), 7.54-7.52 (m, 3H) 7.30 (d, J= 7.8 Hz, 2H), 7.16-7.08 (m, 3H), 6.80-6.74 (m, 4H) , 6.70-6.54 (m, 8H) , 6.23-6.14 (m, 3H), 6.11 (d, J= 8.0 Hz, 1H), 6.05 (d, J= 7.8 Hz, 1H). ¹³C-NMR (75.4 MHz, [D₆]DMSO): δ 163.53, 163.41, 163.17, 162.12, 161.17, 160.09, 142.37, 141.68, 141.46, 137.07, 136.50, 136.27, 135.80, 135.36, 134.53, 133.68, 129.23, 129.10, 129.01, 126.74, 125.62, 124.92, 124.62, 123.41, 123.31, 123.12, 122.55, 122.27, 122.17, 122.04, 119.00, 118.90, 118.76, 115.13, 114.59, 112.09, 112.03, 110.77, 46.79. ESI-MS (pos ion mode, DMSO): m/z = 931.2300 ([M+H])⁺. Anal. Calcd. for C₄₇H₃₂F₃IrN₆: C, 60.70; H, 3.47; N, 9.04. Found: C, 60.81; H, 3.38; N, 8.98 (%).



E3. CELL-FREE ASSAYS

E3.1. Solution stability of the new complexes

Stability of complexes in DMSO or DMF was studied by ^1H -NMR, verifying that no new signals appear after 1, 3 or even 7 days. Stability in cell culture medium was checked by HPLC (50 μM , 5% DMSO) in chapters 2 and 3, and by UV/Vis spectrophotometry (10 μM , 1% DMSO or DMF) in chapters 4, 5 and 6. The stability of **35** at physiological pH values was checked by UV/Vis in water/DMF (50:50) solutions (10 μM) with pH adjustment using 0.1M NaOH and HCl (10%) solutions. UV/Vis also checked the stability of **37** at different pHs in disodium hydrogen phosphate/citric acid buffer solutions (pH 3.3, 5.4, 6.3, and 7.4) with 10% of DMSO.

E3.2. Photostability analyses

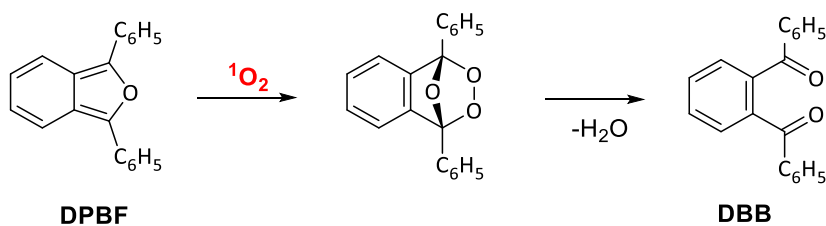
Chapter 2. The UV/Vis spectrum of a solution of complex **10** in water (10 μM , 1% DMSO) was recorded. The sample in the cuvette was irradiated at 420 nm for 15 min with a 450 W xenon lamp. After this time, the UV/Vis spectrum was collected again.

Chapter 3. The UV/Vis spectra of **21a**, **21b**, and **21c** solutions (10 μM) in water (1% DMSO) were recorded. The samples were irradiated for 1 h with a blue-centered LED (450 nm) and subsequently, the UV/Vis spectra were recorded again.

Chapter 4. Solutions of **26-29** (10 μM) in water (1% DMSO) were prepared and their UV/Vis spectra were recorded. The samples were irradiated for 1 h with a photoreactor Luzchem EXPO-LED with blue light led lamp (465 nm, 8.4 mW/cm²) and subsequently, the UV/Vis spectra were recorded again. The measures of light power were realized using Thorlabs Digital Optical Power and Energy Meter PM100D with S120VC photodiode power sensor.

E3.3. Singlet oxygen detection using 1,3-diphenylisobenzofuran (DPBF)

Singlet oxygen was detected by the standard method using DPBF as the $^1\text{O}_2$ indicator through monitoring the decrease in the absorption at 418 nm of 1,3-diphenylisobenzofuran (DPBF) due to its photooxidation to *o*-dibenzoylbenzene (DBB) under blue light irradiation^{24,25} (**Scheme E3.1**). The registration of the absorbance was carried out on a PerkinElmer Lambda 750 S spectrometer with operating software.



Scheme E3.1. The reaction of DPBF with singlet oxygen.

The PBS solutions (1 % DMSO) of **21a**, **21b**, and **21c** (10 μM) containing 10 μM of DPBF, was prepared in the dark and irradiated with blue light (450 nm centered LED). The absorbance of DPBF at 418 nm was recorded every 15 s. A PBS solution (1 % DMSO) of DPBF without the photosensitizer was used as a negative control. The plots shown in **Figure 3.14** reveal that **21a** and **21c** are efficient ${}^1\text{O}_2$ generators whereas **21b** not, which is consistent with the results demonstrating the involvement of ${}^1\text{O}_2$ in the photopotential of Ir(III) complexes **21a-c** in cells.

E3.4. Photo-oxidation of NADH.

Reactions between complexes **26-29** (5 μM) and NADH (100 μM) at different ratios were monitored by UV/Vis in the dark or on irradiation with 465 nm light (8.5 mW/cm^2) in PBS (0.5 % DMSO). Turnover number (TON) was defined as the number of moles of NADH that the Ir compound could convert in 30 min. Turnover frequency (TOF) was calculated from the concentration of oxidized NADH (calculated by the difference of concentration of NADH) after 30 min divided by the concentration of complexes. The concentration of NADH was obtained using the extinction coefficient²⁶ ($\epsilon_{339}=6220 \text{ M}^{-1}\text{cm}^{-1}$). For the measures in hypoxic conditions, the cuvette was degassed by bubbling N_2 for 30 min.

E3.5. Log *P* determination

Log *P* values of **26-29** were calculated using an HPLC-based method validated by Keppler *et al.*²⁷ The iridium complexes were dissolved in 1 mL of water/methanol (1:1) and the solutions were filtered through a 0.45- μm filter. Uracil was employed as an internal standard (1 $\mu\text{mol L}^{-1}$) for determining the system dead time (t_0). The obtained samples were directly analyzed by RP-HPLC. The RP-HPLC experiments were performed in isocratic modes at least in duplicates, employing 3 different water/methanol compositions per complex (30/70, 25/75, and 20/80). The eluent flow rate was 0.7 mL/min. The capacity factors $k = (t_r - t_0)/t_0$, where t_r is the retention time of the analyte, were determined for the

three investigated eluent compositions. Then, we used the linear Soczewinski–Snyder relationship:

$$\text{Log } k = \text{Log } k_w - S\phi$$

Log k is the capacity factor in a specific mobile phase composition

ϕ is the volume fraction of the methanol in the eluent

S is a constant for a given analyte and HPLC system

By representing Log k against their correspondent methanol percentages in the eluent (**Figure S4.18**), we obtained the chromatographic hydrophobicity parameter ϕ_0 , corresponding to Log $k = 0$. Log P was calculated through the equation obtained by representing the log P of the most lipophilic complexes found by Keppler *et al.*²⁷ and their corresponding values of ϕ_0 (**Figure S4.19**). The method was checked first comparing with the log P measured using the “shake flask” method for the Ir complex previously described by us¹ (**Table S4.1**).

E3.6. Interaction with biomolecules

Solutions of **35** (10 μM) were prepared in water (1% DMF) and 10 mM of glutathione or histidine was added. UV/Vis spectra were recorded every 15 min for 90 min at RT.

E3.7. Encapsulation of iridium complexes and characterization of NPs (collaboration with Dr. Marchán)

The synthesis of amphiphilic cationic polymer and Ir-loaded amphoteric NPs (**NPs-38** and **NPs-40**) and the characterization of nanoparticles were carried out by Joaquin Bonelli Blasco (University of Barcelona, Nanobiotechnological Polymers Division, Ecopol Tech). The polymerization reaction progress for NPs synthesis, and the encapsulation reaction was controlled by IR spectroscopy. IR spectra were acquired in a Smart ATR (Nicolet iS10, Thermo Scientific, Raleigh, USA) using a transmittance mode (16 scans) and OMNIC software. For the monitoring of solvent-based samples, one drop was deposited onto the diamond crystal and the solvent was left to dry by evaporation. IR spectra were recorded from a dry film of the sample for the reaction control after emulsification.

The size distribution of the NPs was analyzed on a Zetasizer Nano ZS90 (Malvern, Worcestershire, UK) in Milli Q water at 25 °C at a concentration of 0.5 mg/mL. The Z-pot of the NPs was analyzed on a Zetasizer Nano ZS90 (Malvern, Worcestershire, UK) in Milli Q water at 25 °C at a concentration of 1 mg/mL, measured at different pH values.

The amount of Ir complexes inside the NPs was determined by ICP-MS analysis. A fixed volume of NPs emulsion (previously dialyzed) was diluted in 500 µL of concentrated 72% (v/v) nitric acid into Wheaton v-vials (Sigma-Aldrich) and heated in an oven at 373 K for 18 h. The vials were then allowed to cool, and each sample solution was transferred into a volumetric tube and combined with Milli-Q water washings (1.5 mL). Digested samples were diluted 5 times with Milli-Q to obtain a final HNO₃ concentration of approximately 3.6% v/v. Iridium content was analyzed on an ICP-MS Perkin Elmer Elan 6000 series instrument at the Centres Científics i Tecnològics of the Universitat de Barcelona. The solvent used for all ICP-MS experiments was Milli-Q water with 1% HNO₃. The iridium standard (High-Purity Standards, 1000 µg/mL ± 5 µg/mL in 5% HNO₃) was diluted with 1% HNO₃ to 10 ppb. Iridium standards were freshly prepared in Milli-Q water with 1% HNO₃ before each experiment. The concentrations used for the calibration curve were in all cases 0, 0.2, 0.4, 1, and 2 ppb. The isotope detected was ¹⁹³Ir and readings were made in triplicate for each sample. Rhodium was added as an internal standard at a concentration of 10 ppb in all samples.

The following equations were used to calculate Encapsulation Efficiency EE (%) and Drug Loading DL (%):

$$\% EE = \frac{\text{amount of Ir complex incorporated in the nanocapsule}}{\text{total amount of Ir complex added in the aqueous dispersion}} * 100$$

$$\% DL = \frac{\text{amount of Ir complex incorporated in the nanocapsule}}{\text{total amount of dried nanocapsules}} * 100$$

E4. IN VITRO BIOLOGICAL STUDIES (Collaboration with Prof. Brabec)

E4.1. Cell culture

The A2780 and A2780cisR cells were grown in RPMI 1640 medium supplemented with gentamycin (50 µg mL⁻¹) and 10% heat-inactivated fetal bovine serum. The acquired resistance of A2780cisR cells was maintained by supplementing the medium with 1 µM

cisplatin every second passage. The MCF-7, HeLa, MCF-10A, A375, 518.A2, J774.A1 and BGM cells were grown in DMEM medium (high glucose, 4.5 g L^{-1}) supplemented with gentamycin ($50 \text{ } \mu\text{g mL}^{-1}$) and 10% heat-inactivated fetal bovine serum (Biosera). The medium for MCF-10A cells was supplemented with 100 ng mL^{-1} cholera toxin. CT26 murine fibroblasts were cultured in RPMI growth medium (Biosera) supplemented with gentamycin (50 mg mL^{-1}) and 10% heat-inactivated FBS. Non-tumorigenic chinese hamster ovary cells, CHO, were grown in FK-12 medium supplemented with 10 % FBS and 2 mM L-glutamine .

E4.2. Cytotoxicity assay

The MTT assay is used to measure cellular metabolic activity as an indicator of cell viability, proliferation, and cytotoxicity. This colorimetric assay is based on the reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to purple formazan crystals (**Figure E4.1**). Viable cells with active metabolism contain NAD(P)H-dependent oxidoreductase and convert MTT into purple-colored insoluble formazan crystals with an absorbance maximum near 570 nm . When cells die, they lose the ability to convert MTT into formazan, thus color formation serves as a useful and convenient marker of only the viable cells.

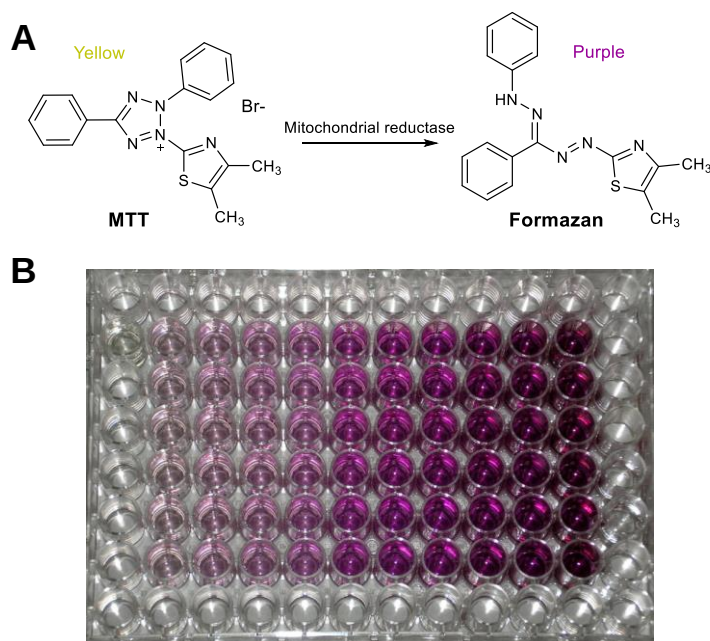


Figure E4.1. Metabolism of MTT to formazan by viable cells as shown in a A) chemical reaction and B) in a 96-well plate.

Cells were seeded on 96-well tissue culture plates at a density of 10^4 cells/well for A2780 and A2780cisR cells or 5×10^3 cells/well for HeLa and MCF7 cells in 100 μL of growth medium and incubated at 37 °C in a humidified 5% CO_2 atmosphere for 16 h. After the incubation period, the cells were treated with the compounds and kept in the incubator for additional 72 h. The stock solutions of metal-based compounds were always freshly prepared in DMSO, DMF (for complexes **34** and **35**) or H_2O (for cisplatin) before use. The final concentration of DMSO or DMF in the cell culture medium did not exceed 0.1% (v/v), which was shown not to affect cell growth. The final concentrations of tested compounds were in the range of 0–50 μM in a volume of 200 μL /well. Subsequently, 10 μL of MTT solution (5 mg mL^{-1}) was added to each well, and plates were incubated for 4 h. At the end of the incubation time, the medium was removed, and the formazan product was dissolved in 100 μL of DMSO per well. Cell viability was evaluated by measuring the absorbance at 570 nm (reference wavelength at 630 nm) using an absorbance reader Synergy Mx (Biotek, USA). The IC_{50} values were calculated based on the inhibitory rate curves using the next the equation:

$$I = \frac{I_{\max}}{1 + \left(\frac{\text{IC}_{50}}{C}\right)^n}$$

Where I represent the percentage inhibition of viability observed, $I_{\max}$ is the maximal inhibitory effect, IC_{50} is the concentration that inhibits 50% of maximal growth, C is the concentration of the treatment and n is the slope of the semi-logarithmic dose-response sigmoidal curves (**Figure E4.2**).

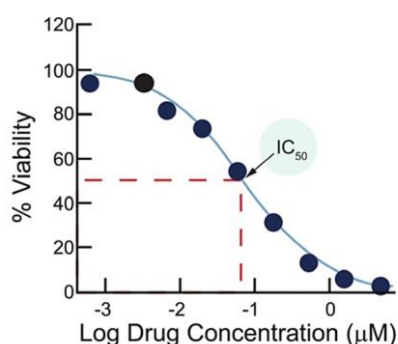


Figure E4.2. Curves constructed by plotting cell survival (%) versus drug concentration (μM) for the calculation of IC_{50} values.

E4.3. Photo-toxicity testing

The phototoxic activity of the new Ir(III) complexes was determined against HeLa cells (in Chapters 2, 3 and 4) and against A375 and 518.A2 (in Chapter 4). Briefly, at a density of 5×10^3 cells/well were cultured under normoxic (21% O₂, 5% CO₂) or hypoxic (2% O₂, 5% CO₂) conditions in a humidified incubator. After overnight incubation, the tested compound was added in EBSS, and the cells were irradiated for 1 h ($\lambda_{\text{max}} = 420$ nm, 7.7 ± 3 mW/m²) or left in the dark. After the irradiation, cells were washed and incubated in the normoxic or hypoxic conditions in a complete drug-free culture medium. The viability of cells was determined 72 h after irradiation using standard MTT assay. The Neutral red assay was also used in Chapter 4 for comparative purposes.

E4.4. Cellular accumulation

The cells were seeded on 100 mm tissue culture dishes (1.5×10^6 cells/dish in 8 mL of growth medium). After 48 h of incubation, the cells were treated with tested compounds at equimolar concentrations for 5 or 24 h. The attached cells were harvested by trypsinization, and the cell pellets were washed twice with cold PBS. The pellets were digested by using a microwave acid (HCl, 11 M) digestion system (MARS5, CEM) to give a fully homogenized solution. Final iridium or platinum content in the samples was determined by inductively coupled plasma mass spectrometry (ICP-MS).

In **Chapter 7**, cellular accumulation from iridium complexes (**38** and **40**) and from iridium-containing nanocapsules (**NPs-38** and **NPs-40**) was measured in HeLa cells. The cells were seeded in 6-well plates at a density of 6×10^5 cells/well in 1.8 mL of complete growth medium and incubated for 48 h before treatment. Cells were then treated with 5 μ M of the iridium complexes or 2 mg/mL of the nanocapsules for 30 min. After trypsinization, cells were counted and the pellets digested using Suprapur® nitric acid 30 % for 1 h. The amount of iridium was determined using ICP-MS. Two independent measurements were conducted (n=2 replicate).

E4.5. Cellular localization

HeLa, A375 or A2780 cells were seeded on 35 mm glass-bottom confocal culture dishes at the density of 2×10^5 cells/dish and incubated overnight. The next day, the cells were treated with tested compounds (5 μ M). After the 2 h incubation, cells were co-stained with ER-Tracker™ (red, BODIPY™ TR glibenclamide) at the concentration of 1 μ M

and/or Mitotracker Green FM (0.1 μ M) for 30 min. Then, cells were analyzed on confocal laser-scanning microscope Leica TCS SP8 SMD. The complexes were excited at 405 nm. Samples were scanned sequentially, and the emission parameters were carefully set to omit possible fluorescence overlaps. Co-localization analysis was performed as described previously²⁸ using the Coloc2 plugin in ImageJ software. Briefly, the Pearson coefficient of correlation (PCC) was measured for entire images by default, and the Costes regression method for estimation of the threshold was used.

E4.6. Real-time cell electronic sensing

The Real-time Cell Analyzer (RTCA) equipment (xCELLigence RTCA SP instrument, Roche) was calibrated for the background impedance with 100 μ L of fresh RPMI-1640 medium. A2780 cells and HeLa cells were added at the density of 8000 and 4000 cells/well, respectively, and incubated for 24 h at 37°C in a humidified 5% CO₂ atmosphere. Subsequently, the cells were treated with **8** and **12**. Cell-sensor impedance called cell index is defined as $(R_t - R_b)/15$, where R_t is the impedance of the cells at defined time points, and R_b is the background impedance of the culture medium. Impedance was monitored 5-minute sweeps for all the time during the experiment. Cell index was normalized at the time of treatment (24 h). Each sample was plated on the E-plate as a quadruplicate. The concentrations of treated compounds in the media used for treatment were verified by FAAS.

E4.7. Effect on translation

HeLa cells were seeded in 6-w plates (5×10^3 cell/well) and grown for 24 h. The medium was replaced with a fresh medium with the absence and/or presence of Ir(III) compounds (**8** and **12**) and the cells were incubated for 1 h. The medium was removed and the cells were washed twice with PBS and incubated in the medium containing [³⁵S]methionine (5 μ Ci/sample) for 2 h. A negative control sample was incubated without [³⁵S]methionine. The medium was removed and the cells were washed with PBS thrice, trypsinized, counted, and pelleted. Cell pellets were lysed in ice-cold RIPA buffer (1 mL/30 min). The lysates were cleared by centrifugation (15 000g/15 min). 40 μ L of individual lysates were transferred onto a 0.22 μ m filter and washed thrice with 5% TFA. The samples were analyzed using Scintillation analyzer Tri-Carb 2800TR.

E4.8. Cell-death studies

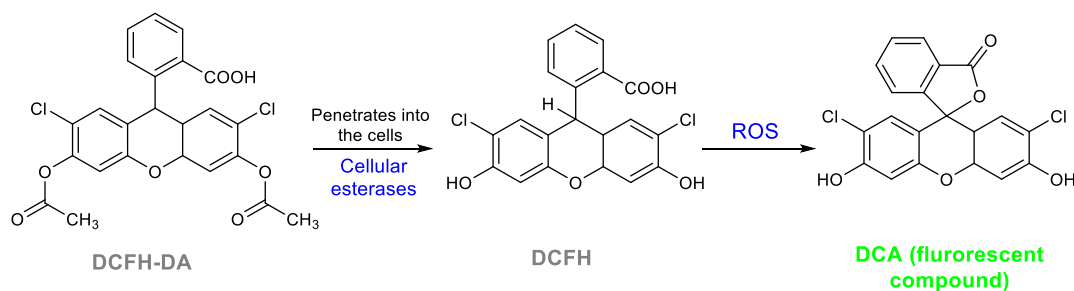
Chapter 2. The cell death detection ELISA plus kit was used to determine the extent of apoptosis and necrosis in cancer cells treated with **8** and **12**. In this assay, internucleosomal DNA fragmentation was quantitatively assayed by antibody-mediated capture and detection of cytoplasmic mononucleosome- and oligonucleosome-associated histone-DNA complexes. HeLa cells were treated with concentrations of complexes corresponding to their IC₂₀, IC₅₀ and IC₈₀ determined after 72 h in these cells (0.36, 1.76, and 4.68 μ M for **8**, and 7.6, 23, and 36 μ M for **12**). Then, the cells were centrifuged (300g, 10 min) and the experiment was performed as already described²⁹ according to the manufacturer's protocol.

Chapter 3. The seven-stage electronic gating strategy was used to identify six different populations of the cells undergoing cell death as described previously.³⁰ HeLa cells were seeded on 6-well plates at the density of 1.5×10^5 cells per well and incubated overnight. Then, the cells were treated with the investigated compounds (10 μ M) and incubated for 1 h in a humidified 5% CO₂ atmosphere. Cells were placed on a LZC-ICH2 photoreactor from Luzchem (Canada) and irradiated with blue light ($\lambda_{\text{max}} = 420$ nm, 77 ± 3 W m⁻²) for another 1 h. Subsequently, cells at different stages of cell death were harvested and stained with Annexin-V-FITC and TO-PRO®-3. Thirty thousand events were analyzed on the flow cytometer (BD FACSVerse). The seven-stage electronic gating strategy was performed by using FCS Express 6 (DeNovo software; Glendale, CA).

Chapters 5 and 6. The impact on cell death induction of the iridium complex on A2780 cells was evaluated using the Annexin V/Propidium Iodide labeling method. First, 3×10^5 A2780 cells were seeded and incubated 24 h (37 °C, 5% CO₂) in complete medium in flat-bottom 12-well plates and treated with the compounds at indicated concentrations. After 24 h treatment, cells were trypsinized, centrifuged, and stained with a mixture of 5 μ L Annexin V-FITC and 10 μ L propidium iodide already diluted in binding buffer for 15 min at RT. Finally, cells were acquired at a total number of 10,000 cells on a FACS Calibur™ flow cytometer. Data were analyzed using FlowingSoftware version 2.5.1. The assay was performed in two independent experiences (n= 2 per replicate), yielding similar results.

E4.9. Intracellular reactive oxygen species (ROS) determination

Chapters 2, 3 and 6. Cells were seeded on 96-well black plates at a density of 1×10^4 cells/well and incubated at 37 °C in a humidified 5% CO₂ atmosphere overnight. Subsequently, the cells were treated with tested compounds in EBSS at equimolar concentrations (1, 5, 10, 20 μ M) and held under cultivation conditions for 1 h. Then cells were irradiated at 420 nm for 1 h or kept in the dark for this period. Afterward, intracellular ROS were quantified using a method developed by Robinson et al.^{31–33} Briefly, 10 μ M of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was added to cells, and samples were incubated for 30 min at 37 °C. After cell uptake, cellular esterases deacetylate DCFH-DA, yielding a non-fluorescent compound and then, ROS oxidize DCFH-DA into 2',7'-dichlorofluorescein (DCF), a fluorescent compound (**Scheme E4.1**). The fluorescence intensity was evaluated by measuring the fluorescence (excitation/emission wavelengths: 504 nm/529 nm) using a fluorescence reader Synergy Mx (Biotek, USA). Three independent experiments were performed.



Scheme E4.1. Mechanism of DCFH-DA probe inside the cell.

Chapter 4. A375 cells were seeded on 6-well plates at a density of 2×10^5 cells per well and were incubated (37°C, 5% CO₂) overnight. Subsequently, cells were treated with tested compounds in EBSS at equimolar concentrations (5, 10, and 20 μ M) and irradiated as described above. The cells were then washed with PBS, harvested by trypsinization, and 5 μ M CellROX®Deep Red reagent was added to the cells and incubated for 30 min at 37°C. This dye penetrates the cell and is not fluorescent when in a reduced state, but presents a red photostable fluorescence after oxidation ROS. Cells were washed with PBS, and the fluorescence intensity (exc: 640nm, em: 660nm) was analyzed by flow cytometer. Data were analyzed by using FCS Express 6. It was verified experimentally that the free complexes (in cell-free media) do not contribute to the final fluorescence signal.

E4.10. Characterization of reactive oxygen species

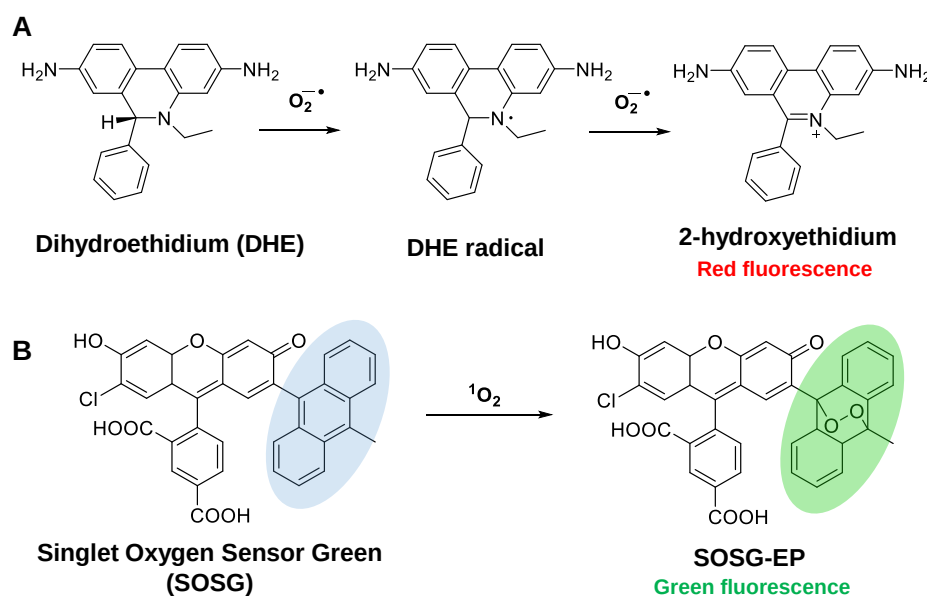
HeLa cells were seeded at the density of 1.5×10^3 cells per cm^2 on 6-well plates and incubated overnight. For the determination of specific ROS generated in HeLa cells after the treatment with compounds **21a–c**, cells were pre-treated with selective ROS scavengers and incubated for 1 h at 37 °C before the treatment with 10 μM of the investigated compounds. We used 10 mM of sodium pyruvate for hydrogen peroxide (H_2O_2),³⁴ 50 mM of D-mannitol for hydroxyl radicals ($\cdot\text{OH}$),^{35,36} 5 mM of tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt monohydrate) for superoxide anions ($\text{O}_2^{\cdot-}$),^{37,38} 5 mM of sodium azide to eliminate singlet oxygen ($^1\text{O}_2$),³⁶ and 50 μM of ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) to prevent the generation of peroxynitrite anion species.^{39–41} The antioxidants remained throughout the experiment. After the pre-treatment with selective ROS scavengers, the cells were treated for 2 h with 10 μM of **21a–c** (1 h of incubation in the dark followed by 1 h of irradiation with 420 nm; $77 \pm 3 \text{ W m}^{-2}$). After irradiation the cells were collected, stained by using CellRox® Oxidative stress Deep red reagent and analyzed on a flow cytometer (BD FACSVerser). Data were analyzed by using FCS Express 6.

E4.11. ROS detection in cells by confocal microscopy

Cells were seeded at a density of 1.5×10^5 cells per confocal 35 mm dish and incubated overnight under normoxic (21% O_2 , 5% CO_2) or hypoxic (2% O_2 , 5% CO_2) conditions in a humidified incubator. After that, cells were treated with compounds at appropriate concentrations and incubated for 1 h in the dark. Subsequently, samples were irradiated with 420 nm or kept in the dark for another 1 h. Cells were stained with 15 μM of dihydroethidium (DHE, $\cdot\text{O}_2^-$ specific probe, Sigma) or with 5 μM of singlet oxygen sensor green (SOSG, $^1\text{O}_2$ specific probe, Thermo Fisher Scientific) for 15 min and then visualized on a confocal microscope Leica TCS SP8 SMD. In Chapter 6, N-acetyl-cysteine (NAC) was used at 5 mM for ROS scavenging as negative control whereas **21b** (5 μM) was used as a positive control for superoxide generation. Cells were analyzed by flow cytometry (Fortessa X-20; $5 \cdot 10^3$ events/sample) using Ex515/Em630 parameters to obtain fluorescence intensities that were normalized to untreated controls to calculate relative superoxide levels.

We should observe a red fluorescence related to the oxidation of dihydroethidium to 2-hydroxyethidium by superoxide anion radicals (**Scheme E4.2A**). Instead, the SOSG

indicator initially exhibits weak blue fluorescence, but in the presence of singlet oxygen, it emits a green fluorescence due to the formation of endoperoxide SOSG-EP (**Scheme E4.2B**).



Scheme E4.2. A) Oxidation of DHE to 2-hydroxyethidium by superoxide anion radicals. B) Formation of green fluorescent endoperoxide by reaction of weak blue fluorescent SOSG with singlet oxygen.

E4.12. Mitochondrial membrane potential

The mitochondrial membrane potential was analyzed by using tetramethylrhodamine ethyl ester (TMRE) that readily accumulates in active mitochondria due to a mitochondrial relative negative charge. Damaged, depolarized, or inactive mitochondria decreased the membrane potential and failed to sequester TMRE. HeLa cells were seeded at the density of 1.5×10^5 cells per well and incubated overnight. Then, the cells were treated with **10**, **20c** and **21c** (10 μM) or cisplatin and incubated for 5 h in a humidified 5% CO_2 atmosphere. Then, the TMRE solution (100 nM) was applied to the cells and incubated for another 30 min at 37 $^\circ\text{C}$. Thirty thousand single cells were analyzed on the flow cytometer followed by data analysis in FCS Express 6.

E4.13. Analysis of the extracellular ATP release

A375 cells were seeded on the 96-well black plate (Corning) at the density of 2×10^4 cells/well and incubated overnight. Cells were then treated with increasing concentration of **29** (1, 5, 10 μM), doxorubicin (0.5 μM), or oxaliplatin (200 μM), irradiated (1 h, $\lambda_{\text{max}} = 420 \text{ nm}$, $77 \pm 3 \text{ W m}^{-2}$), or kept in the dark for 1 h. The samples were washed and incubated

for 24 h in a drug-free medium. Cells were centrifuged (200 g, 5 min), and cell supernatants were analyzed using ATP bioluminescence assay kit CLSII (Roche) according to manufacturer instructions. Luminescence was measured on the SPARK reader (Tecan).

E4.14. Analysis of the extracellular HMGB1 release

A375 cells were seeded on the 96-well plates (TPP) at the density of 10^4 cells/well and incubated for 48 h. The cells were then treated with the indicated concentration of **29**, doxorubicin or oxaliplatin, irradiated with blue light (1 h, $\lambda_{\text{max}} = 420$ nm, 77 ± 3 W m⁻²), washed, and incubated for 24 h in a drug-free medium. Cells were centrifuged (200 g, 5 min), and cell supernatants were analyzed using a colorimetric HMGB1 ELISA kit (IBL International) according to the manufacturer's instructions. The absorbance of the resulting product was determined by the SPARK reader (Tecan).

E4.15. Detection of calreticulin exposure by flow cytometry

Exposure of calreticulin was quantitatively analyzed by using flow cytometry. A375 cells were seeded on the 6-well plate at the density of 4×10^5 cells/well and incubated overnight. The cells were then treated with the indicated concentration of **29**, doxorubicin, or oxaliplatin, and irradiated as described above. The treatment was followed by 20 h of recovery in a drug-free medium. The cells were fixed with 4% formaldehyde and stained with antiCALR-Alexa fluor 488 conjugate (Abcam) (24 h, 4°C). Before analysis on flow cytometry, cells were co-stained with propidium iodide. Single cells (1×10^4) were analyzed and plotted on the histogram with a bi-exponential scale. Data were analyzed by using FCS Express 6.

E4.16. Detection of calreticulin exposure by confocal microscopy

A375 cells were seeded on the 50 mm glass-bottom culture dishes at the density of 2×10^5 cells/dish and incubated overnight. Then the cells were treated with **29**, doxorubicin, or oxaliplatin at indicated concentrations and irradiated. The treatment was followed by 20 h of recovery in a drug-free medium. After that, the cells were fixed with 4% formaldehyde and stained with antiCALR-Alexa fluor 488 conjugate (Abcam) (24 h, 4°C). Samples were visualized on a confocal microscope Leica TCS SP8 SMD.

E4.17. *In vitro* phagocytosis assay

CT26 murine fibroblasts were cultured in RPMI growth medium supplemented with gentamycin (50 mgmL⁻¹) and 10% heat-inactivated FBS. CT26 cells were seeded in a 6-well plate at the density of 2×10^5 cells/well and incubated overnight. Cells were then treated with **29**, doxorubicin or oxaliplatin and irradiated (or kept in the dark) as described above. After this procedure, CT26 cells were stained with Cell Tracker™ green CMFDA (5 μ M, 45 min, 37°C, Invitrogen). Moreover, J774.A1 macrophages were stained with Cell Tracker™ red CMTPIX (15 μ M, 45 min, 37°C, Invitrogen). Both cells were washed and co-incubated at the 1:5 (J774.A1:CT26) ratio for 3 h. After the incubation period, the cells were harvested, washed, and fixed with 4% formaldehyde (10 min, RT). The samples were analyzed by flow cytometry, and the data were evaluated by FCS Express 6.

E4.18. Isolation of Cancer Stem Cells and anti-CSCs activity

A375 cells were used as the primary culture for sorting of CSCs. These human melanoma cancer cells were stained by CD20-APC antibody and subsequently incubated with MACS® anti-APC microbeads. The stained cells were then sorted on a MACS magnetic separator (Miltenyi, Biotec).

The viability of melanospheres was determined by CellTiter-Glo® 3D cell viability assay. The A375CD20+ and A375CD20- cells were transferred to 96-well ultra-low attachment plates (Corning, NY, USA) in tumorsphere medium (DMEM/F12 medium supplemented with B27, hEGF (20 ngmL⁻¹), hFGF2 (10 ngmL⁻¹) and BSA (0.4 %) and cultivated for 4 days to form melanospheres. The melanospheres were then treated with increasing concentrations of **29** for 1 hour. After the incubation period, the cells were irradiated for 1 h ($\lambda_{\text{max}} = 420$ nm, 7.7 ± 3 mW/m²) or left in the dark. Subsequently, the spheroids were washed and cultured in a drug-free medium for 72 h. Finally, the Cell-Titer Glo® 3D Reagent was added, the plates were shaken and incubated at room temperature for 30 min. The viability of cells was evaluated by measuring the luminescence using a fluorescence/luminescence reader Tecan SPARK.

E4.19. Penetration of **29** into A375 melanospheres

Monitoring Penetration of **29** into A375 melanospheres by Confocal Microscopy. Unsorted A375 or A375CD20+ and A375CD20- cells were seeded on 24w plates under ultra-low attachment conditions in tumorsphere formation medium and cultured for 96 h to

form spheroids. The spheroids were subsequently treated with complex **29** (10 μ M) for 2 h or 24 h (dark). The spheroids were then harvested, washed with PBS, and fixed with 4% paraformaldehyde. The fixed samples were stained with CD20 antibody conjugated with APC (recombinant, Miltenyi Biotec). After washing with PBS, the samples were replaced on 35 mm confocal dish and mounted with Prolong Diamond Mounting medium. Samples were analyzed and visualized with a confocal microscope Leica TCS SP8 SMD.

E4.20. Metal accumulation in isolated DNA

HeLa cells were harvested at a density of 1×10^6 per well in a 6-well plate. After 24 h incubation, cells were exposed to 5 μ M of **34**, **35** and cisplatin, and additional 2 h incubation was allowed. For genomic DNA isolation, cells were lysed in DNAzol reagent (DNAzol®, Merck) contained RNase A (100 mg/mL). The DNA was precipitated by adding 100% ethanol followed by twice washing with 75% ethanol and then solubilized in 8 mM NaOH. The DNA content was determined using UV spectrophotometry measuring A260 (NanoDrop ND-1000 spectrophotometer) and the amount of platinum and iridium was quantified by ICP-MS.

E4.21. Cell cycle arrest analysis

For cell cycle analysis, A2780 cells were harvested into a 12-well plate at 3×10^5 per well and grown overnight in optimal condition. The following day cells were exposed to compounds **34**, **35** and cisplatin, at IC₅₀ value for 24 h. After trypsinization and centrifuge, cells were fixed in cold 70% EtOH and stained with propidium iodide for 30 min in the dark and analyzed with flow cytometry ($\lambda_{\text{exc}} = 488$ nm and $\lambda_{\text{em}} = 530$). All experiments were performed in duplicate.

E5. IN VIVO STUDIES IN *C. ELEGANS* (Chapter 6, collaboration with Dr. Gandía-Herrero)

E5.1. *Caenorhabditis elegans* strains and maintenance

The strains used in this work were obtained from the *Caenorhabditis* Genetic Center (CGC, St Paul, MN, USA), supported by the National Institutes of Health - Office of Research Infrastructure Programs (P40 OD010440). Wild-type N2 and JK1466 (*gld-1(q485)/dpy-5(e61) unc-13(e51)*) strains were used. The strains were maintained and

synchronized following the published methods.^{42,43} *Escherichia coli* OP50 concentrated 10x in M9 buffer was used to feed all the strains except the JK1466. The JK1466 strain was feed with the *E. coli* strain HT115 (DE3) with the homologous DNA sequence for the *gld-1* (T23G11.3) gene inside the vector L4440 (pPD129.36) was obtained from Source BioScience (sourcebioscience.com) from the library “RNAi Library (Ahringer)” to assure that all the worms had the tumoral phenotype following the published method.⁴⁴

E5.2. Assimilation *in vivo*

Age synchronized L4 larvae of wild-type worms were feed with *E. coli* OP50 and different concentrations of **37** (0.1, 1, 10 and 100 μ M) in S basal medium for 48 hours at 20 °C. Then worms were washed with M9 buffer three and transferred to microscope slides containing sodium azide (10 mM) as anesthetic for its visualization. Fluorescent images of the animals were taken using the A filter cube of a Leica DM 2500 LED microscope fitted with a Leica DFC550 camera (Leica Microsystems, Wetzlar, Germany) with the 10x lens. The quantification of the **37** inside the worms was performed using ImageJ software.⁴⁵ The images were split in RGB channels and only the red channel was used to measure the luminescence of the compound inside the worms using the threshold tool of the software. Two independent assays were performed with $n \geq 10$ and the significance of the obtained results was estimated by ANOVA test.

E5.3. Antitumoral evaluation of **37** in the *C. elegans* tumoral strain JK1466

The evaluation of the antitumoral potential of the metal complex was performed as published before using cisplatin as a positive control.⁴⁴ Briefly, JK1466 age synchronized L1 larvae was treated with **37** at different concentrations (0.1, 1, 10 and 100 μ M) in S basal medium supplemented with previously induced *E. coli* HT115 *gld-1* at 20 °C under orbital shaking. DMSO (0.4 %) and water were used as negative controls and cisplatin (100 μ M) as positive control. At the four day of adulthood worms were washed in M9 buffer and visualized under the bright-field microscope. Images of the tumoral gonads were taken at 40x and the size of the tumor was evaluated using the imageJ software. Two independent assays were performed with $n \geq 10$ and the significance of the obtained results was estimated by ANOVA test.

E5.4. Toxicity evaluation of **37** *in vivo*

The effect of **37** on the *C. elegans* lifespan, both the tumoral strain, and the wild-type strain was performed automatically using the lifespan machine⁴⁶ following the modifications of Guerrero-Rubio and coauthors.⁴³ Age-synchronized L4 larvae (30-40 individuals) were transferred to analysis plates containing Nematode Growth Medium (NGM) supplemented with 30 µg/mL of nystatin, 30 µg/mL of carbenicillin, FUdR (2'-deoxy-5-fluorouridine) at 10 µg/mL and IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added to a final concentration of 1 mM to induce *E. coli* HT115 *gld-1* bacterial cells only when using the JK1466 strain. Different concentrations of the compound (0.1, 1, 10 and 100 µM) were added for the test plates or DMSO (0.4 %) for the control plates. The plates for the wild-type assay were seeded with heat-inactivated *E. coli* OP50 and the plates used for the JK1466 strain contained IPTG (1 mM) and were seeded with HT115 *gld-1*. Two independent assays were performed, mathematical analysis of the obtained data was performed using the online application for survival analysis OASIS 2,⁴⁷ with the parameters Kaplan-Meier estimator and Survival Time F-Test.

E5.5. ROS measurement *in vivo*

The effect of **37** in the production of reactive oxygen species (ROS) was evaluated using DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) fluorescent probe. Age synchronized N2 young adults treated with the compound (100 µM), cisplatin (100 µM), DMSO (0.4 %) or juglone (20 µM) for 48 hours in liquid medium at 20 °C were washed with M9 buffer three times and stained with DCFH-DA (10 µM) in S medium at 37 °C for one hour. Then the animals were washed twice with M9 to avoid background fluorescence, mounted onto glass slides and visualized under fluorescent light using the filter cube I3. The obtained images were analyzed using ImageJ software, the images were separated into channels, and the measurement was performed in the green channel using the thresholding tool. Two independent assays were performed with $n \geq 10$, and the significance of the obtained results was estimated by the ANOVA test.

E5.6. *In vivo* superoxide levels measurement

The level of superoxide species generated by **37** was measured with DHE (dihydroethidium) following the published protocol of Dues and coauthors⁴⁸ with slight modifications. Briefly, synchronized wild-type L1 larvae were treated with the metallic

complex (100 μM), cisplatin (100 μM), DMSO (0.4 %) and paraquat (methyl viologen 200 μM) for 72 hours at 20 $^{\circ}\text{C}$. Then the animals were collected and washed three times with PBS buffer and stained with 500 μL of a DHE solution (30 μM in PBS) for one hour at 37 $^{\circ}\text{C}$ under orbital shaking. Following the staining procedure, the animals were visualized under fluorescent light using the N2.1 filter cube and the 40x lens. The analysis of the images was performed with ImageJ software using only the red channel. Two independent assays were performed with $n \geq 10$ and the significance of the obtained results was estimated by ANOVA test.

E5.7. Morphology. Acridine orange staining

Morphology of gonad cells was visualized using the *in vivo* stain acridine orange.⁴⁹ Adult animals (4 days) were washed in M9 buffer and transferred to 5 mL of fresh M9 containing 100 μL of concentrated *E. coli* OP50 and 5 $\mu\text{g/mL}$ of acridine orange and left to stain for 1 hour at 20 $^{\circ}\text{C}$ under orbital shaking. After one hour, the animals were washed with M9 three times and left in 5 mL of clean M9 for another hour to reduce the stain accumulated in the digestive tube. Two independent assays were performed with $n \geq 10$.

E6. X-RAY CRYSTALLOGRAPHY

The diffraction data were collected in the University of Murcia's crystallography service and the resolution of the structure was carried out by Prof. Christoph Janiak in Germany.

Chapter 2. Single crystals suitable for X-ray diffraction analysis were obtained for complex **9** from $\text{CH}_2\text{Cl}_2/\text{hexane}$ and complex **10** from $1,2\text{-C}_2\text{H}_4\text{Cl}_2/\text{hexane}$. A summary of crystal data collection and refinement parameters for all compounds are given in **Tables S2.1-S2.6** in the Supporting Information. Crystals were mounted on glass fibers and transferred to the cold gas stream of the diffractometer Bruker Smart APEX. Data were recorded with Mo $\text{K}\alpha$ radiation ($\lambda = 0.71073 \text{ \AA}$) in ω scan mode. Absorption correction for the compound was based on multi-scans. Both structures were solved by direct methods (SHELXS-97⁵⁰); refinement was done by full-matrix least-squares on F^2 using the SHELXL-97 program suite;⁵⁰ empirical (multi-scan) absorption correction with SADABS (Bruker). All non-hydrogen positions were refined with anisotropic temperature factors. Hydrogen atoms for aromatic CH , aliphatic CH_2 and CH_3 groups were positioned

geometrically (C–H = 0.95 Å for aromatic CH, C–H = 0.99 Å for CH₂, C–H = 0.98 Å for CH₃) and refined using a riding model (AFIX 43 for aromatic CH, AFIX 23 for CH₂, AFIX 137 for CH₃), with Uiso(H) = 1.2Ueq(CH, CH₂) and Uiso(H) = 1.5Ueq(CH₃). Graphics were drawn with DIAMOND (Version 4) [DIAMOND 4 for Windows; Crystal Impact Gbr, B, 2016, Germany, <http://www.crystalimpact.com/diamond>]. The structural data have been deposited with the Cambridge Crystallographic Data Center (CCDC 1584720 (**9**) and 1584721 (**10**) contain the supplementary crystallographic data for this chapter).

Chapter 3. Suitable crystals **20b** were grown from 1,2-C₂H₄Cl₂/hexane. Details of the X-ray structure determinations and refinement parameters for the compound are given in **Tables S3.1–S3.3** in the Supporting Information. Crystals were mounted on glass fibers and transferred to the cold gas stream of the diffractometer Bruker Smart APEX. Data were recorded with Mo K α radiation (λ = 0.71073 Å) in the ω scan mode. The structure was solved by direct methods; refinement was done by full-matrix least-squares on F² using the SHELXL program suite⁵⁰ and empirical (multi-scan) absorption correction with SADABS (Bruker). Graphics were drawn with DIAMOND [DIAMOND4 for Windows; Crystal Impact Gbr, Bonn, Germany, 2016, <http://www.crystalimpact.com/diamond>].

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RESUMEN EN CASTELLANO

La finalidad de esta tesis doctoral ha sido la síntesis y caracterización de nuevos complejos octaédricos de Ir(III) y el estudio de su potencial como fármacos anticancerígenos en terapia y fototerapia del cáncer. Este trabajo consta de ocho capítulos entre los que se incluye una introducción general (capítulo 1) y las conclusiones finales (capítulo 8). En los capítulos 2, 3, y 4, se ha realizado un estudio de relación estructura-actividad (SAR) de complejos catiónicos de Ir(III), donde se han introducido modificaciones tanto en los ligandos ciclometalados como en los ligandos auxiliares, para estudiar el efecto sobre su actividad fotodinámica. En los siguientes capítulos se ha estudiado el efecto sinérgico que puede tener el uso de dos metales en una misma molécula (capítulo 5), la ventaja de utilizar modelos *in vivo* en la investigación de un nuevo fármaco (capítulo 6) y la necesidad de usar nanopartículas para aumentar la acumulación y biocompatibilidad de complejos neutros de iridio (capítulo 7).

Este campo de investigación es claramente multidisciplinar, por lo que los resultados biológicos obtenidos tanto *in vitro* como *in vivo*, y la preparación de nanopartículas cargadas con algunos complejos de iridio seleccionados, han requerido la colaboración de otros grupos de investigación con experiencia en estos campos, pertenecientes al Instituto de Biofísica de Brno (República Checa), a la Universidad de Murcia (Departamento de Bioquímica y Biología Molecular) y a la Universidad de Barcelona (Departamento de Química Orgánica).

CAPÍTULO 2

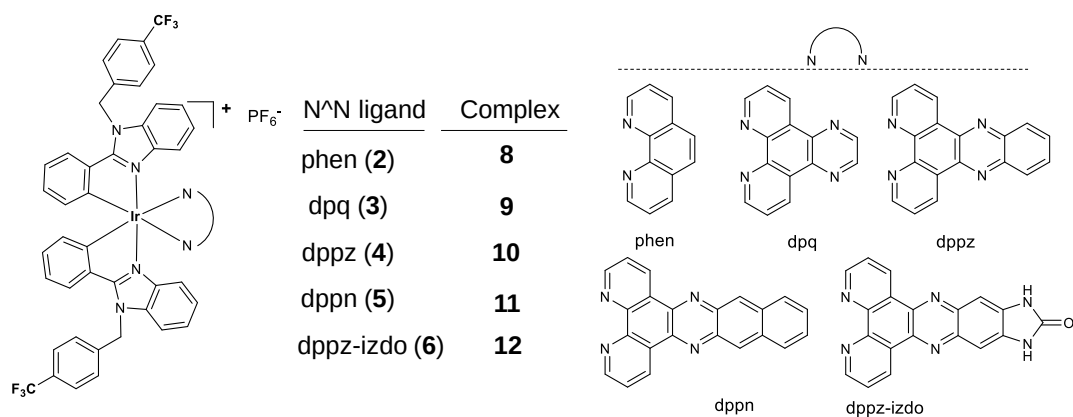
La actividad anticancerígena del fármaco en uso clínico cisplatino surge de la capacidad de este complejo para generar aductos covalentes con el ADN nuclear, lo que finalmente desencadena la muerte celular. Existen muchos metalofármacos con este modo de acción tradicional, pero los principales efectos secundarios observados en los pacientes sometidos a este tipo de quimioterapia, se deben entre otras causas a la falta de selectividad de esta unión covalente. Este hecho ha provocado el desarrollo de fármacos con nuevos mecanismos de acción.

La traducción es la actividad que más energía consume dentro de las células. Debido a las demandas metabólicas y a la alta proliferación, las células cancerígenas requieren tasas elevadas de síntesis de proteínas. Por tanto, dirigirse a este proceso de proteosíntesis directa o indirectamente, ya sea aumentando el estrés del retículo endoplasmático o

interfiriendo con otras vías de estrés que detengan la traducción, representa una estrategia terapéutica emergente para la terapia contra el cáncer.

Por otro lado, los complejos bis-ciclometalados de Ir(III) han despertado un gran interés como posibles agentes en terapia fotodinámica (PDT) debido a sus excelentes propiedades fotofísicas modulables. Estos compuestos exhiben desplazamientos de Stokes significativamente mayores que los agentes de PDT orgánicos, lo que reduce en gran medida la interferencia entre la excitación y la emisión. Sorprendentemente, las propiedades fotofísicas como la longitud de onda de absorción, los rendimientos cuánticos y los tiempos de vida media de emisión pueden ajustarse modificando el ligando ciclometalado y/o el ligando auxiliar para optimizar los requisitos de esta terapia. Además, los complejos de Ir(III) también muestran una alta estabilidad química y fotoquímica, en contraste con la rápida fotodegradación de los derivados de porfirina utilizados en PDT.

En este capítulo, se han desarrollado una serie de complejos organometálicos luminiscentes de iridio(III) (**8-12**) del tipo $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{N}^{\wedge}\text{N})][\text{PF}_6]$ (**Esquema 1**), en los que el ligando ciclometalado basado en benzimidazol se ha mantenido fijo, y el ligando de auxiliar se ha variado $\{\text{N}^{\wedge}\text{N} = \text{phen}$ (**2**), dpq (**3**), dppz (**4**), dppn (**5**) y dppz-izdo (**6**), con el objetivo de ajustar las propiedades fotofísicas y citotóxicas de este tipo de compuestos prometedores.



Esquema 1

Se ha observado que los complejos exhiben diferentes propiedades fotofísicas dependiendo de la longitud y el grado de conjugación π del ligando $\text{N}^{\wedge}\text{N}$; los complejos con los ligandos más largos (**10**, **11** y **12**) muestran un efecto de extinción de la luminiscencia en agua causado por agregación. En cuanto al estudio de citotoxicidad, los complejos menos lipofílicos **8** y **9** (con el nivel más bajo de acumulación celular) exhiben las mayores toxicidades en las células cancerígenas de ovario, cuello uterino y mama (A2780, HeLa y MCF-7 receptivamente) y son marcadamente más eficientes que

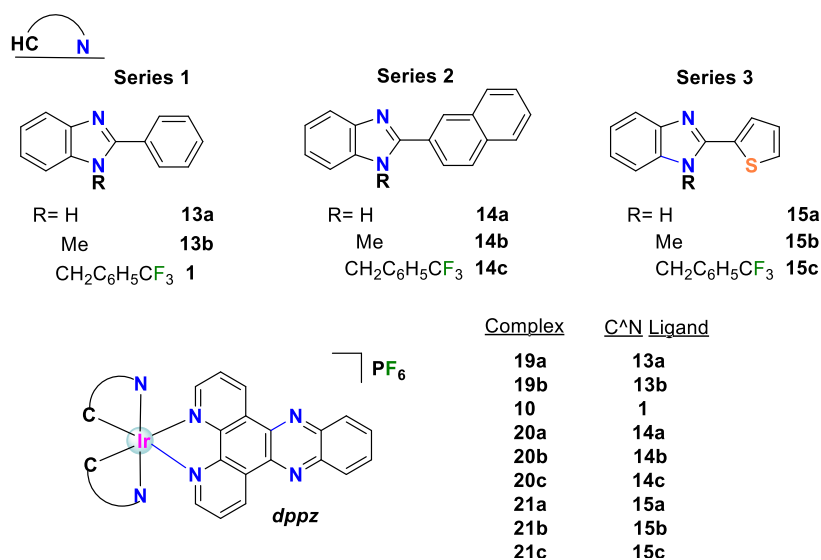
cisplatino. Por el contrario, las citotoxicidades de los complejos más lipofílicos **11** y **12**, disminuyen considerablemente, indicando que la citotoxicidad de los compuestos no refleja su acumulación celular. Cabe destacar que los agentes investigados son capaces de superar la resistencia adquirida e inherente al cisplatino (en las células A2780cisR y MCF-7, respectivamente) y son más selectivos hacia las células de cáncer de mama que hacia las células sanas. También se ha demostrado que los compuestos se dirigen preferentemente al retículo endoplásmico. Así, los perfiles de respuesta celular dependientes del tiempo y las estimaciones de la incorporación de metionina- $[^{35}\text{S}]$ como medida de la actividad de traducción de las células en presencia de los complejos, sugieren que un posible mecanismo de acción implica la inhibición de la síntesis de proteínas. Además, se ha encontrado que los complejos **8-12** inducen apoptosis de forma similar a otros metalofármacos antitumorales. Por otro lado, el estudio de citotoxicidad irradiando con luz azul ($\lambda = 420$ nm) demuestra que el efecto tóxico de todos los complejos en las células HeLa es capaz de elevarse en más de dos órdenes de magnitud. Esta fotopotenciación se atribuye a un marcado aumento (~ 10 a 30 veces) de las especies reactivas de oxígeno (ROS) en el interior celular. Hasta donde sabemos, los complejos de Ir(III) investigados en este estudio representan los primeros compuestos de iridio que inducen la muerte de las células tumorales mediante la inhibición de la traducción celular, y cuya actividad está marcadamente fotopotenciada.

CAPÍTULO 3

Como modalidad de tratamiento emergente, la PDT muestra varias ventajas: control temporal y espacial de la fotoirradiación en el sitio diana, mínima invasividad, baja toxicidad general en los órganos y falta de resistencia inicial. Actualmente, la mayoría de los fármacos desarrollados funcionan mediante un mecanismo de tipo II, en el que el fármaco (también llamado fotosensibilizador, PS) transfiere la energía del estado excitado al oxígeno circundante para producir oxígeno singlete. Sin embargo, el microambiente nativo en los tumores sólidos es hipóxico (0.02 - 2% O_2) debido al rápido crecimiento tumoral y al suministro insuficiente de oxígeno. Además, un consumo rápido de oxígeno durante la terapia agrava aún más la condición hipóxica y restringe el efecto terapéutico. Como resultado, el ambiente hipóxico parece ser una limitación significativa de la PDT y resolver este talón de Aquiles ha despertado un gran interés entre la comunidad científica.

Entre las diferentes estrategias, el mecanismo de PDT tipo I, parece ser una forma de superar la limitación de la hipoxia: el hecho mismo de que se puedan generar diferentes ROS (radicales hidroxilo, aniones superóxido, peróxido de hidrógeno o radicales peroxilo) y estos no se basen en un único mecanismo de acción, dificulta que las células desarrollen resistencia. Además, algunas de estas ROS, como los radicales superóxido, pueden ser menos dependientes de O₂ con reacciones de desproporción o reacciones de Fenton/Haber-Weiss que compensarían este consumo de oxígeno.

En este capítulo se han diseñado y sintetizado nueve complejos octaédricos de Ir(III) (**Esquema 2**) de fórmula general [Ir(C[^]N)₂(dppz)][PF₆] (dppz = dipirido [3,2-a: 2', 3'-c] fenazina), con modificaciones en los ligandos ciclometalados como la incorporación de un heteroátomo de azufre o un aumento de la conjugación π , para ajustar las propiedades fotofísicas, la lipofilia, la eficacia de captación celular y la actividad de fototerapia incluso bajo hipoxia.



Esquema 2

Se ha observado que el sustituyente en la unidad imidazol del ligando ciclometalado juega un papel fundamental en las propiedades fotofísicas de los complejos y también en su actividad antiproliferativa en células cancerígenas de cuello uterino humano, bajo normoxia e hipoxia: los derivados metílicos (**19b**, **20b** y **21b**) son los más activos en la oscuridad, mientras que los complejos con el grupo CH₂C₆H₄CF₃-p (**10**, **20c** y **21c**) exhiben una menor toxicidad en la oscuridad y un alto índice de fototoxicidad. Por otro lado, la sustitución en la posición 2 del benzimidazol parece estar directamente relacionada con la ubicación en la célula: el complejo **21c** que contiene azufre, se localiza principalmente en la mitocondria, mientras que los complejos 2-aryl-benzimidazol se localizan en el retículo

endoplasmático. La generación de ROS estudiada mediante ensayos de tinción con dihidroetidio (DHE) y sensor de oxígeno singlete verde (SOSG) ha mostrado que la generación de radicales superóxido compite con la producción de oxígeno singlete en las células en normoxia, pero se vuelve dominante bajo hipoxia. La importancia del cambio de mecanismo fotoquímico de tipo II a tipo I por parte de los nuevos complejos de Ir(III), indica que podrían ser activos en todas las áreas de un tumor sólido, adaptando su mecanismo de acción según la concentración de oxígeno, superando así el talón de Aquiles de la PDT.

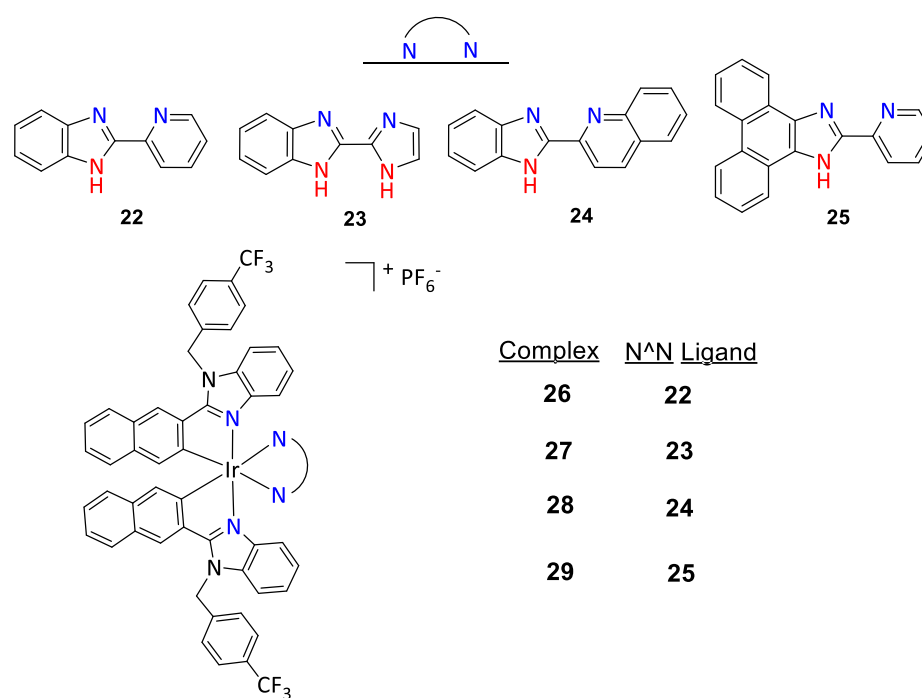
CAPÍTULO 4

La recurrencia del cáncer y la metástasis se han relacionado estrechamente con la existencia de células madre cancerígenas (CSC). Estas células son responsables de la autorrenovación y dan lugar a otras células más diferenciadas que forman la mayor parte del tumor, y hay pruebas contundentes de CSC en cánceres sólidos, como mama, cerebro, colon, páncreas y melanoma. Las CSC son capaces de sobrevivir a la quimioterapia, la radioterapia y el ataque inmunológico que desencadenan la recaída del tumor. La investigación actual sugiere que las estrategias inmunoterapéuticas dirigidas a las CSC pueden mejorar la eficacia del tratamiento del cáncer cuando se usan en combinación con terapias citotóxicas tradicionales. Por tanto, el desarrollo de nuevos agentes inmunoquimioterapéuticos capaces de reducir la masa tumoral mediante mecanismos citotóxicos y eliminar las CSC residuales mediante activación inmunológica de forma simultánea, mantiene la esperanza de mejorar la supervivencia y la calidad de vida de los pacientes con cáncer, incluidos los que se encuentran en la etapa metastásica de la enfermedad. Uno de los enfoques mediante los cuales los quimioterapéuticos pueden inducir una respuesta inmune dirigida al tumor es a través de la muerte celular inmunogénica (ICD).

En los últimos años, se ha demostrado que la PDT puede inducir ICD en células cancerígenas. Recientemente, algunos estudios preclínicos y clínicos han demostrado que, además de su capacidad destructora de tumores, la PDT es capaz de realizar funciones inmunomoduladoras. Esta fototerapia puede estimular las respuestas inmunitarias tanto innatas como adaptativas, destruyendo células tumorales distantes no tratadas (en sitios

primarios o metastásicos) y conduciendo al desarrollo de inmunidad antitumoral que puede proteger contra la recurrencia del tumor.

En este capítulo se ha sintetizado y caracterizado cuatro complejos catiónicos de Ir(III) luminiscentes **26-29** (**Esquema 3**) para investigar el efecto de modificar el ligando auxiliar en su actividad fototerapéutica.



Esquema 3

El ligando ciclometalado se ha elegido porque proporcionó una baja toxicidad en la oscuridad y uno de los índices de fototoxicidad más altos en los complejos del Capítulo 3. La elección de los ligandos auxiliares se basa en un esqueleto común de benzimidazol que contiene uno o dos grupos NH en su estructura. De acuerdo con la bibliografía, estos grupos generan una baja citotoxicidad de sus correspondientes complejos en la oscuridad, una condición crucial para el diseño ideal de un fotosensibilizador.

En medio libre de células, **26-29** pueden oxidar fotocatalíticamente el NADH bajo irradiación de luz azul. Además, los nuevos complejos exhiben una toxicidad muy baja en la oscuridad incluso a altas concentraciones (300 μM) contra células humanas de melanoma (A375 y 518.A2) y de adenocarcinoma de cuello uterino (HeLa). Todos los complejos, excepto **27**, muestran una alta fototoxicidad por irradiación con luz azul en condiciones normóxicas e hipóxicas, siendo **29** el más activo de los complejos, con los índices de fototoxicidad más altos (46-100). Este complejo también presenta la mayor acumulación en células cancerígenas y la generación de ROS fotoinducida más elevada. Por otro lado,

29 se acumula preferentemente en la mitocondria, aunque el estudio de co-localización con ER-Tracker sugiere que este complejo también podría estar asociado parcialmente con el retículo endoplasmático.

Asimismo, los datos presentados en este capítulo indican que el complejo **29** induce la muerte celular inmunogénica en el melanoma en la misma medida que los inductores de ICD conocidos (oxaliplatino y doxorrubicina), pero solo si las células se irradian con luz. Por otro lado, **29** inhibe potencialmente el crecimiento de células madre de melanoma después de la irradiación con luz, siendo aproximadamente tan eficaz como el fármaco específico de CSC abamectina (en la oscuridad), pero presentando una notable mayor selectividad hacia las CSC.

Hasta donde sabemos, **29** es el primer complejo de iridio que induce ICD en células de melanoma y se dirige selectivamente a las CSC mediante irradiación con luz azul. Por tanto, este resultado representa un avance científico significativo en el campo de los agentes quimioterapéuticos oncológicos con mecanismo de acción no convencional. El complejo **29** es un prometedor candidato dirigido a CSC para fototerapia de inmunoestimulación, que podría suprimir los efectos secundarios con una activación selectiva con luz y luchar contra las resistencias adquiridas de cánceres metastásicos como el melanoma.

CAPÍTULO 5

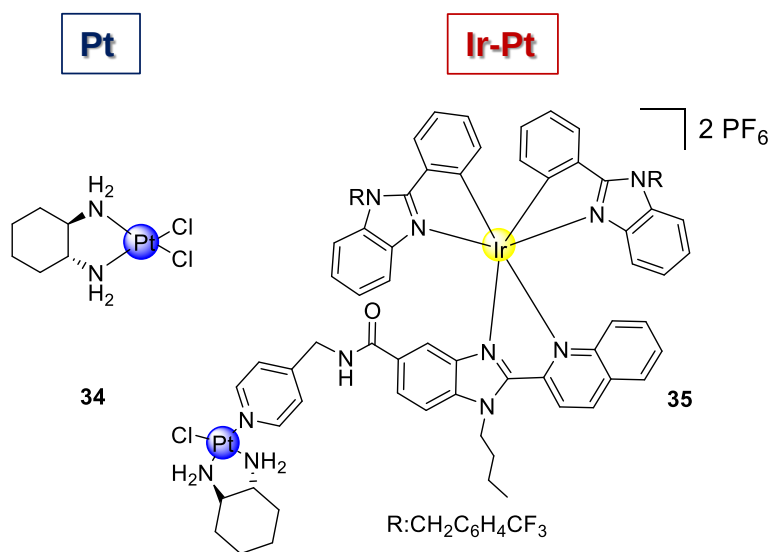
El descubrimiento del cisplatino y sus análogos marcó el comienzo del desarrollo de fármacos antitumorales basados en metales. El cisplatino sigue siendo uno de los fármacos más utilizados en la actualidad, pero su eficacia está limitada por su baja selectividad por las células tumorales y por la resistencia adquirida o intrínseca que desarrollan algunos tipos de cáncer a él. Recientemente se han investigado diferentes enfoques para superar esta resistencia: nanomateriales basados en platino, profármacos de Pt(IV) y complejos heteronucleares.

La combinación de dos o más metales de transición mediante química de coordinación puede mejorar la actividad antitumoral de los metalofármacos. Estos complejos heterobimetálicos o multimetálicos pueden inducir efectos aditivos o incluso sinérgicos utilizando dos o más metales en una sola molécula. En contraste con el desarrollo de complejos anticancerígenos mononucleares, los complejos metálicos dinucleares se han estudiado menos. Sin embargo, estas especies bimetálicas pueden mejorar las propiedades de los monómeros como resultado de la modificación de los ligandos puente, los dos

centros metálicos o los ligandos ciclometalantes. El diseño de un nuevo complejo dinuclear puede resultar difícil por los siguientes desafíos: bajos rendimientos de producción debido a procesos sintéticos complicados, propiedades ópticas deficientes con rendimientos cuánticos de luminiscencia bajos causados por la gran contribución de estados excitados no radiativos de los ligandos puente, grandes pesos moleculares que pueden disminuir su solubilidad en medios biológicos, etc. No obstante, se han logrado avances importantes en este campo.

Dada la importancia del conocimiento del mecanismo de acción para el desarrollo de fármacos metálicos más efectivos, una adecuada selección de diferentes fragmentos metálicos permitiría mejorar no solo la actividad terapéutica con efecto sinérgico sino también incorporar características clave como la trazabilidad. En este sentido, los complejos bis-ciclometalados de Ir(III) han demostrado ser unos excelentes candidatos para su uso en teranostia.

Así, inspirados por la necesidad de superar las desventajas de los fármacos de platino actuales y en las excelentes propiedades fotofísicas de los complejos de iridio, en este capítulo, se ha diseñado y sintetizado un nuevo complejo dinuclear de Ir(III)-Pt(II) (**Esquema 4**) para investigar su potencial como fármaco antitumoral. Este complejo heterobimetálico presenta una fosforescencia roja intensa en agua otorgada por el fragmento de Ir(III). Además, los estudios de estabilidad en disolución e interacción con biomoléculas muestran la resistencia del ligando cloruro a reacciones de sustitución e hidrólisis.



Esquema 4

El estudio antiproliferativo ha revelado la capacidad citotóxica de **35** hacia células de cáncer de cuello uterino y ovario resistentes a cisplatino. Además, el complejo dinuclear es más selectivo hacia las células cancerígenas que cisplatino y el complejo precursor de Pt(II) **34**. Otros estudios sobre la inducción de la muerte celular revelan que, a diferencia del cisplatino, que induce apoptosis en células de A2780, el complejo dinuclear causa necrosis después de 24 h. Esta inducción de muerte celular necrótica podría ser una de las causas responsables de vencer la resistencia a cisplatino.

Este estudio revela la capacidad sinérgica que puede tener el uso de diferentes metales en una sola molécula para superar la resistencia a fármacos, y representa un paso más en el desarrollo de nuevos complejos heterodinucleares Ir-Pt con propiedades duales antitumoral y luminiscente, un área que está actualmente poco desarrollada.

CAPÍTULO 6

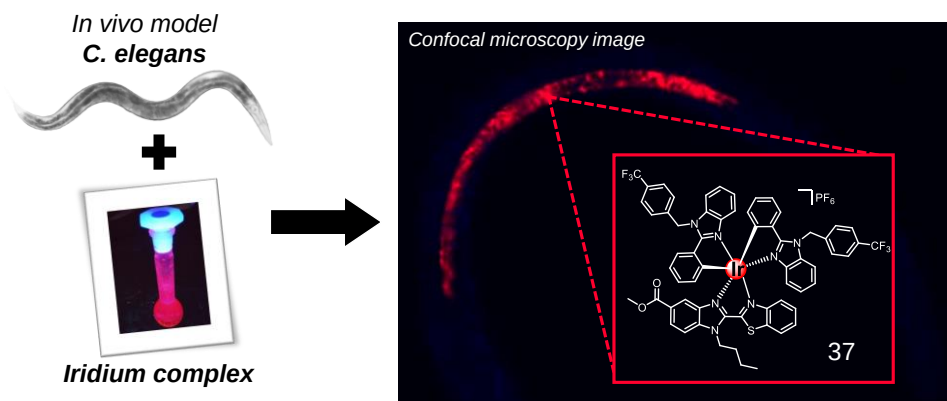
Como ya se ha comentado anteriormente, las excelentes propiedades fotofísicas de los complejos de Ir(III) han suscitado un creciente interés en el desarrollo de estos complejos como sensores. Este hecho ha permitido estudios más profundos del mecanismo de acción dentro de las células o incluso dentro de organismos transparentes como el pez cebra.

Para el desarrollo de nuevos fármacos contra el cáncer son cruciales los estudios con modelos oncológicos preclínicos, los cuales se han extendido significativamente en las últimas décadas. Dichos modelos permiten la demostración de la eficacia *in vivo*, de su baja toxicidad, junto con la confirmación del mecanismo de acción y la diana principal del fármaco candidato.

A pesar de su aparente simplicidad, el gusano nematodo *Caenorhabditis elegans* (*C. elegans*) se ha convertido en un importante modelo animal ampliamente utilizado en la investigación bioquímica, farmacéutica y médica, principalmente en la caracterización de nuevas dianas farmacológicas. Esto se debe a que este modelo animal tiene un organismo completo (con sistemas digestivo, reproductivo y nervioso), y su transparencia también permite la visualización de tejidos marcados con fluorescencia en cepas mutantes. Además, la mayoría de los genes y las vías de las enfermedades humanas están presentes en *C. elegans*. Aunque el uso de mamíferos para estudios *in vivo* es común, probar primero en *C. elegans* supone una reducción en el uso de mamíferos en la investigación, de acuerdo con los principios 3R (Reducción, Refinamiento y Reemplazo) en pruebas con animales.

Así, el modelo tumoral de *Caenorhabditis elegans* desarrollado recientemente, ha sido utilizado en este trabajo.

En este capítulo se ha sintetizado un nuevo complejo de iridio(III) (**Esquema 5**) del tipo $[\text{Ir}(\text{C}^{\wedge}\text{N})_2(\text{N}^{\wedge}\text{N})][\text{PF}_6]$ (**37**), que contiene un nuevo núcleo de 2-benzotiazol-il benzimidazol con un grupo éster como ligando $\text{N}^{\wedge}\text{N}$. Este complejo posee una intensa luminiscencia roja con un alto rendimiento cuántico en agua, lo que resulta conveniente para la visualización del complejo *in vivo*.



Esquema 5

Los efectos potenciales de este fármaco se han evaluado *in vitro* e *in vivo*. El complejo **37** reduce el tamaño del tumor en el modelo tumoral de *C. elegans* en la misma medida que cisplatino, siendo el complejo de iridio menos tóxico para los animales. En las líneas celulares de cáncer de ovario, la citotoxicidad de **37** es mucho más baja que la del cisplatino, mientras que el factor de selectividad del compuesto es mayor. Curiosamente, **37** induce necrosis en lugar de apoptosis en las células A2780. Por otro lado, la medida de aniones superóxido muestra niveles aumentados tanto en líneas celulares de ovario como en *C. elegans*, lo que sugiere que estos radicales juegan un papel importante en el mecanismo de acción de **37**. Todo lo anterior indica que el mecanismo de acción antitumoral del complejo evaluado *in vitro* se corresponde bastante bien con el estudio *in vivo*, y por tanto muestra la prevalencia de la acción antitumoral del fármaco al pasar de modelos *in vitro* bidimensionales al entorno *in vivo*. Este trabajo supone un paso adelante en la investigación de nuevos fármacos quimioterapéuticos con propiedades mejoradas.

CAPÍTULO 7

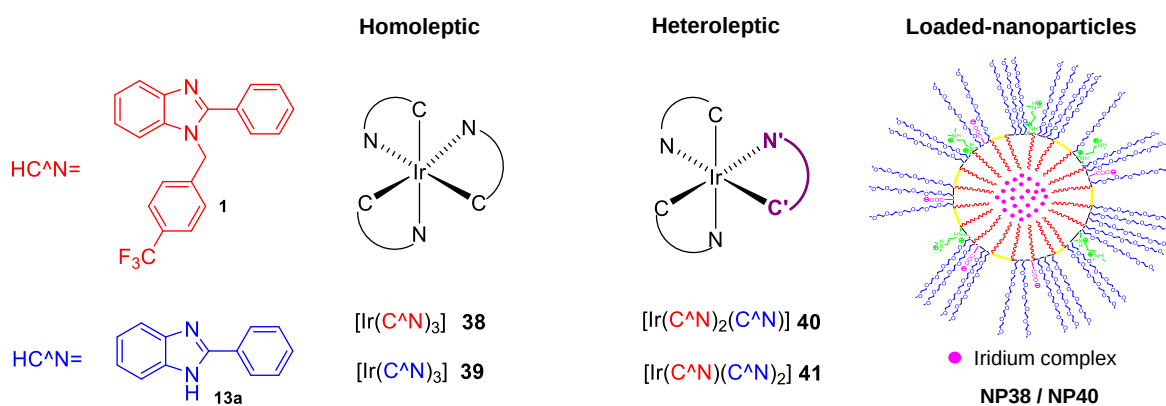
Hasta la fecha, los complejos de Ir(III) neutros son los materiales fosforescentes más estudiados debido a sus excelentes propiedades fotofísicas: tiempos de vida de emisión de microsegundos, que garantizan una alta eficiencia en los diodos emisores de luz orgánicos fabricados (OLEDs); altos rendimientos cuánticos de emisión; y color de emisión modulable. Estos compuestos neutros fosforescentes se pueden dividir en dos categorías principales: bis- y tris-ciclometalados.

Los complejos tris-ciclometalados de Ir(III) muestran isomería geométrica *fac-mer*. De los dos posibles isómeros, el isómero *fac* se favorece termodinámicamente mientras que el isómero *mer* es el favorecido cinéticamente. La menor estabilidad termodinámica del isómero meridional se debe principalmente al hecho de que dos átomos de carbono (con un fuerte efecto trans) de los ligandos ciclometalados están mutuamente en una disposición trans. Esto ha provocado que en aplicaciones terapéuticas, el isómero *fac* se utilice principalmente aunque, por lo general, se obtiene con menor rendimiento. La aplicación de estos compuestos neutros altamente luminiscentes en los sistemas vivos es limitada debido a su falta de carga, lo que dificulta su entrada a la célula. Así, las estructuras de los complejos se han modificado con grupos que aumentan la solubilidad en agua, péptidos catiónicos o grupos sensibles al pH. Además, otra estrategia emergente es el uso de nanopartículas que puedan transportar los complejos al interior de las células.

En la terapia contra el cáncer, las propiedades de las nanopartículas (NP), incluido su pequeño tamaño, gran superficie, perfiles favorables de liberación de fármacos, solubilidad en agua y modificaciones de dirección, pueden permitirles alcanzar mejor el tejido tumoral diana y liberar fármacos de forma estable y controlada. Las NP pueden proteger las cargas útiles de la degradación y la liberación prematura, y pueden acumularse en el tumor y, al mismo tiempo, preservar las células sanas gracias al efecto de permeabilidad y retención mejorada (EPR). Este efecto aprovecha la arquitectura vascular atípica y un sistema de drenaje linfático deteriorado del tejido tumoral, resultando en la retención eficiente en el intersticio del tumor.

En este capítulo se han sintetizado cuatro complejos neutros de Ir(III) tris-ciclometalados (dos homolépticos y dos heterolépticos) (**Esquema 6**). Todos los complejos presentan una emisión fosforescente verde muy alta tanto en diclorometano como en DMSO. Sin embargo, los complejos de iridio no pueden penetrar al interior celular debido a su falta de carga, como se ha demostrado por microscopía confocal. Por tanto, **38** y **40** se

han encapsulado utilizando nanopartículas híbridas poliuretano-poliurea para mejorar su biocompatibilidad y acumulación celular. La absorción celular de los compuestos de Ir(III) determinada por ICP-MS, muestra que el contenido de iridio dentro de las células cancerígenas tratadas con **NP-38** y **NP-40** fue de 6 a 21 veces mayor que las tratadas con los complejos libres. El estudio antiproliferativo confirma la acción citotóxica eficaz de **NP-38** y **NP-40** contra células cancerígenas de cáncer de cuello uterino (HeLa). Además, los agentes cargados en las NP muestran una baja citotoxicidad en las células renales no tumorigénicas BGM, lo que indica la capacidad de **NP-38** y **NP-40** para eliminar preferentemente las células tumorales sobre las células normales. Esta incorporación de complejos de Ir(III) neutros en nanopartículas podría ofrecer una mejora emocionante en la administración, focalización y captación celular para una terapia más segura y exitosa del cáncer de cuello uterino.



Esquema 6

Esta Tesis Doctoral ha sido posible gracias a la financiación de los siguientes programas e instituciones:

- Ministerio de Economía y Competitividad de España y FEDER (Proyecto CTQ2015-64319-R).
- COST CM1105 y red MetDrugs (CTQ2015-70371-REDT).
- Ministerio de Ciencia e Innovación y fondos FEDER (Proyecto RTI2018-096891-b-I00).
- Red MultiMetDrugs (RED2018-102471-T).
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- Contrato predoctoral FPU de la universidad de Murcia (R-1034/2016).

Supporting Information

Chapter 2

Effect of polypyridyl ligands on the anticancer
activity of phosphorescent
Ir(III) complexes under dark and light conditions

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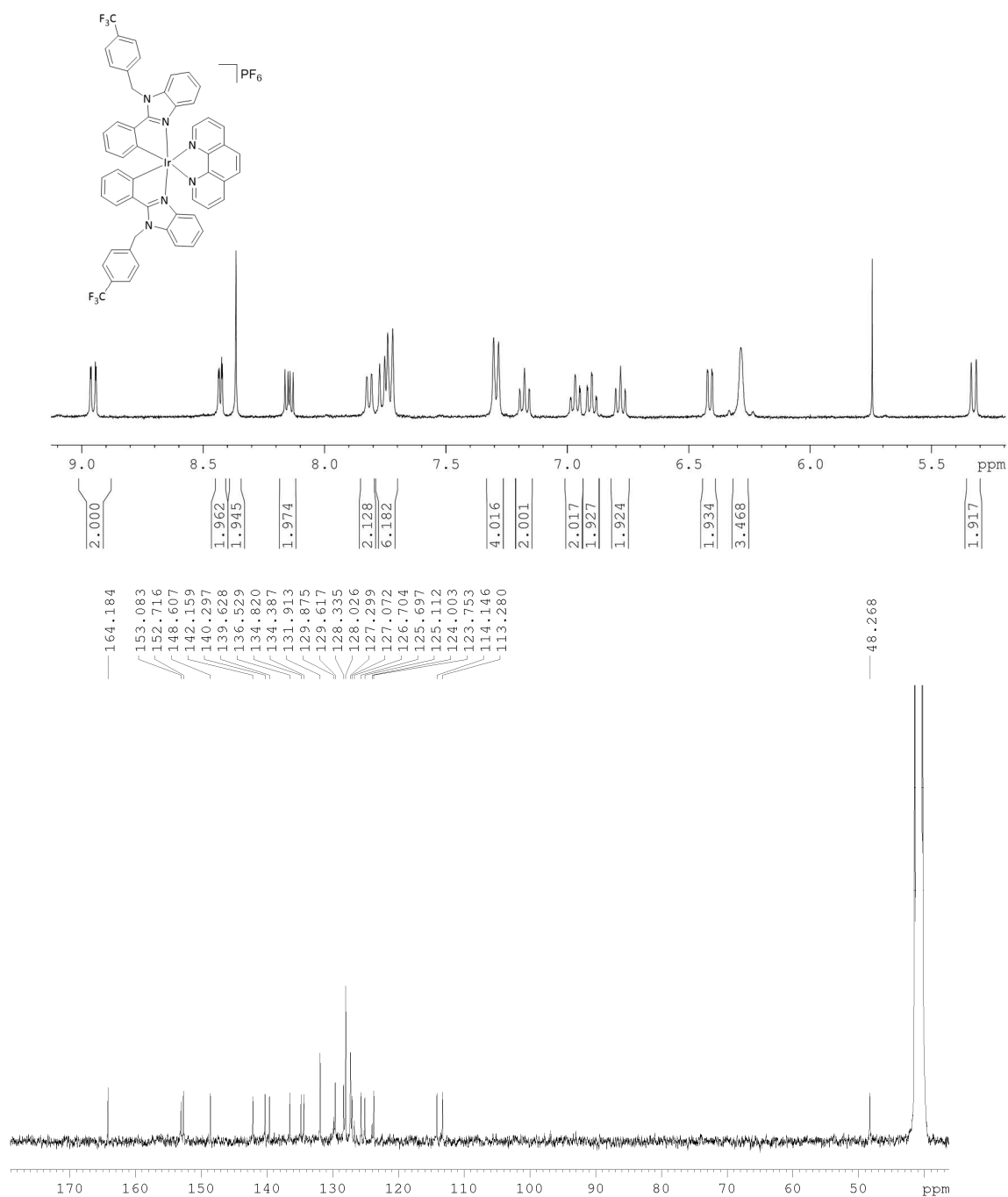


Figure S2.1. ^1H and ^{13}C NMR spectra of **8** (400 MHz, $[\text{D}_6]\text{DMSO}$).

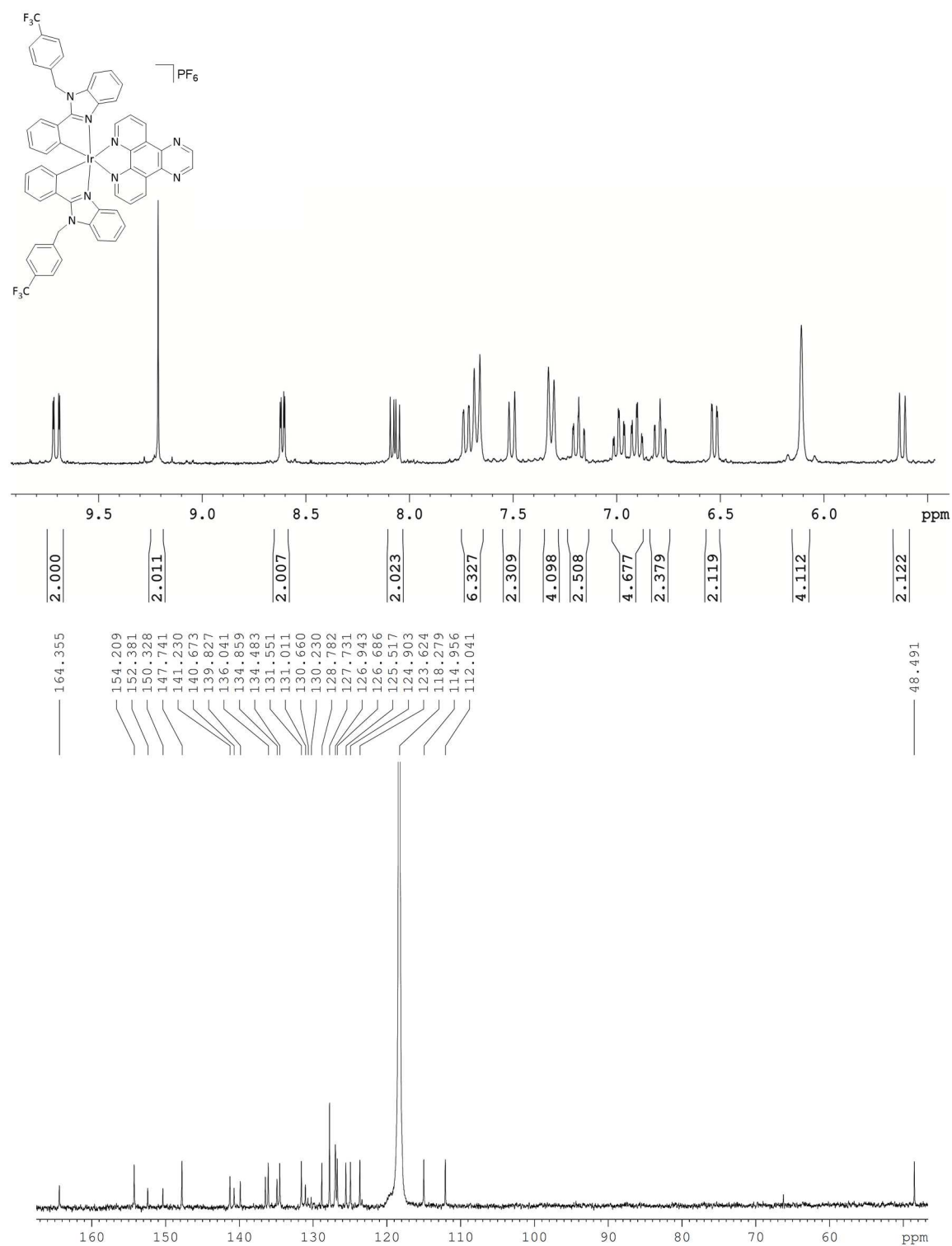


Figure S2.2. ^1H and ^{13}C NMR spectra of **9** (300 MHz, CD_3CN).

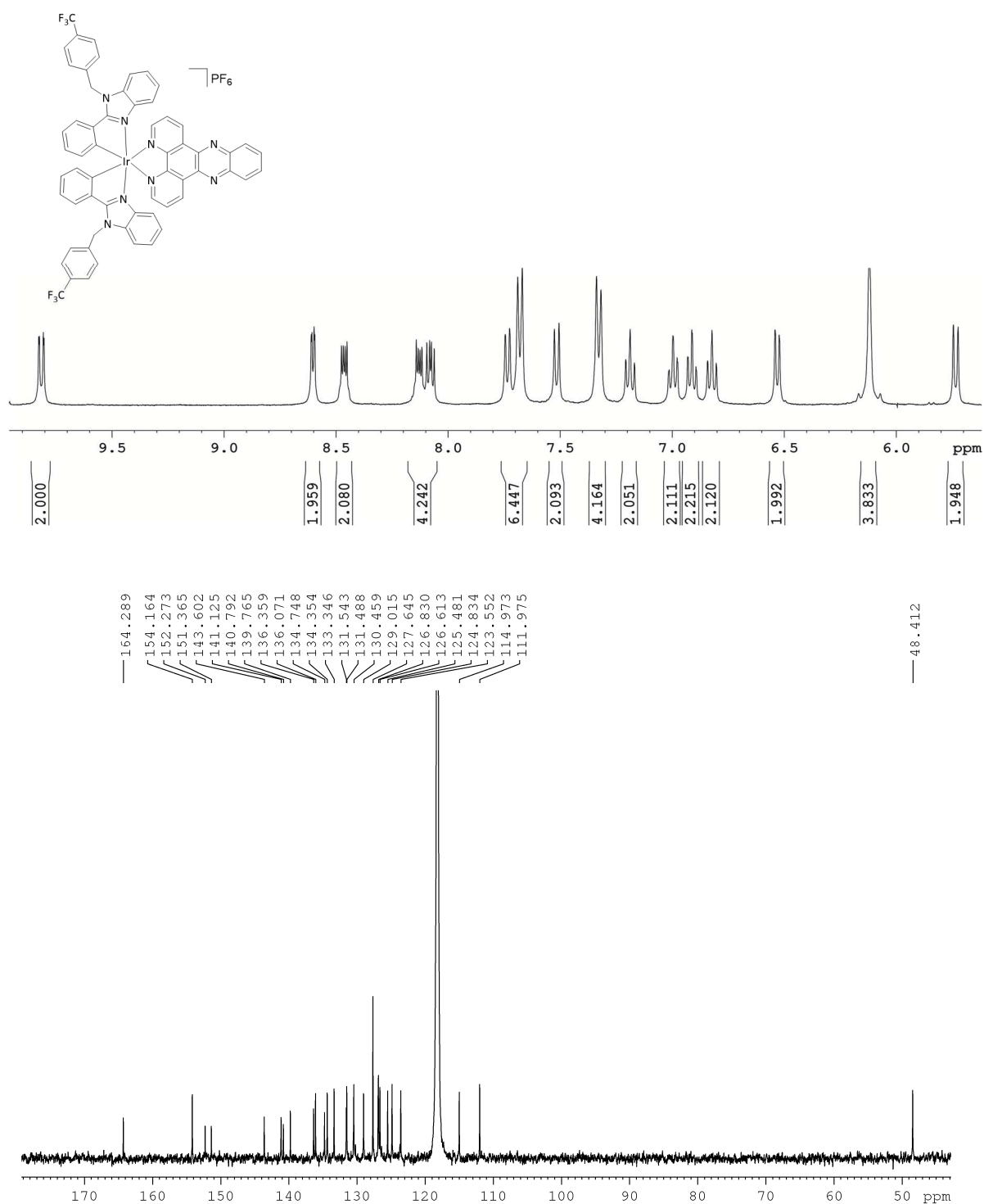


Figure S2.3. ^1H and ^{13}C NMR spectra of **10** (400 MHz, CD_3CN).

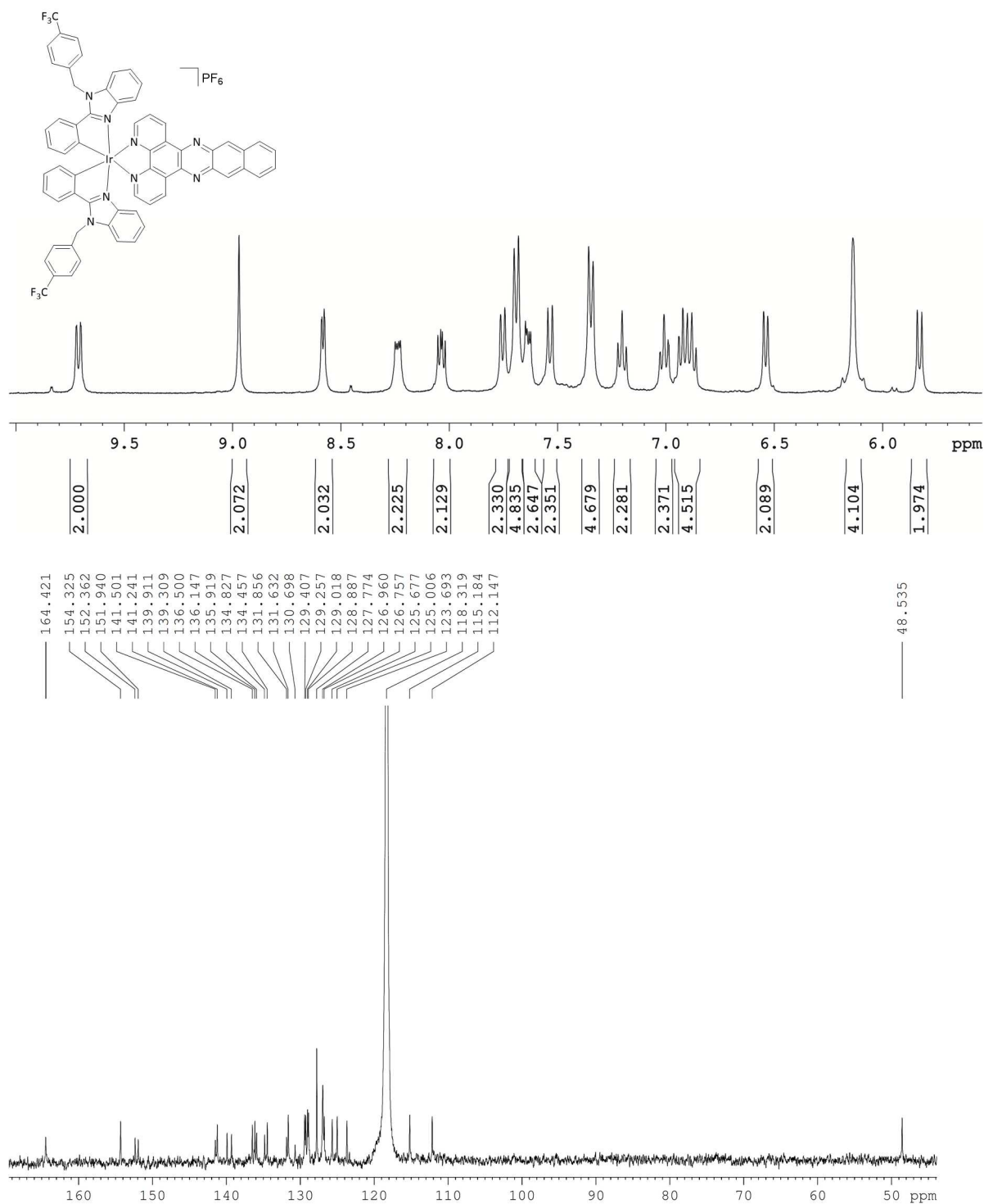


Figure S2.4. ^1H and ^{13}C NMR spectra of **11** (CD_3CN , 400 MHz and 300 MHz respectively).

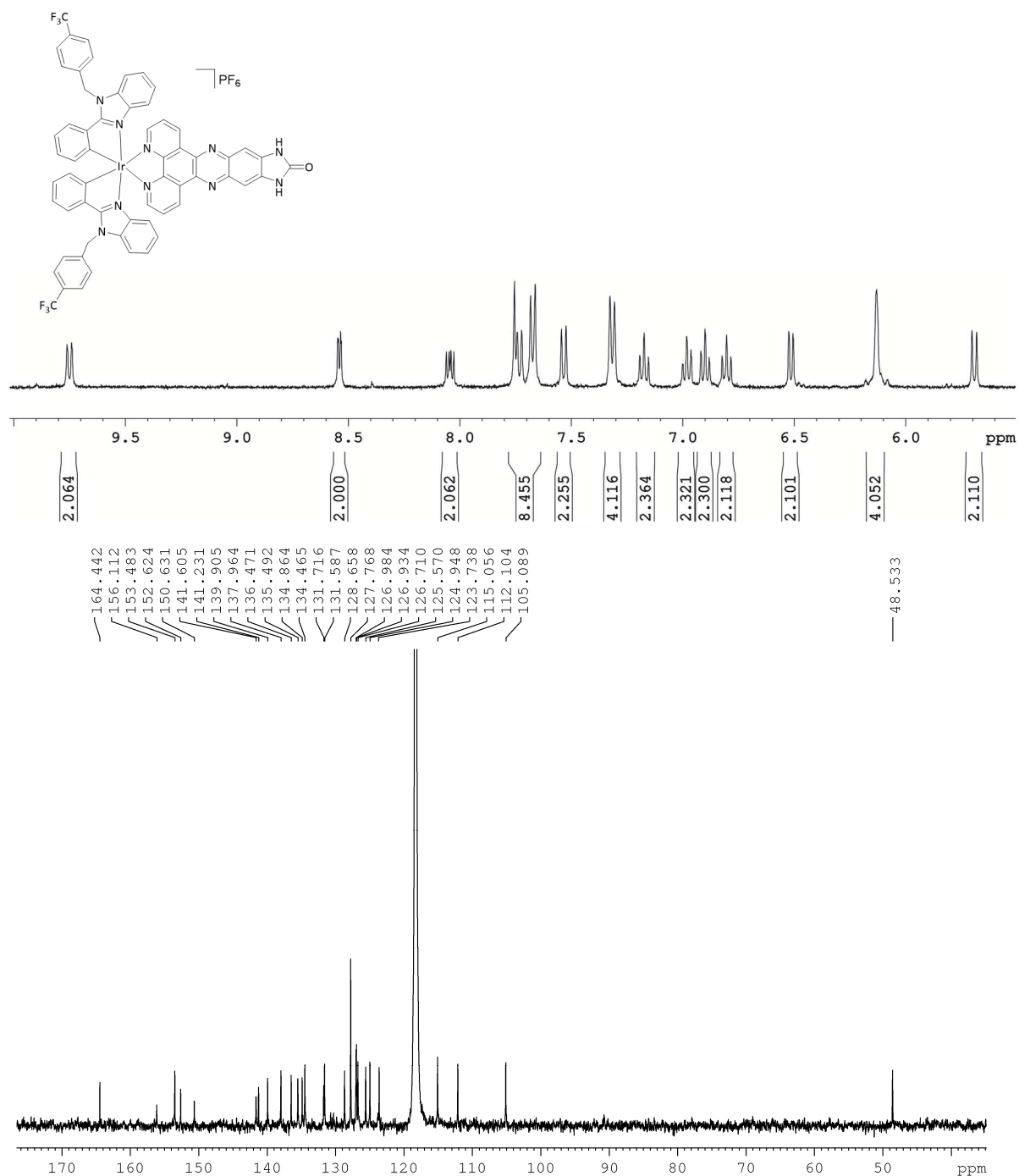


Figure S2.5. ¹H and ¹³C NMR spectra of **12** (CD₃CN, 400 MHz and 300 MHz respectively).

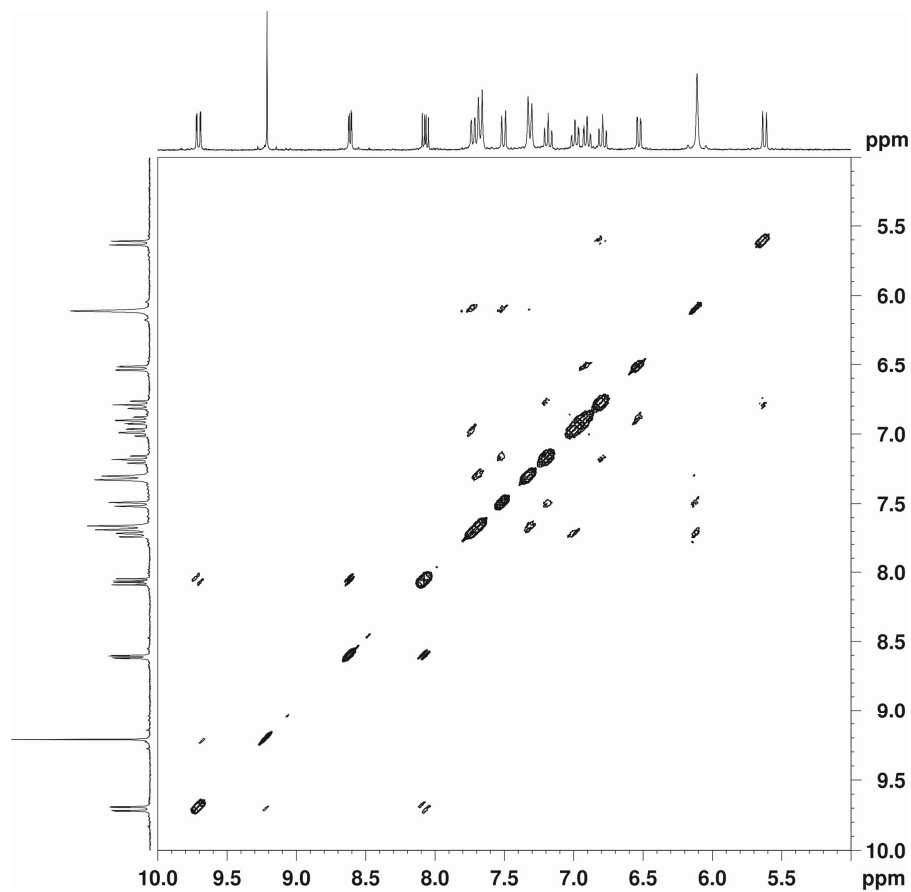


Figure S2.6. NOESY 2D ^1H - ^1H NMR of **9** (CD_3CN).

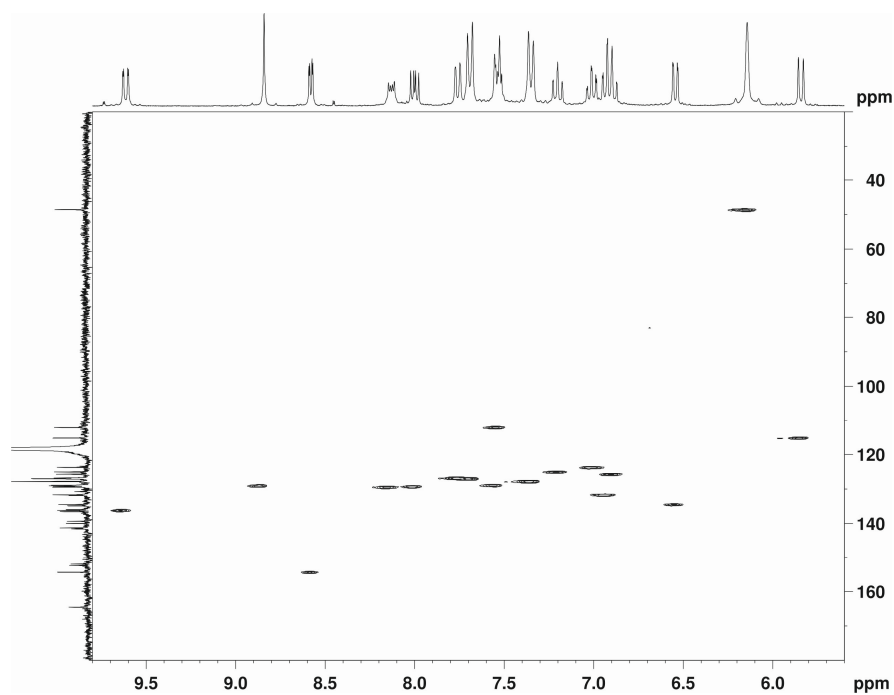


Figure S2.7. HSQC 2D ^1H - ^{13}C NMR of **11** (CD_3CN).

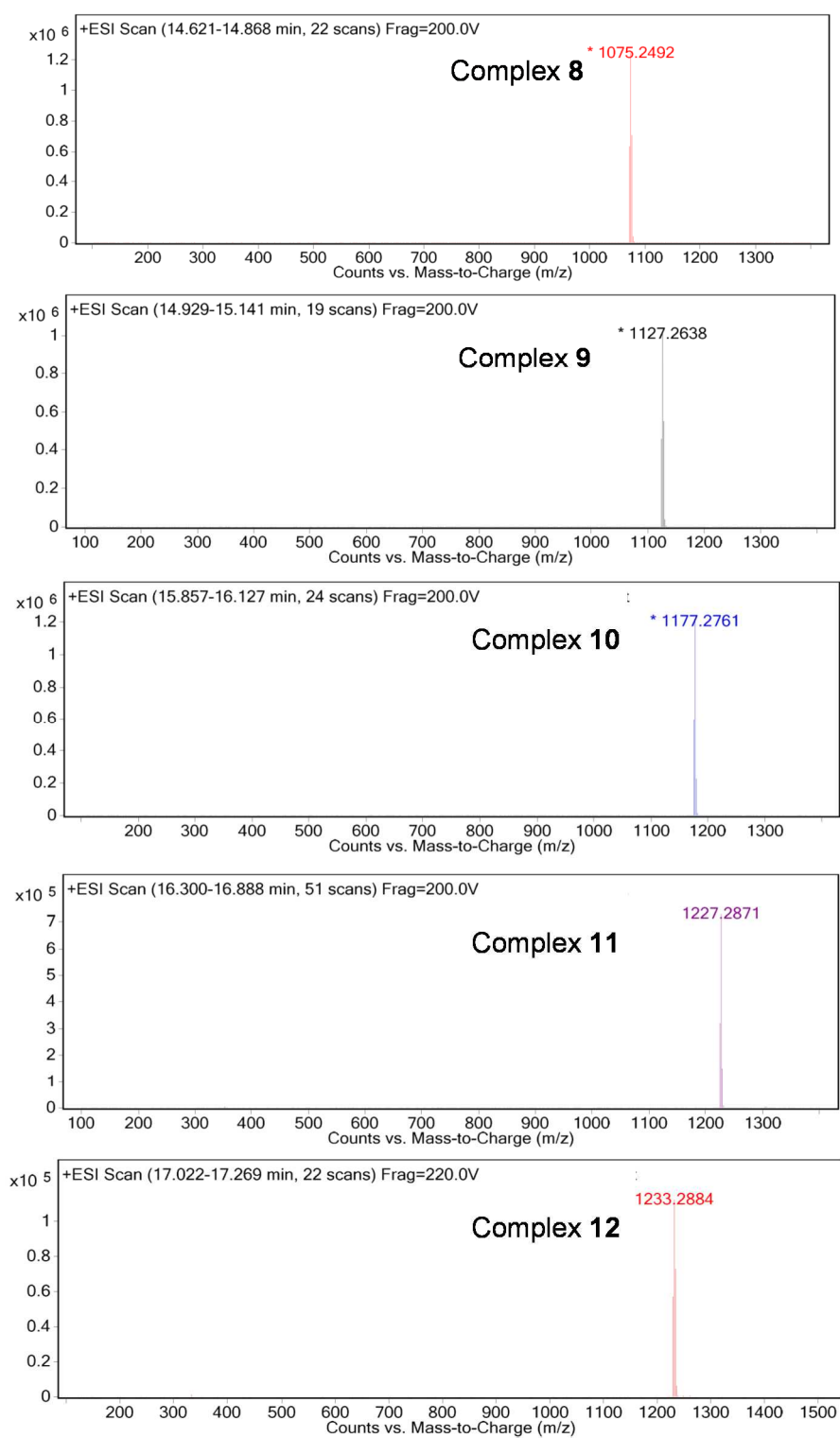


Figure S2.8. Positive-ion ESI-MS of complexes 8-12.

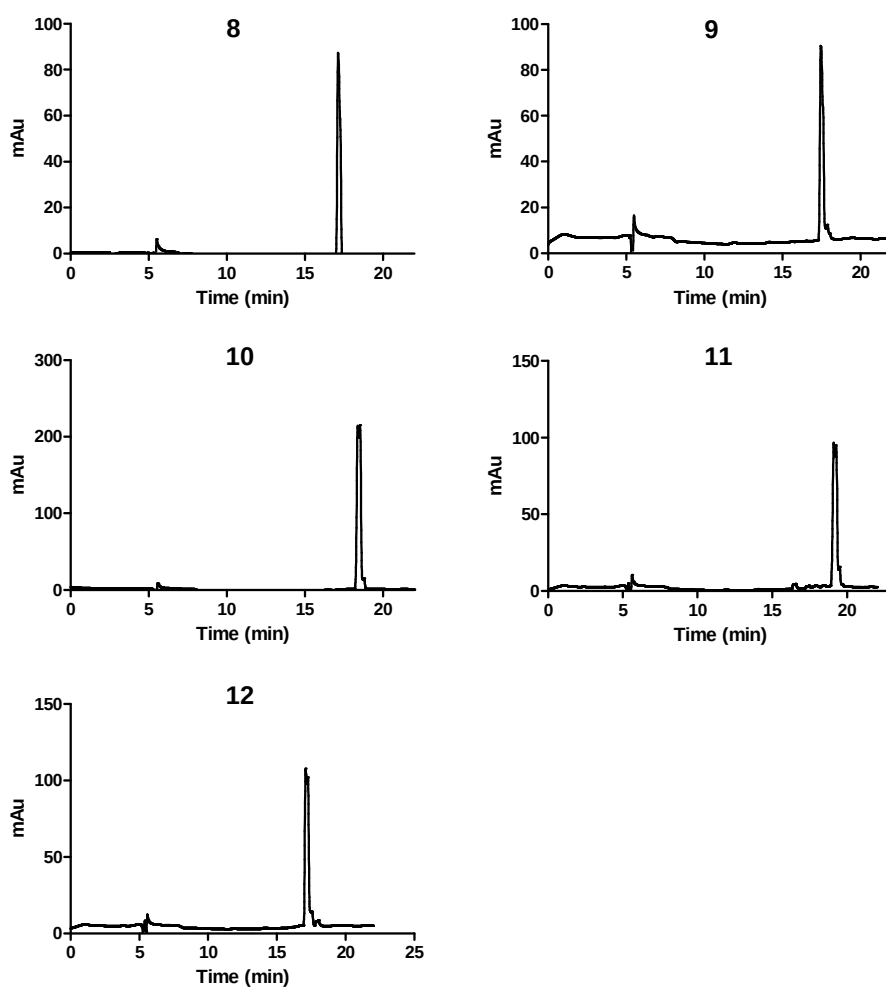


Figure S2.9. HPLC chromatograms of **8–12** in DMSO.

Table S2.1. Crystal data for **9**

$2(\text{C}_{56}\text{H}_{36}\text{F}_6\text{IrN}_8) \cdot 2(\text{F}_6\text{P}) \cdot \text{C}_{0.50}\text{HCl} \cdot 3(\text{CH}_2\text{Cl}_2)$	$Z = 1$
$M_r = 2841.43$	$F(000) = 1403$
Triclinic, P^-1	$D_x = 1.676 \text{ Mg m}^{-3}$
$a = 12.9785 (11) \text{ \AA}$	Mo $K\alpha$ radiation, $\lambda = 0.71073 \text{ \AA}$
$b = 13.2427 (11) \text{ \AA}$	Cell parameters from 9171 reflections
$c = 18.6875 (16) \text{ \AA}$	$\theta = 2.2\text{--}28.3^\circ$
$\alpha = 86.168 (3)^\circ$	$\mu = 2.65 \text{ mm}^{-1}$
$\beta = 80.313 (3)^\circ$	$T = 100 \text{ K}$
$\gamma = 62.762 (3)^\circ$	Block, orange
$V = 2814.8 (4) \text{ \AA}^3$	$0.31 \times 0.21 \times 0.12 \text{ mm}$

Table S2.2. Data collection for **9**

Bruker D8 Quest CCD diffractometer	11326 reflections with $I > 2\sigma(I)$
Radiation source: fine-focus sealed tube	$R_{\text{int}} = 0.047$
ω and ϕ scans	$\theta_{\text{max}} = 26.9^\circ$, $\theta_{\text{min}} = 2.0^\circ$
Absorption correction: multi-scan (SADABS; Sheldrick, 1996)	$h = -16 \rightarrow 16$
$T_{\text{min}} = 0.514$, $T_{\text{max}} = 0.746$	$k = -16 \rightarrow 16$
122280 measured reflections	$l = -23 \rightarrow 23$
12091 independent reflections	

Table S2.3. Refinement data for **9**

Refinement on F^2	0 restraints
Least-squares matrix: full	Hydrogen site location: inferred from neighbouring sites
$R[F^2 > 2\sigma(F^2)] = 0.032$	H-atom parameters constrained
$wR(F^2) = 0.085$	$w = 1/[\sigma^2(F_o^2) + (0.0447P)^2 + 9.2106P]$ where $P = (F_o^2 + 2F_c^2)/3$
$S = 1.05$	$(\Delta/\sigma)_{\text{max}} < 0.001$
12091 reflections	$\Delta_{\text{max}} = 2.84 \text{ e } \text{\AA}^{-3}$
770 parameters	$\Delta_{\text{min}} = -1.29 \text{ e } \text{\AA}^{-3}$

Table S2.4. Crystal data for **10**

$\text{C}_{60}\text{H}_{38}\text{F}_6\text{IrN}_8 \cdot \text{F}_6\text{P} \cdot 2.375(\text{C}_2\text{H}_4\text{Cl}_2)$	$Z = 2$
$M_r = 1557.16$	$F(000) = 1546$
Triclinic, P^-1	$D_x = 1.578 \text{ Mg m}^{-3}$
$a = 11.4643 (18) \text{ \AA}$	Mo $K\alpha$ radiation, $\lambda = 0.71073 \text{ \AA}$
$b = 16.517 (3) \text{ \AA}$	Cell parameters from 9653 reflections
$c = 17.657 (3) \text{ \AA}$	$\theta = 2.4\text{--}28.0^\circ$
$\alpha = 79.448 (5)^\circ$	$\mu = 2.34 \text{ mm}^{-1}$
$\beta = 85.642 (5)^\circ$	$T = 100 \text{ K}$
$\gamma = 89.333 (5)^\circ$	Block, orange
$V = 3277.4 (10) \text{ \AA}^3$	$0.24 \times 0.14 \times 0.07 \text{ mm}$

Table S2.5. Data collection for **10**

Bruker D8 Quest CCD diffractometer	10168 reflections with $I > 2\sigma(I)$
Radiation source: fine-focus sealed tube	$R_{\text{int}} = 0.060$
ω and ϕ scans	$\theta_{\text{max}} = 25.2^\circ$, $\theta_{\text{min}} = 1.9^\circ$
Absorption correction: multi-scan (SADABS; Sheldrick, 1996)	$h = -13 \rightarrow 13$
$T_{\text{min}} = 0.621$, $T_{\text{max}} = 0.746$	$k = -19 \rightarrow 19$

114715 measured reflections	$l = -21 \rightarrow 21$
11795 independent reflections	

Table S2.6. Refinement data for **10**

Refinement on F^2	0 restraints
Least-squares matrix: full	Hydrogen site location: inferred from neighbouring sites
$R[F^2 > 2\sigma(F^2)] = 0.058$	H-atom parameters constrained
$wR(F^2) = 0.160$	$w = 1/[\sigma^2(F_o^2) + (0.0828P)^2 + 22.6994P]$ where $P = (F_o^2 + 2F_c^2)/3$
$S = 1.04$	$(\Delta/\sigma)_{\max} = 0.001$
11795 reflections	$\Delta_{\max} = 3.23 \text{ e } \text{\AA}^{-3}$
838 parameters	$\Delta_{\min} = -2.00 \text{ e } \text{\AA}^{-3}$

Table S2.7. Selected bond lengths and bond angles of **9** and **10**

Compound	9	10
Bond lengths (Å)	Ir—C44 = 2.020 (4)	Ir—C27 = 2.006 (7)
	Ir—C23 = 2.020 (3)	Ir—C48 = 2.015 (8)
	Ir—N5 = 2.039 (3)	Ir—N5 = 2.042 (6)
	Ir—N7 = 2.045 (3)	Ir—N7 = 2.049 (5)
	Ir—N1 = 2.132 (3)	Ir—N1 = 2.123 (6)
	Ir—N4 = 2.133 (3)	Ir—N4 = 2.134 (6)
Bond angles (deg)	C44—Ir—C23 = 92.69 (13)	C27—Ir—C48 = 91.0 (3)
	C44—Ir—N5 = 94.74 (13)	C27—Ir—N5 = 80.1 (3)
	C23—Ir—N5 = 79.29 (13)	C48—Ir—N5 = 95.0 (3)
	C44—Ir—N7 = 79.39 (13)	C27—Ir—N7 = 95.1 (2)
	C23—Ir—N7 = 93.32 (13)	C48—Ir—N7 = 79.7 (3)
	N5—Ir—N7 = 170.41 (11)	N5—Ir—N7 = 172.9 (3)
	C44—Ir—N1 = 95.06 (12)	C27—Ir—N1 = 173.6 (3)
	C23—Ir—N1 = 171.45 (12)	C48—Ir—N1 = 94.9 (3)
	N5—Ir—N1 = 96.44 (11)	N5—Ir—N1 = 96.8 (3)
	N7—Ir—N1 = 91.66 (11)	N7—Ir—N1 = 88.4 (2)
	C44—Ir—N4 = 172.57 (12)	C27—Ir—N4 = 96.4 (2)
	C23—Ir—N4 = 94.69 (12)	C48—Ir—N4 = 172.0 (3)
	N5—Ir—N4 = 87.40 (11)	N5—Ir—N4 = 89.1 (2)
	N7—Ir—N4 = 99.38 (11)	N7—Ir—N4 = 96.7 (2)
	N1—Ir—N4 = 77.62 (11)	N1—Ir—N4 = 77.8 (2)

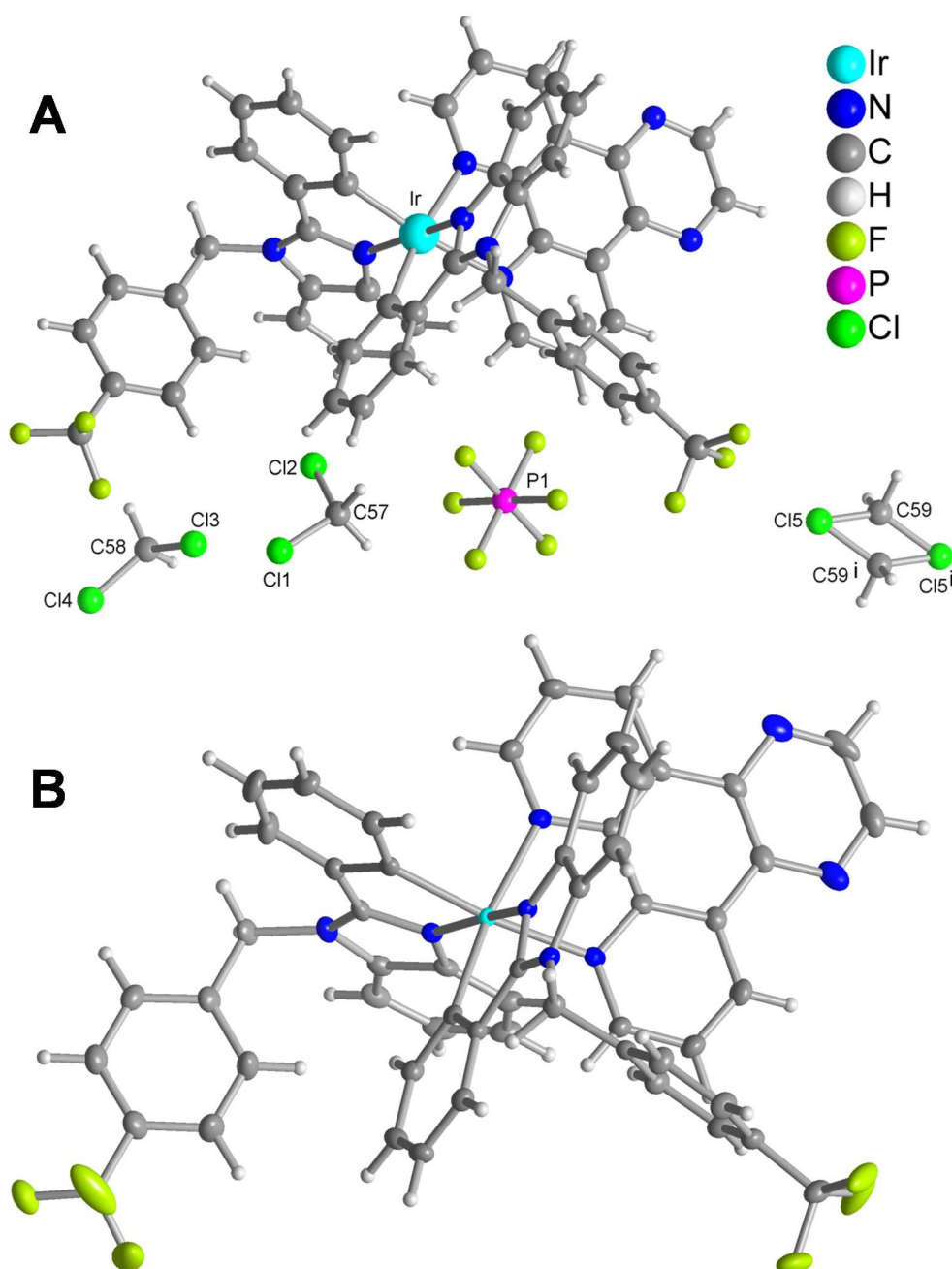


Figure S2.10. Molecular structure within the crystal of **9**, (a) showing the iridium cation, the PF₆ anion and the three partially occupied methylene chloride solvent molecules of crystallization. All three CH₂Cl₂ molecules are not fully occupied; occupation factors were refined to about 0.9 (for C57-Cl1-Cl2) and 0.6 (C58-Cl3-Cl4) and 0.5 (C59-Cl5). After occupation factor refinement they were kept fixed during the last refinement cycles. One CH₂Cl₂ sits on the inversion center and is half occupied with the CH₂ part (occupation factor 0.25) split over two positions. Symmetry transformation $i = -x, 2-y, 1-z$. (b) showing only the iridium cation (50% thermal ellipsoids, H atoms with arbitrary radii).

Table S2.8. C-H $\cdots$ F Hydrogen-bond geometry ($\text{\AA}$, $^\circ$) for **9**.^a

Analysis of Potential Hydrogen Bonds and Schemes with $d(\text{D}\cdots\text{A}) < R(\text{D})+R(\text{A})+0.50$, $d(\text{H}\cdots\text{A}) < R(\text{H})+R(\text{A})-0.12$ Ang., $\text{D-H}\cdots\text{A} > 100.0$ Deg

Note: - ARU codes in [] are with reference to the Coordinates printed above (Possibly transformed, when MOVE .NE. 1.555)

D --- H...A H...A	[ARU] D...A	D - H D - H...A
C(6) --H(6) ..F(9)	[2566.02] 0.95 2.37 2.937(5)	118
C(11) --H(11) ..F(8)	[1555.02] 0.95 2.48 3.225(6)	136
C(17) --H(17) ..F(1)	[2467.01] 0.95 2.40 3.176(5)	138
C(55) --H(55) ..F(9)	[1545.02] 0.95 2.46 3.328(6)	152
C(55) --H(55) ..F(10)	[1545.02] 0.95 2.50 3.381(5)	154
C(57) --H(57A) ..F(10)	[1555.02] 0.99 2.46 3.283(6)	141

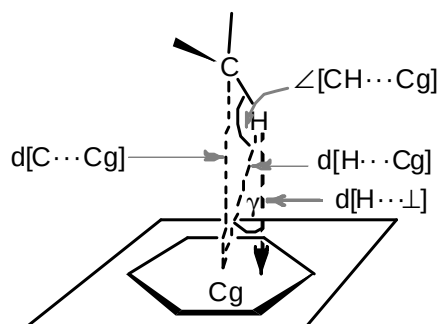
[2467.] = [2_467] = -1-x,1-y,2-z

[2566.] = [2_566] = -x,1-y,1-z

[1545.] = [1_545] = x,-1+y,z

^a D = Donor, A = Acceptor. For found and refined H atoms the standard deviations are given for their hydrogen bond distances and angles.

Significant intermolecular C-H $\cdots\pi$ contacts start below around 2.7 $\text{\AA}$ for the (C-)H $\cdots$ ring centroid distances with H-perp also starting at below 2.6-2.7 $\text{\AA}$ and C-H $\cdots$ Cg $> 145^\circ$.¹



Scheme S2.1. Graphical presentation of the parameters used for the description of CH- π interactions.

Table S2.9. Analysis of intermolecular C-H...Cg(Pi-Ring) Interactions (H...Cg < 3.0 Å, -Gamma < 30.0 Deg)

- Cg(J) = Center of gravity of ring J (Plane number above)
 - H-Perp = Perpendicular distance of H to ring plane J
 - Gamma = Angle between Cg-H vector and ring J normal
 - C-H...Cg = C-H-Cg angle (degrees)
 - C...Cg = Distance of X to Cg (Å)
 - C-H, Pi = Angle of the X-H bond with the Pi-plane (i.e. Perpendicular = 90 degrees, Parallel = 0 degrees)

C-H(I)	Cg(J)	[ARU(J)]	C-H, Pi	H...Cg	H-Perp	Gamma	C-H...Cg	C...Cg
C(28)-H(28B)	Cg(11)	[2557.01]	2.94	2.78	19.21	107	3.368(4)	36
C(30)-H(30)	Cg(15)	[2557.01]	2.98	2.90	13.44	153	3.852(4)	56

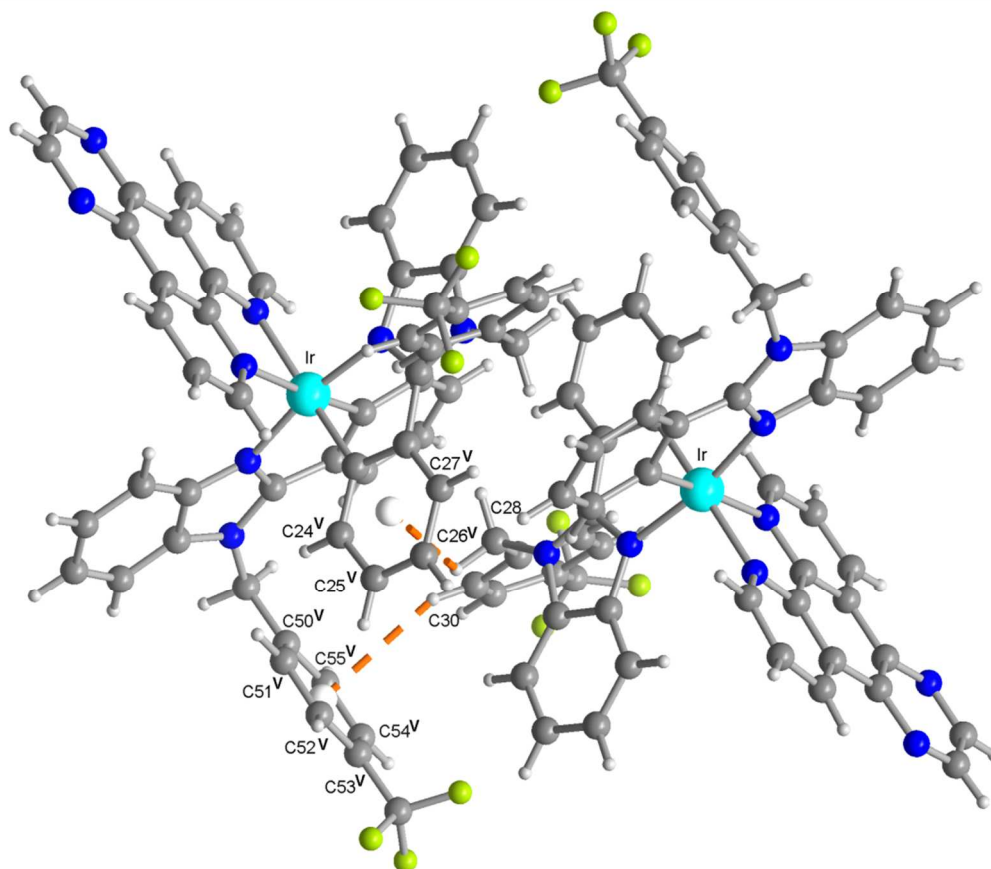
[2557] = -X,-Y,2-Z

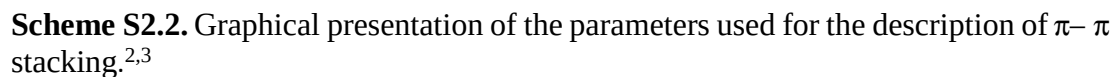
The Cg(I) refer to the Ring Centre-of-Gravity numbers given in

Cg(11) = Ring C22-C23-C24-C25-C26-C27

Cg(15) = Ring C50-C51-C52-C53-C54-C55

[2557] = -X,-Y,2-Z

**Figure S2.11.** Intermolecular C-H... π interactions in **9** indicated as dashed orange lines. Symmetry transformation: v = -X, -Y, 2-Z



Analysis of Short Ring-Interactions with Cg-Cg Distances < 6.0 Angstrom and Beta < 60.0 Deg.

Cg(I)	Cg(J)	[ARU(J)]	Cg-Cg	Alpha	Beta	Gamma	CgI_Perp
		CgJ_Perp	Slippage				
Cg(7)	·Cg(15)	[1465.01]	3.719(2)	11.0(2)	12.3	19.8	3.4991(16)
		3.6335(17)	0.795				
Cg(15)	··Cg(7)	[1645.01]	3.719(2)	11.0(2)	19.8	12.3	3.6336(17)
		3.4991(16)	1.261				

The Cg(I) refer to the Ring Centre-of-Gravity numbers given in
Cg(7) = Ring N2-N3-NC5-C6-C7-C8
Cg(15) = Ring C50-C51-C52-C53-C54-C55

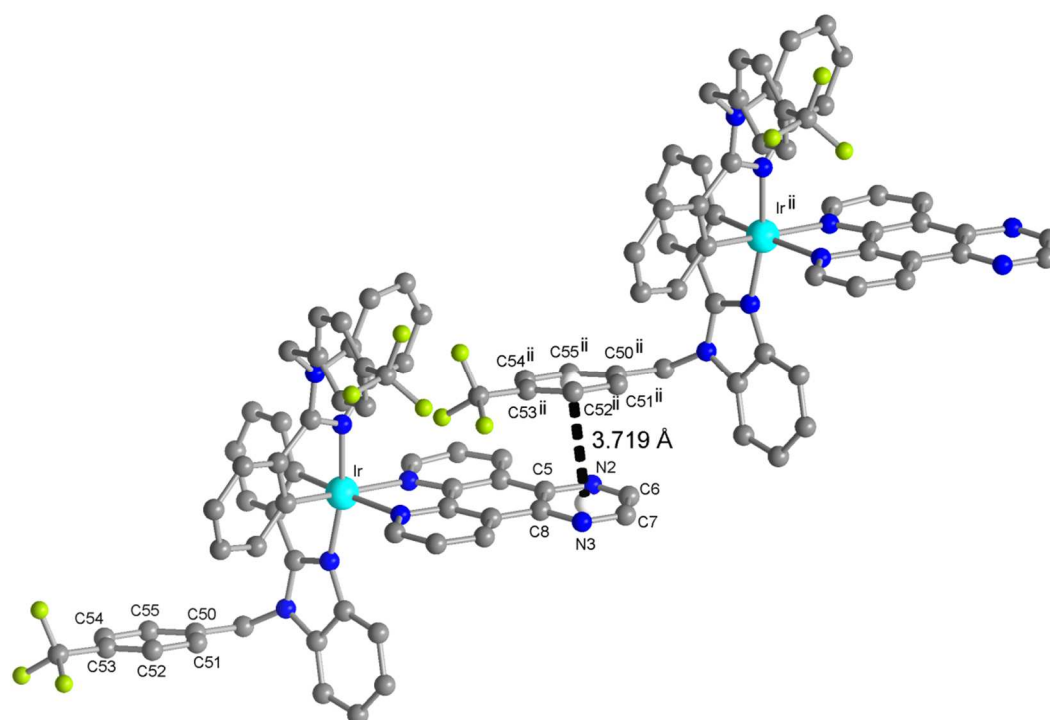


Figure S2.12. π - π interaction in the packing of **9** in the crystal, indicated as dashed black line. Symmetry transformations to other molecule: $-1+X$, $1+Y$, Z .

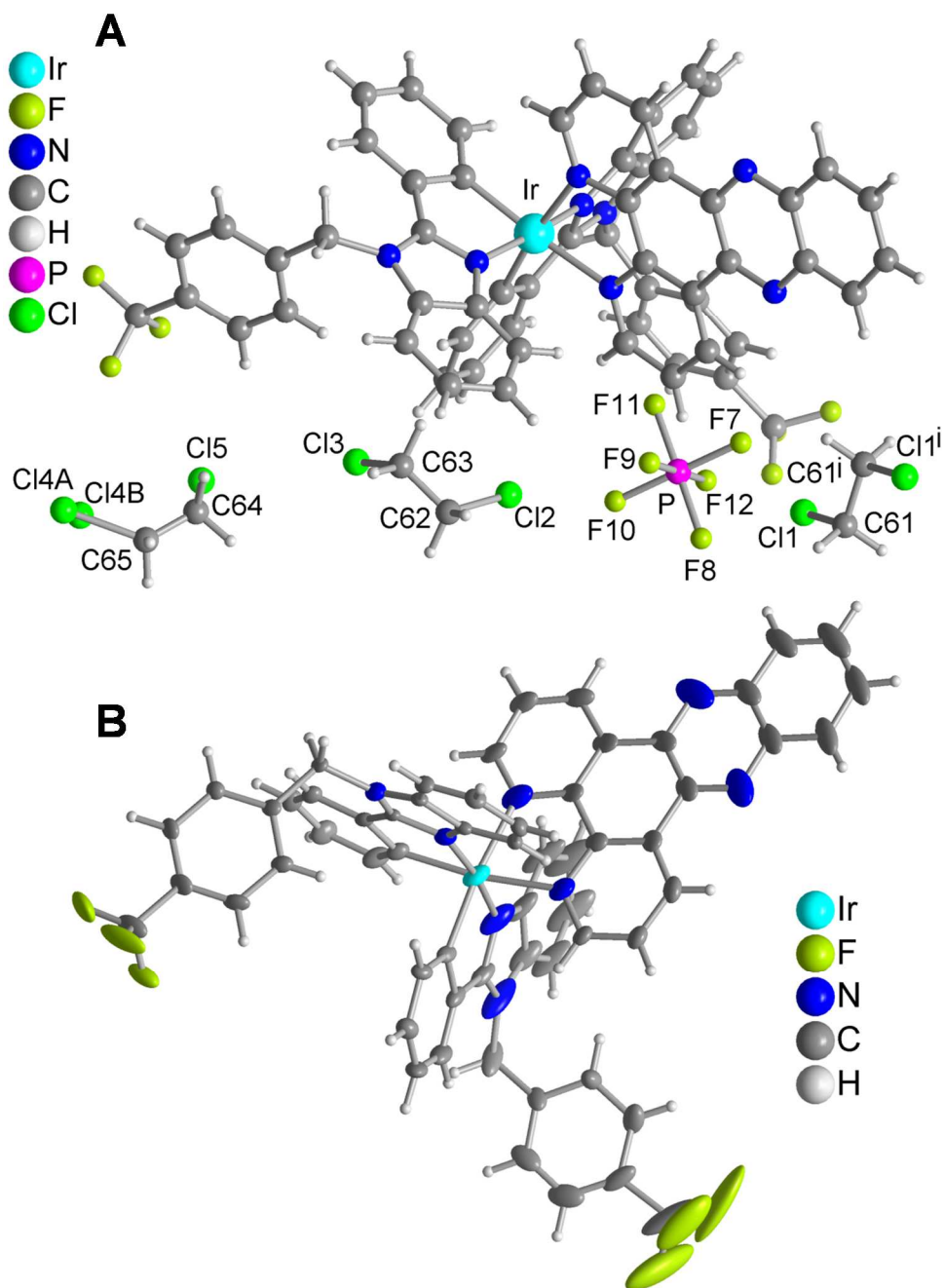


Figure S2.13. Molecular structure within the crystal of **10** (a) showing the iridium cation, the PF₆ anion and the three 1,2-dichloroethane solvent molecules of crystallization. The C₂H₄Cl₂ molecule with C61 and Cl1 sits on a special position and is not fully occupied; occupation factors were refined to about 0.75. After occupation factor refinement they were kept fixed during the last refinement cycles. Symmetry transformation $i = -x, -y, -z+1$. (b) showing only the iridium cation (50% thermal ellipsoids for Ir and N, 30% for C and F, H atoms with arbitrary radii).

Table S2.11. C-H...F and C-H...F Hydrogen-bond geometry (Å, °) for **10**.^a

Analysis of Potential Hydrogen Bonds and Schemes with $d(D...A) < R(D)+R(A)+0.50$, $d(H...A) < R(H)+R(A)-0.12$ Ang., $D-H...A > 100.0$ Deg

Note: - ARU codes in [] are with reference to the Coordinates printed above (Possibly transformed, when MOVE .NE. 1.555)

D --- H...A	[ARU]	D - H	H...A	D...A	D - H...A	
C(3) --H(3) ..F(8)		[1655.02]	0.95	2.50	3.301(13)	142
C(7) --H(7) ..Cl(4B)		[2666.03]	0.95	2.76	3.56(2)	142
C(35) --H(35) ..F(4)		[1545.01]	0.95	2.45	3.192(14)	135
C(42) --H(42) ..F(10)		[1555.02]	0.95	2.53	3.453(9)	164
C(63) --H(63B) ..F(10)		[2566.02]	0.99	2.55	3.323(12)	135

ii = [1655.] = [1_655] = 1+x,y,z

iii = [2666.] = [2_666] = 1-x,1-y,1-z

iv = [1545.] = [1_545] = x,-1+y,z

v = [2566.] = [2_566] = -x,1-y,1-z

^a D = Donor, A = Acceptor. For found and refined H atoms the standard deviations are given for their hydrogen bond distances and angles.

Significant intermolecular C-H... π contacts start below around 2.7 Å for the (C-)H...ring centroid distances with H-perp also starting (Scheme S1) at below 2.6-2.7 Å and C-H...Cg > 145°.⁴⁻⁷

Table S2.12. Analysis of intermolecular C-H...Cg(Pi-Ring) Interactions (H..Cg < 3.0 Ang. - Gamma < 30.0 Deg) in **10**

- Cg(J) = Center of gravity of ring J (Plane number above)
 - H-Perp = Perpendicular distance of H to ring plane J
 - Gamma = Angle between Cg-H vector and ring J normal
 - C-H..Cg = C-H-Cg angle (degrees)
 - C..Cg = Distance of X to Cg (Angstrom)
 - C-H, Pi = Angle of the X-H bond with the Pi-plane (i.e.' Perpendicular = 90 degrees, Parallel = 0 degrees)

C--H(I)	Cg(J) [ARU(J)]	H..Cg	H-Perp	Gamma	C-H..Cg	C..Cg	C-H,Pi
C(9)-H(9)->	Cg(11) [2656.01]	2.84	2.59	23.94	164	3.758(18)	73
C(44)-H(44)->	Cg(6) [2666.01]		2.80	2.77	9.12	138	3.560(8)57
-----		Min or Max	2.580	2.750	3.5	164	3.237 73

vi = [2656] = 1-X,-Y,1-Z

iii = [2666] = 1-X,1-Y,1-Z

The Cg(I) refer to the Ring Centre-of-Gravity numbers given in

Cg(6) = Ring N1-C1-C2-C3-C4-C17

Cg(11) = Ring C19-C20-C21-C22-C23-C24

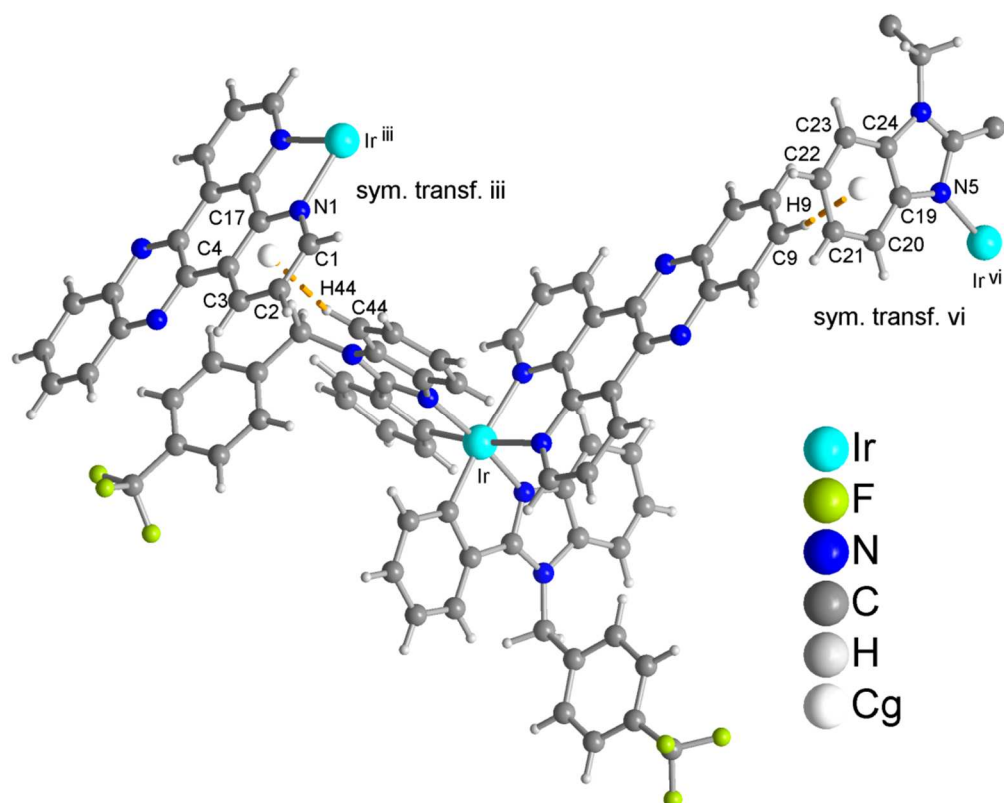


Figure S2.14. Intermolecular C-H $\cdots\pi$ interactions in **10** indicated as dashed orange lines. The two symmetry related cations are only partly drawn for clarity. Details of these hydrogen bonds including the symmetry transformations are given in Table 2 (Cg = ring centroid).

In comparison, significant π -stacking show rather short centroid-centroid contacts (<3.8 Å), near parallel ring planes ($\alpha < 10^\circ$ to $\sim 0^\circ$ or even exactly 0° by symmetry), small slip angles (β , $\gamma < 25^\circ$) and vertical displacements (slippage < 1.5 Å) which translate into a sizable overlap of the aryl-plane areas (Scheme S2).^{8–10}

Table S2.13. Analysis of possible intermolecular(!) π - π interactions in **10** with short centroid-centroid contacts (<4.0 Å).

Analysis of Short Ring-Interactions with Cg-Cg Distances < 6.0 Angstrom and Beta < 60.0 Deg.

- Cg(I) = Plane number I (= ring number in () above)
- Alpha = Dihedral Angle between Planes I and J (Deg)
- Beta = Angle Cg(I) $\rightarrow$ Cg(J) or Cg(I) $\rightarrow$ Me vector and normal to plane I (Deg)
- Gamma = Angle Cg(I) $\rightarrow$ Cg(J) vector and normal to plane J (Deg)
- Cg-Cg = Distance between ring Centroids (Ang.)
- CgI_Perp = Perpendicular distance of Cg(I) on ring J (Ang.)
- CgJ_Perp = Perpendicular distance of Cg(J) on ring I (Ang.)
- Slippage = Distance between Cg(I) and Perpendicular Projection of Cg(J) on Ring I (Ang.).

Cg(I)	Cg(J) [	ARU(J)]	Cg-Cg	Alpha	Beta	Gamma	CgI_Perp
		CgJ_Perp	Slippage				

Cg(5)->Cg(14) [2666.01]	3.724(4)	0.3(4)	25.0	24.8	3.381(3)
3.374(3)	1.576				
Cg(7)->Cg(7) [2656.01]	3.764(5)	0.0(4)	26.0	26.0	- 3.383(4)
3.383(4)	1.650				
Cg(8)->Cg(10) [2656.01]	3.970(6)	3.9(5)	31.4	32.6	- 3.344(3)
3.390(5)	2.066				
Cg(9)->Cg(10) [2656.01]	3.803(6)	3.1(5)	27.7	24.6	3.458(3)
3.367(5)	1.768				
Cg(10)->Cg(8) [2656.01]	3.970(6)	3.9(5)	32.6	31.4	3.390(5)
3.344(3)	2.141				
Cg(10)->Cg(9) [2656.01]	3.803(6)	3.1(5)	24.6	27.7	3.367(5)
3.458(3)	1.585				
Cg(14)->Cg(5) [2666.01]	3.724(4)	0.3(4)	24.8	25.0	3.374(3)
3.381(3)	1.560				
Cg(14)->Cg(14) [2666.01]	3.863(4)	0.0(3)	29.0	29.0	3.380(3)
3.380(3)	1.870				

vi = [2656] = 1-X,-Y,1-Z

iii = [2666] = 1-X,1-Y,1-Z

(unique contacts are highlighted in yellow – reciprocal contacts are not highlighted)

The Cg(I) refer to the Ring Centre-of-Gravity numbers given in

Cg(5) = Ring N7-N8-C40-C45-C46

Cg(7) = Ring N2-N3-C5-C6-C11-C12

Cg(8) = Ring N4-C13-C14-C15-C16-C18

Cg(9) = Ring C4-C5-C12-C13-C17-C18

Cg(10) = Ring C6-C7-C8-C9-C10-C11

Cg(14) = Ring C40-C41-C42-C43-C44-C45

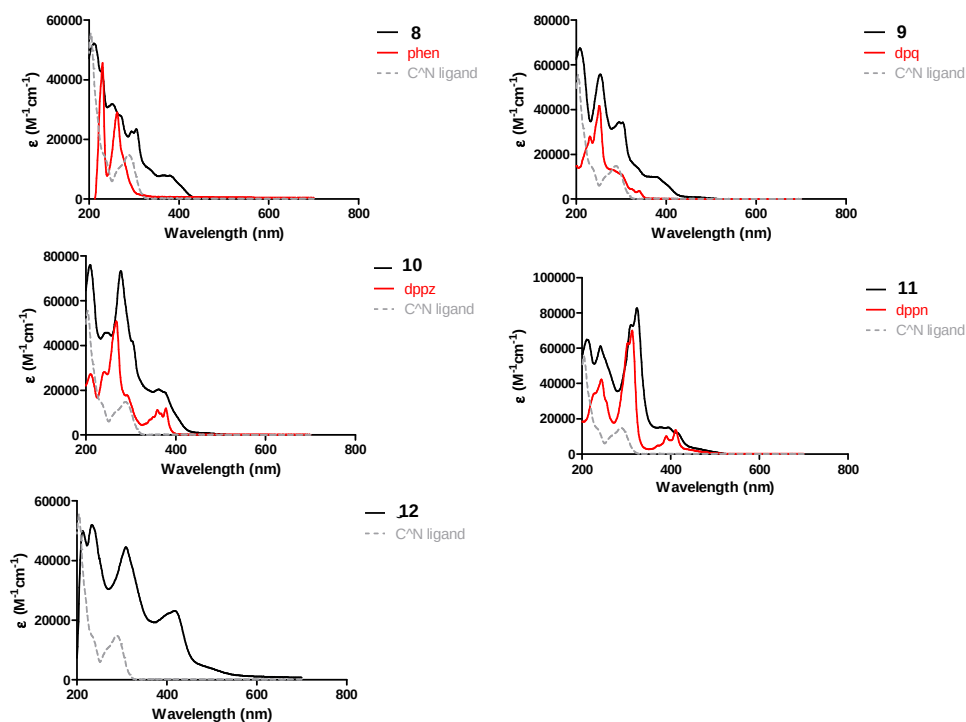


Figure S2.15. UV/Vis absorption spectra comparison of complexes and ligands in acetonitrile. The ligand dppz-izdo was not soluble in this medium.

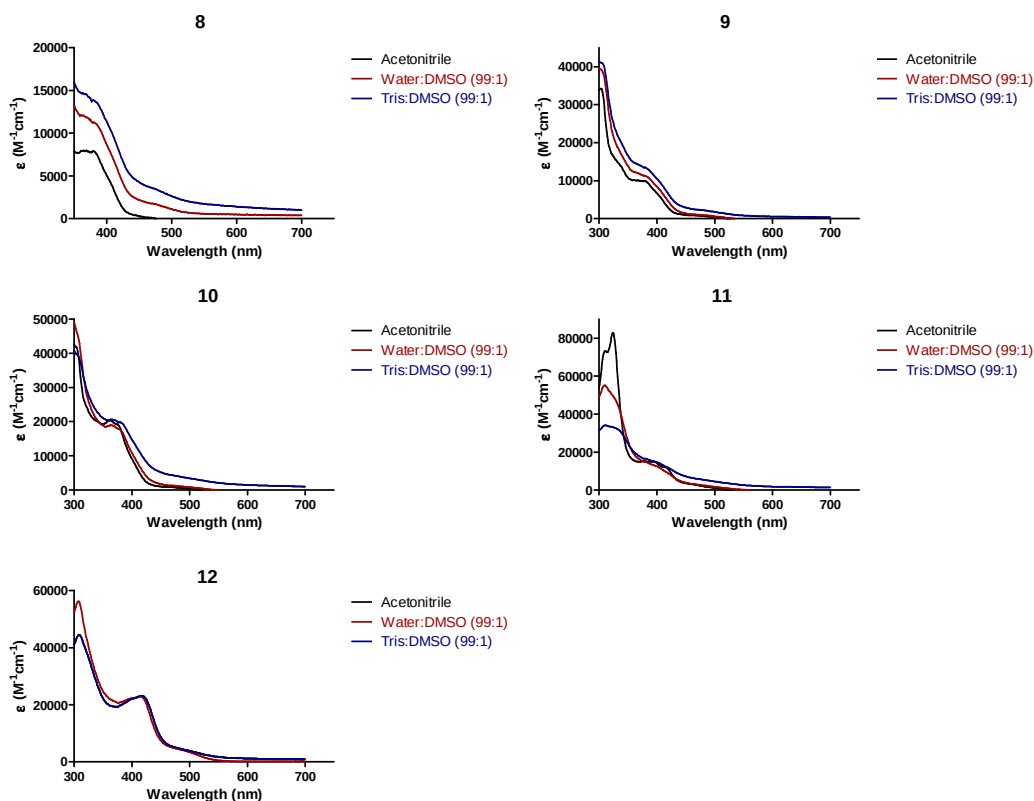


Figure S2.16. UV/Vis absorption changes of the MLCT band of complexes in different solvents.

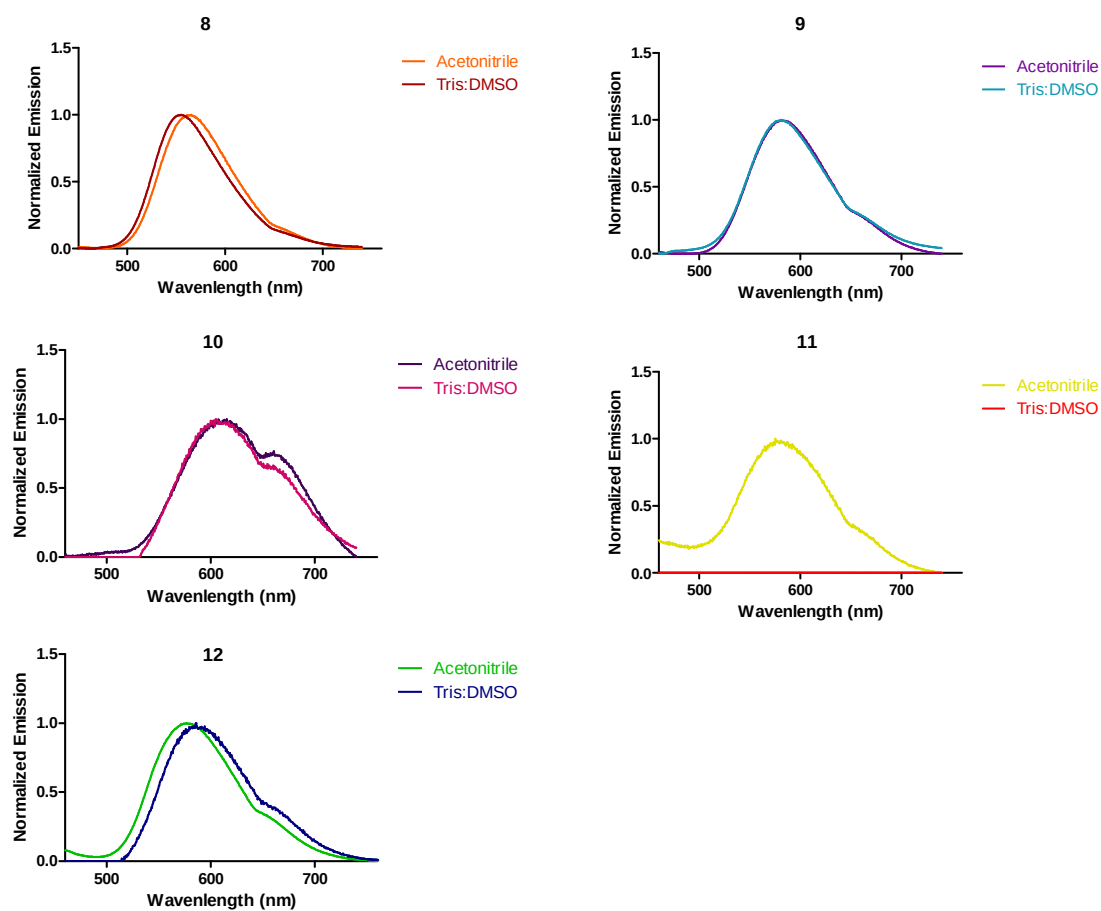


Figure S2.17. Normalized emission spectra of complexes **8–12** in acetonitrile and Tris-HCl buffer.

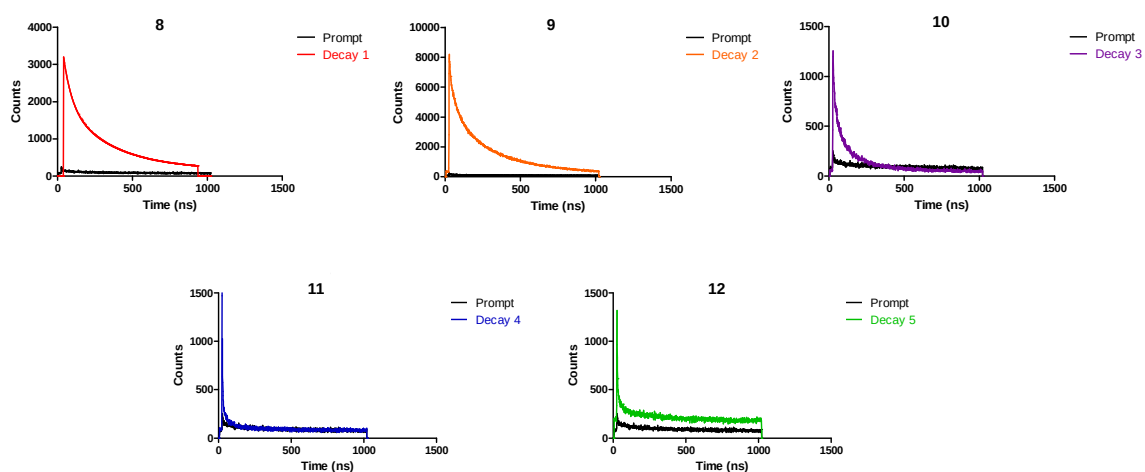


Figure S2.18. Fluorescence emission decay kinetics of **8–12** in water (1% DMSO)

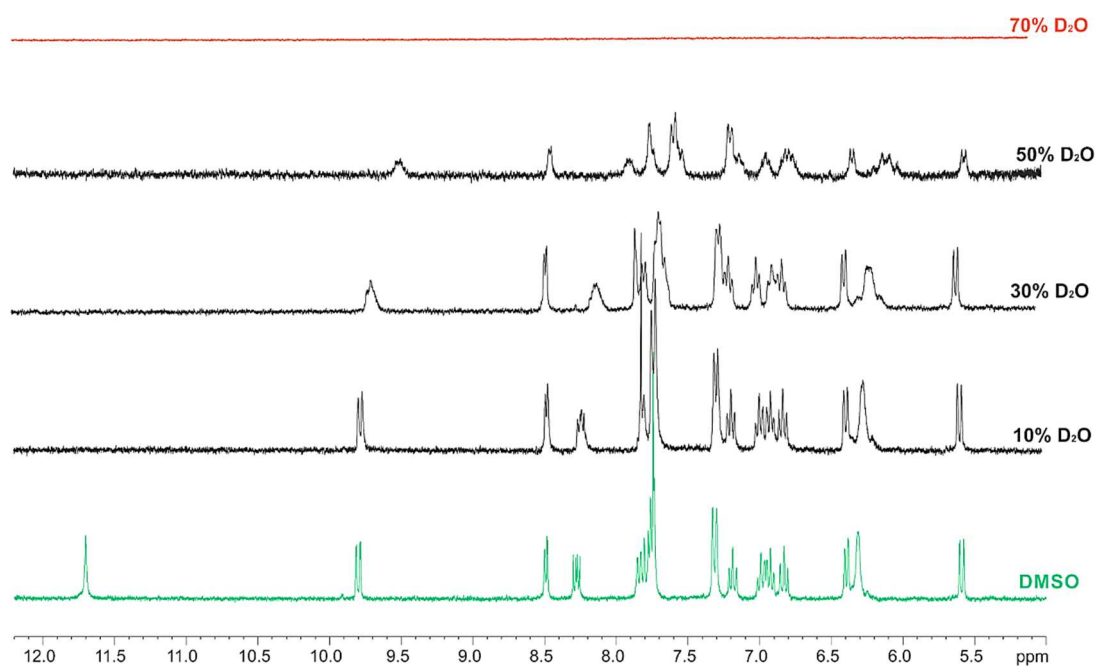


Figure S2.19. ¹H NMR traces of **12** at different composition of D₂O in [D₆]DMSO (1 × 10⁻³ M, 400 MHz, 25 °C). The proton signals show an upfield shift when the percentage of water increases from 0 to 50%, indicating the occurrence of aggregation. A further increase of the water content up to 70% gives as broad signals that lead to the virtual disappearance of spectra.

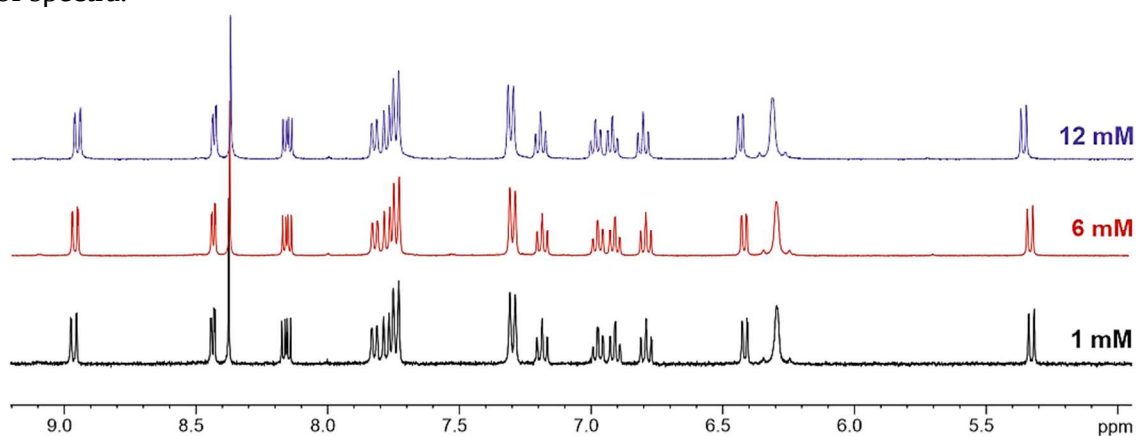


Figure S2.20. ¹H NMR spectral traces of **8** at different concentration in [D₆]DMSO (400 MHz, 25 °C).

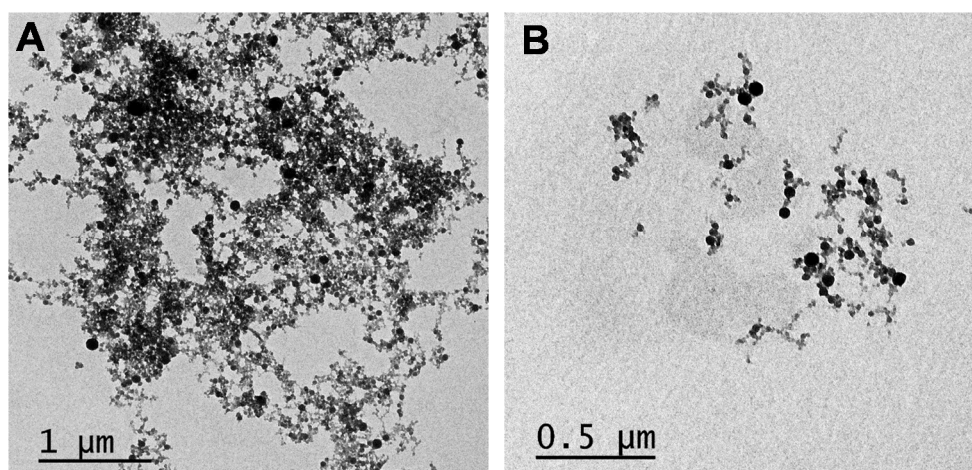


Figure S2.21. TEM images of the aggregates prepared A) and B) for **11** in water:DMSO (99:1) (10^{-5}M)

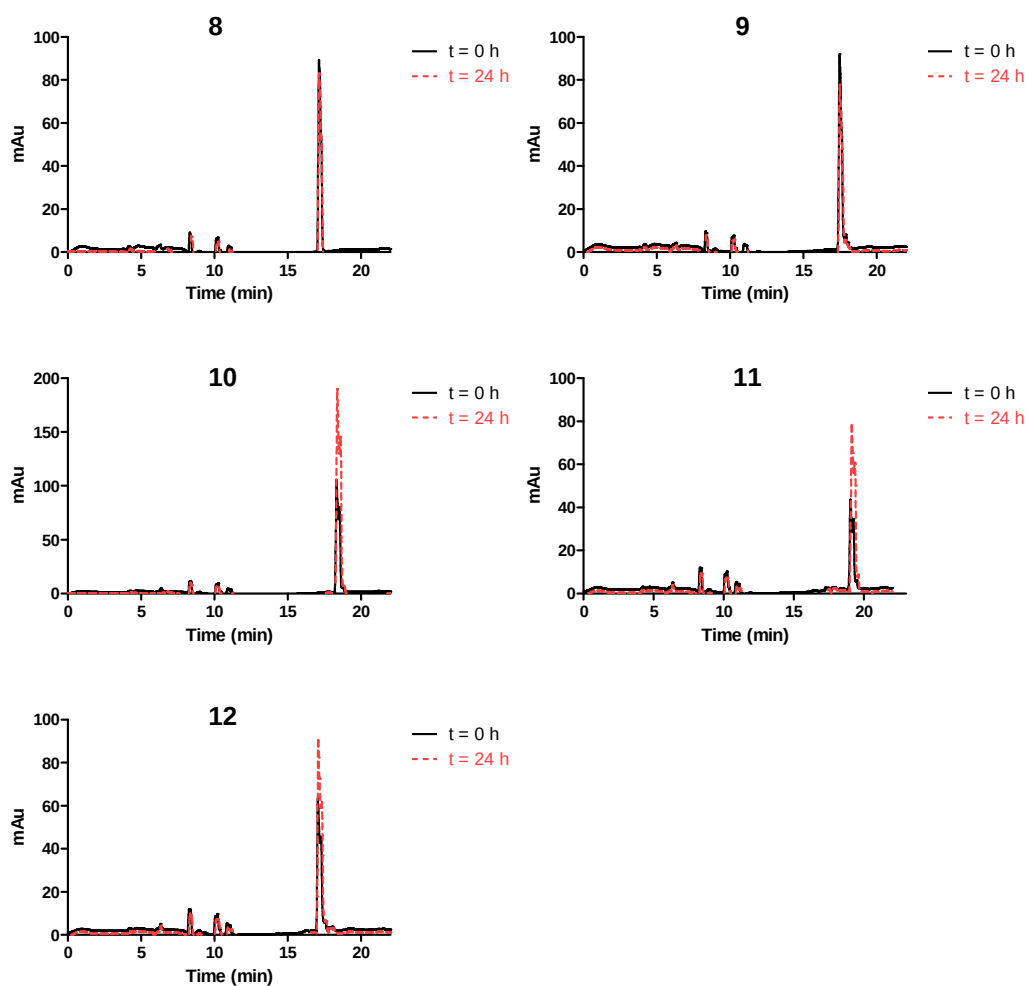


Figure S2.22. HPLC chromatograms of complexes **8–12** in RPMI culture medium at $t = 0$ h (—) and $t = 24$ h (---) incubation. Each sample contain 5% DMSO.

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Chapter 3

Modifying the cyclometalated ligand in
dipyridophenazine Ir(III) complexes to study their
photodynamic activity in normoxia and hypoxia

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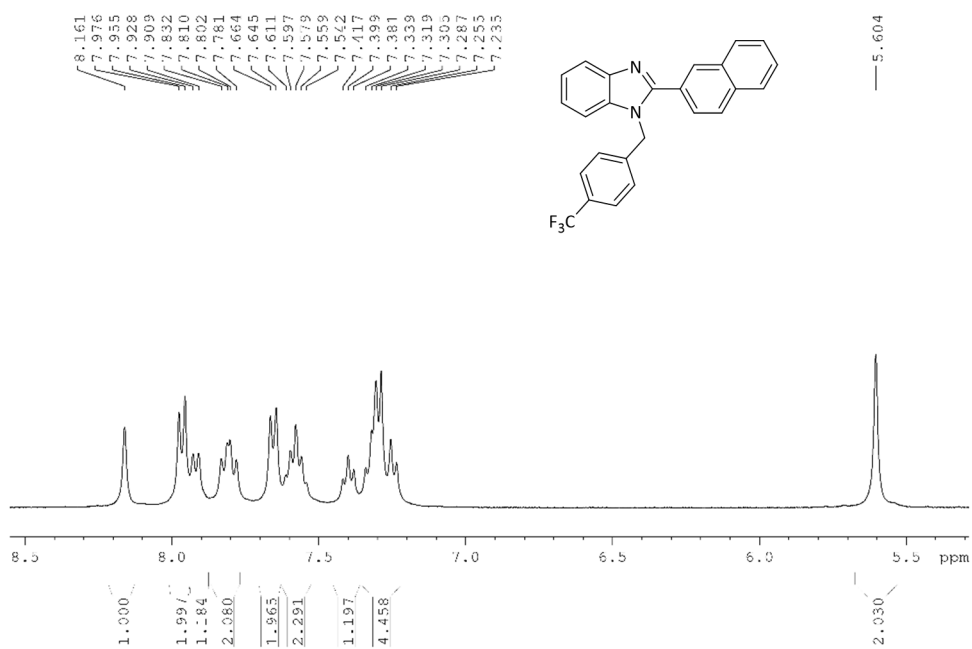


Figure S3.1. ¹H NMR in CDCl₃ for **14c**.

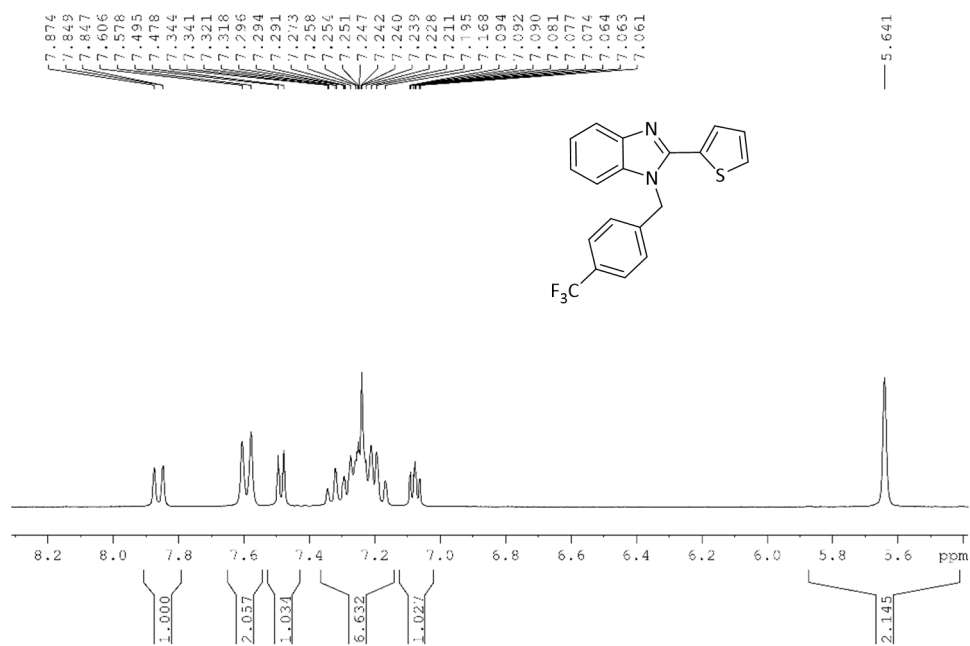


Figure S3.2. ¹H NMR in CDCl₃ for **15c**.

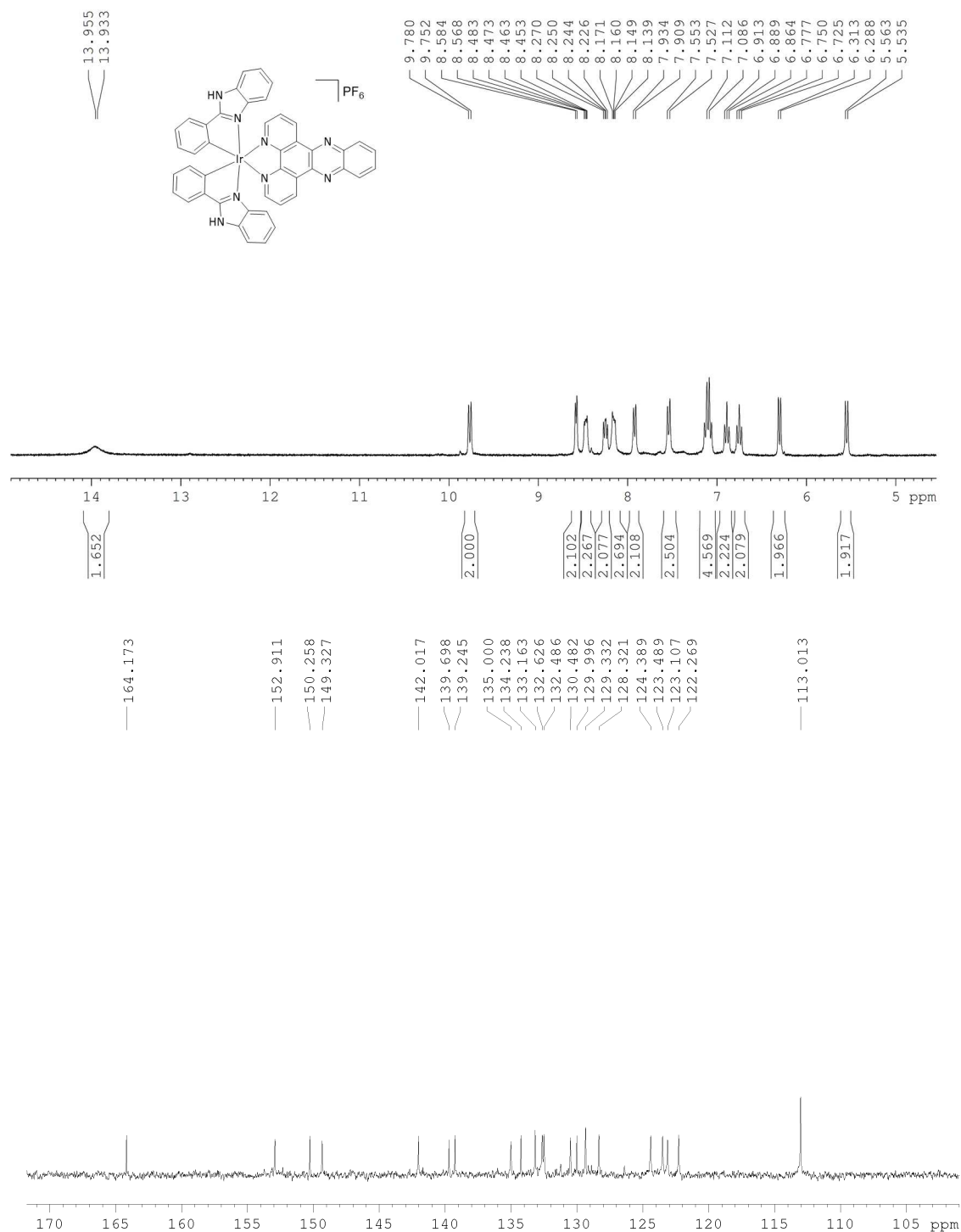


Figure S3.3. ¹H and ¹³C NMR in [D₆]DMSO for **19a**.

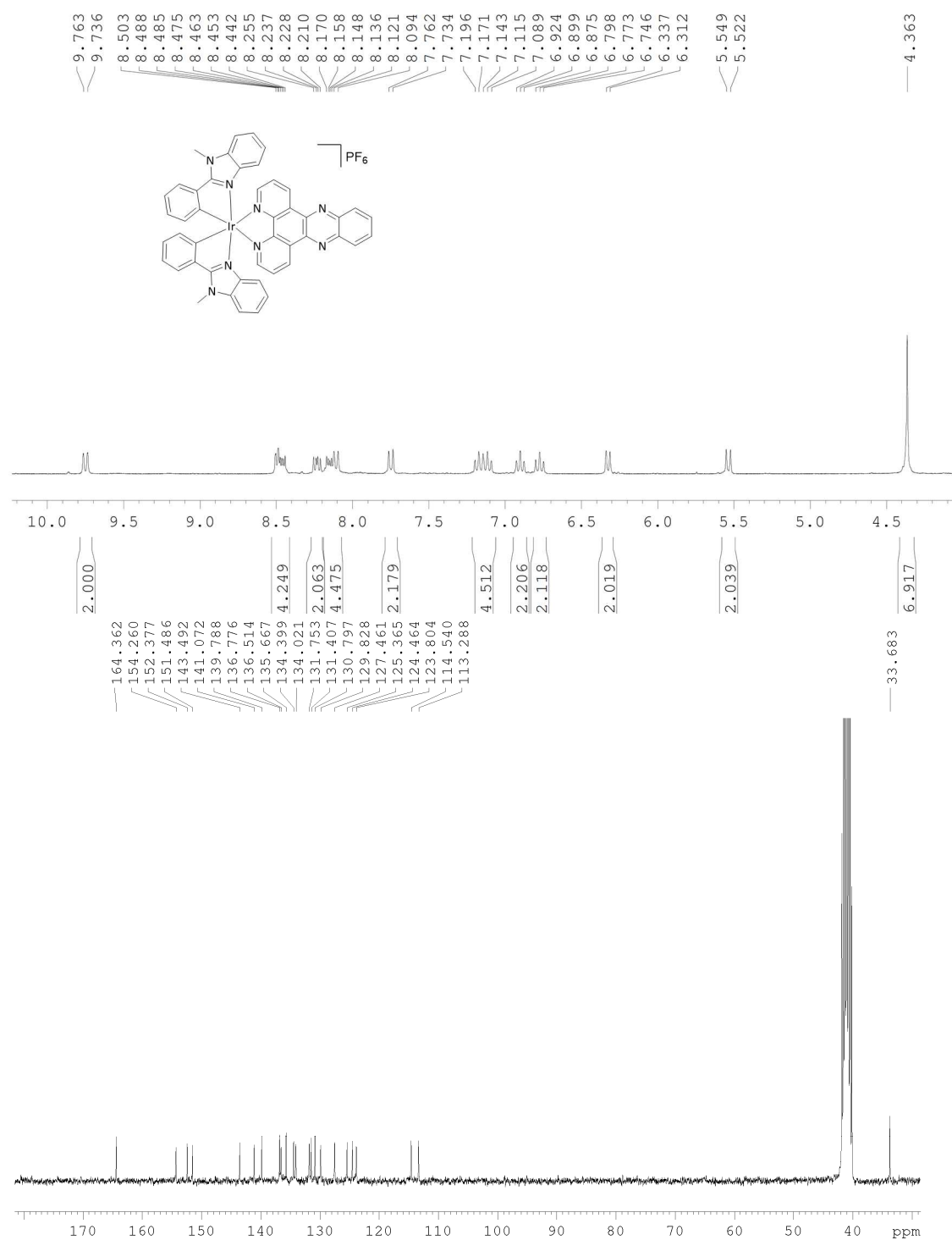


Figure S3.4. ^1H and ^{13}C NMR in $[\text{D}_6]\text{DMSO}$ for **19b**.

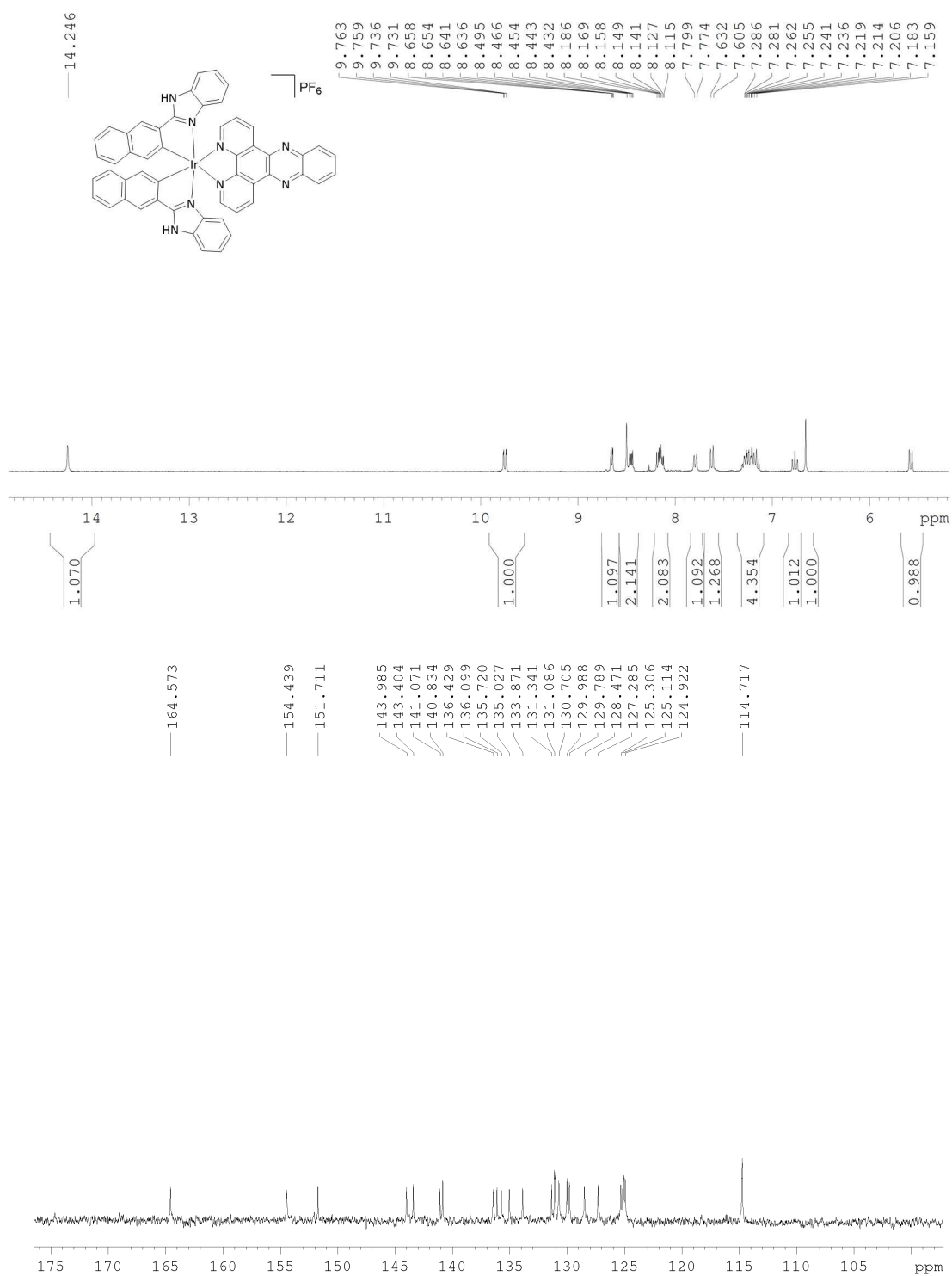


Figure S3.5. ^1H and ^{13}C NMR in $[\text{D}_6]\text{DMSO}$ for **20a**.

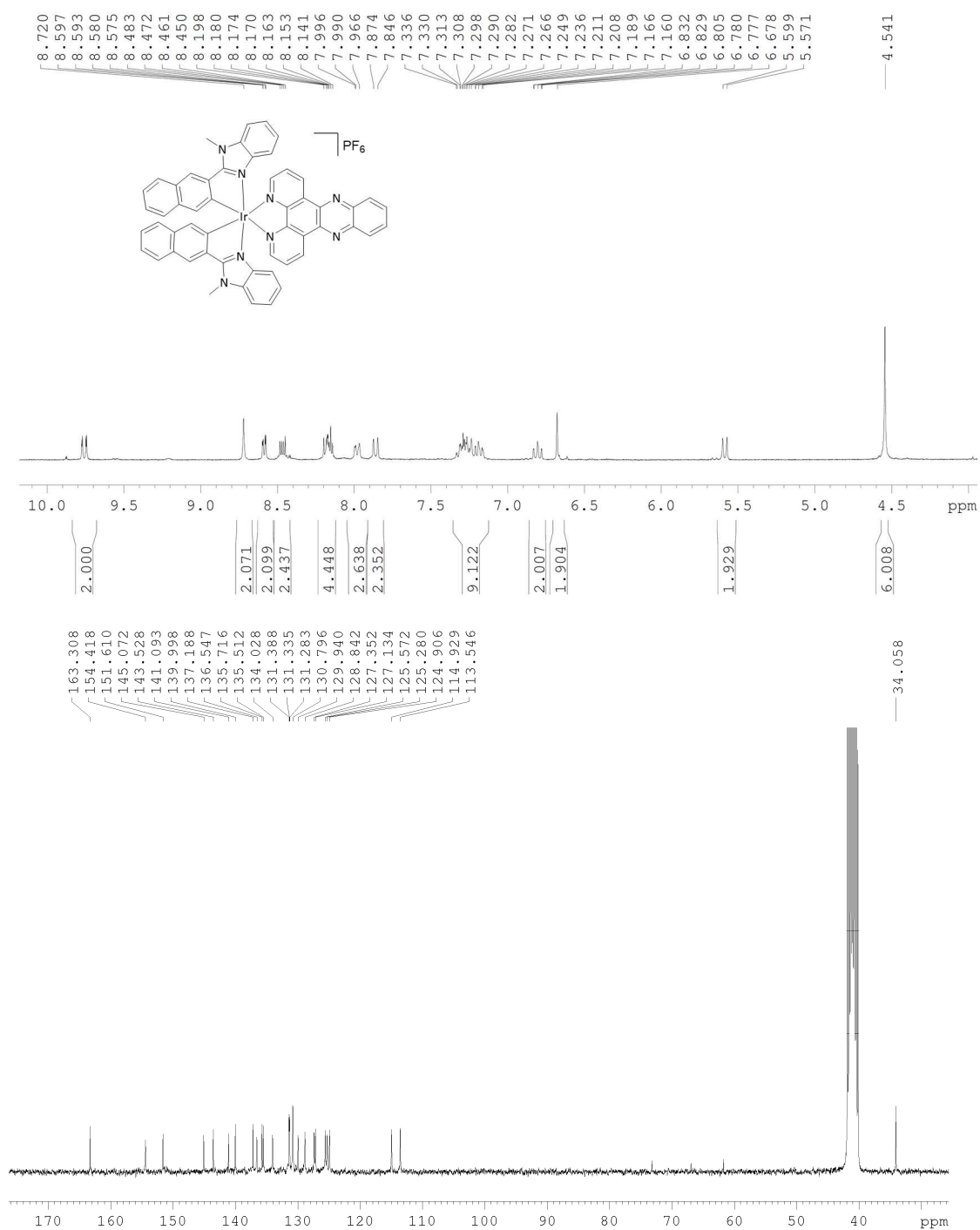


Figure S3.6. ^1H and ^{13}C NMR in $[\text{D}_6]\text{DMSO}$ for **20b**.

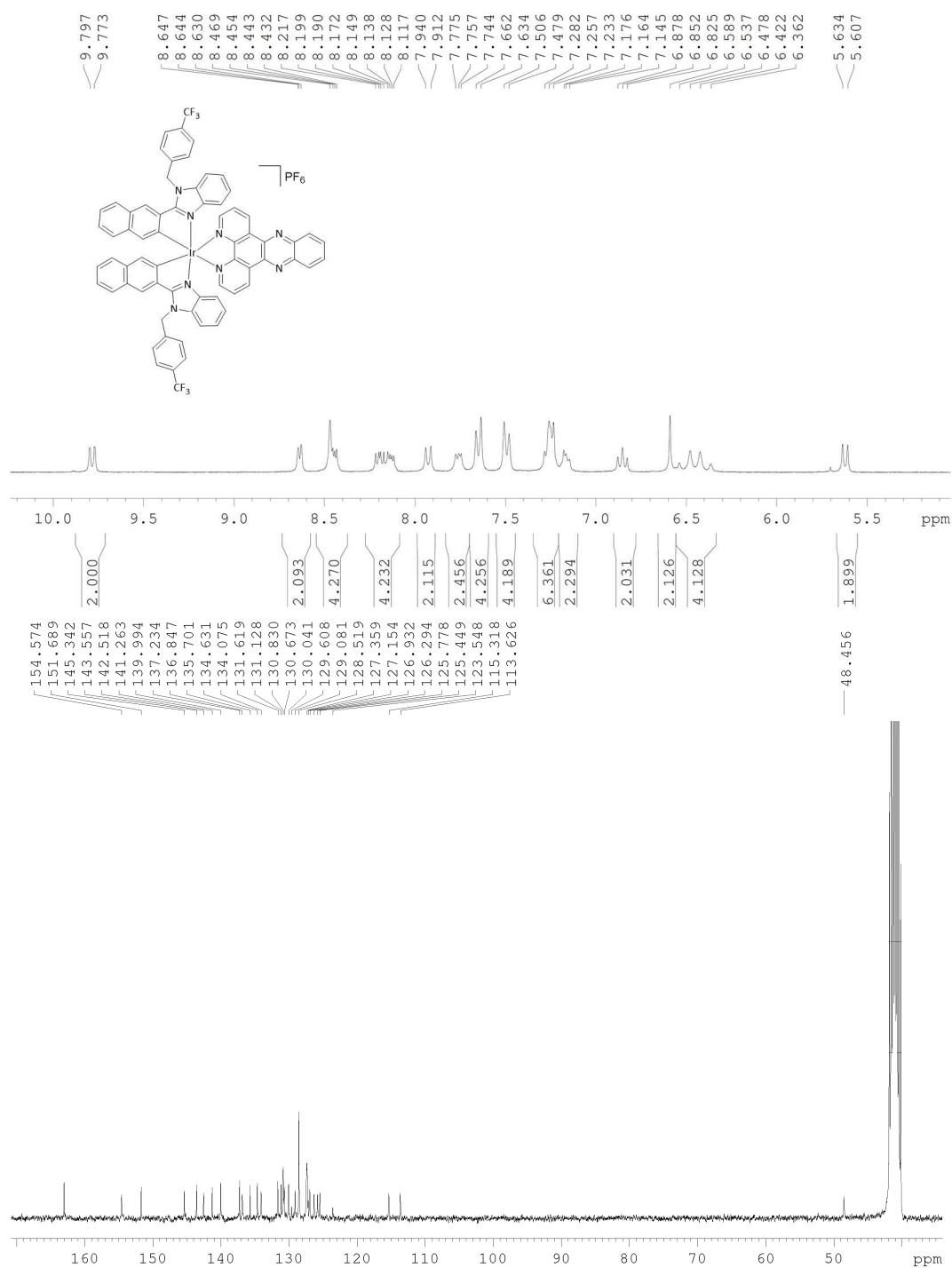


Figure S3.7. ¹H and ¹³C NMR in [D₆]DMSO for **20c**.

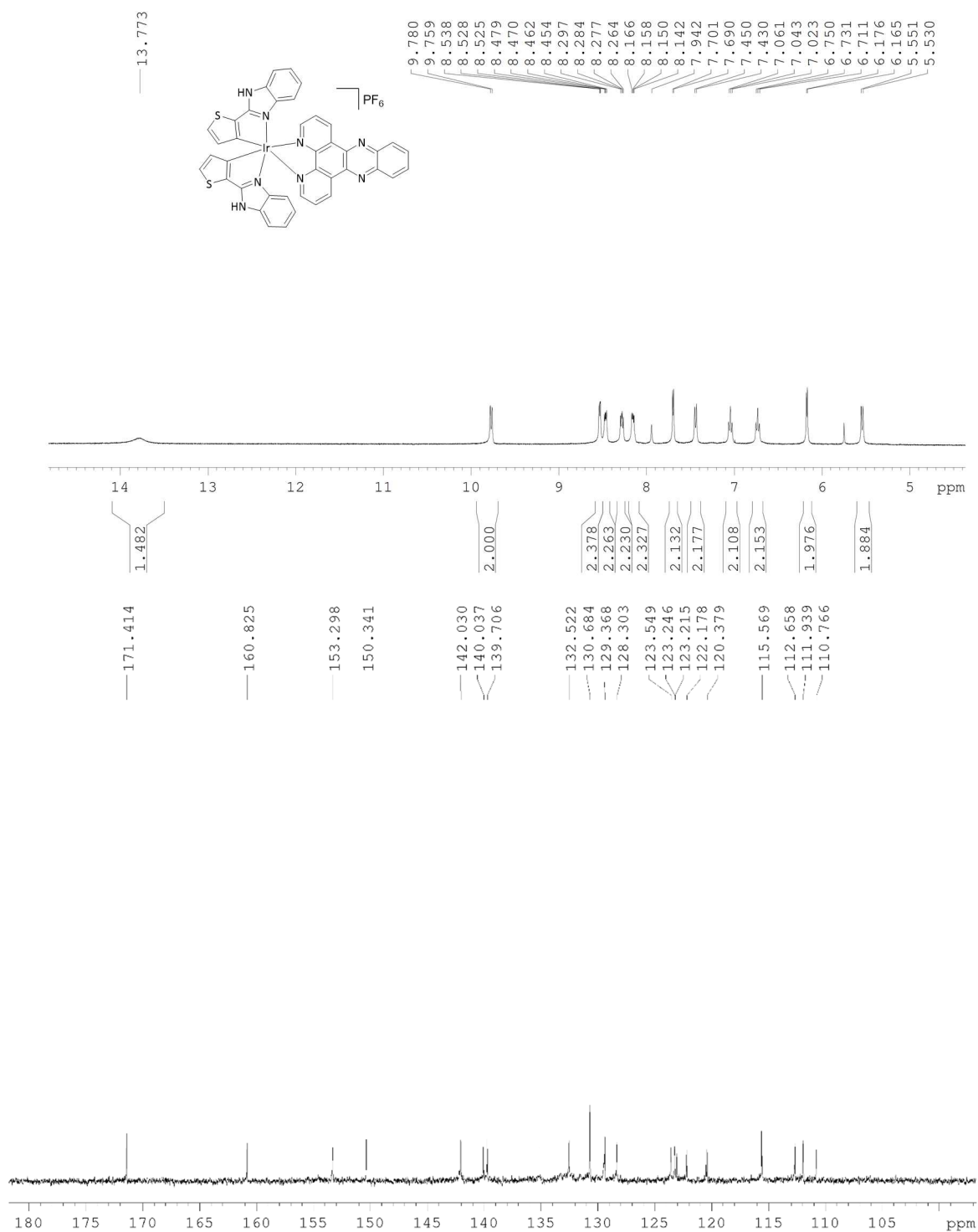


Figure S3.8. ^1H and ^{13}C NMR in $[\text{D}_6]\text{DMSO}$ for **21a**.

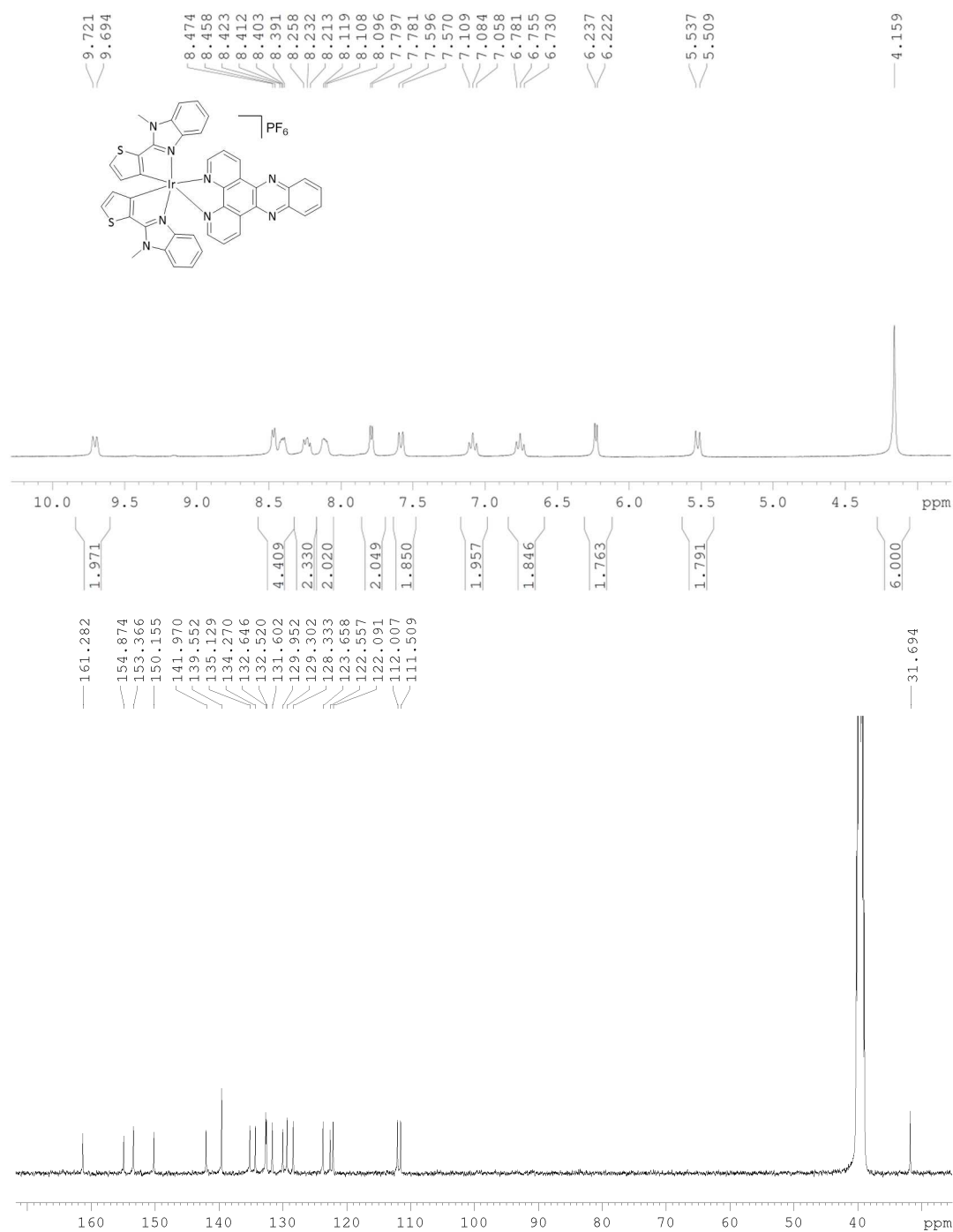


Figure S3.9. ^1H and ^{13}C NMR in $[\text{D}_6]\text{DMSO}$ for **21b**.

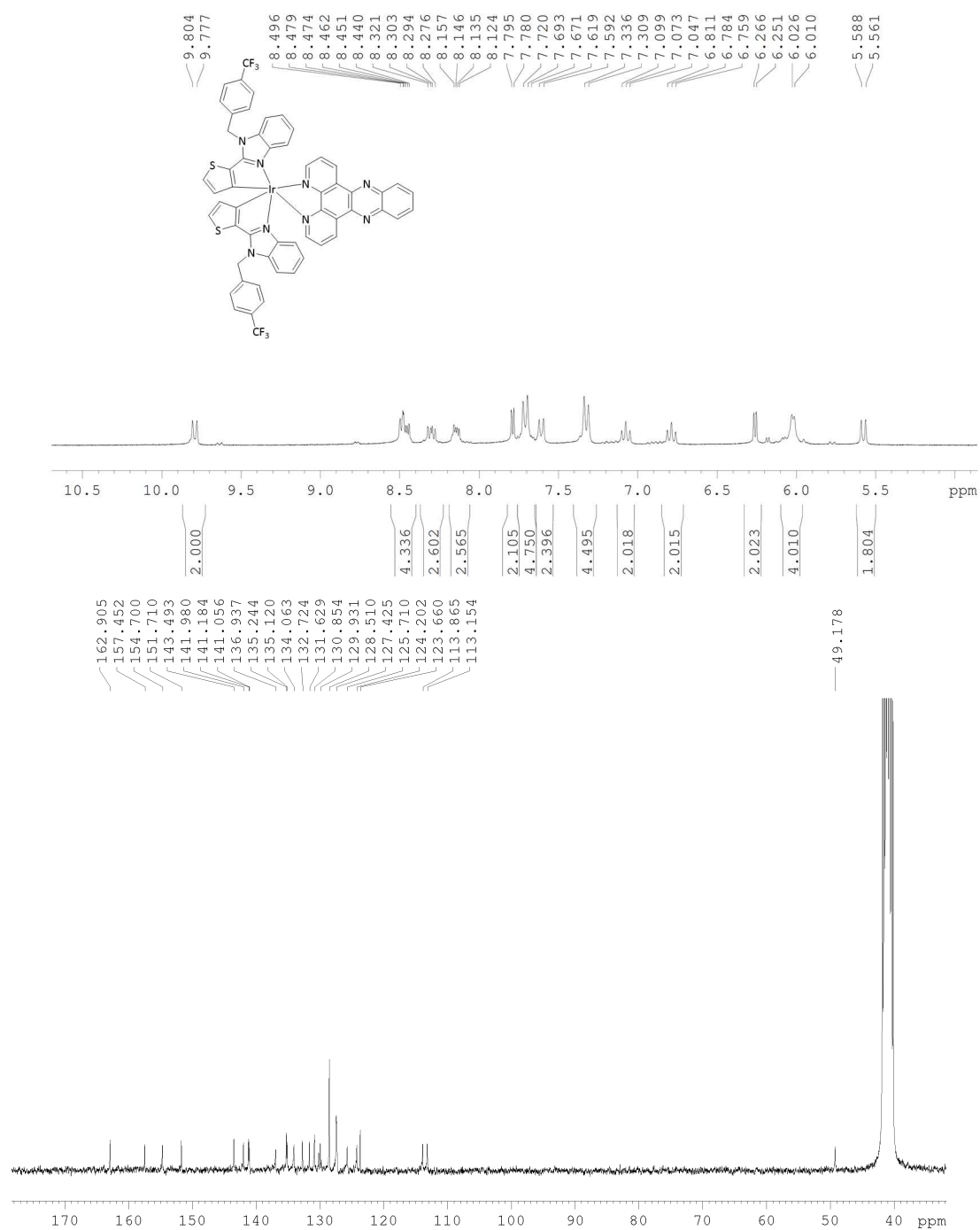


Figure S3.10. ^1H and ^{13}C NMR in $[\text{D}_6]\text{DMSO}$ for **21c**.

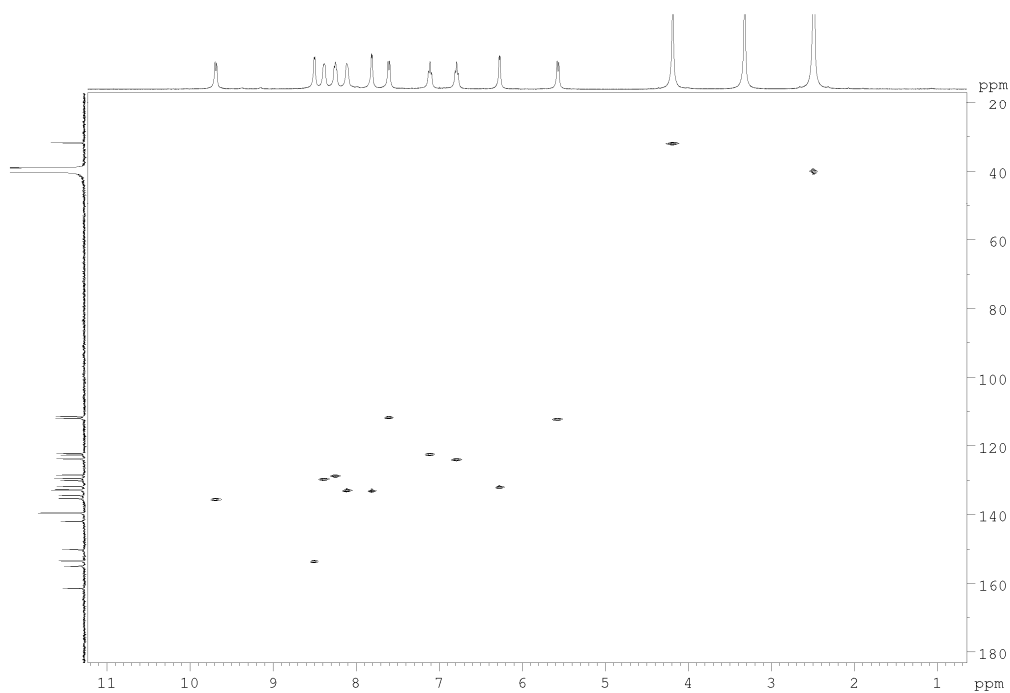


Figure S3.11. HSQC 2D ^1H - ^{13}C NMR of complex **21b**.

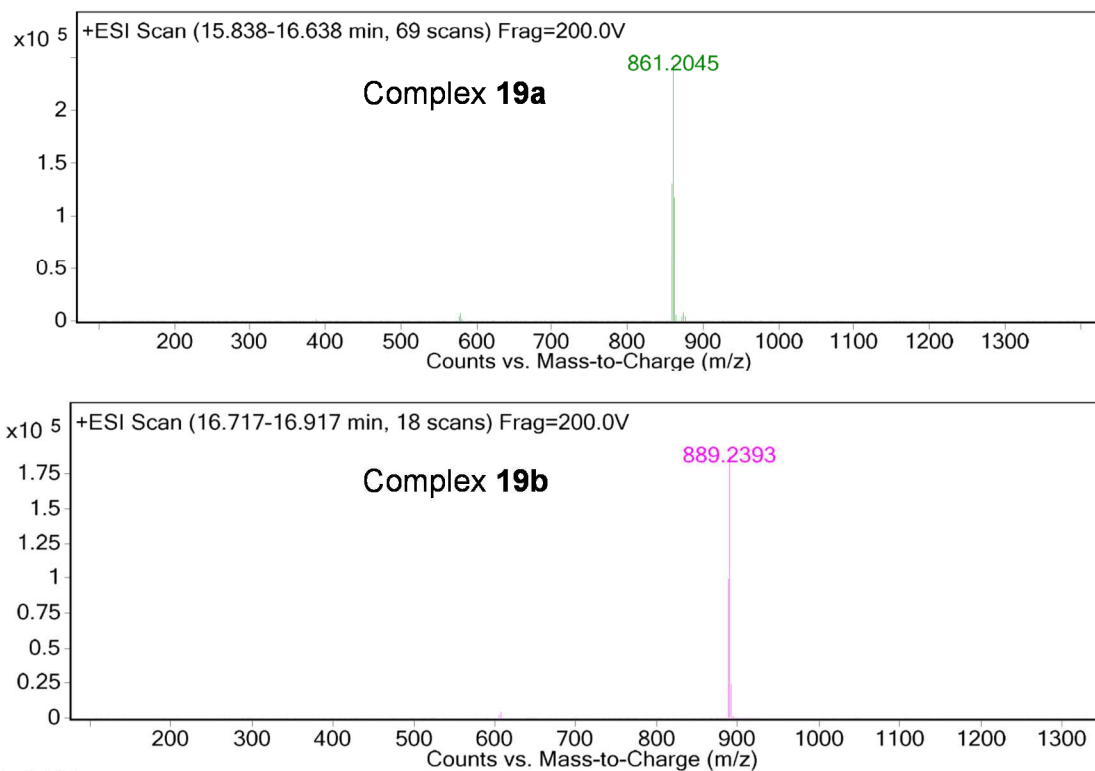


Figure S3.12. Positive-ion ESI-MS of complexes **19a** and **19b**.

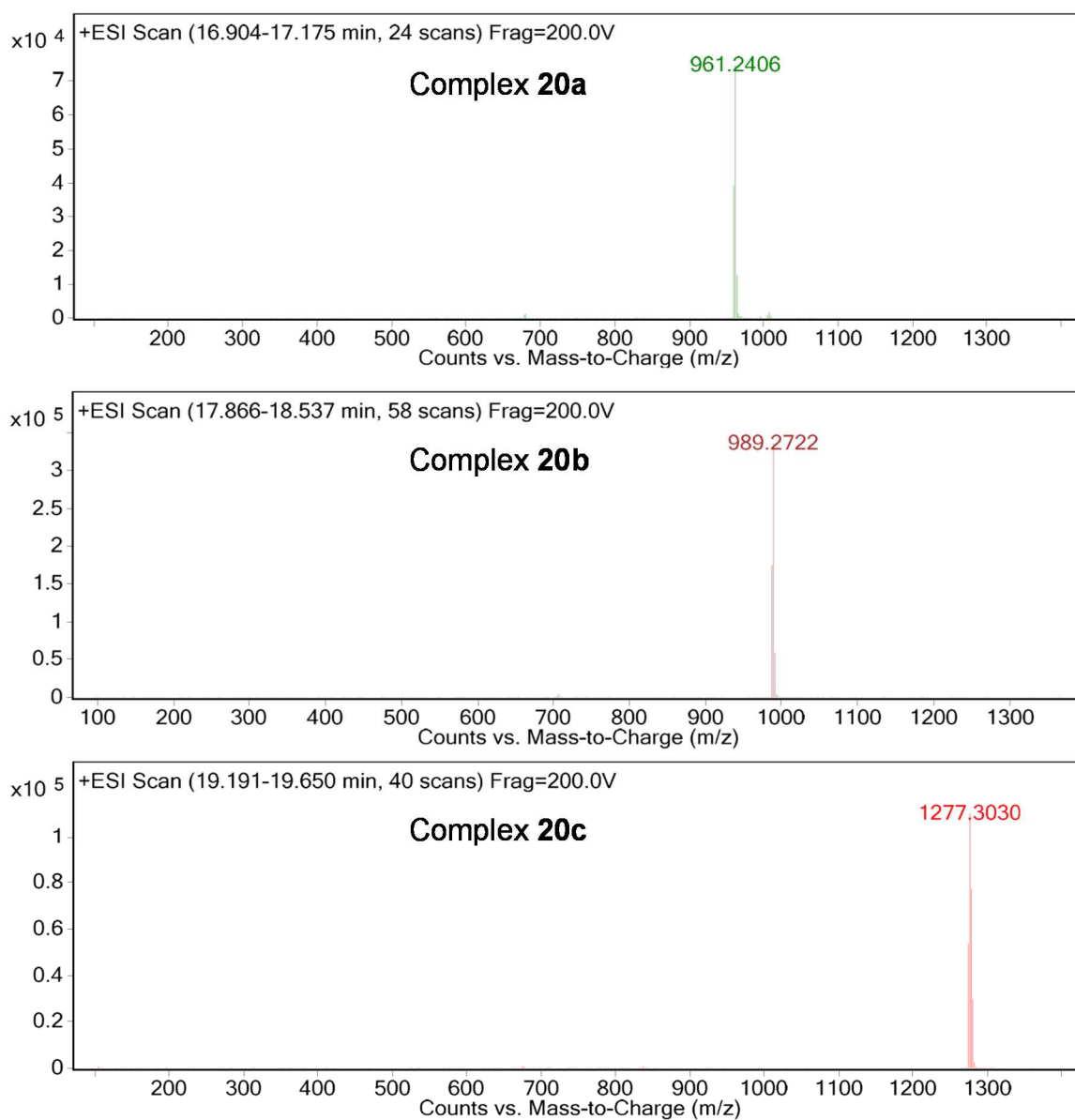


Figure S3.13. Positive-ion ESI-MS of series 2.

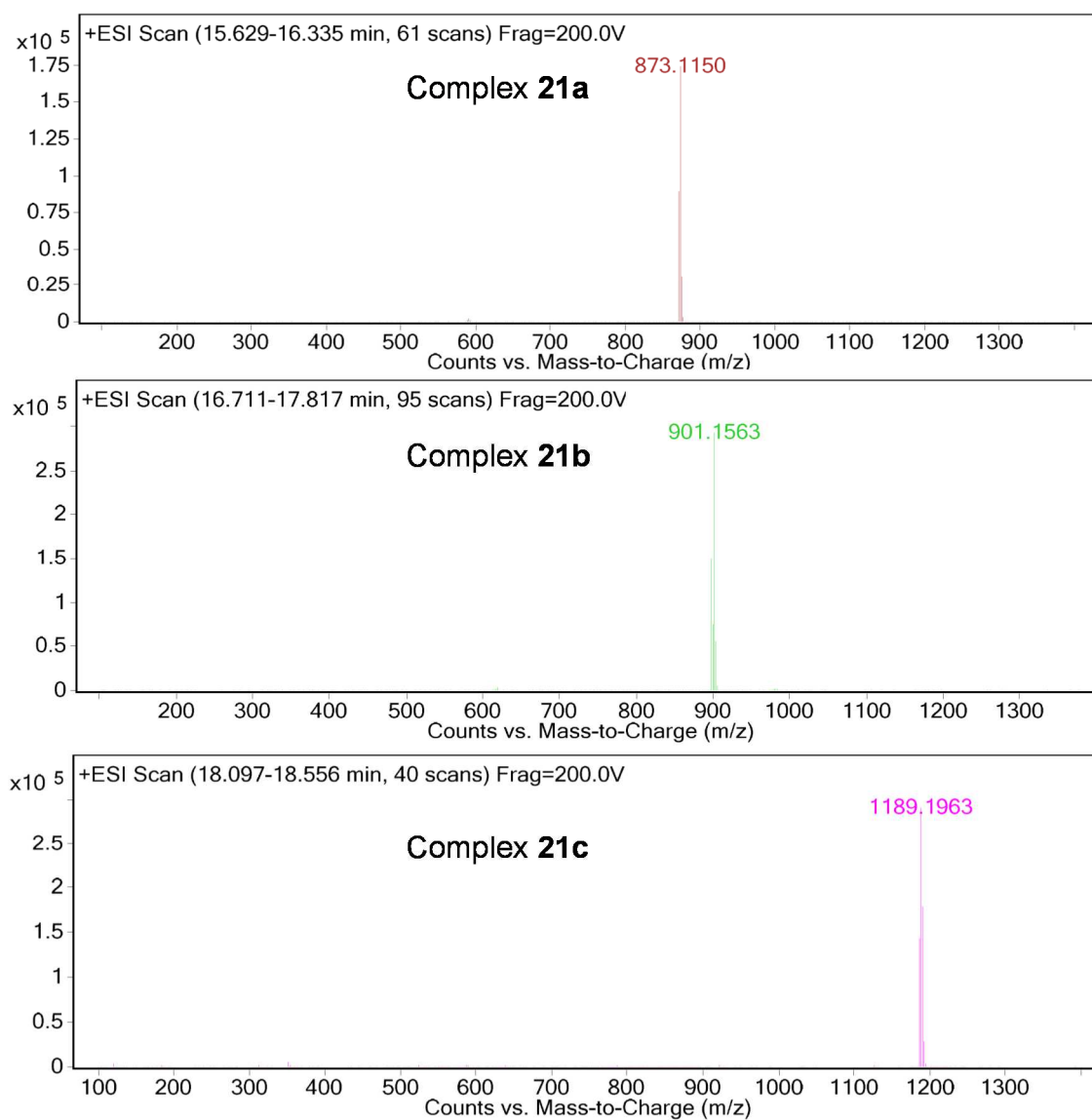


Figure S3.14. Positive-ion ESI-MS of series 3.

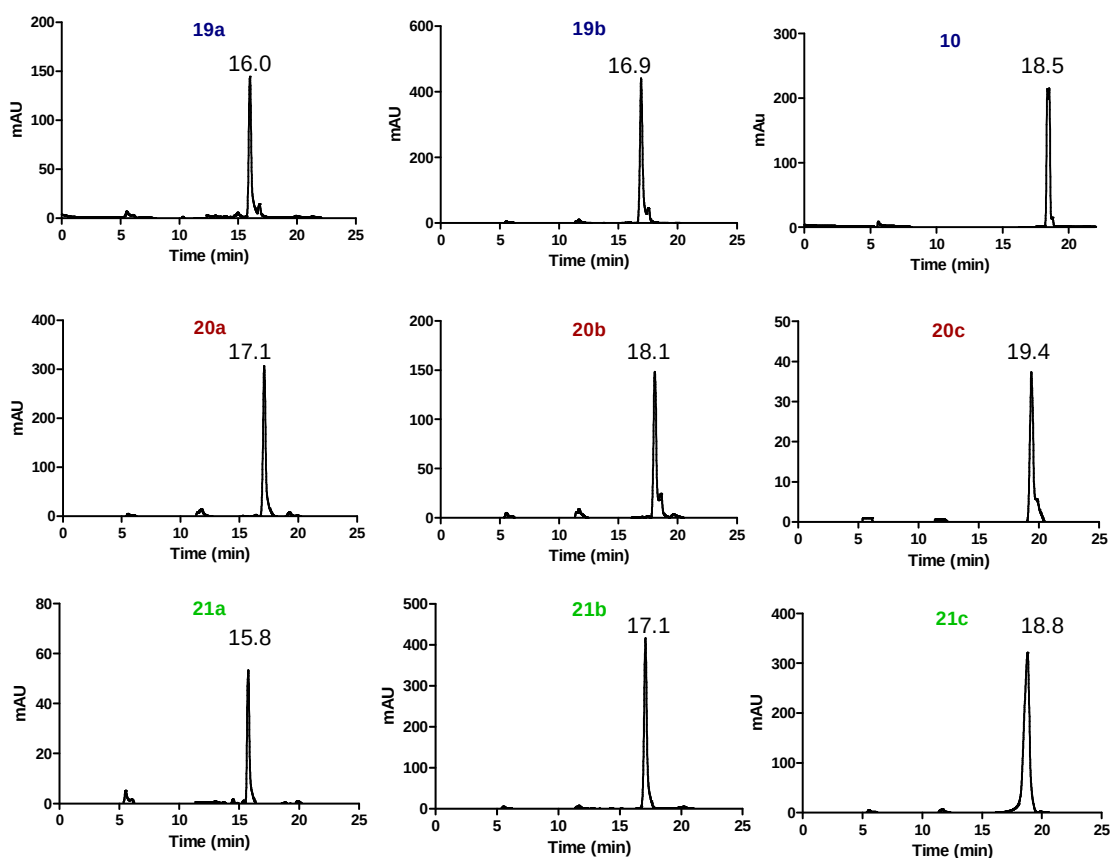


Figure S3.15. HPLC chromatograms of series **1**, **2** and **3** in DMSO.

Table S3.1. Crystal data for **20b**.

$\text{C}_{54}\text{H}_{36}\text{IrN}_8 \cdot 0.5(\text{C}_{7.28}\text{H}_{6.56}\text{Cl}_{3.28}) \cdot \text{F}_6\text{P} \cdot 2.18(\text{C}_2\text{H}_4\text{Cl}_2)$	$Z = 2$
$M_r = 1454.95$	$F(000) = 1448$
Triclinic, $P\bar{1}$	$D_x = 1.595 \text{ Mg m}^{-3}$
$a = 14.2342 (14) \text{ \AA}$	Mo $K\alpha$ radiation, $\lambda = 0.71073 \text{ \AA}$
$b = 15.3701 (15) \text{ \AA}$	Cell parameters from 9790 reflections
$c = 15.5819 (15) \text{ \AA}$	$\theta = 2.3\text{--}29.6^\circ$
$\alpha = 65.102 (3)^\circ$	$\mu = 2.56 \text{ mm}^{-1}$
$\beta = 83.431 (3)^\circ$	$T = 100 \text{ K}$
$\gamma = 78.541 (3)^\circ$	Block, orange
$V = 3028.7 (5) \text{ \AA}^3$	$0.29 \times 0.20 \times 0.13 \text{ mm}$

Table S3.2. Data collection for **20b**.

Bruker D8 Quest CCD diffractometer	11041 reflections with $I > 2\sigma(I)$
Radiation source: fine-focus sealed tube	$R_{\text{int}} = 0.032$
ω and ϕ scans	$\theta_{\text{max}} = 25.6^\circ$, $\theta_{\text{min}} = 1.9^\circ$
Absorption correction: multi-scan	$h = -17 \rightarrow 17$

(SADABS; Sheldrick, 1996)	
$T_{\min} = 0.610$, $T_{\max} = 0.746$	$k = -18 \rightarrow 18$
330048 measured reflections	$l = -18 \rightarrow 18$
11401 independent reflections	

Table S3.3. Refinement data for **20b**.

Refinement on F^2	0 restraints
Least-squares matrix: full	Hydrogen site location: inferred from neighbouring sites
$R[F^2 > 2\sigma(F^2)] = 0.0412$ $wR[F^2 > 2\sigma(F^2)] = 0.1123$	H-atom parameters constrained
$R(F^2) = 0.0424$ $wR(F^2) = 0.1133$	$w = 1/[\sigma^2(F_o^2) + (0.0609P)^2 + 17.7437P]$ where $P = (F_o^2 + 2F_c^2)/3$
$S = 1.08$	$(\Delta/\sigma)_{\max} = 0.001$
11401 reflections	$\Delta_{\max} = 3.24 \text{ e } \text{\AA}^{-3}$
774 parameters	$\Delta_{\min} = -1.27 \text{ e } \text{\AA}^{-3}$

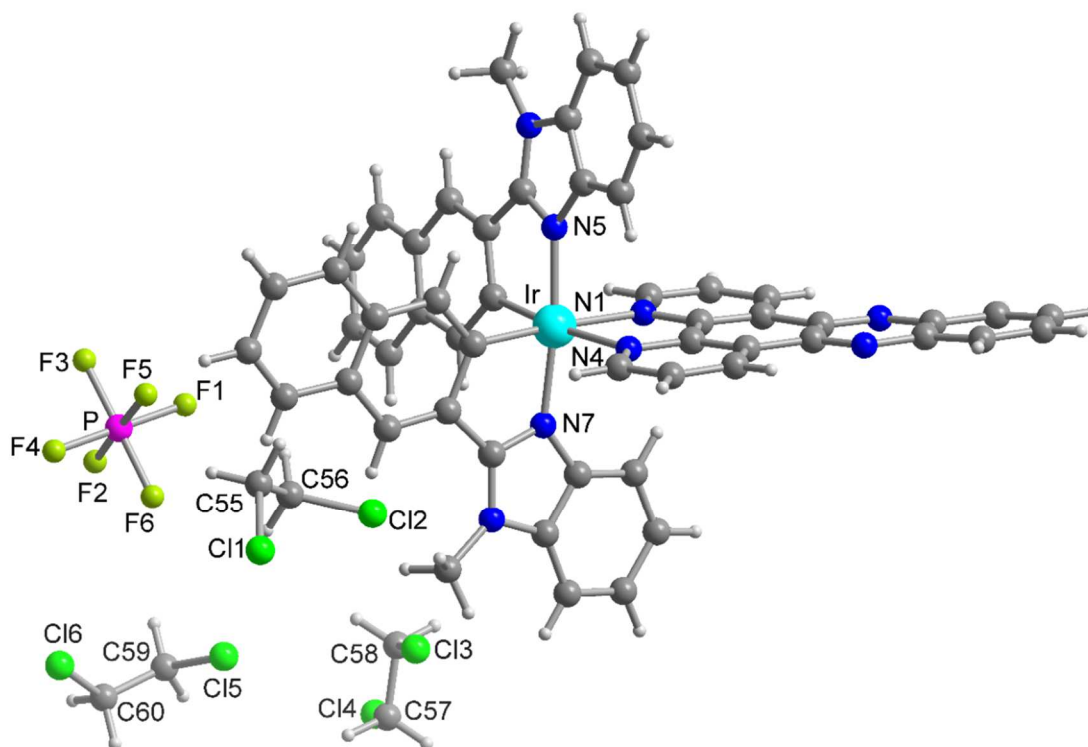
**Figure S3.16.** Molecular structure within the crystal of **20b**, (showing the iridium cation, the PF_6^- anion and three 1,2-dichloroethane solvent molecules of crystallization. The disordered hexane and site-sharing $\text{C}_2\text{H}_4\text{Cl}_4$ molecule were not shown for clarity.

Table S3.4 C-H...F Hydrogen-bond geometry (Å, °) for **20b**.^a

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=====

Analysis of Potential Hydrogen Bonds and Schemes with $d(D...A) < R(D)+R(A)+0.50$, $d(H...A) < R(H)+R(A)-0.12$ Ang., $D-H...A > 100.0$ Deg

=====

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Note: - ARU codes in [] are with reference to the Coordinates printed above (Possibly transformed, when MOVE .NE. 1.555)

=====

=====

D --- H....A	[ARU]	D - H	H...A	D...A	D - H...A

C(15) --H(15) ..F(3)	[1556.03]	0.95	2.50	3.437(7)	167
C(22) --H(22) ..F(1)	[2566.03]	0.95	2.50	3.280(8)	139
C(22) --H(22) ..F(3)	[2566.03]	0.95	2.53	3.251(8)	133
C(41) --H(41) ..F(4)	[2656.03]	0.95	2.38	3.228(8)	148
C(49) --H(49) ..F(4)	[2556.03]	0.95	2.44	3.141(8)	130
C(54) --H(54C) ..F(2)	[2656.03]	0.98	2.48	3.418(8)	161
C(55) --H(55A) ..F(5)	[1555.03]	0.99	2.41	3.247(12)	141
C(55) --H(55A) ..F(6)	[1555.03]	0.99	2.55	3.203(14)	124
C(56) --H(56B) ..F(6)	[1555.03]	0.99	2.50	2.990(13)	110
C(57) --H(57B) ..F(3)	[1655.03]	0.99	2.50	3.355(13)	145
[2566.] = [2_566]	= -x, 1-y, 1-z				
[2656.] = [2_656]	= 1-x, -y, 1-z				
[2556.] = [2_556]	= -x, -y, 1-z				
[1556.] = [1_556]	= x, y, 1+z				
[1655.] = [1_655]	= 1+x, y, z				

^a D = Donor, A = Acceptor. For found and refined H atoms the standard deviations are given for their hydrogen bond distances and angles.

Table S3.5. Analysis of *intermolecular* C-H...Cg(Pi-Ring) Interactions (H..Cg < 3.0 Ang.

- Gamma < 30.0 Deg) in **20b** (see Scheme S6 for explanation).

- Cg(J) = Center of gravity of ring J (Plane number above)
- H-Perp = Perpendicular distance of H to ring plane J
- Gamma = Angle between Cg-H vector and ring J normal
- C-H..Cg = C-H-Cg angle (degrees)
- C..Cg = Distance of X to Cg (Angstrom)
- C-H, Pi = Angle of the X-H bond with the Pi-plane (i.e.' Perpendicular = 90 degrees, Parallel = 0 degrees)

C--H(I)	Cg(J) [ARU(J)]	H..Cg	H-Perp	Gamma	C-H..Cg	C..Cg	C-H,Pi
C(9)-H(9) -> Cg(14)	[2667.01]	2.83	2.75	13.39	155	3.710(7)	69
C(36)-H(36C) -> Cg(12)	[2566.01]		2.77	2.65	16.91	127	3.448(6) 52
C(39)-H(39) -> Cg(12)	[2666.01]		2.85	2.57	25.83	152	3.718(6)88
C(39)-H(39) -> Cg(13)	[2666.01]		2.78	2.57	22.61	156	3.670(7)89

[2667] = 1-X,1-Y,2-Z

[2566] = -X,1-Y,1-Z

[2666] = 1-X,1-Y,1-Z

Cg(14) = Ring C37-C38-C39-C40-C41-C42

Table S3.6. Packing Analysis for **20b** for possible π - π interactions (see Scheme S7 for explanation).

```

=====
Analysis of Short Ring-Interactions with Cg-Cg Distances < 6.0 Angstrom and Beta < 60.0Deg.
=====
=====
- Cg(I) = Plane number I (= ring number in () above)
- Alpha = Dihedral Angle between Planes I and J (Deg)
- Beta = Angle Cg(I)-->Cg(J) or Cg(I)-->Me vector and normal to plane I (Deg)
- Gamma = Angle Cg(I)-->Cg(J) vector and normal to plane J (Deg)
- Cg-Cg = Distance between ring Centroids (Ang.)
- CgI_Perp = Perpendicular distance of Cg(I) on ring J (Ang.)
- CgJ_Perp = Perpendicular distance of Cg(J) on ring I (Ang.)
- Slippage = Distance between Cg(I) and Perpendicular Projection of Cg(J) on Ring I (Ang.)

Cg(I) Res(I) Cg(J) [ ARU(J)] Cg-Cg Alpha Beta Gamma CgI_Perp CgJ_Perp
slippage
Cg(8) [ 1] -> Cg(10) [ 2667.01] 3.796(3) 5.1(3) 26.5 23.6 3.479(2) 3.398(3)
1.692
Cg(10) [ 1] -> Cg(8) [ 2667.01] 3.796(3) 5.1(3) 23.6 26.5 3.398(3) 3.479(2)
1.517
Cg(10) [ 1] -> Cg(22) [ 2667.01] 3.898(3) 4.7(2) 25.3 30.0 3.375(2)
3.5224(14) 1.668
Cg(22) [ 1] -> Cg(10) [ 2667.01] 3.897(3) 4.7(2) 30.0 25.3 3.5224(14) 3.375(2) 1.950

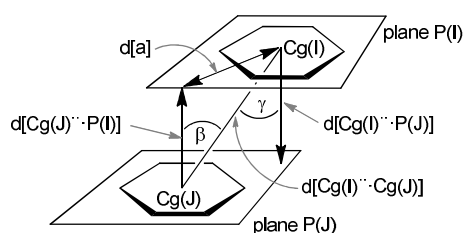
[ 2667] = 1-X,1-Y,2-Z
Cg8 = centroid of ring N4-C13-C14-C15-C16-C18
Cg10 = centroid of ring C6-C7-C8-C9-C10-C11
Cg22 = centroid of ring N4-C4-C5-C12-C13-C14-C15-C16-C17-C18

```

The PLATON-listing "**Analysis of Short Ring-Interactions**" for possible π -stacking interactions yielded mainly rather long centroid-centroid distances (>4.0 Å) together with non-parallel ring planes ($\alpha \gg 0^\circ$) and large slip angles ($\beta, \gamma > 30^\circ$).

In comparison, significant π -stacking show rather short centroid-centroid contacts (<3.8 Å), near parallel ring planes ($\alpha < 10^\circ$ to $\sim 0^\circ$ or even exactly 0° by symmetry), small slip angles ($\beta, \gamma < 25^\circ$) and vertical displacements (slippage <1.5 Å) which translate into a sizable overlap of the aryl-plane areas.²

π -Interactions between pyridyl-type ligands for comparison:^{3–6}



Scheme S3.2. Graphical presentation of the parameters used for the description of π - π stacking.²

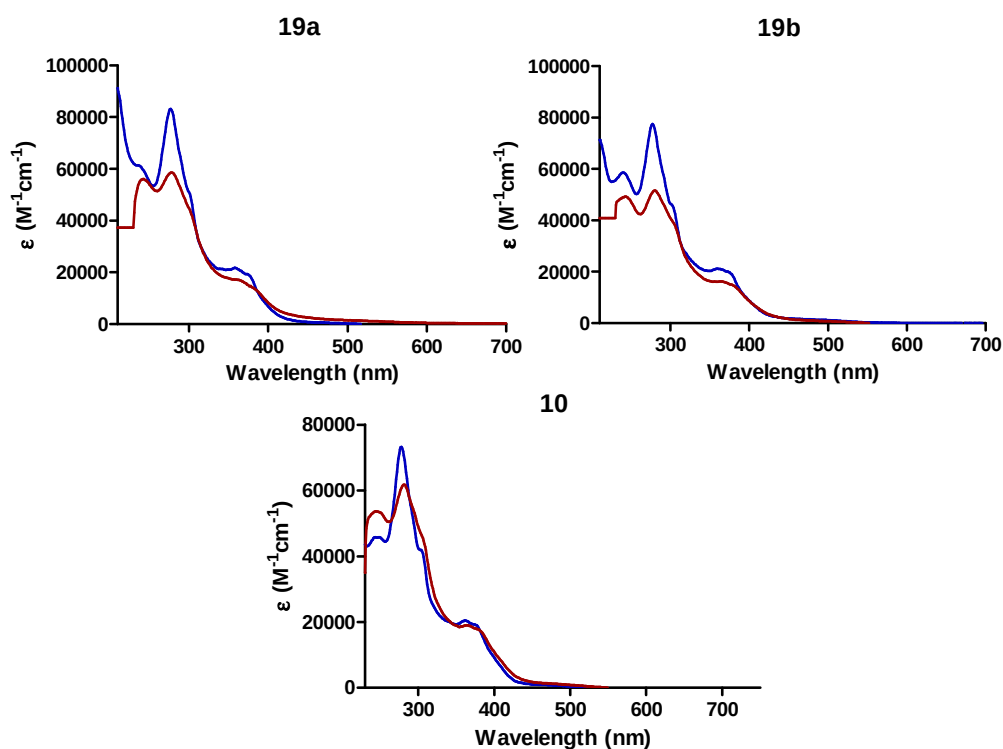


Figure S3.17. UV/Vis absorption spectra of complexes **19a**, **19b** and **10** in acetonitrile (blue) and water (1% DMSO) (red) at room temperature.

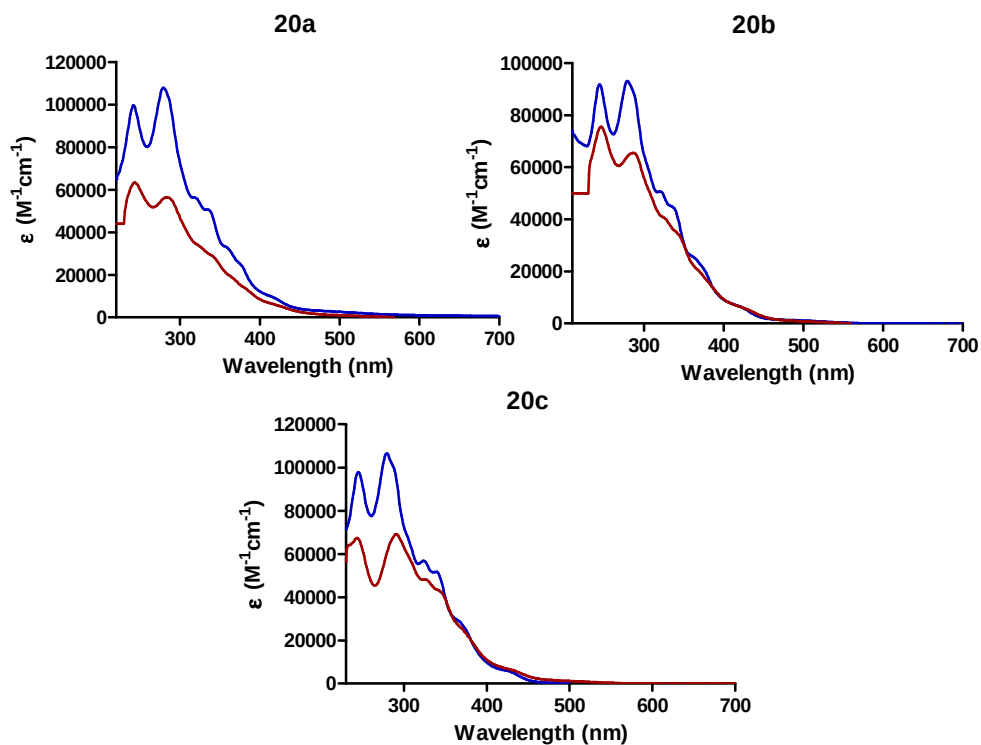


Figure S3.18. UV/Vis absorption spectra of complexes **20a–c** in acetonitrile (blue) and water (1% DMSO) (red) at room temperature.

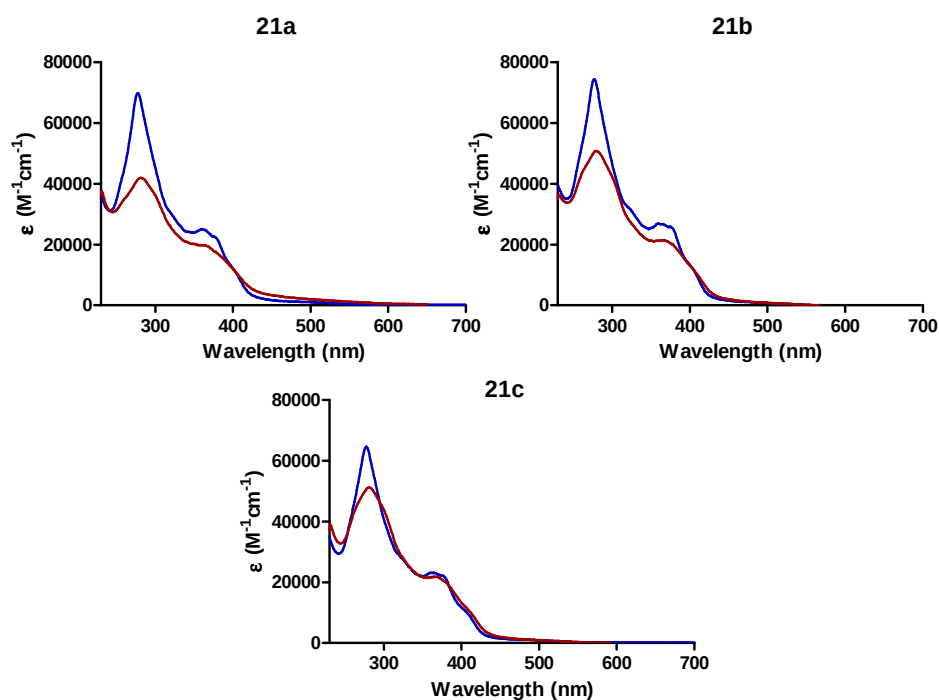
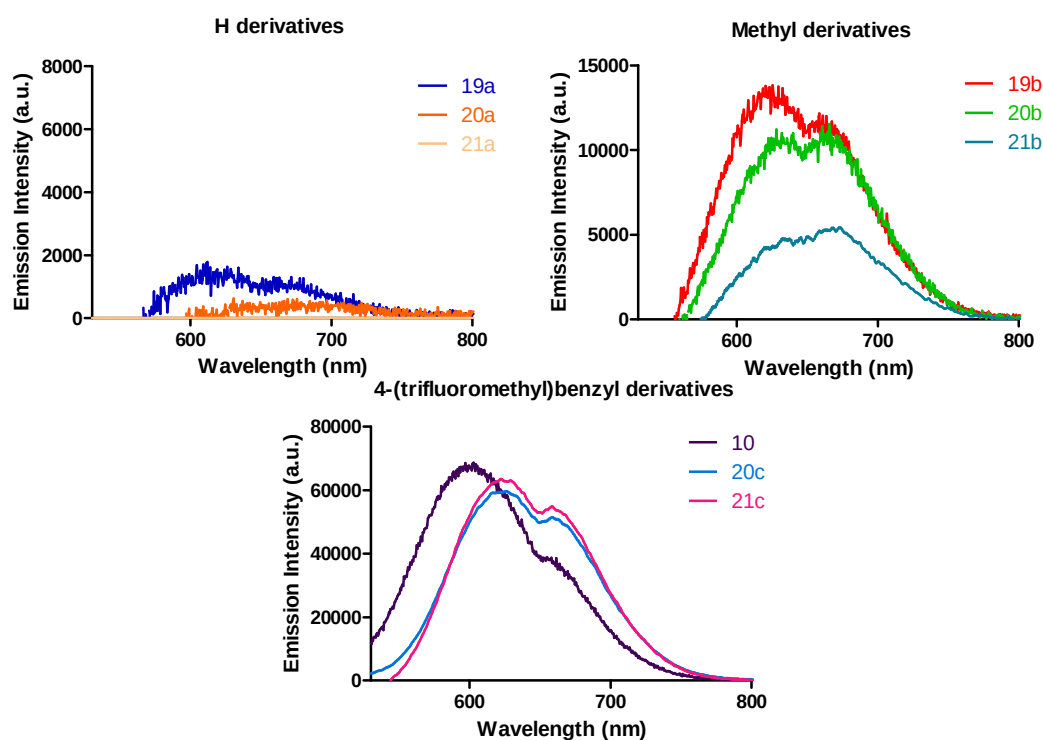


Figure S3.19. UV/Vis absorption spectra of complexes **21a–c** in acetonitrile (blue) and water (1% DMSO) (red) at room temperature.

Table S3.7. UV/Vis absorption bands and extinction coefficients for **19a–21c** in water^a.

Compound	Wavelength, nm (ϵ average, $M^{-1} cm^{-1}$)				
19a	246	282	369	385	426
	(55540)	(57550)	(16240)	(13100)	(3850)
19b	248	285	369	381	429
	(48230)	(50020)	(15970)	(14440)	(2930)
10	245	281	370	380	425
	(54130)	(61820)	(18640)	(17773)	(4220)
20a	247	288	342	385	432
	(62220)	(55779)	(28407)	(12790)	(4500)
20b	249	291	347	400	436
	(74540)	(64490)	(32340)	(9340)	(4530)
20c	247	294	345	380	436
	(65670)	(67910)	(42340)	(20690)	(5590)
21a	286	369	380	429	
	(41550)	(19020)	(16830)	(4790)	
21b	285	372	390	431	
	(49690)	(20980)	(16610)	(3520)	
21c	284	375	385	428	
	(50750)	(20690)	(18220)	(4450)	

^a 1% DMSO**Figure S3.20.** Effect of substituent in the emission of Ir complexes in water (1% DMSO), 10 μM . λ_{exc} = 420 nm.

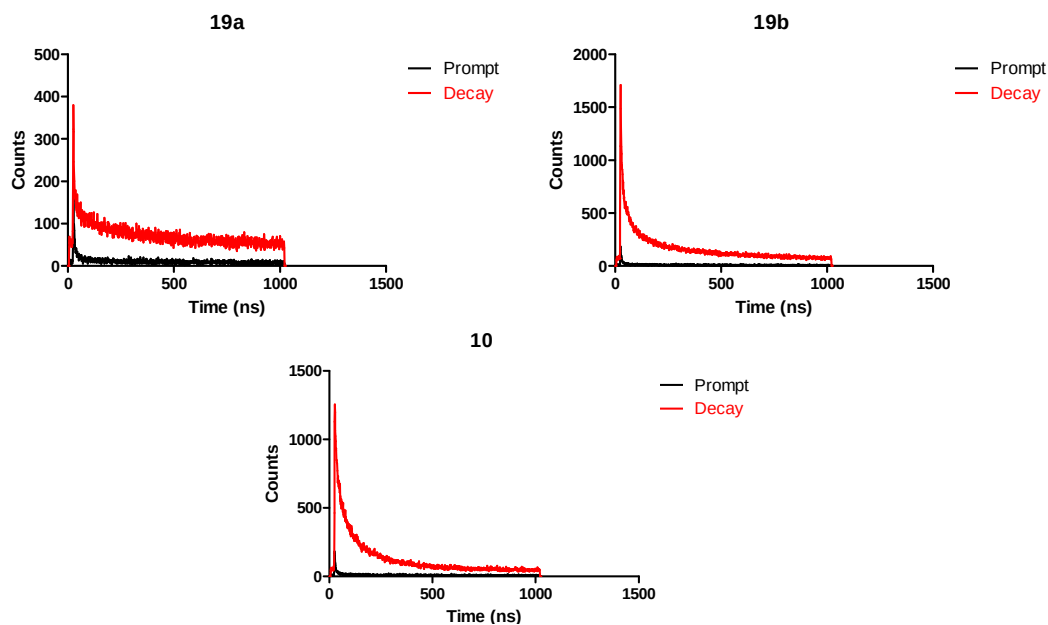


Figure S3.21. Fluorescence emission decay kinetics of series 1 in water (1% DMSO). Prompt: background.

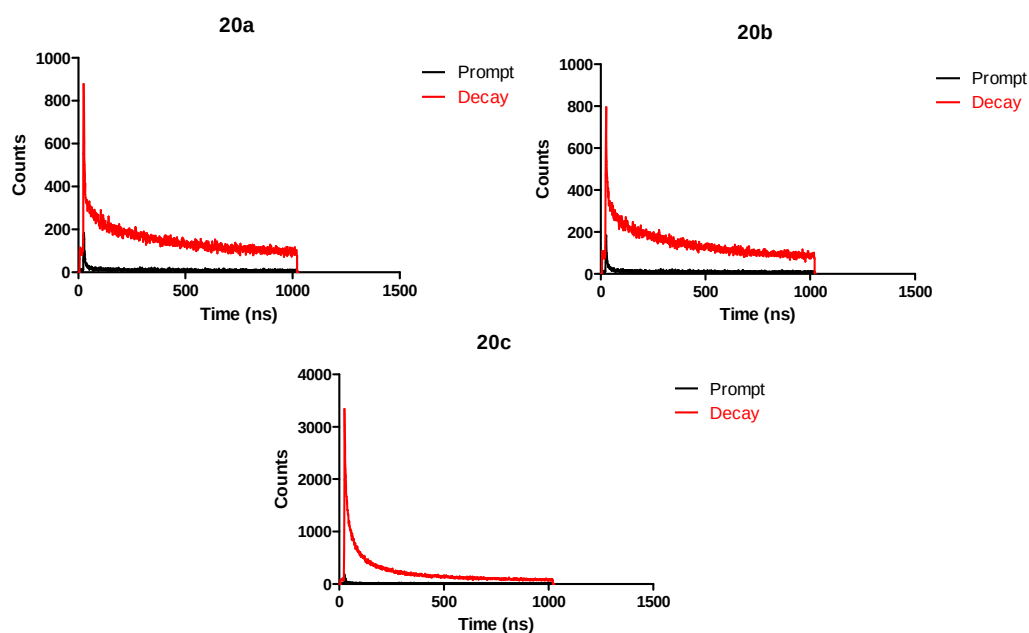


Figure S3.22. Fluorescence emission decay kinetics of series 2 in water (1% DMSO).

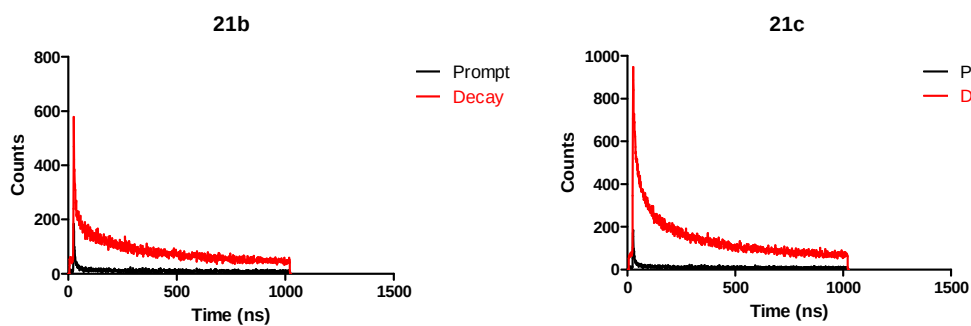


Figure S3.23. Fluorescence emission decay kinetics of series 3 in water (1% DMSO).

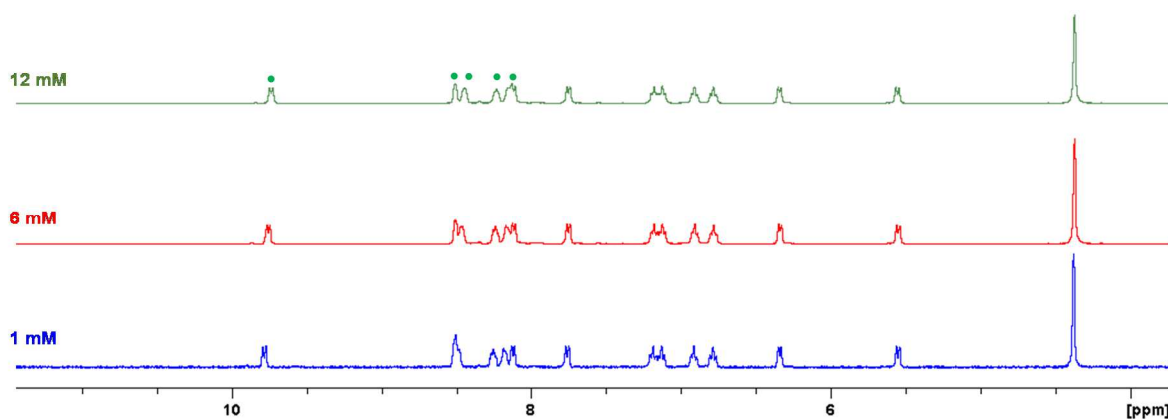


Figure S3.24. ^1H NMR spectral traces of **19b** at different concentration in $[\text{D}_6]\text{DMSO}$ (400 MHz, 298 K). Some proton signals of dppz ligand show an upfield shift when the concentration increases.

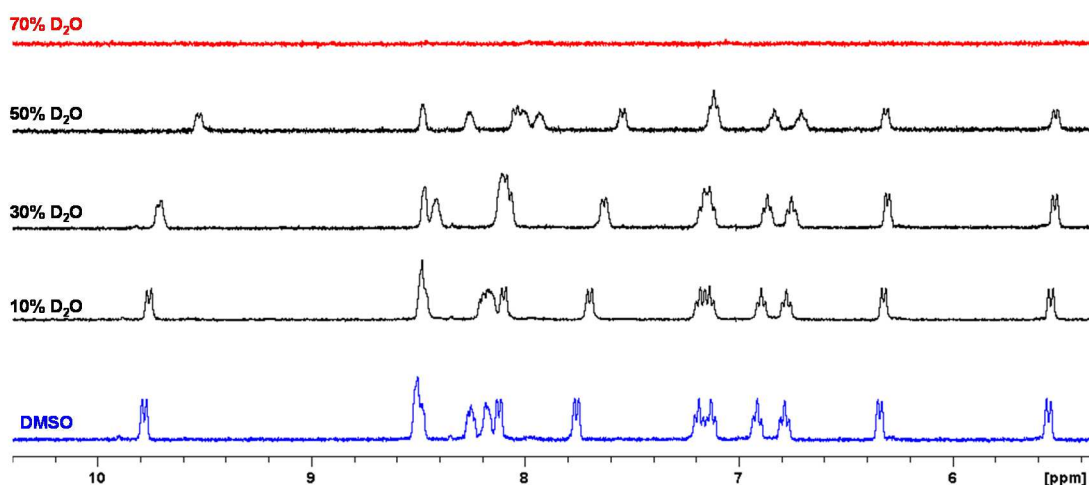


Figure S3.25. ^1H NMR spectral traces of **19b** at different composition of D_2O in $[\text{D}_6]\text{DMSO}$ (1×10^{-3} M, 400 MHz, 298 K). The proton signals show an upfield shift when the percentage of water increases from 0 to 50%, indicating the occurrence of aggregation. The increase of the water content up to 70% gives as broad signals that lead to the virtual disappearance of spectra.

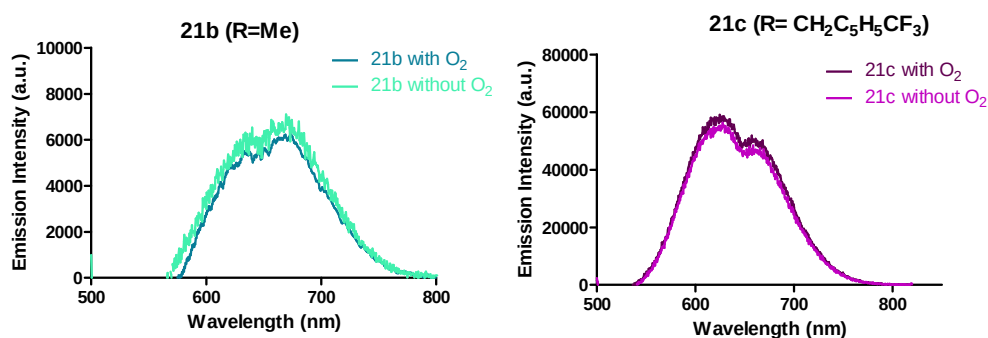


Figure S3.26. Emission spectra of **21b** and **21c** in aerated and deaerated water (1% DMSO), 10 μM . $\lambda_{\text{exc}} = 420$ nm.

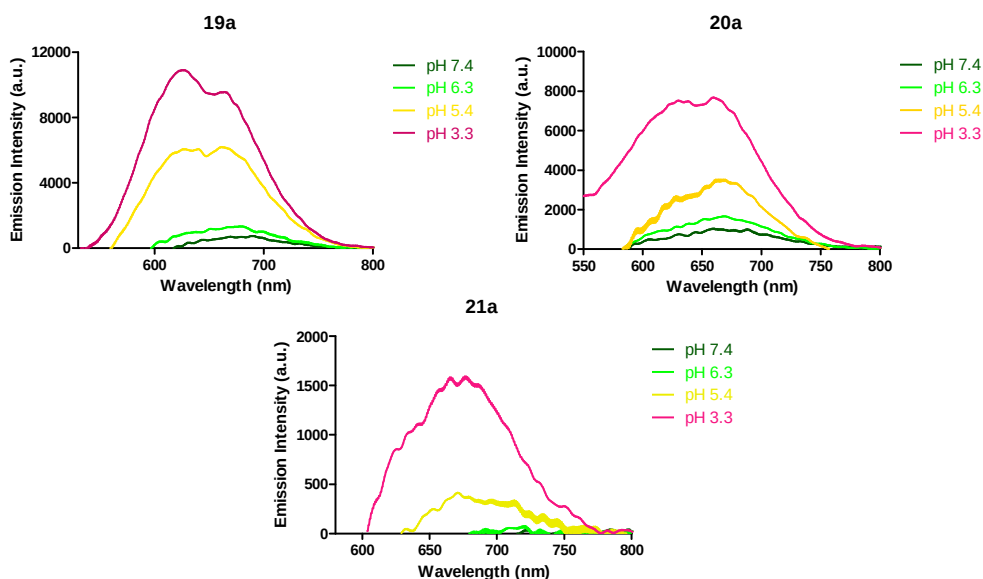


Figure S3.27. pH-sensitive emission of complexes **19a**, **20a** and **21a** in buffer/DMSO (99:1), 10 μ M. λ_{exc} = 420 nm.

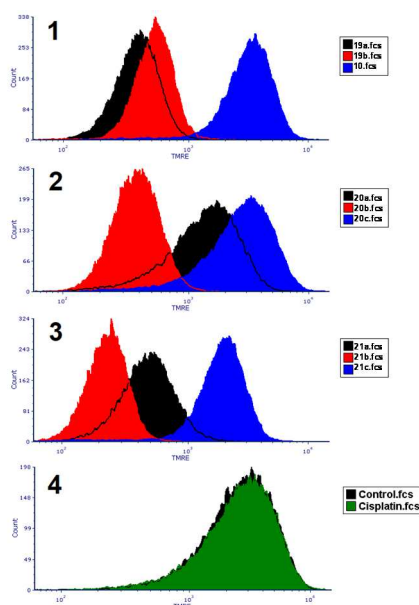


Figure S3.28. Representative overlaid histograms displaying the measurement of mitochondrial membrane potential. HeLa cells were treated with the investigated compounds (10 μ M) and incubated for 5 h in the dark. Panels: 1 - Series **1**; 2 – Series **2**; 3 – Series **3**; 4 – untreated control (black), cisplatin (green). Colors in panels 1-3: “a” compounds – black color, “b” – red color, “c” – blue color. Mitochondrial membrane potential was determined by TMRE staining with subsequent analysis by flow cytometry. Histograms are the representatives of two independent experiments, expressed at the bi-exponential scale.

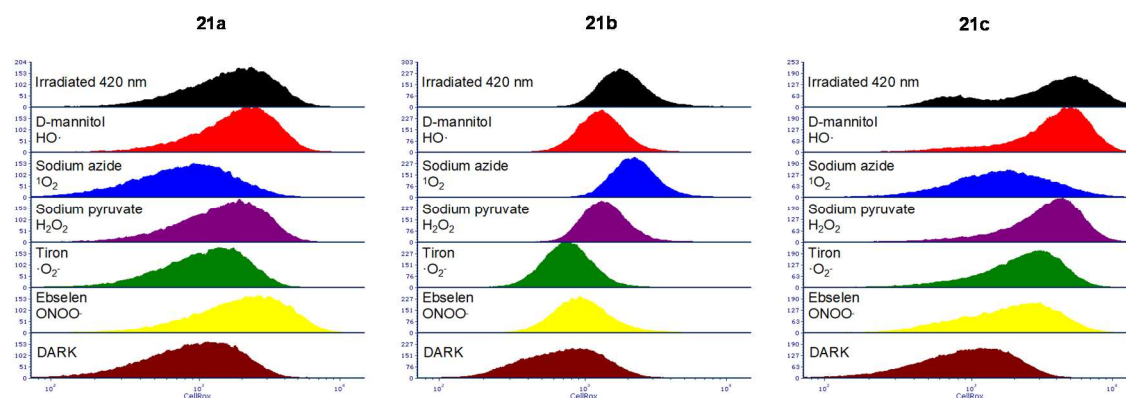


Figure S3.29. Representative histograms displaying determination of ROS in HeLa cells treated with **21a-c**, as determined by the flow cytometry. Cells were pre-incubated for 1 h with specific ROS scavengers: D-mannitol (50 mM), sodium azide (5 mM), sodium pyruvate (10 mM), tiron (5 mM), and ebselen (50 μ M). Cells were treated with the investigated compounds (10 μ M), incubated for 1 h in the dark followed by 1 h incubation under the irradiation with 420 nm or 2 h incubation in the dark (samples marked as „dark“). The controls were treated with an appropriate amount of DMSO. Scavengers remained throughout the duration of the whole experiment. Data are the representatives of three independent experiments expressed on the histograms with bi-exponential scale.

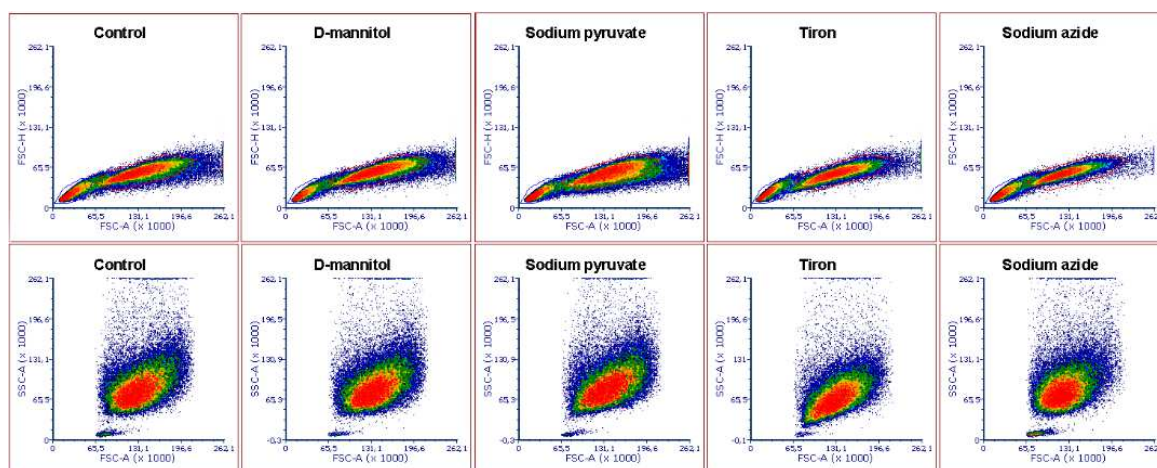


Figure S3.30. Flow cytometric analysis of HeLa cells treated with ROS scavengers. Cells were treated with D-mannitol (50 mM), sodium azide (5 mM), sodium pyruvate (10 mM), tiron (5mM) for 3h to check possible toxic effects of individual scavengers on the cells demonstrated by the number of debris/particles in the sample (top panels) or by cellular granularity (bottom panels). Top panels: forward scatter area vs. forward scatter high; bottom panels: forward scatter area vs. side scatter area.

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Chapter 4

Photoactivated Ir(III) complexes that target cancer stem cells and induce immunogenic cell death in melanoma cells

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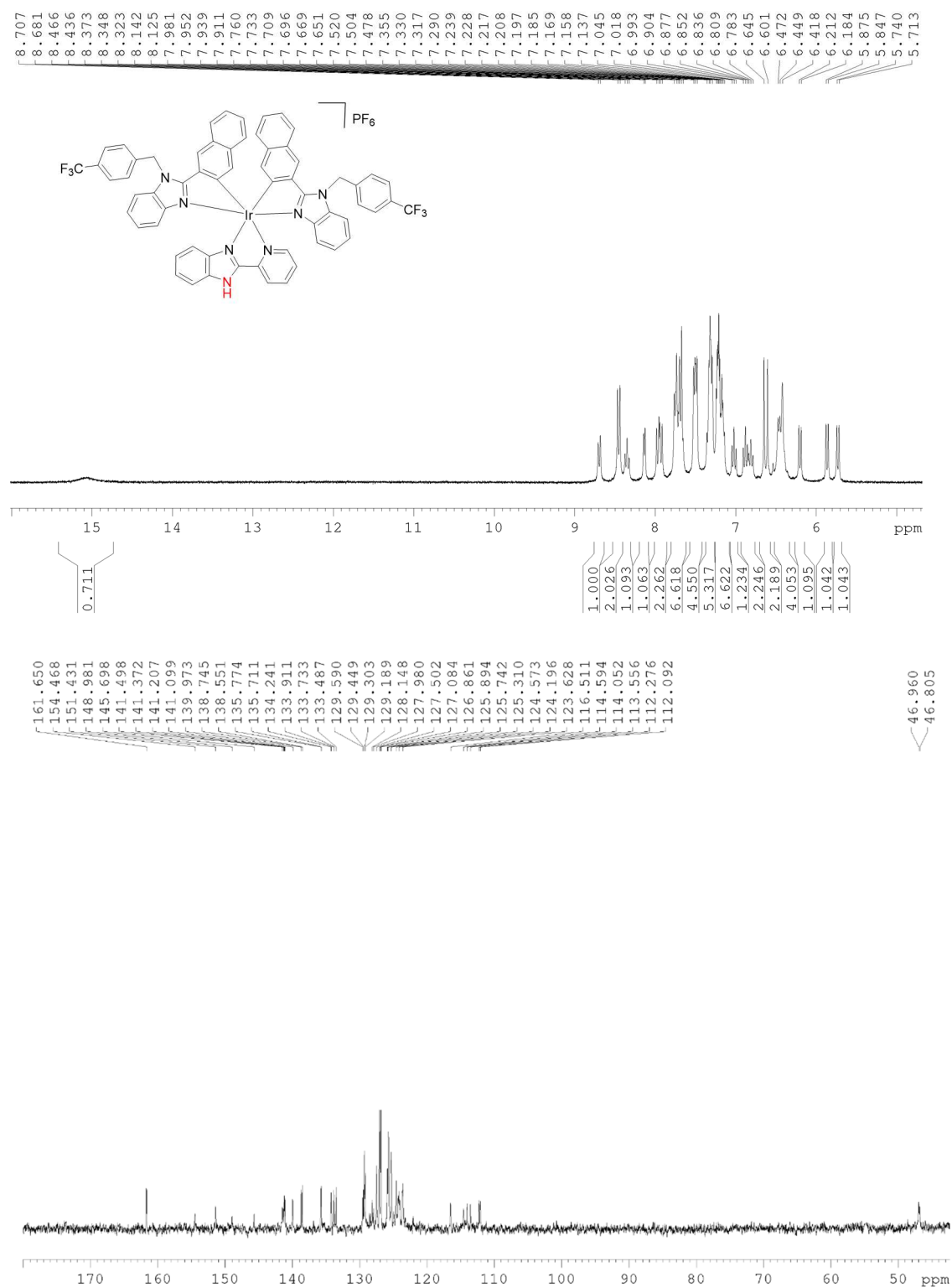


Figure S4.1. ¹H and ¹³C NMR in [D₆]DMSO for 26.

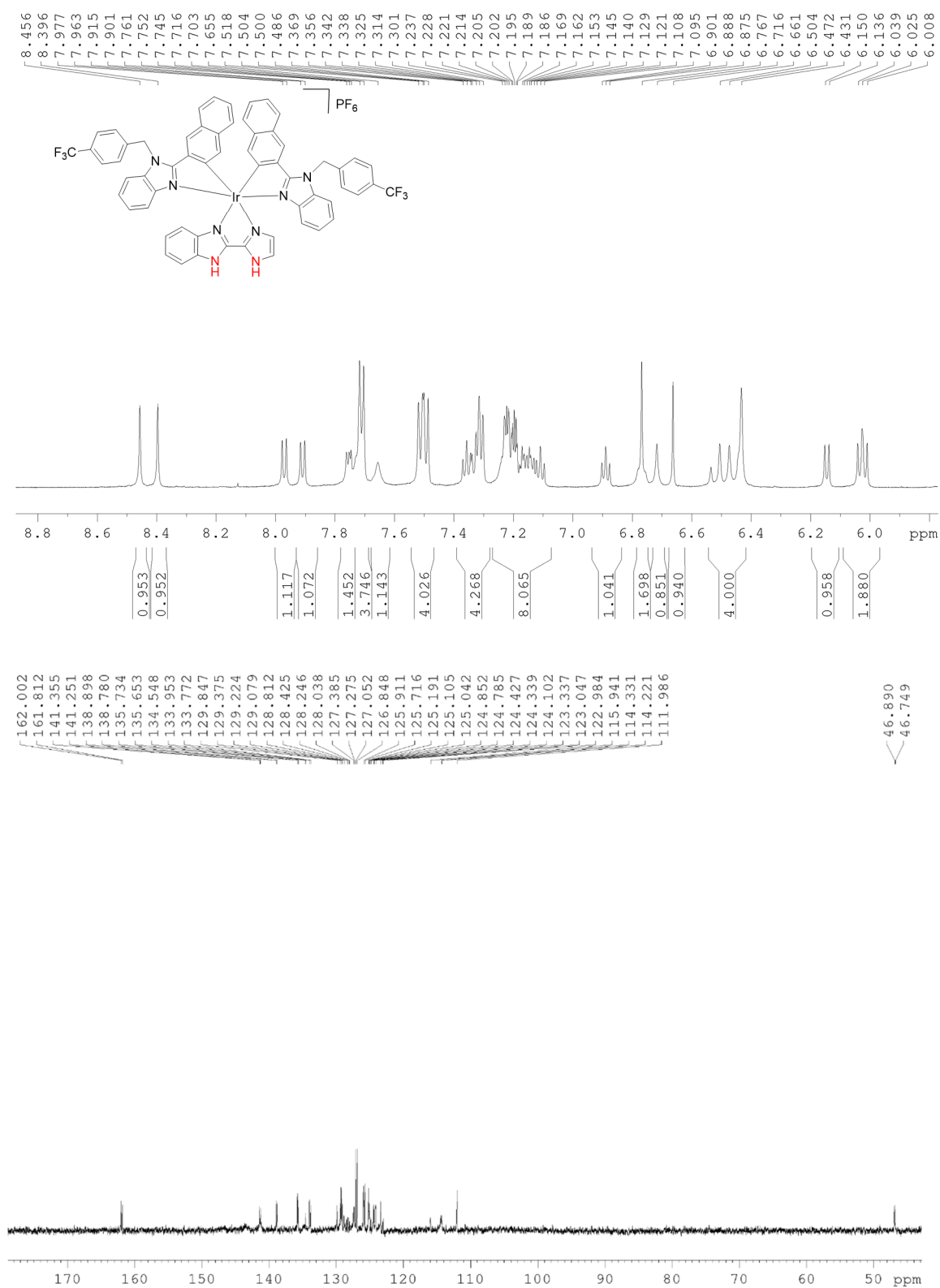


Figure S4.2. ¹H and ¹³C NMR in [D₆]DMSO for 27.

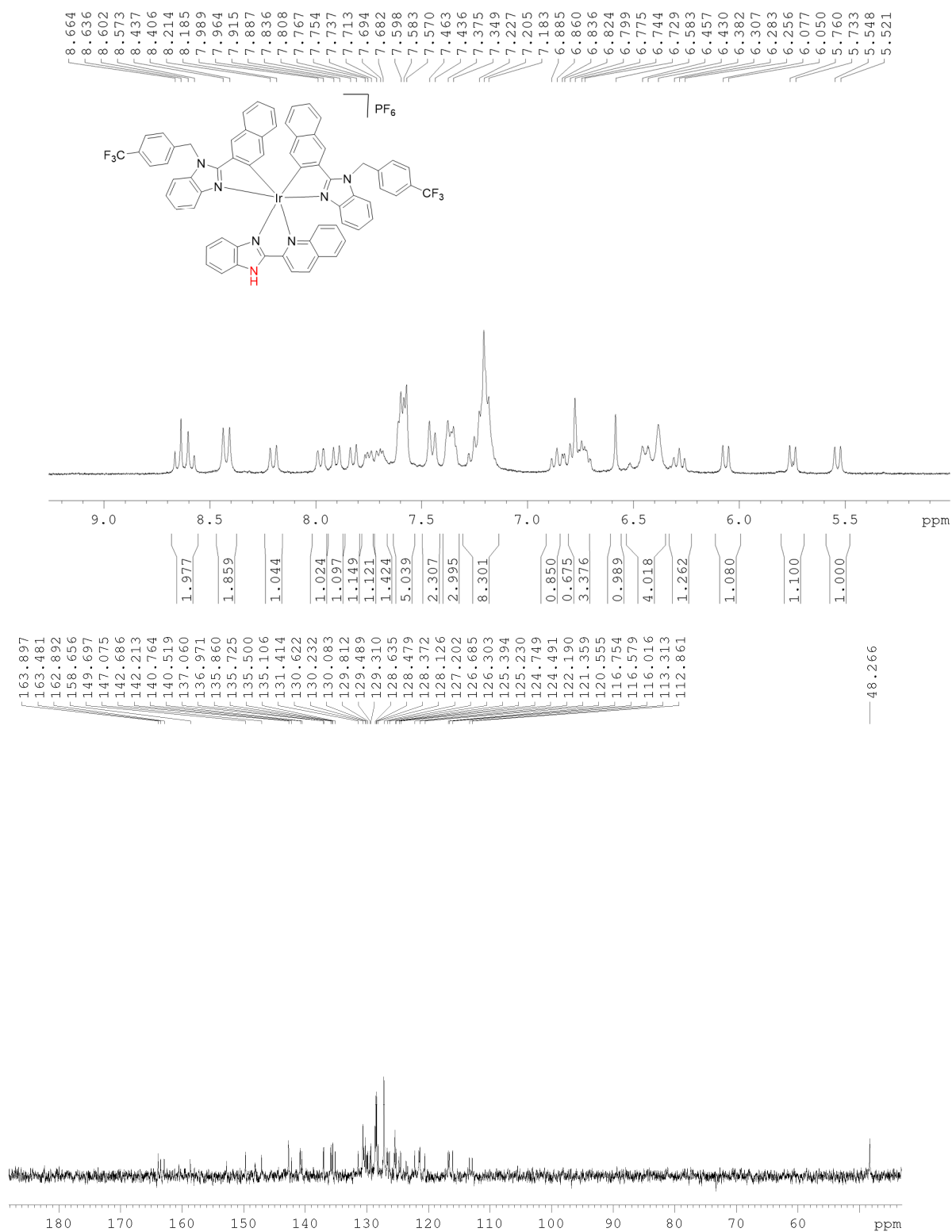


Figure S4.3. ¹H and ¹³C NMR in [D₆]DMSO for **28**.

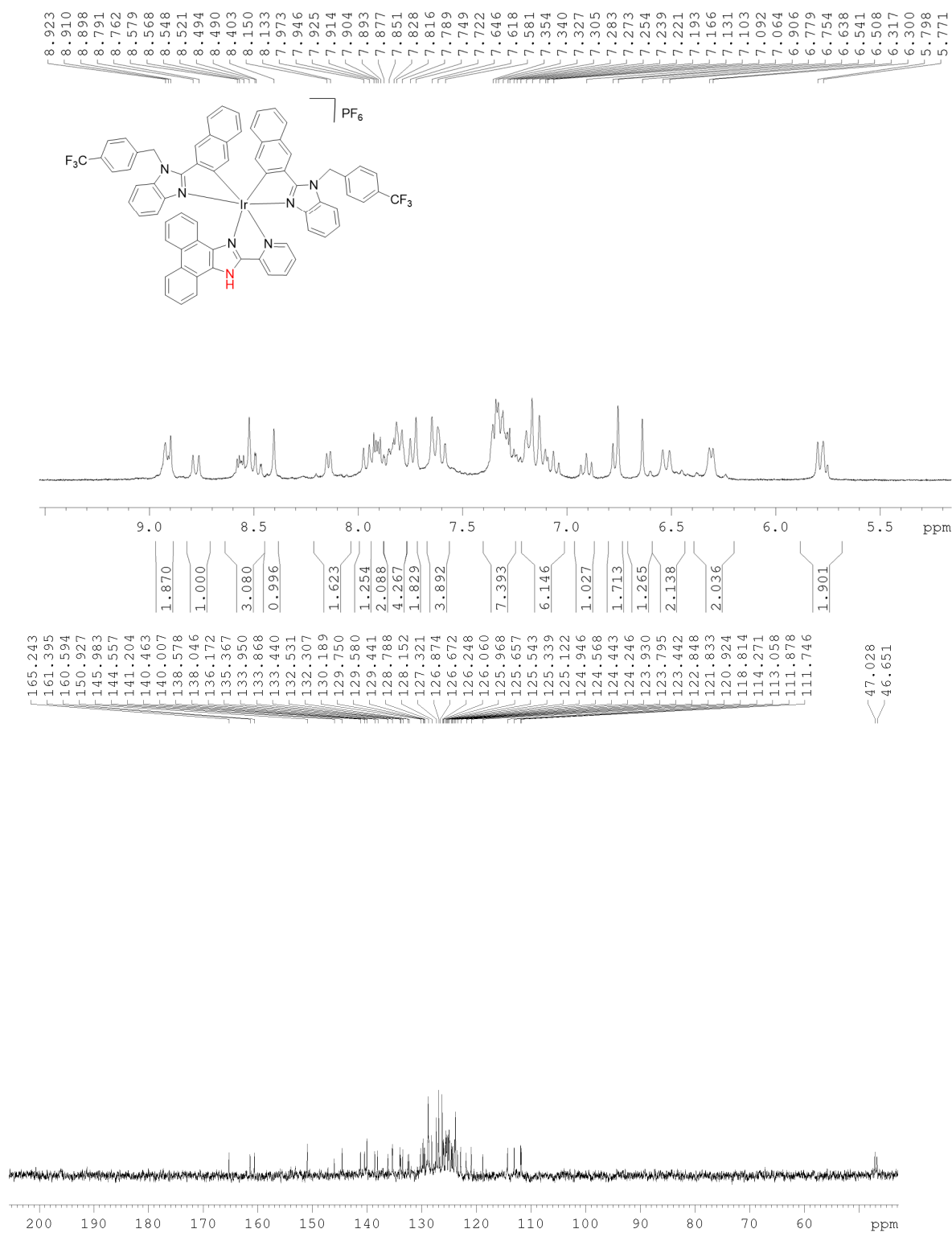


Figure S4.4. ^1H and ^{13}C NMR in $[\text{D}_6]\text{DMSO}$ for **29** at 70°C .

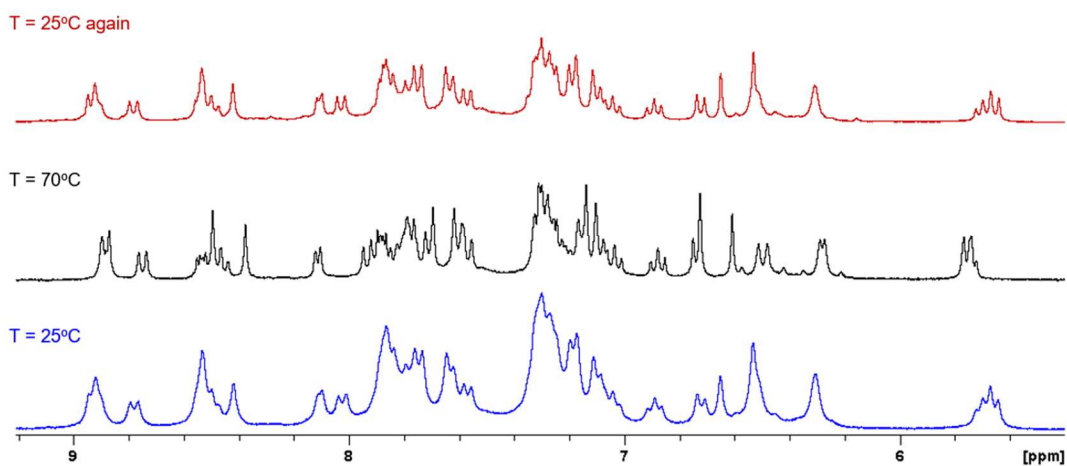


Figure S4.5. ^1H NMR in $[\text{D}_6]\text{DMSO}$ for **29** at 25°C, 70°C and cooled to 25°C again.

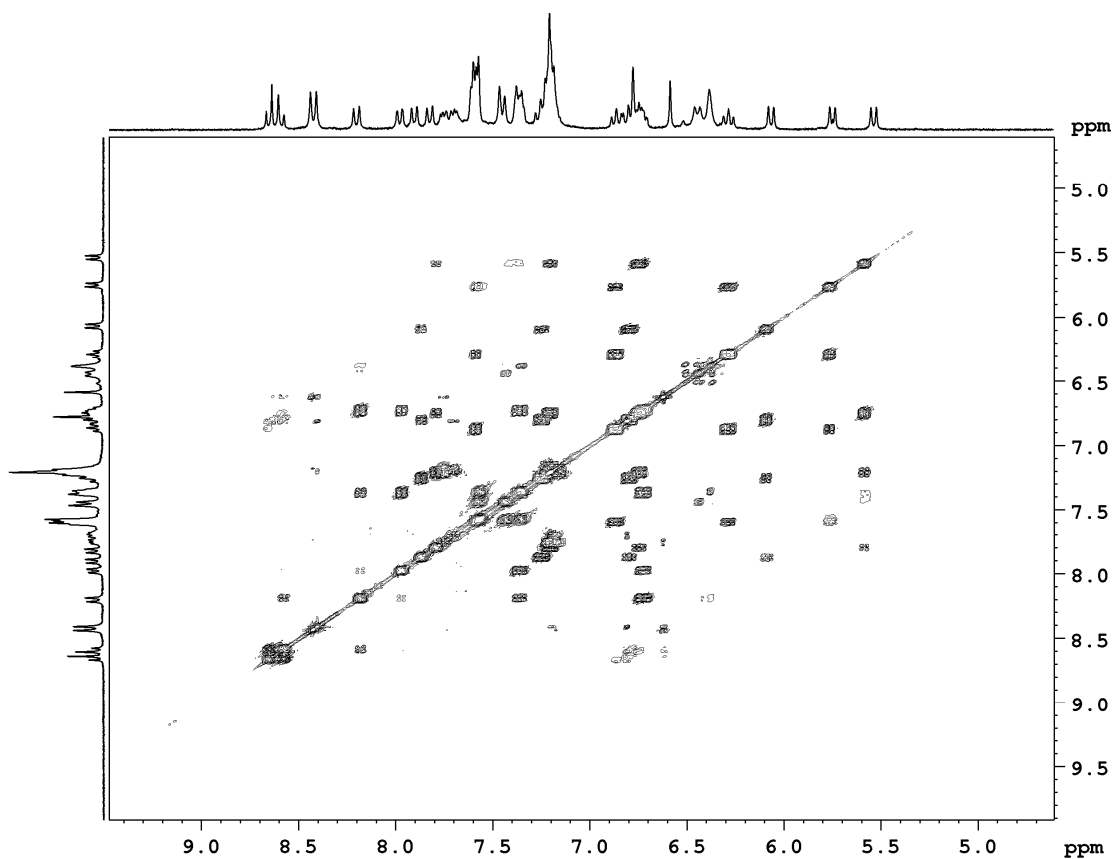


Figure S4.6. COSY 2D ^1H - ^1H NMR of complex **28**.

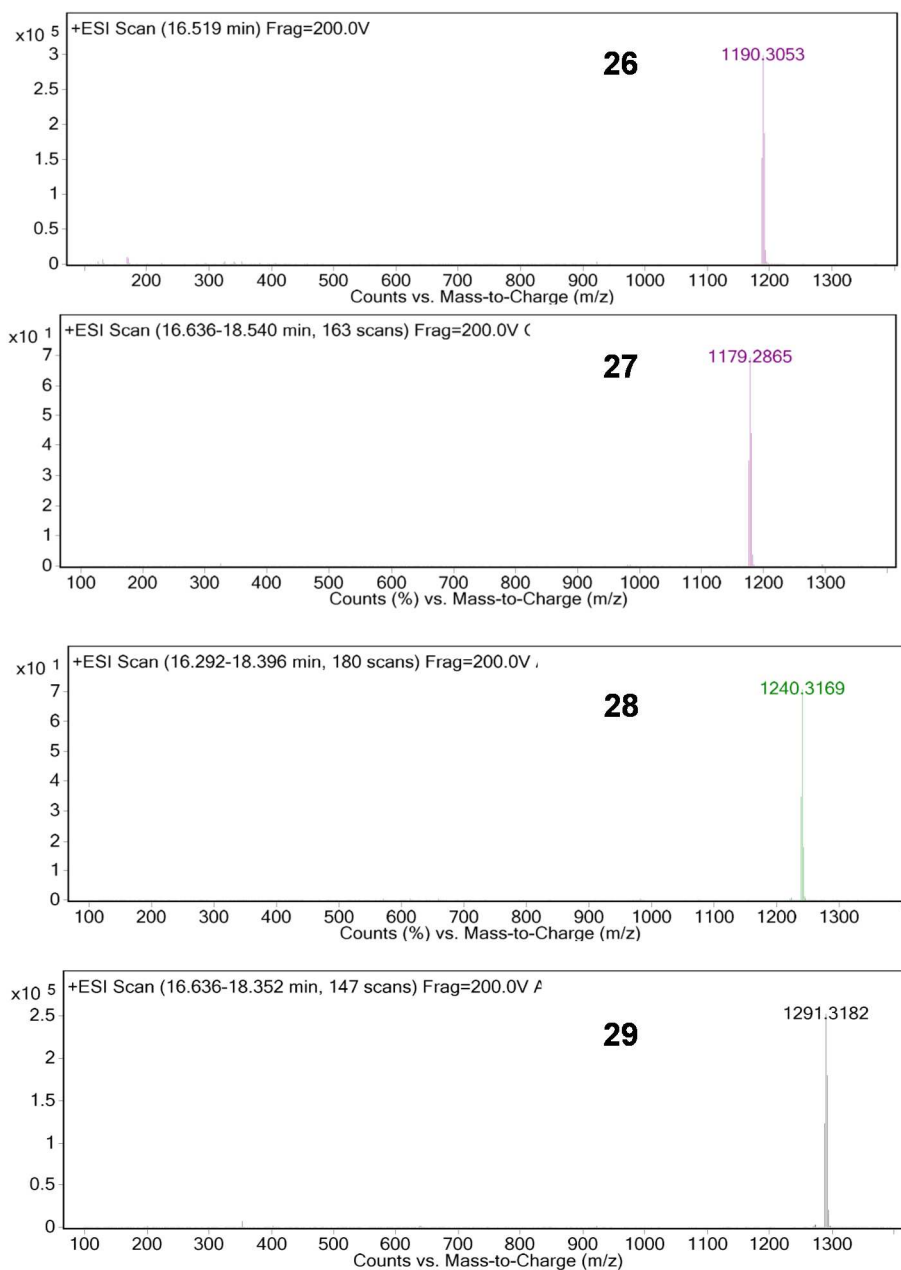


Figure S4.7. Positive-ion ESI-MS of complexes 26-29.

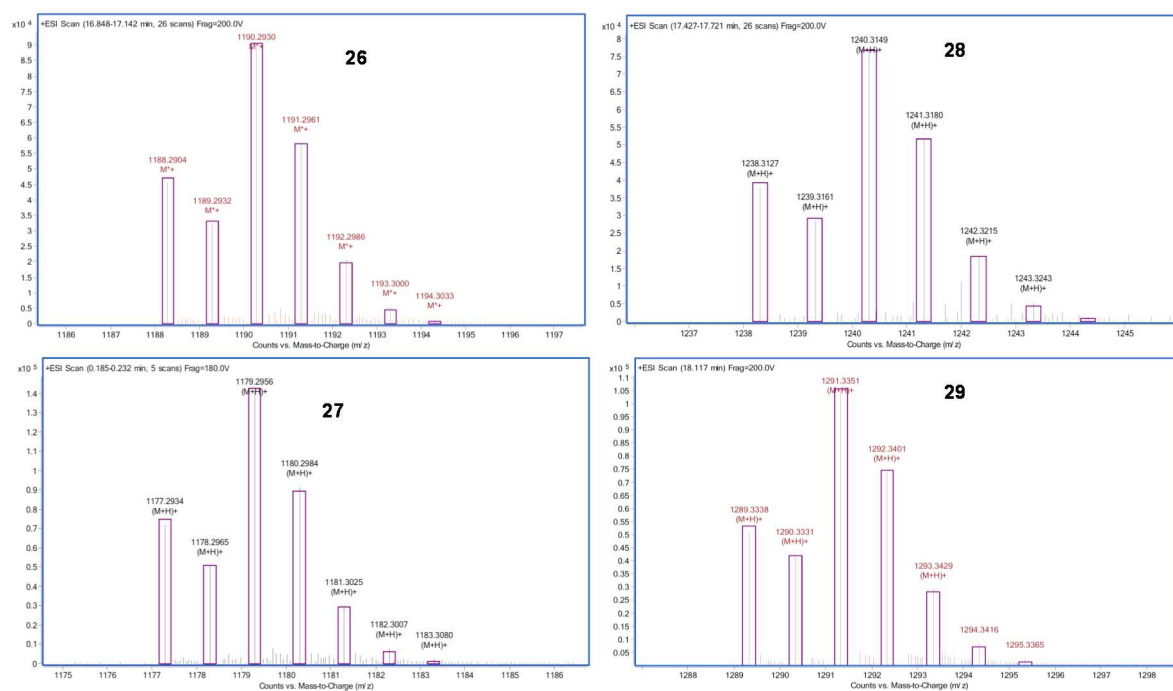


Figure S4.8. Theoric vs experimental molecular ion $[M-PF_6]^+$ of 26-29.

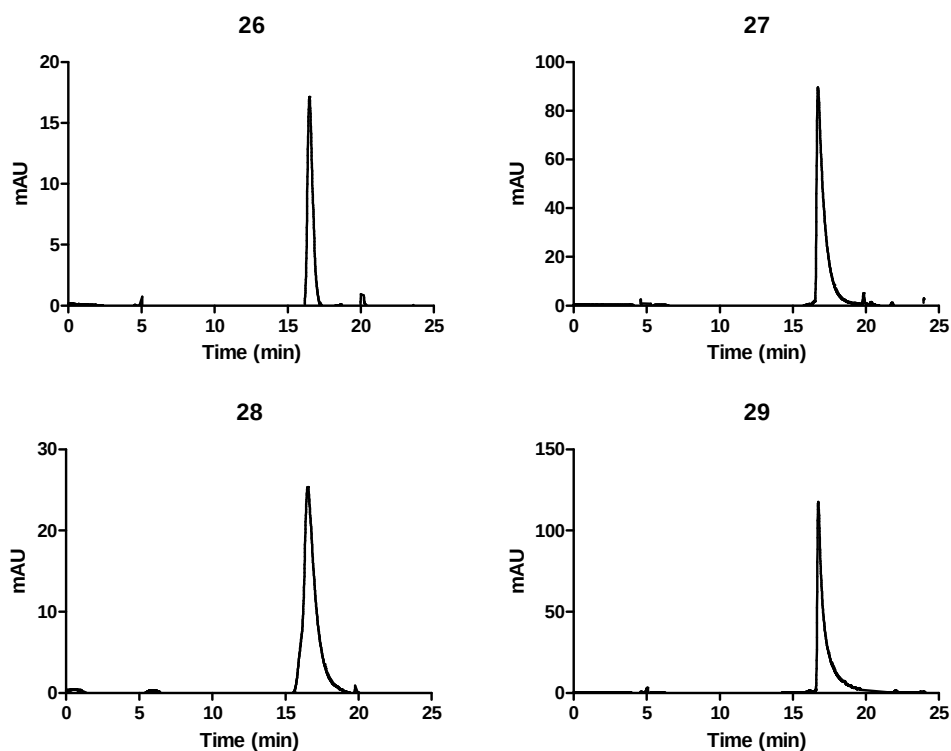


Figure S4.9. HPLC chromatograms of 26-29 in DMSO.

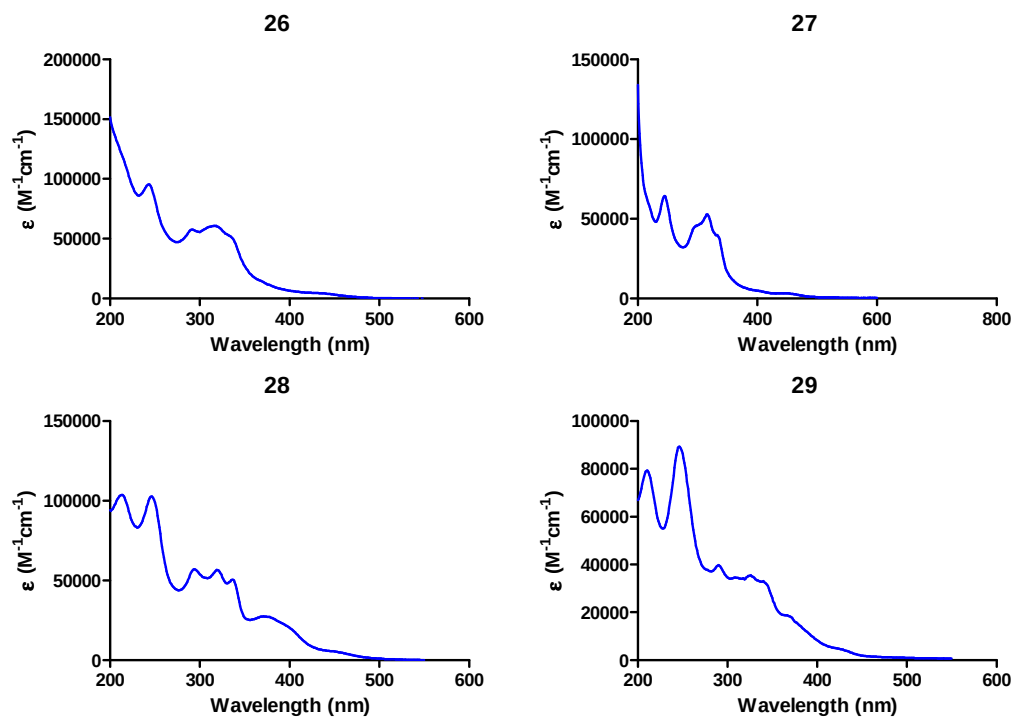


Figure S4.10. UV/Vis absorption spectra of **9-12** in acetonitrile.

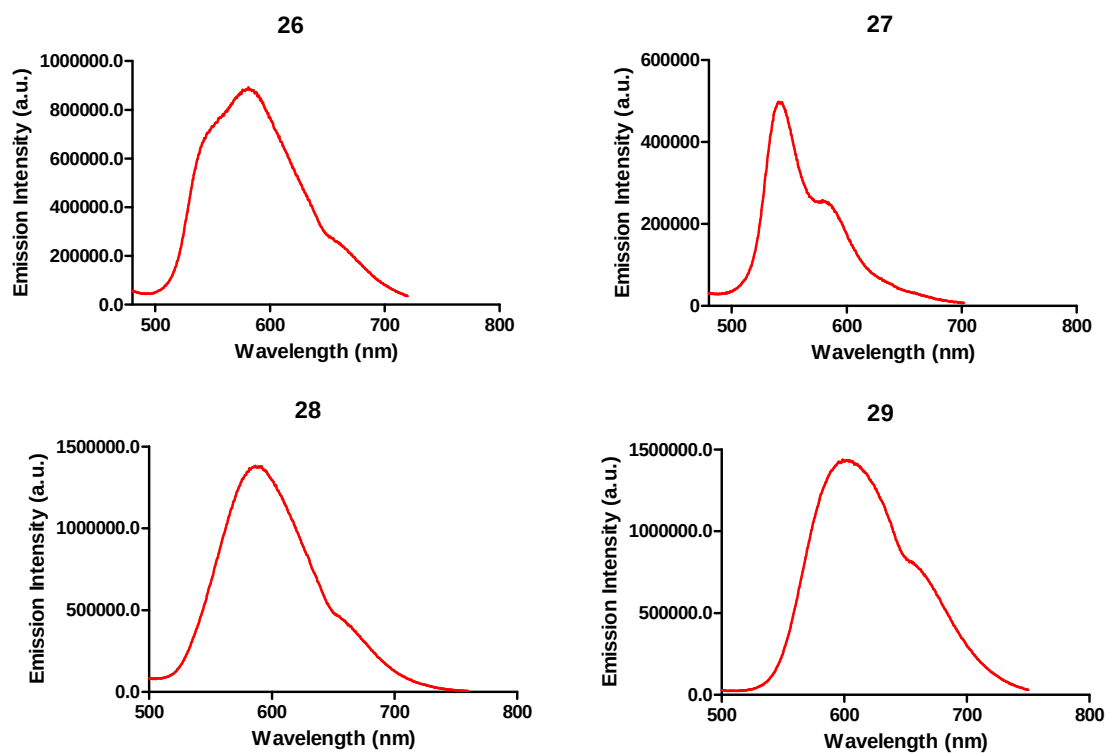


Figure S4.11. Emission spectra of complexes in aerated acetonitrile. λ_{exc} = 370, 360, 390 and 403 nm respectively.

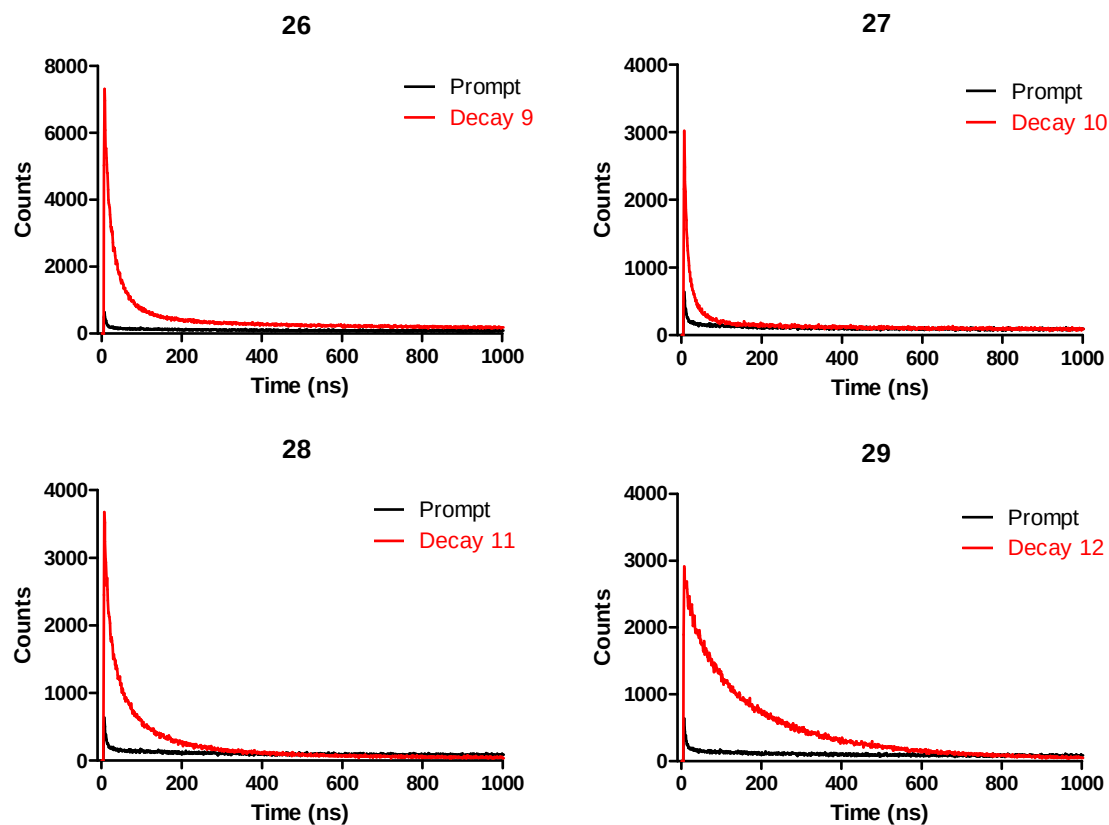


Figure S4.12. Fluorescence emission decay kinetics of complexes in water (1% DMSO).

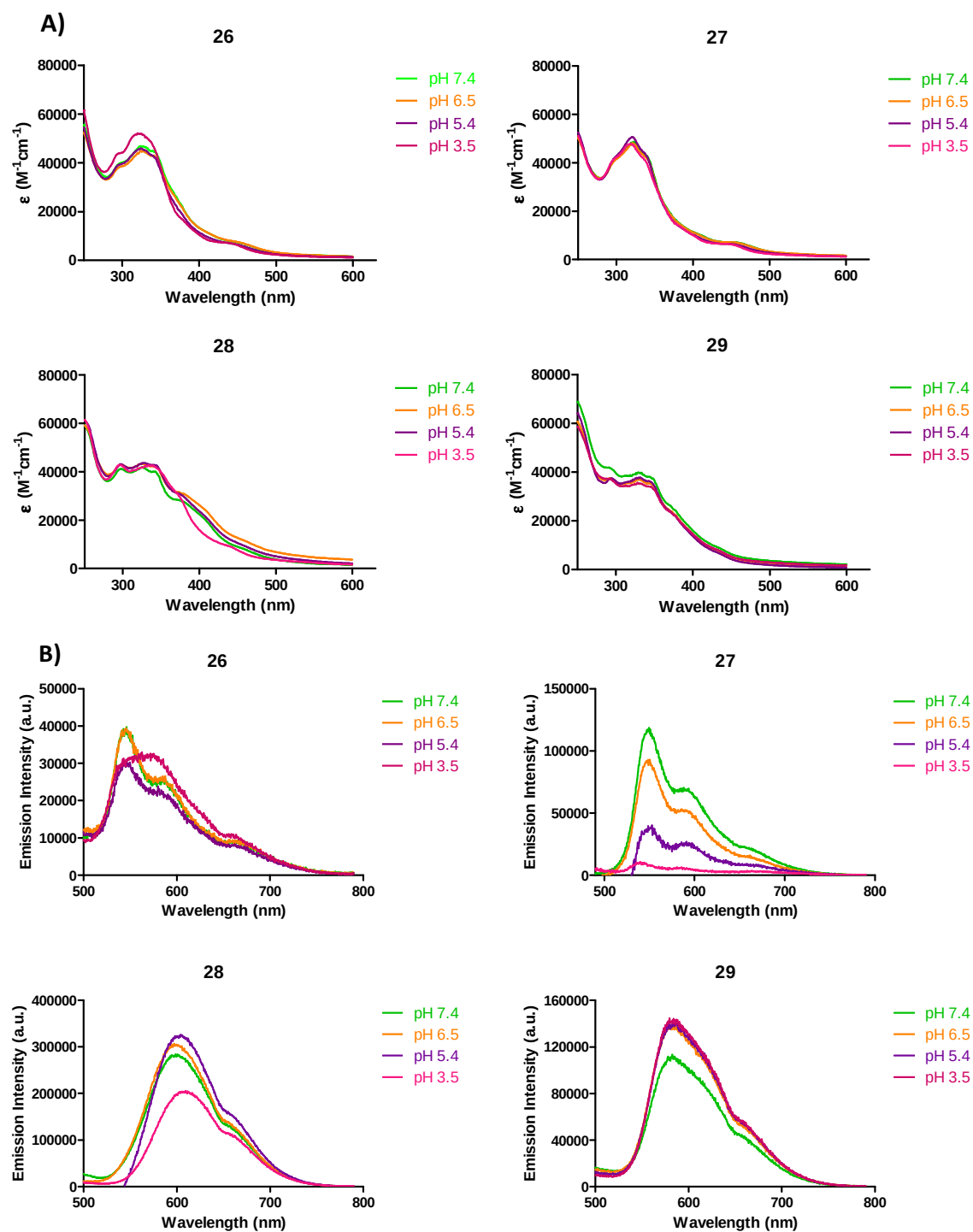


Figure S4.13. A) UV/Vis absorption spectra and B) emission spectra of **26-29** in buffer/DMSO (99:1) at different pH. The excitation wavelength was 405 nm.

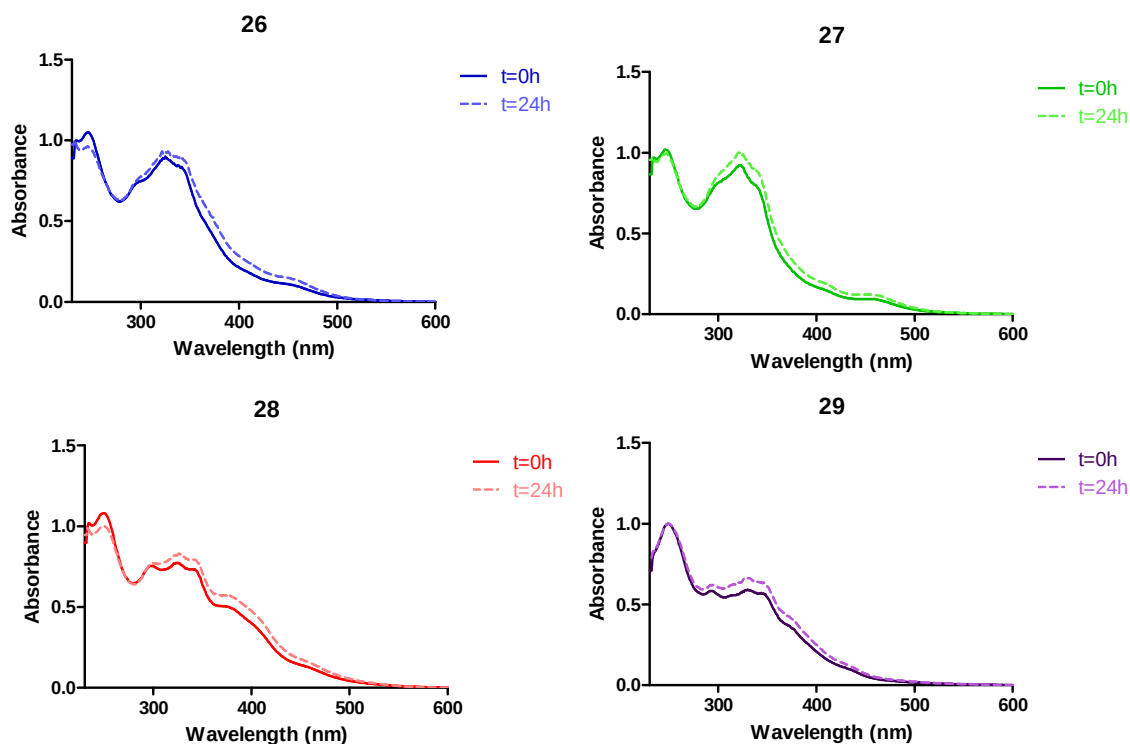


Figure S4.14. UV/Vis spectra of complexes **26-29** showing the stability in RPMI cell culture medium after 24h.

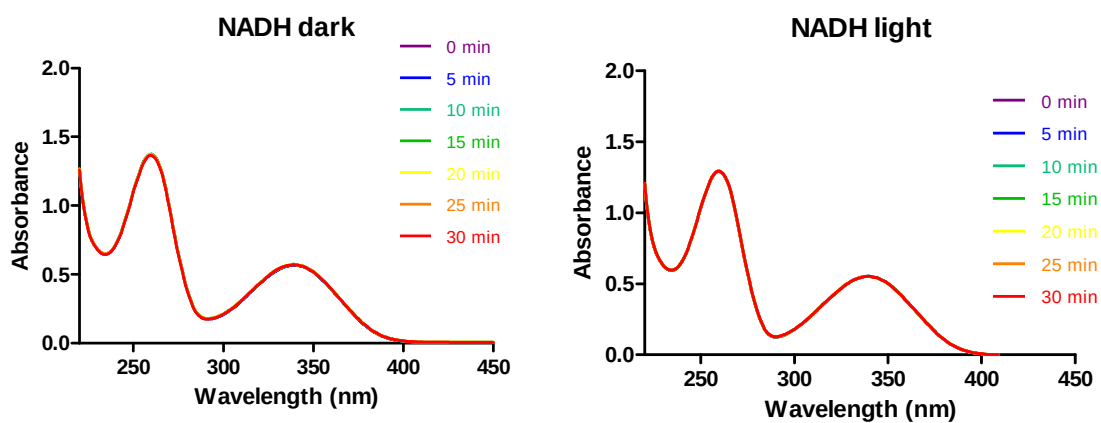


Figure S4.15. Stability of NADH in the dark and under light irradiation (465 nm, 8.4 mW/cm²) after 30 min.

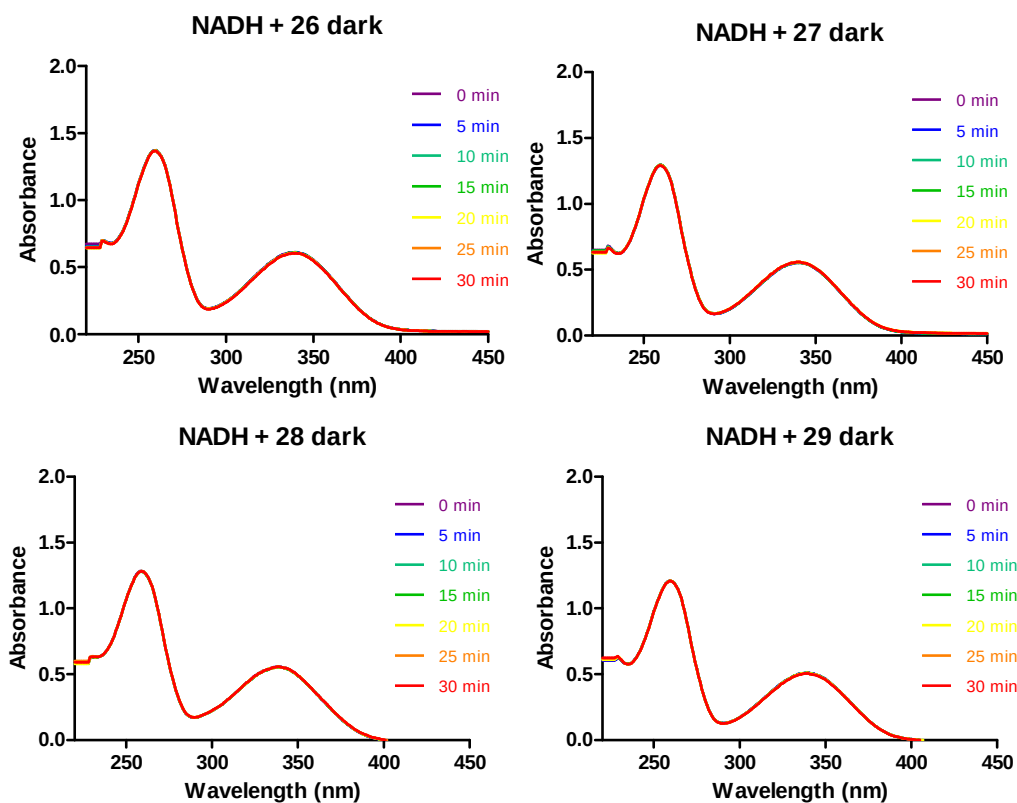


Figure S4.16. UV/Vis spectra of NADH (100 μ M) in presence of complexes (5 μ M) in the dark for 30 min.

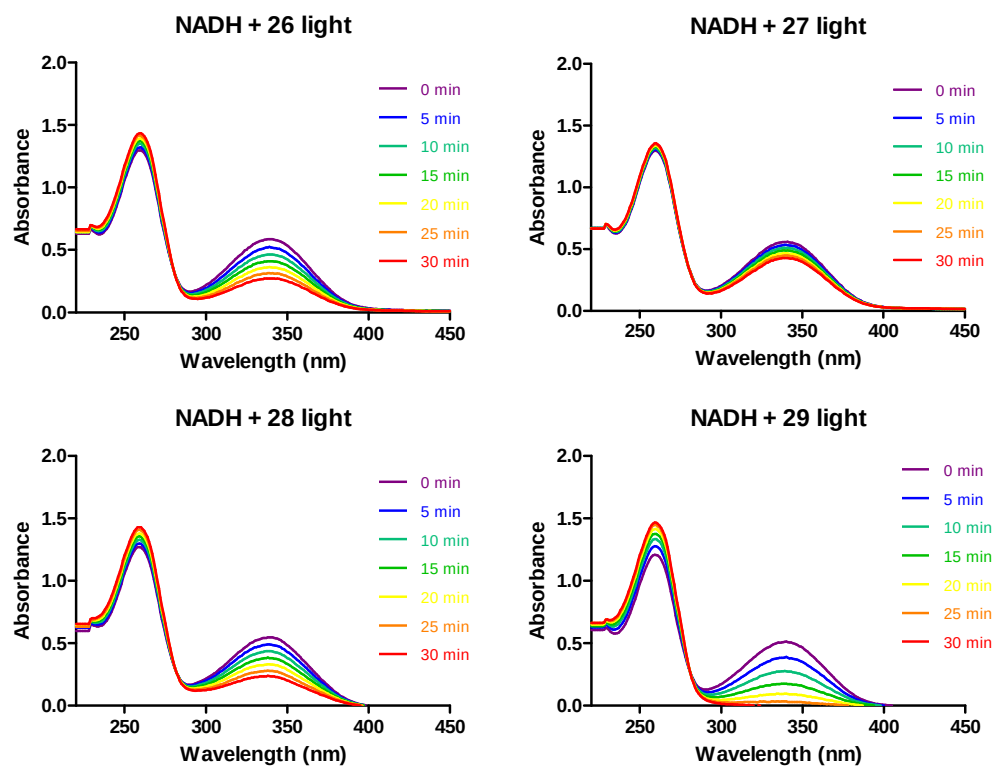
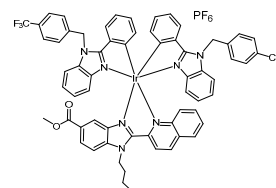


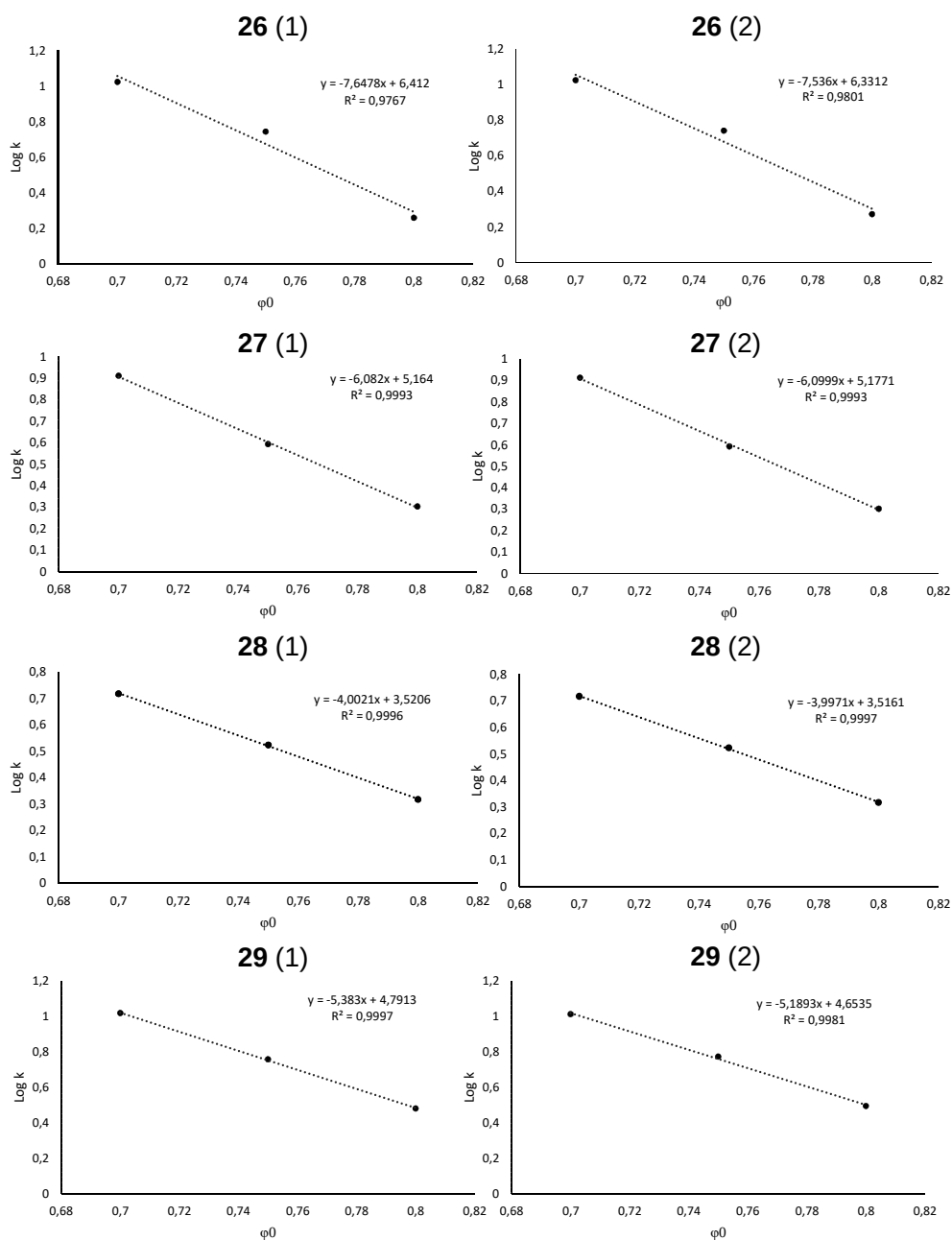
Figure S4.17. UV/Vis spectra of NADH (100 μ M) in presence of complexes (5 μ M) under 465 nm blue light irradiation for 30 min.

Table S4.1. Log *P* values of an iridium complex reported by us¹ measured using the “shake flask” method and the HPLC-method designed by Keppler *et al.*² at room temperature.

Method	φ_0	Log <i>P</i> ^a
Shake flask (ICP-MS)	-	2.6 ± 0.1
HPLC-based method	76.7	2.3 ± 0.1



^aResults are expressed as the mean ± SD from two independent experiments.

**Figure S4.18.** Soczewinski–Snyder relationship for **26–29** of two independent experiments.

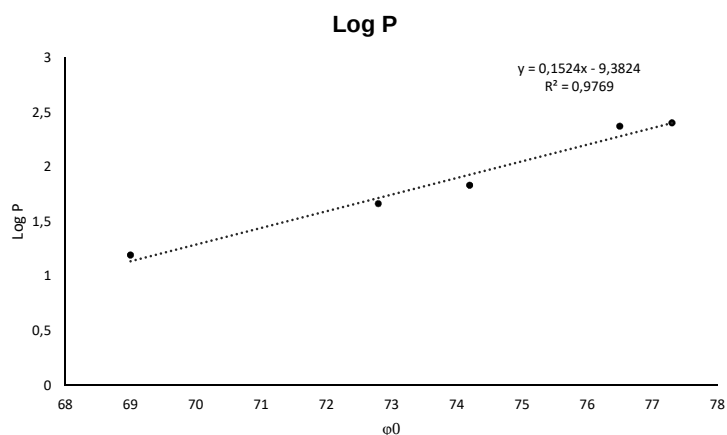


Figure S4.19. Graphic representation of the log P of the most lipophilic complexes found by Keppler *et al.* and their corresponding values of ϕ_0 .

Table S4.2. IC₅₀ values (μM) obtained for melanoma and HeLa cervical cancer cells treated with the investigated Ir complexes in the dark or after irradiation by visible light (1 h, λ_{max} = 420 nm, $7.7 \pm 3 \text{ mW/m}^2$) determined by Neutral Red assay in normoxic condition.

	A375		518.A2		HeLa	
	dark	420 nm	dark	420 nm	dark	420 nm
1	>50	16 ± 2	>50	11.4 ± 0.5	>50	20 ± 3
2	>50	>50	>50	48 ± 2	>50	>50
3	>50	13.8 ± 0.8	>50	8 ± 1	>50	17 ± 4
4	>50	5.9 ± 0.1	>50	3.3 ± 0.2	>50	6.5 ± 0.9

^aData represent the mean $\pm$ SD from two independent experiments, each made in triplicate.

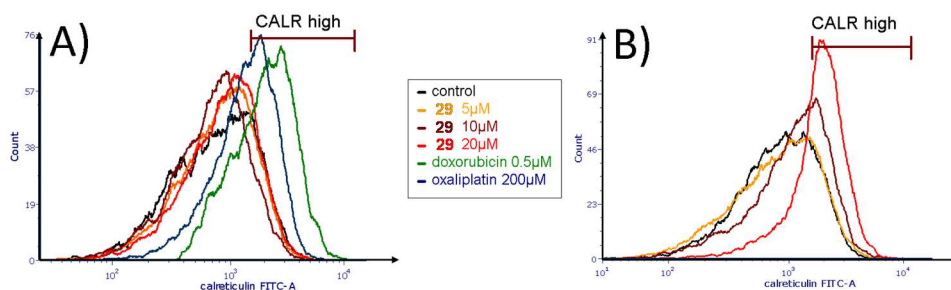


Figure S4.20. Representative flow cytometry histograms of ecto-Calreticulin exposure for A375 cells treated with the tested complexes (**29**, doxorubicin, oxaliplatin) A) in the dark B) after irradiation.

Table S4.3. Ecto-Calreticulin exposing population (%) obtained from flow cytometry histograms.

	DARK	IRRADIATED
control	16.8	16.6
29 5 μM	14.7	17.2
29 10 μM	9.9	27.9
29 20 μM	16.6	60.1
doxorubicin 0.5 μM	63.7	ND
oxaliplatin 200 μM	41.2	ND

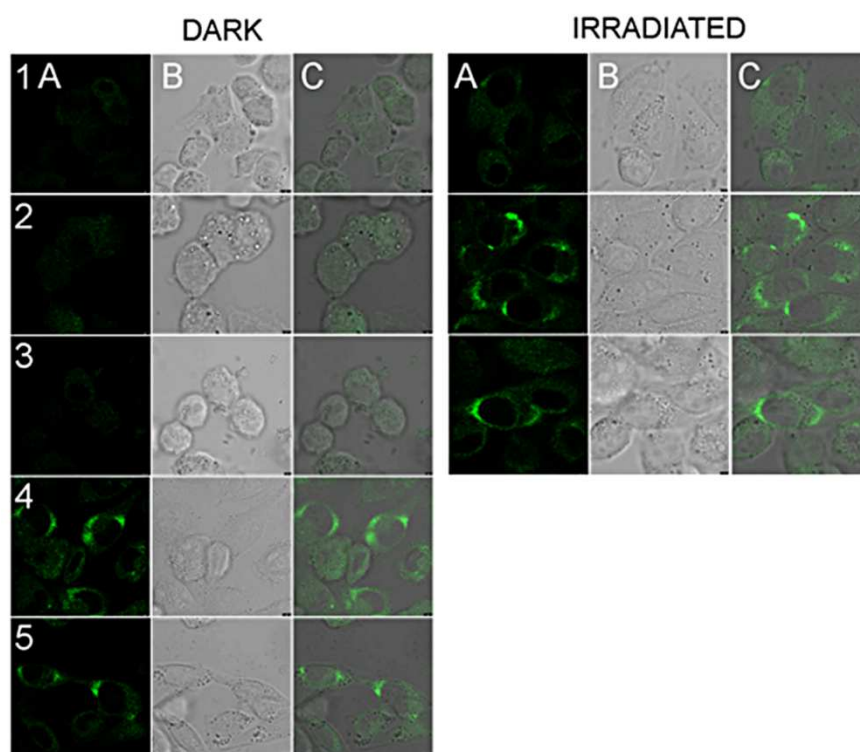


Figure S4.21. Immunofluorescence analysis of calreticulin exposure by confocal microscopy. A375 cells were untreated (panels 1) or treated with **29** (10 μ M panels 2, 20 μ M panels 3), doxorubicin (0,5 μ M – panels 4) or oxaliplatin (200 μ M – panels 5) followed by irradiation with blue light (420nm, 1 h) or kept in the dark with subsequent incubation for 22 h in the drug-free medium in the dark. Channels: A) Fluorescence signal from CRT-Alexa fluor 488 conjugate. B) Bright field. C) Merge of the fluorescence and bright field channels.

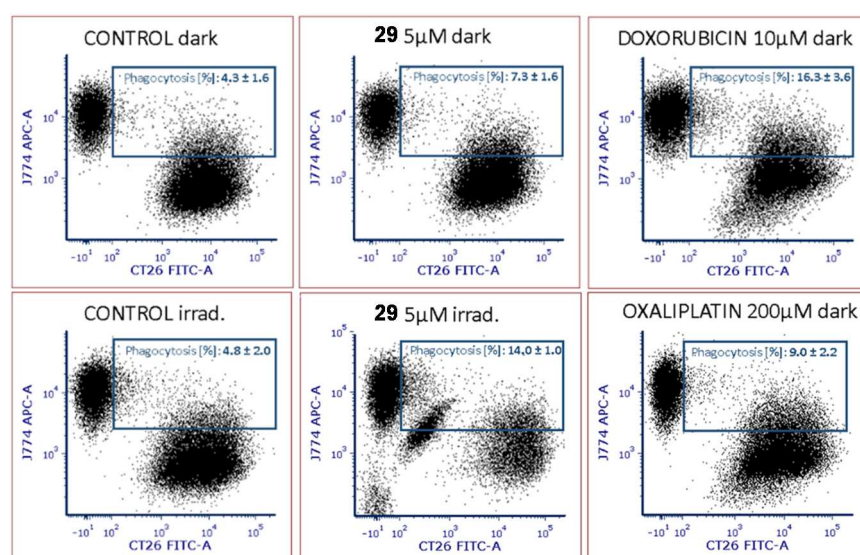


Figure S4.22. Phagocytosis assay CT26/J774.A1. Murine cancer cells CT26 (non-treated or pretreated with **29**, doxorubicin, or oxaliplatin) were stained with CellTracker™ green CMFDA and co-incubated with pre-stained (CellTracker™ red CMTPIX) murine macrophages J774.A1. The cell mixture was analyzed by flow cytometry. Double-stained macrophages indicate phagocytosis.

References

- (1) Yellol, J.; Pérez, S. A.; Yellol, G.; Zajac, J.; Donaire, A.; Viguera, G.; Novohradsky, V.; Janiak, C.; Brabec, V.; Ruiz, J. Highly Potent Extranuclear-Targeted Luminescent Iridium(III) Antitumor Agents Containing Benzimidazole-Based Ligands with a Handle for Functionalization. *Chem. Commun.* **2016**, 52 (98), 14165–14168. <https://doi.org/10.1039/C6CC07909A>.
- (2) Klose, M. H. M.; Theiner, S.; Varbanov, H. P.; Hoefer, D.; Pichler, V.; Galanski, M.; Meier-Menches, S. M.; Keppler, B. K. Development and Validation of Liquid Chromatography-Based Methods to Assess the Lipophilicity of Cytotoxic Platinum(IV) Complexes. *Inorganics* **2018**, 6 (4). <https://doi.org/10.3390/inorganics6040130>.

Chapter 5

Synthesis and characterization of a new
heterobimetallic Ir(III)-Pt(II) complex that
overcomes cisplatin resistance

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¹ H NMR spectra of the N [^] N ligand	S83
¹ H and ¹³ C NMR spectra of monomeric complexes	S83
¹ H and ¹³ C and ¹⁹⁵ Pt NMR spectra of heterobimetallic complex	S87
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NMR comparison of 32 and 33 in [D ₆]DMSO	S91
Stability of 35 in [D ₆]DMSO	S91
Emission decay kinetics of dinuclear complex 35	S91

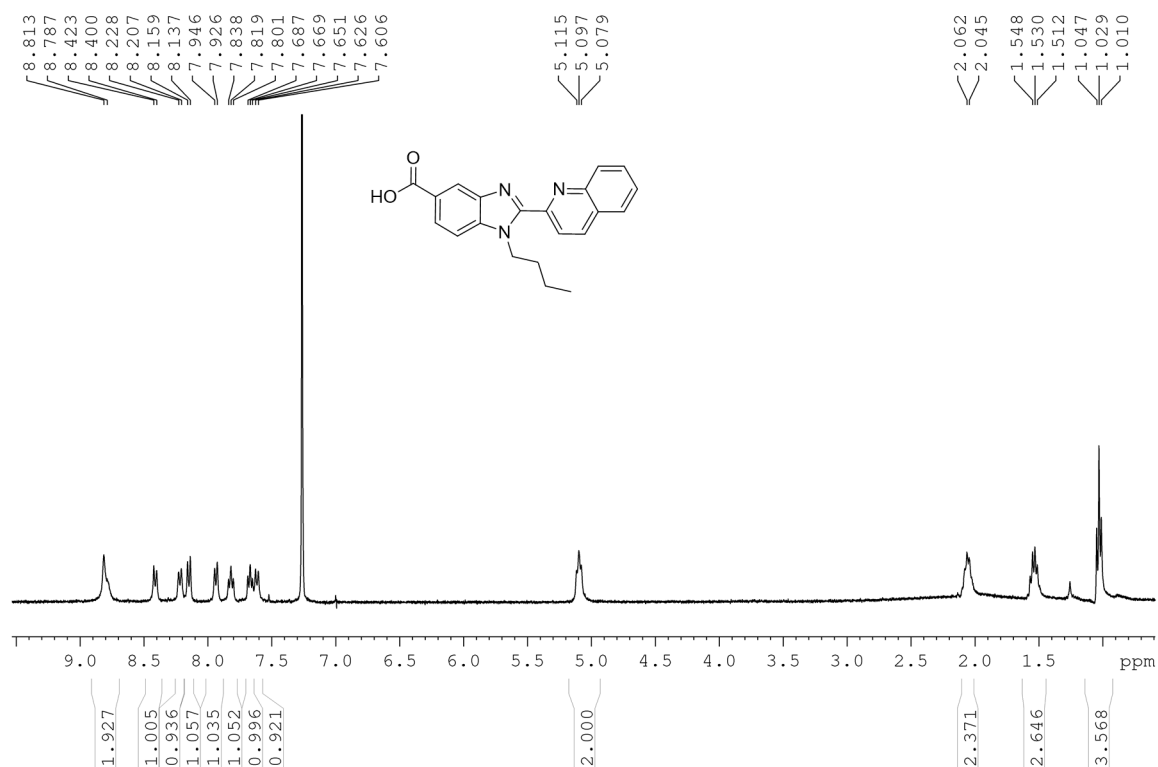


Figure S5.1. ¹H NMR in CDCl₃ for N^N ligand **31**.

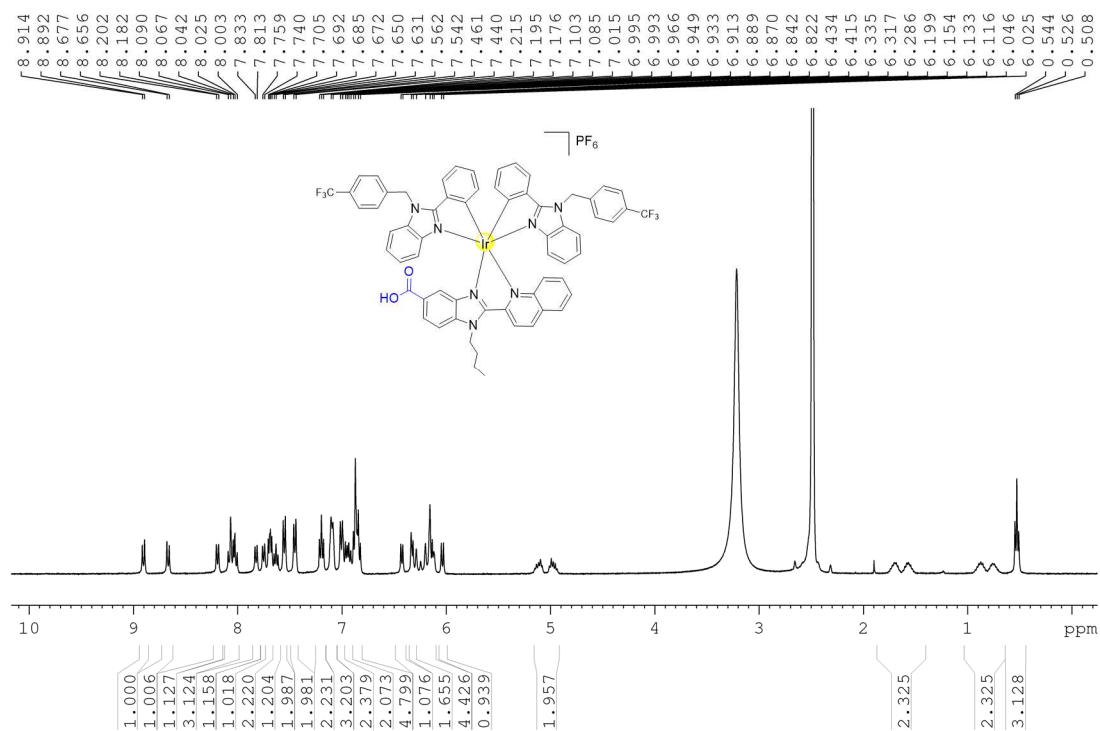


Figure S5.2. ¹H NMR of complex **32** in [D₆]DMSO.

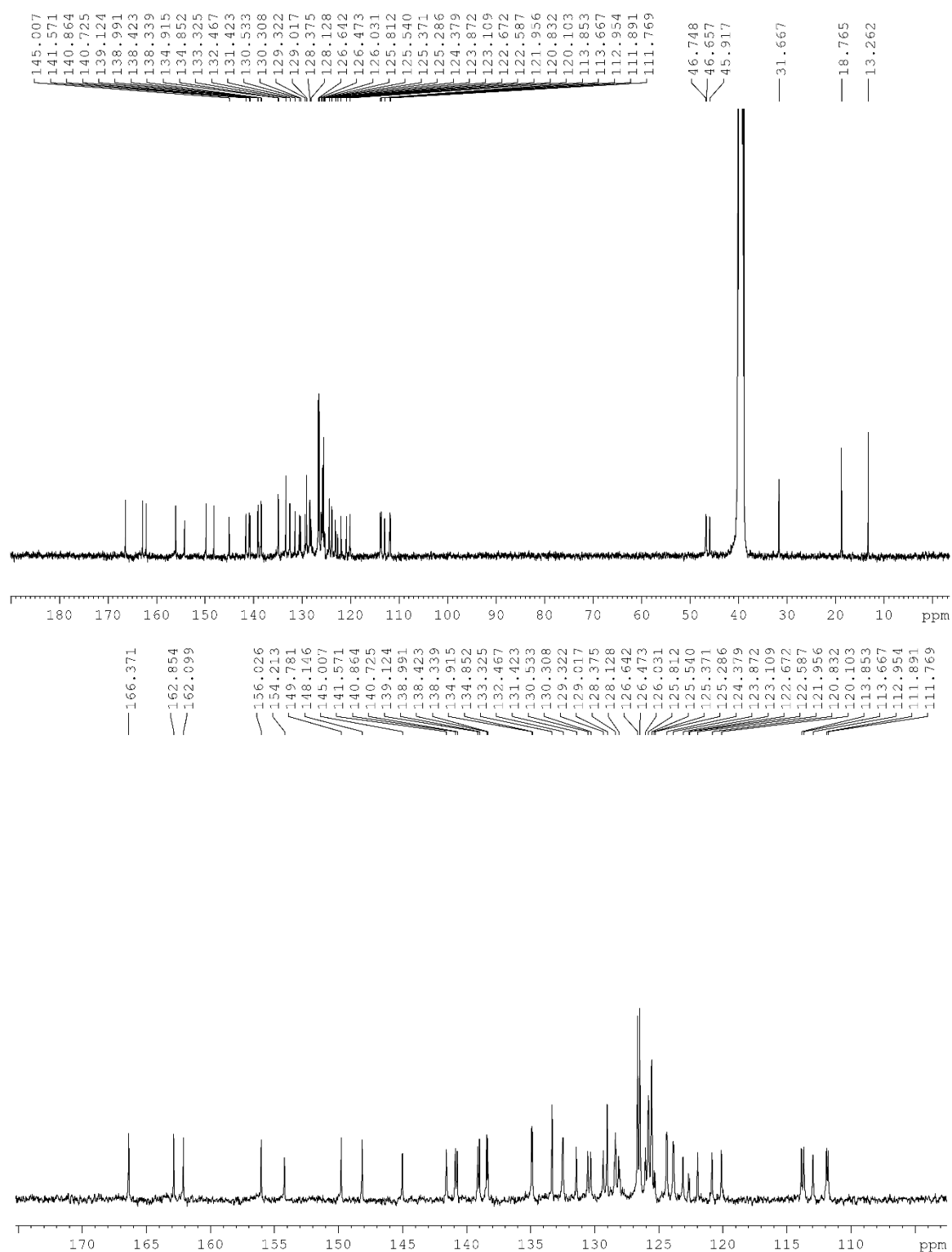


Figure S5.3. ^{13}C NMR of complex 32 in $[\text{D}_6]\text{DMSO}$.

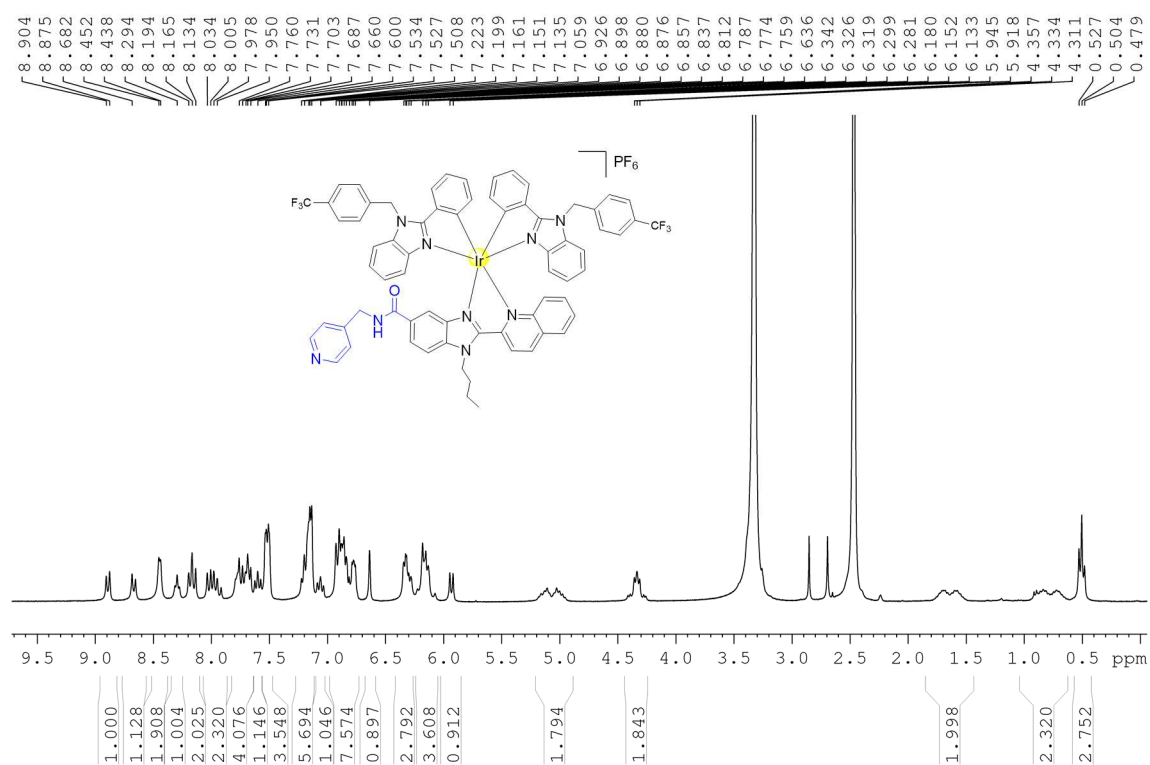


Figure S5.4. ¹H NMR of complex **33** in [D₆]DMSO.

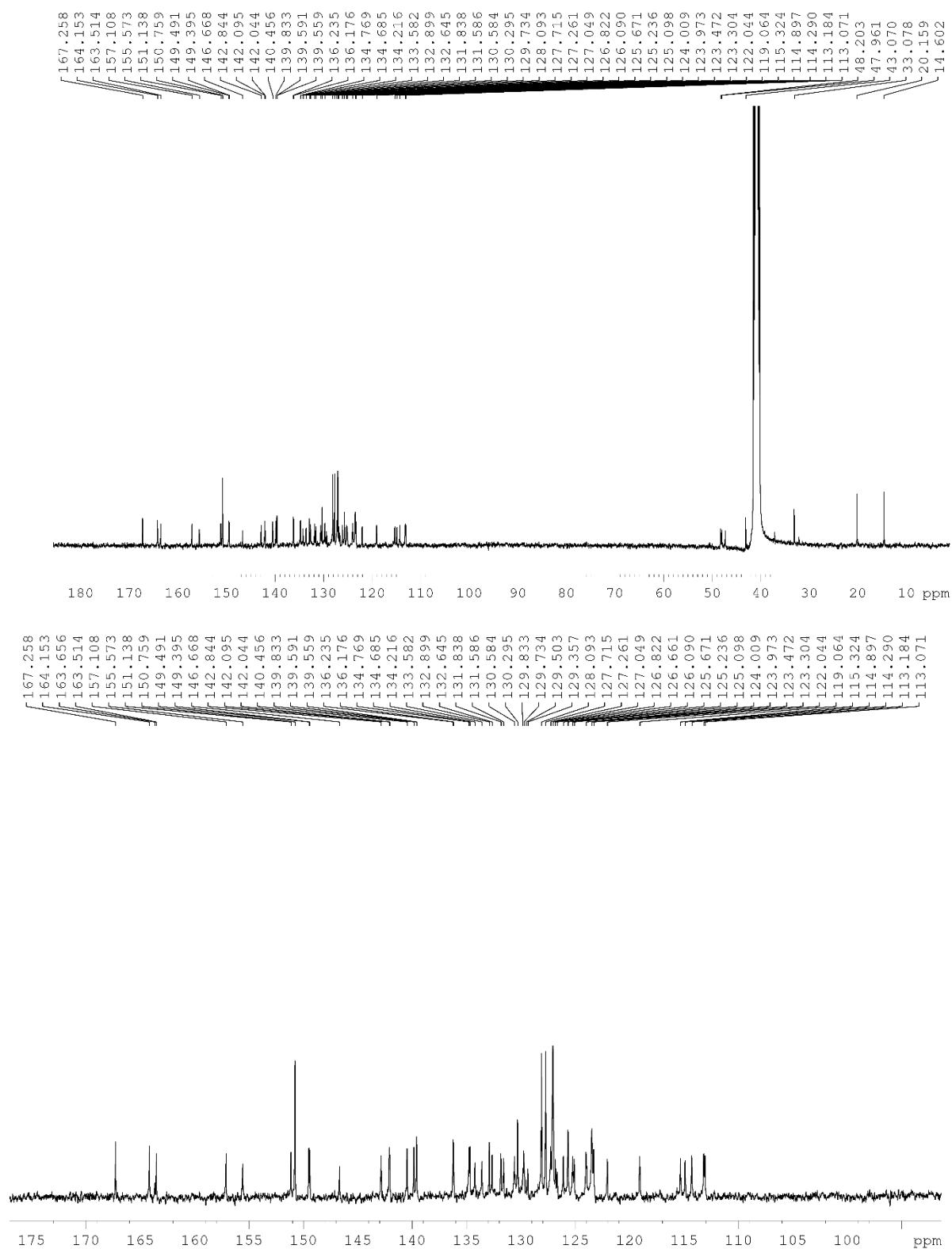


Figure S5.5. ^{13}C NMR of complex 33 in $[\text{D}_6]\text{DMSO}$.

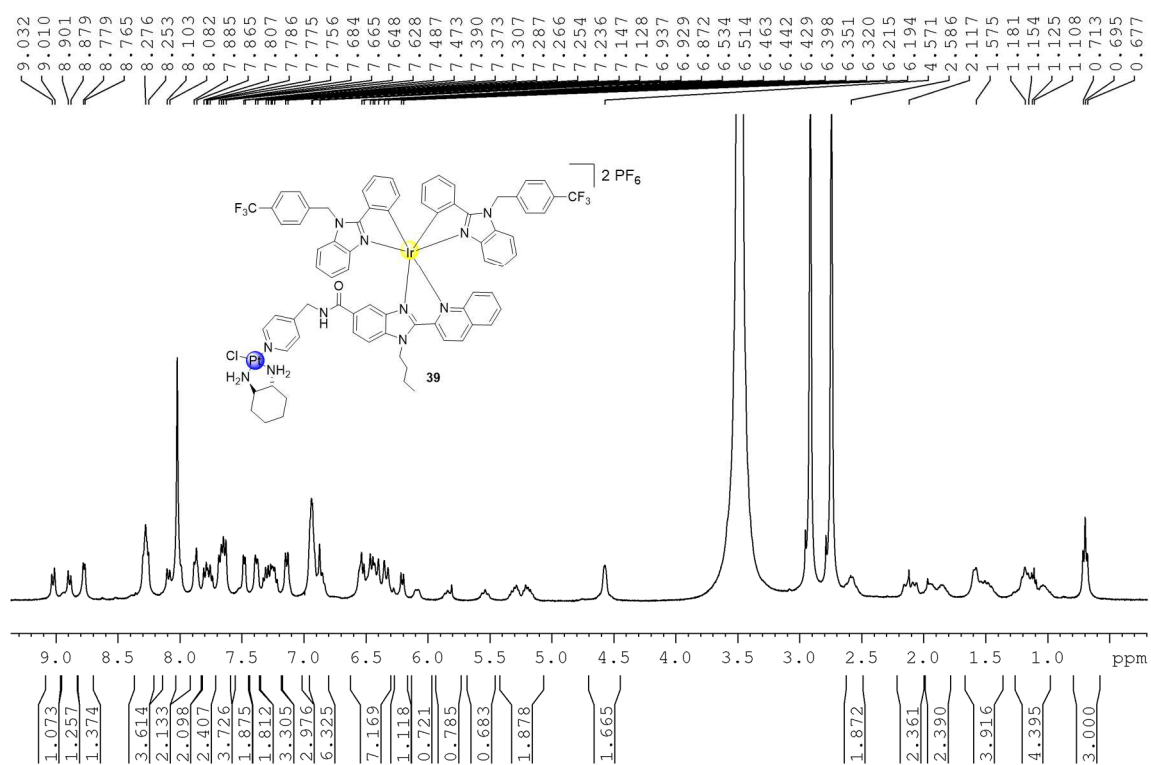


Figure S5.6. ^1H NMR of complex **35** in $[\text{D}_7]\text{DMF}$.

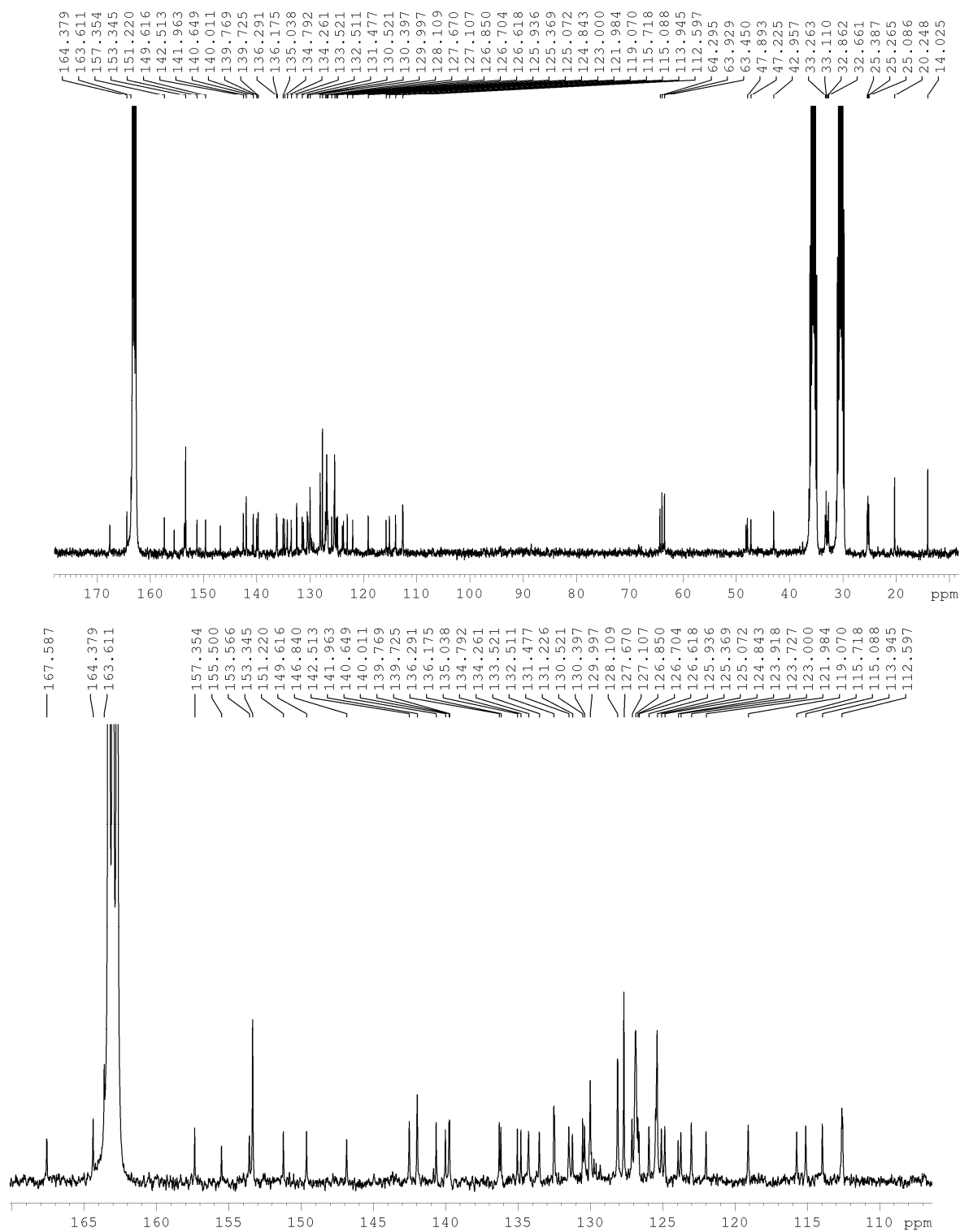


Figure S5.7. ^{13}C NMR of complex 35 in $[\text{D}_7]\text{DMF}$.

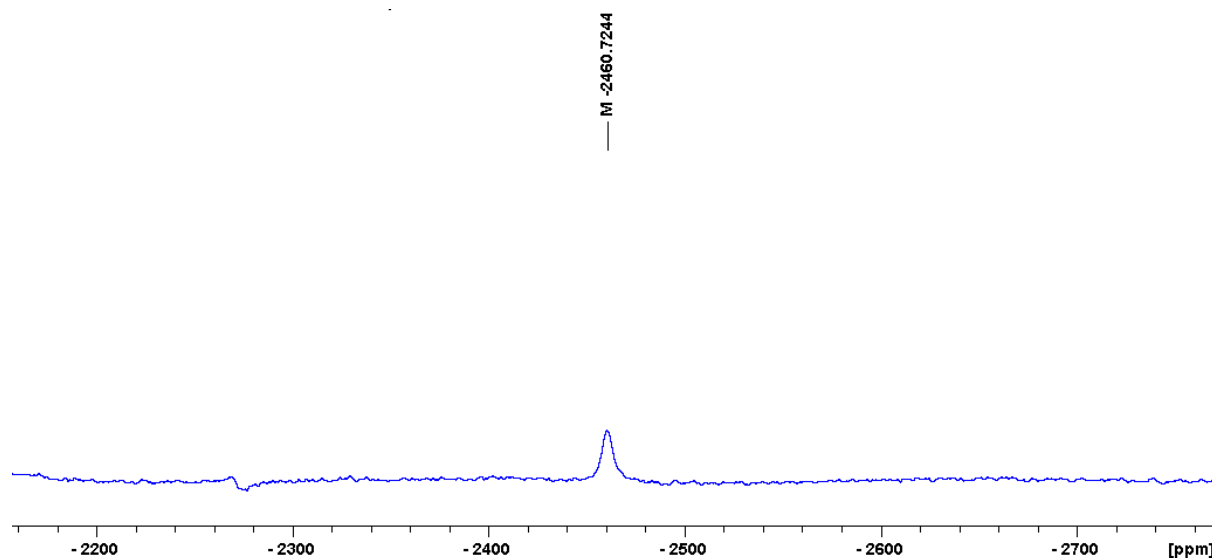


Figure S5.8. ^{195}Pt NMR of complex **35** in $[\text{D}_7]\text{DMF}$.

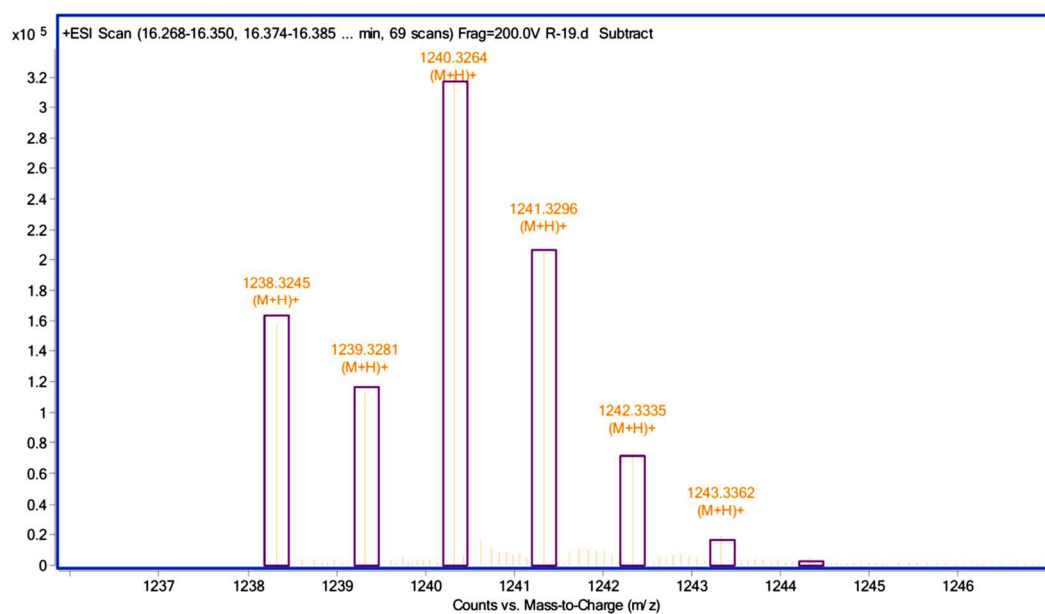


Figure S5.9. Positive-ion ESI-MS of complex **32**.

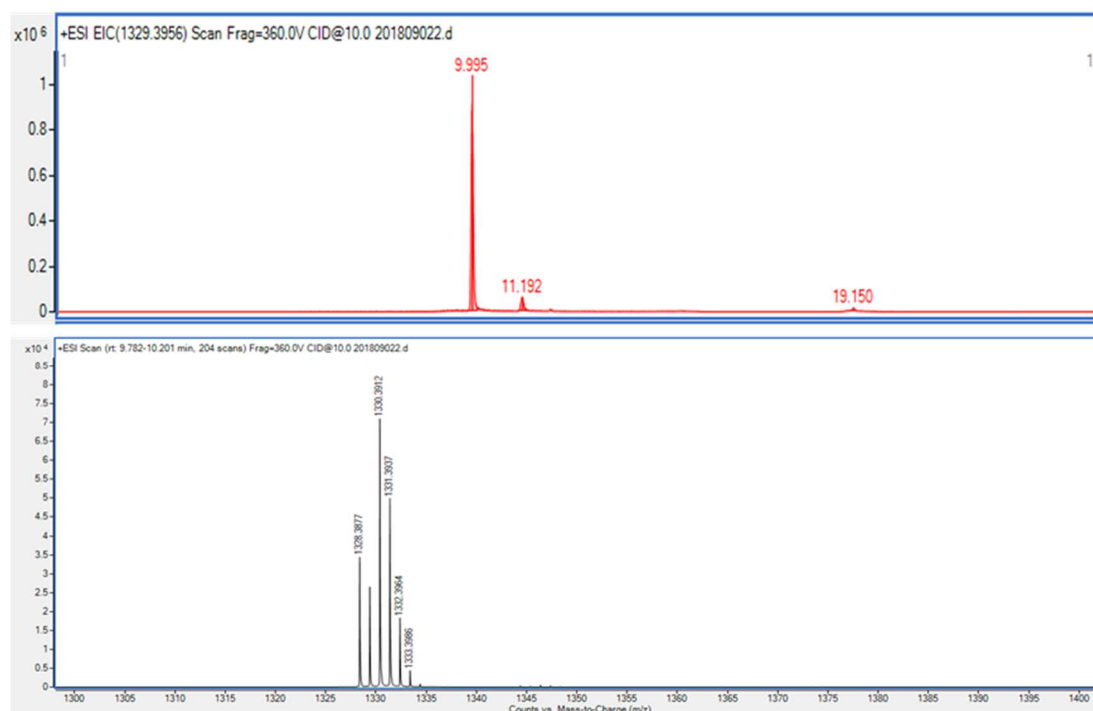


Figure S5.10. HPLC/MS of complex 33.

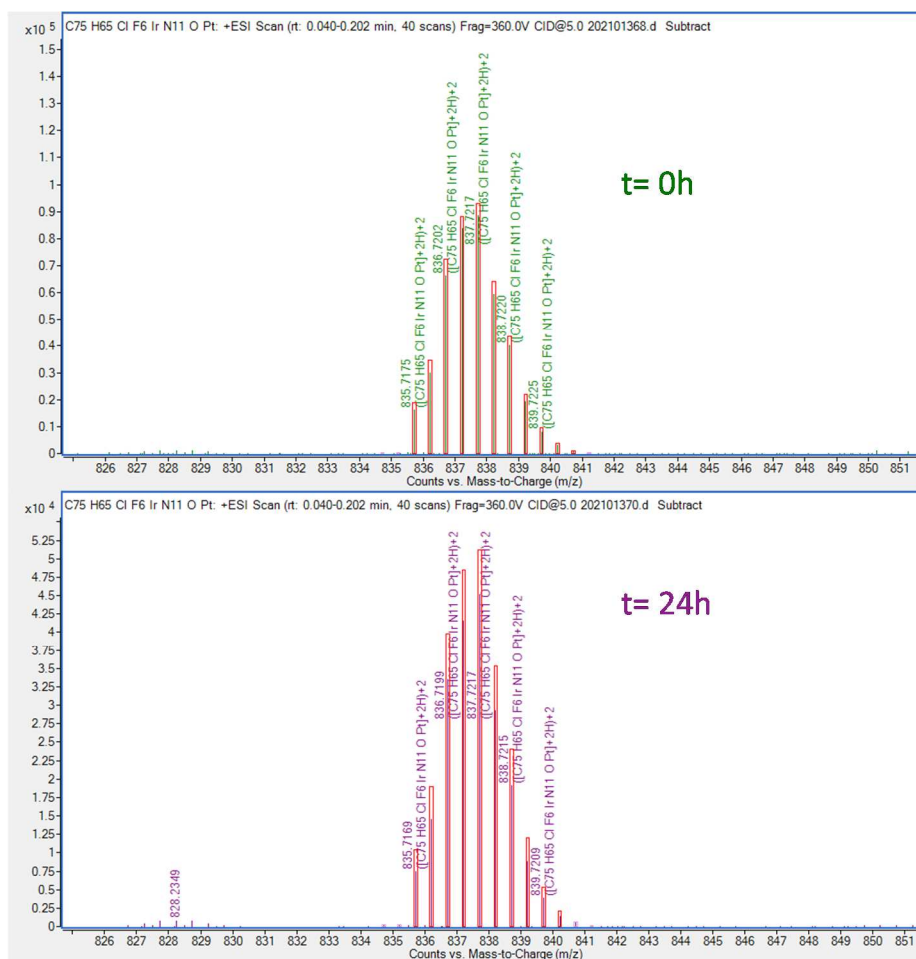


Figure S5.11. MS spectra of Ir-Pt complex 35 at t=0 and 24 h in water (1% DMF). Theoric vs experimental molecular ion.

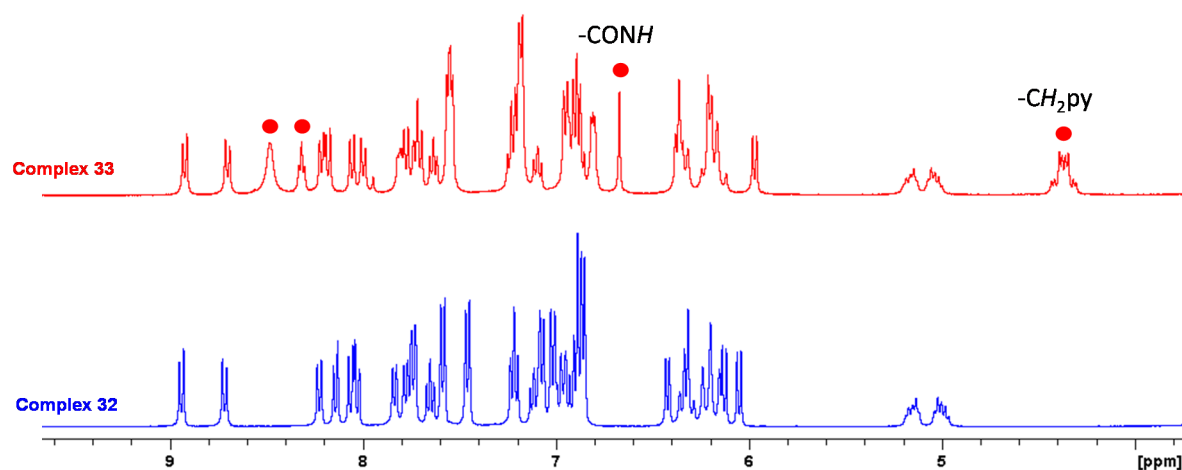


Figure S5.12. ^1H NMR comparison of **32** and **33** in $\text{DMSO}[D_6]$ (aromatic region).

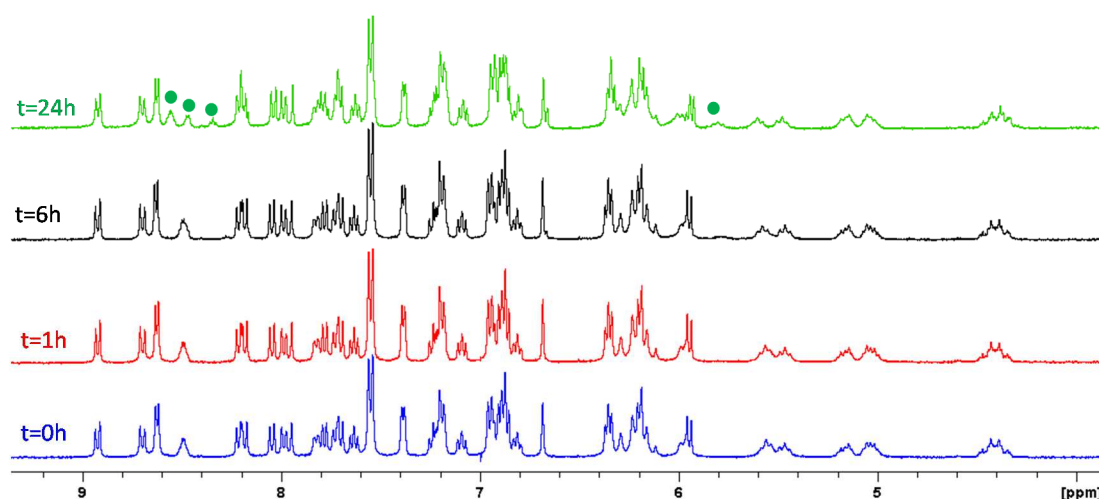


Figure S5.13. ^1H NMR spectra of **35** measured after dissolving immediately in $[D_6]\text{DMSO}$ (bottom) and after 1, 6 and 24 hours at RT. New signals appear on the spectrum after 24 h (green dots).

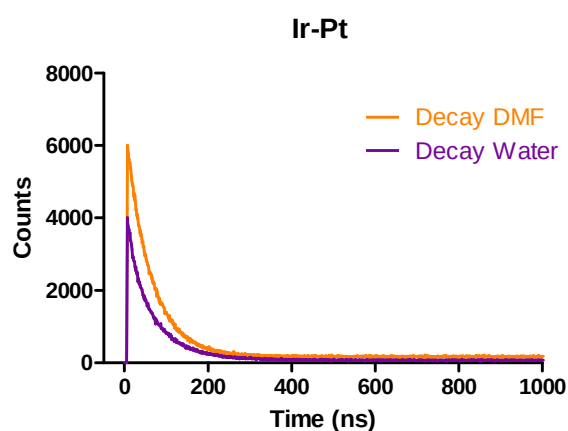


Figure S5.14. Emission decay kinetics of dinuclear complex **35** (Ir-Pt) in DMF and water (1% DMF).

Chapter 6

A red phosphorescent iridium(III) complex as
a promising antitumor agent: *in vivo* studies in
C. elegans

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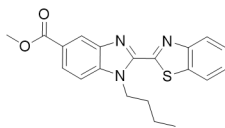


Figure S6.1. ^1H NMR in CDCl_3 for N^N ligand **36**.

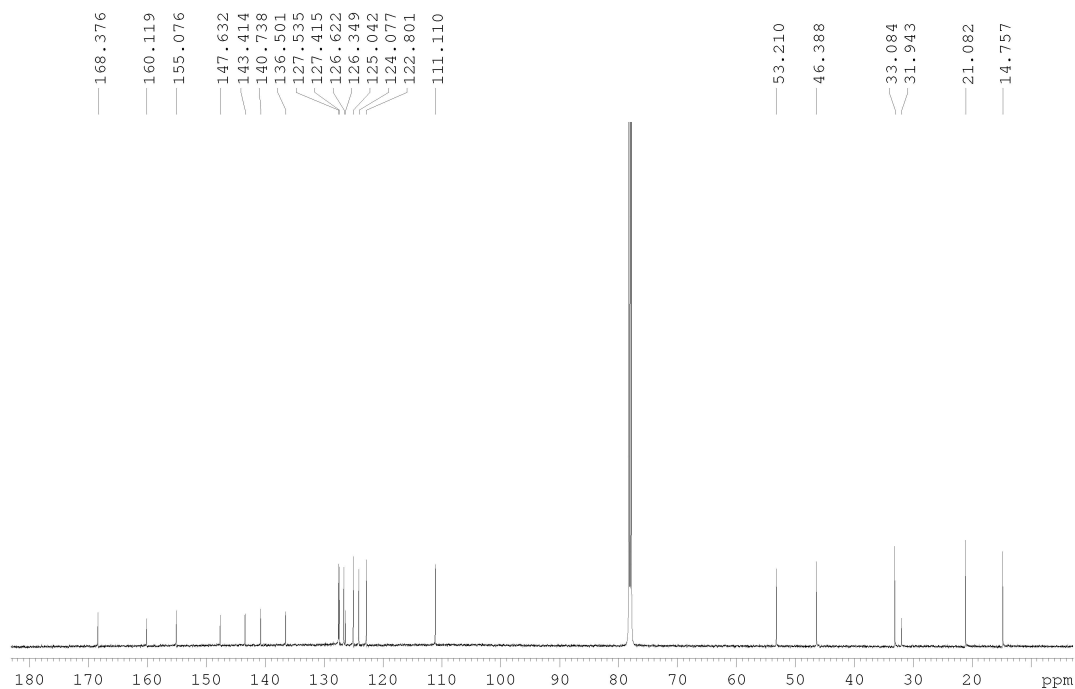


Figure S6.2. ^{13}C NMR in CDCl_3 for N^N ligand **36**.

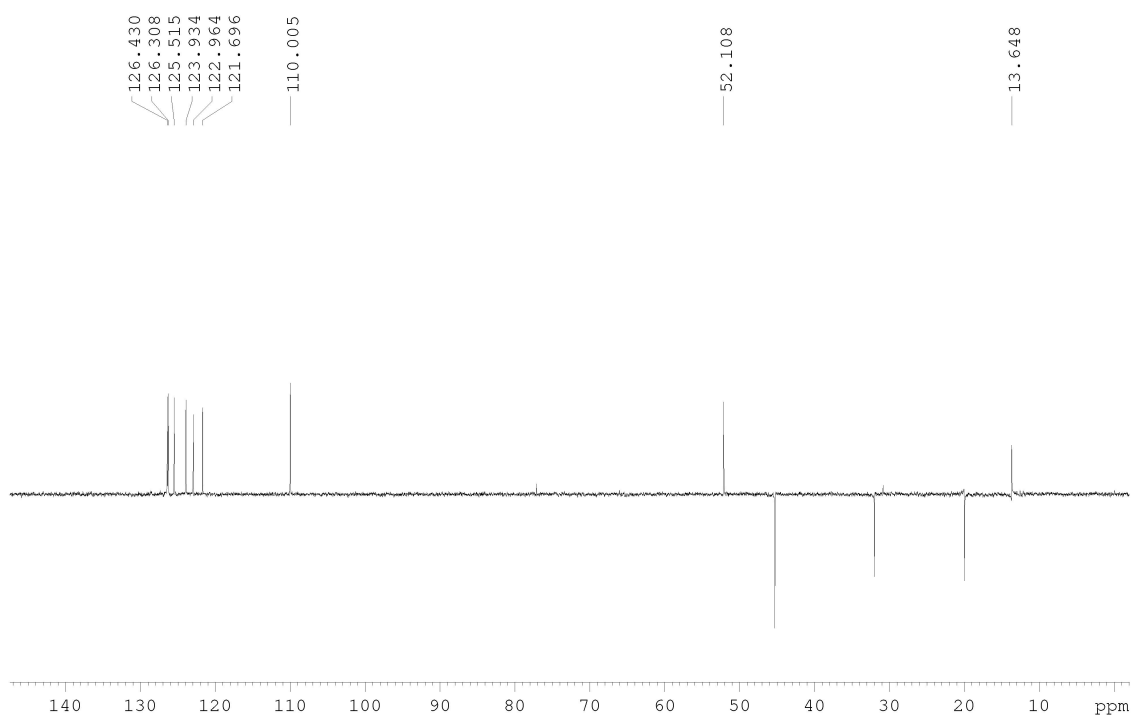


Figure S6.3. DEPT spectrum in CDCl_3 for N^{N} ligand **36**.

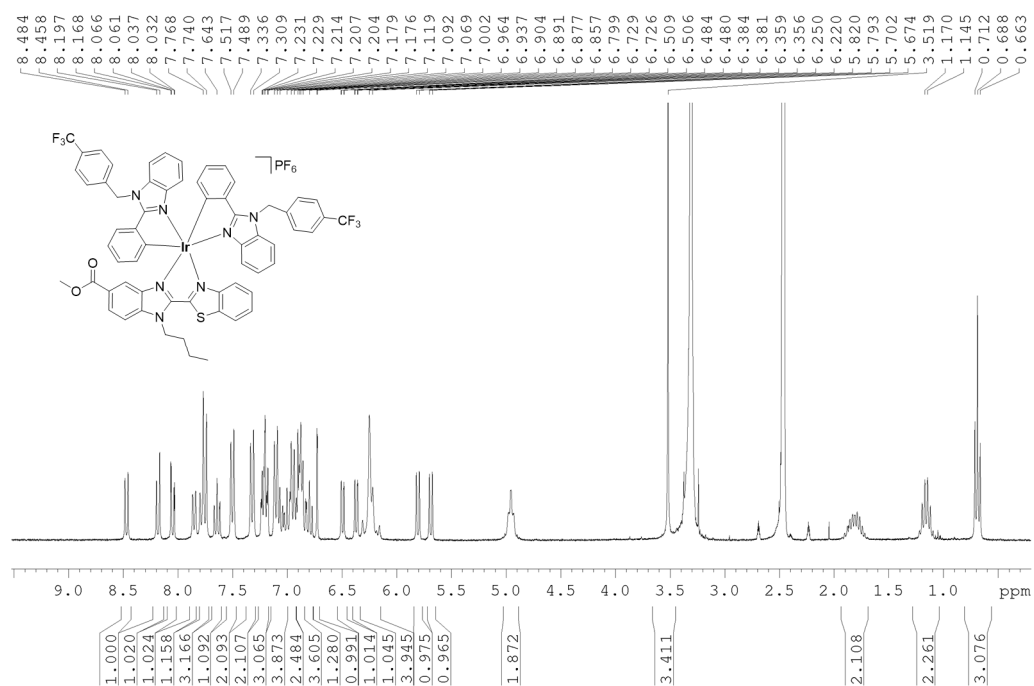


Figure S6.4. ^1H NMR of **37** in $[\text{D}_6]\text{DMSO}$.

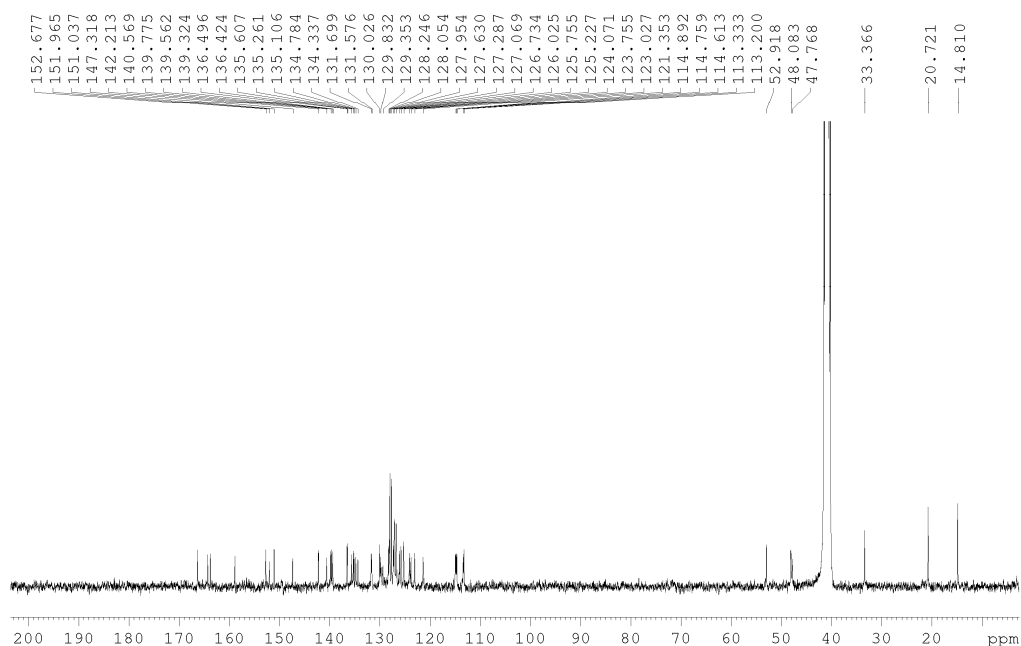


Figure S6.5. ¹³C NMR of 37 in [D₆]DMSO.

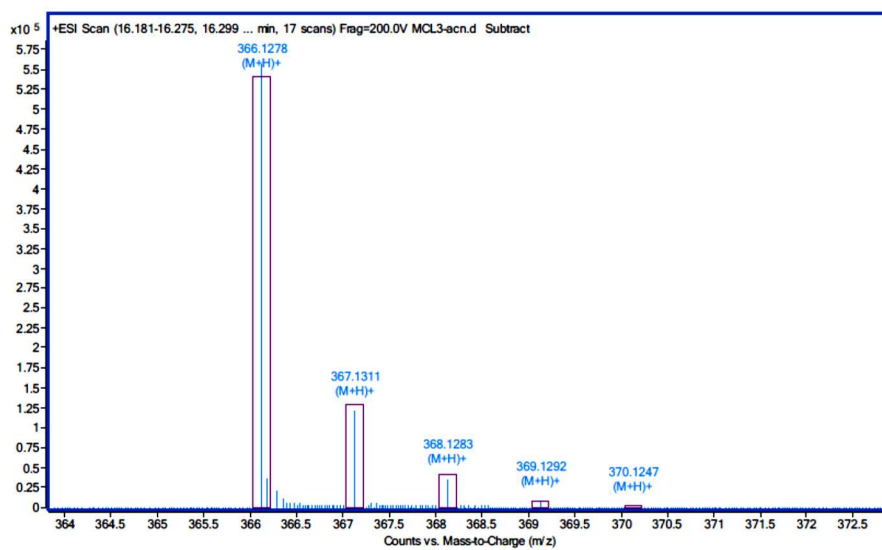


Figure S6.6 Positive-ion ESI-MS of the new N[^]N ligand 36.

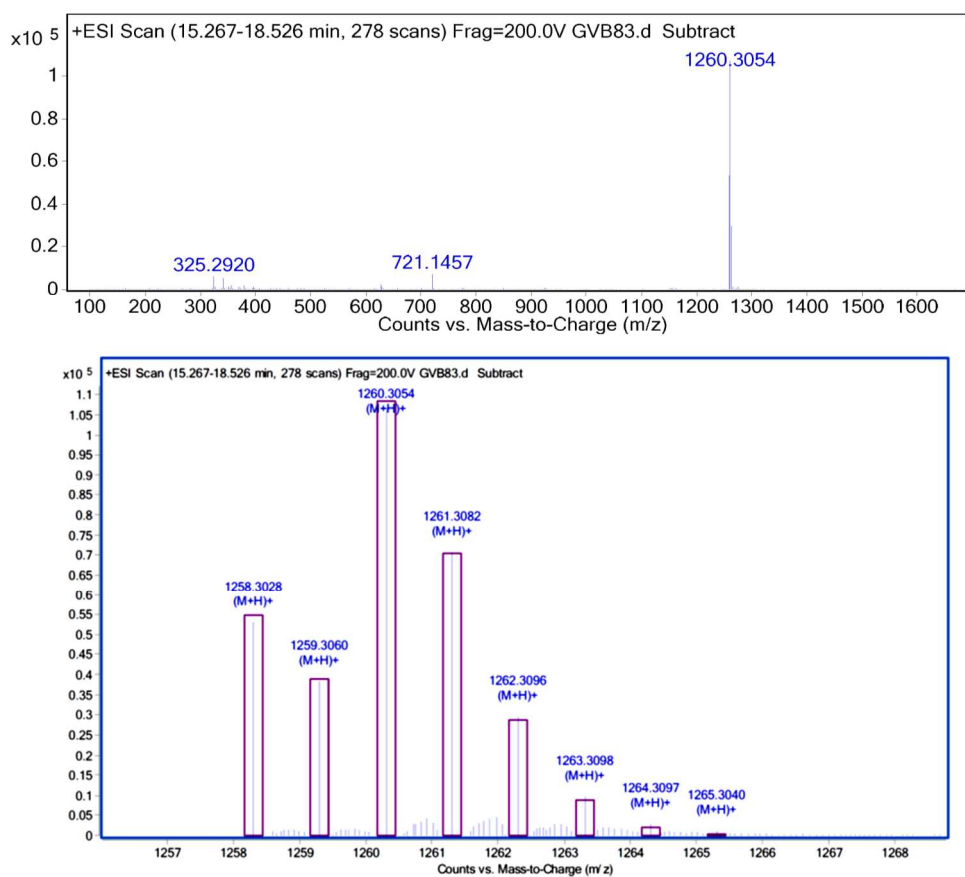


Figure S6.7. Positive-ion ESI-MS of complex 37.

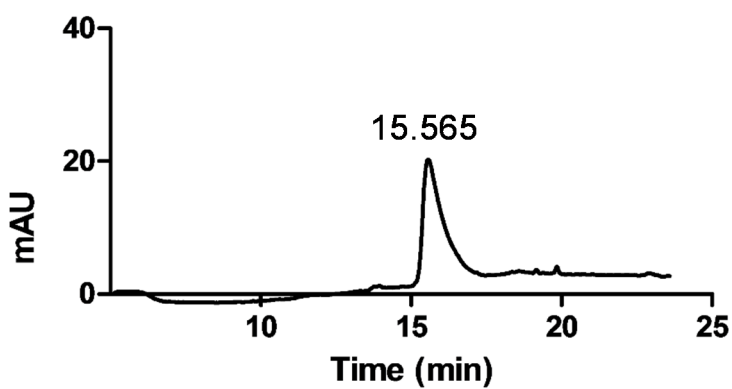


Figure S6.8. HPLC chromatogram of 37.

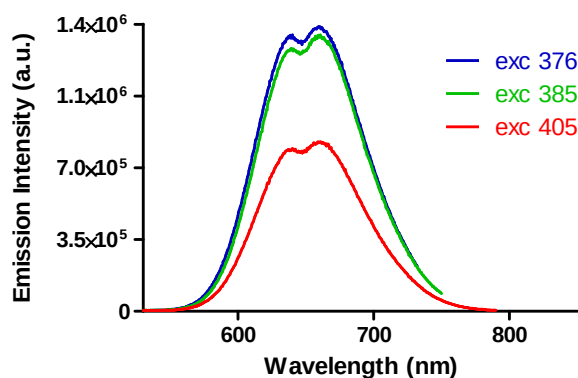


Figure S6.9. Emission spectra of **37** in water at different excitation wavelengths.

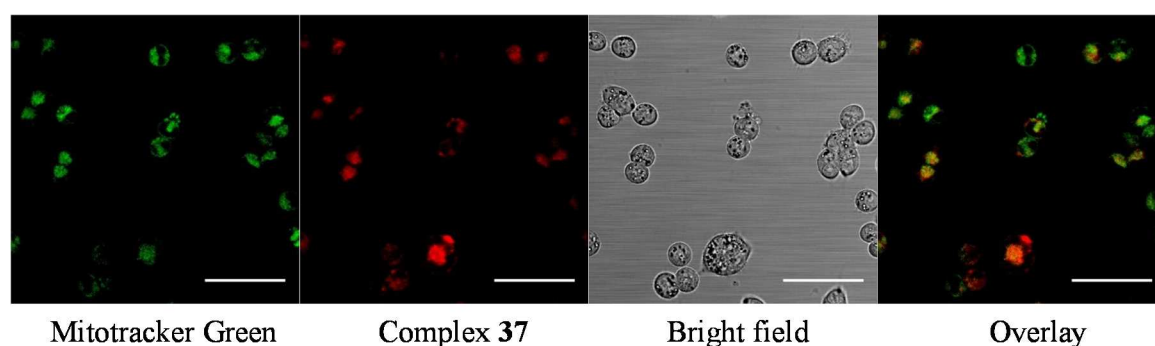


Figure S6.10. Confocal microscopy images of A2780 cells treated with complex **37** (5 μM) for 2 h and co-stained with Mitotracker Green FM (0.1 μM) for 30 min. Co-localization was calculated as Pearson correlation coefficient using the JACoP plugin in ImageJ software and averaged for 10 cells.

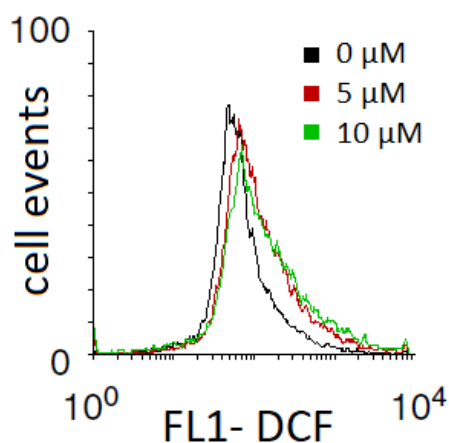
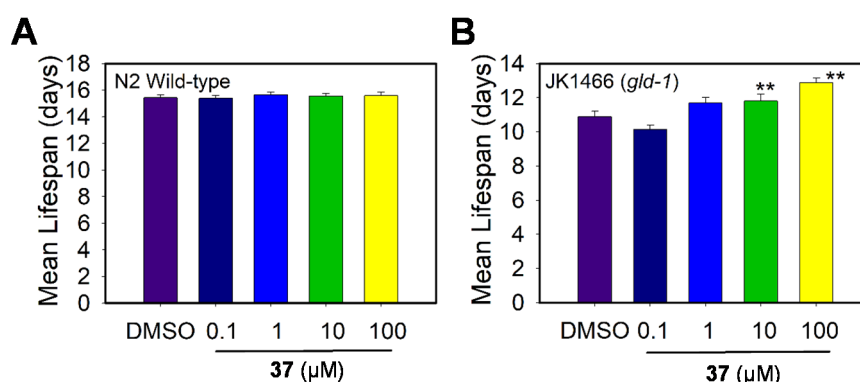
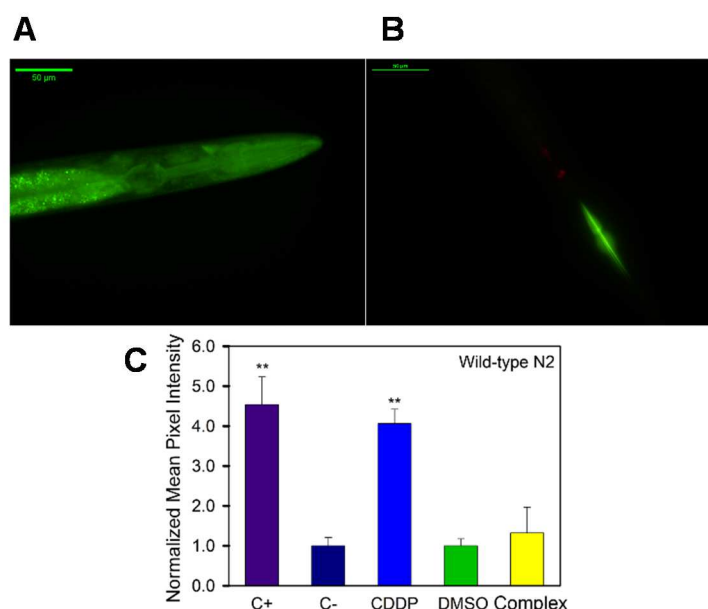


Figure S6.11. Representative flow cytometry histograms of ROS levels in A2780 cells after treatment with **37** for 2 h at indicated concentration as measured by 2',7'-dichlorofluorescein (DCF) staining in FL1-H channel.

Table S6.1. *In vivo* measurements of tumor size

	C (μ M)	n	Tumor Area (μ m ²)	S.D	Reduction (%)	p value vs control
Control		39	14169.02	2613.06	0.00	
CDDP	100	24	8149.27	1072.96	-42.49	<0.00001
DMSO (0.4 %)		20	13622.75	1287.38	0.00	0.099197 ^a
37	0.1	47	12193.85	2703.41	-10.49	<0.00001
	1	21	9517.95	1558.20	-30.13	<0.00001
	10	20	8794.06	1461.13	-35.45	<0.00001
	100	42	8033.38	2067.55	-41.03	<0.00001

^a Control vs DMSO control**Figure S6.12.** A) Mean lifespan of N2 animals treated with different concentrations of the iridium complex, B) Mean lifespan of JK1466 strain treated with different concentrations of 37. Data represented as mean lifespan $\pm$ E.S, **significantly at $p \leq 0.05$ by Log Rank test.**Figure S6.13.** Representative images of wild-type animals, treated with A) juglone as positive control (20 μ M) or B) 100 μ M of complex 37. C) Quantification of the fluorescence of the assays is shown, being data represented as mean $\pm$ S.D, $n > 15$, two independent experiments were performed, **significantly at $p \leq 0.05$ by ANOVA test. Scale bars 50 μ m.

Chapter 7

Vehiculation of highly luminescent neutral Ir(III) complexes to improve their biocompatibility and activity against HeLa cells

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¹ H and ¹³ C NMR spectra of new Ir(III) compounds	S107
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Table S7.2. Drug loading and EE parameters for NPs-38 and NPs-40.	S114
Emission decay kinetics of nanoparticles	S115
Table S7.3. IC ₅₀ values (mg/mL) obtained for nanoparticles in HeLa cells	S115
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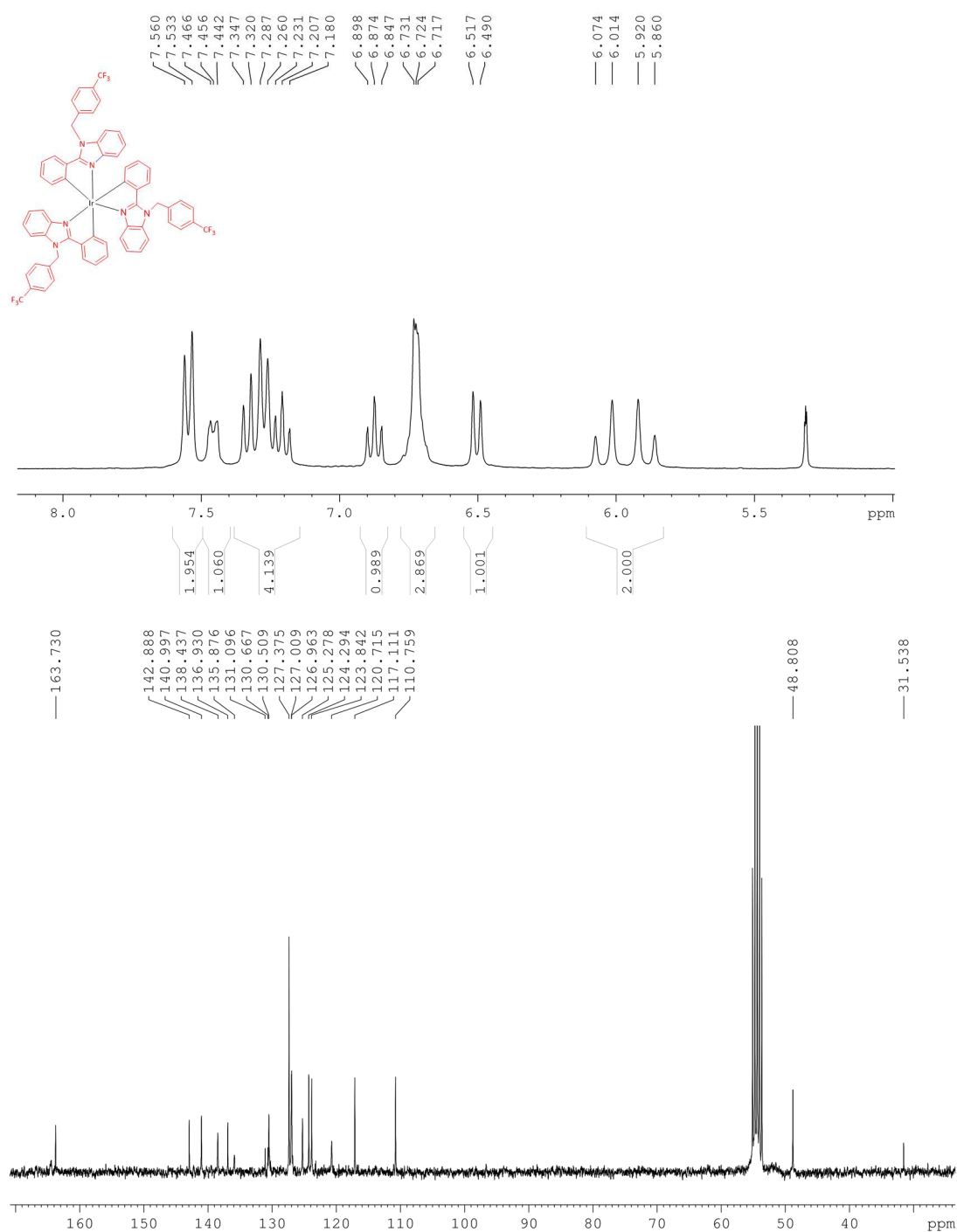


Figure S7.1. ¹H and ¹³C NMR in CD₂Cl₂ for **38**.

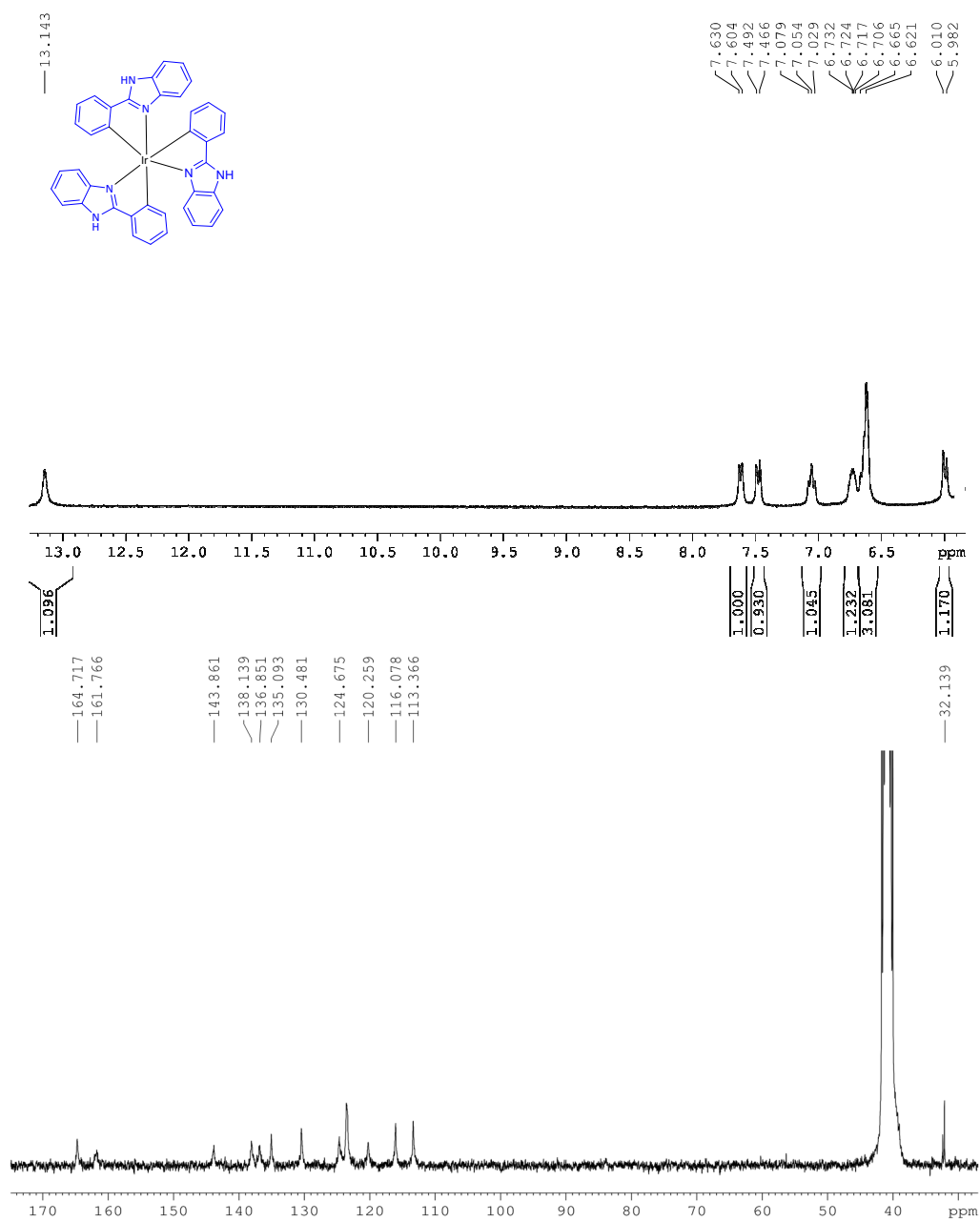


Figure S7.2. ^1H and ^{13}C NMR in $[\text{D}_6]\text{DMSO}$ for **39**.

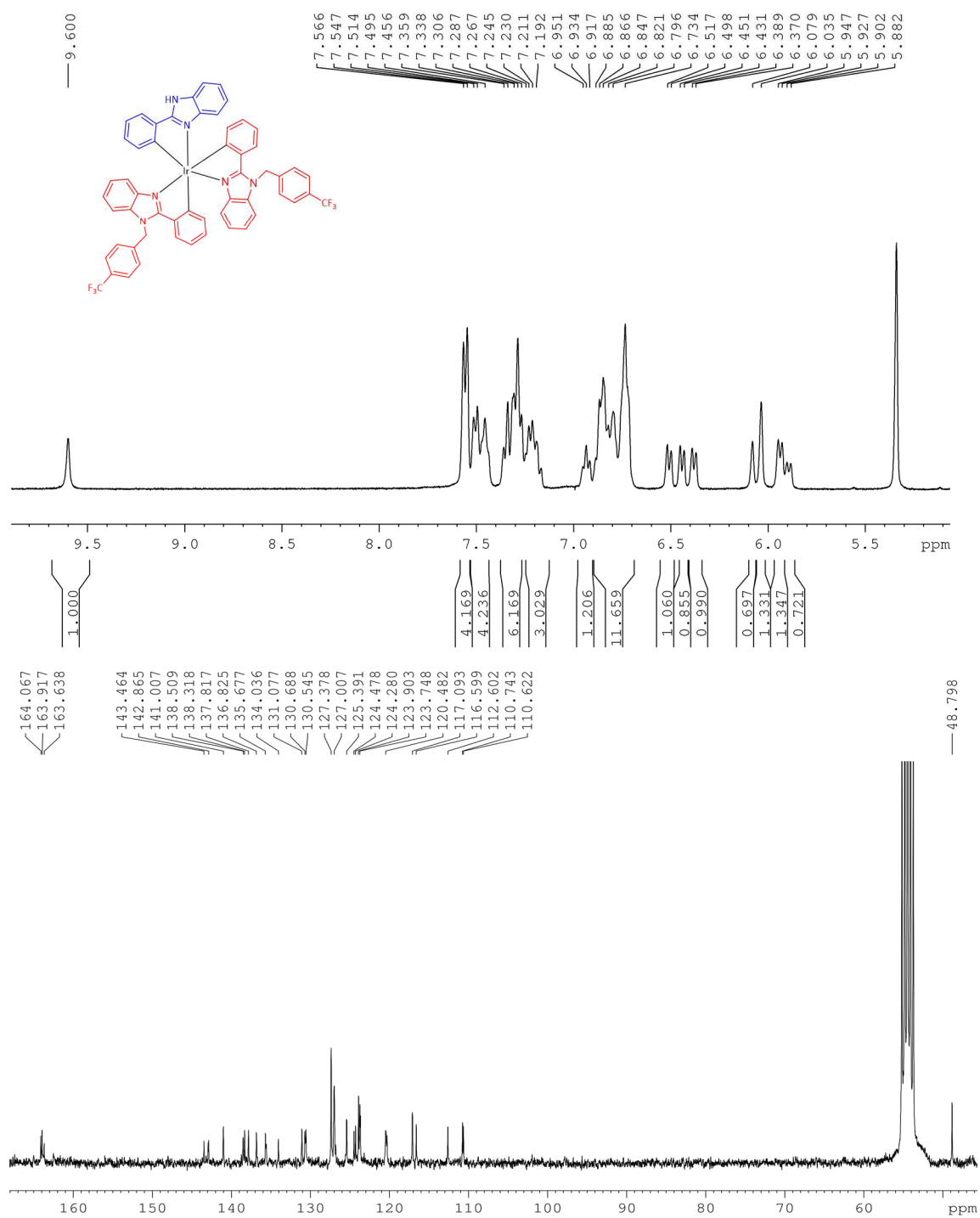


Figure S7.3. ¹H and ¹³C NMR in CD₂Cl₂ for **40**.

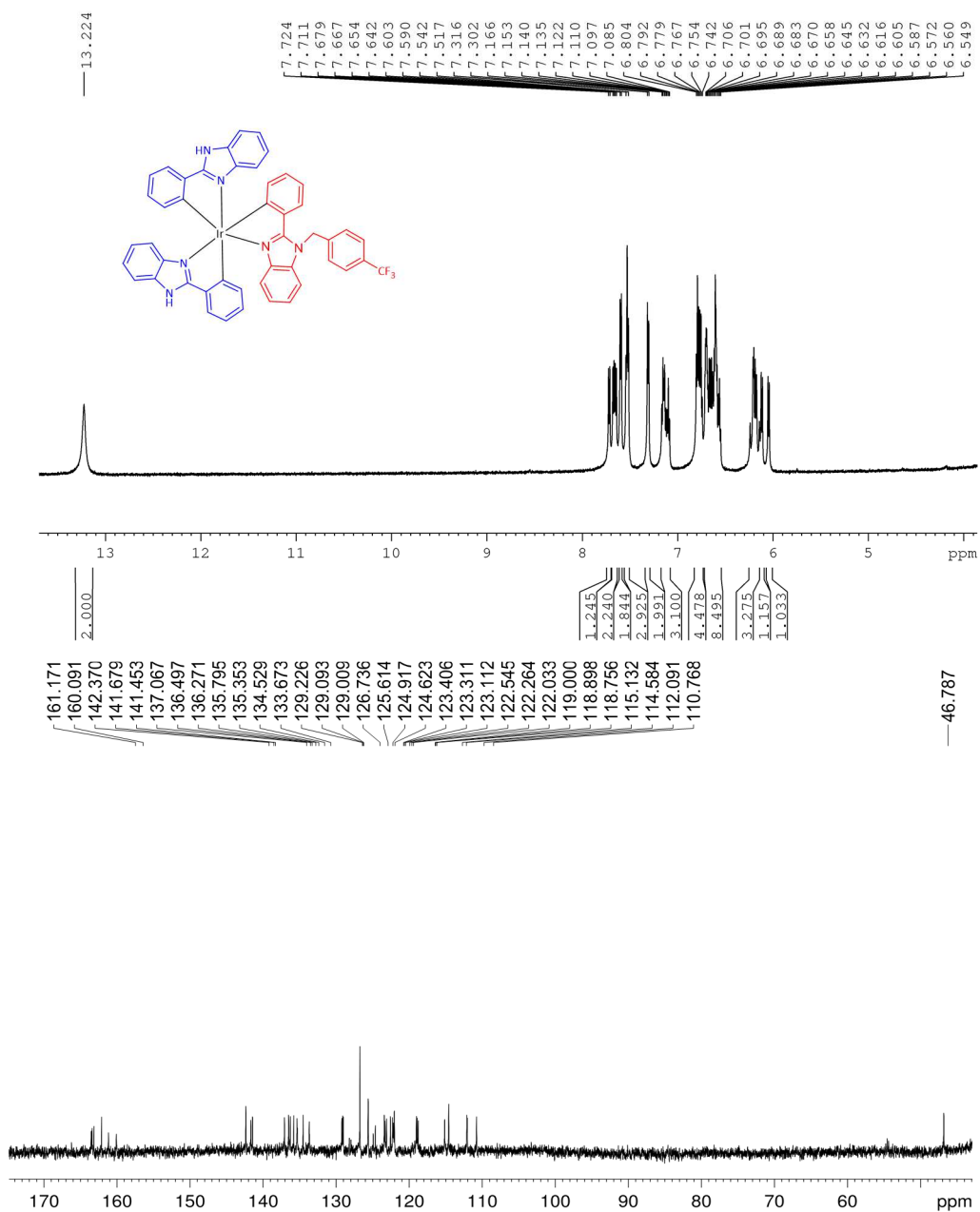


Figure S7.4. ¹H and ¹³C NMR in [D₆]DMSO for **41**.

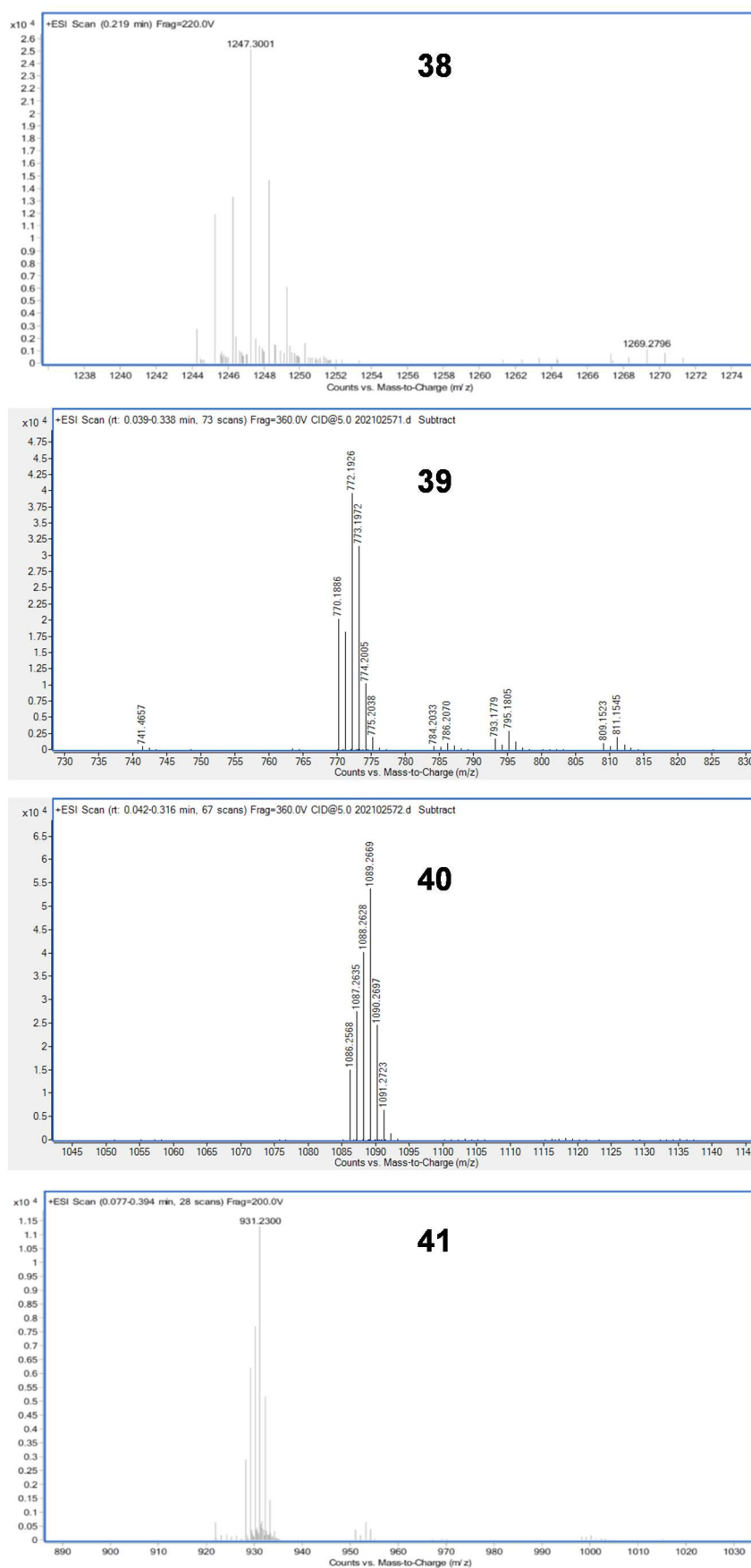


Figure S7.5. Positive-ion ESI-MS of complexes 38-41.

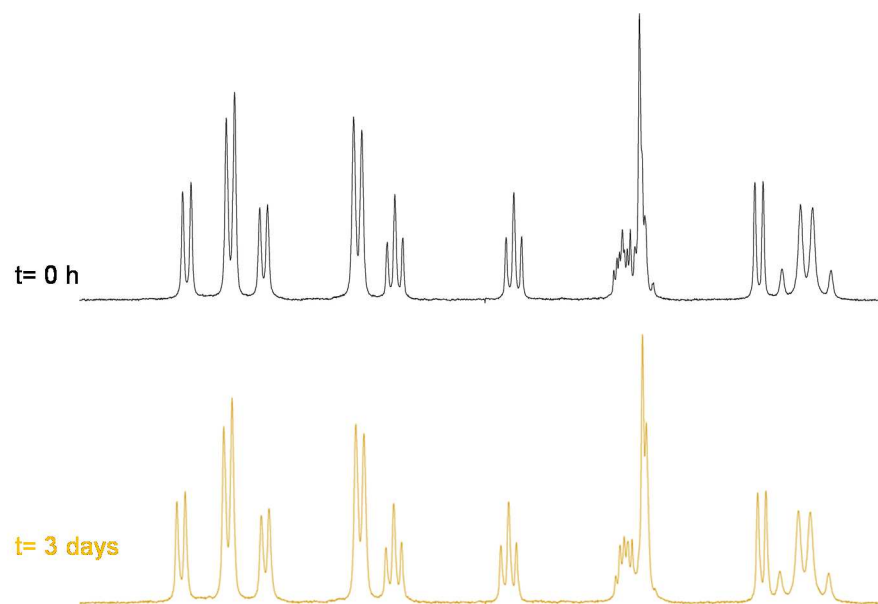


Figure S7.6. ^1H NMR spectra of **38** measured after dissolving immediately in $[\text{D}_6]\text{DMSO}$ (top) and after 3 days (bottom) at RT.

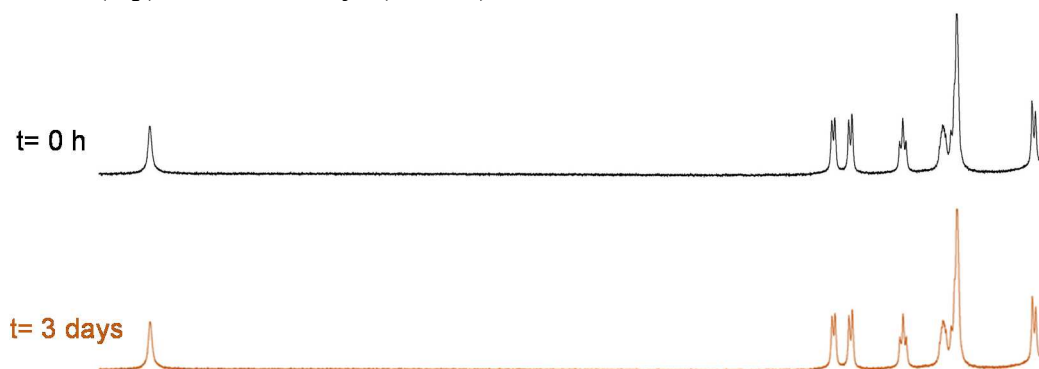


Figure S7.7. ^1H NMR spectra of **39** measured after dissolving immediately in $[\text{D}_6]\text{DMSO}$ (top) and after 3 days (bottom) at RT.

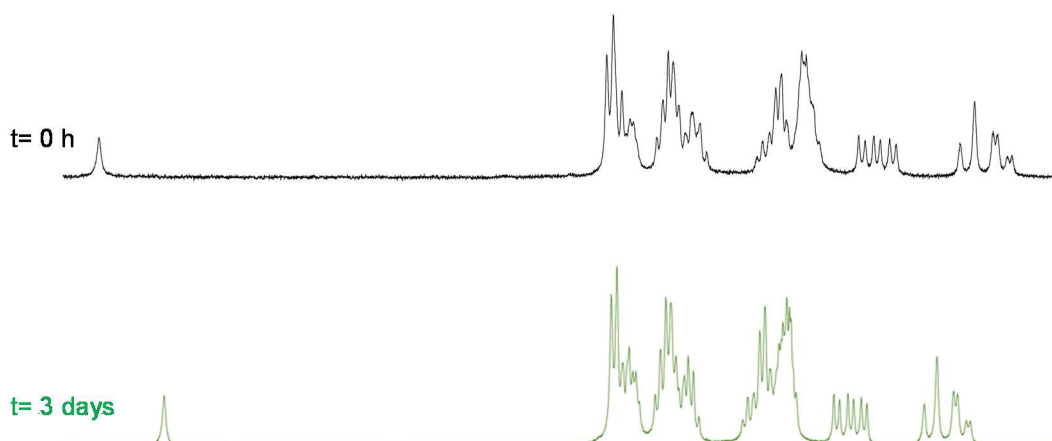


Figure S7.8. ^1H NMR spectra of **40** measured after dissolving immediately in $[\text{D}_6]\text{DMSO}$ (top) and after 3 days (bottom) at RT.

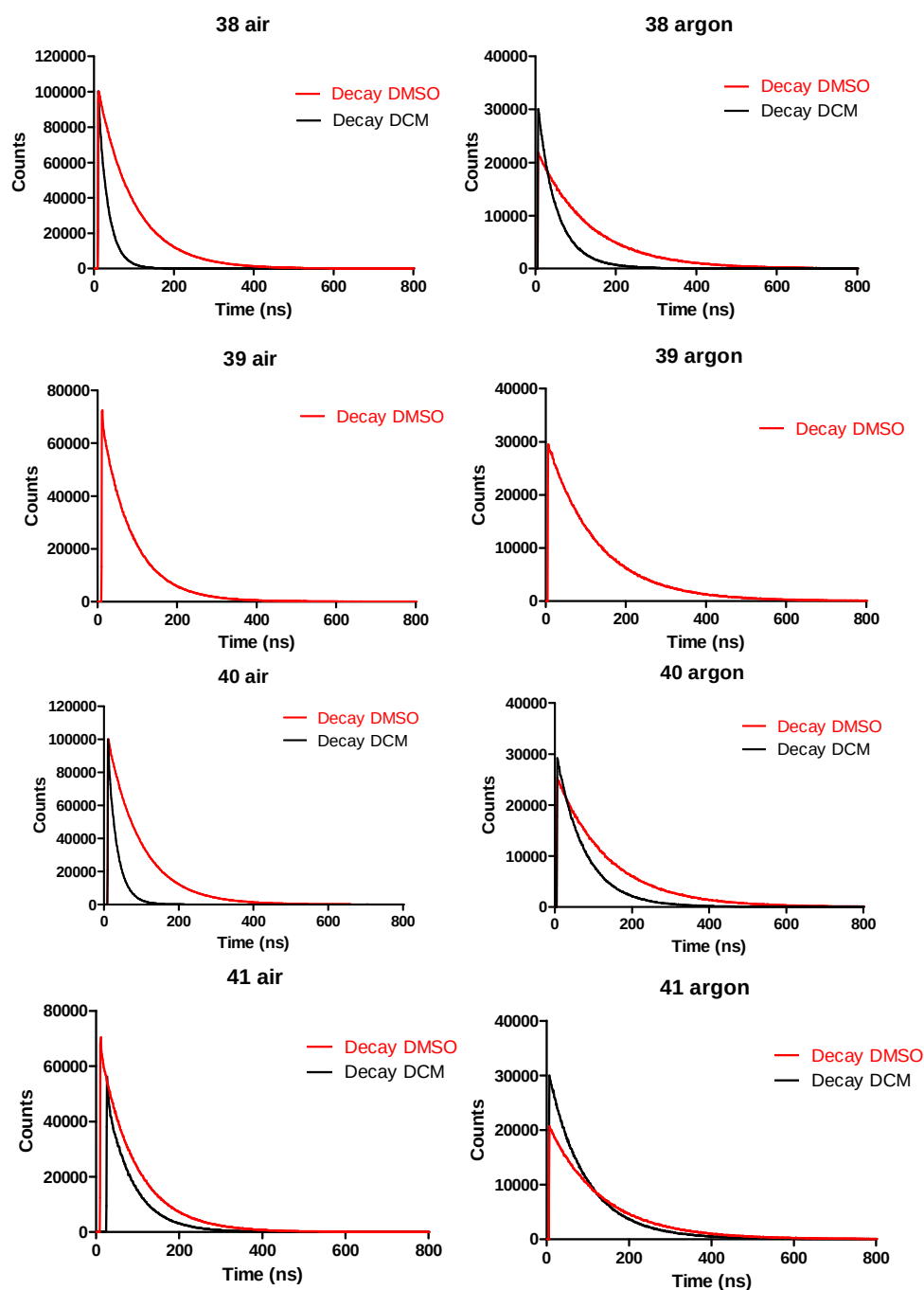


Figure S7.9. Emission decay kinetics of complexes in dichloromethane and DMSO.

Table S7.1. Average particle size of NPs

NPs	22.48 ± 3.95
NPs-38	18.61 ± 0.39
NPs-40	18.45 ± 0.39

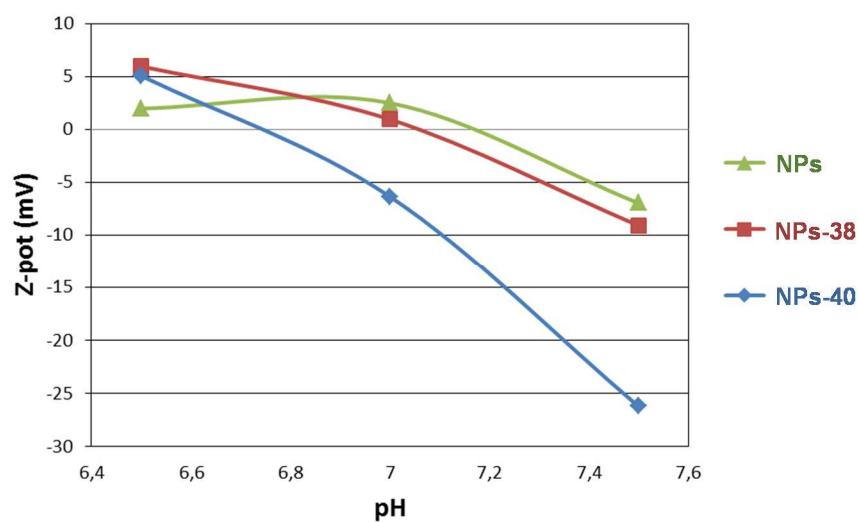


Figure S7.10. Z-potential measurements under different pH conditions of non-loaded NPs and **38** and **40** loaded NPs.

Table S7.2. Drug loading and EE parameters for NPs-**38** and NPs-**40**.

	[NPs] (mg/mL)	Drug Loading (DL, %)	Drug Loading, (μ M)	Encapsulation Efficiency (EE, %)
NPs	64.8 ± 0.5	-	-	-
NPs- 38	45.4 ± 3.1	0.36%	130.5 ± 16.2	41.4%
NPs- 40	64.8 ± 0.5	0.34%	206.2 ± 16.8	40.0%

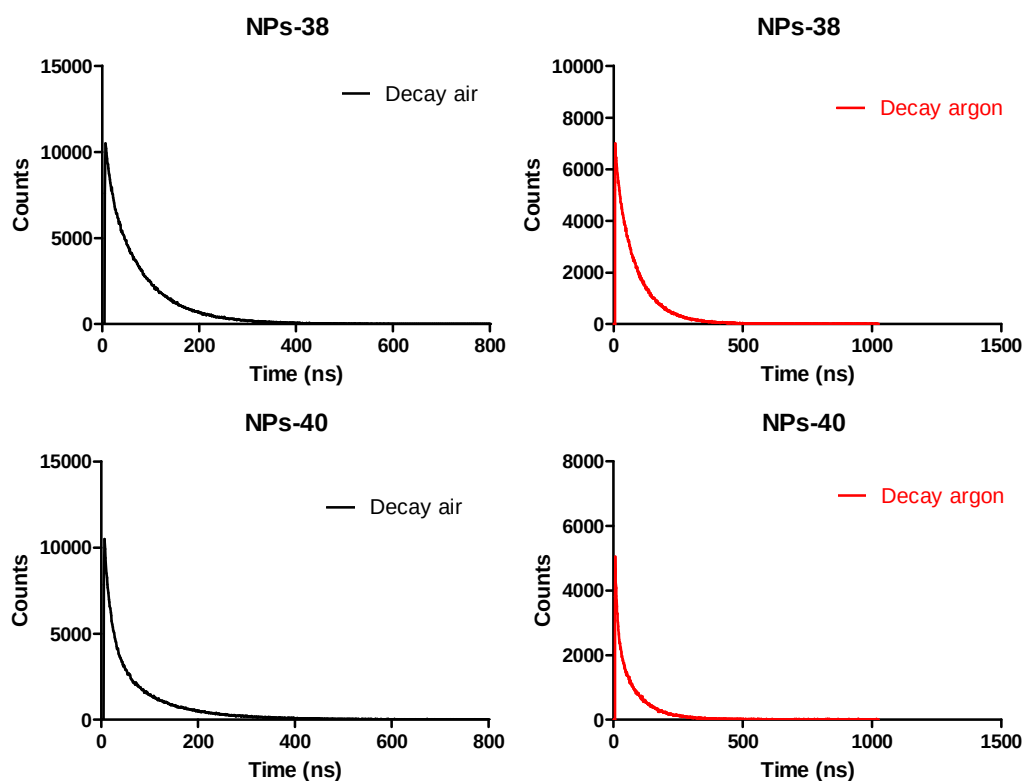


Figure S7.11. Emission decay kinetics of nanoparticles in water.

Table S7.3. Cytotoxicity [IC_{50} mean values (mg/mL)] obtained for nanoparticles in HeLa cells.^a

	HeLa			BGM
	2 h	24 h	48 h	24h
NPs	>30	>30	>30	>30
NPs-38	8.2 ± 0.4	1.01 ± 0.07	0.62 ± 0.02	>30
NPs-38	15 ± 2	1.12 ± 0.06	0.92 ± 0.04	>30

^a The results are expressed as mean values $\pm$ SD of two independent experiments. The terms >30 indicate that no IC_{50} was reached up to that concentration.

References

- (1) Chen, T. R. Microscopic Demonstration of Mycoplasma Contamination in Cell Cultures and Cell Culture Media. *TCA manual / Tissue Culture Association* **1975**, 1 (4), 229–232. <https://doi.org/10.1007/BF00917008>.

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