Iminophosphoranes As Useful Precursors To Potential Transition Metal-Based Cancer Chemotherapeutics

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IMINOPHOSPHORANES AS USEFUL PRECURSORS TO POTENTIAL TRANSITION METAL-BASED CANCER CHEMOTHERAPEUTICS

by

MALGORZATA FRIK

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ABSTRACT

IMINOPHOSPHORANES AS USEFUL PRECURSORS TO POTENTIAL TRANSITION METAL-BASED CANCER CHEMOTHERAPEUTICS

by

Malgorzata Frik

Adviser: Professor Maria Contel

During the past two decades, gold(III), platinum(II), palladium(II) and ruthenium(II) compounds have been investigated as potential anticancer drugs. Our group at Brooklyn College reported on the cytotoxic properties of neutral and cationic gold(III), palladium(II) and platinum(II) complexes with iminophosphoranes (IM) or iminophosphine ligands of the general formula $R_3P=NR'$. These IM ligands have been very useful to synthesize and stabilize compounds of $d^8$ transition metals which displayed higher toxicity against leukemia, prostate cancer and ovarian cancer cells when compared to normal T-lymphocytes. They also seemed to have a mode of action different from that of cisplatin.

This thesis describes the synthesis of coordination and organometallic, gold(III), platinum(II), palladium(II) and ruthenium(II) complexes with different iminophosphorane ligands, the study of their stability in solution by different techniques and of their interaction with biological targets (mostly DNA and HSA). I have also included data on the biological activity of these compounds (in vitro and for selected complexes in vivo) to understand their potential as cancer chemotherapeutics. Most of these compounds have displayed excellent anticancer properties by a mode of action different from that currently accepted for cisplatin and, in some cases, have displayed a lower toxicity, better activity or better permeability in vivo. These results are described in three different chapters as summarized below.
In Chapter III, I describe the synthesis and characterization of a series of coordination gold(III), palladium(II), and platinum(II) complexes with a luminescent IM ligand derived from 8-aminoquinoline \([\text{Ph}_3\text{P}=\text{N}-\text{C}_9\text{H}_6\text{N}]\). The coordination palladium(II) and platinum(II) compounds can evolve further, under appropriate conditions, to give stable cyclometalated \textit{endo} species \([\text{M}\{\kappa^3-\text{C},\text{N}-\text{C}_6\text{H}_4(\text{PPh}_2=\text{N}-8-\text{C}_9\text{H}_6\text{N})\}\text{Cl}](\text{M} = \text{Pd}, \text{Pt})\) by C-H activation of the phenyl group of the PPh$_3$ fragment. The compounds have been evaluated for their antiproliferative properties in a human ovarian cancer cell line (A2780S), in human lung cancer cells (A-549) and in a non-tumorigenic human embryonic kidney cell line (HEK-293T). Most compounds have been more toxic to the ovarian cancer cell line than to the non-tumorigenic cell line. The new complexes interact with human serum albumin (HSA) faster than cisplatin. Studies of the interactions of the compounds with DNA indicate that, in some cases, they exert anticancer effects \textit{in vitro} based on different mechanisms of action with respect to cisplatin. The stability of cyclometallated compounds is markedly higher than that of coordination complexes.

In Chapter IV, I describe the synthesis, characterization and stability studies of new organometallic gold(II) and platinum(II) complexes containing cyclometalated IM ligands. Most compounds are more cytotoxic to a number of human cancer cell lines than cisplatin. A cationic Pt(II) derivative \([\{\text{Pt}\{\kappa^2-\text{C},\text{N}-\text{C}_6\text{H}_4(\text{PPh}_2=\text{N}(\text{C}_6\text{H}_5})(\text{COD})\}(\text{PF}_6)\}]\) displays IC$_{50}$ values in the sub-micromolar range. Its cell death mechanism is mainly through caspase-dependent apoptosis but it triggers caspase-independent cell death when apoptosis is blocked. Permeability studies by two different assays: \textit{in vitro} caco-2 monolayers and a rat perfusion model have revealed a high permeability profile for this compound (comparable to that of metoprolol or caffeine) and an estimated oral fraction absorbed of 100% which potentially makes it a good candidate for oral administration.
Lastly in Chapter V, I describe the synthesis, characterization and stability studies of a series of organometallic ruthenium(II) complexes containing iminophosphorane ligands. These cationic compounds with chloride as counterion are highly soluble in water (70-100 mg/mL). Most compounds (especially the highly water-soluble compound- \( \text{[((} \eta^6\text{-p-cymene})\text{Ru}\{\text{Ph}_3\text{P=NO}_2\text{-N-C}_5\text{H}_4}\text{-κ-N,O}\text{]}\text{Cl}]\text{Cl} \)) are more cytotoxic to a number of human cancer cell lines than cisplatin. Initial mechanistic studies indicate that the cell death type for these compounds is mainly through canonical or caspase-dependent apoptosis, non-dependent on p53, and that the compounds do not interact with DNA or inhibit protease cathepsin B. *In vivo* experiments of \( \text{[((} \eta^6\text{-p-cymene})\text{Ru}\{\text{Ph}_3\text{P=NO}_2\text{-N-C}_5\text{H}_4}\text{-κ-N,O}\text{]}\text{Cl}]\text{Cl} \) on MDA-MB-231 xenografts in NOD.CB17-Prkdc SCID/J mice showed an impressive tumor reduction (shrinkage) of 56% after 28 days of treatment (14 doses of 5 mg/kg every other day) with low systemic toxicity. Pharmacokinetic studies showed a quick absorption in plasma with preferential accumulation in the breast tumor tissues when compared to kidney and liver, which may explain its high efficacy *in vivo*. 
DEDICATION

To my wonderful, loving and caring parents, Maria and Henryk Frik, for their support, encouragement, wisdom and motivation throughout the years.

&

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&

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TABLE OF CONTENTS

LIST OF FIGURES ............................................................................................................................................... xv
LIST OF TABLES ................................................................................................................................................... xxiii
LIST OF SCHEMES ................................................................................................................................................ xxv
LIST OF CHARTS .................................................................................................................................................. xxvi
ABBREVIATIONS AND ACCRONYMYS ................................................................................................................ xxvii

CHAPTER

I. BACKGROUND AND SIGNIFICANCE ................................................................................................................. 1

1.1. Cancer, statistics and current treatment .............................................................................................................. 1

1.1.1. Prostate cancer ........................................................................................................................................... 3
1.1.2. Triple-negative breast cancer ......................................................................................................................... 3
1.1.3. Lung cancer .............................................................................................................................................. 4
1.1.4. Leukemia ................................................................................................................................................... 5
1.1.5. Ovarian cancer .......................................................................................................................................... 5
1.1.6. Pancreas cancer ...................................................................................................................................... 6
1.1.7. Limitations of the current existing chemotherapeutic treatments for the above described cancers .............................................................................................................................................. 6

1.2. The foundation of platinum-based drugs, as cancer chemotherapeutics. Limitations and resistance ............................................................................................................................................. 7

1.3. Platinum(II), gold (III), palladium(II) and ruthenium(II and III) compounds as alternative-chemotherapeutics agents ........................................................................................................................................... 11

1.3.1. Platinum(II) compounds ............................................................................................................................. 11
1.3.2. Gold(III) compounds ................................................................................................................................. 13
1.3.3. Palladium(II) compounds .......................................................................................................................... 15
1.3.4. Ruthenium(II and III) compounds ............................................................................................................... 17

1.4. New drug design with iminophosphorane ligands .............................................................................................. 19

1.5. Previous studies in our group using iminophosphorane ligands ......................................................................... 21

1.6. Bibliography .................................................................................................................................................. 24
II. OBJECTIVES ........................................................................................................... 30
   2.1. Ultimate goal of the work described in this Thesis .......................................... 30
   2.2. Hypothesis .................................................................................................. 30
   2.3. Specific Aims of this Thesis .................................................................... 31

III. SYNTHESIS OF COORDINATION AND ORGANOMETALLIC GOLD(III),
PALLADIUM(II) AND PLATINUM(II) COMPOUNDS CONTAINING A
LUMINESCENT IMINOPHOSPHORANE LIGAND. IN VITRO EVALUATION AND
PRELIMINARY MECHANISTIC INSIGHTS ...................................................... 33
   3.1. Potential of metal-based theranostics ......................................................... 33
   3.2. Synthesis and characterization ................................................................ 34
   3.3. Luminescence studies .............................................................................. 39
   3.4. Antiproliferation studies ........................................................................... 42
   3.5. Interaction with plasmid (pBR322) DNA .................................................. 44
   3.6. Interaction with human serum albumin (HSA) ........................................... 46
   3.7. Conclusion ................................................................................................ 48
   3.8. Experimental section .............................................................................. 48
   3.9 Appendixes ................................................................................................ 56
      3.9.1. Crystallographic Data for Compounds 3 and 4 ................................. 56
      3.9.2. Luminescence Studies for Ligand 1 and compounds 2-7 ............. 58
      3.9.3. Stability of compounds 2-7 in DMSO-\textit{d}_6 solution overtime assessed by $^{31}\text{P}\{^1\text{H}\}$
           NMR spectroscopy. Selected $^{31}\text{P}\{^1\text{H}\}$ NMR spectra for compounds 4 and 7 ... 67
   3.10. Bibliography ............................................................................................ 69

IV. SYNTHESIS OF CYCLOMETALLATED IMINOPHOSPHORANE GOLD(III) AND
PLATINUM(II) COMPLEXES. IN VITRO EVALUATION. PERMEABILITY AND
PRELIMINARY MECHANISTIC STUDIES ..................................................... 73
   4.1. Cyclometallated gold(III) and platinum(II) compounds as potential anticancer agents .. 73
   4.2. Synthesis and characterization .................................................................. 76
   4.3. Biological activity \textit{in vitro} ........................................................................ 83
4.3.1. Antiproliferative studies *in vitro* .......................................................... 83
4.3.2. Mechanism of cell death for compound 5 ............................................... 85
4.3.3. Lipophilicity and permeability assays .................................................... 91
4.4. Interactions with DNA ............................................................................... 96
  4.4.1. Interaction of complexes 1-5 with plasmid (pBR322) DNA ...................... 96
  4.4.2. Interaction with *Calf Thymus* DNA ...................................................... 97
4.5. Interaction with HSA .................................................................................. 99
4.6. Conclusions ............................................................................................... 101
4.7. Experimental section ................................................................................. 102
4.8. Appendix ..................................................................................................... 113
  4.8.1. Crystallographic Data for Compounds 2 and 4 ........................................ 113
  4.8.2. Stability of complexes in DMSO-\textit{d}_6 and D_2O solution overtime assessed by
          \textsuperscript{31}P\{\textsuperscript{1}H\} NMR spectroscopy ...................................... 116
  4.8.3. Selected \textsuperscript{1}H and \textsuperscript{31}P\{\textsuperscript{1}H\} NMR spectra showing the stability of complexed in
          DMSO-\textit{d}_6 overtime ................................................................................. 116
  4.8.4. UV-Vis spectra of compounds 4 and 5 in CH\textsubscript{2}Cl\textsubscript{2} and in DMSO, and in 1%
          DMSO-PBS solution overtime .................................................................. 120
4.9. Bibliography .............................................................................................. 125

V. RUTHENIUM(II) COMPOUNDS WITH N,N- CHELATING AND C,N- (CYCLOMETALLATED) IMINOPHOSPHORANE LIGANDS. \textit{IN VITRO} AND \textit{IN VIVO} EVALUATION AND PRELIMINARY MECHANISTIC STUDIES ......................... 132
5.1. Ruthenium(II and III) as potential anticancer agents ...................................... 132
5.2. Synthesis and characterization of ruthenium(II) organometallic complexes containing
      iminophosphorane ligands ........................................................................... 135
5.3. Biological activity \textit{in vitro} .......................................................................... 140
  5.3.1. Antiproliferation studies \textit{in vitro} ........................................................ 140
  5.3.2. Mechanism of cell death ....................................................................... 142
5.4. Reactivity with biomolecules .................................................................. 147
  5.4.1. Interaction with DNA ........................................................................... 147
  5.4.2. Lack of inhibition of capthensin B ........................................................ 151
5.4.3. Interaction with HSA ................................................................. 151

5.5. Effects on tumor growth in vivo with compound 2 .................................. 153
  5.5.1. Evaluation of the lethal and maximum tolerated doses ...................... 153
  5.5.2. Effects of 2 in MDA-MB-231 mouse xenografts ............................... 153
  5.5.3. Pharmacokinetic study .......................................................... 155

5.6. Conclusions ................................................................................. 158

5.7. Experimental section ................................................................. 159

5.8. Appendix .................................................................................. 175
  5.8.1. Crystallographic Data for Compound 1 ......................................... 175
  5.8.2. $^1$H NMR spectra of compounds 2-4, 7 and 8 .......................... 176
  5.8.3. Stability of compounds 1-4, 8 and 9 in DMSO-$d_6$ and D$_2$O solution overtime assessed by $^{31}$P{$^1$H} NMR spectroscopy ......................................................... 180
  5.8.4. $^{31}$P{$^1$H} NMR spectra showing the stability of compounds 1-4, 8 and 9 in DMSO-$d_6$ overtime ............................................................... 181
  5.8.5. $^{31}$P{$^1$H}, $^1$H NMR and $^{13}$C NMR spectra of compounds 2, 3 and 4 in D$_2$O overtime ................................................................. 184
  5.8.6. $^{31}$P{$^1$H} NMR spectra of compounds 2 in a 100 mM NaCl/D$_2$O solution overtime ............................................................... 188
  5.8.7. $^{31}$P{$^1$H} and $^1$H NMR spectra of compounds 2 and 3 in a D$_2$O solution at 80°C during 1 h ............................................................... 188
  5.8.8. Mass spectra (ESI+) of compound 2 in H$_2$O solution overtime (5 days) ...... 189
  5.8.9. Study of the effect of 2 in the levels of proteins of the Bcl-2 family ............ 192
  5.8.10. Experiments to assess the interaction of compounds 2-4 with CT DNA by circular dichroism ................................................................. 193

5.9. Bibliography .............................................................................. 194

VI. CONCLUSIONS AND FUTURE DIRECTIONS ........................................ 203

VII. PUBLICATIONS, PATENTS AND CONFERENCE PRESENTATIONS WHICH HAVE RESULTED FROM THIS WORK ........................................ 206
LIST OF FIGURES

CHAPTER I

**Figure 1.** Current therapeutic treatments for leukemia, prostate, pancreas, lung, ovarian and triple negative-breast cancer ................................................................. 4

**Figure 2.** Platinum-based chemotherapeutic agents .................................................. 8

**Figure 3.** Binding of cisplatin to guanine bases of the DNA through intrastrand and interstands crosslink ................................................................. 9

**Figure 4.** Recently developed platinum anticancer agents ........................................ 12

**Figure 5.** Representative gold(III) dithiocarbamate and cycloaurated complexes .... 14

**Figure 6.** Representative C,N-palladium(II) complexes .......................................... 16

**Figure 7.** Selected ruthenium(III) compounds with important antitumor and/or antimetastatic properties ................................................................. 18

**Figure 8.** Examples of iminophosphorane ligands ..................................................... 20

**Figure 9.** Resonance from the stabilized iminophosphorane ligand ......................... 20

**Figure 10.** Selected cytotoxic gold(III), platinum(II) and palladium(II) complexes with IM ligands (1-6) prepared in our research group .................................................. 22

CHAPTER III

**Figure 1.** Molecular structure of the cation in compound [Au((Ph$_3$P=N-8-C$_9$H$_6$N)-κ-N,N)Cl$_2$]ClO$_4$ 3 and of the compound [Pd((Ph$_3$P=N-8-C$_9$H$_6$N)-κ-N,N)Cl$_2$] 4 with the atomic numbering scheme ................................................................. 36

**Figure 2.** Study of the luminescence of compound 6 in DMSO solution 5 x 10$^{-4}$ M at RT over time (24 hours) ................................................................. 41

**Figure 3.** Electrophoresis mobility shift assays for cisplatin and compounds 2-7 (see Experimental for details). DNA refers to untreated plasmid pBR322. A, B, C and D correspond to metal/DNAbp ratios of 0.25, 0.5, 1.0 and 2.0 respectively ........................................ 44

**Figure 4.** (A) Fluorescence titration curve of HSA with compound 3. Arrow indicates the increase of quencher concentration. (B) Stern-Volmer plot for HSA fluorescence quenching observed with compounds 2-7 and cisplatin ................................................................. 47
Figure 5. Absorption spectra of ligand 1 and gold compounds 2 and 3 in DMSO solution (5 x 10^{-4} M) at RT ........................................................................................................ 58

Figure 6. Absorption spectra of ligand 1 and palladium compounds 4 and 6 in DMSO solution (5 x 10^{-4} M) at RT ........................................................................................................ 58

Figure 7. Absorption spectra of ligand 1 and platinum compounds 5 and 7 in DMSO solution (5 x 10^{-4} M) at RT ........................................................................................................ 59

Figure 8. Excitation (blue) and emission (red) spectra of compound 1 in DMSO solution (5 x 10^{-4} M) at RT ........................................................................................................ 59

Figure 9. Luminescence of compound 1 in DMSO solution (5 x 10^{-4} M) at RT over time (24 h) ........................................................................................................ 60

Figure 10. Excitation (blue) and emission (red) spectra of compound 2 in DMSO solution (5 x 10^{-4} M) at RT ........................................................................................................ 60

Figure 11. Luminescence of compound 2 in DMSO solution (5 x 10^{-4} M) at RT over time (24 h) ........................................................................................................ 61

Figure 12. Excitation (blue) and emission (red) spectra of compound 3 in DMSO solution (5 x 10^{-4} M) at RT ........................................................................................................ 61

Figure 13. Luminescence of compound 3 in DMSO solution (5 x 10^{-4} M) at RT over time (24 h) ........................................................................................................ 62

Figure 14. Excitation (blue and green) and emission (red) spectra of compound 4 in DMSO solution (5 x 10^{-4} M) at RT ........................................................................................................ 62

Figure 15. Luminescence of compound 4 in DMSO solution (5 x 10^{-4} M) at RT over time (24 h) ........................................................................................................ 63

Figure 16. Excitation (blue and green) and emission (red) spectra of compound 5 in DMSO solution (5 x 10^{-4} M) at RT ........................................................................................................ 63

Figure 17. Excitation (blue) and emission (red) spectra of compound 5 in DMSO solution (5 x 10^{-4} M) at RT immediately after the first measurement of luminescence ............................................................................. 64

Figure 18. Excitation (blue) and emission (red) spectra of compound 6 in DMSO solution (5 x 10^{-4} M) at RT ........................................................................................................ 64

Figure 19. Luminescence of compound 6 in DMSO solution (5 x 10^{-4} M) at RT over time (24 h) ........................................................................................................ 65
Figure 20. Excitation (blue) and emission (red) spectra of compound 7 in DMSO solution (5 x 10^{-4} M) at RT …………………………………………………………………………………………………… 65

Figure 21. Luminescence of compound 7 in DMSO solution (5 x 10^{-4} M) at RT over time (24 h) …………………………………………………………………………………………… 66

Figure 22. Excitation (blue) and emission (red) spectra of compound 7 in DMSO:H_{2}O (50:50) solution (5 x 10^{-4} M) at RT …………………………………………………………………………………………… 66

Figure 23. Luminescence of compound 7 in DMSO:H_{2}O (50:50) solution (5 x 10^{-4} M) at RT over time (24h) …………………………………………………………………………………………… 67

Figure 24. Conversion of coordination palladium compound [Pd((Ph_{3}P=N-8-C_{9}H_{6}N)-κ-N,N)Cl_{2}] 4 (δ = 30.0 ppm) into cyclometalated [Pd{κ^{3}-C,N,N-C_{6}H_{4}(PPh_{3}=N-8-C_{9}H_{6}N)}Cl] 6 (δ = 44.6 ppm) in d^{6}-DMSO solution over time. Small peak at δ = 29.4 ppm corresponds to PPh_{3}=O ……………………………………………………………………………………………………… 68

Figure 25. Study of the stability of cycloplatinated compound [Pt{κ^{3}-C,N,N-C_{6}H_{4}(PPh_{2}=N-8-C_{9}H_{6}N)}Cl] 7 (δ = 45.2 ppm) by $^{31}$P-{^{1}H} NMR spectroscopy in d^{6}-DMSO solution over time …………………………………………………………………………………. 68

CHAPTER IV

Figure 1. Molecular structure of compound 2 …………………………………………………………………………………………………………………………………………………………………………………………… 78

Figure 2. Molecular structure of the cation in compound 4. The anion [Hg_{2}Cl_{6}]^{2-} is omitted for clarity ……………………………………………………………………………………………………… 81

Figure 3. Role of caspases on cell death induced by compound 5 in A595 cells. Cells were cultured for 24 h in the presence of 5 at the indicated concentrations, alone (solid lines) or combined with the general caspase inhibitor z-VAD-fmk (dashed lines). Subsequently, phosphatidylserine exposure (triangles) and cell membrane permeabilization (squares) were analyzed by flow cytometry after staining with annexin V-DY634 and 7-AAD respectively. Results are mean+/−SD of two independent experiments with duplicates …….. 86

Figure 4. Caspase implication in mitochondrial effects of compound 5 in A549 cells. Cells were cultured for 24 h in the presence of compound 5 at the indicated concentrations, alone (solid line) or combined with the general caspase inhibitor z-VAD-fmk (dashed line). Then, transmembrane mitochondrial potential was analyzed by flow cytometry after staining with the probe DiOC6(3). Results are mean+/−SD of two independent experiments with duplicates ………………… 87
Figure 5. Implication of caspases in cell death induced by compound 5 in Jurkat cells. Cells were treated with compound 5 for 6 or 24 h in the presence or in the absence of the general caspase inhibitor z-VAD-fmk. Membrane integrity was analyzed by flow cytometry after staining with 7-AAD, respectively, as indicated in the Experimental Section. Results are mean±SD of two independent experiments .......................................................... 88

Figure 6. Jurkat cells were treated with 5 or 5+z-VAD for 24 h and then harvested, washed and seeded in fresh medium. After further 24 h in fresh medium mitochondrial transmembrane potential (ΔΨm) was analyzed as indicated in the Experimental Section. Results are mean+/−SD of three independent experiments .......................................................... 89

Figure 7. Compound 5 induces apoptosis in Jurkat (upper panels) and A549 cells (bottom panels). Cells were cultured for 24 h in the presence of compound 5 (0.5 µM), alone or combined with the general caspase inhibitor z-VAD-fmk or left untreated (Control). Nuclei were stained with Hoechst 33342 (10 µg/ml) and cells were photographed under UV light. Magnification x400 .................................................................................................................. 89

Figure 8. Jurkat-pLVTHM (control) and Jurkat-shBak cells were treated with compound 5 for 24 h. Mitochondrial transmembrane potential was analyzed as indicated in the Experimental Section. Results are mean±SD of three independent experiments ............................................. 90

Figure 9. Permeability values obtained from apical to basal (Pab) and from basal to apical (Pba) of Cisplatin (at different concentrations), cycloplatinated 4, 5 and permeability reference compounds (Metoprolol, Cimetidine and Lucifer Yellow) at 20 µM in Caco-2 cells. Data correspond to the averaged values for three independent experiments ............................................. 92

Figure 10. Absorption rate coefficients in rats ................................................................. 94

Figure 11. Correlation between oral fractions absorbed vs permeability values obtained from Caco-2 cell monolayers transport assay in apical to basal direction (Pab). Gray diamonds correspond to the internally validated correlation (IVC).54 Triangles correspond to permeability reference compounds (metoprolol/caffeine for high permeability, Cimetidine for intermediate permeability and Lucifer Yellow for low permeability). Light grey squares correspond on tested compounds 4 and 5 ............................................................................................................. 95

Figure 12. Electrophoresis mobility shift assays for cisplatin and compounds 1-5 (see Experimental Section for details). DNA refers to untreated plasmid pBR322. A, B, C and D correspond to metal/DNAbp ratios of 0.25, 0.5, 1.0 and 2.0 respectively ............................................. 97
Figure 13. CD spectra of CT DNA (195 µM) and CT DNA incubated with 0.1, 0.25, 0.5 and 1.0 equivalents of compounds 3 (A), 4 (B), 5 (C) and cisplatin (D) for 20 h at 37°C ………… 98

Figure 14. (A) Fluorescence titration curve of HSA for compound 3. Arrow indicates the increase of quencher concentration. Stern-Volmer plot for HSA fluorescence quenching observed with compounds 1-5 and cisplatin (B), 2-4 and cisplatin (C) and 1, 5 and cisplatin (D) ……………………………………………………………………………………………. 100

Figure 15. $^{31}$P{$^1$H} NMR spectrum of compound 1 in DMSO-d$_6$ ($\delta$ 33.57 (s) ppm) over time …………………………………………………………………………………………………………………. 116

Figure 16. $^{31}$P{$^1$H} NMR spectrum of compound 2 in DMSO-d$_6$ ($\delta$ -2.66 (s) ppm) over time …………………………………………………………………………………………………………………. 117

Figure 17. $^{31}$P{$^1$H} NMR spectrum of compound 3 in DMSO-d$_6$ ($\delta$ -10.26 (s) ppm) over time …………………………………………………………………………………………………………………. 117

Figure 18. $^1$H NMR spectrum of compound 3 in DMSO-d$_6$ over time …………………… 118

Figure 19. $^{31}$P{$^1$H} NMR spectrum of compound 4 in DMSO-d$_6$ ($\delta$ -62.57 (s) ppm) over time …………………………………………………………………………………………………………………. 118

Figure 20. $^1$H NMR spectrum of compound 4 in DMSO-d$_6$ over time …………………… 119

Figure 21. $^{31}$P{$^1$H} NMR spectra of compound 5 in DMSO-d$_6$ ($\delta$ 63.10 (s) and –148.60 (hept) ppm) over time………………………………………………………………………………………………………………. 119

Figure 22. $^1$H NMR spectrum of compound 5 in DMSO-d$_6$ over time …………………… 120

Figure 23. UV-visible spectrum of compound 4 (4.0 µM) in dichloromethane …………… 120

Figure 24. UV-visible spectrum of compound 4 (50.0 µM) in DMSO recorded over time ….. 121

Figure 25. UV-visible spectrum of compound 4 (15.0 µM) in 1:99 DMSO/PBS-1X (pH 7.4) recorded over time, incubation at RT ………………………………………………………………………………… 121

Figure 26. UV-visible spectrum of compound 5 (10.0 µM) in dichloromethane …………… 122

Figure 27. UV-visible spectrum of compound 5 (50.0 µM) in DMSO recorded over time ….. 122

Figure 28. UV-visible spectrum of compound 5 (50.0 µM) in 1:99 DMSO/PBS-1X (pH 7.4) recorded over time, incubation at RT ………………………………………………………………………………… 123

Figure 29. UV-visible spectrum of iminophosphorane ligand Ph$_3$P=NPh (50.0 µM) in DMSO………………………………………………………………………………………………………………. 123

Figure 30. UV-visible spectrum of iminophosphorane ligand Ph$_3$P=NPh (50.0 µM) in 1:99 DMSO/PBS-1X (pH 7.4) ………………………………………………………………………………… 124
CHAPTER V

Figure 1. Selected ruthenium(III) and (II) compounds with important antitumor and/or antimetastatic properties ................................................................. 133

Figure 2. Molecular structure of the cation of compound 1 ........................................ 187

Figure 3. Nuclei morphology after treatment of Jurkat cells with compounds 2 and 3 …… 142

Figure 4. Dose-response quantification of PS exposure (A) and time-course analysis of PS exposure and ΔΨ_m loss (B) caused by 2 and 3 in Jurkat cells ................................. 143

Figure 5. Effect of the general caspase inhibitor z-VAD-fmk in apoptotic features induced by 2 and 3 .................................................................................. 144

Figure 6. Analysis of long-term protection by z-VAD-fmk ........................................... 145

Figure 7. p53 protein levels after short-term incubation of A549 cells with 2. B-Actin levels were determined in the same membranes as a total protein loading control ......................... 146

Figure 8. Electrophoresis mobility shift assays for cisplatin, [Ru(η^6-p-cymene)Cl]_2 and compounds 1-4 and 8, 9. DNA refers to untreated plasmid pBR322. A, B, C and D correspond to metal/DNAbp ratios of 0.25, 0.5, 1.0 and 2.0 respectively ........................................................ 149

Figure 9. (A) Fluorescence titration curve of HSA with compound 4. Arrow indicates the increase of quencher concentration. (B) Stern-Volmer plot for HSA fluorescence quenching observed with compounds 1-4, 8, 9 and cisplatin .................................................. 152

Figure 10. % of reduction of tumor burden in a cohort of 12 female NOD.CB17-Prkd scid/J mice inoculated subcutaneously with 5x10^6 MDA-MB-231 cells. The treatment started when tumors were palpable (5-6 mm diameter). 6 mice were treated with compound 2 (pink bars), 6 were treated with the vehicle 100 µl Normal Saline (0.9% NaCl) (black bars). 2 was administered in the amount of 5 mg/kg/every other day ........................................... 154

Figure 11. Concentration of Compound 2 in plasma at various intervals after the first dose …157

Figure 12. Compound 2 ruthenium content in tissues at the end of efficacy study. Data represents mean ± SD. N = 3; * indicates P < 0.05 ................................................................. 157

Figure 13. ^1H NMR spectra of compound 2 in CDCl_3 ............................................... 176

Figure 14. ^1H NMR spectra of compound 3 in CDCl_3 ............................................... 176

Figure 15. ^1H NMR spectra of compound 4 in CDCl_3 ............................................... 177

Figure 16. Variable temperature ^1H NMR spectra of compound 4 in CDCl_3 (magnification zone 6.1-3.6 ppm) ................................................................. 177
Figure 17. Variable temperature $^1$H spectra of compound 4 in CDCl$_3$ (magnification zone 2.9-0.1 ppm) ................................................................. 178

Figure 18. Variable temperature $^{31}$P{$^1$H} NMR spectra of compound 4 in CDCl$_3$ (δ 46.45 (s) ppm) ........................................................................................................... 178

Figure 19. $^1$H NMR spectra of compound 7 in CDCl$_3$ ................................................................. 179

Figure 20. $^1$H NMR spectra of compound 8 in CDCl$_3$ ................................................................. 179

Figure 21. $^{31}$P{$^1$H} NMR spectra of compound 1 in DMSO-d$_6$ (δ 24.27 (s) and –144.20 (h) ppm) overtime ................................................................................................................. 181

Figure 22. $^{31}$P{$^1$H} NMR spectra of compound 2 in DMSO-d$_6$ (δ 24.26 (s) ppm) overtime ... 181

Figure 23. $^{31}$P{$^1$H} NMR spectra of compound 3 in DMSO-d$_6$ (δ 37.84 (s) ppm) overtime .... 182

Figure 24. $^{31}$P{$^1$H} NMR spectra of compound 4 in DMSO-d$_6$ (δ 45.99 (s) ppm) overtime ... 182

Figure 25. $^{31}$P{$^1$H} NMR spectra of compound 8 in DMSO-d$_6$ (δ 21.09 (s) ppm) overtime ... 183

Figure 26. $^{31}$P{$^1$H} NMR spectra of compound 9 in DMSO-d$_6$ (δ -11.85 (s) ppm) overtime ... 183

Figure 27. $^{31}$P{$^1$H} NMR spectra of compound 2 in D$_2$O (δ 26.37 (s) ppm) overtime ........... 184

Figure 28. $^1$H NMR spectra of compound 2 in D$_2$O at t = 0 and after 5 days .......................... 184

Figure 29. $^{13}$C NMR spectra of compound 2 in D$_2$O at t = 0 and after 5 days .......................... 185

Figure 30. $^{13}$C NMR spectra of compound 2 in D$_2$O at t = 0 and after 5 days .......................... 185

Figure 31. $^{31}$P{$^1$H} NMR spectra of compound 3 in D$_2$O (δ 37.79 (s) ppm) overtime ........... 186

Figure 32. $^1$H NMR spectra of compound 3 in D$_2$O overtime ............................................... 186

Figure 33. $^1$H NMR spectra of compound 3 in D$_2$O overtime ............................................... 187

Figure 34. $^{31}$P{$^1$H} NMR spectra of compound 4 in D$_2$O (δ 46.95 (s) ppm) overtime ........... 187

Figure 35. $^{31}$P{$^1$H} NMR spectra of compound 2 in 100mM NaCl/D$_2$O (δ 26.21 (s) ppm) overtime ....................................................................................................................... 188

Figure 36. $^{31}$P{$^1$H} NMR spectra of compound 2 in D$_2$O (δ 26.48 (s) ppm) after heating at 80°C for one hour ....................................................................................................................... 188

Figure 37. $^{31}$P{$^1$H} NMR spectra of compound 3 in D$_2$O (δ 37.84 (s) ppm) after heating at 80°C for one hour ....................................................................................................................... 189

Figure 38. MS ESI+ of compound 2 in H$_2$O solution at t = 0 ....................................................... 189

Figure 39. Magnification of [m/z] from 600 to 665 of compound 2 in H$_2$O solution at t = 0 ....................................................................................................................... 189
Figure 40. MS ESI+ of compound 2 in H₂O solution at t = 5 days ........................................ 190

Figure 41. Magnification of [m/z] from 600 to 665 of compound 2 in H₂O solution at
t = 5 days .................................................................................................................. 191

Figure 42. Magnification of peak at [m/z]: 617.1 corresponding to species
[(η⁶-p-cymene)Ru(IM-k-C,N-C₆H₄(PPh₂=N-CO-2-N-C₅H₄)]⁺ in the MS ESI+ spectrum of
compound 2 in H₂O solution at t = 5 days. Insert: theoretical isotopic distribution ............... 191

Figure 43. Effect of 2 in the levels of proteins of the Bcl-2 family. Jurkat cells were left
untreated (control) or incubated for 6 h with compound 2 (1 µM). At the end of incubations total
protein extracts were prepared as described in the Experimental section and analyzed by Western
Blot with specific antibodies as indicated. Blots are representative of three independent
experiments ................................................................................................................................ 192

Figure 44. CD spectra of CT DNA (48 µM) and CT DNA incubated with 0.1, 0.25 and 0.5
equivalents of compounds 1 (A), 2 (B), 3 (C) and 4 (D) for 20 h at 37 °C ............................... 193
LIST OF TABLES

CHAPTER I
Table 1. Ten leading cancer types for the estimated new cancer cases and deaths by sex in the United States for 2015 ................................................................. 2

CHAPTER III
Table 1. Selected bond lengths [Å] and angles [°] for complexes 3 and 4 ......................... 37
Table 2. Luminescent spectral data and lifetime measurement for the compounds 1-7 .......... 40
Table 3. IC_{50} (µM) of ligand 1, PPh_{3} oxide and metal complexes 2-7, and cisplatin in human cell lines ..................................................................................................................... 43
Table 4. Crystal Data and Structure Refinement for Complexes 3 and 4 ......................... 57

CHAPTER IV
Table 1. Selected Structural Parameters of complex 2 obtained from X-ray single crystal diffraction studies. Bond lengths in [Å] and angles in [°] ......................................................... 79
Table 2. Selected Structural Parameters of the cation in complex 4 obtained from X-ray single crystal diffraction studies. Bond lengths in [Å] and angles in [°] ......................................................... 82
Table 3. IC_{50} (µM) of metal complexes 1-5, ligand COD and cisplatin in human cell lines. All compounds were dissolved in 1% of DMSO and diluted with water before addition to cell culture medium for a 24 h incubation period. Cisplatin was dissolved in H_{2}O ......................... 84
Table 4. Partition Coefficients (ratio n-octanol: phosphate buffer) of Compounds 4 and 5 and reference Metoprolol .......................................................... 91
Table 5. Permeability values obtained by the Caco-2 cell monolayers assay. Metoprolol, Cimetidine and Lucifer Yellow were used as model compounds of high, medium and low oral permeability. Data correspond to the averaged values for three independent experiments ....... 93
Table 6. Absorption rate coefficients, Ka, and permeability values obtained from in situ rat assays. Metoprolol, Cimetidine and Atenolol were used as model compounds of high, medium and low oral permeability. Data correspond to values of six independent experiments .......... 94
Table 7. Crystal data for compounds 2 and 4 ............................................................... 113
Table 8. Selected Structural Parameters of complex 2 obtained from X-ray single crystal
diffraction studies (see drawing above). Bond lengths are given in [Å] and angles in [°] ...... 114
Table 9. Selected Structural Parameters of complex 4 (see drawing incorporating the anion
above) obtained from X-ray single crystal diffraction studies. Bond lengths are given in [Å] and
angles in [°] .................................................................................................................................................. 115

CHAPTER V
Table 1. Selected Structural Parameters of complex 1 obtained from X-ray single crystal
diffraction studies (see drawing above). Bond lengths are given in [Å] and angles in [°] ...... 138
Table 2. IC\(_{50}\) (µM) of metal complexes 1-4, 8-9, [(η\(^6\)-p-cymene)Ru(μ-Cl)Cl]\(_2\)\(^a\), and cisplatin in
human cell lines.\(^b\) All compounds were dissolved in 1% of DMSO and diluted with water before
addition to cell culture medium for a 24 h incubation period. Cisplatin was dissolved in
H\(_2\)O .................................................................................................................................................. 141
Table 3. Effects of 2 on the tumor growth of MDA-MB-231 mammary carcinoma in
NOD.CB17-Prkdc scid/J mice ........................................................................................................ 155
Table 4. Pharmacokinetic parameters of compound 2 after first injection in NOD.CB17-Prkdc
SCID/J mice ........................................................................................................................................ 156
Table 5. Crystal Data and Structure Refinement for compound 1 ........................................ 175
LIST OF SCHEMES

CHAPTER I

Scheme 1. Staudinger and Pomerantz reaction ................................................................. 21

CHAPTER III

Scheme 1. Preparation of the new luminescent metallic complexes 2-7 ......................... 35

CHAPTER IV

Scheme 1. Previously described synthesis of organomercury compounds containing the semi-stabilized IM ligands PPh$_3$=N-CO-2-C$_6$H$_4$ and PTA=N-CO-2-C$_6$H$_4$ which will be described in Chapter V of this Thesis ...................................................................................................................... 77

Scheme 2. Synthesis of gold(III) and platinum (II) cyclometallated exo iminophosphorane complexes. Compound [Au(2-C$_6$H$_4$C(O)N=PPh$_3$)Cl$_2$] (1) had been previously reported ........ 77

Scheme 3. Synthesis of the new platinum (II) cyclometallated endo iminophosphorane complexes 4 and 5 ............................................................................................................................................. 80

CHAPTER V

Scheme 1. Preparation of cationic ruthenium(II) compounds containing IM ligands ........ 136

Scheme 2. Preparation of the new cycloruthenated compounds 8 and 9 containing IM ligands ..................................................................................................................................................... 137
LIST OF CHARTS

CHAPTER I
Chart 1. New drug design with iminophosphorane ligands ........................................ 19

CHAPTER IV
Chart 1. Possible cyclometallation positions in organometallic iminophosphorane
derivatives ....................................................................................................................... 74
Chart 2. Endo gold(II) and exo palladium(II) complexes previously prepared in our group .... 75
Å - angstrom
A-549 - human lung cancer cell lines
A172 - glioblastoma
A2780S - human ovarian cancer cell line
BSA - Bovine Serum Albumin
cat B - cathepsin B
CCC - covalently closed or supercoiled form
CD - circular dichroism
CD₂Cl₂ - deuterated dichloromethane
CD₃CN - deuterated acetonitrile
COD - 1,5-Cyclooctadiene
CT - Calf Thymus
CT scan - computerized tomography scan
DMF - dimethylformamide
DMSO - dimethyl sulfoxide
DNA - deoxyribonucleic acid
DNAbp – deoxyribonucleic acid base pair
DU-145 – prostate cancer cell lines
EMA - European Medicines Agencies
Et₂O - diethyl ether
F₀ and F - observed fluorescence in the absence and presence of the quencher
FDA - Federal Drug Administration
FT-IR - fourier transform infrared spectroscopy
gpNMB - glycoprotein(transmembrane) NMB
GSH - glutathione
HCC - hepatocellular carcinoma
HCT116 - colon carcinoma
HEK-293T - non-tumorigenic human embryonic kidney cell
HSA - Human Serum Albumin
Hz - hertz
IC₅₀ - half maximal inhibitory concentration
ICP-MS - inductively coupled plasma mass spectrometry
IVC - internally validated correlation
Jurkat-T - leukemia cancer cell lines
K - Kelvin
Kₜᵥ - the Stern-Volmer constant
IM - iminophosphorane
MDA-MB-231 - triple negative breast cancer cell lines
MiaPaca2 - pancreas cancer cell lines
MLCT - metal-to-ligand-charge-transfer
mM - milimolar
MS - mass spectrometry
MTD - maximum tolerated dose
MTT assay - cell viability kit assay
NaClO₄ - sodium perchlorate
Nm - nanaomolar
NMR - Nuclear Magnetic Resonance
NOD.CB17-Prkdc SCID/J - non-obese diabetic–severe combined immunodeficiency
OC - open circular or relaxed form
PARP - poly(ADP-ribose) polymerase
PBMC - peripheral blood mononuclear cells
PBS - phosphate buffer saline
Ph - phenyl
Phe - phenylalanine
ppm - parts per million
PTA - 1,3,5-Triaza-7-phosphaadamantane (TPA)
[Q] - quencher concentration
RNA - ribonucleic acid
ROS - reactive oxygen species
RPTC - human renal proximal tubular cells
RT – room temperature
'BuDAD - di-tert-butylazodicarboxylate
TMS - tetramethyilsilane
Tris/HCl - tris-hydrochloride
Trp - tryptophan
TrxR - thioredoxin reductases
Tyr - tyrosine
UV-Vis - Ultraviolet-visible
XTT assay - cell proliferation kit assay
z-VAD-fmk - cell-permeant pan caspase inhibitor
ΔΨm - mitochondrial transmembrane potential
µM - micro molar
µs - microsecond
λ em - emission wavelength
λ exc - excitation wavelength
CHAPTER I

BACKGROUND AND SIGNIFICANCE

1.1. Cancer, statistics and current treatment

Cancer is a major public health problem in the United States and all over the world. It is currently the second leading cause of death in the United States, and is expected to surpass heart disease as the leading cause of death in the nearby future.\(^1\) In the U.S. alone, more than one million people get cancer each year. Although there are many kinds of cancers, cancer starts when normal cells in a part of the body grow out of control. Cells become cancerous due to DNA damage. In normal cells when DNA gets damaged, the cells either repair the damage or die. In cancer cells however, the damaged DNA is not repaired and the cells do not die like the normal cells. The cells go instead on making new ones that the body does not need. In most cases, the cancer cells form a tumor and over time the tumors can replace normal tissues, crowd it or push it aside. Very often cancer cells travel to other parts of the body where they can grow and form new tumors in a process called metastasis.\(^2\)

Nevertheless, not every cancer is the same. Some cancers, like leukemia, rarely form tumors and instead involve the blood and the blood-forming organs and circulate through other tissues where they grow. Moreover, different cancers can behave very differently. They can grow at different rates and respond to different treatments. Prostate cancer for instance is the second leading cause of cancer death among men in the US, whereas breast cancer is more common in women. At the same time, one particular cancer can differ in how it affects a particular organ. Lung cancer for example, has three main types, such as non-small cell lung cancer, which is the most common type of lung cancer accounting for about 85% of all lung cancers; small cell lung cancer, also called oat cell cancer accounting for about 10-15% of all lung cancers and this type of cancer tends to spread more quickly; and lung carcinoid tumor which occurs for fewer than 5% of lung cancers. When it comes to breast cancer, different types can be identified depending how the cancerous cells look under the microscope. Most breast cancers are carcinomas that start in the cells that line organs and tissues like the breast.\(^2\)
**Table 1.** Ten leading cancer types for the estimated new cancer cases and deaths by sex in the United States for 2015.

<table>
<thead>
<tr>
<th>Cancer Type</th>
<th>Estimated New Cases</th>
<th></th>
<th>Estimated Deaths</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td><strong>Females</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Estimated New Cases</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostate</td>
<td>220,800</td>
<td>26%</td>
<td>Breast</td>
<td>231,840</td>
</tr>
<tr>
<td>Lung &amp; bronchus</td>
<td>115,610</td>
<td>14%</td>
<td>Lung &amp; bronchus</td>
<td>105,590</td>
</tr>
<tr>
<td>Colon &amp; rectum</td>
<td>69,090</td>
<td>8%</td>
<td>Colon &amp; rectum</td>
<td>63,610</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>56,320</td>
<td>7%</td>
<td>Uterine corpus</td>
<td>54,870</td>
</tr>
<tr>
<td>Malenoma of the skin</td>
<td>42,670</td>
<td>5%</td>
<td>Thyroid</td>
<td>47,230</td>
</tr>
<tr>
<td>Non-Hodgkin lymphoma</td>
<td>39,850</td>
<td>5%</td>
<td>Non-Hodgkin lymphoma</td>
<td>32,000</td>
</tr>
<tr>
<td>Kidney &amp; renal pelvis</td>
<td>38,270</td>
<td>5%</td>
<td>Malenoma of the skin</td>
<td>31,200</td>
</tr>
<tr>
<td>Oral cavity &amp; pharynx</td>
<td>32,670</td>
<td>4%</td>
<td>Pancreas</td>
<td>24,120</td>
</tr>
<tr>
<td>Leukemia</td>
<td>30,900</td>
<td>4%</td>
<td>Leukemia</td>
<td>23,370</td>
</tr>
<tr>
<td>Liver &amp; intrahepatic bile duct</td>
<td>25,510</td>
<td>3%</td>
<td>Kidney &amp; renal pelvis</td>
<td>23,290</td>
</tr>
<tr>
<td><strong>All sites</strong></td>
<td>848,200</td>
<td>100%</td>
<td><strong>All sites</strong></td>
<td>810,170</td>
</tr>
<tr>
<td><strong>Estimated Deaths</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung &amp; bronchus</td>
<td>86,380</td>
<td>28%</td>
<td>Lung &amp; bronchus</td>
<td>71,660</td>
</tr>
<tr>
<td>Prostate</td>
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<td>9%</td>
<td>Breast</td>
<td>40,290</td>
</tr>
<tr>
<td>Colon &amp; rectum</td>
<td>26,100</td>
<td>8%</td>
<td>Colon &amp; rectum</td>
<td>23,600</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>20,710</td>
<td>7%</td>
<td>Pancreatic</td>
<td>19,850</td>
</tr>
<tr>
<td>Liver &amp; intrahepatic bile duct</td>
<td>17,030</td>
<td>5%</td>
<td>Ovary</td>
<td>14,180</td>
</tr>
<tr>
<td>Leukemia</td>
<td>14,210</td>
<td>5%</td>
<td>Leukemia</td>
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<tr>
<td>Esophagus</td>
<td>12,600</td>
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<td>Uterine corpus</td>
<td>10,170</td>
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<tr>
<td>Urinary bladder</td>
<td>11,510</td>
<td>4%</td>
<td>Non-Hodgkin lymphoma</td>
<td>8,310</td>
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<tr>
<td>Non-Hodgkin lymphoma</td>
<td>11,480</td>
<td>4%</td>
<td>Liver &amp; intrahepatic bile duct</td>
<td>7,520</td>
</tr>
<tr>
<td>Kidney &amp; renal pelvis</td>
<td>9,070</td>
<td>3%</td>
<td>Brain &amp; other nervous system</td>
<td>6,380</td>
</tr>
<tr>
<td><strong>All sites</strong></td>
<td>312,150</td>
<td>100%</td>
<td><strong>All sites</strong></td>
<td>277,280</td>
</tr>
</tbody>
</table>
1.1.1. Prostate cancer

According to the latest American Cancer Society (ACS) estimates in the United States for 2015, about 220,800 new cases of prostate cancer will be diagnosed and about 27,540 men will die of prostate cancer (Table 1). The mortality due to prostate cancer has however steadily declined between 1999 and 2003, which among several factors, may be attributable to earlier detection and improved treatment of cancer. In the past few years, several targeted therapeutic agents have been developed and clinically used for the treatment of prostate cancer. Current therapeutic treatments for metastatic castration-resistant prostate cancer includes abiraterone (a, Zytiga® in Figure 1). Abiraterone was approved by the FDA in April 2011 and by the EMA since September 2011 for the treatment of metastatic castration-resistant prostate cancer and has been found to be effective as a second-line treatment with increased survival.

1.1.2. Triple-negative breast cancer

Over the past several years, progress has been made in breast cancer research, including the discovery of the role of three proteins associated with breast cancer growth. These proteins include estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2). Researchers have been successful in creating therapies specifically for those three proteins, called targeted therapy. Patients diagnosed with triple-negative breast cancer lack these three proteins on the tumor cells that are responsible for breast cancer growth. This makes the triple-negative breast cancer difficult to treat. Moreover, triple-negative breast cancer is more aggressive than other cancers and more likely to recur than any other types of breast cancer. One of promising chemotherapeutic drugs for triple-negative breast cancer is a drug called glembatumumab vedotin (CDX-011, b in Figure 1), an antibody-drug conjugate that targets gpNMB-containing cells, with the goal of killing the cancer cells. gpNMB is a specific protein over-expressed in breast cancer and other tumor types that is believed to be involved in cancer growth and the spread of cancer to other parts of the body.
Figure 1. Current therapeutic treatments for leukemia, prostate, pancreas, lung, ovarian and triple-negative breast cancer.

1.1.3. Lung cancer

Lung cancer, both small cell and non-small cell, is the second most common cancer in both men and women in the United States. Lung cancer accounts for about 13% of all new cancer cases. According to the ACS estimates in the US, in 2015 about 221,200 new cases of lung cancer will occur and an estimate of 158,040 people will die of lung cancer (Table 1). Lung cancer mainly occurs in older people, with 2 in 3 diagnosed people being 65 of age or older and fewer than 2% of all lung cancer cases are found in people younger than 45. The survival
statistics in people with lung cancer vary depending on the stage of the cancer when it is diagnosed. Chemotherapeutics drugs used for small- and non-small cell lung cancer include cisplatin (c), carboplatin (d, Paraplatin®) and etoposide (e, Toposar®). Paclitaxel (f, Taxol®) is also used for treatment of non-small cell lung cancer (Figure 1). While all the drugs are administrated intravenously, etoposide can be administrated also orally. Drugs that are administrated through a vein as an infusion could be irritants that can cause inflammation of the vein through which it is given. If the medication escapes from the vein it can cause tissue damage. More detailed explanation and description on platinum drugs will be addressed in section 1.2.

1.1.4. Leukemia

There are four different types of leukemia, depending on the type of cells where they initiate, how quickly they grow, which people they affect (adults or children) and how they are treated. The types of leukemia include: acute lymphocytic (or lymphoblastic) leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myeloid (or myelogenous) leukemia (AML) and chronic myeloid leukemia (CML). Acute lymphoblastic leukemia is the most common type of leukemia among children and the risk of developing is highest under the age of 5. According to the American Cancer Society in the United States, in 2015 there will about 54,270 new cases of all kinds of leukemia and approximately 24,450 deaths (Table 1). One of the promising drugs for treatment of chronic lymphocytic leukemia CTT is bendamustine (g, Treanda®) (Figure 1), while etoposide (e) is used for ALL and sometimes for CLL along with oxaliplatin (h, Eloxatin®).

1.1.5. Ovarian cancer

The ovaries are made up of three main kinds of cells and each type of cell can develop into a different type of tumor. These tumors include epithelial, germ cell, and stromal tumors. Most ovarian tumors are epithelial cell tumors. The majority of these tumors are benign (non-cancerous) and never spread beyond the ovary and can be treated by removing either the ovary or the part of the ovary that contains the tumor. Approximately 21,000 women each year are affected by ovarian cancer in the Unites States (Table 1) and according to the ACS statistics in the United
States, in 2015 there will about 14,180 women will die of ovarian cancer. Chemotherapeutics used for ovarian cancer include cisplatin (c), carbobatin (d), etoposide (e) and albumin bound paclitaxel (f, nab-paclitaxel, Abraxane®).²

1.1.6. Pancreatic cancer

Not all growths in the pancreas are cancerous. Some of them are benign while others might become cancer over time if left untreated. Since more people are getting imaging tests such as CT scans more frequently than in the past, these types of pancreatic growths are now being found more often. Pancreatic cancer involves two types of cells, exocrine and endocrine cells, which form different types of tumors. These tumors have distinct risk factors and causes, have different signs and symptoms, are diagnosed using different tests, are treated in different ways, and have different outlooks. Therefore it is important to distinguish between exocrine and endocrine cancers of the pancreas in order to address appropriate treatment. Approximately 48,000 people each year are affected by pancreatic cancer in the United States; both male and female (Table 1) and according to the ACS statistics in the United States, in 2015 there will about 40,560 people will die of pancreas cancer. Chemotherapeutics used for pancreas cancer include cisplatin (c), oxaliplatin (h), albumin bound paclitaxel and paclitaxel (i).²

1.1.7. Limitations of the current existing chemotherapeutic treatments for the above described cancers

The preparation of some of the listed chemotherapeutics can be sometimes challenging. For instance, the current commercial preparation of bendamustine entails at least nine synthetic steps, involving the use of several hazardous reagents such as thionyl chloride. The treatment cost for bendamustine is extremely costly as well. Single dose of the drug cost aproximetly $4.320 and the entire treatment could be as expensive as $80,000.⁸ The process of preparation of abiretarone involves multiple steps, including the use of Pd(II) catalysts through Suzuki or Negashi coupling, and long purification processes. Even though a more recent advancement in the preparation of abiretarone has been made with a reduced reaction time, it involves multiple crystallizations in order to enable large-scale synthesis. Furthermore, the process also engages the use of pyridine in the final step and the excess pyridine, removal of reagents such as acetic
anhydride on a rotary evaporator at higher temperature, leading to a laborious work-up with decrease in the yield and purity of the product.9

In addition, patients who received Zytiga® and prednisone combination have a median overall survival of 14.8 months, which is only 4 months more from the patients that did not receive the treatment.10 Clinical trials for Taxol® in advanced ovarian cancer, showed that the survival impact is an average prolongation of one year in survival, which is a modest dramatic result for patients with advanced disease. In case of Taxol® for non-small-cell lung cancer, it prolongs the survival by approximately two months compared to a standard chemotherapy treatment consisting of cisplatin and etoposide.11

Additionally, the major drawbacks of these drugs are side effects, such as nausea, vomiting, hair loss or vision problems. Cisplatin and bendamustine can cause major damage to the kidneys2,12,13 and because of the way etoposide acts on cells in the body, it may increase the risk of getting a second type of cancer, such as leukemia.2 Paclitaxel can cause allergic reactions or condition known as hand-foot syndrome, causing pain, numbness or swelling in the hands or feet, while albumin bound paclitaxel can affect heart rhythm, as well as lung inflammation.2 There is therefore, a real need for new chemotherapeutics that can overcome the major drawbacks of already existing drugs for the cancers listed above.

1.2. The foundation of platinum-based drugs, as cancer chemotherapeutics - limitations and resistance

The accidental discovery of cisplatin (cis-diamminedichloroplatinum(II)) over 40 years ago by Barnett Rosenberg (a Brooklyn College ‘62 alumnus) and its clinical approval in 1978, revolutionized cancer chemotherapy that represents a major landmark in the history of successful anticancer drugs.13,14 This discovery led to the synthesis and biological evaluation of hundreds of cisplatin analogues, such as carboplatin, oxaliplatin, picoplatin, as well as nedaplatin and lobaplatin (the last two being widely used in Asia) (Figure 2).15 However, only cisplatin and the follow-on drugs carboplatin (Paraplatin®) and oxaliplatin (Eloxatin®) have been approved worldwide as anticancer drugs and are still used to treat 40–80% of cancer patients.12,15 At present, cisplatin is one of the most effective drugs employed to treat testicular and ovarian cancer. Carboplatin has been approved by the FDA in 1989 to treat ovarian cancer and
oxaliplatin has been approved by the FDA in 2002 for treating colorectal cancer. Despite the precious metal component, cisplatin is actually one of the cheapest drugs in the cancer chemotherapy market. 

Figure 2. Platinum-based chemotherapeutic agents.

The primary target for cisplatin is DNA (Figure 3). The major mechanism of action is that cisplatin becomes activated intracellularly by the aquation of one of the two chloride leaving groups which allows cisplatin to covalently bind to guanines of the DNA, forming a DNA adduct. This activates various signal-transduction pathways, such as those involved in DNA-damage recognition and repair, cell-cycle arrest and programmed cell death/apoptosis. 

13
Figure 3. Binding of cisplatin to guanine bases of the DNA through intrastrand and interstands crosslink.\textsuperscript{13}

Despite therapeutic success in the treatment of several types of tumors, the high effectiveness of platinum-derived drugs has faced certain limitations. Since DNA is the primary target for cisplatin, it can affect both sick and healthy cells. In addition, due to the affinity of platinum for the sulfur and selenium donor that are present in many proteins in the plasma and in the cellular environment, cisplatin can interact and disrupt the function of different proteins and enzymes, as confirmed by the fact that only 1\% of the intravenously administrated drug actually reaches DNA. This produces a variety of side effects such as neurotoxicity, nephrotoxicity, hepatotoxicity, ototoxicity, gastrointestinal disturbances, bone marrow suppression, hair loss and anemia.\textsuperscript{13,15}

Another important concern is that cisplatin may induce acquired or intrinsic resistance.\textsuperscript{15,16} Cisplatin can bind to DNA through both interstrand and intrastrand cross-linking, thus hindering RNA transcription and DNA replication which subsequently triggers the cell death pathway. Unfortunately, its efficacy is frequently lost after several chemotherapeutic-cycles due to tumor cells resistance. Platinum drugs such as carboplatin, that bind to DNA in the same manner and generate the same kind of adduct as cisplatin probably induce cross-resistance to cisplatin. Another drawback is the fact that cisplatin only reaches DNA in low amounts where the majority of the complex is dispersed within the plasma and the cells.\textsuperscript{15}
A number of platinum analogues have entered clinical trials in the past 30 years with two (carboplatin and oxaliplatin) having been approved by the FDA. Satraplatin and picoplatin have emerged after the development of carboplatin. Satraplatin was originally developed as an orally active version of carboplatin and early clinical trials demonstrated the feasibility of administrating a platin by the oral route. Satraplatin showed promising clinical activity in a trial of 50 patients with hormone-refractory prostate cancer. Phase III with this drug, involving 900 patients who had failed previous chemotherapy, showed significant reduction of the risk in disease progression. The most recent data for Satraplatin has been submitted as a new drug application to the FDA and awaits approval.\textsuperscript{13}

In addition, picoplatin was designed to provide steric bulk around the platinum centre and showed to retain activity against a wide range of cisplatin-resistant and oxaliplatin-resistant cells \textit{in vitro}. Picoplatin also demonstrated \textit{in vivo} antitumor activity by both the intravenous and oral routes, as well as promising antitumor activity in phase II trials of ‘platinum sensitive’ ovarian cancer and cisplatin-resistant small cell lung cancer. However, it failed in phase III trials for small-cell lung cancer.\textsuperscript{17}

Overcoming these limitations can be quite challenging in pharmaceutical research. Development of more active platinum-derived drugs\textsuperscript{14,18-26} with improved properties and with less side effects that can address the clinical problems\textsuperscript{2,12,13,15} related to the use of cisplatin and their follow-on drugs, carboplatin and oxaliplatin is extremely important. Nanotechnology may provide several advantages for drug delivery and accumulation of platinum compounds\textsuperscript{27} such as: 1) Control of drug solubility; 2) Modulation of drug distribution; 3) Targeting of specific cells; and 4) Multidrug delivery and therasnotics. In this context, a few selected Pt(II) compounds \textit{(nanoplatin,\textsuperscript{28} cisplatin,\textsuperscript{29} \{Pt(NH\textsubscript{3})\textsubscript{2}\}\textsuperscript{2+}, \textsuperscript{30} mitaplatin,\textsuperscript{31} \{di-DAHC-Pt\textsubscript{2}\}\textsuperscript{4+}, \textsuperscript{32} and \{DACH-Pt\}\textsuperscript{2+}}\textsuperscript{33,34} have been encapsulated into polymer nanoparticles and tested \textit{in vitro} or \textit{in vivo} as potential cancer chemotherapeutics. All these formulations were proven to be beneficial with better activities \textit{in vitro} or \textit{in vivo} and lower nephrotoxicity than cisplatin. The synthetic strategies involved are nevertheless not straight forward and certainly not cheap. It would also be desirable to have metallodrugs more selective and effective than cisplatin to be delivered this way.

A wide range of other transition-metal based drugs emerged as potential candidates and are currently investigated for future advancement.\textsuperscript{14,15,35,36,39-43,45-51,53-57} Within this context,
organometallic compounds with properties bridged somewhat between inorganic and organic drugs have been recently considered as promising alternatives as cancer chemotherapeutics. One reason is that these cross-compounds combine the features of a metallic center and organic scaffolds and may be endowed with versatile stereochemistry having geometries ranging from linear to octahedral and sometimes even beyond, with a large number of stereoisomers in some cases. Another reason is that the redox properties of the resulting metal cations \textit{in vitro} along with their ability to bind covalently to biological targets can make them good candidates in medicinal chemistry. Lastly, organometallic species are relatively lipophilic (more permeable) and can be functionalized with a variety of organic ligands that contain specific reactivity.\textsuperscript{14}

In the following subsections, new coordination and mostly organometallic platinum(II), gold(III), palladium(II) and ruthenium(II and III) complexes are described as potential cancer chemotherapeutics.

1.3. Platinum (II), gold(III), palladium(II) and ruthenium (II and III) compounds as alternatives chemotherapeutic agents

1.3.1. Platinum(II) compounds

From the different strategies employed (trans compounds, platinum(IV) pro-drugs, multinuclear platinum complexes), organometallic platinum(II) compounds have emerged as attractive anticancer agents.\textsuperscript{14} Multiple efforts have been made to synthesize various and structurally different organoplatinum complexes exhibiting antitumor properties and with different mechanisms of action. Within this context, novel cyclometalated platinum-based complexes have been designed, synthesized and studied, with an emphasis on their potential interaction with DNA and favorable cytotoxicity against different cancer cell lines.\textsuperscript{14} The early development of this type of compounds for anticancer purposes focused on different bidentate C-N cycloplatinated complexes having the general formula Pt(C-N)LX. By changing the structure of C-N ligands and other ancillary ligands, the kinetic, structural and electronic properties of cycloplatinated compounds can be appropriately tuned to enhance their activity.\textsuperscript{18,19} One such example is five coordinated cyclometalated Pt(II) complexes (A, Figure 4) containing chelating biphosphine ligands, that exhibited more potent anticancer activity \textit{in vitro} and \textit{in vivo} than
cisplatin. Moreover, this compound displayed proteasome inhibitory activity in human breast cancer cells associated with apoptosis induction.\textsuperscript{20}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure_4}
\caption{Recently developed platinum anticancer agents.\textsuperscript{14,20-22}}
\end{figure}

Cycloplatinated tridentate C-N-N complexes that are more stable under physiological conditions than cisplatin and its derivatives have been recently described by Che’s \textit{et al.} (like B in Figure 4). This N-heterocyclic carbene platinum complex displayed \textit{in vitro} cytotoxicity activity higher than that of cisplatin and inhibited tumor growth in a nude mice model. Surprisingly, B did not accumulate in the vicinity of DNA but preferentially accumulated in cytoplasmic structures including sites where active survivin, an inhibitor of apoptosis, is located. Most importantly, \textit{in vivo} results showed that injection at 3 mg/kg considerably inhibited NCI-H460 tumor growth, did not cause death of mice and induced no significant weight loss.\textsuperscript{21}

Moreover, a rhomboidal Pt(II)-based complex C was recently evaluated \textit{in vivo} on the tumor growth rate in a breast cancer (MDA-MB-231) mouse xenograft model. Interestingly, substantial 64\% reduction of the average tumor volume reduction burden was observed on the last day of the experiment. In addition, the emissive properties of this complex open a possibility for future development in image-guided drug delivery.\textsuperscript{22}
1.3.2. Gold(III) compounds

The successful development of antitumor platinum drugs has paved the way for studying other metal-based chemotherapeutic compounds with potentially improved properties. Gold was one of the first metals used as a treatment for disease thousands of years ago; however its exploitation in modern medicine was mainly restricted to the cure of rheumatoid arthritis since the mid-1930s.\(^{15}\) During the past decade gold complexes have gained increasing attention due to their strong tumor cell growth inhibiting effects generally achieved by exploiting non-cisplatin-like pharmacodynamic and pharmacokinetic properties and mechanisms of action.\(^{31-33}\) Gold(III) compounds were initially chosen as an alternative to cisplatin since Au(III) is isoelectronic with Pt(II) and has a d\(^8\) configuration resulting in square-planar tetracoordinate complexes, which in theory should make them interact with DNA in a similar manner. The ligand exchange kinetics are relatively slow for both metals although they are slightly faster for Au(III) complexes. Unfortunately, their relatively poor chemical stability in solution slowed down research in this field until the mid-1990s when complexes stable in physiologically relevant conditions were developed.\(^{15}\) Since then, improved classes of gold(III) compounds have been reported with anticancer activities. They not only display high cytotoxicity against representative human tumor cell lines in vitro and tumors in vivo, but also show a mode of action different from that of cisplatin which allows them to be active in specific cell lines resistant to cisplatin. It has been proposed that the mechanism of action for Au(III) complexes is either purely DNA-independent or that the interaction with DNA in the intercalative mode is partly responsible for the cell death. Moreover, in most cases, Au(III) compounds display interactions with a variety of target proteins isolated or inside cells. Thus, they form effective bonds with serum proteins, such as albumin or with sulfur-containing proteins such as GSH and thioredoxin reductases (TrxR).\(^{14,15}\) The two main mechanisms known to date that are responsible for the biological activity of gold(III) compounds include reductive oxygen species (ROS) production and direct metal binding to the proteasome.\(^{34}\)

Among these compounds, gold(III)-dithiocarbamato AuD6 (E) and AuD8 (F) (Figure 5) were designed as anticancer gold(III)-based peptidomimetics. Both compounds were highly active against human MDA-MB-231 breast cancer cell lines in vitro, with AuD8 being more potent than AuD6 (IC\(_{50}\) = 6.5±0.6 µM). In vivo studies showed slightly higher antitumor activity for AuD8, at a low dose of 1.0 mg/kg/day, with 53% inhibition of xenograft growth after 27
days of treatment. In some mice, 85% inhibition after 13 days was observed. AuD68 was also a more potent proteasome inhibitor both \textit{in vitro} and \textit{in vivo}.\textsuperscript{35}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Representative gold(III) dithiocarbamate and cycloaurated complexes.\textsuperscript{14,35-39}}
\end{figure}

Cinellu, Messori \textit{et al.} developed a C,N,N-gold(III) complex (G, in Figure 5) that was stable under pseudo-physiological conditions and was cytotoxic against different ovarian cancer cell lines.\textsuperscript{36} Cycloaurated compound G displayed good activity and high selectivity against a panel of 12 human tumor cell lines while H was considerably more cytotoxic and had a high degree of selectivity against panel of 36 human tumor cell lines.\textsuperscript{37} Studies on the inhibitory effects on TrxR suggested that mitochondrial pathways were directly involved in the apoptotic process and the cytotoxicity. The interaction of G with \textit{Calf Thymus} DNA showed to be weak and reversible in nature. Bovine serum albumin (BSA) studies reveled that Au-protein adduct was formed, which suggested coordination of the complex at the level of surface histidines. Interestingly, the gold(III) compounds formed small amounts of metal-protein adducts with cytochrome c and lysozyme in which gold(III) center and the C-N-N tridentate ligand were conserved.\textsuperscript{14,37,38}
In vivo studies in rats bearing HCC orthografts with the dinuclear gold(III) complex I (Figure 5) reported by Che et al. showed significant inhibition in tumor growth (77%). It was revealed that the complex inhibited thioredoxin reductase and induced endoplasmic reticulum (ER) stress. Particularly, I has also been subjected to acute and sub-chronic toxicity evaluation in nude mice and in beagle dogs, reveling no severe and irreversible side-effects in association with the consumption of the compound at its effective concentrations in these animal models. Furthermore, gold(III) complex did not induce obvious genotoxicity.\textsuperscript{14,38,39}

All these results indicate that gold(III) derivatives are therefore good candidates for further studies and some may be even suitable to enter phase I clinical trials.

1.3.3. Palladium(II) compounds

The coordination chemistry of palladium(II) is very similar to that of platinum(II); therefore palladium derivatives have been explored as an alternative to platinum-based compounds due to the structural and thermodynamic similarity between the two metal-based complexes. One of the features that distinguishes Pd(II) complexes from those of Pt(II) is the ligand exchange mechanism, which is $10^5$ times higher for Pd(II)-derivatives. This can cause fast hydrolysis of Pd(II)-based complexes associated with ligand dissociation by generating very active species that can easily interact with donor groups encountered in the bloodstream and cellular environment; thus preventing the drug from reaching its target. This makes palladium complexes generally inactive and somewhat toxic due to their higher reactivity. This process can be however avoided by using bulky chelating ligands and achieving higher stabilization with strongly coordinated ligands.\textsuperscript{14,15} Palladium(II) compounds have been therefore studied as anticancer agents in the past 15 years with relevant examples found in the family of organometallic compounds.

The advantage of using Pd(II)-derived complexes over Pt(II) is their higher solubility.\textsuperscript{15} Moreover, the higher stability of cyclopalladated compounds in physiological media and a lower toxicity to normal cells are all promising features for their biological applications.\textsuperscript{40} Organometallic Pd(II) complexes have been reported to have cytotoxicity activity against human cervical epitheloid carcinoma, human chronicmyelogenous leukemia, osteogenic sarcoma, malignant melanoma, breast cancer, lung cancer, glioma, human colorectal adeno-carcinoma, head and neck squamous cancer, prostate cancer and ovarian cancer.\textsuperscript{15}
Figure 6. Representative C,N-palladium(II) complexes.\textsuperscript{14,41-47}

Cyclopalladated complexes are less toxic overall, which makes them very promising as antitumor compounds. A good example of such cyclopalladated compound is J in Figure 6, which showed to induce apoptotic cell death in human leukemia cells (HL-60 and Jurkat) by rapture of lysosomal membranes and release of cathepsin B into the cytoplasm\textsuperscript{14,41,42}

Travassos et al. reported on the biphosphinic cyclopalladated complex (K, C7a) that displayed promising antitumor properties against murine and cisplatin-resistant human tumor cells both \textit{in vitro} and \textit{in vivo}. This compound induced the central apoptotic pathway by a strong effect on mitochondria and interacted with thiol-containing protein groups in the mitochondrial membrane, by inducing Bax translocation from cytosol, colocalizing with mitochondrial tracker. Increase in cytosolic calcium concentration, mainly from intracellular compartment was observed, as well as a significant decrease of adenosine triphosphate (ATP) and caspse/endonuclease activation. Moreover, in preclinical studies of melanoma model, this dimeric complex was effective against primary and metastatic tumors, having low toxicity. Thus, cyclopalladated complex K is a promising tumor chemotherapeutic agent and was selected for further preclinical studies that include a gene therapy protocols in conjugation with plasmids\textsuperscript{43-45}

Another study conducted on C7a (K) investigated therapeutic efficacy in patient-derived xenograft model of adult T-cell leukemia lymphoma (ATLL) and the mechanism of action in the human T lymphotrophic virus type 1-positive and negative-T cell lines (HTLV-1). \textit{In vivo} survival studies led to significant increase of survival in the treated mice. Moreover, C7a inhibited more than 60\% of the \textit{ex vivo} proliferation of PBMC from HTLV-1-infected individuals\textsuperscript{46}
Furthermore, this compound was also found to be highly effective to promote cell death in the K562 human leukemia cells through apoptotic cell death and associated to cytochrome c release and caspace activation. These results prove that C7a compound is a promising drug in the chronic myeloid leukemia (CML) antitumor chemotherapy.\textsuperscript{47}

1.3.4. Ruthenium(II and III) compounds

In the 1970s, Clarke \textit{et al.} reported on a ruthenium complex, [Ru(NH\textsubscript{3})\textsubscript{5}(purine)]\textsuperscript{3+}, capable of inhibiting DNA and proteins synthesis in human nasopharyngeal carcinoma cells \textit{in vitro}.\textsuperscript{48} This subsequently initiated interest in ruthenium complexes as potential anticancer agents and without a doubt, ruthenium became the star metal in the present search for chemotherapeutic alternatives to cisplatin.\textsuperscript{15} In the 1980s, chloro-ammine-Ru(III) compounds were found to have anticancer activity against rats, but their poor solubility limited their action. Soon after, a DMSO-Ru(II) species, \textit{cis}-[RuCl\textsubscript{2}(dmso)\textsubscript{4}] was shown to be active against both primary and metastatic cancers, though less effective than cisplatin, but with fewer side effects compared with the platinum-based drugs. Since then a number of active ruthenium compounds \textit{in vitro} and \textit{in vivo} two of which have already entered clinical trials.\textsuperscript{15}

The emergence of ruthenium complexes as some of the most promising candidates for cancer therapy with metallodrugs can be attributed to their specific characteristics. First of all, ruthenium compounds can easily access three different oxidation states (II, III and possibly IV) in physiological solutions.\textsuperscript{15,49} Ruthenium II and III states can form six-coordinate octahedral species that can facilitate finer tuning of the steric and electronic properties of the complexes by intervening with the two “extra” axial ligands. They can also interact with macromolecules in a different manner that those derived from platinum.\textsuperscript{49} The rate of ligand exchange in ruthenium complexes is comparable to that for platinum, ranging from 10\textsuperscript{-2} to 10\textsuperscript{-3} per second, which gives the molecules high kinetic stability and preventing rapid equilibrium reactions. This helps the molecule to stay intact on its way to the target and it also remains viable throughout its interaction with the cell.\textsuperscript{15} And most importantly, ruthenium compounds are able to mimic iron in binding biologically relevant molecules such as albumin and transferrin, and as a consequence their toxicity is much lower than that of platinum therapies.\textsuperscript{15,49}
A number of ruthenium(III)-arene complexes showed high in vivo antitumor activity and have successfully completed phase I clinical trials and entered phase II.\textsuperscript{14} Those compounds include NAMI-A,\textsuperscript{50,51} developed by Sava et al., KP1019 and its analogue containing Na\textsuperscript{+}, KP1339\textsuperscript{50,52} developed by Keppler et al. (Figure 7). KP1019 (indazolium trans-[tetrachloridobis(1H-indazole)ruthenate(III)]) was found to be highly active against the rat colon cancer model, yielded efficacy with up to 95\% reduction of tumor volume without any mortality (0\%) and without any considerable weight loss (6\%).\textsuperscript{53} KP1339 was also found to exhibit cytotoxic activities in various tumor types, such as colon carcinomas.\textsuperscript{52}

NAMI-A (imidazolium trans-[tetrachloridobis(1H-imidazole)(S-dimethyl sulfoxide)ruthenate(III)]) demonstrated to affect the process of metastasizing, rather than acting against the primary tumors or already established metastases. Its activity is most likely based on enhanced cell adhesion, inhibition of cancer cell motility and invasiveness, as well as on inhibition of neoangiogenesis in the tumor tissues.\textsuperscript{53}

I will detail in Chapter V a number of organometallic ruthenium(II) derivatives that have been subjected to advanced pre-clinical studies.
1.4. New drug design with iminophosphorane ligands

Iminophosphorane (IM) ligands of the general formula $R_3P=NR'$ are attractive substrates for coordination (N,N-) or cyclometallation (C,N-). They provide a C,N- or N,N- chelating backbone that stabilizes the resulting square-planar structure of $d^8$ transition metal complexes and can also coordinate to $d^6$ metals like Ru(II) (Chart 1). Incorporating phosphines in the skeleton of the IM pincer ligand is important since the phosphorus atom can serve as a spectroscopic “marker” for $^{31}P\{^1H\}$ NMR spectroscopy in order to study the stability and oxidation state of the compounds in vitro in different deuterated solvents. Furthermore, modifications to X ancillary ligands in these metallic compounds permits further functionality by ligand substitution.

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**Chart 1.** New drug design with iminophosphorane ligands.
Iminophosphoranes can be divided into three classes: stabilized, semi-stabilized and non-stabilized (Figure 8), as a function of the carbanionic charge delocalization degree and the ability to delocalize this charge which is closely related to the nature of the substituents (Figure 9). For example, the stabilized iminophosphorane ligands are characterized by the presence of the C=O next to the P=N bond. This results in more stable structures due to the conjugation. The semi-stabilized and non-stabilized classes lack the C=O and are therefore less stable. The main focus remains on using stabilized and semi-stabilized iminophosphorane ligands avoiding the least stable third class of IM ligands.

There are many different methods in which iminophosphorane ligands can be prepared. The most common of them is the Staudinger reaction in which a tertiary phosphine reacts with an
organic azide in the presence of non-polar solvent, such a dichloromethane, at room temperature, releasing nitrogen gas and forming semi-stabilized IM (\(R_3^3P + N_3R' \rightarrow R_3^3P=NR'\)).\(^{54}\) The second method is the Pomerantz reaction, in which a tertiary phosphine reacts with a primary amide in the presence of di-\(\text{tert}\)-butylazodicarboxylate (\(^1\text{BuDAD}\)) and polar solvent, such a tetrahydrofuran at 0ºC, forming stabilized IM (\(R_3^3P + H_3^3NC(O)R' \rightarrow R_3^3P=NC(O)R'\)) (Scheme 1).\(^{55}\) A number of our IM were prepared by Pomerantz method which is the method of choice when amides are used.

![Scheme 1. Staudinger and Pomerantz reaction.](image)

1.5. Previous studies using iminophosphorane ligands

Our research group in Brooklyn College has reported that non-toxic iminophosphorane or iminophosphane compounds serve as stabilizing (C,N- or N,N) chelating ligands in the preparation of anticancer organometallic and coordination \(d^8\) metal complexes (Figure 10).\(^{56-60}\) A series of neutral and cationic organo-gold(III) iminophosphorane complexes (like 1, in Figure 10) showed higher toxicity against leukemia cells, including cells from patients with chronic leukemia CCL, while being less toxic to normal T-lymphocytes with IC\(_{50}\) values in the nanomolar range (60-100 nM). It was found that gold compounds do not interact with Calf
Thymus DNA and induce intracellular oxidative stress that provokes mitochondrial dysfunction. Complex 1 induces necrotic cell death and reactive oxygen species (ROS) production at the mitochondrial level which was a critical step in its antitumor effect.\textsuperscript{56,57}

\textbf{Figure 10.} Selected cytotoxic gold(III), platinum(II) and palladium(II) complexes with IM ligands (1-6) prepared in our research group.\textsuperscript{56-60}

Another study conducted in our research group involved the coordination of a watersoluble IM ligand PTA=N-C(O)-2-N\textsubscript{5}H\textsubscript{4}(N,N-IM) to d\textsuperscript{8} metals, affording hydrophilic stable Pd(II) and Pt(II) compounds (like 2, in Figure 10). Compound 2 was highly cytotoxic \textit{in vitro} against human T-cell leukemia Jurkat while being less toxic to normal T-lymphocytes (PBMC). It was also 15 times more toxic to Jurkat shBak cell lines than cisplatin, indicating a cell death pathway that may be different from that of cisplatin. The interactions of compound 2 with \textit{Calf Thymus} and plasmid (pBR322) DNA, revealed differences compared to cisplatin and with faster binding to HSA. It was demonstrated that compound 2 interacted with HSA in a reversible way which could be potentially beneficial to the antitumor activity \textit{in vivo} of this compound.\textsuperscript{58}

In another project, a series of water-soluble and highly lipophilic organometallic Pd(II) complexes including the orthopalladated dimer [Pd(μ-Br){C\textsubscript{6}H\textsubscript{4}(C(O)N=TPA-kC,N)-2}]\textsubscript{2} (3) and a mononuclear complex [Pd{C\textsubscript{6}H\textsubscript{4}(C(O)N=TPA-kC,N)-2}(acac)] (4) (Figure 10) were
synthesized. Palladium compounds 3 and 4 displayed high cytotoxicity in vitro against human Jurkat-T acute lymphoblastic leukemia cells, and DU-145 human prostate cancer cells while being less toxic to normal T-lymphocytes. Notably 3 and, particularly 4, were very toxic to cisplatin-resistant Jurkat shBak cells (Bax/Bak-deficient Jurkat cells), indicating a cell death pathway that may be different from that of cisplatin. The interaction of these Pd(II) complexes with plasmid (pBR322) DNA was very weak (3) or non-existing at all (4), indicating an alternative biomolecular target for these cytotoxic compounds.59

The more recent project in our research lab involved the synthesis of Au(III) and Pd(II) heterometallic complexes with IM ligands derived from ferrocenyl-phosphanes (Figure 10).60 The trimetallic derivatives M2Fe (M = Au, Pd, 5 and 6) exhibited important cytotoxic effects in the low micromolar range in all the studied cells and being slightly less toxic to non-tumorigenic human embryonic kidney cell line (HEK-293T). The interaction of Pd(II) derivative 6 with plasmid (pBR322) DNA showed a strong interaction demonstrating significant retardation of the faster-running supercoiled form, while Au(III) complex 5 showed no interaction. However, Au(III) complex showed to be a good inhibitor of the zinc-finger protein PARP-1, while the Pd(II) complex was noticeably less potent. Our results support the idea of different reactivity of the complexes with the investigated biomolecules depending on the metal ion and not only on the ligand set.60

One of the outcomes of our studies with IM ligands was that cyclometallated monometallic gold(III) and palladium(II) compounds proved to be more stable than coordination derivatives in physiological media. It seems that the skeleton C,N- is a better alternative to stabilize mononuclear metal centers than the N,N- backbone when iminophosphorane ligands are used. However, stable mononuclear Pt(II)58 and trimetallic coordination Fe-Au(III)2 and Fe-Pd(II)260 coordination compounds which display important antitumoral effects were prepared.

The successful results of studies with cyclometallated and coordination compounds conducted in our research group, led us to further explore the synthesis and biological activities of gold(III), platinum(II), palladium(II) and ruthenim(II) with different IM ligands.
1.6. Bibliography


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2.1. Ultimate goal of the work described in this Thesis

The ultimate goal of my research is to develop effective potential cancer chemotherapeutics based on metallic complexes with a mode of action different from that of cisplatin and related compounds in order to overcome some of their drawbacks.

2.2. Hypothesis

Selected iminophosphorane ligands will stabilize $d^8$ and $d^6$ transition metal centers in physiological media and modifications of these ligands will afford compounds with improved antitumor properties and/or properties to better understand their mode of action. We envisioned these modifications in the following way: i) incorporation of water soluble phosphines in the skeleton to increase the hydrophilicity of the complexes; ii) incorporation of luminescent molecules in the ligand to ideally be able to track these complexes inside the cells by techniques like fluorescence microscopy; iii) incorporation of a second different metal in the ligand (like ferrocene) to prepare heterometallic complexes to improve their activity as anti-tumor agents by the interaction of different metals with multiple biological targets or by the improved chemicophysical properties of the resulting heterometallic compound.
2.3. Specific Aims of this Thesis

Aim 1: *Synthesis and study of the stability in solution of compounds with iminophosphorane-containing ligands which may behave as potential anticancer agents.*

a) Synthesis of water-soluble, ferrocene-based and luminescent iminophosphorane ligands to use them in the preparation of gold(III), platinum(II), palladium(II) and ruthenium(II) coordination and organometallic complexes.

b) Study of the stability of the compounds in deuterated solvents or buffers by \(^{31}\text{P}^{1\text{H}},^{1\text{H}}\text{NMR}\) and UV-vis spectroscopy.

Aim 2: *Study of the mechanism of action of the new iminophosphorane compounds.*

Study of the interaction of these compounds with different bimolecular targets like DNA and HSA by different techniques (gel electrophoresis, circular dichroism, thermal denaturation experiments and fluorescence spectroscopy).

Since this is an interdisciplinary project while my doctoral work has focused on the specific aims described above our group, along with the groups of our collaborators, has worked in the biological and mechanistic evaluation of these compounds in order:

1) To study the antiproliferative properties of the new compounds in different human cancer cell lines and in non-tumorigenic human cell lines such as a human embryonic kidney cell line (HEK-293T) or a human renal proximal tubular cell line (RPTC).

2) To investigate the cell death pathway (apoptosis, necrosis, necroptosis) of selected compounds and explore their mode of action by additional studies (inhibition of capthesin, involvement of caspases and/or p-53 in the cell death pathway).

In the case of the luminescent compounds, to study (by fluorescence microscopy) their distribution inside the cancer cells.
3) To study the *in vivo* activity in mice (including pharmacokinetic studies) of the most promising water-soluble ruthenium candidate discovered so far.

I will include in this thesis complete data on the biological evaluation of these compounds as this is relevant to understand the outcomes and significance of my research but I will detail in every chapter the contributions of other scientists. *My research* has consisted on the *rational design and the synthesis and complete characterization of the ligands and metallic compounds described in this thesis, the study of the stability in solution of the new metallic complexes and the study of the interactions of these compounds with biomolecular targets like DNA and Human Serum Albumin* as outlined in the specific aims 1 and 2.
CHAPTER III

SYNTHESIS OF COORDINATION AND ORGANOMETALLIC GOLD(III), PALLADIUM(II) AND PLATINUM(II) COMPOUNDS CONTAINING A LUMINESCENT IMINOPHOSPHORANE LIGAND. *IN VITRO* EVALUATION AND PRELIMINARY MECHANISTIC INSIGHTS.

3.1. Potential of metal-based theranostics

As explained in the introduction, during the past two decades, a large number of metal-based complexes with promising anticancer activities have been reported. In the last five years, a shift has taken place in the field, with more emphasis being placed on understanding how these metallodrugs work. Mechanistic approach may be of help to design improved metal-based drugs by suppressing the side effects and clinical problems related to the use of existing platinum-derived drugs such as cisplatin. One approach is to design new active metal-based compounds that can be tracked *in vitro* and *in vivo* in order to “visualize” the cellular location and fate of these complexes.

Many transition-metal complexes display very interesting photophysical properties that make them suitable as therapeutics agents for targeting and detection of specific biomolecules. These type of compounds are considered to be potential metal-based theranostic, a term which is defined as the combination of therapy and diagnostic imagining into a single modality. The advantage of using this type of approach is that the therapeutic and detection abilities can be combined within a single metal complex, allowing the progress of any disease to be monitored without the requirement for an additional labeling or imaging agent.

Phosphorescent metal complexes have been widely applied for sensing, bio-imaging and organic light-emitting diode applications. They possess several advantageous features that make them suitable as sensing or imaging probes. Photophysical properties of metal complexes make them sensitive to changes in their environment. Moreover, they usually display significant Stokes shifts which can prevent self quenching and allow for easy resolution of the excitation and
emission light. In addition, the long phosphorescence lifetime of metal complexes allows their phosphorescence to be readily distinguished in the presence of endogenous fluorophores likely to be present in biological environments by the use of fluorescence.\(^6\)

Within this context, we aimed to explore the anticancer properties of iminophosphorane d\(^8\) transition metal complexes incorporating a luminescent molecule, as well as to determine their intracellular distribution by using fluorescence microscopy.\(^3,7,8\)

The following chapter reports on the synthesis, characterization and luminescence studies of new gold(III), palladium(II) and platinum(II) derivatives containing previously reported\(^9\) iminophosphorane ligand derived from 8-aminoquinoline [Ph\(_3\)P=N-C\(_9\)H\(_6\)N] (1). These metal-complexes were evaluated for their antiproliferative properties in a human ovarian cancer cell line (A2780S), in human lung cancer cells (A-549) and in a non-tumorigenic human embryonic kidney cell line (HEK-293T). The compounds were also tested for their possible interactions with plasmid (pBR322) DNA used as a model nucleic acid, and for their reactivity with the transport protein human serum albumin (HSA).

### 3.2. Synthesis and characterization

The iminophosphorane ligand [Ph\(_3\)P=N-C\(_9\)H\(_6\)N] (1) can be prepared by a modification of a previously reported procedure.\(^8\) 8-azidoquinoline was obtained by reaction of 8-aminoquinoline with trifly azide\(^9\) and subsequently reacted with triphenylphosphine (PPh\(_3\)) in CH\(_2\)Cl\(_2\) at room temperature for one hour, using the Staudinger method.\(^10\) Upon addition of ligand 1 to a \textit{in situ} prepared CH\(_3\)CN solution of [AuCl\(_2\)(CH\(_3\)CN)]\(_2\)A (A = ClO\(_4^−\), PF\(_6^−\)), or to CH\(_2\)Cl\(_2\) solutions/suspensions of PdCl\(_2\)(COD) and PtCl\(_2\), coordination complexes of gold(III) (2, 3) palladium(II) (4) and platinum(II) (5) were obtained in moderate to high yields (Scheme 1).
Scheme 1. Preparation of the new luminescent metallic complexes 2-7.

All of the complexes were characterized by spectroscopic methods, including $^1$H, $^{31}$P{$^1$H}, $^{13}$C{$^1$H} and/or $^{195}$Pt{$^1$H} NMR, mass spectrometry, conductivity and elemental analysis. The crystal structures of the gold(III) (3) and the palladium(II) compound (4) were determined by an X-ray analysis (Figure 1). Selected bond lengths and angles for both compounds are collected in Table 1 and described below.
The geometry about the gold(III) and palladium(II) centers is pseudo-square planar with the N(2)-M(1)-N(1) angle of 80.5(4)° (3) and 80.5(2)° (4) suggesting a rigid ‘bite’ angle of the chelating ligand. The Au and Pd centers are on an almost ideal plane with negligible deviations from the least-squares plane.

**Figure 1.** Molecular structure of the cation in compound [Au((Ph3P=N-8-C9H6N)-κ-N,N)Cl2]ClO4 3 and of the compound [Pd((Ph3P=N-8-C9H6N)-κ-N,N)Cl2]4 with the atomic numbering scheme.

The main distances M-N(1) or M-N(iminic) and M-N(2) found for the Pd complex 4 are similar to that in related IM complexes, such as [PdCl2(PTA=N(O)-2-NC5H4)], [Pd{κ2-C,N-C6H4(PPh2=NC6H4Me-4’)-2}μ-OAc}2, [Pd{κ2-C,N-C6H4(PPh2=NC6H4Me-4’)-2}(tmeda)]ClO4, [Pd(C6H4CH2NMMe2)(Ph3PNC(O)-2-NC5H4)]ClO4, [(Cp-P(Ph2)=N-CH2-2-NC5H4)PdCl2]2Fe and [(Cp-P(Ph2)=N-CH2-2-NC5H4)PdCl2]Fe(Cp). The distances Au-N(1), Au-M(2), Au-Cl(1) and Au-Cl(2) in 3 are similar to the ones obtained by X-ray crystallographic analysis or DFT calculations for [AuCl2(Ph2PyP=NC(O)-Ph)]ClO4, [(Ph3P=N-CH2-2-NC5H4)AuCl2]ClO4, [AuCl2(PTA=N-C(O)-2-NC5H4)]ClO4, [(Cp-P(Ph2)=N-CH2-2-
NC₅H₄)AuCl₂)₂Fe(ClO₄)₂ and [(Cp-P(Ph₃)=N-CH₂=NC₅H₄)AuCl₂]Fe(Cp)ClO₄. The distances P=N are shorter than in compounds which incorporate a carbonyl group bonded to the iminic nitrogen, which is due to a smaller delocalized charge density as compared to the CO group.

Table 1. Selected bond lengths [Å] and angles [°] for complexes 3 and 4.

<table>
<thead>
<tr>
<th></th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au(1)-N(2)</td>
<td>1.998(8)</td>
<td>Pd(1)-N(2)</td>
</tr>
<tr>
<td>Au(1)-N(1)</td>
<td>2.045(13)</td>
<td>Pd(1)-N(1)</td>
</tr>
<tr>
<td>Au(1)-Cl(2)</td>
<td>2.254(3)</td>
<td>Pd(1)-Cl(2)</td>
</tr>
<tr>
<td>Au(1)-Cl(1)</td>
<td>2.278(5)</td>
<td>Pd(1)-Cl(1)</td>
</tr>
<tr>
<td>P(1)-N(1)</td>
<td>1.656(11)</td>
<td>P(1)-N(1)</td>
</tr>
<tr>
<td>N(2)-Au(1)-N(1)</td>
<td>80.5(4)</td>
<td>N(2)-Pd(1)-N(1)</td>
</tr>
<tr>
<td>N(2)-Au(1)-Cl(2)</td>
<td>172.7(3)</td>
<td>N(2)-Pd(1)-Cl(2)</td>
</tr>
<tr>
<td>N(2)-Au(1)-Cl(1)</td>
<td>95.1(3)</td>
<td>N(2)-Pd(1)-Cl(1)</td>
</tr>
<tr>
<td>N(1)-Au(1)-Cl(2)</td>
<td>95.4(3)</td>
<td>N(1)-Pd(1)-Cl(2)</td>
</tr>
<tr>
<td>N(1)-Au(1)-Cl(2)</td>
<td>175.7(3)</td>
<td>N(1)-Pd(1)-Cl(2)</td>
</tr>
<tr>
<td>Cl(2)-Au(1)-Cl(1)</td>
<td>88.88(15)</td>
<td>Cl(2)-Pd(1)-Cl(1)</td>
</tr>
</tbody>
</table>

The stability of the compounds in different solution was assessed by ³¹P{¹H} NMR spectroscopy in deuterated solvents. Most compounds are stable for weeks in CD₃CN or CD₂Cl₂ solution. The stability in DMSO-d₆ solution was also evaluated for all of the compounds (see Appendix for more details). Gold(III) compounds 2 and 3 have half-lives of 4 (2) or 6 (3) hours, after which they decompose to phosphane oxide (Ph₃P=O) and the coordination gold(III) compound based on the deprotonated uninegative bidentate 8-aminoquinoline fragment [Au(N-8-C₉H₇N)-κ-N,N]Cl₂]. The poor stability for some monometallic Au(III) iminophosphorane coordination complexes¹⁴,¹⁶ and their decomposition to biologically active gold(III) amino derivatives¹⁴ has been described before. The coordination Pd(II) compound 4 in DMSO-d₆ solution has a half-life of about 1.5 hours and over time 4 evolves to the endo cyclometalated species [Pd{k3,C,N,N-C₆H₄(PPh₂=N-8-C₉H₆N)}Cl] (6 in Scheme 1) by activation of the C-H of one aryl group from the PPh₃ fragment of the iminophosphorane ligand. The coordination Pt(II)
compound 5 evolves to a cyclometalated analogue of 6 to give species [Pt{κ^3-C,N,N-C_6H_4(PPh_2=N-8-C_9H_6N)}Cl] (7 in Scheme 2) by heating it in DMSO.

The cyclometalated Pd(II) (6) and Pt(II) (7) species have been fully characterized by spectroscopic methods (including $^{195}$Pt$^1$H NMR), mass spectrometry, conductivity and elemental analysis (see the Experimental Section). These species can also be obtained as pure compounds in high yields (Scheme 1) by reaction of IM ligand 1 with Pd(AcO)$_2$ in CH$_2$Cl$_2$ at room temperature, followed by treatment with LiCl in CH$_3$OH (6) or by refluxing the coordination platinum(II) compound 5 in CH$_2$Cl$_2$ for 3 days (7). The cyclometalated species 6 and 7 are stable in DMSO-$d_6$ solution with half-lives of several weeks. The fact that 6 and 7 are orthometalated species is clearly inferred from the analysis of their NMR spectra. 6 and 7 display downfield shifts in their $^{31}$P$^1$H NMR signals with respect to the coordination compounds 4 and 5, respectively. In the $^1$H and $^{13}$C$^1$H NMR spectra of 6 and 7 there are also new signals due to the presence of 4 protons (a-d) of the cyclometalated aryl ring and six well resolved peaks, for the carbons of this C$_6$H$_4$- fragment. Moreover, the $^2$J$_{Pt-P}$ coupling constant of 426 Hz is visible only for the cyclometalated derivative 7 displaying a signal at 44.7 ppm in $^{31}$P$^1$H NMR with $^{195}$Pt satellites while the signal in the $^{195}$Pt$^1$H NMR is a doublet at -2899.7 ppm. A $^3$J$_{Pt-P}$ coupling constant is not observable for the coordination iminophosphorane compound 5. This coupling constant has not been observed for other coordination iminophosphorane platinum derivatives either.\textsuperscript{11}

The iminophosphorane ligand in compounds 6 and 7 acts as a C,N,N- pincer ligand. Urriolabeitia et al. have reported on the selective C-H activation by palladium(II) complexes in iminophosphoranes based on type of IM ligand, solvents that were used and the temperature at which the cyclometalation reaction takes place giving either endo or exo derivatives.\textsuperscript{17-20} The semi-stabilized IM ligand [Ph$_3$P=N-C$_9$H$_6$N] (1) coordinates to a palladium or platinum center (II) in compounds 4 and 5 affording cyclometalated endo derivatives with the IM acting as a C,N,N-pincer ligand in 6 and 7. The only example of a Pt(II) cyclometalated compound containing an iminophosphorane ligand reported so far is the endo [Pt(C$_6$H$_4$-2-PPh$_2$=N-C(O)-2-NC$_5$H$_4$-κ-C,N,N)Cl] derivative obtained by reaction of [PtCl$_2$(NCPh)$_2$] with Ph$_3$P=N-C(O)-2-NC$_5$H$_4$ in refluxing 2-methoxyethanol.\textsuperscript{20}
3.3. Luminescence studies

*These studies were performed by Dr. Josefina Jiménez and Elena Gascón at the University of Zaragoza, Spain.*

The luminescence studies of the metallic complexes 2-7 and of the previously described IM ligand 1\(^8\) whose luminescence had not been reported before were carried out. All the compounds are luminescent in DMSO solution (5 x 10\(^{-4}\) M) at room temperature.

The excitation and emission data as well as the lifetimes for excited states are summarized in Table 2. The lifetimes are all relatively long (7-12 \(\mu s\)), which indicate the emission transitions are all forbidden and phosphorescent. Ligand 1, non-cyclometalated complexes 2-4 and cyclometalated palladium complex 6 all show similar optical behavior. The spectra show a broad band with the emission maximum at around 486-521 nm, upon excitation between 290-410 nm (see Appendix for more details). Consequently, an intraligand transition modified by the coordination to metal is probably responsible for the luminescence in all of these complexes 2-4 and 6. The luminescence profile and features (excitation and emission maxima) of the cyclometalated platinum complex 7 are different. For this compound, an emission band showing some evidence of vibrational structure is observed, whose maximum is red-shifted from 521 nm in the free ligand 1 to 677 nm (see also Appendix). This spectral shift suggests a \(^3\)MLCT character in the excited state of 7.\(^{21}\) On the other hand, unlike what we observed for 1 and complexes 2-6, the absorption spectrum of 7 exhibits a lower-energy band in the 400-500 nm region (see Appendix) at substantially longer wavelength than the absorption of the free ligand. This new band leads to the observed emissions and is likely to arise from charge-transfer transitions involving the metal, as it has been described for other Pt(II) complexes. Thus, a triplet state of mixed \(\pi^*/\)MLCT character is probably responsible for the phosphorescence in 7.\(^{21-25}\)

Non-cyclometalated platinum complex 5 deserves special attention. A DMSO diluted solution of this pure compound is brightly emissive yellow under a common UV lamp (ex 365 nm) at room temperature. However, the spectrum shows two emissions, whose intensities depend on the excitation wavelength and the time of the luminescence measurement (see Figures 20 and 21 in Appendix). At 390 nm, it exhibits two emission bands, a broad one with the maximum at 513 nm (similar to that observed for ligand 1 and complexes 2-4 and 6) and another one with a
profile and features similar to those observed for the cyclometalated platinum complex 7, with a maximum at 677 nm. Excitation at a longer wavelength, 450 nm, only gives the emission band at 677 nm. When the luminescence of this solution is measured immediately afterwards the spectrum only shows the emission band at 677 nm. Under a common UV lamp (ex 365 nm) we observed an orange-red emission for the irradiated area while the non-irradiated area remained yellow. This observation seems indicative of the transformation of coordination complex 5 into cyclometalated 7 upon UV irradiation, which has been demonstrated by $^{31}$P{$^{1}$H} and $^{1}$H NMR spectroscopy. The transformation is not clean, though, and the NMR spectra of the DMSO solution after irradiation show other signals besides those corresponding to 5 and 7, which have not been identified.

**Table 2.** Luminescent spectral data and lifetime measurement for the compounds 1-7.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{max}$, solution (298 K)$^a$</th>
<th>$\lambda_{exc}$</th>
<th>$\lambda_{em}$ [s ($\mu$s)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>296, 336, 370, 407</td>
<td>521 [10]</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>294, 337, 370, 404</td>
<td>517 [10]</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>298 sh, 342, 368 sh</td>
<td>496 [7]</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>306, 343, 377 sh, 390</td>
<td>513 [12]</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>294, 341 sh, 378, 402</td>
<td>486 [10]</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>348 sh, 373, 440</td>
<td>645, 677 [8], 702 sh</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Data of wavelength are given in nm. Data using DMSO as solvent at room temperature, $5 \times 10^{-4}$ M.

2 and 3 are rare examples of non-cyclometalated gold(III) complexes with chlorido ligands which emit in a fluid solution at room temperature. Two essential control parameters to obtain luminescent (square-planar) gold(III) complexes have been highlighted: the ligand field strength and the rigidity of chelating ligands.$^{26,27}$ These parameters are also relevant for the preparation of luminescent platinum(II) complexes.$^{21}$ The iminophosphorane ligands are strong $\sigma$-donors, and this is expected to lead to an increase in the ligand field strength, which, in turn, decreases the probability for the thermal population of non-emissive d-d states. Importantly, for
all complexes, the energy of the excitation and emission bands are potentially of suitable wavelengths for cellular distribution studies.\(^7\)

The luminescence properties of the compounds in DMSO solution was also studied over time. As expected, the most stable cyclometalated palladium and platinum compounds in DMSO solution (6, 7) and ligand 1 did not change their luminescence significantly over time. Figure 2 shows the change of the luminescence properties of compound 6 in DSMO solution at room temperature over 24 hour period time. The other figures are collected in the Appendix.

![Figure 2](image.png)

**Figure 2.** Study of the luminescence of compound 6 in DMSO solution 5 x 10\(^{-4}\) M at RT over time (24 hours).

In the case of coordination Au(III) complexes, 2 and 3, and coordination Pd(II) complex, 4, the study of the luminescence overtime affords results that are in agreement with the stability of these compounds in DMSO solution. As mentioned before, 2 and 3 decompose (with half-lives of ca. 4 (2) and 6 (3) hours in DMSO-\(d_6\)) to phosphane oxide (Ph\(_3\)P=O) and the coordination Au(III) compound [Au(N-8-C\(_9\)H\(_7\)N)-κ-N,N,N]Cl\(_2\)]. Compound 2 shows a shift of the emission band at 513 nm to lower wavelength (473 nm) in concomitance to the appearance of two new bands of lower intensity at 634 and 683 nm after 3.5 hours.
The spectrum is identical 24 hours later and therefore it may correspond to that of decomposition product [Au(8-C9H7N)-κ-N,N)Cl2]. Compound 3 is slightly more stable and the change in intensity and position of the bands to give spectra identical to that of 2 over time occurs 8 hours later. Coordination Pd(II) complex 4 shows a shift of the emission band at 496 nm to lower wavelength (486 nm), which corresponds to that of the endo cyclometalated species 6 decomposition product.

The study of the luminescence of compound 7 in a mixture DMSO:H2O (50:50) was carried out and a broad emission band at 669 nm was observed, which is similar to that obtained for 7 in DMSO (see Figure 25 in the Appendix). However, the spectrum was completely different after 6 h, showing two broad emission bands at 430 and 630 nm, which may correspond to decomposition products. NMR studies of solutions of mixtures of 7 in DMSO:H2O close to 1:1 ratio showed decomposition to different products over time including most plausibly cyclometalated species 7 with a Cl displaced by DMSO and hydrolysed species. Importantly, the solutions containing these products are still luminescent after 24 hours and their excitation and emission bands are of suitable wavelengths for intramolecular cellular distributions studies.

These preliminary luminescence studies support the idea to select the most intense compounds 3 and 7 as candidates for further intramolecular cellular distribution studies by fluorescence microscopy.

3.4. Antiproliferative studies

These studies were performed by Dr. Angela Casini and Andreia de Almeida at the University of Groningen, the Netherlands.

The antiproliferative properties of ligand 1 and metallic complexes 2-7 were assayed by monitoring their ability to inhibit cell growth using the MTT assay. The cytotoxic activity of the compounds was determined as described in the Experimental Section in the human ovarian cancer A2780 cell line, and in the human lung cancer cell line A549, in comparison to cisplatin. The results are summarized in Table 3. Ligand 1 and its PPh3-based decomposition product (Ph3P=O) are not cytotoxic in all tested cell lines. The coordination complexes 2 (Au(III)) and 5 (Pt(II)) and cyclometalated monometallic Pt(II) complex 7 are as cytotoxic or more cytotoxic...
than cisplatin toward the A2780 cell line (low micromolar range). However, these compounds are poorly selective, being very cytotoxic also towards the non-tumorigenic HEK-293T cell line. The selectivity towards the A2780 cells is more pronounced for the coordination and cyclometalated Pd(II) compounds 4 and 6. Most compounds are poorly cytotoxic towards the human lung cancer cell line A459, with the exception of the cyclometalated monometallic Pt(II) compound 7 which is ca. 3-fold more cytotoxic than cisplatin. However 7 is quite toxic to HEK-293T cell lines as well.

Table 3. IC₅₀ (µM) of ligand 1, PPh₃ oxide and metal complexes 2-7, and cisplatin in human cell lines.a

<table>
<thead>
<tr>
<th></th>
<th>A2780</th>
<th>A549</th>
<th>HEK-293T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ph₃P=O</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>1</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>2*</td>
<td>6.35 ± 0.69</td>
<td>25.1 ± 3.8</td>
<td>21.6 ± 5.1</td>
</tr>
<tr>
<td>4</td>
<td>11.0 ± 1.5</td>
<td>62.5 ± 3.7</td>
<td>53.5 ± 10.4</td>
</tr>
<tr>
<td>5</td>
<td>3.33 ± 0.14</td>
<td>19.5 ± 6.5</td>
<td>10.8 ± 0.8</td>
</tr>
<tr>
<td>6</td>
<td>13.2 ± 2.1</td>
<td>86.5 ± 2.5</td>
<td>66.0 ± 5.5</td>
</tr>
<tr>
<td>7</td>
<td>3.56 ± 0.70</td>
<td>4.60 ± 0.50</td>
<td>2.64 ± 0.87</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>3.90 ± 1.80</td>
<td>8.0 ± 0.5</td>
<td>11.0 ± 2.9</td>
</tr>
</tbody>
</table>

MTT assay (see experimental). All compounds were dissolved in 1% of DMSO and diluted with water before addition to cell culture medium for a 72 h incubation period. Cisplatin was dissolved in H₂O. Since the biological activity of cationic iminophosphorane gold compounds is due to the cation, 2 was used to evaluate the biological activity of compounds 2 and 3 with identical cations.

Studies of intracellular distribution on A2780 cells were attempted with selected compounds (e.g. 3, and 7) using fluorescence microscopy, but unfortunately, and in spite of the different conditions tested (different compounds’ concentrations and incubation time with cells), it was impossible to observe any fluorescence.
3.5. Interaction with plasmid (pBR322) DNA

The key role of DNA in cell replication makes this biomolecule one of the most interesting targets in cancer chemotherapy. It is known that most cytotoxic platinum drugs can form strong covalent bonds with the DNA bases.\textsuperscript{28} However, a variety of platinum compounds act as DNA intercalators upon coordination to the appropriate ancillary ligands, while most gold(III) compounds display reduced affinity for DNA.\textsuperscript{29,30} There are also reports of palladium derivatives interacting with DNA in covalent\textsuperscript{31,32} and non-covalent ways.\textsuperscript{33,34} Conversely, most gold-based compounds do not display strong interaction with DNA.\textsuperscript{35,36} Therefore, agarose gel electrophoresis studies were performed to unravel the effects of compounds 2-7 on plasmid (pBR322) DNA (Figure 3).

\textbf{Figure 3.} Electrophoresis mobility shift assays for cisplatin and compounds 2-7 (see Experimental Section for details). DNA refers to untreated plasmid pBR322. A, B, C and D correspond to metal/DNAbp ratios of 0.25, 0.5, 1.0 and 2.0 respectively.
Plasmid (pBR322) DNA has two main forms: OC (open circular or relaxed form, Form II) and CCC (covalently closed circular or supercoiled form, Form I). Generally, the larger the retardation of supercoiled DNA (CCC, Form I), the greater the DNA unwinding produced by the drug. Changes in electrophoretic mobility of both forms are usually taken as evidence of direct metal-DNA interactions, such as covalently binding the drug to DNA. Binding of cisplatin to plasmid DNA, for instance, results in a decrease in mobility of the CCC form and an increase in mobility if the OC form (lanes A-D for cisplatin, Figure 3).

Treatment of plasmid (pBR322) DNA with increasing amounts of compounds 3-7 do not affect the mobility of the faster-running supercoiled form (Form I or CCC) at ratios up to 1.0 metal/DNA bp. In the case of the coordination compounds of gold(III) (3) and platinum(II) (5), at the highest ratio of 2.0 metal/DNA bp, there is retardation of the faster-running supercoiled form (CCC). It can be, however, concluded that these complexes have no (4, 6, 7) or weak interaction (3, 5) with plasmid (pBR322) DNA.

On the other hand, the coordination gold(III) compound 2 modifies the electrophoretic mobility and the retardation of the faster-running supercoiled form (CCC) in the same way as described previously for other coordination gold(III) compounds containing iminophosphorane ligands. This is due to the poor or lack of stability of these complexes in DMSO/buffer solutions that causes a cleavage of the P=N from the IM ligand, subsequently decomposing the Au(III)-IM complex into Ph₃P=O and cytotoxic square-planar gold(III) compounds, which are known to intercalate in DNA. Compound 3 is slightly more stable than 2 in solution and therefore this effect is less noticeable. However, oxidative damage of the DNA produced by the metal center cannot be ruled out. The rest of compounds which are more stable (coordination and cyclometalated compounds of palladium(II) and platinum(II) have no or a weak interaction with plasmid (pBR322) DNA. It has been established to be the case for some coordination and organometallic compounds of gold(III) and organometallic derivatives of palladium(II) containing iminophosphorane ligands. Moreover, coordination iminophosphorane complexes of palladium and platinum have displayed interactions with DNA stronger but of a different nature that those exerted by cisplatin.
3.6. Interaction with human serum albumin (HSA)

Human serum albumin (HSA) is the most abundant carrier protein in plasma, making about 60% of the total serum protein concentration. The main function of HSA include maintaining osmotic blood pressure, serving as a depot protein, and transporting diverse endogenous and exogenous ligands including fatty acids, bilirubin, drug molecules and metal ions. Furthermore, HSA is able to bind a variety of substrates including metal cations, hormones and most therapeutic drugs. It has been demonstrated that the distribution, the free concentration and the metabolism of various drugs can be significantly altered as a result of their binding to the protein. HSA possesses three fluorophores, these being tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) residues, with Trp214 being the major contributor to the intrinsic fluorescence of HSA. This Trp fluorescence is sensitive to the environment and binding of substrates, as well as changes in conformation that can result in quenching (either dynamic or static).

We recorded the fluorescence spectra of HSA in the presence of increasing amounts of compounds 2-7 and cisplatin in the range of 300-450 nm upon excitation of the tryptophan residue at 295 nm (Figure 4). The compounds caused a concentration dependent quenching of fluorescence without changing the emission maximum or shape of the peaks, as seen in Figure 4 (A) for compound 3. Quenching measurements are a valuable source to reveal the accessibility of fluorophores to quenchers since a decrease in intensity at a specific wavelength is observed in emission spectra upon quencher addition. However, there are a number of quenching mechanisms that are available. By the addition of other molecules, excited fluorophores experience energy loss due to collision between molecules. In the case of collisional (dynamic) quenching, the quencher must diffuse to the fluorophores and contact the excited fluorophore physically, which returns to the ground state after the energy of the excitation previously absorbed is taken away by the quencher. When the quencher binds to a fluorescent molecule, quenching due to the ground-state complex formation between two molecules occurs. For that reason, static quenching is a valuable source of information about the binding between the fluorescence molecule and the quencher.
The fluorescence data were analyzed by the Stern-Volmer equation. While a linear Stern-Volmer plot is indicative of a single quenching mechanism, either dynamic or static, the positive deviation observed in the plots of $F_0/F$ versus $[Q]$ of our compounds (Figure 4) suggests the presence of different binding sites in the protein. Of note, a similar behaviour was observed in the case of some coordination and organometallic iminophosphorane complexes of d$^8$ metals for which it was also reported as concentration dependent fluorescence quenching. In this graph higher quenching by the iminophosphorane complexes was observed compared to that of cisplatin under the chosen conditions and without changing the emission maximum or shape of the peaks.

In the case of $[\text{MCl}_2(\text{PTA} = \text{N-C(O)}-2-\text{NC}_5\text{H}_4)]$ (M = Pd, Pt) isothermal titration calorimetry (ITC) showed two different binding interactions which explained the lack of linearity observed in the fluorescence quenching studies, as the Stern-Volmer method assumes all binding sites to be equivalent. We believe that a similar binding takes place for the iminophosphorane compounds described here.

**Figure 4.** (A) Fluorescence titration curve of HSA with compound 3. Arrow indicates the increase of quencher concentration. (B) Stern-Volmer plot for HSA fluorescence quenching observed with compounds 2-7 and cisplatin.
3.7. Conclusions

In conclusion we have prepared a series of luminescent iminophosphorane complexes of gold(III), palladium(II) and platinum(II) derived from 8-aminoquinoline. The coordination palladium(II) and platinum(II) compounds can evolve further, under appropriate conditions, to give stable cyclometalated endo species \([M\{κ^3-C,N,N-C_6H_4(PPh_2=N-8-C_9H_6N)\}Cl]\) (M = Pd, Pt) by C-H activation of the phenyl group of the PPh\(_3\) fragment. All the compounds exhibit important cytotoxic effects in the low micromolar range in a human ovarian cancer cell line (A2780S), and in particular the Pd(II) derivatives (monometallic coordination 4 and cyclopaladated 6) show promising selectivity being poorly toxic for the non-tumorigenic human embryonic kidney cell line (HEK-293T).

Studies of the interactions of the compounds with plasmid (pBR322) DNA indicate that, unless they lose the IM skeleton in solution, they have none or little interaction with DNA supporting the idea of different mechanisms of action than cisplatin. The complexes display a concentration dependent fluorescence quenching of HSA which has been correlated to a faster binding of our compounds with HSA compared to cisplatin.

Even though the luminescence properties of the compounds did not allow for the detection inside the cells via fluorescence microscopy, these results will allow us to design new IM complexes in order to achieve better stability in biological media and to increase the fluorescence detection limits. More particularly, the focus will be on the synthesis of cyclometalated IM derivatives containing luminescent phosphines.

3.8. Experimental section

Materials and methods

All manipulations involving air-free syntheses were performed at an nitrogen vacuum manifold using standard Schlenk-line techniques under an argon atmosphere or in a glove-box MBraun MOD System. Solvents were purified by use of a PureSolv purification unit from Innovative Technology, Inc. The phosphine substrate PPh\(_3\) was purchased from Sigma-Aldrich, K[AuCl\(_4\)], AgClO\(_4\), [PdCl\(_2\)(COD)], Pd(OAc)\(_2\) and PtCl\(_2\) were purchased from Strem chemicals and used
without further purification. The purity of the compounds, based on elemental analysis, is \( \geq 99.5\% \). NMR spectra were recorded in a Bruker AV400 (\(^1\)H NMR at 400 MHz, \(^{13}\)C NMR at 100.6 MHz, \(^{31}\)P NMR at 161.9 MHz, \(^{195}\)Pt NMR at 85.8 MHz). Chemical shifts (\(\delta\)) are given in ppm using CD\(_3\)CN, CDCl\(_3\), DMSO-\(d_6\) or D\(_2\)O as solvents. \(^1\)H and \(^{13}\)C resonances were measured relative to solvent peaks considering TMS = 0 ppm. \(^{31}\)P{\(^1\)H} was externally referenced to H\(_3\)PO\(_4\) (85\%) and \(^{195}\)Pt{\(^1\)H} was referenced to [PtCl\(_6\)]\(^2\). Infrared spectra (4000-250 cm\(^{-1}\)) were recorded on a Nicolet 6700 FT-IR spectrophotometer from nujol mulls between polyethylene sheets. Elemental analyses were performed on a Perkin Elmer 2400 CHNS/O Analyzer, Series II. Mass spectra (ESI) were performed on an Agilent Analyzer and on a Bruker Analyzer. Conductivity was measured in an OAKTON PC 700 pH/conductivity meter in acetone solution. Electrophoresis experiments were carried out in a Bio-Rad Mini sub-cell GT horizontal electrophoresis system connected to a Bio-Rad Power Pac 300 power supply. Photographs of the gels were taken with an Alpha Innotech FluorChem 8900 camera. Fluorescence intensity measurements were carried out on a PTI QM-4/206 SE Spectrofluorometer (PTI, Birmingham, NJ) with right angle detection of fluorescence using a 1 cm path length quartz cuvette.

**Synthesis of 1**

\( \text{Ph}_3\text{P}=N-8-\text{C}_9\text{H}_6\text{N} \)

An alternative method of synthesis was used: a solution of PPh\(_3\) (2.59 mmol) in CH\(_2\)Cl\(_2\) was added dropwise to a solution of 8-Azidoquinoline\(^9\) (2.59 mmol) in CH\(_2\)Cl\(_2\) at RT and allowed to react for 1 h. The solvent was reduced in vacuo to 2 mL and Et\(_2\)O (10 mL) was added. A pale-yellow solid precipitated and was filtered off and dried in vacuo. \( 1 \) was used without further purification. Yield: 0.857 g, 82\%.

**Synthesis of 2 and 3**

\[ \text{[Au(Ph}_3\text{P}=N-8-\text{C}_9\text{H}_6\text{N})-\kappa-N,N)]\text{Cl}_2\text{X} \quad \text{[X = PF}_6^{-}\quad (2); \quad \text{ClO}_4^{-}\quad (3)] \]

To K[AuCl\(_4\)] (0.076 g, 0.2 mmol) in dry MeCN (10 mL), AgClO\(_4\) (0.091 g, 0.44 mmol) for the obtention of 2 or AgPF\(_6\) (0.111 g, 0.44 mmol) for the obtention of 3 in dry CH\(_3\)CN (5 mL) was added. The reaction mixture was stirred in the darkness for 30 min and subsequently filtered through celite (to remove AgCl). \( 1 \) (0.081 g, 0.2 mmol) in CH\(_2\)Cl\(_2\) (1 mL) was added and the
yellow solutions became purple instantly. After stirring for 30 min the reaction mixture was filtered through celite (to remove KClO₄) and then the solvent was reduced in vacuo to ~2 mL. Upon addition of Et₂O (10 mL), a purple solid was obtained, which was washed with CH₃CN (<1 mL at a time) and Et₂O and dried in vacuo.

2: Yield: 0.1198 g, 73%. Anal. Calcd. for C₂₇H₂₁AuCl₂N₂P₂ (817.28): C, 39.68; H, 2.59; N, 3.43. Found: C, 39.21; H, 2.36; N, 3.21. MS(ESI+) [m/z]: 671.0 [M - PF₆]⁺. ³¹P{¹H} NMR (CD₃CN): δ 39.6 (s), -142.5 (sept, PF₆). ¹H NMR (CD₃CN): δ 6.66 (1H, d, J = 7.8 Hz, H₇), 7.15 (1H, t, J = 8.0 Hz, H₆), 7.62 (1H, d, J = 8.2 Hz, H₅), 7.62-7.82 (9H, m, Hpara + Hmeta), 8.04 (1H, dd, J = 8.3, 5.7 Hz, H₃), 8.13 (6H, dd, J = 12.6, 7.6 Hz, Hortho), 8.85 (1H, d, J = 8.3 Hz, H₄), 9.44 (1H, d, J = 5.6 Hz, H₂). ¹³C{¹H} NMR (CD₃CN): δ 121.6 (s, C₉H₆N), 122.4 (s, C₉H₆N), 122.6 (s, C₇), 124.1 (s, C₅), 124.2 (s, C₃), 128.4 (s, C₆), 130.2 (d, J = 13.3 Hz, Cmeta), 131.5 (s, Cipso), 134.1 (d, J = 10.5 Hz, Cortho), 135.1 (d, J = 2.8 Hz, Cpara), 144.0 (s, C₄), 145.8 (s, CN=P), 149.0 (s, C₂). IR (cm⁻¹): ν 368 (Au-Cl), 1083 (ν br) and 622 (ClO₄⁻), 1267 (P=N). Conductivity (acetone): Λ = 110 µS/cm.

3: Yield: 0.109 g, 69%. Anal. Calcd. for C₂₇H₂₁AuCl₃N₂O₄P (771.77): C, 42.02; H, 2.74; N, 3.63. Found: C, 42.08; H, 2.96; N, 3.66. MS(ESI+) [m/z]: 671.1 [M - ClO₄]⁺. ³¹P{¹H} NMR (CD₃CN): δ 39.6 (s). ¹H NMR (CD₃CN): δ 6.48 (1H, d, J = 7.7 Hz, H₇), 7.06 (1H, t, J = 8.0 Hz, H₆), 7.49 (1H, d, J = 8.1 Hz, H₅), 7.62-7.82 (9H, m, Hpara + Hmeta), 7.93 (1H, dd, J = 8.0, 5.6 Hz, H₃), 8.16 (6H, dd, J = 12.6, 7.6 Hz, Hortho), 8.67 (1H, d, J = 8.3 Hz, H₄), 9.41 (1H, d, J = 5.3 Hz, H₂). ¹³C{¹H} NMR (CD₃CN): δ 121.5 (s, C₉H₆N), 122.4 (s, C₉H₆N), 122.6 (s, C₅), 124.1 (s, C₃), 124.2 (s, C₆), 130.2 (d, J = 13.3 Hz, Cmeta), 131.5 (s, Cipso), 134.1 (d, J = 10.5 Hz, Cortho), 135.1 (d, J = 2.8 Hz, Cpara), 144.0 (s, C₄), 145.8 (s, CN=P), 149.0 (s, C₂). IR (cm⁻¹): ν 368 (Au-Cl), 1083 (ν br) and 622 (ClO₄⁻), 1267 (P=N). Conductivity (acetone): Λ = 128 µS/cm.

**Synthesis of 4**

[Pd((Ph₃P=⁻N-8-C₉H₆N)-κ-N₄N)Cl₂]

[PdCl₂(COD)] (0.041 g, 0.15 mmol) and 1 (0.042 g, 0.15 mmol) were dissolved in dry and degassed CH₂Cl₂ (4 mL) and left to react for 3 h at room temperature after which an orange
precipitate had formed. The solvent was then reduced to a minimum in vacuo and upon addition of Et₂O a pale orange solid was obtained which was washed twice with CHCl₃ and dried in vacuo. Solution of 1 (0.081 g, 0.2 mmol) in CH₂Cl₂ was dropwise added to the a solution of PdCl₂(COD) (0.2 mmol) in CH₂Cl₂ while stirring at RT and allowed to react for 30 min. The product was then concentrated under vacuum and precipitated with diethyl ether, producing a light orange solid. Yield: 0.098 g, 84%. Anal. Calcd. for C₂₇H₂₁Cl₂N₂PPd (581.77): C, 55.74; H, 3.64; N, 4.82. Found: C, 55.38; H, 3.65; N, 4.90. MS(ESI+) 509.0 [M-Cl]²⁺, 544.4 [M-Cl]⁺. ³¹P{¹H} NMR (CDCl₃): δ 30.0 (s). ¹H NMR (CDCl₃): δ 6.27 (1H, d, J = 7.8 Hz, H₇), 6.88 (1H, t, J = 7.9 Hz, H₆), 7.13 (1H, d, J = 8.0 Hz, H₅), 7.47 (1H, dd, J = 8.3, 5.2 Hz, H₃), 7.58-7.65 (9H, m, H para + H meta), 8.22 (1H, d, J = 8.3 Hz, H₄), 9.26 (1H, dd, J = 5.2, 1.4 Hz, H₂). ¹³C{¹H} NMR (CDCl₃): δ 118.6 (s, C₇), 118.7 (s, C₅), 121.8 (s, C₃), 125.2 (s, C₉_H₆_N), 126.2 (s, C₉_H₆_N), 127.2 (s, C₆), 129.0 (d, J = 12.9 Hz, C meta), 130.0 (s, C ipso), 133.2 (d, J = 3.0 Hz, C para), 133.8 (d, J = 9.8 Hz, C ortho), 138.2 (s, C₄), 149.7 (s, CN=P), 150.2 (s, C₂). IR (cm⁻¹): ν 342 (Pd-Cl), 1268 (P=N). Conductivity (acetone): Λ = 3.4 µS/cm.

Synthesis of 5

[Pt(Ph₃P=N-8-C₉H₆N)-κ-N,N)Cl₂]

A solution of 1 (0.1616 g, 0.4 mmol) in 20 mL of CH₂Cl₂ was added to a suspension of PtCl₂ (0.1064 g, 0.4 mmol) in 15 mL and refluxed for 3 h. Upon completion a brown side-product was filtered off and the resulting yellow solution was concentrated under vacuum. Upon addition of diethyl ether, a bright yellow solid was obtained. Yield: 0.1662 g, 62%. Anal. Calcd. for C₂₇H₂₁Cl₂N₂PPt (670.43): C, 48.37; H, 3.16; N, 4.18. Found: C, 48.18; H, 3.14; N, 4.15. MS(ESI+) [m/z]: 635.1 [M - Cl]⁺. ³¹P{¹H} NMR (CDCl₃): δ 31.3 (s). ¹⁹⁵Pt{¹H} NMR (CDCl₃): δ -1930.3 (s). ¹H NMR (CDCl₃): δ 6.23 (1H, d, J = 7.9 Hz, H7), 6.83 (1H, t, J = 7.9 Hz, H6), 7.11 (1H, d, J = 8.0 Hz, H5), 7.46 (1H, t, J = 8.3 Hz, H3), 7.57-7.67 (9H, m, H para + H meta), 8.14 (6H, dd, J = 8.0, 8.2 Hz, H ortho), 8.30 (1H, d, J = 8.1 Hz, H4), 9.63 (1H, d, J = 5.0 Hz, H2). ¹³C{¹H} NMR (CDCl₃): δ 118.8 (d, J = 5.9 Hz, C₂), 119.2 (s,
52

C₅), 122.1 (s, C₅), 125.0 (s, C₆H₅N), 126.0 (s, C₆H₅N), 127.0 (s, C₆), 129.0 (d, J = 12.6 Hz, Cmeta), 130.2 (s, Cipso), 133.2 (d, J = 2.9 Hz, Cpara), 133.8 (d, J = 9.9 Hz, Cortho), 137.3 (s, C₄), 148.6 (s, C₂), 151.0 (s, CN=P). IR (cm⁻¹): ν 341 (Pt-Cl), 1266 (P=N). Conductivity (acetone): Λ = 1.2 µS/cm.

**Synthesis of 6**

[Pd[κ³-C,N,N-C₆H₄(PPh₂=N-8-C₉H₆N)Cl]

A solution of 1 (0.121 g, 0.3 mmol) in 20 mL of CH₂Cl₂ was added to Pd(OAc)₂ (0.067 g, 0.3 mmol) in 10 mL CH₂Cl₂. The mixture was allowed react overnight (15 h) while stirring at RT. Excess LiCl (0.020 g, 0.47 mmol) in CH₃OH was then added and the mixture was left to react for 30 min. Concentration under vacuum and precipitation with diethyl ether afforded an orange solid.

Yield: 0.133 g, 81%. Anal. Calcd. for C₂₇H₂₀ClN₂PPd (545.31): C, 59.47; H, 3.70; N, 5.14. Found: C, 58.98; H, 3.72; N, 5.20. MS(ESI+) [m/z]: 544.4 [M], 509.3 [M - Cl]+. ³¹P{¹H} NMR (CDCl₃): δ 43.4 (s). ¹H NMR (CDCl₃): δ 6.73 (1H, dd, J = 6.6, 1.7 Hz, H₇), 6.90 (1H, ddd, J = 11.3, 7.7, 1.4 Hz, H₄), 7.05 (1H, ddd, J = 12.8, 7.4, 1.1 Hz, H₃), 7.14-7.20 (2H, m, H₅ + H₆), 7.26 (1H, t, J = 7.7 Hz, H₉), 7.45 (1H, dd, J = 8.3, 4.8 Hz, H₃), 7.53-7.61 (4H, m, Hmeta), 7.65-7.61 (2H, m, Hpara), 7.91-7.99 (4H, m, Hortho), 8.17 (1H, dd, J = 8.3, 1.5 Hz, H₄), 8.24 (1H, dd, J = 7.9, 1.6 Hz, H₉), 9.19 (1H, dd, J = 4.7, 1.6 Hz, H₂). ¹³C{¹H} NMR (CDCl₃): δ 115.7 (d, J = 7.9 Hz, C₇), 117.2 (s, C₅ + C₆), 121.9 (s, C₃), 124.6 (d, J = 15.6 Hz, Cₙ), 125.5 (s, C₆H₅N), 126.4 (s, C₆H₅N), 127.4 (s, C₅ + C₆), 128.8 (d, J = 4.7 Hz, Cc), 129.4 (d, J = 11.9 Hz, Cmeta), 130.7 (s, Cipso), 131.3 (d, J = 3.1 Hz, Cb), 133.1 (d, J = 10.4 Hz, Cortho), 133.6 (d, J = 2.8 Hz, Cpara), 137.7 (s, C₄), 138.6 (d, J = 14.7 Hz, Ca), 143.5 (d, J = 144.7 Hz, Ce, C-P), 147.2 (s, CN=P), 148.4 (s, C₂), 154.5 (d, J = 19.1 Hz, Cf, C-Pd). IR (cm⁻¹): ν 307 (Pd-Cl), 1285 (P=N). Conductivity (acetone): Λ = 30.5 µS/cm
Synthesis of 7
[Pt{κ$_3$-C,N,N-C$_6$H$_4$(PPh$_2$=N-8-C$_9$H$_6$N)}Cl]

The coordination platinum(II) complex 5 (0.167 g, 0.25 mmol) was dissolved in CH$_2$Cl$_2$ and the resulting solution refluxed for 3 days. After cooling at RT, the solution was concentrated under vacuum and 20 mL of diethyl ether were added. A yellow solid precipitated (7) which was filtered off, dried *in vacuo* and used without further purification.

Yield: 0.1353 g, 87%. Anal. Calcd. for C$_{27}$H$_{20}$ClN$_2$Pt·H$_2$O (651.08): C, 49.74; H, 3.40; N, 4.30. Found: C, 49.77; H, 3.37; N, 4.20. MS (ESI+) [m/z]: 634.08 [M+H]$^+$.

$^{31}$P{$^1$H} NMR (CDCl$_3$): $\delta$ 44.7 (s, $^2$J$_{P$-Pt} = 426 Hz).

$^{195}$Pt{$^1$H} NMR (CDCl$_3$): $\delta$ -2899.69 (d, $^2$J$_{P$-Pt} = 419 Hz).

$^1$H NMR (CDCl$_3$): $\delta$ 6.74 (1H, t, $J = 4.3$ Hz, H$_7$), 6.93 (1H, dd, $J = 11.7$, 7.6 Hz, H$_d$), 7.06 (1H, dd, $J = 13.4$, 6.9 Hz, H$_c$), 7.14-7.20 (2H, m, H$_5$ + H$_6$), 7.31 (1H, dd, $J = 7.6$ Hz, H$_b$), 7.51 (1H, dd, $J = 8.3$, 4.8 Hz, H$_3$), 7.55-7.60 (4H, m, H$_{meta}$), 7.69 (2H, t, $J = 7.2$ Hz, H$_{para}$), 7.95 (4H, dd, $J = 12.3$, 7.6 Hz, H$_{ortho}$), 8.23-8.29 (2H, m, H$_4$+H$_a$), 9.47 (1H, d, $J = 4.3$ Hz, H$_2$).

$^{13}$C{$^1$H} NMR (CDCl$_3$): $\delta$ 116.1 (s, C$_7$), 117.8 (s, C$_5$ + C$_6$), 122.1 (s, C$_3$), 124.0 (d, $J = 15.6$ Hz, C$_c$), 125.2 (s, C$_9$H$_6$N), 126.0 (s, C$_9$H$_6$N), 127.5 (s, C$_5$ + C$_6$), 129.1 (d, $J = 4.7$ Hz, C$_d$), 129.5 (d, $J = 12.2$ Hz, C$_{meta}$), 131.1 (d, $J = 3.2$ Hz, C$_b$), 131.3 (s, C$_{ipso}$), 133.2 (d, $J = 10.5$ Hz, C$_{ortho}$), 133.7 (d, $J = 2.8$ Hz, C$_{para}$), 137.1 (d, $J = 13.8$Hz, C$_a$), 137.6 (s, C$_4$), 142.7 (d, $J = 138.4$ Hz, C$_e$, C-P), 147.4 (s, CN=P), 147.7 (s, C$_2$), signal corresponding to C-Pt (Cf) not observable. IR (cm$^{-1}$): $\nu$ 319 (Pt-Cl), 1286 (P=N). Conductivity (acetone): $\Lambda = 5.7$ $\mu$S/cm.

**Luminescence Studies**

Absorption spectra in solution were recorded with a Unicam UV-4 spectrophotometer. Steady-state photoluminescence spectra were recorded with a Jobin-Yvon Horiba Fluorolog FL-3-11 spectrofluorimeter using band pathways of 3 nm for both excitation and emission. Phosphorescence lifetimes were recorded with a Fluoromax phosphorimeter accessory containing a UV xenon flash tube at a flash rate between 0.05 and 25 Hz. The lifetime data were fit using the Jobin-Yvon software package and the Origin 7.0 program.
Cell culture and inhibition of cell growth

The human lung cancer cell line A549 and the human ovarian cancer cell line A2780 and the human (obtained from the European Centre of Cell Cultures ECACC, Salisbury, UK) were cultured in DMEM (Dulbecco’s Modified Eagle Medium) and RPMI, respectively, containing GlutaMaxI supplemented with 10% FBS and 1% penicillin/streptomycin (all from Invitrogen), at 37°C in a humidified atmosphere of 95% of air and 5% CO₂ (Heraeus, Germany). Non-tumoral human embryonic kidney cells HEK293 were kindly provided by Dr. Maria Pia Rigobello (CNRS, Padova, Italy), and were cultivated in DMEM medium, added with GlutaMaxI (containing 10% FBS and 1% penicillin/streptomycin (all from Invitrogen) and incubated at 37°C and 5% CO₂. For evaluation of growth inhibition, cells were seeded in 96-well plates (Costar, Integra Biosciences, Cambridge, MA) and grown for 24 h in complete medium. Solutions of the compounds were prepared by diluting a freshly prepared stock solution (in DMSO) of the corresponding compound in aqueous media. Afterwards, the intermediate dilutions of the compounds were added to the wells (100 μL) to obtain a final concentration ranging from 0 to 150 μM, and the cells were incubated for 72 h. DMSO at comparable concentrations did not show any effects on cell cytotoxicity. Following 72 h drug exposure, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the cells at a final concentration of 0.25 mg ml⁻¹ incubated for 2 h, then the culture medium was removed and the violet formazan (artificial chromogenic precipitate of the reduction of tetrazolium salts by dehydrogenases and reductases) dissolved in DMSO. The optical density of each well (96-well plates) was quantified three times in tetraplicates at 540 nm using a multiwell plate reader, and the percentage of surviving cells was calculated from the ratio of absorbance of treated to untreated cells. The IC₅₀ value was calculated as the concentration reducing the proliferation of the cells by 50% and is presented as a mean (± SE) of at least three independent experiments.
Interaction of of compounds 2-7 and cisplatin with plasmid (pBR322) DNA by 
Electrophoresis 
(Shift Mobility Assay)

10 µL aliquots of pBR322 plasmid DNA (20 µg/mL) in buffer (5 mM Tris/HCl, 50 mM NaClO₄, pH = 7.39) were incubated with molar ratios between 0.25 and 2.0 of the compounds at 37 °C for 20 h in the darkness. Samples of free DNA and cisplatin-DNA adduct were prepared also as controls. After the incubation period, 2 µL of loading dye were added to the samples and of these mixtures, only 7 µL were finally loaded into the gel. The samples were separated by electrophoresis in 1%(w/v) agarose gel for 1.5 h at 84 V in Tris-acetate/EDTA buffer (TAE). Afterwards, the gel was dyed for 30 min with a solution of GelRed Nucleic Acid stain.

Interaction of metal complexes with HSA by Fluorescence Spectroscopy

The excitation wavelength was adjusted at 295 nm, and the emission spectra were recorded at room temperature in the range of 300 to 450 nm. The fluorescence intensities of the new compounds, the buffer and the DMSO are negligible under these conditions, and so is the effect of additions of pure DMSO to the fluorescence of HSA. An 8 mM solution of each compound in DMSO was prepared and ten aliquots of 2.5 µL were added successively to a solution of HSA (10 µM) in phosphate buffer (pH = 7.39), the fluorescence being measured after each addition.

The data was analyzed using the classical Stern-Volmer equation \( F_0/F = 1 + K_{sv}[Q] \).
3.9. Appendix

*Table with the crystal data and structure refinement for complexes 3 and 4, luminescence profiles and studies of the luminescence overtime in solution for ligand 1 and compounds 2-7; Stability of compounds 2-7 in DMSO-d₆ solution overtime by $^{31}$P{$^1$H} NMR spectroscopy.*


Single crystals of 3a and 3d (see details below) were mounted on a glass fiber in a random orientation. Data collection was performed at room temperature on a Kappa CCD diffractometer using graphite monochromated Mo-Ka radiation (λ=0.71073 Å). Space group assignments were based on systematic absences, E statistics and successful refinement of the structures. The structures were solved by direct methods with the aid of successive difference Fourier maps and were refined using the SHELXTL 6.1 software package. All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were assigned to ideal positions and refined using a riding model. Details of the crystallographic data are given in Table S1 (below). These data can be obtained free of charge from The Cambridge Crystallographic Data Center via www.ccdc.cam.ac.uk/data_request/cif. (CCDC 977990 for compound 3, and 977991 for compound 4).

**3**: Crystals of 3 (purple prisms with approximate dimensions 0.25 x 0.23 x 0.23mm) were obtained from a solution of 3 in CH₃CN by slow diffusion of Et₂O at RT. **4**: Crystals of 4 (orange prisms with approximate dimensions 0.25 x 0.24 x 0.22mm) were obtained from a solution of 4 in CH₂Cl₂ by slow diffusion of Et₂O at RT.
Table 4. Crystal Data and Structure Refinement for Complexes 3 and 4.

<table>
<thead>
<tr>
<th>Compound formula</th>
<th>3</th>
<th>4</th>
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<tr>
<td>fw</td>
<td>828.81</td>
<td>939.83</td>
</tr>
<tr>
<td>T [K]</td>
<td>293(2)</td>
<td>293(2)</td>
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<tr>
<td>λ (MoKα)[Å]</td>
<td>0.71073</td>
<td>0.71073</td>
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<tr>
<td>crystal system</td>
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<td>P-1</td>
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<td>7.894(5)</td>
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<tr>
<td>b [Å]</td>
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<tr>
<td>c [Å]</td>
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<td>γ [°]</td>
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<td>V [Å³]</td>
<td>3125(2)</td>
<td>3738.5(13)</td>
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<tr>
<td>Z</td>
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<td>0.1509</td>
<td>0.1343</td>
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</table>
3.9.2. Luminescence Studies for Ligand 1 and compounds 2-7.

**Figure 5.** Absorption spectra of ligand 1 and gold compounds 2 and 3 in DMSO solution (5 x 10^{-4} M) at RT.

**Figure 6.** Absorption spectra of ligand 1 and palladium compounds 4 and 6 in DMSO solution (5 x 10^{-4} M) at RT.
Figure 7. Absorption spectra of ligand 1 and platinum compounds 5 and 7 in DMSO solution (5 x 10^{-4} M) at RT.

Figure 8. Excitation (blue) and emission (red) spectra of compound 1 in DMSO solution (5 x 10^{-4} M) at RT.
Figure 9. Luminescence of compound 1 in DMSO solution (5 x 10^{-4} M) at RT over time (24 h).

Figure 10. Excitation (blue) and emission (red) spectra of compound 2 in DMSO solution (5 x 10^{-4} M) at RT.
**Figure 11.** Luminescence of compound 2 in DMSO solution (5 x 10^{-4} M) at RT over time (24 h).

**Figure 12.** Excitation (blue) and emission (red) spectra of compound 3 in DMSO solution (5 x 10^{-4} M) at RT.
Figure 13. Luminescence of compound 3 in DMSO solution (5 x 10^{-4} M) at RT over time (24 h).

Figure 14. Excitation (blue) and emission (red) spectra of compound 4 in DMSO solution (5 x 10^{-4} M) at RT.
**Figure 15.** Luminescence of compound 4 in DMSO solution ($5 \times 10^{-4} \text{ M}$) at RT over time (24 h).

**Figure 16.** Excitation (blue and green) and emission (red) spectra of compound 5 in DMSO solution ($5 \times 10^{-4} \text{ M}$) at RT.
**Figure 17.** Excitation (blue) and emission (red) spectra of compound 5 in DMSO solution (5 x $10^{-4}$ M) at RT immediately after the first measurement of luminescence.

**Figure 18.** Excitation (blue) and emission (red) spectra of compound 6 in DMSO solution (5 x $10^{-4}$ M) at RT.
Figure 19. Luminescence of compound 6 in DMSO solution (5 x 10^{-4} M) at RT over time (24 h).

Figure 20. Excitation (blue) and emission (red) spectra of compound 7 in DMSO solution (5 x 10^{-4} M) at RT.
Figure 21. Luminescence of compound 7 in DMSO solution (5 x 10^{-4} M) at RT over time (24 h).

Figure 22. Excitation (blue) and emission (red) spectra of compound 7 in DMSO:H_{2}O (50:50) solution (5 x 10^{-4} M) at RT.
Figure 23. Luminescence of compound 7 in DMSO:H₂O (50:50) solution (5 x 10⁻⁴ M) at RT over time (24h).

3.9.3. Stability of compounds 2-7 in DMSO-d₆ solution overtime assessed by ³¹P{¹H} NMR spectroscopy. Selected ³¹P{¹H} NMR spectra for compounds 4 and 7.

<table>
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<tr>
<th>Compound</th>
<th>Time (DMSO-d₆)</th>
<th>Half life (50%)</th>
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<td>4 h</td>
<td>53%</td>
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<tr>
<td></td>
<td>1 d</td>
<td>13%</td>
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<td></td>
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<tr>
<td></td>
<td>0%</td>
<td></td>
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<tr>
<td></td>
<td>1.5 h</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>&gt;99%</td>
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<td></td>
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<td>7</td>
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</tr>
<tr>
<td></td>
<td>&gt;99%</td>
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</tbody>
</table>

% of decomposition of initial compound determined by integration of all the signals appearing in the ³¹P{¹H} NMR spectra, the sum being set to 100%.
Figure 24. Conversion of coordination palladium compound \([\text{Pd}(\text{Ph}_3\text{P} = \text{N}-8\text{-C}_9\text{H}_6\text{N}) - \kappa\text{-N,N})\text{Cl}_2]\) 4 \((\delta = 30.0 \text{ ppm})\) into cyclometalated \(\text{[Pd}\{\kappa^3\text{-C,N,N-}C_6\text{H}_4(\text{PPh}_2 = \text{N}-8\text{-C}_9\text{H}_6\text{N})\}\text{Cl}]\) 6 \((\delta = 44.6 \text{ ppm})\) in DMSO-\(d_6\) solution over time. Small peak at \(\delta = 29.4 \text{ ppm}\) corresponds to \(\text{Ph}_3\text{P}=\text{O}\).

Figure 25. Study of the stability of cycloplatinated compound \(\text{[Pt}\{\kappa^3\text{-C,N,N-}C_6\text{H}_4(\text{PPh}_2 = \text{N}-8\text{-C}_9\text{H}_6\text{N})\}\text{Cl}]\) 7 \((\delta = 45.2 \text{ ppm})\) by \(^{31}\text{P}\{^1\text{H}\}\) NMR spectroscopy in DMSO-\(d_6\) solution over time.
3.10. Bibliography


22. Kozhevnikov, D.N.; Kozhevnikov, V.N.; Shafikov, M.Z.; Prokhorov, A.M.; Bruce, D.W.; Williams, J.A.G. Phosphorescence vs. fluorescence in cyclometalated platinum(II) and


4.1. Cyclometallated gold(III) and platinum(II) compounds as potential anticancer agents

From the various strategies and approaches that have been employed in the search of more effective and selective potential anticancer metallodrugs, organometallic compounds have emerged as attractive candidates. It has been reported that organometallic platinum compounds perform better than non-organometallic derivatives. Overall, organometallic compounds are more stable in physiological environments and more lipophilic than coordination metal complexes, which allows for the design of anticancer metallodrugs with improved properties. In the last five years, several reviews on the anticancer activity of organometallic compounds of different transition metals have appeared in the literature.

In particular, gold(III) and platinum(II) organometallic compounds have been studied as potential anticancer agents. A number of complexes containing the [Pt(COD)] fragment and different ligands, such as alkyls, alkynyls, and nucleosides have been recently described. Platinum COD alkynyl compounds showed high toxicity against HT-29 colon carcinoma and MCF-7 breast adenocarcinoma cell lines while [PtMe(R-COD)L] compounds with different ligands (halides, alkyl, aryl, alkynyl) revealed higher toxicity to HeLa cells in comparison to that of cisplatin. In the case of gold(III), it is well known that pincer ligands containing carbon- and nitrogen-ligands stabilize the metal center against reduction to gold(I) and gold(0) species in physiological media. Some cyclometallated gold(III) complexes based on C-N-N and C-N-C pincer complexes have displayed notable anticancer activity in vitro and in vivo by a mode of action different than that of cisplatin. The presence of
the σ(M-C) bond in these complexes, potentially increases the stability allowing the organometallic fragment to reach the cell unaltered. Additionally, for platinum-based compounds the presence of aromatic groups in the cyclometallated ligand might favor intercalative binding to DNA (π-π stacking), while the labile positions in the coordination sphere of the metal may favor covalent coordination to DNA (similar to that for cisplatin). Very recently, a luminescent DNA intercalator cyclometallated platinum(II) complex, [Pt(C^N^N)(C-NtBu)]ClO₄ (HC^N^N=6-phenyl-2,2’-bipyridyl) with a potent inhibitory effect in human cancer cells in vitro and in a xenograft model in mice has been described.²⁹ The stabilization of the topoisomerase I–DNA complex with resulting DNA damage by the cyclometallated compound is suggested to contribute to its anticancer activity.

![Chart 1](image.png)

**Chart 1.** Possible cyclometallation positions in organometallic iminophosphorane derivatives.

Iminophosphorane ligands can be cyclometallated at different positions generating stable organometallic compounds containing pincer C,N- and C,N,N- ligands.³⁰-³² Urriolabeitia and co-workers described cyclometallation processes for palladium(II) compounds for which exo or endo products could be obtained depending on the ligand, the solvent and the temperature employed. Endo derivatives are those generated by C-H activation of an aryl group (H in ortho to the phosphorous atom) in the phosphine fragment of the R₃P=NR iminophosphorane moiety (see
Chart 1). *Exo* derivatives are those generated by C-H activation on an aryl group contained in the imino or amido fragment of the IM ligand. Additionally, organometallic *exo* compounds can be prepared by exchange of a bromine or chlorine atom by a metal in the imino or amido fragment of the iminophosphorane. By modification of synthetic conditions and choice of appropriate ligands we can potentially prepare *exo* or *endo* cyclometallated complexes. Most *endo* derivatives involve the use of semi-stabilized IM ligands (Chart 1). This synthetic route limits the use of phosphines to those containing at least one aryl group. This becomes important if we want to use alternative phosphines in order to improve the hydrophilicity of the final product or to tune electronic/steric properties. On the other hand, the preparation of *exo* derivatives allows for the use of a variety of different phosphines. The best substrates for this purpose are stabilized iminophosphoranes containing CO or -CH2- between the aryl group to be metallated in the ortho position and the N=PR3 fragment (Chart 1).

**Chart 2.** *Endo* gold(III) and *exo* palladium(II) complexes previously prepared in our group.33,34

As mentioned in the introduction, our group prepared *endo* cyclometallated compounds of gold(III) with the IM ligand Ph3P=NPh33,35 In the previous chapter we also described cyclometallation processes happening at high or room temperature in polar solvents with the ligand PPh3=N-8-C9H8N to afford *endo* derivatives of palladium(II) and platinum(II) in which
the iminophosphorane moiety behaved as a C,N,N- pincer ligand. In order to obtain organometallic compounds of palladium(II) in water, we synthesized an iminophosphorane ligand (PTA=N-C(O)-2BrC₆H₄ (C,N-IM)) containing PTA. Exo palladium(II) complexes were obtained by oxidative addition of a palladium(0) derivative on the C-Br bond (Chart 2). All our studies with iminophosphorane metal compounds indicated that in most cases the cyclometallated compounds were more stable in physiological media than coordination N,N-compounds, and that they displayed relevant anticancer properties (especially cationic compounds). Within this frame work, we aimed to prepare exo cyclometallated IM compounds of gold(III) and platinum(II) in a systematic way in order to expand the range of phosphines incorporated into the final molecule to tune the hydrophilicity and/or electronic/steric properties of the resulting complexes.

This chapter reports on the synthesis of novel exo cyclometallated C,N-IM compounds of gold(III) and platinum(II) containing the water-soluble phosphine PTA (1,3,5-Triaza-7-phosphaadamantane) and the synthesis of endo C,N-IM compounds of platinum(II) derivatives never described before. These metal-complexes along with previously described exo derivative [Au(2-C₆H₄C(O)N=PPh₃)Cl₂] (1) and cisplatin have been evaluated against a number of human cancer cell lines in vitro. We studied the interaction of these complexes with plasmid (pBR322) DNA and HSA and the interaction of the platinum(II) compounds with Calf Thymus DNA by circular dichroism. Initial cell death mechanistic insights of the most active compounds in cancer cells are also described in this chapter. Additionally, the permeability of selected platinum(II) compounds was evaluated by two different assays: in vitro caco-2 monolayers and rat perfusion assay, in order to make comparisons with cisplatin and drugs or compounds that can be orally administered.

4.2. Synthesis and characterization

The synthesis of the exo cyclometallated gold(III) and platinum(II) compounds was based on the preparation of [Hg(Ph₃P=N-CO-2-C₆H₄)Cl] by Nicholson et al. The C-H activation at the N-CO-Ph fragments takes place at a manganese center and by transmetallation of the resulting cyclometallated iminophosphorane manganese compounds to HgCl₂, the organomercury derivatives with PPh₃, [Hg(Ph₃P=N-CO-2-C₆H₄)Cl], or water soluble
phosphine PTA, [Hg(PTA=N-CO-2-C₆H₄)Cl]³⁷ (whose synthesis and characterization will be described in Chapter V) are obtained in high yields (Scheme 1).

\[ R_3P=N \xrightarrow{\text{PhCH}_2\text{Mn(CO)}_5} \Delta, n\text{-hexane} \rightarrow \xrightarrow{\text{HgCl}_2} \Delta, \text{MeOH} \]

\( PR_3 = \text{PPh}_3, \text{PTA} \)

**Scheme 1.** Previously described synthesis of organomercury compounds containing the semi-stabilized IM ligands PPh₃=N-CO-2-C₆H₄³⁸ and PTA=N-CO-2-C₆H₄³⁷ which will be described in Chapter V of this Thesis.

Transmetallation reactions of [Hg(PR₃=N-CO-2-C₆H₄)Cl] (PR₃ = PPh₃; PTA³⁷) with NMe₄[AuCl₄] or [PtCl₂(COD)] afforded previously described compound [Au(2-C₆H₄C(O)N=PPh₃)Cl₂] (1)³⁶ and new cyclometallated *exo* iminophosphorane complexes of gold(III) and platinum(II) of the type [Au(2-C₆H₄C(O)N=PTA)Cl₂] (2) and [Pt(2-C₆H₄C(O)N=PTA)(COD)]₂[Hg₄Cl₁₀] (3) (Scheme 2) in moderate to high yields.

\[ \text{Au} \xrightarrow{\text{NMe}_4[\text{AuCl}_4]} \xrightarrow{\text{NMe}_4[\text{Cl}]} \Delta, \text{CH}_3\text{CN, 3d} \]

\( PR_3 = \text{PPh}_3 (1), \text{PTA} (2) \)

\[ \text{Hg} \xrightarrow{\text{[PtCl}_2(\text{COD})]} \Delta, \text{CH}_3\text{CN, 2h} \]

\( PR_3 = \text{PTA} (3) \)

**Scheme 2.** Synthesis of gold(III) and platinum (II) cyclometallated *exo* iminophosphorane complexes. Compound [Au(2-C₆H₄C(O)N=PPh₃)Cl₂] (1) had been previously reported.³⁶
The reaction of \([\text{Hg(PPh}_3=\text{N-CO-2-C}_6\text{H}_4]\text{Cl}]\) with \([\text{PtCl}_2(\text{COD})]\) did not afford a pure cycloplatinated compound. Different synthetic conditions were tried and in most cases abundant Pt(0) decomposition took place while unreacted \([\text{Hg(PPh}_3=\text{N-CO-2-C}_6\text{H}_4]\text{Cl}]\) and \(\text{Ph}_3\text{P}=\text{O}\) were the observed products along with free COD. Longer refluxing times in polar solvents afforded small amounts (4-10\%) of a possible cyclometallated product along with \([\text{Hg(PPh}_3=\text{N-CO-2-C}_6\text{H}_4]\text{Cl}]\) and \(\text{Ph}_3\text{P}=\text{O}\).

New compounds 2 and 3 are obtained as air-stable yellow and white solids, respectively. Compound 2 is neutral whereas the Pt(II) derivative 3 is cationic (2:1 ions) as confirmed by conductivity measurements (see Experimental Section). Compound 3 is only soluble in solvents such as DMSO or DMF. We found that the COD ligand in 3 is immediately exchanged by DMSO molecules in DMSO-\(d_6\) solution at room temperature and that the new IM-cycloplatinated species did not change in DMSO-\(d_6\) over time (see Appendix, Figure 18). This was surprising since a COD ligand is not easily replaceable and usually requires thermal activation. The structures of these compounds have been proposed on the basis of elemental analysis, NMR and IR spectroscopy and MS spectrometry. Both compounds are soluble in mixtures 1:99 DMSO:\(\text{H}_2\text{O}\) at micromolar concentrations, relevant for biological studies.

**Figure 1.** Molecular structure of compound 2.
The structure of 2 has been determined by an X-ray analysis and it is very similar to that of previously reported\textsuperscript{36} compound \([\text{Au}(2\text{-C}_6\text{H}_4\text{C(O)N}=\text{PPh}_3)\text{Cl}_2] (1)\)\textsuperscript{36} with very close distances and angles. The molecular structure of 2 is depicted in Figure 1 while selected structural parameters are collected in Table 1. The analysis confirms the square-planar arrangement around the gold(III) center with a bite angle of 81.68(8)\(^\circ\). Like in other C,N-IM cycloaurated complexes\textsuperscript{32,33,36,39} the Au-Cl(1) bond \textit{trans} to the carbon is longer (2.3834(5) Å) than the Au-Cl(2) \textit{trans} to the nitrogen (2.2798(5) Å) due to the higher \textit{trans} influence of the C donor atom. As observed in compound 1, upon coordination to the gold there is an increase in both the P-N and N–C bond lengths when compared to the uncoordinated ligand\textsuperscript{40} (P-N: 1.626(3) Å in ligand, 1.6658 (18) Å in 2; N–C:1.353(5) Å in ligand, 1.401(3) Å in 2). This effect is also observed in the IR spectra of compound 2 for which band corresponding to the P-N bond appears at a lower frequency than that for the free ligand (1,289 cm\(^{-1}\) \textit{versus} 1,374 cm\(^{-1}\)). As described in the structure of compound 1, a decrease of the C=O bond length was observed (from 1.245(5) Å in the ligand to 1.213(3) Å in the cycloaurated complex).

\textbf{Table 1.} Selected Structural Parameters of complex 2 obtained from X-ray single crystal diffraction studies. Bond lengths in [Å] and angles in [°].

<table>
<thead>
<tr>
<th>Bond</th>
<th>Length/Angle</th>
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<td>C(7)-O(1)</td>
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<td>C(7)-C(6)</td>
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<td>178.28(6)</td>
<td>N(1)-P(1)-C(8)</td>
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</table>
The biological activity of the endo iminophosphorane compound \([\text{Au}\{\kappa^2-\text{C,N}}\text{C}_6\text{H}_4(\text{PPh}_2=\text{N(}\text{C}_6\text{H}_5)-2\}\text{Cl}_2]\)^32 and some of its cationic derivatives like \([\text{Au}\{\kappa^2-\text{C,N}}\text{C}_6\text{H}_4(\text{PPh}_2=\text{N(}\text{C}_6\text{H}_5)-2\}\{\text{S}_2\text{CN(}\text{CH}_3)\}_2]\)PF_6\(^33\) had been described by our research group at Brooklyn College.\(^33,35\) We had never synthesized Pt(II) endo compounds with the IM Ph-N=PPh_3 ligand or evaluated their biological activity. The reaction of \([\text{Hg}\{\text{C}_6\text{H}_4(\text{PPh}_2=\text{N(}\text{C}_6\text{H}_5)-2\}\text{Cl}\}]\(^32,39\) with \([\text{PtCl}_2(\text{COD})]\) was carried out and a cationic species (4) was obtained. Compound 4 (as for the exo compound 3) has a mercury chloride containing anion (in this case \([\text{Hg}_2\text{Cl}_6]\)^2-). In order to avoid the use of organomercury compounds and the presence of mercury in the resulting compound, a “greener” synthetic approach\(^41\) based of the transmetallation with an organogold(I)phosphine compound \([\text{Au}\{\text{C}_6\text{H}_4(\text{PPh}_2=\text{N(}\text{C}_6\text{H}_5)-2\}\}\text{(PPh}_3)\] described previously\(^32\) was employed (Scheme 3).

Scheme 3. Synthesis of the new platinum(II) cyclometallated endo iminophosphorane complexes 4 and 5.

This mercury-free approach had been used to obtain the endo gold(III) cyclometallated compound \([\text{Au}\{\kappa^2-\text{C,N}}\text{C}_6\text{H}_4(\text{PPh}_2=\text{N(}\text{C}_6\text{H}_5)-2\}\text{Cl}_2]\)^32 The reaction described here with \([\text{PtCl}_2(\text{COD})]\) proceeds much faster and in much milder conditions than that for the synthesis of 4 (25 min at RT in CH_2Cl_2 instead of 5 days in refluxing acetone). Compound 5 is obtained in moderate yield (58%). In order to avoid the formation of a neutral platinum(II) dimer with chloride bridges \([\text{Pt}\{\text{C}_6\text{H}_4(\text{PPh}_2=\text{N(}\text{C}_6\text{H}_5)-2\}\text{Cl}_2]\) observed while performing this reaction,
NH₄PF₆ was added. In this way, we obtained compound 5, an analogue of cationic compound 4 with a mercury-free anion (PF₆⁻). The structures of these compounds have been confirmed by elemental analysis, NMR (including ¹⁹⁵Pt NMR) and IR spectroscopy, and MS spectrometry studies. In this case the compounds do not exchange the COD ligand for DMSO molecules at RT in DMSO-d₆ solution as in the case of compound 3 which may have some implications for the biological activity of the compounds. Compounds 4 and 5 are also soluble in mixtures 1:99 DMSO:H₂O solutions at micromolar concentrations which are relevant for biological studies. A mercury-free analogue of compound 3 could not be obtained since the preparation of the appropriate Au(I) transmetallation agent from the organomanganese compound (Scheme 1) was not successful so far.

Figure 2. Molecular structure of the cation in compound 4. The anion [Hg₂Cl₆]²⁻ is omitted for clarity.

The number of cycloplatinated iminophosphorane compounds described previously is limited to two examples of endo neutral derivatives [Pt(C₆H₄-2-PPh₂=N-C(O)-2-NC₅H₄-κ-C,N,N)Cl]⁴² and [Pt{κ³-C,N,N-C₆H₄(PPh₂=N-8-C₉H₆N)Cl}]⁴³ in which the iminophosphorane fragment acts as a C,N,N- pincer ligand. In compounds 3-5 the IM ligand is cyclometallated in
either an *exo* (3) or *endo* (4, 5) position acting as a C,N-pincer ligand. The other two coordination positions for the Pt(II) center are occupied by the COD ligand. The molecular structure for compound 4 was determined by X-ray crystallography confirming the proposed structure. The molecular structure of the cation in 4 is depicted in Figure 2 while selected structural parameters are collected in Table 2. A complete drawing of the crystal structure of 4 including the [Hg₂Cl₆]²⁻ anion and crystallization molecules along with a more complete Table of distances and angles are provided in the Appendix, section 4.8.1, Tables 7-9.

The coordination geometry around the platinum atom is slightly distorted from square-planarity, with the C(1)-Pt(1)-N(1) angle of 85.31(9)° suggesting a rigid ‘bite’ angle. The X(1)-Pt(1)-N(1) also deviates (94.14(9)°). The distance Pt-N(1) 2.039(2) Å is shorter than that for other C,N-cyclometallated Pt(II) derivatives such as dmba (dimethylbenzylamine) compounds (ca. 2.1230-2.1340 Å). The distances Pt-N(1) and Pt-C(1) both of 2.039(2) Å are almost identical to those found for Au-N(1) and Au-C(1) (both 2.035(4) Å) in a cyclometallated compound with the same IM ligand such as [Au{C₆H₄(PPh₂=N(C₆H₅))-2}(PPh₃)]. The distances Pt-X(1) and Pt-X(2) to the centroids of the COD ligand are 2.169(3) and 2.039(3) Å, respectively, which reflects the higher *trans*-influence of C versus N (longer Pt-X(1) distance).

**Table 2.** Selected Structural Parameters of the cation in complex 4 obtained from X-ray single crystal diffraction studies. Bond lengths in [Å] and angles in [°].

<table>
<thead>
<tr>
<th>Bond Description</th>
<th>Length (Å)</th>
<th>Angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt(1)-C(1)</td>
<td>2.039(2)</td>
<td>N(1)-Pt(1)-C(1) 85.31(9)</td>
</tr>
<tr>
<td>Pt(1)-N(1)</td>
<td>2.039(2)</td>
<td>C(1)-Pt(1)-X(1) 178.95(9)</td>
</tr>
<tr>
<td>Pt(1)-X(1)</td>
<td>2.169(3)</td>
<td>C(1)-Pt(1)-X(2) 94.79(10)</td>
</tr>
<tr>
<td>Pt(1)-X(2)</td>
<td>2.039(2)</td>
<td>N(1)-Pt(1)-X(1) 94.14(9)</td>
</tr>
<tr>
<td>P(1)-N(1)</td>
<td>1.622(2)</td>
<td>N(1)-Pt(1)-X(2) 179.01(9)</td>
</tr>
<tr>
<td>P(1)-C(2)</td>
<td>1.773(2)</td>
<td>X(1)-Pt(1)-X(2) 85.78(10)</td>
</tr>
<tr>
<td>P(1)-C(7)</td>
<td>1.797(3)</td>
<td>C(19)-N(1)-Pt(1) 125.82(16)</td>
</tr>
<tr>
<td>P(1)-C(13)</td>
<td>1.797(3)</td>
<td>C(19)-N(1)-P(1) 116.21(16)</td>
</tr>
<tr>
<td>N(1)-C(19)</td>
<td>1.444(3)</td>
<td>C(19)-N(1)-P(1) 116.21(16)</td>
</tr>
<tr>
<td>C(1)-C(2)</td>
<td>1.409(3)</td>
<td></td>
</tr>
</tbody>
</table>
The stability of 1 and of the new compounds 2-5 was evaluated in DMSO-$d_6$ solution by $^{31}\text{P}\{^1\text{H}\}$ and $^1\text{H}$ NMR spectroscopy. All the complexes are stable for months in DMSO-$d_6$ solution (see spectra and stability table in the Appendix, sections 4.8.2 and 4.8.3). As mentioned before, compound 3 exchanges the COD ligand by DMSO immediately when dissolved in DMSO-$d_6$ (free COD is clearly visible along with coordinated DMSO). In the case of compounds 4 and 5, this exchange is extremely slow and after one week the percentage of free uncoordinated COD observed is around 6%. Compounds 4 and 5 are stable in DMSO and mixtures 1:99 DMSO:PBS for 24 hours as established by UV-Vis spectroscopy. The UV-Vis band shifts from 262 nm in DMSO to 225 nm in 1:99 DMSO:PBS for compound 4, and from 264 nm in DMSO to 225 nm in 1:99 DMSO:PBS for compound 5. The UV-Vis band observed for the free iminophosphrane ligand $\text{Ph}_3\text{P}=$NPh in DMSO appears at 257 nm, while in DMSO:PBS there are two visible bands, a major band at 230 nm and a minor band at 265 nm. This might explain the shift observed for compounds 4 and 5 in the UV-VIS spectra in DMSO:PBS solution when compared to DMSO solution (see Addpendix).

4.3. Biological activity in vitro

These studies were performed by Dr. Isabel Marzo, Oscar Gonzalo and Alfonso Serrano del Valle at the University of Zaragoza, Spain. The studies on non-carcinogenic RPTC cell lines were performed by PhD student Benelita T. Elie in our laboratory.

4.3.1. Antiproliferative studies in vitro

The antiproliferative properties of the gold(III) and platinum(II) complexes 1-5 and ligand COD were assessed by monitoring their ability to inhibit cell growth using the MTT assay (see Experimental Section). The cytotoxicity activity of the compounds was determined in several human cancer cell lines: leukemia Jurkat-T, lung A549, prostate DU-145, pancreas MiaPaca2, and triple negative breast MDA-MB-231, in comparison to cisplatin. The results are summarized in Table 3. The COD ligand is poorly cytotoxic in all tested cell lines ($\text{IC}_{50} >125 \, \mu\text{M}$). The IM ligands are known to be poorly cytotoxic ($\text{IC}_{50}$ in different cell lines $>100-500 \, \mu\text{M}$).
Cyclometallated neutral gold(III) compound showed similar cytotoxicity to cisplatin while compound 2 was less cytotoxic for all the studied cell lines with the exception of the leukemia Jurkat cell line. We have found previously that the replacement of PPh\(_3\) by PTA in IM-cyclometallated complexes decreases the cytotoxicity.\(^3^7\) The IC\(_{50}\) value for Jurkat for compound 1 is very similar to that obtained for the neutral iminophosphorane \textit{endo} derivative \([\text{Au}\{\kappa^2\text{-C},\text{N}-\text{C}_6\text{H}_4(\text{PPh}_2=\text{N}(\text{C}_6\text{H}_5))-2\}\text{Cl}_2]\).\(^3^2\) Cationic gold(III) complexes containing IM ligands are more cytotoxic than neutral derivatives.\(^3^3,^3^5\) The cationic cyclometallated platinum compounds described here 3 and especially 4 and 5 were considerably more cytotoxic than cisplatin in all the cell lines studied. 4 and 5 (same cation) display almost identical IC\(_{50}\) values with the exception of A549 and MDA-MB-231 for which compound 4 containing the \(\text{Hg}_2\text{Cl}_6^{2-}\) anion is twice as active than 5. The data indicates that cytotoxicity for these compounds comes mainly from the cationic platinum fragment.

\textbf{Table 3.} IC\(_{50}\) (µM) of metal complexes 1-5, ligand COD and cisplatin in human cell lines.\(^a\) All compounds were dissolved in 1\% of DMSO and diluted with water before addition to cell culture medium for a 24 h incubation period. Cisplatin was dissolved in H\(_2\)O.

<table>
<thead>
<tr>
<th></th>
<th>Jurkat</th>
<th>A549</th>
<th>DU-145</th>
<th>MiaPaca2</th>
<th>MDA-MB-231</th>
<th>HEK-293T</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.4 ± 0.5</td>
<td>85.3 ± 5.9</td>
<td>40 ± 8.1</td>
<td>81.8 ± 2.6</td>
<td>101.8 ± 16</td>
<td>14.6 ± 1.4</td>
</tr>
<tr>
<td>2</td>
<td>9.5 ± 0.07</td>
<td>&gt;125</td>
<td>&gt;125</td>
<td>&gt;125</td>
<td>&gt;125</td>
<td>&gt;125</td>
</tr>
<tr>
<td>3</td>
<td>2.13 ± 0.24</td>
<td>20.8 ± 1.7</td>
<td>22.5 ± 4.2</td>
<td>7.53 ± 5.0</td>
<td>14.6 ± 3.7</td>
<td>4.0 ± 0.42</td>
</tr>
<tr>
<td>4</td>
<td>0.43 ± 0.06</td>
<td>0.85 ± 0.29</td>
<td>0.93 ± 0.43</td>
<td>0.79 ± 0.09</td>
<td>0.39 ± 0.05</td>
<td>1.25 ± 0.25</td>
</tr>
<tr>
<td>5</td>
<td>0.53 ± 0.13</td>
<td>2.01 ± 0.89</td>
<td>0.81 ± 0.07</td>
<td>1.03 ± 0.06</td>
<td>0.84 ± 0.29</td>
<td>0.94 ± 0.07</td>
</tr>
<tr>
<td>COD</td>
<td>&gt;125</td>
<td>&gt;125</td>
<td>&gt;125</td>
<td>&gt;125</td>
<td>&gt;125</td>
<td>&gt;125</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>10.8 ± 1.2</td>
<td>114.2 ± 9.1</td>
<td>112.5 ± 33</td>
<td>76.5 ± 7.4</td>
<td>131.2 ± 18</td>
<td>69.0 ± 6.7</td>
</tr>
</tbody>
</table>

\(^a\)Data are expressed as mean ± SD (n =4).

In order to assess the compounds’ selectivity for cancerous cells with respect to normal cell lines, they were also screened for their antiproliferative effects on the non-tumorigenic
human embryonic kidney cells HEK293T. In most cases the cytotoxicity is comparable for the
cancerous and HEK293T cells. All compounds are more toxic to leukemia than to HEK293T cell
lines (2 to 12 times) and compound 4 is more toxic to all the cell lines than to the HEK293T cell
lines. The toxicity of mercury-free compound 5 to HEK293T is comparable to that in the human
cancer cell lines. As HEK293T cell lines (immortalized cells) can display a higher sensitivity to
chemicals we measured the effect of compound 5 on human renal proximal tubular cells (RPTC).
Renal proximal tubular cells in primary culture have been described as an in vitro model to study
nephrotoxicity. The IC_{50} value (XTT assay 24 hours see SI for details) for 5 in this cell line was
2.77±0.83 µM making 5 more sensitive to cancerous cell lines than to RPTCs. In addition, we
will describe in chapter V an IM ruthenium compound \([\eta^5_{\text{p-cymene}}\text{Ru}\{(\text{Ph}_3\text{P}=\text{N-CO-2-N-}
\text{C}_3\text{H}_4)-\kappa-N,O}\}\text{Cl}]\text{Cl}\) which displayed similar IC_{50} values in vitro for all the human cancer cell
lines described above and HEK293T but which was very effective in vivo on MDA-MB-231
xenografts in NOD.CB17-Prkdc SCID/J mice while having low toxicity.

4.3.2. Mechanism of cell death for compound 5

The mechanism of cell death induced by mercury-free cytotoxic cycloplatinated
compound 5 was analyzed in two cell lines of different origin: A549 lung carcinoma and Jurkat
T-cell leukemia. Phosphatidyl serine exposure, plasma membrane damage and nuclear
morphology were assessed in both cell lines after treatment with 5. Caspase implication in the
toxicity of 5 was studied using the general caspase inhibitor z-VAD-fmk. In A549 cells we found
that z-VAD-fmk protected cells from 5 at doses up to 0.5 µM (Figure 3) inhibiting both
phosphatidylserine exposure (annexin V binding) and plasma membrane permeabilization (7-
ADD uptake). As expected, phosphatidylserine exposure was more dependent on caspase
activity. At higher concentrations 7-AAD staining, but not annexin V binding, increased,
suggesting that cell death was necrotic.
Figure 3. Role of caspases on cell death induced by compound 5 in A595 cells. Cells were cultured for 24 h in the presence of 5 at the indicated concentrations, alone (solid lines) or combined with the general caspase inhibitor z-VAD-fmk (dashed lines). Subsequent, phosphatidylserine exposure (triangles) and cell membrane permeabilization (squares) were analyzed by flow cytometry after staining with annexin V-DY634 and 7-AAD respectively. Results are mean+/-SD of two independent experiments with duplicates.

The apparent decrease in the percentage of annexinV positive cells could reflect cell disintegration caused by necrosis. Consistently, z-VAD-fmk did not inhibit cell death at 1 µM (Figure 3). When an early event of apoptosis, loss of mitochondrial transmembrane potential, was analyzed we also observed that caspase inhibition by z-VAD-fmk only partially reduced ∆Ψm loss caused by 5 (Figure 4), further suggesting that compound 5 can induce caspase-dependent and caspase-independent cell death in A549 cells.
Figure 4. Caspase implication in mitochondrial effects of compound 5 in A549 cells. Cells were cultured for 24 h in the presence of compound 5 at the indicated concentrations, alone (solid line) or combined with the general caspase inhibitor z-VAD-fmk (dashed line). Then, transmembrane mitochondrial potential was analyzed by flow cytometry after staining with the probe DiOC6(3). Results are mean+/−SD of two independent experiments with duplicates.

Jurkat cells were more sensitive to 5 than A549 cells, with an IC_{50} of 0.6 µM, even though this cell line does not express functional p53, discarding an essential role of this protein in the activity of compound 5. In these cells the percentage of 7-AAD (Figure 5) and that of Annexin V-positive (data not shown) cells were the same in every assay. High sensitivity of Jurkat cells was confirmed in short-term experiments as we observed that 5 at 0.5 µM induced cell death in almost 100% of cells even at 6 h. Caspase inhibition by z-VAD-fmk completely avoided cell death at 6 h. However, longer treatment with 5 induced both caspase-dependent and caspase-independent cell death (Figure 5).
Figure 5. Implication of caspases in cell death induced by compound 5 in Jurkat cells. Cells were treated with compound 5 for 6 or 24 h in the presence or in the absence of the general caspase inhibitor z-VAD-fmk. Membrane integrity was analyzed by flow cytometry after staining with 7-AAD, respectively, as indicated in the Experimental Section. Results are mean±SD of two independent experiments.

Mitochondrial damage was also analyzed in Jurkat cells (Figure 6). At 24 h treatment with 5 caused a decrease in ΔΨm in 80% of cells, compared to 32% in cells treated with 5 in the presence of the general caspase inhibitor z-VAD-fmk. In order to determine whether mitochondrial damage caused by 5 was irreversible and committed cells to death, cells were washed and re-suspended in fresh medium. After a further 24 h incubation in fresh medium ΔΨm collapse was observed in nearly 100% of cells (Figure 6). These results indicate that caspase inhibition only delays cell death in Jurkat cells and 5 induces cell damage leading to cell death independently of caspase activation. Thus, these experiments confirm that alternative caspase-independent cell death mechanisms are activated by this compound, as observed in A549 cells.
Jurkat cells were treated with 5 or 5+z-VAD for 24 h and then harvested, washed and seeded in fresh medium. After further 24 h in fresh medium mitochondrial transmembrane potential (ΔΨₘ) was analyzed as indicated in the Experimental Section. Results are mean+/−SD of three independent experiments.

On the other hand analysis of nuclear morphology indicated that 5 induced typical apoptotic features (chromatin condensation and fragmentation) that were prevented by z-VAD-fmk in both cell lines (Figure 7). However, some nuclei of cells treated with 5 + zVAD displayed an altered morphology when compared to controls. This morphology could be caused by necroptosis⁴⁸ or AIF-mediated cell death.⁴⁹

Figure 7. Compound 5 induces apoptosis in Jurkat (upper panels) and A549 cells (bottom panels). Cells were cultured for 24 h in the presence of compound 5 (0.5 μM), alone or combined with the general caspase inhibitor z-VAD-fmk or left untreated (Control). Nuclei were stained with Hoechst 33342 (10 μg/ml) and cells were photographed under UV light. Magnification x400.
Finally, the implication of mitochondria in the toxicity of compound 5 was analyzed. Jurkat-shBak cells, obtained by RNAi of Bak\textsuperscript{49} were employed. Since Jurkat cells do not express Bax, Jurkat-shBak cell line constitutes a model of human leukemia cells deficient in the intrinsic (mitochondrial) pathway of apoptosis. A cell line transfected with a non-specific shRNA was used as a control (Jurkat pLVTHM). As shown in Figure 8, Jurkat-shBak cells were less sensitive to 5 than control cells. However, high concentrations of 5 induced Bax/Bak independent cell death in Jurkat-shBak cells, suggesting that this compound could be useful in the treatment of tumors with alterations in the intrinsic pathway of apoptosis.

![Graph](image)

**Figure 8.** Jurkat-pLVTHM (control) and Jurkat-shBak cells were treated with compound 5 for 24 h. Mitochondrial transmembrane potential was analyzed as indicated in the Experimental Section. Results are mean±SD of three independent experiments.

To summarize, from these initial mechanistic studies it seems clear that the cell death type for compound 5 is mainly through caspase-dependent apoptosis but that it triggers caspase-independent cell death when apoptosis is blocked pointing out to a mode of action different from that of cisplatin.
4.3.3. Lipophilicity and permeability assays

These studies were performed by Víctor Mangas-Sanjuán, Dr. Marta González-Alvarez, Dr. Isabel González-Alvarez and Prof. Marival Bermejo at the University Miguel Hernández, Spain.

The lipophilicity of the most active cycloplatinated 4 and 5 was determined by calculation the partition coefficients (see Table 4 and Experimental Section) between $n$-octanol and phosphate buffer (pH 7.00). Partition coefficients have been used to predict the permeability of drugs since there is a good correlation between intestinal permeability and physicochemical parameters such as lipophilicity.

Table 4. Partition Coefficients (ratio $n$-octanol: phosphate buffer) of Compounds 4 and 5 and reference Metoprolol.

<table>
<thead>
<tr>
<th>Compound</th>
<th>P</th>
<th>logP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metoprolol</td>
<td>0.20 ± 0.02</td>
<td>-0.68</td>
</tr>
<tr>
<td>4</td>
<td>0.54 ± 0.03</td>
<td>-0.26</td>
</tr>
<tr>
<td>5</td>
<td>1.05 ± 0.05</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Metoprolol was chosen as the reference compound for permeability since 95% of the drug is known to be absorbed from the gastrointestinal tract. Thus, drugs that exhibit partition coefficients and human intestinal permeability values greater than or equal to the corresponding value for metoprolol are considered high-permeability drugs. Drugs with estimated partition coefficients and human intestinal permeability values less than the corresponding value for metoprolol are classified as low-permeability drugs. This type of correlation is a suitable source of information on the passive and also possible carrier mediated absorption mechanism. From this data we can state that compound 5 is more lipophilic than 4 and metoprolol.
Figure 9. Permeability values obtained from apical to basal (Pab) and from basal to apical (Pba) of Cisplatin (at different concentrations), cycloplatinated 4, 5 and permeability reference compounds (Metoprolol, Cimetidine and Lucifer Yellow) at 20 µM in Caco-2 cells. Data correspond to the averaged values for three independent experiments.

Subsequently, the permeability of cisplatin as commercialized parent compound, cycloplatinated 4 and 5 as test compounds and Metoprolol, Cimetidine and Atenolol/Lucifer Yellow as reference compounds of high, intermediate and low permeability respectively, were determined using an *in vitro* cell model based on the measurement of the permeability of the compounds through Caco-2 monolayers\textsuperscript{50,51} and an *in situ* method by performing a rat perfusion assay.\textsuperscript{52,53} Results from the *in vitro* cell assay are shown in Figure 9 and data collected in Table 5 while the results in the rat model are depicted in Figure 10 and data collected in Table 6.

Pab is the value corresponding to the permeability from apical to basolateral chamber that simulates the permeability in the physiological sense from intestine to plasma. The Pba value corresponds to the permeability form basolateral to apical chamber. This Pba value would be the hypothetical value for the permeability “from plasma to intestine”. Despite Pba value has not physiological sense, this parameter and the ratio Pab/Pba can help to elucidate the mechanism of drug transport across the intestinal barrier.\textsuperscript{54}
**Table 5.** Permeability values obtained by the Caco-2 cell monolayers assay. Metoprolol, Cimetidine and Lucifer Yellow were used as model compounds of high, medium and low oral permeability. Data correspond to the averaged values for three independent experiments.

<table>
<thead>
<tr>
<th>Compound (20 µM)</th>
<th>P (cm/s)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>$5.44 \times 10^{-07}$</td>
<td>$4.66 \times 10^{-07}$</td>
</tr>
<tr>
<td><strong>4</strong></td>
<td>$4.62 \times 10^{-06}$</td>
<td>$3.54 \times 10^{-06}$</td>
</tr>
<tr>
<td><strong>5</strong></td>
<td>$2.71 \times 10^{-05}$</td>
<td>$5.00 \times 10^{-06}$</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>$2.32 \times 10^{-05}$</td>
<td>$1.75 \times 10^{-06}$</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>$1.86 \times 10^{-06}$</td>
<td>$3.71 \times 10^{-07}$</td>
</tr>
<tr>
<td>Lucifer Yellow</td>
<td>$1.90 \times 10^{-07}$</td>
<td>$4.98 \times 10^{-08}$</td>
</tr>
</tbody>
</table>

Cell transport assays reveal that cisplatin is a compound with very low permeability. This permeability value ($5.44 \times 10^{-07}$ cm/s) indicates that cisplatin is not a drug suitable for oral route administration if the objective is to obtain therapeutic plasma values. However, organoplatinum compounds 4 and 5 show higher permeability values than cisplatin. In fact, the permeability value of compound 4 is ten-fold higher than that of cisplatin at the same concentration and the permeability of compound 5 is fifty-fold higher. The permeability of compound 4 is higher than Lucifer Yellow and Cimetidine but lower than Metoprolol. 4 can be considered a compound of medium oral permeability. However, the permeability of compound 5 is higher than that of compound 4 and even higher than Metoprolol (in accordance with the lipophilicity data) indicating that it can be considered a highly permeable compound. The high permeability of active principles is a crucial condition for oral administration.
Results from the *in situ* rat model assays confirm those obtained by the *in vitro* cell experiments. The permeability of compound 4 is higher than that of Atenolol, slightly higher that the permeability of Cimetidine but lower than that of Metoprolol. Compound 4 can be considered a compound of intermediate permeability. Compound 5 exhibits higher permeability than compound 4 and slightly higher than Metoprolol indicating that 5 is a highly permeable compound. Both compounds 4 and 5 display a much better absorption profile than cisplatin.

**Table 6.** Absorption rate coefficients, $K_a$, and permeability values obtained from *in situ* rat assays. Metoprolol, Cimetidine and Atenolol were used as model compounds of high, medium and low oral permeability. Data correspond to values of six independent experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_a$ (h$^{-1}$)</th>
<th>SD</th>
<th>Peff (cm/s)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin$^a$</td>
<td>Non detectable</td>
<td>-</td>
<td>Non detectable</td>
<td>-</td>
</tr>
<tr>
<td>4$^a$</td>
<td>2.00 ± 0.11</td>
<td>4.72 × 10$^{-05}$</td>
<td>± 2.60 × 10$^{-06}$</td>
<td></td>
</tr>
<tr>
<td>5$^a$</td>
<td>2.12 ± 0.22</td>
<td>5.50 × 10$^{-05}$</td>
<td>± 5.40 × 10$^{-06}$</td>
<td></td>
</tr>
<tr>
<td>Metoprolol$^b$</td>
<td>2.30 ± 0.15</td>
<td>5.40 × 10$^{-05}$</td>
<td>± 3.54 × 10$^{-06}$</td>
<td></td>
</tr>
<tr>
<td>Cimetidine$^b$</td>
<td>1.68 ± 0.12</td>
<td>3.97 × 10$^{-05}$</td>
<td>± 3.04 × 10$^{-06}$</td>
<td></td>
</tr>
<tr>
<td>Atenolol$^b$</td>
<td>0.22 ± 0.02</td>
<td>5.19 × 10$^{-06}$</td>
<td>± 4.72 × 10$^{-07}$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ 20 µM.  
$^b$ 100 µM.
In addition, the relationship between caco-2 cells permeability and oral fraction absorbed in the experimental system (represented in Figure 11) had been validated and previously used for fraction absorbed predictions. The permeabilities of cisplatin and derivatives 4 and 5 were included in this correlation. The oral fraction absorbed predicted is more than 60% for compound 4 and almost 100% for compound 5 demonstrating its improved absorbability properties with respect to cisplatin. In the absence of solubility or dissolution limitations the absorption of these compounds would be almost complete, thus with the adequate formulation strategy they represent promising candidates for oral administration.

**Figure 11.** Correlation between oral fractions absorbed vs permeability values obtained from Caco-2 cell monolayers transport assay in apical to basal direction (Pab). Gray diamonds correspond to the internally validated correlation (IVC). Triangles correspond to permeability reference compounds (metoprolol/caffeine for high permeability, Cimetidine for intermediate permeability and Lucifer Yellow for low permeability). Light grey squares correspond on tested compounds 4 and 5.
4.4. Interactions with DNA

Since DNA replication is the key event for cell division, it is among critically important targets in cancer chemotherapy. Most cytotoxic platinum drugs form strong covalent bonds with DNA bases.\textsuperscript{55} However, a variety of platinum compounds act as DNA intercalators upon coordination to the appropriate ancillary ligands.\textsuperscript{56} It has been reported that most gold(III) compounds display reduced affinity for DNA\textsuperscript{33} although there are a number of Au(III) porphyrin complexes\textsuperscript{5,25,57} and cyclometallated species with C-N-C pincer ligands\textsuperscript{5,25,58} that act as DNA intercalators and, in some cases as DNA topoisomerase inhibitors. We investigated the interaction of the gold(III) and platinum(II) complexes with plasmid (pBR322) DNA and with \textit{Calf Thymus} DNA and directly compared to that of cisplatin.

4.4.1. Interaction of complexes 1-5 with plasmid (pBR322) DNA

To gain insight into the nature of the compound-DNA interactions, gel electrophoresis studies were performed with gold(III) (1 and 2) and platinum(II) (3-5) complexes on plasmid (pBR322) DNA (Figure 12). Plasmid (pBR322) presents two main forms, OC (open circular or relaxed) and CCC (covalently closed circle or supercoiled), which display different electrophoretic mobility. Changes in the electrophoretic mobility of any of the forms upon incubation of the plasmid DNA with a compound are usually interpreted as evidence of drug-DNA interaction. Generally, a drug that induces unwinding of the CCC form will produce a retardation of the electrophoretic mobility, while coiling of the OC form will result in increased mobility. Figure 12 shows the effect of cisplatin and compounds 1-5 on plasmid (pBR322) DNA after incubation at 37ºC for 20 h in Tris/HCl buffer at different drug/DNA ratios. As previously reported, cisplatin is able to both increase and decrease the mobility of the OC and the CCC forms, respectively.\textsuperscript{59} Treatment with increasing amounts of compounds 1, 2, 4 and 5 do not cause any shift for either form, consistent with no unwinding or other changes in topology under the chosen conditions. Treatment with increasing amounts of 3 retards the mobility of the faster-running supercoiled form (Form I) especially at higher molar ratios. In order to understand the interaction of 3 with DNA, platinum compounds 3-5 were incubated with \textit{Calf Thymus} DNA and analyzed by circular dichroism.
**Figure 12.** Electrophoresis mobility shift assays for cisplatin and compounds 1-5 (see Experimental Section for details). DNA refers to untreated plasmid pBR322. A, B, C and D correspond to metal/DNAbp ratios of 0.25, 0.5, 1.0 and 2.0 respectively.

### 4.4.2. Interaction with Calf Thymus DNA

More detailed DNA conformational changes can be detected by means of Circular Dichroism (CD) spectroscopy. CD spectral technique is very sensitive to diagnose alteration of the secondary structure of DNA that results from DNA-drug interactions. A typical CD spectrum of Calf Thymus DNA shows a positive band with a maximum at 273 nm due to base stacking, and negative band with a minimum at 242 nm due to helipticity, characteristic of the B conformation of DNA. Therefore, changes in the CD signals can be assigned to corresponding changes in DNA secondary structures. In addition, it is known that simple groove binding or electrostatic interaction of small molecules causes little or no alteration to any of the CD bands when compared to major perturbation induced by covalent binding or intercalation. The most dramatic changes in the CD spectrum of CT DNA can be observed with compound 3 (Figure 13A). Upon addition of increasing amounts of the complex, the intensity of the positive band diminishes and a new negative band at 287 nm and a positive band at 251 nm appears. This type of modification in CD spectrum of CT DNA is characteristic of conformational changes in DNA from B, usual right-handed form of DNA, to Z, left-handed form of DNA. The formation of left-handed helix of Z-form DNA structure is similar to the transition seen in purely electrostatic environments such as those provided by HgCl$_2$ and Hg(ClO$_4$)$_2$. Thus, the presence of
[Hg₄Cl₁₀]²⁻ anion in compound 3 seems to lead to the conformational change from B form to Z form.

Cycloplatinated endo compound 4 leads to minor changes of the B-type CD spectrum (Figure 13B), with slight decrease of the intensities of the positive bands and with no modification in the negative region. This points out that the DNA binding of complex 4 induces conformational changes including conversion from a more B-like to a more C-like structure within the DNA molecule. This conformational change is indicative of a non-intercalative mode of binding of the complex and offers support for either groove binding or electrostatic in nature and it might be due to the lower concentration of Hg²⁺ released by compound 4, [Hg₂Cl₆]²⁻ (compared to that released by compound 3, with a [Hg₄Cl₁₀]²⁻ anion) although the influence of the more lipophilic Pt(II) cation could not be completely ruled out.

**Figure 13.** CD spectra of CT DNA (195 µM) and CT DNA incubated with 0.1, 0.25, 0.5 and 1.0 equivalents of compounds 3 (A), 4 (B), 5 (C) and cisplatin (D) for 20 h at 37°C.
Finally, as shown in Figure 13C, compound 5 leads to no modification of the DNA bands with respect to untreated CT DNA, suggesting that the interaction of compound 5 with CT DNA is almost non-existent. This is in good agreement with our findings described above about the influence of the mercury anion in compounds 3 and 4 in their interaction with CT DNA since the anion in compound 5 is PF$_6^-$.

In conclusion, the experiments of DNA-drug interactions have shown that compound 3 induces the formation of left-handed helix of Z-form DNA through strong electrostatic interactions and compound 4 appears to be either groove binding or electrostatic in nature. This is supported by two main facts: 1) retardation of the plasmid (pBR322) DNA electrophoretic mobility is observed only for compound 3; and 2) results obtained by CD spectroscopy. Importantly, the mercury-free cationic organoplatinum compound 5 does not seem to interact with DNA indicating that as for other transition-metal IM complexes$^{33-35,37,43,46,47}$ its antitumor properties are due to non-DNA related mechanisms/factors.

4.5. Interaction with HSA

As mentioned in Chapter III, human serum albumin (HSA) is the most abundant carrier protein in plasma and is able to bind a variety of substrates including metal cations, hormones and most therapeutic drugs. It has been demonstrated that the distribution, the free concentration and the metabolism of various drugs can be significantly altered as a result of their binding to this protein.$^{67}$ HSA possesses three fluorophores, namely tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) residues, with Trp214 being the major contributor to the intrinsic fluorescence of HSA. This Trp fluorescence is sensitive to the environment and binding of substrates, as well as changes in conformation that can result in quenching (either dynamic or static).

Thus, the fluorescence spectra of HSA in the presence of increasing amounts of the compounds 1-5 and cisplatin were recorded in the 300-450 nm range upon excitation of the tryptophan residue at 295 nm. The compounds caused a concentration dependent quenching of fluorescence without changing the emission maximum or the shape of the peak, as seen on Figure 14A for compound 3. All these data indicate an interaction of the compounds with HSA. The fluorescence data was analyzed by the Stern-Volmer equation (Figure 14B). While a linear
Stern-Volmer plot is indicative of a single quenching mechanism, either dynamic or static, the positive deviation observed in the plots of $F_0/F$ versus $[Q]$ of compounds 2-4 (Figure 14C) suggests the presence of different binding sites in the protein with different binding affinities.\textsuperscript{68} Of note, a similar behavior was observed in the case of coordination iminophosphorane complexes of d\textsuperscript{8} metals for which we also reported a concentration dependent fluorescence quenching.\textsuperscript{34,37,46,47} On the other hand, the Stern-Volmer plot for complexes 1 and 5 shows a linear relationship (Figure 14D), suggesting the existence of a single quenching mechanism, most likely dynamic, and a single binding affinity. The Stern-Volmer constants for complexes 1 and 5 are $4.58 \times 10^6$ and $3.67 \times 10^6$ M\textsuperscript{-1}, respectively.

In general, higher quenching by the iminophosphorane complexes was observed compared to that of cisplatin under the chosen conditions, most likely due to the faster binding of our compounds with HSA, as compared to cisplatin.

![Figure 14](image.png)

**Figure 14.** (A) Fluorescence titration curve of HSA for compound 3. Arrow indicates the increase of quencher concentration. Stern-Volmer plot for HSA fluorescence quenching observed with compounds 1-5 and cisplatin (B), 2-4 and cisplatin (C) and 1, 5 and cisplatin(D).
4.6. Conclusions

We have shown in this chapter a systematic way to synthesize novel \textit{exo} cyclometallated C,N-IM compounds of gold(III) and platinum(II) containing the water-soluble phosphine PTA. This synthetic method could be expanded to incorporate different phosphines in the IM ligand to tune the hydrophilicity and/or electronic/steric properties of the resulting metal complexes. I also prepared an \textit{endo} C,N-IM compound of platinum(II) for comparison purposes. These complexes resulted stable in solution of DMSO-$d_6$ and in DMSO:PBS buffer (1:99) overtime as demonstrated by NMR and UV-vis spectroscopic studies. The compounds, along with \textit{exo} derivative [Au(2-C$_6$H$_4$C(O)N=PPh$_3$)Cl$_2$], were evaluated against a number of human cancer cell lines \textit{in vitro}. The platinum compounds 3 and particularly, 4 and 5 were significantly more cytotoxic than cisplatin in all the cell lines studied; indicating that cytotoxicity of 4 and 5 comes mainly from the cationic fragment. The interaction of complexes 3-5 with plasmid (pBR322) and calf thymus DNA by circular dichroism demonstrated different modes of action. Compound 3 induces the formation of left-handed helix of Z-form DNA through strong electrostatic interactions, while compound 4 appears to be either groove binding or electrostatic in nature. In addition, higher quenching of has fluorescence was observed compared to that of cisplatin under the chosen conditions, most likely due to the faster reactivity of the complexes with HSA, as compared to cisplatin.

All the above mentioned results have helped us to identify a mercury-free lipophilic cationic cycloplatinated iminophosphorane compound (5) as a good candidate for subsequent advanced preclinical studies. This compound is not only active against a number of cisplatin resistant cell lines, but also less toxic on human renal proximal tubular cell lines. Initial mechanistic studies carried out by our collaborators in Spain indicate that the cell death type for compound 5 is mainly through caspase-dependent apoptosis but that it triggers caspase-independent cell death when apoptosis is blocked. These facts along with the lack of interaction observed for 5 with plasmid (pBR322) and CT DNA points to a mode of action different from that of cisplatin. Permeability studies of 5 by two different assays: \textit{in vitro} caco-2 monolayers and a rat perfusion model, have revealed its high permeability profile (comparable to that of metoprolol or caffeine) and an estimated oral fraction absorbed of 100% which potentially makes it a good candidate for oral administration. The results obtained in this chapter support the idea
that cyclometallated iminophosphorane compounds containing d$^8$ metals (especially cationic species) are more stable in physiological media and display relevant anticancer properties.

4.7. Experimental section

Materials and methods

All manipulations involving air-free syntheses were performed using standard Schlenk-line techniques under a nitrogen atmosphere or in a glove-box MBraun MOD System. Solvents were purified by use of a PureSolv purification unit from Innovative Technology, Inc. The phosphine substrate PTA and PPh$_3$ were purchased from Sigma-Aldrich, [Mn$_2$(CO)$_{10}$] and [PtCl$_2$(COD)] were purchased from Strem chemicals, Na/Hg were purchased from Fisher Scientific and used without further purification. Compounds [PhCH$_2$Mn(CO)$_3$],$^{68}$ [Hg(2-C$_6$H$_4$(O)N=PPh$_3$)Cl],$^{36}$ [Hg(2-C$_6$H$_4$(O)N=PTA)Cl],$^{37}$ and IM ligands Ph$_3$P=N-CO-2-N-C$_5$H$_4$,$^{38}$ were prepared by reported methods. The purity of the compounds, based on elemental analysis, is ≥99.5%. NMR spectra were recorded in a Bruker AV400 ($_1$H NMR at 400 MHz, $^{13}$C NMR at 100.6 MHz, $^{31}$P NMR at 161.9 MHz, $^{195}$Pt NMR at 85.7 Hz). Chemical shifts (δ) are given in ppm using CDCl$_3$ or DMSO-d$_6$ as solvent, unless otherwise stated. Elemental analyses were performed on a Perkin Elmer 2400 CHNS/O Analyzer, Series II. Mass spectra HR-ESI (high-resolution electrospray ionization) or MALDI (matrix-assisted laser desorption/ionization) were performed on an Agilent Analyzer or a Bruker Analyzer. Conductivity was measured in an OAKTON pH/conductivity meter in acetone solution (10$^{-3}$ M). X-ray collection was performed at room temperature on using graphite-monochromated and 0.5 mm-MonoCap-collimated Mo-K$\alpha$ radiation ($\lambda = 0.71073$ Å) with the ω scan method. DNA thermal denaturation experiments were performed with a Cary 100 Bio UV-visible spectrophotometer. Circular Dichroism spectra were recorded using a Chirascan CD Spectrometer equipped with a thermostated cuvette holder. Electrophoresis experiments were carried out in a Bio-Rad Mini sub-cell GT horizontal electrophoresis system connected to a Bio-Rad Power Pac 300 power supply. Photographs of the gels were taken with an Alpha Innotech FluorChem 8900 camera. Fluorescence intensity measurements were carried out on a PTI QM-4/206 SE Spectrofluorometer (PTI, Birmingham, NJ) with right angle detection of fluorescence using a 1 cm path length quartz cuvette.
Synthesis of 2

\[\text{[Au(2-C_6H_4(C(O)N=PTA)Cl] (0.15 g, 0.3 mmol), [NMe}_4][\text{AuCl}_4] (0.12 g, 0.2 mmol) and [NMe}_4\text{Cl (0.035 g, 0.32 mmol) were stirred at RT in CH}_2\text{Cl}_2 (15 mL) for 1 day in a foil-covered flask. The solvent was removed under reduced pressure. The fraction containing compound 2 was then extracted from the solid residue with CHCl}_3 (3 x 10 mL) and the resulting yellow solution filtered through a column of celite. The volume was reduced (< 3 mL) and upon addition of Et}_2\text{O (20 mL) a pale yellow solid was precipitated. This solid was finally isolated by filtration and dried in vacuo.}

Yield: 0.15 g (93%). Anal. Calc. for C_{13}H_{16}N_4OPCl_2Au (543.14): C, 28.75; H, 2.97; N, 10.32. Found: C, 28.32; H, 3.07; N, 9.93%. ESI-MS: \(m/z: 507.04 (100\%, [M-\text{Cl}]^+, \text{calc. 507.04}).\)

\[^{31}\text{P} \{^{1}\text{H}\} \text{NMR (CDCl}_3): \delta -7.66 (s), \text{(DMSO-}d_6): -2.68 (s); ^{1}\text{H NMR (CDCl}_3): \delta 4.54 (6H, AB system, NCH}_2\text{N), 7.34 (1H, d, }^{3}J_{\text{HH}} = 7.0 \text{ Hz, 6-C}_6\text{H}_4\text{), 7.38 (dd, }^{3}J_{\text{HH}} = 7.8, ^{3}J_{\text{HH}} = 7.8 \text{ Hz, 4-C}_6\text{H}_4\text{), 7.42 (dd, }^{3}J_{\text{HH}} = 7.2, ^{3}J_{\text{HH}} = 7.1 \text{ Hz, 5-C}_6\text{H}_4\text{), 8.04 (d, }^{3}J_{\text{HH}} = 8.1 \text{ Hz, 3-C}_6\text{H}_4\text{).}^{13}\text{C}\{^{1}\text{H}\} \delta 55.78 (d, ^{1}J_{\text{PC}} = 36.8 \text{ Hz, PCH}_2\text{N), 72.37 (d, }^{3}J_{\text{PC}} = 10.8 \text{ Hz, NCH}_2\text{N), 128.64 (s, 2-C}_6\text{H}_4\text{), 129.73 (s, 3-C}_6\text{H}_4\text{), 130.74 (s, 5-C}_6\text{H}_4\text{), 134.42 (s, 4-C}_6\text{H}_4\text{), 143.16 (s, Au-C) ppm. Signals corresponding to NC=O and C_1 not observable. IR (cm}^{-1}): \nu 352 (\text{Au-Cl}), 1299 (\text{N=PTA}) 1654 (\text{C=O}). \text{Conductivity: 37.66 }\mu\text{S/cm (acetone) (neutral).}

Synthesis of 3

\[\text{[Pt(2-C_6H_4(C(O)N=PTA)(COD)]_2Hg}_4\text{Cl}_10\]

[Hg(2-C_6H_4(C(O)N=PTA)Cl] (0.225 g, 0.44 mmol) and [PtCl_2(COD)] (0.165 g, 0.44 mmol) were refluxed in CH_3CN (20 mL) for 2 h giving rise to white solid that was filtrated off and washed with Et}_2\text{O (3 x 10 mL), benzene (2 x 5 mL) and hexane (2 x 10 mL). After drying in vacuo, complex 3 was isolated as a white powder.

Yield: 0.097 g (40%). Anal. Calc. for C_{42}H_{56}Cl_{10}Hg_4N_8O_2P_2Pt_2 (2313.94): C, 21.80; H, 2.44; N, 4.84. Found: C, 21.72; H, 2.58; N, 4.72. ESI-MS: \(m/z: 470.1 ([M-COD- Hg_4Cl]_10^+, \text{calc. 470.4),}

103
578.2 (100%, [M-HgCl₂]⁺, calc. 577.9), 1275.0 (100%, [2M-HgCl₂]²⁺ + CCl₃⁻, calc. 1275.4).

³¹P {¹H} NMR (CDCl₃): δ −7.66 (s), (DMSO-d₆): −10.25 (s). ¹⁹⁵Pt{¹H} NMR (DMSO-d₆): δ -3652.87 (s). ¹H (DMSO-d₆): δ 2.30 (8H, s, COD), 4.39 (6H, s, NCH₂N), 4.91 (6H, d, ²Jₚₜ = 10.3 Hz, PCH₂N), 5.51 (4H, s, COD), 7.07 (1H, m, 4-CH₃), 7.16 (1H, m, 5-CH₃), 7.29 (1H, d, ³Jₚₜ = 7.3 Hz, 6-CH₃), 8.15 (1H, d, ³Jₚₜ = 8.0 Hz, 3-CH₃). ¹³C{¹H}(DMSO-d₆): 27.98 (s, COD), 54.27 (d, ¹JPC = 39.2 Hz, PCH₂N), 71.30 (d, ³JPC = 10.2 Hz, NCH₂N), 124.6 (s, 4-CH₃), 128.1 (s, 6-CH₃), 128.9 (s, COD), 131.9 (s, 5-CH₃), 132.7 (s, 3-CH₃), 135.0 (s, 1-CH₃), 138.0 (d, ²JCP = 10.2 Hz, 2-PtC), 182.1 (d, ²JPC = 5.18 Hz, C=O) ppm. IR (cm⁻¹): ν 1300 (N=P), 1643 (C=O). Conductivity: 129 μS/cm (DMF) (1:2 electrolyte).

Synthesis of 4

[Pt{k⁵-C₅N-C₆H₄(PPh₂=N(C₆H₄))(COD)}₂(Hg₂Cl₆)]

[Hg{C₆H₄(PPh₂=N(C₆H₃))Cl} (0.18 g, 0.3 mmol) and [PtCl₂(COD)] (0.11 g, 0.3 mmol) were refluxed in acetone (30 mL) for 5 d. The solvent was removed under reduced pressure. The final product was extracted with CH₂Cl₂ and the resulting solution filtered through a column of celite giving a light yellow solution. The solution was concentrated (< 3 mL) and upon addition of Et₂O (20 mL), the final product was precipitated as a white solid, isolated by filtration and dried in vacuo.

Yield: 0.20 g (72%). Anal. Calc. for C₆H₆₂N₂P₂C₆lPt₂Hg₂ · CH₂Cl₂ (1961.24): C, 38.84; H, 3.21; N, 1.39. Found: C, 38.84; H, 3.42; N, 1.30%. ESI-MS: m/z: 655.18 (100%), [M]⁺, calc. 655.18). ³¹P {¹H} NMR (CDCl₃): δ 64.51 (s), (DMSO-d₆): 63.19 (s). ¹⁹⁵Pt{¹H} NMR (CDCl₃): δ -3622.47 (d, ²JPPt = 404 Hz). ¹H NMR (CDCl₃): δ 2.30 (8H, s, COD), 5.12-5.30 (4H, m, COD), 6.88 (2H, m, 2-6-NAr), 7.12 (1H, m, 4-NAr), 7.21 (2H, m, 3,5-NAr), 7.35 (1H, m, 4-CH₃), 7.56-7.67 (10H, m, o-,m-,p-C₆H₅), 7.75 (2H, m, 5-CH₃). ¹³C{¹H} CDCl₃: δ 28.24 (s, COD), 31.23 (s, COD), 89.30 (s, COD), 116.12 (s, COD), 124.3 (d, ¹JPC = 91.6 Hz, C₁), 127.1 (d, ⁵JPC = 2.7 Hz, 4-NAr), 127.8 (d, ³JPC = 13.9 Hz, 4-CH₃), 129.5 (d, ³JPC = 2.9, Hz, m-C₆H₅), 129.7 (s, 3,5-NAr), 130.1 (d, J = 4.8 Hz, 2-,6-NAr), 132.7 (d, ²JPC = 14.3 Hz, 3-C₆H₅), 133.3 (m, o-,p-C₆H₅), 134.3 (d, ⁴JPC = 2.3 Hz, 5-C₆H₅), 147.8 (s, Pt-C). IR (cm⁻¹): ν 529 (Pt-N), 1310 (N=P). Conductivity: 102.3 μS/cm (acetone) (1:1 electrolyte).
Synthesis of 5

\[ \text{[Pt}\kappa^2\text{-C,N-}C_6\text{H}_4(\text{PPh}_2=\text{N}(\text{C}_6\text{H}_5))(\text{COD})](\text{PF}_6) \]

[Au{C}_6\text{H}_4(\text{PPh}_2=\text{N}(\text{C}_6\text{H}_5))-2](\text{PPh}_3)] (0.28 g, 0.3 mmol) and PtCl\(_2\)(COD) (0.11 g, 0.3 mmol) were stirred in CH\(_2\)Cl\(_2\) (20 mL) for 25 min at RT, follow by addition of NH\(_4\)PF\(_6\) (0.049g, 0.3 mmol). The resulting solution was stirred for an additional 1 h. The solution was concentrated (<3 mL) and dry Et\(_2\)O (20 mL) was added giving rise to gray precipitate which was stirred for 10 min. The precipitated gray solid was isolated by filtration and washed with water (4 x 2 mL) and a cold mixture (1:8) of CH\(_2\)Cl\(_2\)/Et\(_2\)O (4 x 5 mL) yielding a white solid that was dried in vacuo.

Yield: 0.14 g (58%). Anal. Calc. for C\(_{32}\)H\(_{31}\)F\(_6\)NP\(_2\)Pt (800.63): C, 48.01; H, 3.90; N, 1.75. Found: C, 47.74; H, 4.11; N, 1.74%. ESI-MS: m/z: 655.18 (100%), \([\text{M}]^+\), calc. 655.18).

\(^{31}\text{P}\{^1\text{H}\}\) NMR (CDCl\(_3\)): δ 64.65 (s), (DMSO-\(d_6\)): 63.19 (s).

\(^{195}\text{Pt}\{^1\text{H}\}\) NMR (CDCl\(_3\)): δ -3614.48 (d, \(^2J_{PPt}=406.1\) Hz).

\(^1\text{H}\) NMR (CDCl\(_3\)): δ 2.64 (8H, m, COD), 5.20 (4H, m, COD), 6.88 (2H, m, 2-,6-NAr), 7.09 (1H, m, 4-NAr), 7.20 (2H, m, 3-,5-NAr), 7.23 (1H, m, 3-C\(_6\)H\(_4\)), 7.33 (1H, m, 4-C\(_6\)H\(_4\)), 7.49-7.66 (10H, m, o-,m-,p-C\(_6\)H\(_5\)), 7.72 (2H, m, 5-C\(_6\)H\(_4\)); \(^{13}\text{C}\{^1\text{H}\}\) (CDCl\(_3\)): δ 28.05 (s, COD), 31.02 (s. COD), 89.22 (s, COD), 116.49 (s, COD), 124.3 (s, C\(_\text{ipso}\)), 125.2 (s, C\(_\text{ipso}\)), 127.0 (d, \(^5J_{PC}=2.9\) Hz, 4-NAr), 127.6 (d, \(^5J_{PC}=13.9\) Hz, 4-C\(_6\)H\(_4\)), 129.5 (d, \(^3J_{PC}=2.5\) Hz, m-C\(_6\)H\(_5\)), 129.6 (s, 3-,5-NAr), 130.1 (d, \(^3J_{PC}=4.9\) Hz, 2-,6-NAr), 132.6 (d, \(^2J_{PC}=13.8\) Hz, 3-C\(_6\)H\(_4\)), 133.3 (m, o-,p-C\(_6\)H\(_5\)), 134.2 (d, \(^4J_{PC}=2.9\) Hz, 5-C\(_6\)H\(_4\)), 147.8 (s, Pt-C). IR (cm\(^{-1}\)): ν 566 (Pt-N), 838 (br, PF\(_6^-\)), 1298 (N=P). Conductivity: 106.5 μS/cm (acetone) (1:1 electrolyte).

X-Ray crystallography

A gold block-like crystal with the size of 0.10 × 0.18 × 0.18 mm\(^3\) was selected for geometry and intensity data collection with a Bruker SMART APEXII CCD area detector on a D8 goniometer at 100 K. The temperature during the data collection was controlled with an Oxford Cryosystems Series 700+ instrument. Preliminary lattice parameters and orientation matrices were obtained from three sets of frames. Data were collected using graphite-monochromated and 0.5 mm-MonoCap-collimated Mo-K\(_\alpha\) radiation (λ = 0.71073 Å) with the ω and φ scan method. Data were processed with the INTEGRATE program of the APEX2 software for reduction and cell
refinement. Multi-scan absorption corrections were applied by using the SCALE program for the area detector. The structure was solved by the direct method and refined on \( F^2 \) (SHELXTL)\(^2\). Non-hydrogen atoms were refined with anisotropic displacement parameters, and hydrogen atoms were placed in idealized positions (C-H = 0.95-0.99 Å) and included as riding with \( U_{\text{ISO}}(\text{H}) = 1.2 \) or 1.5 \( U_{\text{eq}}(\text{non-H}) \).

**Cell culture, inhibition of cell growth and cell death analysis**

**(MTT toxicity assays)**

For toxicity assays cells (5 x10\(^4\) for Jurkat cells and 10\(^4\) for adherent cell lines) were seeded in flat-bottom 96-well plates (100 µl/well) in complete medium. Adherent cells were allowed to attach for 24 h prior to addition of cisplatin or tested compounds. Compounds were added at different concentrations in triplicate. Cells were incubated with cisplatin or compounds for 24 h and then cell proliferation was determined by a modification of the MTT-reduction method. Briefly, 10 µl/well of MTT (5 mg/ml in PBS) was added and plates were incubated for 1-3 h at 37 ºC. Finally, formazan crystal was dissolved by adding 100 µl/well \(^1\)PrOH (0.05 M HCl) and gently shaking. The optical density was measured at 570 nm using a 96-well multiscanner autoreader (ELISA).

**Cell death analysis**

Apoptosis/necrosis hallmarks of cells treated with compound 5 were analyzed by measuring mitochondrial membrane potential, plasma membrane integrity and exposure of phosphatidylserine. Cells were treated with different concentrations and at different incubation times as indicated in figure legends. In some experiments the general caspase inhibitor z-VAD-fmk was added at 50 µM 1 h before compounds. For mitochondrial membrane potential determination cells (2.5x10\(^5\) in 200 µl) after treatment with 5 were incubated at 37ºC for 15 minutes in medium containing 5nM DiOC\(_6\)(3) (Molecular Probes). Phosphatidylserine exposure was quantified by labeling cells with AnnexinV-DY634 (Invitrogen) after treatment with 5. AnnexinV was added at a concentration of 0.5 µg/ml in Annexin Binding Buffer (ABB) and cells were incubated at room temperature for 15 min. Plasma membrane integrity was evaluated by staining with 7-Amino-Actinomycin D (7-AAD, Inmunostep). At the end of the treatment with 5, cells were incubated for 15 min in 200 µl of PBS containing 50 ng/µl 7-AAD. In all
cases, cells were diluted to 1 ml with ABB or PBS (Phosphate Buffered Saline) to be analyzed by flow cytometry (FACScan, BD Bioscience, Spain).

**Permeability determinations**
** (Cell culture and transport assays)

Caco-2 cells were grown in Dubelcco’s Modified Eagle’s Media containing L-glutamine, fetal bovine serum and penicillin-streptomycin. To obtain cells monolayers 250000 cells/cm² were seeded on each well with polycarbonate membrane with 4.2 cm² area. Plates were incubated at standard conditions of 37ºC temperature, 90% humidity and 5% CO₂ until confluence. After 19-21 days the integrity of the each cell monolayer was evaluated by measuring the trans-epithelial electrical resistance (TEER). Values ranging 500-750 Ωcm² were considered appropriated.

Transport studies were performed using an orbital environmental shaker at constant temperature (37ºC) and agitation rate (50 rpm). Hank’s balanced salt solution (HBSS) supplemented with HEPES was used to fill the receiver chamber and to prepare the drug solution placed in the donor chamber. Four samples of 200 µL each one were taken from the receiver chamber side at predefined times (15, 30, 60 and 90 minutes) and the same volumen were replaced with fresh buffer. Moreover, two samples of the donor side were taken at the beginning and the end of the experiment. Moreover, the amount of compound in cell membranes and inside the cells was determined at the end of experiments in order to check the mass balance and the percentage of compound retained in the cell compartment was always less than 5%.

Transport studies were performed in both directions, from apical-to-basal (A-to-B) and from basal-to-apical (B-to-A) sides. The volume of donor compartment was 2 mL in A-to-B direction and 3 mL in B-to-A direction.

**Analysis of the samples**

Samples were analyzed by HPLC using a 5 µm, 4 x 200 mm Novapack C18 column. Samples of Cisplatin and compound 4 were analyzed with UV detection (λ=240 nm) and the mobile phase was 95:5, acetonitrile: water, with a flow rate of 1mL/min, and the injected sample volume was 50 µL. Samples of compound 5 were analyzed similarly but using a UV detector at λ=215 nm and a mobile phase of 80:20 acetonitrile: water.
**Data analysis**

The apparent permeability coefficient was calculated following the equation:

\[
C_{\text{receiver}, t} = \frac{Q_{\text{total}}}{V_{\text{receiver}} + V_{\text{donor}}} + \left( C_{\text{receiver}, t-1} \cdot f \right) - \frac{Q_{\text{total}}}{V_{\text{receiver}} + V_{\text{donor}}} e^{-P \cdot S \left( \frac{1}{V_{\text{receiver}}} + \frac{1}{V_{\text{donor}}} \right) \Delta t}
\]

where \( C_{\text{receiver}, t} \) is concentration of compound in the receiver chamber at \( t \) time, \( Q_{\text{total}} \) is the total amount of drug in both chambers, \( V_{\text{receiver}} \) and \( V_{\text{donor}} \) are the volumes corresponding to receiver and donor compartment respectively in each chamber, \( C_{\text{receiver}, t-1} \) is concentration of compound in receiver chamber at previous time, \( f \) is the sample dilution factor due to replaced volume, \( S \) is the surface area of the monolayer, \( \Delta t \) is the time interval and \( P \) is the permeability coefficient. This equation takes into account the continuous change of the donor and receiver concentrations, i.e. non-sink conditions. However, when the transport rate is low, there are not significantly changes between the donor and the receiver concentrations with time. In this conditions sink conditions are assumed and a simpler expression can be used to estimate the permeability coefficient:

\[
P = \frac{dQ/dt}{C \cdot S}
\]

where \( dQ/dt \) is the apparent appearance rate of drug in the receiver side calculated using linear regression of amounts in the receiver chamber versus time, \( S \) is the surface area of the monolayer and \( C \) is the drug concentration in the donor chamber.

The permeability coefficient estimations in sink and non-sink conditions were carried out in an Excel® worksheet.

Studies were performed by triplicate and the data were presented as mean ± SD. Student’s t-test were performed with SPSS 16.0 (SPSS Inc.) in order to determine statistically significant differences between A-to-B and B-to-A permeabilities.
In situ absorption experiments

The absorption experiments were performed using an in situ loop technique described by Doluisio.\textsuperscript{52} The study was approved by the Scientific Committee of the Faculty of Pharmacy and followed the guidelines described in the EC Directive 86/609, the Council of the Europe Convention ETS 123 and Spanish national laws governing the use of animals in research (Real Decreto 223/1988, BOE 67, 18-3-98: 8509-8511). Male Wistar rats weighing 280-320 g were used after 8 h of fasting. Previously to surgical procedure, animals were anesthetized with Diazepam (Valium, Roche) (1,67 mg/kg), Ketamine (Ketolar; parke-Davis) (50mg/kg) and Atropine (atropine sulfate; Braun) (1mg/kg). The body temperature was maintained during the procedure by heating with a lamp. Therefore, a midline abdominal incision were performed and a loop was isolated from duodenal and ileal region of each rat. The proximal ligatures of the duodenal and ileal regions were placed approximately 1 cm from the pylorus and 2 cm above the ileocecal junction. The bile duct was tight up in all experiments. 50 mL of cleaning solution (Solution A (pH 7,4): 9.2 g NaCl, 0.34 g KCL, 0.19 g CaCl\(_2\)*H\(_2\)O and 0.76 g NaH\(_2\)PO\(_4\)*2 H\(_2\)O per liter) were used to flushed out the content of the loop and then 20 mL volume of solution B (NaCl g, NaH\(_2\)PO\(_4\)*2H\(_2\)O 1/15M 3.9 mL, Na\(_2\)HPO\(_4\) 1/15M 6.1 mL and water up to 1L) were perfused to condition the intestinal mucosa prior to the experiments. A catheter was tight up at both intestinal ends and connected to a glass syringe by the use of a stopcock type valve. Under this set up, the intestinal segment is an isolated compartment and the drug solution can be perfused. The drug solutions were prepared freshly day at 20 µM using solution B as solvent and perfused into the loop and then the entire intestine was restored into the abdominal cavity. Samples of the perfusate were carried out every 5 minutes for 30 minutes.

Permeability Calculations

The apparent first order absorption rate coefficients (kapp) were obtained by non-linear fitting of a monoexponential equation to the luminal concentrations versus time.

\[
C = C_0 \cdot e^{-kapp \cdot t} \quad (3)
\]

where the \(C\) is the drug concentration remaining in the lumen, \(kapp\) is the apparent absorption rate constant, and \(C_0\) corresponds to a calculated fraction of the initial perfusion concentration. Test solutions suffer a slight dilution in the intestinal lumen due to the remaining cleaning
solution, the adsorption to the membrane, and the loading process in the enterocyte. So, the intercept, \( C_0 \), is lower than the perfusion concentration. The quasi-steady-state is achieved in the membrane when this process is finished. Under these conditions, the disappearance of the compound from the lumen can be considered as a first order process during the sampling time interval. For these reasons, only the concentrations obtained after 5 min were used for regression analysis. In order to obtain good prediction data water reabsorption correction of was introduced for the concentrations calculations.

The intestinal permeability values were calculated taking into account the relationship between \( k_a \) and \( P_{\text{eff}} \):

\[
P_{\text{eff}} = \frac{(k_a \cdot R)}{2}
\]

where \( R \) is the radius of the intestinal segment, calculated as area/volume ratio. The effective intestinal permeabilities (\( P_{\text{eff}} \)) of the tested compounds (means of at least of three animals) were used as indexes of the absorption effectiveness.

**Interaction of compounds 1-5 and cisplatin with plasmid (pBR322) DNA by Electrophoresis**

(Mobility Shift Assay)

10 \( \mu \)L aliquots of pBR322 plasmid DNA (20 \( \mu \)g/mL) in buffer (5 mM Tris/HCl, 50 mM NaClO₄, pH = 7.39) were incubated with different concentrations of the compounds 1-5 (in the range 0.25 and 2.0 metal complex:DNAbp) at 37 °C for 20 h in the dark. Samples of free DNA and cisplatin-DNA were prepared as controls. After the incubation period, the samples were loaded onto the 1 % agarose gel. The samples were separated by electrophoresis for 1.5 h at 84 V in Tris-acetate/EDTA buffer (TAE). Afterwards, the gel was stained for 30 min with a solution of GelRed Nucleic Acid stain.

**Interaction of compounds 3-5 and cisplatin with Calf Thymus DNA by Circular Dichroism**

Stock solutions (5 mM) of each complex were freshly prepared in water prior to use. The right volume of those solutions was added to 3 ml samples of an also freshly prepared solution of CT DNA (195 \( \mu \)M) in Tris/HCl buffer (5 mM Tris/HCl, 50 mM NaClO₄, pH=7.39) to achieve molar
ratios of 0.1, 0.25, 0.5 and 1.0 drug/DNA. The samples were incubated at 37 °C for a period of 20 h. All CD spectra of DNA and of the DNA-drug adducts were recorded at 25 °C over a range 220-330 nm and finally corrected with a blank and noise reduction. The final data is expressed in molar ellipticity (millidegrees).

**Interaction of compounds 1-5 and cisplatin with HSA by Fluorescence Spectroscopy**

A solution of each compound (8 mM) in DMSO was prepared and ten aliquots of 2.5 µL were added successively to a solution of HSA (10 µM) in phosphate buffer (pH = 7.4) to achieve final metal complex concentrations in the range 10-100 µM. The excitation wavelength was set to 295 nm, and the emission spectra of HSA samples were recorded at room temperature in the range of 300 to 450 nm. The fluorescence intensities of all the metal compounds, the buffer and the DMSO are negligible under these conditions. The fluorescence was measured 240 s after each addition of compound solution. The data were analyzed using the classical Stern-Volmer equation $F_0/F = 1 + K_{SV}[Q]$.

**Cell culture and XTT assay for RPTC cells**

The human Renal Proximal Tubule Cells (RPTC) a non-tumoral human kidney epithelial cell line (obtained from Lifeline Cell Technology, Frederick, Maryland, USA) were cultured in Lifeline's RenaLife Medium containing RenaLife LifeFactors with 2.4 mM L-Glutamine, 5 ñg/mL rh insulin, 1.0 ñg/mL epinephrine, 10 nM triiodothyronine, 0.1 ñg/mL hydrocortisone hemisuccinate, 10 ng/mL rh EGF, 0.5% FBS and 5 ñg/mL transferrin PS (all from Lifeline Cell Technology), at 37°C in a humidified atmosphere of 95% of air and 5% CO₂ (Brooklyn College, CUNY, US). For evaluation of cell viability, cells were seeded at a concentration of 5×10³ cells/well in 90 µl Lifeline's RenaLife complete medium into tissue culture grade 96-well flat bottom microplates (Thermo Scientific BioLite Microwell Plate, Fisher Scientific, Waltham, Massachusetts, USA) and grown for 24 h. Solutions of the compounds were prepared by diluting a freshly prepared stock solution (in H₂O) of the corresponding compound in Lifeline's RenaLife complete medium. Afterwards, the intermediate dilutions of the compounds were added to the wells (10 µL) to obtain a final concentration ranging from 0.1 to 200 µM, and the cells were incubated for 24 h. Following 24 h drug exposure, 50 µL of 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) (Roche Diagnostics, Indianapolis, Indiana,
USA) labeling mixture per well was added to the cells at a final concentration of 0.3 mg/ml and incubated for 4 h at 37°C in a humidified atmosphere of 95% of air and 5% CO₂. The optical density of each well (96-well plates) was quantified using EnVision Multilabel Plate Readers (Perkin Elmer, Waltham Massachusetts, USA) at 450 nm wavelength to measure absorbance. The percentage of surviving cells was calculated from the ratio of absorbance of treated to untreated cells. The IC₅₀ value was calculated as the concentration reducing the proliferation of the cells by 50% and is presented as a mean (± SE) of at least two independent experiments each with triplicates.
4.8. Appendix

Crystallographic data for compounds 2 and 4 including complete drawing of the structure and table of selected distances and angles for compound 4; stability of compounds 1-5 by $^{31}P^{[1]}H$ spectroscopy in DMSO-$d_6$ solution; selected $^1H$ and $^{31}P^{[1]}H$ NMR spectra for compounds 1-5 in DMSO-$d_6$; stability of compounds 4 and 5 in DMSO:PBS (1:99) determined by Uv-Vis.


**Table 7.** Crystal data for compounds 2 and 4.

<table>
<thead>
<tr>
<th>Compound</th>
<th>2</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formula</td>
<td>$C_{13}H_{16}N_4OPCl_2Au$</td>
<td>$C_{66}H_{60}Cl_{10}Hg_2N_2P_2Pt_2$</td>
</tr>
<tr>
<td>Formula weight</td>
<td>543.13</td>
<td>2095.01</td>
</tr>
<tr>
<td>$T$ [K]</td>
<td>100(2)</td>
<td>100(2)</td>
</tr>
<tr>
<td>$\lambda$ (Mo-Kα) [Å]</td>
<td>0.71073</td>
<td>0.71073</td>
</tr>
<tr>
<td>Crystal system</td>
<td>Orthorhombic</td>
<td>Triclinic</td>
</tr>
<tr>
<td>Space group</td>
<td>$Pbca$</td>
<td>$P1$</td>
</tr>
<tr>
<td>$a$ [Å]</td>
<td>12.8453(8)</td>
<td>11.1159(5)</td>
</tr>
<tr>
<td>$b$ [Å]</td>
<td>11.0611(7)</td>
<td>11.8606(5)</td>
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<tr>
<td>$c$ [Å]</td>
<td>22.6045(14)</td>
<td>14.8021(6)</td>
</tr>
<tr>
<td>$\alpha$ [°]</td>
<td>90</td>
<td>92.5060(10)</td>
</tr>
<tr>
<td>$\beta$ [°]</td>
<td>90</td>
<td>99.7520(10)</td>
</tr>
<tr>
<td>$\gamma$ [°]</td>
<td>90</td>
<td>115.8280(10)</td>
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<tr>
<td>$V$ [Å³]</td>
<td>3211.7(3)</td>
<td>1715.94(13)</td>
</tr>
<tr>
<td>$Z$</td>
<td>8</td>
<td>1</td>
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<tr>
<td>$D_{calc}$ (g cm$^{-3}$)</td>
<td>2.247</td>
<td>2.027</td>
</tr>
<tr>
<td>$\mu$ (mm$^{-1}$)</td>
<td>9.599</td>
<td>9.000</td>
</tr>
<tr>
<td>GOF</td>
<td>1.057</td>
<td>1.048</td>
</tr>
<tr>
<td>$R_1$ [$I &gt; 2\sigma(I)$]</td>
<td>0.0142</td>
<td>0.0186</td>
</tr>
<tr>
<td>w$R_2$ (all data)</td>
<td>0.0317</td>
<td>0.0390</td>
</tr>
</tbody>
</table>
Table 8. Selected Structural Parameters of complex 2 obtained from X-ray single crystal diffraction studies (see drawing above). Bond lengths are given in [Å] and angles in [°].

<table>
<thead>
<tr>
<th>Bond</th>
<th>Length [Å]</th>
<th>Angle [°]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au(1)-Cl(1)</td>
<td>2.3834(5)</td>
<td>N(1)-Au(1)-Cl(2) 173.33(5)</td>
</tr>
<tr>
<td>Au(1)-Cl(2)</td>
<td>2.2798(5)</td>
<td>N(1)-Au(1)-Cl(1) 97.32(5)</td>
</tr>
<tr>
<td>Au(1)-C(1)</td>
<td>2.020(2)</td>
<td>Cl(2)-Au(1)-Cl(1) 88.62(2)</td>
</tr>
<tr>
<td>Au(1)-N(1)</td>
<td>2.0497(18)</td>
<td>P(1)-N(1)-Au(1) 126.07(10)</td>
</tr>
<tr>
<td>P(1)-N(1)</td>
<td>1.6658(18)</td>
<td>P(1)-N(1)-C(7) 119.25(15)</td>
</tr>
<tr>
<td>N(1)-C(7)</td>
<td>1.404(3)</td>
<td>N(1)-C(7)-C(6) 112.16(19)</td>
</tr>
<tr>
<td>C(7)-O(1)</td>
<td>1.213(3)</td>
<td>N(1)-C(7)-O(1) 123.72(19)</td>
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<tr>
<td>C(7)-C(6)</td>
<td>1.478(3)</td>
<td>C(7)-N(1)-Au(1) 114.68(14)</td>
</tr>
<tr>
<td>C(6)-C(1)</td>
<td>1.385(3)</td>
<td>C(7)-C(6)-C(1) 118.0(2)</td>
</tr>
<tr>
<td>C(1)-Au(1)-N(1)</td>
<td>81.68(8)</td>
<td>C(6)-C(1)-Au(1) 113.24(16)</td>
</tr>
<tr>
<td>C(1)-Au(1)-Cl(2)</td>
<td>92.44(6)</td>
<td>N(1)-P(1)-C(10) 113.99(10)</td>
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<tr>
<td>C(1)-Au(1)-Cl(1)</td>
<td>178.28(6)</td>
<td>N(1)-P(1)-C(8) 116.45(10)</td>
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<tr>
<td>C(1)-Au(1)-Cl(1)</td>
<td>178.28(6)</td>
<td>N(1)-P(1)-C(13) 117.81(10)</td>
</tr>
</tbody>
</table>
Table 9. Selected Structural Parameters of complex 4 (see drawing incorporating the anion above) obtained from X-ray single crystal diffraction studies. Bond lengths are given in [Å] and angles in [°].

<table>
<thead>
<tr>
<th>Bond</th>
<th>Length (Å)</th>
<th>Angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt(1)-C(1)</td>
<td>2.039(2)</td>
<td>N(1)-Pt(1)-C(1) 85.31(9)</td>
</tr>
<tr>
<td>Pt(1)-N(1)</td>
<td>2.039(2)</td>
<td>N(1)-Pt(1)-C(25) 95.99(9)</td>
</tr>
<tr>
<td>Pt(1)-C(30)</td>
<td>2.150(2)</td>
<td>N(1)-Pt(1)-C(26) 91.92(8)</td>
</tr>
<tr>
<td>Pt(1)-C(29)</td>
<td>2.160(2)</td>
<td>N(1)-Pt(1)-C(29) 162.15(8)</td>
</tr>
<tr>
<td>Pt(1)-C(26)</td>
<td>2.268(2)</td>
<td>N(1)-Pt(1)-C(30) 160.07(9)</td>
</tr>
<tr>
<td>Pt(1)-C(25)</td>
<td>2.287(2)</td>
<td>C(1)-Pt(1)-C(25) 163.39(9)</td>
</tr>
<tr>
<td>P(1)-N(1)</td>
<td>1.622(2)</td>
<td>C(1)-Pt(1)-C(26) 161.34(9)</td>
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<tr>
<td>P(1)-C(2)</td>
<td>1.773(2)</td>
<td>C(1)-Pt(1)-C(29) 96.26(10)</td>
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<td>P(1)-C(7)</td>
<td>1.797(3)</td>
<td>C(1)-Pt(1)-C(30) 92.76(10)</td>
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<tr>
<td>P(1)-C(13)</td>
<td>1.797(3)</td>
<td>P(1)-N(1)-Pt(1) 117.55(11)</td>
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<td>N(1)-C(19)</td>
<td>1.444(3)</td>
<td>C(19)-N(1)-Pt(1) 125.82(16)</td>
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<td>C(1)-C(2)</td>
<td>1.409(3)</td>
<td>C(19)-N(1)-P(1) 116.21(16)</td>
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<td>Hg(1)-Cl(1)</td>
<td>2.6117(7)</td>
<td>Cl(1)-Hg(1)-Cl(2) 107.66(2)</td>
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<tr>
<td>Hg(1)-Cl(2)</td>
<td>2.3808(7)</td>
<td>Cl(2)-Hg(1)-Cl(3) 135.47(3)</td>
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<td>Hg(1)-Cl(3)</td>
<td>2.3865(7)</td>
<td>Cl(3)-Hg(1)-Cl(1) 105.68(2)</td>
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<tr>
<td>Hg(1)-Cl(1)#1</td>
<td>2.6665(7)</td>
<td>Cl(2)-Hg(1)-Cl(1)#1 106.38(2)</td>
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<tr>
<td>Cl(1)-Hg(1)#1</td>
<td>2.6666(7)</td>
<td>Cl(3)-Hg(1)-Cl(1)#1 102.92(3)</td>
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<tr>
<td></td>
<td></td>
<td>Cl(1)-Hg(1)-Cl(1)#1 88.90(2)</td>
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<tr>
<td></td>
<td></td>
<td>Hg(1)-Cl(1)-Hg(1)#1 91.10(2)</td>
</tr>
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</table>
4.8.2. Stability of complexes in DMSO-\textit{d}_\textit{6} solution overtime assessed by $^{31}$P$\{^{1}$H$\}$ NMR spectroscopy.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time (DMSO-\textit{d}_\textit{6})</th>
<th>Half life (50%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$&gt;99%$ 90% 55% 36%</td>
<td>2 months</td>
</tr>
<tr>
<td>2</td>
<td>$&gt;99%$ 90% 50%</td>
<td>3 months</td>
</tr>
<tr>
<td>3</td>
<td>$&gt;99%$</td>
<td>months</td>
</tr>
<tr>
<td>4</td>
<td>$&gt;99%$ 90% 84%</td>
<td>months</td>
</tr>
<tr>
<td>5</td>
<td>$&gt;99%$ 96% 94%</td>
<td>77% months</td>
</tr>
</tbody>
</table>

% of decomposition determined by integration of all the signals present in the $^{31}$P$\{^{1}$H$\}$ NMR spectra, the sum being set to 100%.

4.8.3. Selected $^{1}$H and $^{31}$P$\{^{1}$H$\}$ NMR spectra showing the stability of complexes in DMSO-\textit{d}_\textit{6} overtime.

**Figure 15.** $^{31}$P$\{^{1}$H$\}$ NMR spectrum of compound 1 in DMSO-\textit{d}_\textit{6} ($\delta$ 33.57 (s) ppm) overtime.
Figure 16. $^{31}\text{P}^{\{1\text{H}\}}$ NMR spectrum of compound 2 in DMSO-$d_6$ ($\delta$ -2.66 (s ppm) overtime.

Figure 17. $^{31}\text{P}^{\{1\text{H}\}}$ NMR spectrum of compound 3 in DMSO-$d_6$ ($\delta$ -10.26 (s ppm) overtime
Figure 18. $^1$H NMR spectrum of compound 3 in DMSO-$d_6$ overtime.

Figure 19. $^{31}$P{$^1$H} NMR spectrum of compound 4 in DMSO-$d_6$ ($\delta$ -62.57 (s) ppm) overtime.
Figure 20. $^1$H NMR spectrum of compound 4 in DMSO-$d_6$ overtime.

Figure 21. $^{31}$P{$^1$H} NMR spectra of compound 5 in DMSO-$d_6$ ($\delta$ 63.10 (s) and – 148.60 (hept) ppm) overtime.
Figure 22. $^1$H NMR spectrum of compound 5 in DMSO-$d_6$ overtime.

4.8.4. UV-Vis spectra of compounds 4 and 5 in CH$_2$Cl$_2$ and in DMSO, and in 1% DMSO-PBS solution overtime.

Figure 23. UV-visible spectrum of compound 4 (4.0 µM) in dichloromethane.
Figure 24. UV-visible spectrum of compound 4 (50.0 µM) in DMSO recorded overtime.

Figure 25. UV-visible spectrum of compound 4 (15.0 µM) in 1:99 DMSO/PBS-1X (pH 7.4) recorded overtime, incubation at RT.
Figure 26. UV-visible spectrum of compound 5 (10.0 µM) in dichloromethane.

Figure 27. UV-visible spectrum of compound 5 (50.0 µM) in DMSO recorded overtime.
Figure 28. UV-visible spectrum of compound 5 (50.0 µM) in 1:99 DMSO/PBS-1X (pH 7.4) recorded overtime, incubation at RT.

Figure 29. UV-visible spectrum of iminophosphorane ligand Ph₃P=NPh (50.0 µM) in DMSO.
Figure 30. UV-visible spectrum of iminophosphorane ligand Ph$_3$P=NPh (50.0 µM) in 1:99 DMSO/PBS-1X (pH 7.4).


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CHAPTER V
RUTHENIUM(II) COMPOUNDS WITH N,N- CHELATING AND C,N- (CYCLOMETALLATED) IMINOPHOSPHORANE LIGANDS.
IN VITRO AND IN VIVO EVALUATION AND PRELIMINARY MECHANISTIC STUDIES.

5.1. Ruthenium(II and III) as potential anticancer agents

As mentioned in chapter I, ruthenium compounds have emerged as promising candidates for metal based cancer chemotherapeutics.¹⁻⁵ Like platinum-based drugs, ruthenium compounds can exchange N- and O-donor molecules with the added advantage of the possibility of forming octahedral complexes (of interest in reactions with DNA). As described in Chapter I, three ruthenium(III) coordination complexes have successfully completed phase I clinical trials and entered phase II (NAMI-A,⁴,⁶ KP1019 and KP1339⁴,⁷,⁸).

More recently, ruthenium (II)-arene complexes (with a piano-stool structure) have also been described as promising candidates.⁴,⁹⁻¹⁸ The two most relevant examples, which have undergone advanced preclinical studies are RM175, described by Sadler et al. and RAPTA-T reported by Dyson et al. (Figure 1). RAPTA-T, containing the hydrophilic and monodentante PTA phosphate ligand, showed to reduce the growth of lung metastases in mice bearing mammary carcinoma and was able to inhibit highly invasive breast cancer. RM175 demonstrated to be active in vivo against triple-negative breast cancer cell lines.¹³,¹⁹ Pfeffer-Gaiddon and co-workers reported on C,N-cyclo metallated compounds (such as RDC11 in Figure 1) that inhibited the growth of human cancer xenografts in mice more efficiently than cisplatin.⁴,¹⁰,²⁰⁻²³ Notably, it did not cause severe side effects in liver, kidneys, neuronal sensory system or blood cells qualifying as a promising candidate for future clinical trials in cisplatin-resistant cancers.⁴,²⁰,²²
Figure 1. Selected ruthenium(III) and (II) compounds with important antitumor and/or antimetastatic properties⁴⁻²², ³⁵⁻³⁷ and refs. therein.

Very recently, Dyson et al. showed that RAPTA-C reduces the growth of primary tumors in preclinical models for ovarian and colorectal carcinomas through an antiangiogenic mechanism, most likely through binding to the histone protein core in chromatin rather than to the DNA.²⁴,²⁵ Another recent report by the same group described Ru(II)-arene complex with a perfluoroalkyl-modified ligand (A, in Figure 1) that displayed antiproliferative activity against four cancer cell lines in vitro and with no toxic effect to non-tumoral cells. Furthermore, in vivo studies of A in a xenografted ovarian carcinoma (A2780) grown on the chicken chorioallantoic membrane model (CAM) led to a 90% reduction in tumor growth, displaying a stronger antivasual effect than RAPTA-C.²⁴

Dyson et al. also reported on the evaluation of a similar Ru(II)-arene complex, with a long fluororous chain (B, in Figure 1), which exhibited a synergistic tumor growth reduction in dual-therapy with local hyperthermia showing tumor growth reduction by 90%. It has been
shown that combining chemotherapy with regional mild hyperthermia, applying heat locally to rise the tumor temperature between 40-42ºC, can sensitize tumor tissue to anticancer agents resulting in improved local control, treatment efficacy and overall survival prolongation.\textsuperscript{26}

The recent strategy to bind a drug of well-known therapeutic value (such as curcumin, ketoconazole, clotrimazole, hydroxyflavones, hydroxyquinolinones, letrozole, indolobenzazepins or aspirin) to ruthenium centers has rendered a number of complexes with improved properties with respect to the parent organic drugs for cancer.\textsuperscript{27-34} In this context, ruthenium compounds resembling staurosporine (like DW1/2 in Figure 1) developed by Meggers and co-workers are relevant examples of potential chemotherapeutics targeting protein kinases.\textsuperscript{4,10,35-37}

Hartinger \textit{et al.} explored new routes to develop metallodrugs by coordinating pharmacophores to metal fragments with established anticancer properties. In their study, oxicam backbone that is found in the nonsteroidal anti-inflammatory drugs, was incorporated into an organometallic Ru(II)-arene compound. The most lipophilic compound in this study (C in Figure 1) displayed the most cytotoxicity against human colon carcinoma (HCT116) cell lines \textit{in vitro}. Even though the IC\textsubscript{50} of the compound was not significantly low, this and other compounds in this study could be further explored to fully understand their mode of action. In comparison, other ruthenium compounds complexes, such as NAMI-A and RAPTA derivatives, were noncytotoxic \textit{in vitro} but exhibited high activity against metastases \textit{in vivo}.\textsuperscript{38}

In a recent study done by Süss-Fink and co-workers, dinuclear \textit{p}-cymene ruthenium complex was synthesized and evaluated \textit{in vitro} for its anticancer activity in series of cancer cell lines (diruthenium-1 in Figure 1). Diruthenium-1 was found to be highly cytotoxic towards ovarian cancer (A2780) cell lines \textit{in vitro}, in low nanomolar range (30 nM). The complex was further studied \textit{in vivo} in tumor-bearing mice at a dose of 0.6 mg/kg and showed to significantly prolonged the survival rate of tumor-bearing mice as compared to the untreated control group.\textsuperscript{39}

Nowadays, there are examples of multinuclear ruthenium compounds,\textsuperscript{40,41} ruthenium derivatives which can be activated by light,\textsuperscript{42} that are thermoresponsive,\textsuperscript{43} that can be obtained by a combinatorial approach,\textsuperscript{44} as well as ruthenium compounds that can be delivered to tumor sites more efficiently by binding to polymers,\textsuperscript{45} nanocarriers,\textsuperscript{46,47} peptides,\textsuperscript{48,49} or transport proteins.\textsuperscript{50} However, there is still a need to find the ultimate target(s) for these ruthenium compounds as well as to get a better knowledge on the detailed molecular mechanism of action
in order to develop more powerful and selective chemotherapeutics.\textsuperscript{4} In addition, more \textit{in vivo} data is needed to make more reliable predictions of structure-biological activity correlations.\textsuperscript{17,19}

Within this context, we aimed to explore the potential of IM complexes containing metals other than d\textsuperscript{8} transition metals (Au(III), Pd(II) and Pt(II)) as anticancer agents and we focused on ruthenium(II) compounds. The following chapter reports on the preparation, characterization and stability studies of organometallic ruthenium(II) compounds containing different iminophosphorane ligands. Preliminary biochemical and biochemical studies (\textit{in vitro} effects on human cancer cells, type of cell death and permeability studies on selected compounds) were performed by our collaborators. I studied the interactions of the compounds with plasmid (pBP322) DNA and of selected compounds on \textit{Calf Thymus} DNA by different techniques (melting point, CD circular dichroism). Finally the effects of a water-soluble compound with the best pharmacological profiles on human cancer xenografts in mice (performed by a PhD student from our laboratory) will be reported and discussed to put the results into context and discuss the potential of these ruthenium compounds as cancer chemotherapeutics.

5.2. Synthesis and characterization of ruthenium(II) organometallic complexes containing iminophosphorane ligands

The synthesis of ruthenium(II) piano-stool complexes containing iminophosphorane ligands has been described by Urriolabeitia and co-workers.\textsuperscript{51} We have employed here \textit{p}-cymene as the arene group coordinated to the ruthenium centers. Thus, compounds 1-4 can be easily obtained in high yields by the addition of different IM ligands previously described\textsuperscript{52-55,56} to [(\textit{η}\textsuperscript{6}-\textit{p}-cymene)Ru(μ-Cl)Cl]\textsubscript{2} \textsuperscript{57} (Scheme 1). 4 is an example of a new heterometallic ruthenium complex containing a ferrocene fragment.

Ruthenium(II) complexes with cyclometallated IM (pincer C,N-) ligands have been prepared by transmetallation with organomercury derivatives.\textsuperscript{51} However the nature of the IM ligand played a crucial role and compounds with semi-stabilized IM ligands containing carbonyl groups like (2-C\textsubscript{6}H\textsubscript{4})Ph\textsubscript{2}P=N-CO-Ph could not be obtained due to steric reasons.\textsuperscript{51}
Scheme 1. Preparation of cationic ruthenium(II) compounds containing IM ligands.

In addition, we also aimed to prepare cycloruthenated compounds in which the aryl group of the imino fragment is coordinated to the metal center (exo derivatives) as opposed to an aryl group of the phosphine fragment (endo derivatives) in order to be able to incorporate different phosphines into the final molecule to tune electronic/steric properties of the resulting complexes. As described in Chapter IV, cyclometalated iminophosphorane exo derivatives can be prepared by reaction of the appropriate metal compound with the organomercury derivative Hg(Ph₃P=N-CO-2-C₆H₄)Cl.58 As mentioned in the previous chapter, C-H activation at the N-CO-Ph fragments takes place at a manganese center and by transmetallation of the resulting
cyclometalated iminophosphorane manganese compounds to HgCl$_2$, the organomercury derivatives with PPh$_3$ [Hg(Ph$_3$P=N-CO-2-C$_6$H$_4$)Cl]$^{58}$ or water soluble phosphine PTA [Hg(PTA=N-CO-2-C$_6$H$_4$)Cl] (7) are obtained in high yields. Transmetallation reactions of [Hg(Ph$_3$P=N-CO-2-C$_6$H$_4$)Cl]$^{58}$ and 7 with [(η$^6$-p-cymene)Ru(μ-Cl)Cl]$_2$ afford new cyclometallated compounds [(η$^6$-p-cymene)Ru(IM-k-C,N)Cl] (IM = Ph$_3$P=N-CO-2-C$_6$H$_4$ 8; PTA=N-CO-2-C$_6$H$_4$ 9) in high yields (Scheme 2).

Scheme 2. Preparation of the new cycloruthenated compounds 8 and 9 containing IM ligands.

All the compounds are obtained as air stable solids in moderate to high yields. Their structures have been proposed on the basis of microanalytical, spectroscopic (IR and NMR), conductivity and MS spectrometry data. Some of the compounds are slightly hygroscopic. As previously proposed by Urriolabeitia and co-workers,$^{51}$ the IM ligand in compounds 1 and 2 is bonded as a chelate giving a fac-Cl,N,O arrangement while in compounds 3 and 4 the arrangement is fac-Cl,N,N. This can be clearly inferred from the IR data. There is a strong absorption at 1531 cm$^{-1}$ due to νCO stretch for 1 and 2 shifted to lower frequencies with respect to that of the free ligand at 1598 cm$^{-1}$. For 3 and 4 the signal that can be assigned to νPN stretch is shifted to lower frequency than that of the free ligands (see Experimental Section). As previously described for compounds containing benzene and some IM ligands,$^{51}$ the $^{31}$P{$_1$H} NMR signals for 1 and 2 resemble that of the free ligand whereas for 3 and 4 the signals are strongly shifted to low field with respect to the free ligands, indicating iminic N-bonding. In addition, the ortho protons of the quinoline (H$_2$) and pyridine (H$_6$) in the $^1$H NMR spectra for 3 and pyridine (H$_6$) for 4 are shifted downfield supporting the idea of N-coordination. Compound 4 shows fluxional behavior at room temperature (giving rise to broad signals in the $^1$H NMR.
spectrum in the area of the $p$-cymene and Cp rings) and its variable temperature NMR spectra are collected in the Appendix.

The structure of 1 has been determined by an X-ray analysis and it is very similar to that of previously reported$^{51}$ [($\eta^6$-C$_6$H$_6$)Ru(k-N,O-Ph$_3$P=N-CO-2-N-C$_5$H$_4$)]PF$_6$ with very similar distances and angles. The molecular structure for the cation of 1 is depicted in Figure 2. Selected bond lengths and angles for compound 1 are collected in Table 1.

![Figure 2. Molecular structure of the cation of compound 1.](image)

**Table 1.** Selected Structural Parameters of complex 1 obtained from X-ray single crystal diffraction studies (see drawing above). Bond lengths are given in [Å] and angles in [°].

<table>
<thead>
<tr>
<th>Bond Lengths (Å)</th>
<th>Angles (°)</th>
</tr>
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<tbody>
<tr>
<td>Ru(1)-O(1)</td>
<td>2.110(3)</td>
</tr>
<tr>
<td>Ru(1)-N(1)</td>
<td>2.095(4)</td>
</tr>
<tr>
<td>Ru(1)-C(10)</td>
<td>2.153(5)</td>
</tr>
<tr>
<td>Ru(1)-C(6)</td>
<td>2.154(5)</td>
</tr>
<tr>
<td>Ru(1)-C(7)</td>
<td>2.171(5)</td>
</tr>
<tr>
<td>Ru(1)-C(9)</td>
<td>2.187(5)</td>
</tr>
<tr>
<td>Ru(1)-C(11)</td>
<td>2.191(5)</td>
</tr>
<tr>
<td>Ru(1)-C(8)</td>
<td>2.204(5)</td>
</tr>
</tbody>
</table>
The analysis confirms the piano-stool structure around the ruthenium center as well as the coordination of the IM ligand through the N and O atoms. Ru-C distances are on average slightly longer and the O(1)-Ru(1)-N(1) and N(1)-Ru(1)-Cl(1) angles slightly smaller for 1 than for the compound with \(\eta^6\)-C\(_6\)H\(_6\)^{51} as expected.

Compounds 8 and 9 are cycloruthenated neutral species with the IM ligand in an \(exo\) disposition \([(\eta^6-p\text{-cymene})\text{Ru}([\text{IM-k-C,N}])]\) (IM = Ph\(_3\)P=N-CO-2-C\(_6\)H\(_4\) 8; PTA=N-CO-2-C\(_6\)H\(_4\) 9). Their \(^1\)H NMR spectra show signals due to \(\eta^6\)-arene as well as the C\(_6\)H\(_4\)P units. In addition, the \(^{13}\)C\({^1}\)H NMR spectrum shows six well-resolved peaks due to the C\(_6\)H\(_4\)P unit. The signals in the \(^{31}\)P\({^1}\)H NMR spectra are strongly shifted downfield with respect of those for the free ligand as reported before for \(endo\) cycloruthenated species.\(^{51}\)

The cationic compounds with chloride as counterion (2, 3, and 4) are highly soluble in water (70 to 100 mg/mL). The cycloruthenated derivative 9 with a water-soluble phosphine PTA (9) is much less soluble in water (1 mg/mL 9). 1, and cyclorutenated 8 and 9 are soluble in micromolar concentrations in 1:99 DMSO:H\(_2\)O mixtures. All the complexes but 9 are stable for weeks in DMSO-\(d_6\) solution (see spectra and stability table in the Appendix). The stability of the water-soluble complexes was studied by \(^{31}\)P\({^1}\)H and \(^1\)H NMR spectroscopy in D\(_2\)O. The spectrum in D\(_2\)O for compound \([(\eta^6-p\text{-cymene})\text{Ru}\{\text{C}-\text{P}(\text{Ph}_2)=\text{N}-\text{CH}_2-2-\text{NC}_5\text{H}_4}\}\text{Fe}(<\text{Cp})]\) Cl (4) does not change for over 3 days but after that time the compound precipitates. The \(^{31}\)P\({^1}\)H NMR spectrum in D\(_2\)O for 3 (\(\delta=37.73\) ppm) shows an additional signal (\(\delta=38.34\) ppm) that may be assigned to a hydrolyzed species of the type \([(\eta^6-p\text{-cymene})\text{Ru}(\text{Ph}_3\text{P}=\text{N}-8-C\text{H}_8\text{N})(\text{OH}_2)]^{2+}\). The integration of these signals is ca. 45:55 and the spectrum does not change much over time (days).

In the case of compound 2 (\(\delta\) in D\(_2\)O 26.33 ppm) a signal (attributable to \([(\eta^6-p\text{-cymene})\text{Ru}(\text{Ph}_3\text{P}=\text{N}-\text{CO}-2\text{-N}-\text{C}_5\text{H}_4)(\text{OH}_2)]^{+}\) is also visible in D\(_2\)O (\(\delta=26.65\) ppm) along with another signal (\(\delta=43.79\) ppm), which may correspond to the cyclometalated species \([(\eta^6-p\text{-cymene})\text{Ru}(\text{IM-k-C,N-C}_6\text{H}_4(\text{PPh}_2)=\text{N}-\text{CO}-2\text{-N}-\text{C}_5\text{H}_4)\text{Cl}]\) or \([(\eta^6-p\text{-cymene})\text{Ru}(\text{IM-k-C,N-C}_6\text{H}_4(\text{PPh}_2)=\text{N}-\text{CO}-2\text{-N}-\text{C}_5\text{H}_4)(\text{OH}_2)]^{+}\) and grows overtime. The \(^1\)H NMR spectrum of 2 in D\(_2\)O after 5 days shows (in addition to those of compound 2) signals due to new species containing \(\eta^6\)-arene as well as the C\(_6\)H\(_4\)P unit indicating the cyclometalation of the PPh\(_3\) ring. In addition, the \(^{13}\)C\({^1}\)H NMR spectrum shows most of the six peaks due to this C\(_6\)H\(_4\)P unit. The doublet that can be assigned to the C=O peak for these new cyclometalated species appears at 176.06 ppm (versus 178.08 ppm for 2) an it is closer to the chemical shift observed when C=O is not
coordinated to the ruthenium center (like in the case of the free ligand which displays a doublet at 175.34). The MS spectra of 2 in H₂O overtime shows a peak at m/z = 617 with a pattern fitting that of cationic species [(η⁶-p-cymene)Ru(IM-k-C,N-C₆H₄(PPh₂=N-CO-2-N-C₅H₄)]⁺. The cyclometalation processes at room temperature in DMSO of aryl groups from PPh₃ in IM coordination complexes of Pd and Pt with the Ph₂P=N-8-C₉H₆N IM ligand have been reported before. The half-life for 2 (14.5 mM) in D₂O is 2.5 days (although the process slows down for more concentrated samples). The cyclometalation process for 2 proceeds faster in a 100 mM NaCl solution in D₂O (half-life ca. 10 hours for a concentration of 2 of 14.5 mM) and by increasing the temperature (60% after 1 hour at 80°C). However, as it will be explained in section 5.3, the biological activity of compound 2 is very fast (in 8 hours it induces 80% of apoptosis on Jurkat cells) and thus we believe that the biological activity observed comes mainly from coordination compound 2 or its hydrolysis product.

5.3. Biological activity in vitro

These studies were performed by Dr. Isabel Marzo, Oscar Gonzalo and Daniel Ramírez de Mingo at the University of Zaragoza, Spain. The studies on non-carcinogenic RPTC cell lines were performed by PhD student Benelita T. Elie in our laboratory.

5.3.1. Antiproliferation studies in vitro

The antiproliferative properties of the new ruthenium complexes 1-4, 8 and 9 and of the starting material [(η⁶-p-cymene)Ru(μ-Cl)Cl]₂ were assayed by monitoring their ability to inhibit cell growth using the MTT assay (see Experimental Section). Cytotoxic activity of the compounds was determined in several human cancer cell lines: leukemia Jurkat-T, lung A549, prostate DU-145, pancreas MiaPaca2, and triple negative breast MDA-MB-231, in comparison to cisplatin. The results are summarized in Table 2. The starting material [(η⁶-p-cymene)Ru(μ-Cl)Cl]₂ is poorly cytotoxic in all tested cell lines (IC₅₀ >125 µM). The IM ligands coordinated to the ruthenium centers are known to be poorly cytotoxic (IC₅₀ in different cell lines >100-500 µM). Compounds with coordinated IM ligands 1-3 and with the IM-PPh₃ cyclometalated
ligand 8 were considerably more cytotoxic than cisplatin in all the cell lines studied. Compounds 1 and 2 (same cation) display almost identical IC_{50} values but 2 is soluble in H$_2$O. The ruthenium compound based on an iminophosphorane ligand containing a ferrocenyl phosphine Fe-Ru 4 was less cytotoxic than cisplatin. The bimetallic compounds Fe-Au and Fe-Pd also showed higher IC_{50} values when compared to trimetallic derivatives or compounds with different IM ligands. The cycloruthenated compound containing a water soluble IM ligand (IM-PTA) was more cytotoxic than cisplatin for the pancreas MiaPaca2, and triple negative breast MDA-MB-231 cell lines.

**Table 2.** IC$_{50}$ (µM) of metal complexes 1-4, 8-9, [(η$_6$-p-cymene)Ru(μ-Cl)Cl]$_2$$_a$, and cisplatin in human cell lines.$_b$ All compounds were dissolved in 1% of DMSO and diluted with water before addition to cell culture medium for a 24 h incubation period. Cisplatin was dissolved in H$_2$O.

<table>
<thead>
<tr>
<th></th>
<th>Jurkat</th>
<th>A549</th>
<th>DU-145</th>
<th>MiaPaca2</th>
<th>MDA-MB-231</th>
<th>HEK-293T</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.1 ± 0.14</td>
<td>9.9 ± 1.9</td>
<td>1.89 ± 0.64</td>
<td>2.4 ± 0.18</td>
<td>4.91 ± 2.7</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>0.78 ± 0.08</td>
<td>9.5 ± 2.1</td>
<td>1.55 ± 0.21</td>
<td>2.9 ± 0.8</td>
<td>2.61 ± 1.2</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>0.9 ± 0.32</td>
<td>43.3 ± 8.0</td>
<td>6.6 ± 0.85</td>
<td>7.0 ± 0.4</td>
<td>16.2 ± 0.9</td>
<td>2.2 ± 1.1</td>
</tr>
<tr>
<td>4</td>
<td>9.3 ± 0.07</td>
<td>&gt;125</td>
<td>148 ± 33</td>
<td>&gt;125</td>
<td>&gt;125</td>
<td>114.5 ± 14.8</td>
</tr>
<tr>
<td>8</td>
<td>2.39 ± 0.27</td>
<td>29.9 ± 5.8</td>
<td>14.2 ± 4.2</td>
<td>8.2 ± 0.98</td>
<td>7.1 ± 0.11</td>
<td>4.1 ± 0.06</td>
</tr>
<tr>
<td>9</td>
<td>17.7 ± 7.5</td>
<td>&gt;125</td>
<td>125.5 ± 28</td>
<td>54.5 ± 16</td>
<td>75.4 ± 9.8</td>
<td>141.9 ± 13.1</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>10.8 ± 1.2</td>
<td>114.2 ± 9.1</td>
<td>112.5±33</td>
<td>76.5 ± 7.4</td>
<td>131.2 ± 18</td>
<td>69.0 ± 6.7</td>
</tr>
</tbody>
</table>

$_a$ IC$_{50}$ for [(η$_6$-p-cymene)Ru(μ-Cl)Cl]$_2$ >125 µM in all cell lines. $_b$ Data are expressed as mean ± SD (n =4).

In order to assess the compounds’ selectivity for cancerous cells with respect to normal cell lines, they were also screened for their antiproliferative effects on the non-tumorigenic human embryonic kidney cells HEK293T. In most cases the cytotoxicity is comparable for the cancerous and HEK293T cells. All compounds are more toxic to leukemia than to HEK293T cell lines (2 to 8 times) and compounds 1, 2, and 9 are more toxic to the prostate DU-145 cancer cell line than to HEK cell lines. In addition, 9 is more toxic to the pancreas MiaPaca2 cancer cell line.
and to the breast cancer MDA-MB-231 cell line than to HEK293T although the IC$_{50}$ for those cell lines are higher than other compounds in the table.

Importantly as HEK cell lines are immortalized cells that can display a higher sensitivity to chemicals, we measured the effect of compound 2 on human renal proximal tubular cells (RPTC). Renal proximal tubular cells in primary culture have been described as an *in vitro* model to study nephrotoxicity. The IC$_{50}$ value (XTT assay 24 hours see Experimental Section) for 2 in this cell line was 13.84±1.46 µM (the value obtained for cisplatin as control was 46.42±2.46 µM similar to a values previously reported for 24h in rabbit RPTC). Thus, 2 is more toxic to all the cancer cell lines studied than to the “healthy” human renal cell line and markedly more toxic to the leukemia Jurkat-T (17.7-fold), prostate DU145 (9-fold), triple negative breast cancer MDA-MB-231 (5.3-fold), and pancreas MiaPaca (4.7-fold) cancer cell lines.

2 with an IC$_{50}$ of 2.64 µM is 30 times more cytotoxic in MDA-MB-231 breast cancer cell lines than compound RM175 (Figure 1) with an IC$_{50}$ of 62 µM under the same conditions (MTT, 24 h incubation). The toxicity of RM175 in HBL100 human epithelial cell lines (IC$_{50}$= 54 µM) was similar to that in breast cancer cell lines.

5.3.2. Mechanism of cell death

The mechanism of cell death induced by 2 and 3 was explored in Jurkat cells. Nuclei morphology after 24 h incubation with 1 µM solution of 2 or 3 was analysed by Hoechst staining. Typical apoptotic features, chromatin condensation and fragmentation, were detected as shown in Figure 3.

![Figure 3](image.png)

*Figure 3.* Nuclei morphology after treatment of Jurkat cells with compounds 2 and 3.
Other apoptotic parameters such as phosphatidylserine exposure and mitochondrial membrane potential dissipation were analyzed, using fluorescent probes as indicated in the Experimental Section. Dose-response experiments (Figure 4, right panel) confirmed that 2 is more cytotoxic than 3, as also indicated by MTT assays. Time-course experiments indicated that 2 induced apoptosis in around 80% of cells after 8 h treatment (Figure 4, left panel). Trypan blue staining confirmed that cell death was through apoptosis, with very low secondary necrotic cells at 6 h (data not shown). Phosphatidylserine exposure and loss of transmembrane mitochondrial potential occurred in the same percentage of cells, although at longer incubation periods, there was an apparent decrease in the percentage of AnnexinV positive cells that was in fact due to cell disintegration. Apoptosis induction was slower for 3 with the percentage of both AnnexinV$^+$ and $\Delta \Psi_m$ low cells gradually increasing during the 24 h period of the experiments (Figure 4, left panel).

**Figure 4.** Dose-response quantification of PS exposure (A) and time-course analysis of PS exposure and $\Delta \Psi_m$ loss (B) caused by 2 and 3 in Jurkat cells.

Proteins of the Bcl-2 family are key regulators of apoptosis and the levels of some members are modified in early phases of the process. Thus, we analyzed the effect of 2 in the levels of two antiapoptotic proteins of the Bcl-2 family (Bcl-XL and Mcl-1) and three proapoptotic members (Bim, Puma and Noxa) in Jurkat cells (Appendix Figure 43). Jurkat cells express very low levels of the other antiapoptotic member (Bcl-2). The main changes observed after treatment with 2 were a high increase in the levels of the propapoptotic proteins Noxa,
Puma (isoform b) and Bim (isoform beta). Also a slight decrease in the levels of the antiapoptotic members Bcl-XL and Mcl-1 was observed in cells treated with 2. The proapoptotic proteins are damage sensors that induce the activation of Bax and Bak and mitochondrial permeabilization to release cytochrome c, the key event in the intrinsic pathway of apoptosis.61

Taken together these results point to a classical apoptosis mechanism of cell death. This was confirmed by the finding that the cytotoxicity of both compounds was caspase-dependent (Figure 5). The general caspase inhibitor z-VAD-fmk completely abrogated PS exposure but it only partially reduced ΔΨₘ loss (from 80% to 30% for 2 and from 40% to 20% for 3).

Figure 5. Effect of the general caspase inhibitor z-VAD-fmk in apoptotic features induced by 2 and 3.

ΔΨₘ disruption was not completely inhibited by z-VAD-fmk we hypothesize that 2 and 3 could activate caspase-independent pathways acting on mitochondria. In order to determine whether caspase inhibition prevented or just delayed cell death, we performed experiments in which cells were treated with 2 or 3 for 24 h in the presence of z-VAD-fmk and then washed and further cultured in fresh medium for 24 h. PS exposure was analysed after the first 24 h in the presence of compound+z-VAD-fmk and 24 h after washing. Only around 20% of cells were AnnexinV⁺ after 24 h incubation with 2+z-VAD-fmk and 24 h in fresh medium, while 70% of
cells treated with 2 alone were apoptotic at the end of the experiments. Thus, these results indicate that in the case of compound 2, caspase inhibition prevented death commitment in a very high percentage of cells since only 22% were Annexin V+ 24 hours after washing. However, in the case of 3, the percentage of AnnexinV+ cells 24 h after washing (32%) equalled that of cell treated with 3 alone for 24 h (28%), suggesting that in this case caspase inhibition did not prevent irreversible cell damage leading to cell death. These results suggest that 2 and 3 could be acting through different mechanisms, with 3 causing caspase-independent premitochondrial damage. These differences in the mechanism of 2 and 3 could explain why 3 is less selective than 2 for tumor cell lines (Table 2).

Figure 6. Analysis of long-term protection by z-VAD-fmk.

Ruthenium compounds have been reported to induce p53-dependent and –independent cytotoxicity.$^{12, 23}$ In order to determine whether the cytotoxicity of the compounds here is p53-dependent we analyzed the levels of p53 in A549 cells, bearing wt-p53, after a short term treatment with 2. As shown in Figure 7, treatment with 2 did not induce the stabilization of p53, and even at 3 and 6 h we observed a slight decline in p53 levels, probably due to cell death and protein loss. These results differ from that reported with other ruthenium organometallic
compounds that induce short-term p53 accumulation.\textsuperscript{12,23} However, although p53 protein is induced by RM175 in HCT116 (colon carcinoma)\textsuperscript{12} or RDC-9 in A172 (glioblastoma) and HCT116,\textsuperscript{23} genetic inhibition of p53 does not avoid the cytotoxicity of these compounds, clearly indicating that other p53-independent mechanisms can be activated by ruthenium compounds. Moreover, Gaiddon \textit{et al.} have shown that a p53\textsuperscript{-/-} cell line (TK6) exhibits the same sensitivity to RDC-9 than its p53\textsuperscript{+/+} parental cell line (NH32).\textsuperscript{23} Furthermore, compounds 1-3 showed high toxicity against p53 mutated cell lines (Jurkat, MiaPaca2, DU-145 and MDA-MB-231) as shown in Table 2. Since the activity of cisplatin has been reported to be p53-dependent, new organometallic compounds that activate p53-independent pathways could be useful in the treatment of tumors with alterations in p53, the most frequently mutated gene in human cancer.

\textbf{Figure 7.} p53 protein levels after short-term incubation of A549 cells with 2. B-Actin levels were determined in the same membranes as a total protein loading control.
5.4. Reactivity with biomolecules

5.4.1. Interaction with DNA

As described in chapters III and IV, since DNA replication is a key event for cell division, it is among critically important targets in cancer chemotherapy. Most cytotoxic platinum drugs form strong covalent bonds with DNA bases.\textsuperscript{62} However, a variety of platinum compounds act as DNA intercalators upon coordination to the appropriate ancillary ligands.\textsuperscript{63} The more thoroughly studied ruthenium antitumor agents (Figure 1) have displayed differences with respect to their interactions with DNA depending on their structure.\textsuperscript{4} Thus, while NAMI-A is known to have fewer and weaker interactions with DNA than cisplatin,\textsuperscript{4} indazolium bisindazoletetrachlororuthenate (KP1019) undergoes interactions similar to cisplatin but with a lower intensity in terms of DNA-DNA and DNA-protein crosslinks.\textsuperscript{64} Organometallic piano-stool ruthenium(II) compounds based on biphenyl rings RM175 interact strongly with DNA binding to guanines and by intercalation.\textsuperscript{65,66} Organometallic ruthenium(II) RAPTA derivatives, characterized by the presence of water-soluble PTA phosphine, exhibit pH-dependent DNA damage: at the pH typical of hypoxic tumor cells DNA was damaged, whereas at the pH characteristic of healthy cells little or no damage was detected.\textsuperscript{67,68} Cycloruthenated compounds based on pincer C,N ligands (RDC family) displayed a much weaker interaction with plasmid (pBR322) DNA when compared to cisplatin.\textsuperscript{69,70} Complexes of the type [Ru(Cp)(2,2-bipy)(PR\textsubscript{3})][CF\textsubscript{3}SO\textsubscript{3}] have shown no observable interaction with DNA.\textsuperscript{71}

In this context, we evaluated the effect of DNA interactions that could, to some extent, contribute to the observed cytotoxicity of compounds 1-4, 8 and 9, and the apoptotic behaviour of compounds 2 and 3. We followed the interaction with Calf Thymus DNA (CT DNA) by circular dichroism (CD), and with plasmid (pBR322) DNA by electrophoresis in agarose gel. The CD spectral technique is very sensitive to diagnose alterations on the secondary structure of DNA that result from DNA-drug interactions. A typical CD spectrum of CT DNA shows a positive band with a maximum at 275 nm due to base stacking, and a negative band with a minimum at 248 nm due to helicity, characteristic of the B conformation.\textsuperscript{72} Therefore, changes in the CD signals can be assigned to corresponding changes in DNA secondary structure. In addition, it is known that simple groove binding or electrostatic interaction of small molecules
cause little or no alteration in any of the CD bands when compared to major perturbations induced by covalent binding or intercalation.

CD spectra of CT DNA incubated with compounds 1-4 (see Appendix, Figure 44) at 37°C and pH = 7.30 in Tris/HCl buffer up to molar ratio drug/DNA = 0.5 show no modification of the DNA bands with respect to untreated CT DNA, indicating that drug-DNA interactions, if existing, do not induce any observable perturbation on the DNA secondary structure under our experimental conditions.

Higher ratios were also tested, although loss of CD signal was observed due to precipitation of the DNA induced by 1-4, most likely because of phosphate charge neutralization by the cationic compounds, which suggests the existence of an electrostatic attraction. DNA condensation or precipitation by neutralization of backbone charges has been previously described for other ionic ruthenium drugs and confirmed by us for compounds 2 and 3 through ICP-MS analysis of metal content in the DNA precipitate. In this experiment 500 μM concentration DNA solutions were treated with 2 equivalents of ruthenium compounds 2 and 3 for 20 hours at 37°C to promote DNA precipitation. The samples were then centrifuged and the resulting pellets were analyzed for DNA and metal concentration (see Experimental Section for more details). Our results show ruthenium content values of 2.60 ± 0.26 mg Ru/mg DNA for compound 2 and 2.43 ± 0.18 mg Ru/mg DNA for compound 3. This high Ru content in DNA precipitate, especially when compared to similar Ru compounds interacting with DNA through covalent interactions suggests that the key factor promoting the precipitation of DNA is the presence of the cationic ruthenium compound.

Attempts to obtain additional evidence of drug-CT DNA interactions were made by performing thermal denaturation experiments, but resulted in cyclometallation of compounds 1, 2 and hydrolysis of 3 at temperatures above 60°C, as previously discussed, preventing us from obtaining reliable information through this technique.

In order to gain further insights on the nature of the compound-DNA interactions, gel electrophoresis studies were also performed with the ruthenium(II) complexes 1-4, 8 and 9 on plasmid (pBR322) DNA (Figure 8).
Figure 8. Electrophoresis mobility shift assays for cisplatin, \([\text{Ru}(\eta^6-p\text{-cymene})\text{Cl}]_2\) and compounds 1-4 and 8, 9. DNA refers to untreated plasmid pBR322. A, B, C and D correspond to metal/DNAbp ratios of 0.25, 0.5, 1.0 and 2.0 respectively.

For these experiments, cisplatin, all uncoordinated ligands and the starting dimeric organometallic ruthenium(II) complex \([\text{Ru}(\eta^6-p\text{-cymene})\text{Cl}]_2\) were also measured as controls. Plasmid pBR322 presents two main forms, OC (open circular or relaxed) and CCC (covalently closed circular or supercoiled), which display different electrophoretic mobility. Changes in the electrophoretic mobility of any of the forms upon incubation of the plasmid with a compound are usually interpreted as evidence of interaction. Generally, a drug that induces unwinding of the CCC form will produce a retardation of the electrophoretic mobility, while coiling of the OC form will result in increased mobility. Figure 8 shows the effect of cisplatin and compounds 1-4, 8 and 9 on DNA pBR322 after incubation at 37°C for 20 h in Tris/HCl buffer up to drug/DNA ratio 2.0. As previously reported, cisplatin is able to both increase and decrease the mobility of the OC and the CCC forms, respectively. Interestingly, treatment with increasing amounts of 1
and 2 induce retardation of the mobility of the CCC form of plasmid DNA, while the rest of the compounds, the neutral ligands, and the Ru starting dimer do not seem to induce any alteration on the mobility of the plasmid.

The results of CD, ICP-MS and gel electrophoresis taken together suggest that compounds 1-4 undergo only electrostatic interactions with DNA. This conclusion is supported by three main facts: 1) results obtained by CD spectroscopy do not show evidence of CT DNA modifications of secondary structure, suggesting that drug-DNA interactions, if any, are of weak nature, but neither covalent nor intercalation; 2) precipitation of CT DNA is observed in CD experiments at high ratios drug/DNA, and it is further confirmed by ICP-MS analysis of metal content in DNA precipitates, suggesting backbone charge neutralization, and 3) retardation of the plasmid DNA electrophoretic mobility is observed also at high drug/DNA ratios for compounds 1 and 2, but only when plasmid DNA is incubated with the cationic metal compounds and not with the neutral ligands or neutral ruthenium starting material under the same conditions, which could also be consistent with charge neutralization or DNA precipitation. Loss of migration in electrophoresis experiments has been previously reported as a consequence of DNA precipitation for other cationic ruthenium compounds. The electrophoretic mobility results also suggest that the electrostatic interaction between DNA and compounds 1 and 2 is of larger magnitude than that experienced by 3 and 4, since no mobility retardation is observed for the latter compounds up to drug/DNA ratio of 2.0. Further evidence of this could be found in the fact that lower amount of Ru content in DNA is detected for compound 3 when compared to compound 2, according to ICP-MS results.

Thus, we hypothesize that the anti-tumor properties observed for compounds 1-4, 8 and 9 are due to non-DNA related mechanisms/factors, as previously observed for other iminophosphorane complexes described before and some other ruthenium compounds. and refs therein
5.4.2. Lack of inhibition of capthesin B

This assay was performed at Reaction Biology Corporation.

Cathepsin B (cat B) is an abundant and ubiquitously expressed cysteine peptidase of the papain family, which has turned out to be a prognostic marker for several types of cancers.\textsuperscript{77} Cathepsin B seems to be involved (along with other cathepsins) in metastasis, angiogenesis, and tumor progression.\textsuperscript{78} It has been proposed that cat B may be a possible therapeutic target for the control of tumor progression.\textsuperscript{79} RAPTA Ru compounds which inhibit cat B with IC\textsubscript{50} in the low micromolar range can reduce the mass and number of metastases \textit{in vivo}.\textsuperscript{80} We therefore, studied the inhibition of Cat B by compound 2 (see Experimental Section for details). Compound 2 does not inhibit the enzymatic activity of cat B at concentrations up to 100 µM indicating that this protease may not be a target for this type of arene-ruthenium(II) derivatives.

5.4.3. Interaction with HSA

Due to the abundance in plasma and relevance to drug binding\textsuperscript{81} of human serum albumin (HSA) we studied the interactions of the new ruthenium(II) IM complexes with this protein. The fluorescence spectra of HSA in the presence of increasing amounts of the compounds 1-5, 8, 9 and cisplatin were recorded in the 300-450 nm range upon excitation of the tryptophan residue at 295 nm. The compounds caused a concentration dependent quenching of fluorescence without changing the emission maximum or the shape of the peak. All these data indicate an interaction of the compounds with HSA. The fluorescence data was analyzed by the Stern-Volmer equation. While a linear Stern-Volmer plot is indicative of a single quenching mechanism, either dynamic or static, the positive deviation observed in the plots of F\textsubscript{0}/F versus [Q] of compounds 1-4 (Figure 9) suggests the presence of different binding sites in the protein with different binding affinities.\textsuperscript{82} Similar behavior was observed in the case of coordination iminophosphorane complexes of d\textsuperscript{8} metals for which concentration dependent fluorescence quenching was reported.\textsuperscript{52-55} On the other hand, the Stern-Volmer plot for complexes 8 and 9 shows a linear relationship, suggesting the existence of a single quenching mechanism, most likely dynamic, and a single binding affinity. The Stern-Volmer constants for complexes 8 and 9 are 1.81x10\textsuperscript{4} and 3.85x10\textsuperscript{4} M\textsuperscript{-1}, respectively.
Figure 9. (A) Fluorescence titration curve of HSA with compound 4. Arrow indicates the increase of quencher concentration. (B) Stern-Volmer plot for HSA fluorescence quenching observed with compounds 1-4, 8, 9 and cisplatin.

In general, higher quenching by the iminophosphorane complexes was observed compared to that of cisplatin under the chosen conditions, most likely due to the faster binding of our compounds with HSA, as compared to cisplatin.
5.5. Effects on tumor growth in vivo with compound 2

These studies were designed and performed by Benelita T. Elie, and Dr. Maria Contel, at the laboratory of Dr. Joe W. Ramos (University of Hawaii Cancer Center, Honolulu).

5.5.1. Evaluation of the lethal and maximum tolerated doses

The lethal and maximum tolerated doses of compound 2 were evaluated in C57/Black6 mice (see Experimental Section for details). The lethal dose was determined to be 30 mg/kg/day. No biological samples were collected from those mice. The MTD was determined to be 10 mg/kg/day, at which the mice showed no visible signs of distress over the 7 days course of treatment.

Mice lost weight during the trial in a dose dependent manner where mice treated with 5 mg/kg/day, 10 mg/kg/day, or 20 mg/kg/day lost 15%, 19% or 37% body weight respectively, while vehicle treated mice gained 3% body weight over the 7 days of treatment. Mice treated with 20 mg/kg/day were euthanized on day 6 of the trial as they had lost too much body weight and looked in distress.

24 hours after the last dose all the mice used in the MTD study were euthanized and blood plasma, liver, spleen and kidneys were collected and used for histological analysis. Necropsy and histology indicate that mice treated at 20 mg/kg/day had discolored livers and atrophied spleens; at 10 mg/kg/day much less atrophy and minor discoloration was observed, while in mice treated at 5 mg/kg/day there was no detectable liver discoloration and no observable change in spleen size.

Therefore, 5 mg/kg/every other day were chosen as the dose of to conduct the subsequent in vivo trial with compound 2.

5.5.2. Effects of 2 in MDA-MB-231 mouse xenografts

12 female NOD.CB17-Prkdc SCID/J (non-obese diabetic–severe combined immunodeficiency) were selected for the in vivo trial. The mice were inoculated with MDA-MB-231 cells (see Experimental Section for details) and treated when the tumors were palpable
(about 5–6 mm diameter). Each six MDA-MB-231-transplanted animals received compound 2 (5 mg/kg/every other day) or vehicle (0.9% NaCl) intraperitoneally (i.p.). To palliate the weight loss observed in the MTD study all the mice used in this trial were fed a 46% fat-adjusted diet (Harlan Teklad, Madison, WI), plus HydroGel™(Harlan Teklad, Madison, WI) and received subcutaneous injection of 100µl Normal Saline on the off-treatment day.

In the group treated with 2 (see Table 3 and Figure 10) we observed a significant decrease in tumor size (shrinkage) of 56% from the starting volume between day 1 and day 28 of treatment (after a total of 14 doses), while in the control vehicle treated group we observed 200% increase in tumor volume between day 1 and day 28 of treatment. One 2-treated mouse was removed from the trial because it was not feeding itself and showed other signs of distress, none of which were observed in the other mice on trial. No significant weight loss in mice treated with 5 mg/kg/every other day was observed. Mice treated with 2 gained an average weight of 2.88%, while untreated mice gained an average of 18.67% weight (all groups were fed a 46% fat-adjusted diet).

![Figure 10. % of reduction of tumor burden in a cohort of 12 female NOD.CB17-Prkdc scid/J mice inoculated subcutaneously with 5x10⁶ MDA-MB-231 cells. The treatment started when tumors were palpable (5-6 mm diameter). 6 mice were treated with compound 2 (pink bars), 6 were treated with the vehicle 100 µl Normal Saline (0.9% NaCl) (black bars). 2 was administered in the amount of 5 mg/kg/every other day.](image)
Table 3. Effects of 2 on the tumor growth of MDA-MB-231 mammary carcinoma in NOD.CB17-Prkdc scid/J mice.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Primary Tumor (mm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>473.47 ± 45.44</td>
</tr>
<tr>
<td>Compound 2 (5 mg/kg/e.o.d$^a$ x 14)</td>
<td>59.58 ± 8.66</td>
</tr>
</tbody>
</table>

$^a$e.o.d = every other day. Tumor measured on day 28, after the 14th dose.

The results clearly indicate that compound 2 is extremely efficient in vivo since it not only inhibits tumor growth but also results in the decrease in the size of the tumors by 56%. It is interesting to compare these results with those obtained with other arene-ruthenium (II) derivatives. Compound RM175 was reported to have a final primary tumor growth inhibition of 30% at a dose of 7.5 mg/kg/day in an in vivo trial for breast cancer in mice$^{13}$ while the compounds RAPTA-C reduced the growth of lung metastases in CBA mice bearing the MCa mammary carcinoma in the absence of a corresponding action at the site of primary tumor growth.$^{15,16}$ More recently, a ruthenium-arene complex with a perfluoroalkyl-containing amine ligand demonstrated a 90% reduction in the tumor growth in a xenografted ovarian carcinoma tumor (A2780) grown in a chorioallantoic membrane (CAM) assay of chicken embryo.$^{18}$ As stated above compound 2 is able to decrease tumor size.

5.5.3. Pharmacokinetic study

These studies were performed by Dr. Swayam Prabha and Tanmoy Sadhukha at the University of Minnesota.

The pharmacokinetic profile of compound 2 in the NOD.CB17-Prkdc scid/J mice used for the in vivo study described above (Figure 11) is summarized in Table 4. Ruthenium content was determined using Inductively Coupled Plasma - Mass Spectrometry (ICP-MS). Compound 2 was absorbed quickly into plasma ($t_{1/2\,abs} = 0.5$ h) and the peak plasma concentration was reached.
within 2 h of dosing. The drug was eliminated slowly from the blood compartment with an elimination half-life ($t_{1/2\text{e}}$) greater than 12 h. This elimination half-life is similar to that reported for compounds RAPTA-C,$^{13}$ NAMI-A$^{83}$ and KP1019.$^{7}$

During determination of total area under the concentration – time curve (AUC total) only 7% of the AUC was extrapolated from the last time point suggesting a high confidence in the AUC, $V_{app}$ and apparent clearance ($CL_{app}$) determination. Blood concentration, at 6 hours after the last dose of compound 2, was $4.2 \pm 1.3 \mu g/ml$, which is higher ($P < 0.1$) than the $C_{max}$ after the first dose. This suggests an accumulation of compound 2 after each dose. While it is hard to make comparisons with other ruthenium compounds for which a PK analysis has been performed (e.g. RAPTA-C,$^{15}$ NAMI-A,$^{83}$ and KP1019$^{7}$) due to the different structures, oxidation states (see Figure 1) and the amounts employed in these studies (for example 70 mg total of 2 in 14 doses of 5 mg/kg/every other day versus a total of 400 mg or 200 mg for RAPTA-C) there are some differences that can be pointed out. The $V_d$ (volume of distribution) of 2 when compared to that of more structurally related arene-ruthenium(II) RAPTA-C derivative is smaller which may be due to a higher water solubility of 2 or the possibility that it binds strongly to plasma-proteins. Indeed, we have seen in a qualitative way that compound 2 binds faster to HSA than cisplatin (section 5.4.3).

**Table 4.** Pharmacokinetic parameters of compound 2 after first injection in NOD.CB17-Prkdc SCID/J mice.

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{abs}$</td>
<td>$1.39 \text{ h}^{-1}$</td>
</tr>
<tr>
<td>$K_e$</td>
<td>$0.055 \text{ h}^{-1}$</td>
</tr>
<tr>
<td>$t_{1/2\text{e}}$</td>
<td>12.67 h</td>
</tr>
<tr>
<td>$t_{1/2\text{abs}}$</td>
<td>0.50 h</td>
</tr>
<tr>
<td>$t_{\text{max}}$</td>
<td>2.00 h</td>
</tr>
<tr>
<td>$C_{max}$</td>
<td>2.62 $\mu g/ml$</td>
</tr>
<tr>
<td>$AUC_{\text{total}}$</td>
<td>54.47 $\mu g.h/ml$</td>
</tr>
<tr>
<td>$V_{app}$</td>
<td>33.57 ml</td>
</tr>
<tr>
<td>$CL_{app}$</td>
<td>1.84 ml/h</td>
</tr>
</tbody>
</table>
Figure 11. Concentration of Compound 2 in plasma at various intervals after the first dose.

At the end of the study, ruthenium content in liver, kidney and tumor was determined (Figure 12). The level of compound 2 is liver and kidney was less than 5 µg/g tissue weight while the tumor concentration was about 40 µg/g. The high level in tumor suggests enhanced tumor accumulation of compound 2 which may explain the high efficacy observed for this compound in the in vivo studies. The preferential accumulation of some ruthenium complexes in neoplastic masses in comparison with normal tissue has been reported before by different researchers.84,85

Figure 12. Compound 2 ruthenium content in tissues at the end of efficacy study. Data represents mean ± SD. N = 3; * indicates P <0.005.
5.6. Conclusions

I have successfully synthesized a series of organometallic ruthenium(II) complexes containing iminophosphorane ligands. The compounds, along with starting material \( [(\eta^6-p\text{-cymene})\text{Ru}(\mu\text{-Cl})\text{Cl}]_2 \), were evaluated against a number of human cancer cell lines \textit{in vitro} and most compounds were more cytotoxic to all the cell lines than cisplatin. Only the cationic compounds (1 and 2) show interaction with plasmid \((pBR322)\) DNA but it seems weak and electrostatic in nature. Precipitation of \textit{Calf Thymus} DNA observed in CD experiments at high ratios drug/DNA, and further confirmed by ICP-MS analysis of metal content in DNA precipitates, suggesting backbone charge neutralization. Furthermore, higher quenching of HSA was observed compared to that of cisplatin under the chosen conditions, most likely due to the faster reactivity of the complexes with HSA, as compared to cisplatin.

From all the iminophosphorane compounds that I prepared, highly water-soluble ruthenium-arene-iminophosphorane compound (2) was chosen as the best candidate to continue with advanced pre-clinical studies. This compound is active against a number of cisplatin resistant cell lines while been less toxic on human renal proximal tubular cell lines. Initial mechanistic studies indicated that the cell death type for compound 2 is mainly through canonical or caspase-dependent apoptosis. In addition, cell death seems to be independent of p53. The interaction of 2 with DNA is weak and electrostatic in nature. All these facts indicate that 2 has a mode of action different from that of cisplatin which may explain its activity in cancer cell lines quite resistant to cisplatin such as MDA-MB-231, A549 and DU-145. Compound 2 does not inhibit protease cathepsin B in concentrations of 100 \( \mu \text{M} \) or lower. The efficacy of 2 \textit{in vivo} has been demonstrated on xenografted breast carcinoma MDA-MB-231-tumors grown on NOD.CB17-Prkdc scid/J mice. An impressive tumor reduction (shrinkage) of 56\% was observed after 28 days treatment (14 doses of 5 mg/kg every other day) with low systemic toxicity. Pharmacokinetic studies showed a quick absorption of 2 in plasma with an elimination half-life of 12.67 hours (similar to that reported for other ruthenium derivatives). Importantly, 2 accumulated preferentially in the breast tumor tissues when compared to kidney and liver, which may explain its high efficacy \textit{in vivo}. The simple, cheap and accessible synthesis of compound 2 (by simple coordination of an IM ligand to a well-known organometallic ruthenium (II) precursor), its high water-solubility and its encouraging preliminary biological
activity *in vitro* and *in vivo* makes it therefore a good candidate for further evaluation as a potential chemotherapeutic agent.

5.7. Experimental section

**Materials and methods**

All manipulations involving air-free syntheses were performed using standard Schlenk-line techniques under a nitrogen atmosphere or a glove-box MBraun MOD System. Solvents were purified by use of a PureSolv purification unit from Innovative Technology, Inc. Compounds \([\text{PhCH}_2\text{Mn(CO)}_3]_{\text{86}}\) \((\eta^6-\text{p-cymene})\text{Ru(μ-Cl)Cl}]_{\text{2.57}}\) \([\text{Hg(2-C}_6\text{H}_4\text{C(O)N=PPh}_3]\text{Cl}]_{\text{58}}\) and IM ligands \(\text{Ph}_3\text{P=N-CO-2-N-C}_5\text{H}_4\), \(\text{Ph}_3\text{P=N-8-C}_9\text{H}_6\text{N}\), \([\text{Cp-P(Ph}_2]=\text{N-CH}_2-2-\text{NC}_5\text{H}_4}\text{Fe(Cp)}]_{\text{54}}\) and \(\text{Ph}_3\text{P=N-CO-2-C}_6\text{H}_4\) \(\text{56}\) were prepared by reported methods. The purity of the compounds, based on elemental analysis, is ≥99.5%. In the case that the compound crystallizes with solvent the \(^1\text{H}\text{ NMR (CDCl}_3\) spectrum is available in the Appendix. Elemental analyses were performed by Atlantic Microlab Inc. NMR spectra were recorded in a Bruker AV400 (**1**H NMR at 400MHz, **13**C NMR at 100.6 MHz, **31**P NMR at 161.9 MHz). Chemical shifts (δ) are given in ppm using CDCl\(_3\) or DMSO-\(d_6\) as solvent, unless otherwise stated. \(^1\text{H}\) and \(^{13}\text{C}\) chemical shifts were measured relative to solvent peaks considering TMS = 0 ppm; \(^{31}\text{P}\{^1\text{H}\}\) was externally referenced to H\(_3\)PO\(_4\) (85%). Infrared spectra (4000-250 cm\(^{-1}\)) were recorded on a Nicolet 6700 FT-IR spectrophotometer from nujol mulls between polyethylene sheets. Mass spectra (electrospray ionization, ESI) were performed on an Agilent Analyzer, a Bruker Analyzer, or a Waters Q-Tof Ultima analyzer. Conductivity was measured in an OAKTON pH/conductivity meter in CH\(_3\)CN solutions (10\(^{-3}\)M). X-ray collection was performed at room temperature on a Kappa CCD diffractometer using graphite monochromated Mo-K\(\alpha\) radiation (λ=0.71073 Å). Electrophoresis experiments were carried out in a Bio-Rad Mini sub-cell GT horizontal electrophoresis system connected to a Bio-Rad Power Pac 300 power supply. Photographs of the gels were taken with an Alpha Innotech FluorChem 8900 camera. Fluorescence intensity measurements were carried out on a PTI QM-4/206 SE Spectrofluorometer (PTI, Birmingham, NJ) with right angle detection of fluorescence using a 1 cm path length quartz cuvette. Circular dichroism spectra were

159
recorded using a Chirascan CD Spectrometer equipped with a thermostated cuvette holder. The inhibition of Capthesin B experiments were performed by Reaction Biology Corporation.

**Synthesis of 1**

\[
[(\eta^6-p\text{-cymene})\text{Ru}((\text{Ph}_3\text{P}=\text{N}-\text{CO}-2\text{-N}-\text{C}_5\text{H}_4)-\kappa\text{-N,O})\text{Cl}](\text{PF}_6)
\]

\([(\eta^6-p\text{-cymene})\text{Ru}(\mu\text{-Cl})\text{Cl}]_2\) (0.15 g, 0.25 mmol) was dissolved in CH\(_3\)OH (15 mL) and Ph\(_3\)P=N(CO)(C\(_5\)H\(_5\)-2-N) (0.19 g, 0.5 mmol) was added. To the resulting mixture KPF\(_6\) (0.10 g, 0.55 mmol) was added. The mixture was stirred for 4 h. The suspension was then filtered and washed 3 times with Et\(_2\)O (10 mL). The solution was then concentrated and the precipitate was collected by filtration and dried *in vacuo*.

Yield: 0.36 g (90%). Anal. Calc. for C\(_{34}\)H\(_{33}\)N\(_2\)OP\(_2\)F\(_6\)ClRu (798.07): C, 51.17; H, 4.17; N, 3.51. Found: C, 50.95; H, 4.20; N 3.56 %. ESI-MS: m/z: 653.10 (100%, [M-PF\(_6\)]\(^+\), calc. 653.11). \(^{31}\)P {\(^1\)H} NMR (CDCl\(_3\)) \(\delta\) 25.72 (s), -148.26 to -140.19 (septet, PF\(_6\)), (DMSO-\(d_6\)): 24.27 (s), -152.87 to -130.98 (septet, PF\(_6\)) NMR (CDCl\(_3\)): \(\delta\) 1.10 (6H, dd, \(J = 6.9, 18.9\) Hz, CH\(_3\), \(\eta^6\)-p-cymene), 1.69 (3H, s, CH\(_3\), \(\eta^6\)-p-cymene), 1.65 (3H, s, CH\(_3\), \(\eta^6\)-p-cymene), 2.30 (1H, m, CH, \(\eta^6\)-p-cymene), 5.48-5.59 (4H, m, CH, \(\eta^6\)-p-cymene), 7.64-7.78 (16H, m, H\(_5\), H\(_m\) + H\(_o\) + H\(_p\)), 8.03 (1H, d, \(J = 7.7\) Hz, H\(_4\), C\(_5\)H\(_4\)N), 8.35 (1H, d, \(J = 7.5\) Hz, H\(_3\), C\(_5\)H\(_4\)N), 9.21 (1H, d, \(J = 5.3\) Hz, H\(_6\), C\(_5\)H\(_4\)N); \(^{13}\)C {\(^1\)H} (CDCl\(_3\)): \(\delta\) 18.10 (s, CH\(_3\), \(\eta^6\)-p-cymene), 21.87 (s, CH\(_3\), \(\eta^6\)-p-cymene), 22.18 (s, CH\(_3\), \(\eta^6\)-p-cymene), 30.79 (s, CH, \(\eta^6\)-p-cymene), 81.00 (s, CH, \(\eta^6\)-p-cymene), 82.09 (d, CH, \(J = 8.3\) Hz, \(\eta^6\)-p-cymene), 82.41 (s, CH, \(\eta^6\)-p-cymene), 83.92 (s, C, \(\eta^6\)-p-cymene), 124.5 (s, C\(_2\), \(\eta^6\)-p-cymene), 125.5 (d, C\(_{ipso}\), \(J = 100.4\) Hz), 127.7 (s, C\(_3\), C\(_5\)H\(_4\)N), 129.5 (d, C\(_m\), \(J = 13.0\) Hz), 129.8 (s, C\(_5\), C\(_5\)H\(_4\)N), 133.2 (d, C\(_o\), \(J = 10.4\) Hz), 133.8 (s, C\(_p\)), 139.3 (s, C\(_4\), C\(_5\)H\(_4\)N), 153.7 (s, C\(_o\)) ppm. The signal due to C\(_2\) (C\(_5\)H\(_4\)N) and C=O was not observed. IR (cm\(^{-1}\)): \(\nu\) 524 (Ru-N), 834 (v br, PF\(_6\)), 1116 (N=P), 1540 (C=O). Conductivity (acetone): 125.5 \(\mu\)S/cm (1:1 electrolyte).
Synthesis of 2

\[ (\eta^6-p\text{-cymene})\text{Ru}\{(\text{Ph}_3\text{P}=\text{N}-\text{CO}-2\text{-N-C}_8\text{H}_4)\text{-}\kappa\text{-N},\text{O}\}\text{Cl}]\text{Cl} \]

\[ (\eta^6-p\text{-cymene})\text{Ru}(\mu\text{-Cl})\text{Cl}]_2 \text{ (0.15 g, 0.25 mmol) and Ph}_3\text{P}=\text{N( CO)}(\text{C}_8\text{H}_5-2\text{-N}) \text{ (0.19 g, 0.5 mmol) were stirred in acetone (20 mL) for 3 h. The brown solution was concentrated and 30 mL of Et}_2\text{O added dropwise. The orange solid that formed was collected by filtration and dried in vacuo.} \]

Yield: 0.33 g (94%). Anal. Calc. for C\textsubscript{34}H\textsubscript{53}N\textsubscript{2}OPCl\textsubscript{2}Ru·2H\textsubscript{2}O (724.62): C, 56.36; H, 5.15; N, 3.87. Found: C, 56.41; H, 4.99; N 3.87 %. ESI-MS: m/z: 653.1 (100%, [M-Cl]\textsuperscript{+}, calc. 653.01), 618.1 (100%, [M-2Cl]\textsuperscript{2+}, calc. 618.1), 519.0 (100%, [M-p\text{-cymene}-Cl]\textsuperscript{+}, calc. 519.0). \textsuperscript{31}P \{\textsuperscript{1}H\} NMR (CDCl\textsubscript{3}): \delta 25.18 (s), (DMSO-d\textsubscript{6}): 28.99 (s); \textsuperscript{1}H NMR (CDCl\textsubscript{3}): \delta 1.00 (6H, dd, J = 7.0, 24.8, Hz CH\textsubscript{3}, \eta^6-p\text{-cymene}), 2.01 (3H, s, CH\textsubscript{3}, \eta^6-p\text{-cymene}), 2.25 (1H, m, CH, \eta^6-p\text{-cymene}), 5.51-5.71 (4H, m, CH, \eta^6-p\text{-cymene}), 7.57 (6H, m, H\textsubscript{meta}), 7.68 (9H, m, H\textsubscript{ortho} + H\textsubscript{para}), 7.87 (1H, d, J = 6.0 Hz, H\textsubscript{5}, C\textsubscript{3}H\textsubscript{4}N), 8.03 (1H, d, J = 7.7 Hz, H\textsubscript{H}, C\textsubscript{3}H\textsubscript{4}N), 8.32 (1H, d, J = 7.4 Hz, H\textsubscript{3}, C\textsubscript{3}H\textsubscript{4}N), 9.62 (1H, d, J = 5.0 Hz, H\textsubscript{6}, C\textsubscript{3}H\textsubscript{4}N); \textsuperscript{13}C \{\textsuperscript{1}H\} (CDCl\textsubscript{3}): \delta 18.47 (s, CH\textsubscript{3}, \eta^6-p\text{-cymene}), 21.82 (s, CH\textsubscript{3}, \eta^6-p\text{-cymene}), 22.39 (s, CH\textsubscript{3}, \eta^6-p\text{-cymene}), 30.75 (s, CH, \eta^6-p\text{-cymene}), 81.20 (s, CH, \eta^6-p\text{-cymene}), 82.60 (d, CH, J = 8.3 Hz, \eta^6-p\text{-cymene}), 83.65 (s, CH, \eta^6-p\text{-cymene}), 97.87 (s, C, \eta^6-p\text{-cymene}), 102.5 (s, C, \eta^6-p\text{-cymene}), 125.6 (d, C\textsubscript{ips}, J = 101.2 Hz), 127.2 (s, C\textsubscript{i}, C\textsubscript{3}H\textsubscript{4}N), 129.5 (d, C\textsubscript{m}, J = 12.8 Hz), 130.3 (s, C\textsubscript{5}, C\textsubscript{3}H\textsubscript{4}N), 133.3 (d, C\textsubscript{o}, J = 10.2 Hz), 133.9 (s, C\textsubscript{p}), 139.0 (s, C\textsubscript{4}, C\textsubscript{3}H\textsubscript{4}N), 151.6 (d, C\textsubscript{2}, J = 24.0 Hz), 156.3 (s, C\textsubscript{o}), 176.8 (d, C=O, J = 24.14 Hz) ppm. IR (cm\textsuperscript{-1}): \nu 527 (Ru-N), 1114 (N=O), 1535 (C=O). Conductivity (acetone): 124.20 \mu S/cm (1:1 electrolyte). Solubility: 145.3 mM or 100 mg/mL (H\textsubscript{2}O), pH (5 x 10\textsuperscript{-5}M in H\textsubscript{2}O): 5.76.

Synthesis of 3

\[ (\eta^6-p\text{-cymene})\text{Ru}\{(\text{Ph}_3\text{P}=\text{N}-8\text{-C}_6\text{H}_4\text{N})\text{-}\kappa\text{-N},\text{N}\}\text{Cl}]\text{Cl} \]

\[ (\eta^6-p\text{-cymene})\text{Ru}(\mu\text{-Cl})\text{Cl}]_2 \text{ (0.092 g, 0.15 mmol) was dissolved in CH\textsubscript{2}Cl\textsubscript{2} (15 mL) and Ph}_3\text{P}=\text{N-8-C}_6\text{H}_4\text{N} \text{ (0.12 g, 0.3 mmol) was added. The mixture was stirred for 3h. The solution was concentrated to 2 mL and 20 mL of Et}_2\text{O added to precipitate an orange solid, which was filtered and dried in vacuo.} \]
Yield: 0.18 g (84%). Anal. Calc. for C$_{37}$H$_{35}$N$_2$PCl$_2$Ru.2.5H$_2$O (755.68): C, 58.81; H, 5.34; N, 3.71. Found: C, 58.95; H, 4.81; N 3.82 %. ESI-MS: m/z: 675.13 (100%, [M-Cl]$^+$, calc. 675.13), 540.0 (100%, [M-p-cymene-Cl]$^+$, calc. 541.02). $^{31}$P $^1$H NMR (CDCl$_3$): δ 37.68 (s), (DMSO-d$_6$): 37.84 (s); $^1$H NMR (CDCl$_3$): δ 0.73 (3H, d, J = 6.7 Hz, CH$_3$, $^6$-p-cymene), 1.01 (3H, d, J = 6.6 Hz, CH$_3$, $^6$-p-cymene), 1.98 (3H, s, CH$_3$, $^6$-p-cymene), 2.44 (2H, m, CH, $^6$-p-cymene), 5.43 (2H, s, CH, $^6$-p-cymene), 5.72 (2H, s, CH, $^6$-p-cymene), 6.40 (1H, d, J = 8.0 Hz, H$_7$, C$_9$H$_6$N), 6.91 (1H, d, J = 8.1 Hz, H$_7$, C$_9$H$_6$N), 7.15 (1H, d, J = 8.1 Hz, H$_7$, C$_9$H$_6$N), 7.66 (9H, m, H$_m$ + H$_p$), 7.68 (1H, s, H$_3$, C$_9$H$_6$N), 8.00 (6H, m, H$_o$), 8.21 (1H, d, J = 8.4 Hz, H$_4$, C$_9$H$_6$N), 8.21 (1H, s, H$_2$, C$_9$H$_6$N). $^{13}$C $^1$H NMR (CDCl$_3$): δ 19.20 (s, CH$_2$, $^6$-p-cymene), 22.96 (s, CH$_3$, $^6$-p-cymene), 31.62 (s, CH, $^6$-p-cymene), 118.4 (s, C$_5$, $^6$-p-cymene), 121.7 (d, C$_7$, J = 10.3 Hz, $^6$-p-cymene), 124.6 (s, C$_3$, $^6$-p-cymene), 125.8 (s, C$_6$), 129.4 (s, C$_8$), 129.6 (d, C$_m$, J = 12.7 Hz), 130.0 (s, C$_{ipso}$), 134.1 (s, C$_p$), 134.9 (d, C$_o$, J = 9.8 Hz), 138.4 (d, C$_4$), 144.7 (s, C$_9$H$_6$N), 144.9 (s, C$_9$H$_6$N), 149.2 (s, C=NP) ppm. Signals due to the quaternary C atoms were not observed. IR (cm$^{-1}$): ν 519 (Ru-N), 1268 (N=P). Conductivity (acetone): 125.60 μS/cm (1:1 electrolyte). Solubility: 112.6 mM or 80 mg/mL (H$_2$O).

**Synthesis of 4**

$([\eta^6$-p-cymene]$\text{Ru}([\{\text{Cp-P(Ph$_2$)=N-CH$_2$-2-NC$_5$H$_4$}\text{Fe(Cp)}\}]$\kappa-N,N$)$\text{Cl}]$\text{Cl}$

To a solution of $[([\eta^6$-p-cymene]$\text{Ru(µ-Cl)}$]$\text{Cl}_2$ (0.16 g, 0.26 mmol) in CH$_2$Cl$_2$ (10 mL), $[[\text{Cp-P(Ph$_2$)=N-CH$_2$-2-NC$_5$H$_4$}\text{Fe(Cp)}]]$ (0.25 g, 0.52 mmol) in CH$_2$Cl$_2$ (10 mL) was added and stirred for 40 min. The solvent was filtered to dryness under reduced pressure. The solid was dissolved in CH$_2$Cl$_2$ and 25 mL of Et$_2$O were added. The solid formed was then filtered and dried in vacuo.

Yield: 0.30 g (81%). Anal. Calc. for C$_{38}$H$_{39}$N$_2$FePCl$_2$Ru·3.5H$_2$O (845.59): C, 53.98; H, 5.48; N, 3.31. Found: C, 54.02; H, 5.30; N 3.47 %. ESI-MS: m/z: 747.0 (100%, [M-Cl]$^+$, calc. 747.1), 613.0 (100%, [M-p-cymene-Cl], calc. 612.98). $^{31}$P $^1$H NMR (CDCl$_3$): δ 46.40 (s), (DMSO-d$_6$): 45.99 (s), (D$_2$O): 46.95 (s); $^1$H NMR (CDCl$_3$): δ 0.99 (3H, d, J = 5.4 Hz, CH$_3$, $^6$-p-cymene), 1.23 (3H, m, CH$_3$ + CH$_2$, $^6$-p-cymene, NCH$_2$C), 2.03 (3H, s, CH$_3$, $^6$-p-cymene), 3.51 (1H, m,
CH, \( \eta^6 \)-p-cymene), 3.98 (5H, s, \( \text{C}_5\text{H}_5 \)), 4.42-4.78 (6H, m, \( \text{CH}_2 + \text{C}_5\text{H}_4 \)), 4.79 (2H, m, \( \text{Cp} \)), 5.17-5.62 (4H, m, CH, \( \eta^6 \)-p-cymene), 7.45-7.68 (10H, m, H\( _{m+o+p} \)), 7.83 (1H, d, \( J = 7.4 \text{ Hz} \), \( \text{H}_5 \), \( \text{C}_5\text{H}_4\text{N} \)), 7.98 (2H, d, \( J = 7.7 \text{ Hz} \), \( \text{H}_3 + \text{C}_5\text{H}_4\text{N} \)), 9.09 (1H, d, \( J = 4.7 \text{ Hz} \), \( \text{H}_6 \), \( \text{C}_5\text{H}_4\text{N} \)); \( ^{13} \text{C} \{ ^1\text{H} \} \) (CDCl\( _3 \)): \( \delta 18.91 \) (s, \( \text{CH}_3 \), \( \eta^6 \)-p-cymene), 21.99 (d, \( \text{CH}_3 + \text{CH}_3 \), \( \eta^6 \)-p-cymene), 23.25 (s, \( \text{CH}_3 \), \( \eta^6 \)-p-cymene), 31.30 (s, CH, \( \eta^6 \)-p-cymene), 70.55 (s, \( \text{Cp} \)), 72.24 (d, \( J = 9.9 \text{ Hz} \), \( \text{Cp} \)), 73.73 (d, \( J = 9.9 \text{ Hz} \), \( \text{Cp} \)), 75.03 (m, \( \text{Cp} \)), 83.29 (d, \( J = 14.4 \text{ Hz} \), \( \eta^6 \)-p-cymene), 85.98 (d, 2CH, \( J = 8.3 \text{ Hz} \), \( \eta^6 \)-p-cymene), 87.09 (s, CH, \( \eta^6 \)-p-cymene), 99.99 (s, C, \( \eta^6 \)-p-cymene), 103.8 (s, C, \( \eta^6 \)-p-cymene), 124.9 (s, Ph), 128.4-128.8 (m, Ph), 130.2 (s, \( \text{C}_{ipso} \)), 133.0-133.3 (s, Ph), 133.9-134.0 (d, \( \text{C}_3 + \text{C}_4 \), \( J = 10.1 \text{ Hz} \), \( \text{C}_5\text{H}_4\text{N} \)), 138.8 (s, \( \text{C}_4 \), \( \text{C}_5\text{H}_4\text{N} \)), 155.1 (s, \( \text{C}_2 \), \( \text{C}_5\text{H}_4\text{N} \)), 164.1 (s, \( \text{C}_6 \)) ppm. \( \text{IR} \) (cm\(^{-1} \)): \( \nu 488 \) (Ru-N), 1116 (N=P). Conductivity (MeCN): 130.37 \( \mu \text{S/cm} \) (1:1 electrolyte). Solubility: 89.5 mM or 70 mg/mL (H\( _2 \)O).

**Synthesis of 5**

[PTA=N-C(O)-2-C\(_6\)H\(_5\)]

PTA (0.34 g, 2.18 mmol) and benzamide (0.264 g, 2.18 mmol) were placed in a Schlenk flask under nitrogen. Dry, degassed THF (10 mL) was added and to this solution, \( ^3 \text{BuDAD} \) (N,N-bis(tert-butyl)1,4-diazabutadiene) (0.503g, 2.18 mmol) in dry and degassed THF (4 mL) was added dropwise at 0 °C. The reaction was left stirring at RT for 2.5 h. After this period, the solvent was removed to dryness under reduced pressure. The white residue was washed three times with Et\(_2\)O (15 mL) giving a white solid that was filtered and dried \( \text{in vacuo} \).

Yield: 0.53 g (88%). Anal. Calc. for C\(_{13}\)H\(_{17}\)N\(_4\)OP (276.11): C, 56.52; H, 6.20; N, 20.28. Found: C, 55.55; H, 6.16; N 20.69 %. ESI-MS: m/z: 277.12 \( \text{(99.6\%)} \), [M]+, calc. 276.11). \( ^{31} \text{P} \{ ^1 \text{H} \} \) NMR (CDCl\(_3 \)): \( \delta -30.8 \) (s); \( ^1 \text{H} \) NMR (CDCl\(_3 \)): \( \delta 4.32-4.63 \) (12H, m, PTA), 7.38 (2H, t, \( J = 7.6 \text{ Hz} \), \( \text{H}_3 + \text{H}_5 \), \( \text{C}_6\text{H}_5 \)), 7.44 (1H, t, \( J = 7.1 \text{ Hz} \), \( \text{H}_4 \)), \( \text{C}_6\text{H}_5 \)), 8.07 (2H, d, \( J = 7.5 \text{ Hz} \), \( \text{H}_2 + \text{H}_6 \), \( \text{C}_6\text{H}_5 \)); \( ^{13} \text{C} \{ ^1 \text{H} \} \) NMR (CDCl\(_3 \)): \( \delta 55.10 \) (d, \( J = 47.2 \text{ Hz} \), PTA), 72.60 (d, \( J = 8.9 \text{ Hz} \), PTA), 128.08 (s, \( \text{C}_3 + \text{C}_5 \), \( \text{C}_6\text{H}_5 \)), 129.21 (s,\( \text{C}_2 + \text{C}_6 \), \( \text{C}_6\text{H}_5 \)), 131.47 (s, \( \text{C}_4 \)), 136.74 (d, \( J = 17.7 \text{ Hz} \), \( \text{C}_1 \)), 179.28 (s, \( J = 9.6 \text{ Hz} \), \( \text{C}=\text{O} \)). Conductivity (acetone): 2.08 \( \mu \text{S/cm} \) (neutral).
Synthesis of 6
[(CO)₄Mn(2-C₆H₄C(O)=PTA)]

PhCH₂Mn(CO)₅ (0.43 g, 1.5 mmol) and PTA=NC(O)Ph (5) (0.41 g, 1.5 mmol) were refluxed in n-hexane (45 mL) for 4h. The hot solution was filtered and the yellow filtrate reduced in volume until signs of crystallization became evident. Storage at -20 ºC gave yellow crystals of (CO)₄Mn(2-C₆H₄C(O)=PTA).

Yield: 0.59 g (90%). Anal. Calc. for C₁₇H₁₆N₄O₄PMn (442.02): C, 46.07; H, 3.65; N, 12.67. Found: C, 45.69; H, 3.56; N 12.72%. ESI-MS: m/z: 443.03 (100%, [M], calc. 443.03), 415.04 (100%, [M-CO], calc. 415.04), 386.01 (100%, [M-2CO], calc. 387.04), 331.05 (100%, [M-4CO], calc. 331.05). ³¹P {¹H} NMR (CDCl₃): δ −15.27 (s); ¹H NMR (CDCl₃): δ 4.32-4.58 (12H, m, PTA), 7.16 (1H, t, J = 7.5, 14.8 Hz, H₄, C₆H₄), 7.38 (1H, t, J = 7.5, 14.5 Hz, H₃ , C₆H₄), 7.68 (1H, d, J = 7.6 Hz, H₂, C₆H₄), 7.93 (1H, d, J = 7.4 Hz, H₅, C₆H₄); ¹³C{¹H} (CDCl₃): δ 53.30 (d, J = 43.3 Hz, PTA) ppm. 72.54 (d, J = 9.8 Hz, PTA), 124.02 (s, C₄, C₆H₄), 128.60 (s, C₂, C₆H₄), 132.55 (s, C₃, C₆H₄), 139.93 (s, C₁), 141.25 (s, C₅, C₆H₄), 171.36 (s, C=O), 213.66 (s, C=O), 215.97 (s, C=O) ppm. Conductivity (acetone): 1.11 μS/cm (neutral).

Synthesis of 7
[Hg(2-C₆H₄C(O)=PTA)Cl]

(CO)₄Mn(2-C₆H₄C(O)=PTA) (6) (0.44 g, 1.0 mmol) and HgCl₂ (0.54 g, 2.0 mmol) were refluxed in CH₃OH (55 mL) for 5 h during which time the solution turned yellow and a white solid formed. The mixture was cooled in an ice-water bath and subsequently filtered. The white solid formed was washed well with cold methanol. The solid was redissolved in CH₂Cl₂ (<200 mL) and filtered through celite. The resulting clear solution was reduced in volume (<3 mL) and Et₂O was added dropwise until the solution became cloudy. Storage at -20 ºC gave white crystals of 7, which were filtered off, dried and used without further purification.

Yield: 0.33 g (65%). Anal. Calc. for C₁₃H₁₆N₄OPCIHg·0.5CH₂Cl₂ (553.78): C, 29.28; H, 3.09; N, 10.112. Found: C, 29.08; H, 3.02; N, 9.75 %. ESI-MS: m/z: 513.05 (100%, [M+H]⁺, calc.
513.05), 535.03 (100%, [M+N\text{a}]^+), \text{calc. } 535.04). \textsuperscript{31}P \{^1H\} NMR (CDCl\textsubscript{3}): \text{δ} -25.68 (s, J\textsubscript{Hg-P} = 27.4 Hz). \textsuperscript{1}H NMR (CDCl\textsubscript{3}): \text{δ} 4.40 (12H, m, PTA), 7.39 (2H, t, J = 7.6 Hz, H\textsubscript{2} + H\textsubscript{4}, C\textsubscript{6}H\textsubscript{4}), 7.51 (1H, t, J = 7.5 Hz, H\textsubscript{3}, C\textsubscript{6}H\textsubscript{4}), 8.23 (1H, d, J = 8.1 Hz, H\textsubscript{5}, C\textsubscript{6}H\textsubscript{4}); \textsuperscript{13}C\{^1H\} (CDCl\textsubscript{3}): \text{δ} 52.71 (d, J = 12.4 Hz), 129.51 (d, C\textsubscript{6}H\textsubscript{4}), 129.53 (100%, \[M+Na]\]): 535.04). ESI MS: m/z: 616.13 (100%, \[M+Cl\]^+), \text{calc. } 616.13).

\textbf{Synthesis of 8}

\[(\eta^6-p\text{-cymene})\text{Ru}(\text{Ph}_3\text{P}=\text{N-CO-2-C}_6\text{H}_4)\eta-\text{C}_1\text{N})\text{Cl}]\]

\[\text{[Hg(2-C}_6\text{H}_4\text{C(O)N}^{=}\text{PPh}_3)\text{Cl]} (0.12 g, 0.2 mmol) and [(\eta^6-p\text{-cymene})\text{Ru(μ-Cl)Cl}_2] (0.13 g, 0.22 mmol) were refluxed in CH\textsubscript{3}CN (20 mL) for 7 days after which a yellow precipitate formed. The pale yellow solid was filtered off and discarded and the orange solution was concentrated to dryness. The solid was dissolved in CH\textsubscript{2}Cl\textsubscript{2} and filtered through celite. The solvent was removed under reduced pressure to a minimum, followed by addition of Et\textsubscript{2}O (~20 mL). The orange solid was filtered off and dried \textit{in vacuo}.

Yield: 0.049 g (37%). Anal. Calc. for C\textsubscript{35}H\textsubscript{33}NOPCl\textsubscript{2}Ru·2H\textsubscript{2}O (687.18): C, 61.18; H, 5.43; N, 2.04. Found: C, 60.97; H, 5.05; N 2.35 %. ESI-MS: m/z: 616.13 (100%, [M-Cl]^+, \text{calc. } 616.13). \textsuperscript{31}P \{^1H\} NMR (CDCl\textsubscript{3}): \text{δ} 20.63 (s), \text{(DMSO-d\textsubscript{6})}: 21.15 (s); \textsuperscript{1}H NMR (CDCl\textsubscript{3}): \text{δ} 1.31 (6H, d, J = 6.9 Hz, CH\textsubscript{3}, \eta^6-p\text{-cymene}), 2.18 (3H, s, CH\textsubscript{3}, \eta^6-p\text{-cymene}), 2.95 (1H, sept, J = 6.9 Hz, CH, \eta^6-p\text{-cymene}), 5.37 (2H, d, J = 6.0 Hz, CH, \eta^6-p\text{-cymene}), 5.49 (2H, d, J = 6.0 Hz, CH, \eta^6-p\text{-cymene}), 7.42-7.48 (2H, m, H\textsubscript{3} + H\textsubscript{4}), 7.49 -7.53 (6H, m, H\textsubscript{m}), 7.56 -7.61 (3H, m, H\textsubscript{p}), 7.84-7.89 (6H, m, H\textsubscript{o}), 8.36-8.37 (2H, dd, J = 1.4, 8.0 Hz, H\textsubscript{2} + H\textsubscript{3}), \textsuperscript{13}C\{^1H\} (CDCl\textsubscript{3}): \text{δ} 19.02 (s, CH\textsubscript{3}, \eta^6-p\text{-cymene}), 22.21 (s, 2CH\textsubscript{3}, \eta^6-p\text{-cymene}), 30.68 (s, CH, \eta^6-p\text{-cymene}), 80.58 (s, CH, \eta^6-p\text{-cymene}), 81.32 (s, CH, \eta^6-p\text{-cymene}), 96.77 (s, C, \eta^6-p\text{-cymene}), 101.25 (s, C, \eta^6-p\text{-cymene}), 127.65 (s, C\textsubscript{3} + C\textsubscript{4}), 127.92 (s, C\textsubscript{6}), 128.62-128.74 (d, C\textsubscript{m}, J = 12.4 Hz), 128.91 (s, C\textsubscript{ipso}), 129.51-129.53 (d, J = 3.02 Hz, C\textsubscript{2} + C\textsubscript{3}), 130.67 (s, C\textsubscript{3} + C\textsubscript{4}), 132.19-132.22- (d, C\textsubscript{p}, J = 2.9 Hz), 133.13-133.24 (d, C\textsubscript{o}, J = 9.6 Hz), 138.51-138.72 (d, J = 20.6 Hz, C-Ru), 176.26 (s, C=O) ppm. IR (cm\textsuperscript{-1}): ν 517 (Ru-N), 1162 (N=P), 1593 (C=O). Conductivity (acetone): 3.76 (neutral) μS/cm.
Synthesis of 9
[(η⁶-p-cymene)Ru{(PTA=N-CO-2-C₆H₄)-κ-C,N}Cl]

[Hg(2-C₆H₄C(O)N=PTA)Cl] (7) (0.1 g, 0.2 mmol) and [(η⁶-p-cymene)Ru(μ-Cl)Cl]₂ (0.13 g, 0.2 mmol) were refluxed in CH₂Cl₂ (20 mL) for 3 days. Subsequently, the solvent was removed to dryness and the yellow solid obtained was dissolved in CHCl₃ and filtered through celite. The solvent was removed under reduced pressure to a minimum, followed by addition of Et₂O. The yellow solid obtained was collected by filtration and dried in vacuo.

Yield: 0.14 g (66%). Anal. Calc. for C₂₃H₃₀N₄OPClRu (546.01): C, 50.59; H, 5.54; N, 10.26. Found: C, 50.32; H, 5.20; N 10.53 %.

ESI-MS: m/z: 546.0 (100%, [M-Cl]⁺, calc. 546.06), 412.0 (100%, [M-p-cymene]⁻, calc. 412.0). ³¹P {¹H} NMR (CDCl₃): δ -16.3 (s), (DMSO-d₆): -11.98 (s); ¹H NMR (DMSO-d₆): δ 0.61 (3H, d, J = 6.7 Hz, CH₃, η⁶-p-cymene), δ 0.99 (3H, d, J = 6.8 Hz, CH₃, η⁶-p-cymene), 2.34 (3H, s, CH₃, η⁶-p-cymene), 2.24 (1H, sept, J = 7.1 Hz, CH, η⁶-p-cymene), 4.47-4.67 (12H, m PTA), 5.81 (d, 2H, CH, η⁶-p-cymene), 6.06 (d, 1H, J = 6.5 Hz, CH, η⁶-p-cymene), 6.74 (d, 1H, J = 6.9 Hz, CH, η⁶-p-cymene), 7.11 (t, 1H, J = 7.7 Hz, H₄), 7.31 (t, 1H, J = 7.5 Hz, H₃), 7.37 (d, 1H, J = 7.5 Hz, H₃), 7.81 (d, 1H, J = 7.5 Hz, H₂); ¹³C{¹H} (DMSO-d₆): δ 18.70 (s, CH₃, η⁶-p-cymene), 19.97 (s, CH₃, η⁶-p-cymene), 24.13 (s, CH₃, η⁶-p-cymene), 31.00 (s, CH, η⁶-p-cymene), 51.82 (s, PTA), 52.22 (s, PTA), 71.50 (d, J = 40.5 Hz, PTA), 86.72 (s, CH, η⁶-p-cymene), 87.22 (s, CH, η⁶-p-cymene), 95.97 (s, CH, η⁶-p-cymene), 100.6 (s, C, η⁶-p-cymene), 127.65 (s, C₄), 128.6 (s, C₅), 128.6 (s, Cipso), 132.6 (s, C₃), 138.6-138.7 (d, J = 20.6 Hz, C-Ru), 141.1 (s, C₂), 183.9 (s, C=O) ppm. IR (cm⁻¹): ν 562 (Ru-N), 1314 (N=P), 1582 (C=O). Conductivity (acetone): 25.4 (neutral) μS/cm. Solubility: 0.79 mM or 0.43 mg/mL (H₂O).

X-Ray crystallography

Single crystals of 1 (see details in Table 5 in Appendix) were mounted on a glass fiber in a random orientation. Data collection was performed at RT on a Kappa CCD diffractometer using graphite monochromated Mo-Kα radiation (λ=0.71073 Å). Space group assignments were based on systematic absences, E statistics and successful refinement of the structures. The structures were solved by direct methods with the aid of successive difference Fourier maps and were
refined using the SHELXTL 6.1 software package. All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were assigned to ideal positions and refined using a riding model. Details of the crystallographic data are given in Table S1 (SI). These data can be obtained free of charge from The Cambridge Crystallographic Data Center via www.ccdc.cam.ac.uk/data_request/cif. (CCDC 1008354) or in the supporting information. Crystals of 1 (xxx prisms with approximate dimensions 0.26 x 0.24 x 0.21 mm) were obtained from a solution of 1 in CH₂Cl₂ by slow diffusion of Et₂O at RT.

**Cell culture, inhibition of cell growth and cell death analysis**

**Cell culture**

The human T-cell leukemia Jurkat (clone E6.1) and the prostate carcinoma DU-145 were routinely cultured in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS), L-glutamine and penicillin/streptomycin. A549 (lung carcinoma), MiaPaca2 (pancreatic carcinoma), MDA-MB-231 (Triple negative breast carcinoma) and 293T (non-tumoral embryonic kidney cells) were cultured in DMEM medium supplemented with 10% FCS, L-glutamine and penicillin/streptomycin. Media for A549 cells were also supplemented with 2.2 g/l Na₂CO₃, 100 µg/ml pyruvate and 5 ml non-essential amino acids (Invitrogen). All these media will be referred as ‘complete medium’ hereinafter. Cell cultures were maintained in a humidified atmosphere of 95% air / 5% CO₂ at 37 ºC.

**MTT toxicity assays**

For toxicity assays cells (5 x 10⁴ for Jurkat cells and 10⁴ for adherent cell lines) were seeded in flat-bottom 96-well plates (100 µl/well) in complete medium. Adherent cells were allowed to attach for 24 h prior to addition of cisplatin or tested compounds. Compounds were added at different concentrations in triplicate. Cells were incubated with cisplatin or compounds for 24 h and then cell proliferation was determined by a modification of the MTT-reduction method. Briefly, 10 µl/well of MTT (5 mg/ml in PBS) was added and plates were incubated for 1-3 h at 37 ºC. Finally, formazan crystal was dissolved by adding 100 µl/well iPrOH (0.05 M HCl) and gently shaking. The optical density was measured at 570 nm using a 96-well multiscanner.
autoreader (ELISA). In some experiments total cell number and cell viability were determined by the Trypan-blue exclusion test.

**Cell culture and XTT assay for RPTC cells**

The human Renal Proximal Tubule Cells (RPTC) a non-tumoral human kidney epithelial cell line (obtained from Lifeline Cell Technology, Frederick, Maryland, USA) were cultured in Lifeline's RenaLife Medium containing RenaLife LifeFactors with 2.4 mM L-Glutamine, 5 Î¼g/mL rh insulin, 1.0 Î¼M epinephrine, 10 nM triiodothyronine, 0.1 Î¼g/mL hydrocortisone hemisuccinate, 10 ng/mL rh EGF, 0.5% FBS and 5 Î¼g/m transferrin PS (all from Lifeline Cell Technology), at 37°C in a humidified atmosphere of 95% of air and 5% CO2 (University of Hawaii Cancer Center, Honolulu, Hawaii, USA). For evaluation of cell viability, cells were seeded at a concentration of 5×10^3 cells/well in 90 µl Lifeline's RenaLife complete medium into tissue culture grade 96-well flat bottom microplates (Thermo Scientific BioLite Microwell Plate, Fisher Scientific, Waltham, Massachusetts, USA) and grown for 24 h. Solutions of the compounds were prepared by diluting a freshly prepared stock solution (in H2O) of the corresponding compound in Lifeline's RenaLife complete medium. Afterwards, the intermediate dilutions of the compounds were added to the wells (10 µL) to obtain a final concentration ranging from 0.1 to 200 µM, and the cells were incubated for 24 h. Following 24 h drug exposure, 50 µL of 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) (Roche Diagnostics, Indianapolis, Indiana, USA) labeling mixture per well was added to the cells at a final concentration of 0.3 mg/ml and incubated for 4 h at 37°C in a humidified atmosphere of 95% of air and 5% CO2. The optical density of each well (96-well plates) was quantified using EnVision Multilabel Plate Readers (Perkin Elmer, Waltham Massachusetts, USA) at 450 nm wavelength to measure absorbance. The percentage of surviving cells was calculated from the ratio of absorbance of treated to untreated cells. The IC50 value was calculated as the concentration reducing the proliferation of the cells by 50% and is presented as a mean (± SE) of at least two independent experiments each with triplicates.

**Cell death analysis**

Apoptosis/necrosis hallmarks of cells treated with compounds 2 and 3 were analyzed by measuring mitochondrial membrane potential and/or exposure of phosphatidylserine. Cells were
treated with different concentrations and at different incubation times as indicated in figure legends. In some experiments the general caspase inhibitor z-VAD-fmk was added at 50 µM 1 h before compounds. For mitochondrial membrane potential determination cells (2.5x10⁵ in 200 µl) after treatment with compounds were incubated at 37ºC for 15 min. in ABB (140 mM NaCl, 2.5 mM CaCl₂, 10 mM Hepes/NaOH, pH 7.4) containing 60 mM tetramethylrhodamine ethyl ester (TMRE, Molecular Probes). Phosphatidylinerseine exposure was quantified by labeling cells with AnnexinV-PE or AnnexinV-DY636 (Invitrogen) after treatment with compounds. AnnexinV was added at a concentration of 0.5 µg/mL and cells were incubated at room temperature for 15 min. In all cases, cells were diluted to 1 ml with ABB to be analyzed by flow cytometry (FACScan, BD Bioscience, Spain).

**Intracellular ROS quantification**

Oxidative stress induced by compounds 2 and 3 was analyzed by intracellular staining with the fluorescent probe 2-hydroxietidium (2-HE, Molecular Probes). After 16 h of culture in the presence of compounds 1-3, cells were incubated with 2 µM 2-HE at 37ºC for 15 min. Red fluorescence produced by reduction of 2-HE to ethidium was quantified in a flow cytometer.

**Effect of 2 in the levels of proteins of the Bcl-2 family**

Jurkat cells (5x10⁵ cells/ml) were treated with 2 (1 µM) for 6 h. At the end of incubations total protein extracts from 2x10⁶ cells were prepared in lysis buffer and samples (50 micrograms/lane) were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. Then, levels of some members of the Bcl-2 family of proteins were analyzed by Western Blot using specific antibodies: Bcl-XL (Cell Signalling, cat 2764), Bcl-2 (Abcam, Cat AB692), Mcl-1 (Santa Cruz Biotech, cat. SC819), Bim (Calbiochem, cat. 202000), Puma (Cell Signaling, Cat. 4976) and Noxa (Abcam, Cat. 114C307). After incubation with primary antibodies, membranes were incubated with appropriate secondary antibodies conjugated with HRP. Finally, membranes were revealed using a chemiluminiscence substrate (Pierce).

**Inhibition of cathepsin B**

Cathepsin B, purified from human liver (Accession # P07858) and substrate Peptide sequence: Z-FR-AMC [AMC=7-amino-4-methylcoumarin] were dissolved on a buffer: 25 mM MES pH 6,
50 mM NaCl, 0.005% Brij35, 5 mM DTT and 1% DMSO with a final concentration of 10 μM. The enzyme solution was delivered into the reaction well. 2 (1% DMSO solution) was delivered into the enzyme mixture by Acoustic technology (Echo550; nanoliter range), incubate for 10 min. at room temp. The substrate solution was delivered into the reaction well to initiate the reaction. The enzyme activity was monitored (Ex/Em = 355/460 nm) as a time-course measurement of the increase in fluorescence signal from fluorescently-labeled peptide substrate for 120 min. at room temperature. The data was analyzed data by taking slope (signal/time) of linear portion of measurement. The slope is calculated by using Excel, and curve fits are performed using Prism software.

**Interaction of compounds 1-4, 8, 9, [(η⁶-p-cymene)Ru(μ-Cl)Cl]₂ and cisplatin with plasmid (pBR322) DNA by Electrophoresis (Mobility Shift Assay)**

10 µL aliquots of pBR322 plasmid DNA (20 μg/mL) in buffer (5 mM Tris/HCl, 50 mM NaClO₄, pH = 7.39) were incubated with different concentrations of the compounds (1-4, 8, 9, [(η⁶-p-cymene)Ru(μ-Cl)Cl]₂) (in the range 0.25 and 2.0 metal complex:DNAbp) at 37 ºC for 20 h in the dark. Samples of free DNA and cisplatin-DNA were prepared as controls. After the incubation period, the samples were loaded onto the 1 % agarose gel. The samples were separated by electrophoresis for 1.5 h at 84 V in Tris-acetate/EDTA buffer (TAE). Afterwards, the gel was stained for 30 min. with a solution of GelRed Nucleic Acid stain.

**Interaction of compounds 1-4 with Calf Thymus DNA by Circular Dichroism**

Stock solutions (5 mM) of each complex were freshly prepared in water prior to use. The right volume of those solutions was added to 3 ml samples of an also freshly prepared solution of CT DNA (48 µM) in Tris/HCl buffer (5 mM Tris/HCl, 50 mM NaClO₄, pH=7.39) to achieve molar ratios of 0.1, 0.25, 0.5, 1.0 and 2.0 drug/DNA. The samples were incubated at 37ºC for a period of 20 h. All CD spectra of DNA and of the DNA-drug adducts were recorded at 25ºC over a range 220-420 nm and finally corrected with a blank and noise reduction. The final data is expressed in molar ellipticity (millidegrees).
DNA precipitation with compounds 2 and 3 and quantification of ruthenium by ICP-MS

Stock solutions of compounds 2 and 3 (4 mM in water) and CT DNA (11.56 mM in 5 mM Tris/HCl, 50 mM NaClO₄, pH=7.39) were freshly prepared prior to use. 216 µl of DNA stock solution were diluted in 3.53 mL of buffer and 1.25 mL of compound stock solution were then added to achieve 5 mL final volume at concentrations of 500 µM in DNA and 1 mM in metal compound. Each sample was incubated at 37ºC for a period of 20 h, then cooled down to room temperature and centrifuged at 3000 rpm for 15 min. and at 4000 rpm for extra 40 min. The supernatant was separated and analyzed for CT DNA concentration by CD spectroscopy. The resulting pellet was washed twice with ice-cold ethanol (1 mL), centrifuged at RT for 1 min. at 4000 rpm, dried under high vacuum and analyzed for Ru content by ICP-MS. The total amount of DNA in each sample was 0.92 mg. Every experiment was run in duplicate.

Interaction of compounds 1-4, 8, 9, and cisplatin with HSA by Fluorescence Spectroscopy

A solution of each compound (8 mM) in DMSO was prepared and ten aliquots of 2.5 µL were added successively to a solution of HSA (10 µM) in phosphate buffer (pH = 7.4) to achieve final metal complex concentrations in the range 10-100 µM. The excitation wavelength was set to 295 nm, and the emission spectra of HSA samples were recorded at room temperature in the range of 300 to 450 nm. The fluorescence intensities of all the metal compounds, the buffer and the DMSO are negligible under these conditions. The fluorescence was measured 240 s. after each addition of compound solution. The data were analyzed using the classical Stern-Volmer equation \( \frac{F_0}{F} = 1 + K_{SV}[Q] \).

In vivo tests

All animal experiments were performed according to the University of Hawaii Cancer Center regulations and by approval of the responsible authorities (UH IACUC number: A3423-01).

Determination of lethal dose (LD) and maximum tolerated dose (MTD) of 2 in mice

14 female C57/Black 6 from Jackson Laboratory (Bar Harbor, ME and Sacramento, CA, USA) ages 8 to 14 weeks and weighing 18–26 g were used for these experiments. Mice were randomized to treatment groups based on their age to ensure equivalent distribution between the
groups. At trial end-point the mice were sacrificed and liver, spleen, kidney and blood plasma were collected, and then processed for further analysis. Gross and microscopic evaluations of liver, spleen and kidney were conducted. The weight of 2 treated mice compared and that of vehicle-treated mice as measured twice weekly.

The lethal dose (LD) was determined by injecting once mouse i.p. once 5 mg/kg/day, 10 mg/kg/day, 20 mg/kg/day, 30 mg/kg/day, or 50 mg/kg/day, and one vehicle control with 100µl Normal Saline (0.9% NaCl)). The dose that killed the mice within 24 hours was set to be lethal dose. The lethal dose was confirmed by administering that dose to a second mouse. The maximum tolerated dose (MTD) was determined by injecting two mice with 5 mg/kg/day, 10 mg/kg/day, or 20 mg/kg/day over 6 days, or 20 mg/kg/every other day over 6 days and one vehicle control mouse with 100µl Normal Saline (0.9% NaCl). The MTD was confirmed by administering the determined dose to 3 mice over 7 days and three vehicle control mice with 100µl Normal Saline (0.9% NaCl).

**Study of the effects of 2 in MDA-MB-231 xenografts in mice**

12 female NOD.CB17-Prkdc scid/J (non-obese diabetic–severe combined immunodeficiency) from Jackson Laboratory (Bar Harbor, ME and Sacramento, CA, USA) for the xenograft experiment (ages 8 to 12 weeks and weighing 19–24 g, were used. Each mouse received 5x10⁶ tumor cells subcutaneously without anesthesia. Exponentially growing oestrogen-receptor alpha-negative MDA-MB-231 human breast cancer cells were suspended in 1:1 ratio 50 µl phosphate-buffered saline (PBS; pH 7.4) plus 50 µl of matrigel (BD Biosciences, San Jose, CA, USA) were injected subcutaneously on both left and right flank of each mice’. The diameter of the tumors was measured once weekly using an electronic digital caliper and the tumor volume (TV) was calculated according to the empirical equation $TV = (a)(b^2) \times \pi/6$ where $a =$ longest dimension; $b =$ largest dimension orthogonal to $a$. The median values of each group were normalized to the initial tumor volume resulting in the relative tumor volume. Each six MDA-MB-231-transplanted animals received compound 2 (5 mg/kg/every other day) or vehicle (0.9% NaCl) intraperitoneally (i.p.) Treatment started when tumors were palpable (about 5–6 mm diameter). To palliate the weight loss observed in the MTD study the mice were fed a 46% fat-adjusted diet (Harlan Teklad, Madison, WI), plus HydroGel™ (Harlan Teklad, Madison, WI) and received
subcutaneous injection of 100µl Normal Saline (0.9% NaCl) to improve hydration. Mice were randomized to treatment groups based on their starting tumor burden at 12 weeks of age to ensure equivalent distribution between the two groups. At trial end-point the mice were sacrificed and tumors measured again after excision and then processed for further analysis. Histological as well as biochemical evaluations of blood, liver, intestine, kidney, and lung were conducted. Tumor volumes were graphed for (2) treated mice compared to vehicle-treated mice, based on weekly external digital caliper measurements.

**Pharmacokinetic study: determination of ruthenium content in the organs, entire blood, and plasma**

Female NOD.CB17-Prkdc scid/J bearing subcutaneous MDA-MB-231 tumors and treated with compound 2 (5 mg/kg/every other day) intraperitoneally were used for pharmacokinetic evaluation of the drug in blood and other tissues. Blood was collected retroorbitally using heparin coated glass capillary into heparinized blood collection vials on ice at time intervals of 30 min, 2 h, 6 h, 24 h and 48 h. after the first dose. The blood samples were centrifuged at 2800 rpm at 4°C for 15 min. and the supernatant plasma was transferred into 1.5 mL micro-centrifuge tubes and maintained at -80 °C until analysis.

Ruthenium content was determined using Inductively Coupled Plasma - Mass Spectrometry (ICP-MS). Fifty microliter of plasma was transferred into a glass vial and 1 mL of concentrated acid mix (comprising of 75% of 16 N nitric acid and 25% of 12 N hydrochloric acid) was added. The mixture was heated at 90 °C for 5 h. After cooling, the samples were diluted with water, 40 ppb of Indium internal standard was added and analyzed in a Thermo Scientific XSERIES 2 ICP-MS with ESI PC3 Peltier cooled spray chamber with SC-FAST injection loop and SC-4 autosampler. All the elements were analyzed using He/H₂ collision-reaction mode. Plasma from control mice was spiked with the test compound to determine the extraction efficiency.

At the end of the study, liver, kidney and tumor of the animals were harvested, weighed and transferred into glass vials. One ml of water was added to each samples and subjected to ultrasonic tissue disruption at 15W power for 1 min. The tissue homogenates were frozen at -80 °C for 2 h and lyophilized. The lyophilized product was heated at 90 °C with the concentrated acid mix (described above) for 5 h, cooled, diluted with water and analyzed for ruthenium by
ICP-MS. Pharmacokinetic estimates were obtained from the plasma concentration–time profiles by noncompartmental analysis using Phoenix WinNonlin 6.1 (Mountain View, California).
5.8. Appendix


Table 5. Crystal Data and Structure Refinement for compound 1.

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<th>Formula</th>
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<td>$b$ [Å]</td>
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<td>$c$ [Å]</td>
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<tr>
<td>$\gamma$ [$^\circ$]</td>
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<td>$wR_2$(all data)</td>
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5.8.2. $^1$H NMR spectra of compounds 2-4, 7 and 8.

**Figure 13.** $^1$H NMR spectra of compound 2 in CDCl$_3$.

**Figure 14.** $^1$H NMR spectra of compound 3 in CDCl$_3$. 
Figure 15. $^1$H NMR spectra of compound 4 in CDCl$_3$.

Figure 16. Variable temperature $^1$H NMR spectra of compound 4 in CDCl$_3$ (magnification zone 6.1-3.6 ppm).
**Figure 17.** Variable temperature $^1$H spectra of compound 4 in CDCl$_3$ (magnification zone 2.9-0.1 ppm).

**Figure 18.** Variable temperature $^{31}$P($^1$H) NMR spectra of compound 4 in CDCl$_3$ ($\delta$ 46.45 (s) ppm).
Figure 19. $^1$H NMR spectra of compound 7 in CDCl$_3$.

Figure 20. $^1$H NMR spectra of compound 8 in CDCl$_3$. 
5.8.3. Stability of compounds 1-4, 8 and 9 in DMSO-$d_6$ and D$_2$O solution overtime assessed by $^{31}$P{$^1$H} NMR spectroscopy.$^a$

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<th>2 w</th>
<th>3 w</th>
<th>1 m</th>
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<td></td>
<td></td>
<td>58%</td>
<td>25% 0% ~ 9 days</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td>&gt;99%</td>
<td>85%</td>
<td>50%</td>
<td>27%</td>
<td>6%</td>
<td></td>
<td></td>
<td>~ 1 week</td>
</tr>
</tbody>
</table>

a) % of decomposition determined by integration of all the signals present in the $^{31}$P{$^1$H} NMR spectra, the sum being set to 100%.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time (D$_2$O)</th>
<th>3 d</th>
<th>Half life (50%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>insoluble</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>2.5 days</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>b</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>99%</td>
<td>c</td>
</tr>
</tbody>
</table>

a) % of decomposition determined by integration of all the signals present in the $^{31}$P{$^1$H} NMR spectra, the sum being set to 100%.
b) Compound hydrolyzes when dissolved in water (45:55 ratio).
c) Compound precipitates after 3 days.
5.8.4. $^{31}\text{P}^{\{1\text{H}\}}$ NMR spectra showing the stability of compounds 1-4, 8 and 9 in DMSO-$d_6$ overtime.

**Figure 21.** $^{31}\text{P}^{\{1\text{H}\}}$ NMR spectra of compound 1 in DMSO-$d_6$ ($\delta$ 24.27 (s) and –144.20 (h) ppm) overtime.

**Figure 22.** $^{31}\text{P}^{\{1\text{H}\}}$ NMR spectra of compound 2 in DMSO-$d_6$ ($\delta$ 24.26 (s) ppm) overtime.
Figure 23. $^{31}\text{P}\left\{^1\text{H}\right\}$ NMR spectra of compound 3 in DMSO-$d_6$ ($\delta$ 37.84 (s) ppm) overtime.

Figure 24. $^{31}\text{P}\left\{^1\text{H}\right\}$ NMR spectra of compound 4 in DMSO-$d_6$ ($\delta$ 45.99 (s) ppm) overtime.
Figure 25. $^{31}$P{$^1$H} NMR spectra of compound 8 in DMSO-$d_6$ ($\delta$ 21.09 (s) ppm) overtime.

Figure 26. $^{31}$P{$^1$H} NMR spectra of compound 9 in DMSO-$d_6$ ($\delta$ -11.85 (s) ppm) overtime.
5.8.5. $^{31}\text{P}_{^1\text{H}}$, $^1\text{H}$ NMR and $^{13}\text{C}$ NMR spectra of compounds 2, 3 and 4 in D$_2$O overtime.

Figure 27. $^{31}\text{P}_{^1\text{H}}$ NMR spectra of compound 2 in D$_2$O ($\delta$ 26.37 (s) ppm) overtime.

Figure 28. $^1\text{H}$ NMR spectra of compound 2 in D$_2$O at $t = 0$ and after 5 days.
Figure 29. $^{13}$C NMR spectra of compound 2 in D$_2$O at t = 0 and after 5 days.

Figure 30. $^{13}$C NMR spectra of compound 2 in D$_2$O at t = 0 and after 5 days.
**Figure 31.** $^3\text{P}^{[1\text{H}]}$ NMR spectra of compound 3 in D$_2$O ($\delta$ 37.79 (s) ppm) overtime.

**Figure 32.** $^1\text{H}$ NMR spectra of compound 3 in D$_2$O overtime.
Figure 33. $^1$H NMR spectra of compound 3 in D$_2$O overtime.

Figure 34. $^{31}$P($^1$H) NMR spectra of compound 4 in D$_2$O ($\delta$ 46.95 (s) ppm) overtime
5.8.6. $^{31}$P{$^1$H} NMR spectra of compounds 2 in a 100 mM NaCl/D$_2$O solution overtime.

Figure 35. $^{31}$P{$^1$H} NMR spectra of compound 2 in 100mM NaCl/D$_2$O ($\delta$ 26.21 (s) ppm) overtime.

5.8.7. $^{31}$P{$^1$H} and $^1$H NMR spectra of compounds 2 and 3 in a D$_2$O solution at 80°C during 1 h.

Figure 36. $^{31}$P{$^1$H} NMR spectra of compound 2 in D$_2$O ($\delta$ 26.48 (s) ppm) after heating at 80°C for one hour.
Figure 37. $^{31}$P {$^{1}$H} NMR spectra of compound 3 in D$_2$O ($\delta$ 37.84 (s) ppm) after heating at 80°C for one hour.

5.8.8. Mass spectra (ESI+) of compound 2 in H$_2$O solution overtime (5 days).

Figure 38. MS ESI+ of compound 2 in H$_2$O solution at t = 0.
Figure 39. Magnification of [m/z] from 600 to 665 of compound 2 in H$_2$O solution at t = 0.

Figure 40. MS ESI+ of compound 2 in H$_2$O solution at t = 5 days.
Figure 41. Magnification of [m/z] from 600 to 665 of compound 2 in H₂O solution at t = 5 days.

![Magnification of [m/z] from 600 to 665 of compound 2 in H₂O solution at t = 5 days.](image)

Figure 42. Magnification of peak at [m/z]: 617.1 corresponding to species $([\eta^6-p$-cymene]Ru(IM-k-C,N-C₆H₄(PPh₂=N-CO-2-N-C₅H₄))$^+$ in the MS ESI+ spectrum of compound 2 in H₂O solution at t= 5 days. Insert: theoretical isotopic distribution.
5.8.9. Study of the effect of 2 in the levels of proteins of the Bcl-2 family.

**Figure 43.** Effect of 2 in the levels of proteins of the Bcl-2 family. Jurkat cells were left untreated (control) or incubated for 6 h with compound 2 (1 µM). At the end of incubations total protein extracts were prepared as described in the Experimental section and analyzed by Western Blot with specific antibodies as indicated. Blots are representative of three independent experiments.
5.8.10. Experiments to assess the interaction of compounds 2-4 with CT DNA by circular dichroism.

Figure 44. CD spectra of CT DNA (48 µM) and CT DNA incubated with 0.1, 0.25 and 0.5 equivalents of compounds 1 (A), 2 (B), 3 (C) and 4 (D) for 20 h at 37 °C.
5.9. Bibliography


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CHAPTER VI
CONCLUSIONS AND FUTURE DIRECTIONS

- A series of organometallic and coordination gold(III), platinum(II), palladium(II) and ruthenium(II) compounds with water-soluble, ferrocene-based and luminescent iminophosphorane ligands have been successfully synthesized and characterized. A synthetic method for the preparation of cyclometalated exo derivatives of different metals is described. A mercury-free transmetallation with organogold(I) derivatives has been employed for the preparation of endo cycloplatinated compounds.
- In the cases of d⁸ transition metal complexes it was demonstrated that cyclometallated complexes are more stable in physiological media than coordination compounds. Both, coordination and cyclometalated IM ruthenium(II) complexes are quite stable in physiological media or water.
- The compounds exhibit important cytotoxic effects in vitro in the low or sub-micromolar range in selected human cancer cell lines and in most cases are markedly more cytotoxic than cisplatin, while being less toxic on "healthy" cell lines.
- Studies of the interactions of the compounds with plasmid (pBR322) DNA and/or Calf Thymus DNA indicated in most cases no interaction, or a weak interaction electrostatic in nature. In specific cases we were able to demonstrate the formation of left-handed helix of Z-form DNA through strong electrostatic interactions (for a cyclometalated exo-Pt(II) complex (3)) or backbone charge neutralization, further confirmed by ICP-MS analysis of metal content in DNA precipitates for ruthenium(II) complexes.
- Most of the complexes displayed a concentration dependent fluorescence quenching of HSA, indicating faster binding compared to cisplatin.
- Studies of intracellular distribution on human ovarian cancer (A2780) cells with selected compounds (e.g. 3, and 7, Chapter I) using fluorescence microscopy failed.
- Initial mechanistic studies for selected compounds indicated that the cell death type is different than that of cisplatin (e.g. ruthenium(II) compounds cell death is mainly through
caspase-dependent apoptosis, not dependent on p53 while for apoptotic cycloplatinated compound 5, caspase-independent cell death is triggered when apoptosis is blocked).

- Permeability studies for a selected cycloplatinated compound (5) revealed its high permeability profile and an estimated oral fraction absorbed of 100% which potentially makes it a good candidate for oral administration.
- The efficacy of the highly water-soluble Ru(II) complex (2) in vivo on xenografted breast carcinoma MDA-MB-231-tumors grown on NOD.CB17-Prkdc scid/J mice demonstrated a tumor reduction (shrinkage) of 56% after 28 days treatment with low systemic toxicity, and accumulation of the complex preferentially in the breast tumor tissues when compared to kidney and liver. These results make compound 2 the most promising organometallic p-cymene ruthenium(II) anticancer agent described so far and a potential candidate for subsequent more elaborated preclinical studies.
- Overall, our results support the idea that non-toxic iminophosphorane molecules are excellent ligands for the synthesis of organometallic compounds of d^6 and d^8 metals (especially cationic species) with relevant anticancer properties, high permeability and, in some cases, water-solubility.

**Future directions**

The results obtained in chapter III will guide us to design new iminophosphoranes complexes with better luminescence properties in cells in vitro. We will need to design compounds with a better stability in biological media and to increase the fluorescence detection limits. A possible direction involves the synthesis of cyclometalated compounds incorporating modified luminescent iminophosphorane ligands based on substitutions on the 8-aminoquinoline scaffold (methyl or fluorine groups).

The results obtained in chapter IV have made us select endo cycloplatinated compound 5 as a candidate for further in vivo and mechanistic evaluation. The promising water-soluble ruthenium(II) complex (2) will be further evaluated in vivo on different tumors such as prostate, colon and pancreas. More detailed mechanistic studies are underway after its evaluation on the NCI 60-cell panel.
CHAPTER VII

PUBLICATIONS, PATENTS AND CONFERENCE PRESENTATIONS WHICH HAVE RESULTED FROM THIS WORK

PUBLICATIONS


Submitted

PATENTS


CONFERENCE PRESENTATIONS


“248th ACS National Meeting & Exposition”, Indianapolis, IN, USA, September 8-12th, 2013. In vitro and in vivo Evaluation of Water-soluble Iminophosphorane Ruthenium(II) Anticancer Complexes”. (Poster presentation)

Other publications and presentations non related to this thesis with work performed during previous undergraduate studies or during my thesis


“Marie Curie Nobel Centennial Symposium”, New York, NY, USA. **November 15th, 2011**. Luminescent Di and Polynuclear Organometallic Gold(I)-M (Au₂, {Au₂Ag}ₙ and {Au₂Cu}ₙ) Compounds Containing Bidentate Phosphanes as Active Antimicrobial Agents. (Poster presentation)

“ACS Middle Atlantic Regional Meeting, MARM”. College Park, MD, USA. **May 21st-23rd, 2011**. Luminescent Di and Polynuclear Organometallic Gold(I)-M (Au₂, {Au₂Ag}ₙ and {Au₂Cu}ₙ) Compounds Containing Bidentate Phosphanes as Active Antimicrobial Agents. (Poster presentation)

“Younger Chemists Committee Symposium”. New York, NY, USA. **March 19th, 2011**. Luminescent Di and Polynuclear Organometallic Gold(I)-M (Au₂, {Au₂Ag}ₙ and {Au₂Cu}ₙ) Compounds Containing Bidentate Phosphanes as Active Antimicrobial Agents. (Poster presentation)