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# The role of ABC proteins in the mechanism of action of promising ruthenium anticancer agents

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**Mestrado em Química**

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**Resumo**

O cancro é um termo genérico para um vasto grupo de doenças que podem afetar qualquer parte do nosso corpo. Esta doença é definida pela proliferação anormal de células. Estas células anómalas podem invadir outros tecidos e órgãos formando assim metástases. O cancro, considerado uma doença mundial e que afeta diversas faixas etárias, continua a ser uma preocupação para a população e, nomeadamente, para os cientistas. A investigação nesta área já é longa e felizmente conta já com importantes avanços. No entanto, apesar de todos os progressos, continuam a existir obstáculos para o tratamento cem por cento eficaz. Um desses obstáculos é a resistência das células cancerígenas aos fármacos, o que limita consideravelmente a eficácia dos mesmos. Esta resistência deve-se a vários fatores sendo, um deles, a existência de um tipo de proteínas transportadoras, denominadas transportadores ABC, que se encontram sobre expressas nas células cancerígenas e que atuam sobre os fármacos levando ao seu rápido efluxo para fora da célula limitando, assim, a sua capacidade de ação sobre as células cancerígenas. A resistência a fármacos refere-se à capacidade das células cancerígenas para resistirem a uma variedade estrutural de fármacos anticancerígenos, levando a um dos maiores problemas da quimioterapia. Na realidade, este tipo de resistência é responsável pelo fracasso de mais de 90 % dos tratamentos em cancro.

A família ABC (*ATP binding cassette*) é constituída por várias proteínas, sendo que atualmente as mais conhecidas, e aqui estudadas são: P-gp ou ABCB1, MRP1 ou ABCC1, MRP2 ou ABCC2 e ABCG2 ou BCRP. Apesar de existirem várias teorias que procuram explicar os seus mecanismos de ação, a certeza é que estas proteínas transportadoras permitem a expulsão dos fármacos, aumentando, em consequência, a resistências das células cancerígenas a estes fármacos. Os estudos de elucidação dos mecanismos bioquímicos que permitem combater esta resistência aos fármacos têm-se centrado principalmente na identificação de inibidores seletivos destas proteínas que bloqueiem a passagem dos fármacos para o exterior da célula cancerígena. A maior limitação até agora tem sido encontrar inibidores específicos para cada transportador, que ao mesmo tempo apresentem baixa citotoxicidade para células saudáveis e de alta eficiência. Por isso, a investigação nesta área continua a ser uma prioridade. Foi neste âmbito que o Laboratorio de Química Organometálica da Faculdade de Ciências da Universidade de Lisboa, Portugal, juntamente com o "*Drug Resistance and Membrane Proteins team*" em Lyon, França, avaliou, durante a realização desta tese de Mestrado, o papel que diversos transportadores ABC têm no mecanismo de ação de uma família de complexos organometálicos de ruténio ciclopentadienilo, "RuCp" (Cp = $\eta^5$-$C_5H_5$).

Foram estudados sete compostos, todos contendo o fragmento 'Ru($\eta^5$-CpR)($PPh_3$)(bipiridina-R)', com potencial atividade anticancerígena e anteriormente desenvolvidos pelo Laboratório de Química Organometálica. Entre eles, encontram-se os compostos de ruténio-polímero PMC78 e PMC85 que foram escolhidos devido ao seu elevado peso molecular que permite uma maior facilidade de acumulação destes compostos no interior das células pelo efeito de EPR ("enhanced permeation and retention effect"). Para além disso, estes compostos revelaram melhores citotoxicidades que a cisplatina para as linhas celulares do ovário A2780 e mama MCF7 e MDA-MB-231, e parecem ser capazes de ultrapassar os mecanismos de resistência de células cancerígenas (resultados obtidos por comparação entre a linha celular A2780 sensível e A2780CisR, resistente à cisplatina) . Para além destes compostos, foi também escolhido o composto PMC79, composto parental dos anteriores, com a mesma estrutura, mas sem as cadeias de polímero na sua estrutura. O composto PMC79 apresenta uma

boa citotoxicidade relativamente à cisplatina para as mesmas linhas celulares. No entanto, para este composto o nível de acumulação nas células A2780 sensíveis foi muito superior que nas resistentes. Devido a estes resultados, o PMC79 foi também escolhido para este trabalho para se tentar perceber em maior detalhe qual o(s) transportadores ABC responsáveis por este efeito.

O composto LCR134, $[Ru(\eta^5\text{-}Cp)(PPh_3)(bipiridina\text{-}biotina)][CF_3SO_3]$, foi também escolhido uma vez que é baseado no PMC79, mas onde foram adicionadas duas moléculas de biotina (vitamina H ou B7) à bipiridina. A inclusão desta biomolécula poderá ser vantajosa devido à capacidade de se ligar a recetores da membrana celular das células cancerígenas. A biotina é essencial para o nosso organismo e tem sido frequentemente utilizada em diversos estudos reportando a sua facilidade de transporte para dentro das células cancerígenas.

Os três compostos restantes, pertencem à subfamília de ruténio $\eta^5$-metilciclopentadienilo e foram escolhidos com o objetivo de se conseguir obter uma correlação entre a sua atividade biológica e os substituintes na bipiridina.

Desta forma, para se estudar o papel dos transportadores ABC no mecanismo de ação destes compostos, utilizaram-se diversas técnicas, tais como o teste de viabilidade celular para avaliar a citotoxicidade de cada composto através do cálculo do $IC_{50}$, citometria de fluxo para verificar a percentagem de inibição de cada composto para os transportadores ABC, citometria de massa para quantificar a percentagem de acumulação do ruténio nas células, e *docking* molecular para a caracterizar a ligação de compostos ao sitio ativo da proteína P-gp.

Todos os compostos obtiveram bons resultados ao nível da citotoxicidade para a linha celular cancerígena 2008C (1.1 - 4.5 μM), assim como bons níveis de internalização celular de ruténio.

Os resultados obtidos permitiram concluir que compostos mesmo estruturalmente muito similares, possuem atividades biológicas distintas. Verificou-se que os compostos de ruténio-polímero, PMC78 e PMC85, são mais citotóxicos para células sobre expressas com transportadores (P-gp e MRP1, respetivamente) do que sem transportadores. O PMC78 demonstrou também que seria um bom inibidor para a P-gp. Todos estes fatores levaram a indicar que o uso do polilactídeo poderá potenciar a ação anticancerígena de compostos não poliméricos.

Observou-se também que o uso do fragmento da bipiridina funcionalizada com duas moléculas de biotina poderá potenciar a capacidade anticancerígena dos compostos, visto que o complexo LCR134 revelou ser muito bom inibidor da P-gp. Cálculos de *docking* molecular mostram que é possível que haja competição entre o LCR134 e o conhecido substrato Rodamina 123 pelo centro ativo da P-gp .

Os compostos LCR136 e RT11, pertencentes à família $\eta^{5}$-MeCp, foram os compostos que revelaram os melhores resultados ao nível das suas atividades inibidoras e a melhor internalização para as linhas com os transportadores ABC estudados, sugerindo uma correlação entre as suas atividades e a sua internalização celular. Para além disso, revelaram melhor citotoxicidade para células sobre expressas.

Os compostos PMC79 e RT12, são os compostos estruturalmente mais parecidos, onde a única diferença é a existência do grupo metil no ciclopentadienilo para o RT12. Os resultados mostraram que estes dois compostos têm atividades biológicas muito parecidas. Ambos são mais citotóxicos para as células sem sobre expressão de transportadores do que para as células sobre

expressas e parecem não terem qualquer efeito inibitório para este tipo de células resistentes, contrariamente aos outros compostos estudados. Concluindo, pode-se afirmar que o grupo -$CH_2OH$, comum aos dois compostos e que os distingue dos restantes, terá um papel importante no efluxo dos mesmos, tornando-os substratos dos transportadores ABC.

Decorrente da avaliação dos estudos biológicos realizados, foi sintetizado com sucesso um novo complexo de ruténio, [Ru($\eta^5$-(Me-$C_5H_4$)($PPh_3$)(bipiridina-biotina)][$CF_3SO_3$] (**Ru2**). Este composto foi analisado por técnicas espetroscópicas como o RMN ($^1$H, $^{31}$P, $^{13}$C e técnicas bidimensionais), UV-Vis e FT-IR, e a sua pureza foi determinada por análises elementares. O complexo revelou também adequada estabilidade em meio celular (variação menor que 5 % às 24 h) e caráter lipofílico ($logP_{o/w}$= 1,6), o que nos assegurou continuação para os estudos biológicos neste novo composto.

Foi então avaliado, para **Ru2**, a viabilidade celular nas linhas celulares utilizadas anteriormente. Contrariamente aos resultados previamente obtidos, este novo complexo de ruténio é muito menos citotóxico para NIH3T3 WT, NIH3T3-P-gp e 2008C, sendo que não é citotóxico para as outras linhas celulares estudadas. Percebe-se também que este composto é um substrato para a P-gp e não tem qualquer efeito inibitório para esta ou outra proteína transportadora. Concluindo, pode-se afirmar que a coordenação da biotina e do grupo $\eta^5$-MeCp na mesma estrutura parece modificar a capacidade inibitória para P-gp e MRP2 como tinham os compostos LCR134, RT11 e LCR136. Este resultado revelou ser muito interessante, e como tal deve ser explorado em trabalhos futuros.

Deste modo este trabalho apresenta pela primeira vez o estudo de novos compostos de ruténio com fragmento 'Ru($\eta^5$-CpR)($PPh_3$)(bipiridina-R)' em células sobre expressas por transportadores ABC. A descoberta de que estes complexos de ruténio são inibidores para proteínas transportadoras abre novas possibilidades relativamente aos seus mecanismos de ação. Para além disso, tal como observado para outros compostos da literatura, verificou-se que pequenas alterações estruturais desencadeiam respostas biológicas muito diferentes mostrando a importância deste tipo de estudos que relaciona a estrutura com a atividade.

O objetivo deste trabalho foi então concluído com sucesso revelando que compostos de 'Ru($\eta^5$-CpR)($PPh_3$)(bipiridina-R)' poderão constituir uma ferramenta importante para o combate ao cancro, especialmente em cancros resistentes.

**Palavras-Chave**:

Transportadores ABC, resistência a fármacos, compostos organometálicos, complexos de ruténio ciclopentadienilo.

**Abstract**

Cancer is a global disease that affects most of the age ranges and is still one of the biggest concerns for the scientists worldwide. The research in this area is exhaustive and, fortunately, important developments are done year after year. However, there are some obstacles for the successful treatment such as multidrug resistance (MDR) that limits the drug efficacy. The main reason for this resistance lies in one type of proteins called ABC transporters. These proteins are overexpressed in cancer cell lines and allow the efflux of the drug out from the cell.

P-gp or ABCB1, MRP1 or ABCC1, MRP2 or ABCC2 and ABCG2 or BCRP are the most studied proteins belonging to the ABC family. Although the transport mechanism of each pump is still missing, one thing that the scientists are sure is that these proteins are responsible for the efflux of molecules out of the cells. To try to avoid this efflux, the identification of selective inhibitors that block the drugs efflux is being explored. The main challenge of this research is to find compounds that can act as high effective inhibitors while presenting low toxicity for healthy cells. Within this frame, the Organometallic Chemistry Laboratory from *Faculdade de Ciências da Universidade de Lisboa, Portugal*, and the Drug Resistance and Membrane Proteins in Lyon, France, studied the role of several ABC transporters on the mechanism of action of new ruthenium cyclopentadienyl compounds "Ru($\eta^5$-Cp)".

All the complexes were cytotoxic for the cell lines overexpressed and not overexpressed with ABC transporters and also for one cancer cell line, 2008C. Four compounds (PMC78, LCR134, RT11, LCR136) exhibited specific inhibitory activity for some of the ABC transporters studied. The amount of ruthenium internalization on the cell lines was also quantified by mass cytometry (CyTOF), indicating that, in all cases, the compounds are internalized. A molecular docking study was also carried out for one of the structures (LCR134) in P-gp protein revealing that a competition between LCR134 and the P-gp substrate might happen.

With the aim of optimizing the inhibitory activity of this family of compounds, a new ruthenium complex was synthesized, [Ru($\eta^5$-MeCp)($PPh_3$)(bipy-biot)][$CF_3SO_3$] **Ru2**, bearing the structural features inducing the best inhibition effects: a biotin molecule and a $\eta^5$-MeCp ligand. This compound was characterized by the usual techniques (NMR, UV-Vis and IR spectroscopies) and its purity was assessed by elemental analyses. **Ru2** was found to be very stable in cell medium (less than 5% variation over 24 h) and it has an hydrophobic character ($logP_{o/w}$= 1.6), allowing us to carry on with the biological evaluation.

The new compound was evaluated in the same cell lines as the previous compounds. Interestingly, this compound is much less cytotoxic for NIH3T3 WT, NIH3T3-P-gp and 2008C cell lines than the previously compounds studied, and is non-cytotoxic for all the other cell lines. Moreover, it seems that this compound is a substrate for P-gp pumps and does not have any inhibitory effect. To conclude, we can say that the biotin and $\eta^5$-MeCp motifs in the same complex do not improve the inhibitory potential, resulting, in contrast, in the loss of the inhibitory capacity.

Altogether, the proposed aims for this work were successfully achieved and allowed us to unravel an unprecedented mechanism of action for ruthenium cyclopentadienyl complexes that can be used as tool to fight the multidrug resistance in cancer.

**Key-words:**
ABC transporters, multidrug resistance, organometallic compounds, ruthenium cyclopentadienyl complexes.

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**Abbreviations**

**ABC**- ATP-Binding Cassette

**ABCB1**- ATP Binding Cassete protein B1

**ABCC1**-ATP Binding Cassete sub-family C1

**ABCC2**- ATP Binding Cassete sub-family C2

**ABCG2**- ATP-Binding Cassette sub-family G member 2

**ATP**- Adenosine Triphosphate

**BCRP**- Breat Cancer Resistance Protein

**COSY** – Correlation Spectroscopy

**Cp** – Cyclopentadienyl

**d** – dublet

**DMEM**- Dulbecco´s Modified Eagle Medium

**DMSO** – DiMethyl Sulfoxide

**DNA** - Deoxyribonucleic Acid

**DPBS**- Dulbecco´s Phosphate Buffered Saline

**EDC**- N-(3-Dimethylaminopropyl)-N´-ethylcarbodiimide

**EPR**- Enhanced Permeation and Retention

**ε** - Molar absorptivity

**FTIR** – Fourier Transform InfraRed

**GLUT1**- Glucose Transporter 1

**HEK293**-Kidney, Embryo from Human

**HMBC** – Heteronuclear Multiple Bond Correlation

**HSQC** –Heteronuclear Single Quantum Coherence

**IARC**- International Agency for Research on Cancer

**IC50**- Cytotxic Concentration 50

**ILCT**- Intraligand Charge Transference

**IR** – InfraRed

**LMCT**- Ligand-Metal Charge Transference

**m** – multiplet

**M**- Molar

**MCF7**- Michigan Cancer Foundation-7

**MDR**- Multidrug Resistance

**MFS**- Mouse FibroSarcoma

**mL** – millilitre

**MLCT**- Metal-Ligand Charge Transference

**MRP1**- Multidrug Resistance-associated Protein 1

**MRP2**- Multidrug Resistance-associated Protein 2

**MTT**- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

**NBD**- Nucleotide-Binding Domain

**NIH3T3**- National Institute of Health 3-day transfer, inoculum 3 x 105 cells.

**NMR 13C** – Carbon Nuclear Magnetic Resonance

**NMR 1H** – Proton Nuclear Magnetic Resonance

**NMR 31P** – Phosphorus Nuclear Magnetic Resonance

**PBS**- Phosphate Buffered Saline

**PFA**- Paraformaldehyde

**P-gp**- P-glycoprotein

**Ph** –Phenyl

**Pyd**- Pyridine

**RPMI**- Roswell Park Memorial Institute medium

**s** – singlet

**t – triplet**

**TMD**- Transmembrane Domain

**UV-Vis** – Ultraviolet-Visible

**WT**-Wild Type

**δ** – Chemical shift

**λ** – wavelength

**υ** –Absorption frequency

**µL**- microliter

## Introduction

### 1. Cancer

Cancer is a generic term for a large group of diseases that can affect any part of the body. Other terms used are malignant tumors and neoplasms. One defining a feature of cancer is the rapid creation of abnormal cells that grow beyond their usual boundaries, which can then invade adjoining parts of the body and spread to other organs, the latter process is referred to as metastasizing (Fig.1.1) Metastases are a major cause of death from cancer. [1]

According to estimates from the International Agency for Research on Cancer (IARC)[2], cancer is a leading cause of death worldwide, accounting for 8.8 million deaths in 2015. The most common death by cancer are:

- Lung (1.69 million deaths)
- Liver (788 000 deaths)
- Colorectal (774 000 deaths)
- Stomach (754 000 deaths)
- Breast (571 000 deaths)

By 2030, the global burden is expected to grow to 21.7 million new cancer cases and 13 million cancer deaths simply due to the growth and aging of the population.[2] The future burden will probably be even larger because of the adoption of western lifestyles, such as smoking, poor diet, physical inactivity, and fewer childbirths, in economically developing countries.

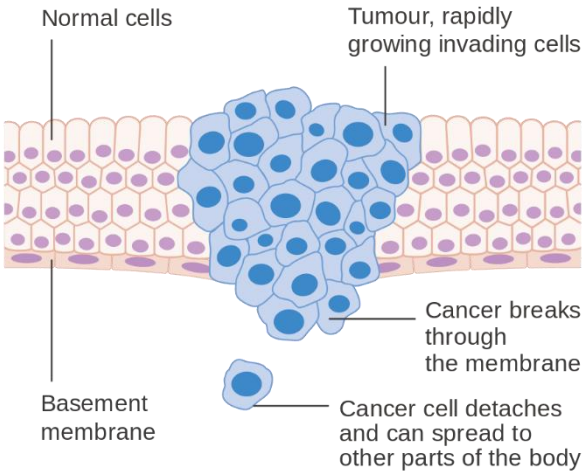



*Figure 1.1*- Cancer cells invading adjoining parts of the body (metastasis)[1].

Cancer arises from the transformation of normal cells into tumor cells in a multistage process that generally progresses from a pre-cancerous lesion to a malignant tumor. These changes are the result of the interaction between a person's genetic factors and three categories of external agents, including: physical carcinogens, such as ultraviolet and ionizing radiation; chemical carcinogens, such as asbestos, components of tobacco smoke, aflatoxin (a food contaminant), and arsenic (a drinking water contaminant); and biological carcinogens, such as infections from certain viruses, bacteria, or parasites.[3]

### 1.1 Barriers to the cancer treatment

Because of all the facts described above, cancer is considered one of the deadliest diseases worldwide. A major concern regarding chemotherapy, one of the first line treatments in cancer therapy, is the rise of drug resistant phenotypes that considerably limit the efficiency of the drugs. Drug resistance arises through several mechanisms, it is either inherent (*i.e.* at the first treatment), or acquired (*i.e.* after subsequent treatments). After a long-term drug use, resistance appears not only to the respective drug but also to a series of structurally-unrelated drugs.[4,5,6]

Multidrug resistance (MDR) refers to the cancer cells ability to resist to a broad variety of structurally and mechanistically different anticancer drugs, which is one of the major clinical obstacles in cancer chemotherapy. MDR is responsible for more than 90% of treatments failure of metastatic cancer using adjuvant chemotherapy. [4]

MDR can be caused by several mechanisms, such as efflux transporters. One of the most common mechanisms of MDR is the overexpression of adenosine triphosphate (ATP)-binding cassette (ABC) superfamily of transporters, which mediate the efflux of anticancer drugs to limit the effective use of chemotherapeutic drugs.[7] Among these ABC transporters, the ABC transporter subfamily B member 1 (ABCB1), subfamily G member 2 (ABCG2) and subfamily C member 1 and 2 (ABCC1/2) have been reported to play important roles in inducing MDR in several cancers, such as lung, breast, colon, ovarian cancers and melanomas.[7] These pumps significantly reduce the intracellular concentration of anticancer compounds. In this frame, developing inhibitors for these transporters is a promising strategy to overcome MDR and retrieve the effective need of conventional anticancer drugs.[8]

#### 1.1.2 ABC Transport Proteins

The ATP-binding cassette (ABC) transporter superfamily is among the largest and the most broadly expressed protein superfamilies known. These proteins are responsible for the active transport of a wide variety of compounds across biological membranes, including phospholipids, ions, peptides, steroids, polysaccharides, amino acids, organic anions, bile acids, drugs, and other xenobiotics.[9]

ABC transporters are widespread in all forms of life and are characterized by two nucleotide-binding domains (NBD) and two transmembrane domains (TMDs). ATP hydrolysis on the NBD drives conformational changes in the TMD, resulting in alternating access from inside and outside of the cell for unidirectional transport across the lipid bilayer.[10] This means that ABC transporters are responsible for the ATP dependent movement of a wide variety of xenobiotics, including drugs, lipids and metabolic products across the plasma and intracellular membranes. Overexpression of certain ABC transporters occurs in cancer cell lines and tumors as an answer to the chemical stress conferring resistance, not only to the anticancer drug used, but also to other drugs transported by the pump, consequently extending the MDR phenotype of the cancer cells.[11]

Their contribution to multidrug resistance in tumor cells is well documented, making them privileged targets to tackle MDR.[12]

### 1.1.3 ABC transporters that confer multidrug resistance

Resistance to multiple anticancer agents is a major impediment for the successful treatment of many forms of malignant disease. In tumor cell lines, multidrug resistance is often associated with an ATP-dependent decrease in cellular drug accumulation which was originally attributed to the overexpression of a single protein, the 170-kDa ABC drug transporter P-glycoprotein (P-gp; encoded by ABCB1). The isolation of a second distantly related protein (MRP1; encoded by ABCC1) facilitated the discovery of more genes, such as MRP2 (encoded ABCC2); a third drug transporter, also distantly related to P-glycoprotein and the MRPs, is the breast cancer resistance protein (BCRP[i]; encoded by ABCG2).[13]

Increased expression of MRP1 and P-glycoprotein has been reported in a variety of haematological and solid tumors, suggesting a significant role for these transport proteins in clinical drug resistance.[14] In addition to their role in drug resistance, MRP1, MRP2, P-gp and ABCG2 (Fig.1.2) are expressed in non-malignant tissues and are believed to be involved in protecting tissues from xenobiotic accumulation. For example, MRP1 was found in high levels in the lung, testis, kidneys and skeletal muscle and ABCG2, P-gp and MRP2 were found in the blood-brain barrier, placenta, liver, gut, and kidney.[9] The problem is that when this xenobiotic accumulation protection happens in cancer cells they will act like pumps and prevent the action of anticancer drugs with the efflux of these drugs out of the cell. In this context, these ABC transporters can confer resistance to anticancer drugs. The involvement of such pumps in chemoresistance requires elucidation of the mechanisms of multidrug export and a targeted inhibition. These subjects will be developped in the next chapters.

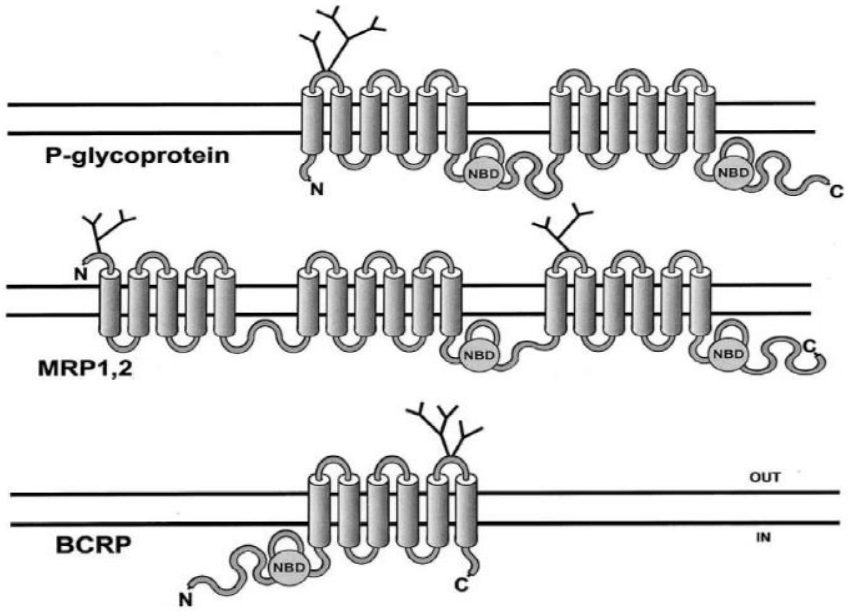



*Figure 1.2*- ABC transporters. Consists of two transmembrane domains, each containing 6 transmembrane segments, and two nucleotide binding domains (NBDs). N and C denote amino- and carboxy-terminal ends of the proteins, respectively. Cytoplasmic (IN) and extracellular (OUT) orientation indicated for BCRP applies to all transporters drawn here. Adapted from [14].

[i] Although commonly referred to as BCRP, there is no evidence at present that this transporter is preferentially expressed in normal or malignant breast tissue and its clinical relevance is not yet well established.[13]

### 1.1.4 Type of ABC transporter and their mechanism

With few exceptions, ABC transporters must transport substrates against a chemical gradient, a process that requires ATP hydrolysis as a driving force. In the case of these transporters, conformational switching of the membrane domain for providing alternating access is driven by the binding of transport substrate and MgATP, followed by ATP hydrolysis and product release.[10] There is little evidence to suggest that all ABC transporters function by the very same mechanism. Figure 1.3 illustrates how the mechanism of ABC transporter works:

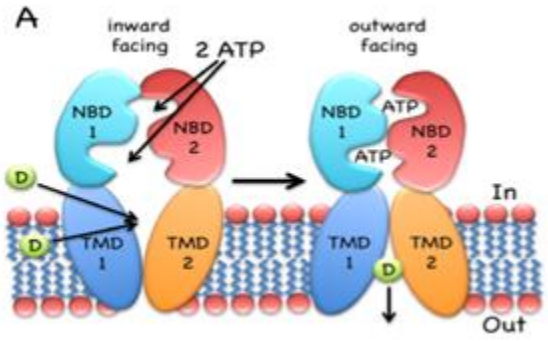



Figure 1.3-The inward-facing exporter binds substrate "D" (drug) from the cytoplasm or the inner leaflet of the bilayer. After binding two molecules of MgATP, the nucleotide-binding domains (NBDs) dimerize and switch the transmembrane domain (TMDs) from the inward- to the outward-facing conformation, followed by the release of the drug to the extracellular milieu. ATP hydrolysis, ADP/Pi release and NBD dissociation reset the transporter to the inward-facing conformation. Adapted from [10].

Even if these protein pumps share similar functions and mechanisms, they also comprise several differences. Thus, it is important to know more about each of these ABC transporters in order to better fight the problem of chemo resistance.

**P-glycoprotein (P-gp)**

The human P-glycoprotein (P-gp/ABCB1) was the first human ABC transporter identified and has been studied extensively. P-gp is an integral membrane protein that actively pumps exogenous compounds out of cells. The expression of P-gp is up-regulated in many cancer cells, where it reduces the intra-cellular concentrations of many chemotherapeutic drugs, thereby conferring multidrug resistance. P-gp is one of the best-known pumps in this context, in addition to multidrug resistance protein 1 and 2 (MRP1/2 or ABCC1/2) and breast cancer resistance protein (BCRP/ABCG2). P-gp transports a broad spectrum of molecules sharing a marked hydrophobicity but structurally divergent.[15]

**MRP2**

Multidrug resistance protein 2 (MRP2/ ABCC2), also referred to as multi-specific organic anion transporter, is a membrane drug efflux pump belonging to the ATP-binding cassette (ABC) transporter subfamily C (ABCC). This ABCC subfamily comprises eight other MRPs, including

at least six drug transporters. MRP2 plays an important role in the membrane transport of various drugs, including organic anions and anticancer agents.[16]

MRP2 transports a diverse set of substrates and endogenous molecules, such as amphipathic chemicals, drug conjugates and has an important role in tissue distribution and elimination. The expression and function of this export pump are highly significant in a lot of tissues such as the renal proximal tubular cells and intestinal epithelial cells that also express MRP2. MRP2 expression is responsive to several drug treatments and is associated with diseases affecting the liver.[17,18]

**ABCG2**

It has been established that ABCG2/ABCRP functions as a high capacity drug transporter with wide substrate specificity. This protein can transport large, hydrophobic, either positively or negatively charged molecules, including cytotoxic compounds and fluorescent dyes.[19]ABCG2 mediates the extrusion of the transported compounds towards the extracellular space through a process energized by ATP hydrolysis.

The overexpression of ABCG2 was observed in certain drug-resistant cell lines and tumors, providing a special multidrug resistant phenotype in these cancer cells. Human ABCG2 was shown to confer resistance against various, clinically relevant compounds. Based on the role of ABCG2 in tumour resistance described above, the selective and sensitive detection of the ABG2 protein, as for the other ABC proteins, has a major importance in cancer diagnostics and treatment.[20]

**MRP1**

Multidrug resistance protein 1 (MRP1/ABCC1), also referred to as multi-specific organic anion transporter, is a membrane drug efflux pump belonging to the ATP-binding cassette (ABC) transporter subfamily C (ABCC). This ABCC subfamily comprises eight other MRPs, as MRP2 already mentioned.[16]

MRP1 is widely expressed in normal tissues and cellular organelles, particularly in the testis, kidneys, placenta and at pharmacological barriers. MRP1's capacity for drug efflux prevents effective treatment of a range of diseases, beyond cancer, including clinical depression and epilepsy. The overexpression of MRP1 across a range of cancers has led to relapse and drastically reduced overall survival in cancer patients.[21,22,23]

### 1.1.5 Targeted Inhibition

In chemoresistance, the ABC transporters act as pumps in the membrane of the cell, effluxing the drugs out of the cell. One way to prevent this chemoresistance of the cancer cells is to find out specific inhibitors of drug efflux. In the absence of an inhibitor, ABC transporters utilize energy derived from the hydrolysis of ATP to efflux the anticancer drug crossing the membrane (Fig.1.4). The specific inhibitors can interact with the proteins and, when the drug

enters the cell, the inhibitor represses the majority of the drug to comes out, by modifying the substrate-binding site of the ABC transporter protein and, consequently, there is a strong decrease of the efflux of substrate drugs by ABC transporter.[4]

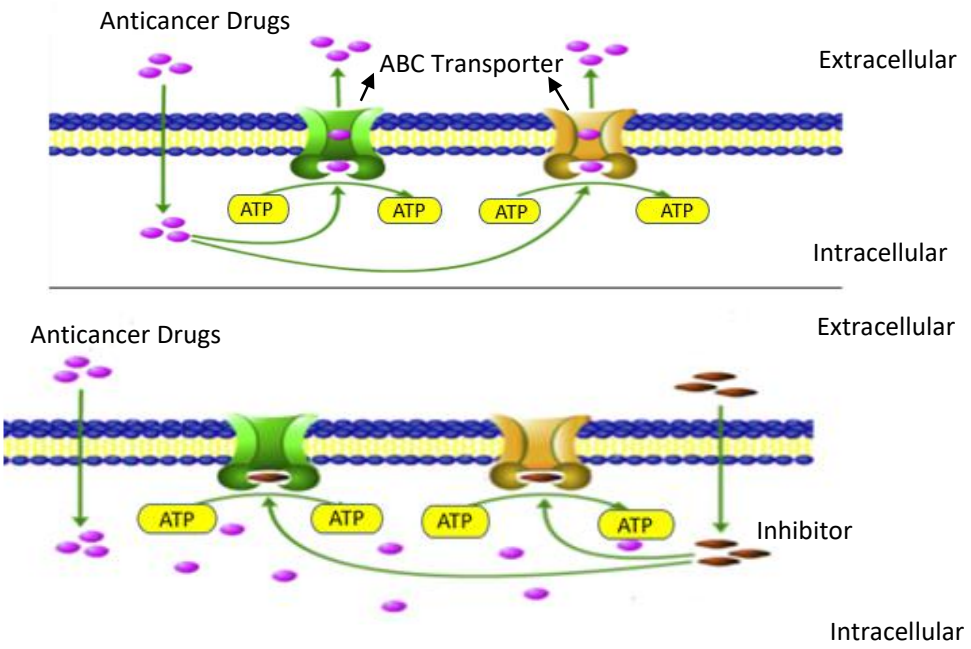



Figure 1. 4-Mechanism of ABC transporters inhibition. Adapted from[4].

The problem is the difficulty in finding adequate inhibitors for each type of ABC transporters. Since 1980, researchers have been searching for specific inhibitors that can reverse MDR in cancer cells.[24] Tremendous efforts have been made to discover and synthetize such inhibitors. Several examples of ABC drug transporter inhibitors have been discovered or synthetized but finding potent inhibitors that are selective, low in intrinsic toxicity and highly effective has been more difficult than expected.[24] So the research in this area continues and it is crucial for new developments against MDR.

#### 1.1.5.1 Substrates and Inhibitors

One of the methods for identifying mechanisms of MDR was to select surviving cancer cells in the presence of cytotoxic drugs and use cellular and molecular biology techniques to identify altered genes that confer drug resistance on native cells.[25] Such studies proved that there were some mechanisms of drug resistance in cells, and one the most commonly encountered was the increased efflux of a broad class of hydrophobic cytotoxic drugs that is mediated by ABC transporters. [19]

The main roles of ABC transporters are based on their ability to expel a wide variety of drugs from cells. Explaining this extremely broad substrate recognition remained a major

challenge to the scientists for decades, even though a better understanding of the molecular mechanism of these transporters is crucial for the generation of structure-based specific drugs and inhibitors. Similar to enzymes, most membrane transporter proteins specifically bind to one or to a limited number of substrates in a well-defined binding pocket. Following substrate recognition, transporter proteins translocate the transported substrate from one side to the other side of the membrane.[19] However, this classical mechanism cannot be directly applied to the multidrug transporters, which recognize an exceptionally large number of chemically unrelated compounds as substrates. Since the transported substrates of the multidrug transporters are mostly lipophilic, the hypothetical models suggested less specific, hydrophobic substrate-transporter interaction within the lipid bilayer of the membrane, due to some experimental data indicated that ABC pumps are capable of extruding their substrates before they reach the cytosol.[26]

The broad substrate specificity and the abundance of ABC transporter proteins might explain the difficulties faced during the past 20 years in attempting to circumvent ABC-mediated MDR *in vivo*. Cancer pharmacologists have worked to develop drugs to inhibit the function of efflux transporters, and although progress in this area has been slow, the rationale for this approach is still strong.[25]

The potential involvement of the overproduction of drug pumps in clinical drug resistance in tumor cells has led to the search for compounds that can be used to inhibit these transporters in cancer patients. These inhibitors should preferably be: i) selective and bind to the transporters with a high affinity, ii) non-toxic for healthy cells, and iii) stable in human plasma. Several compounds have been described that effectively block MDR mediated drug resistance, some of which were tested in the clinic trials, and currently the combination of topotecan and elacridar, that inhibits P-gp and ABCG2, is administrated orally in breast cancer patients.[27,28]

A strategy to understand if some ruthenium organometallic complexes can act as inhibitors for these particular pumps will be presented in this project.

## 1.2 Metallodrugs in cancer therapy

The discovery of the anticancer properties of cisplatin, cis-$Pt(NH_3)_2CL_2$ (Fig.1.5), in 1965 is possibly the most significant and life-changing breakthrough in bioinorganic chemistry.[29] Cisplatin rapidly became one of the most widely used anticancer drugs and it is estimated that it is still used today, in combination with other drugs, in 50-70% of all cancer patients.[29]

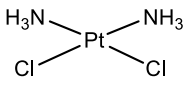
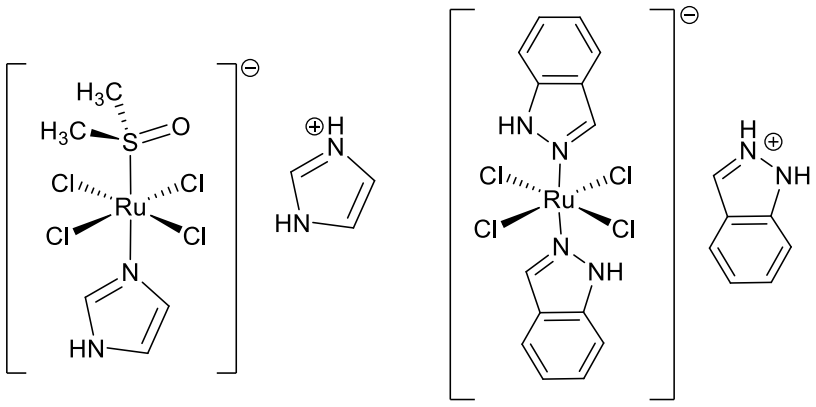



*Figure 1.5*- Cisplatin structure

However, the success of platinum-based drugs for the treatment of cancer is accompanied by high general toxicity, resulting in undesirable side-effects. So, the development of potential alternative non-platinum-based anticancer drugs is crucial.

A vast number of metal complexes, other than platinum, have been evaluated as potential anticancer chemotherapeutics. Some of the most promising metallic compounds as anticancer chemotherapeutics that reached clinic trials include two ruthenium(III) complexes, KP1019 and NAMI-A (Fig.1.6). Although NAMI-A and KP1019 have similar structures, their biological activity is completely different. KP1019 is mostly used against primary tumors and NAMI-A attack the metastases of tumors. Some of the problems encountered for these two compounds in the progression into more advanced clinical trials are related to the aqueous instability of the complexes.[30]




**NAMI-A** **KP1019**

Figure 1.6-Structures of anticancer agents NAMI-A and KP1019, first ruthenium compounds in clinical trials.

During the last decade, other structurally different families of ruthenium compounds have been synthesized, some of them also exhibiting interesting potential.[31]

### 1.2.1 Ruthenium organometallic compounds with the '$\eta^5$-$C_5H_5$' unity

A family of organometallic compounds bearing the {Ru($\eta^5$-cyclopentadienyl} scaffold (Fig.1.7) have been identified as promising anticancer agents.[31] All these organometallic compounds have a piano-stool structure, where three of the coordination sites are occupied by the ($\eta^5$-cyclopentadienyl) ligand, which serves to stabilize the Ru(II) center. The three remaining coordination sites are occupied by diverse *co*-ligands that are able to modulate the cytotoxicity and stability of the compounds.[32] Aromatic cyclopentadienyl ligands π-bonded (Cp) to the metal centre have attracted much attention due to their ability to act as a donor and electron acceptor group. Therefore, they can modify the acceptor/donor character and the reactivity of the other *co*-ligands in the complex. Besides stabilizing the metal centre, the Cp ligand provides a hydrophobic surface which might facilitate passive transport through the cell membrane.[32]

In this frame, over the last years our group has been exploring the potential applications as anticancer agents of half-sandwich compounds based on the "Ru($\eta^5$-Cp)" fragment.[31]Most of these compounds presented cytotoxic activities against a several of human cancer cell lines, such as A2780 (ovarian carcinoma cell line), A2780cisR (cisplatin resistant cell line), MFC7 (breast

cell line) and HL-60 (Human Leukaemia cell line). In addition, these complexes present, in most cases, lower $IC_{50}$ values than cisplatin.[29,5]

In particular, within the ruthenium(II) family of general formula $[Ru^{II}Cp(PP)L]^+$ (Fig.1.7), where L is a nitrogen sigma-bonded N-heterocyclic ligand (1,3,5-triazine, pyridazine) and a PP a phosfane ligand (1,2-bis(ddiphenylphosphane)ethane or triphenylphosphane) exhibited excellent cell viability inhibition of LoVo human colon adenocarcinoma and MiaPaCa pancreatic cell lines.[33,34] A second set of $Ru^{II}(Cp)$ complexes with imidazole, 5-phenyl-1H-tetrazole or N-cyano ligands (benzo[1,2-b; 4,3-b]dithio-phen-2-carbonitrile; [5-(2-thiophen-2-yl)-vinyl]-thiophene-2-carbonitrile) also exhibited excellent activity against HL-60 cells.[35] In addition, all complexes of this set induced cell death mainly by apoptosis.[34,35]

The compound $[Ru(\eta^5\text{-}C_5H_5)(PPh_3)_2Pyd][CF_3SO_3]$, bearing a mono-coordinated N-heteroaromatic ligand was also synthesized and its $IC_{50}$ values were within the lowest observed for three-legged piano-stool ruthenium complexes in MiaPaCa and LoVo cell lines.[34]

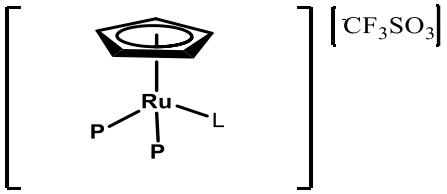



Figure 1.7-General structure of the compounds from the $[Ru^{II}(\eta^5\text{-}Cp)(PP)L][CF_3SO_3]$ family, where PP is mono or bidentade phospane ligand and L=N donor ligand.

Later, interesting features for the complex $[Ru^{II}(\eta^5\text{-}C_5H_5)(bipy)(PPh_3)][CF_3SO_3]$, (bipy = 2,2'-bipyridine), TM34 (Fig.1.8) have been described.[36] This complex was five times more active towards HL-60 cells than cisplatin and was also found to be seventeen times and two hundred times more active than cisplatin in ovarian cancer cell line A2780 and in A2780cisR, respectively. Furthermore, TM34 induced the same percentage of apoptotic and damaged or necrotic cells as cisplatin.[36,37]

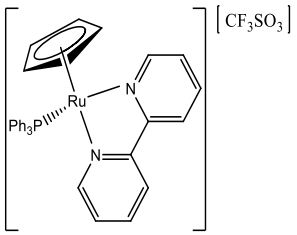



*Figure 1.8*- Structure of $[Ru^{II}(\eta^5\text{-}C_5H_5)(bipy)(PPh_3)][CF_3SO_3]$ (TM34).

Progress in research has been made, combining coordination chemistry with polymerization, to generate polymer-metal complexes (PMCs).[38,39] These macromolecules present several advantages when compared with low molecular weight compounds, such as their easier accumulation in the cancer cells by the "enhanced permeation and retention" (EPR) effect. EPR is a phenomenon by which macromolecules tend to accumulate more in solid tumor tissues

than in normal tissues, increasing the therapeutic index, (the drug concentration in tumor compared to that of the blood) can be as high as 10-100 times.[38]

In this frame, polymer-metal conjugates constitute a promising alternative to the conventional drug approaches in cancer therapy.[39] In fact, the RuPMC compound (Fig.1.9), has shown to enter the MCF7 cancer cells and to be retained in the nucleus fraction, while its low molecular weight related compound TM34 is mainly found in the membrane.[38] Also, RuPMC is more active than other reported polymer-metal conjugates of platinum and ruthenium in MCF7 and A2780 cancer cell lines.[38]

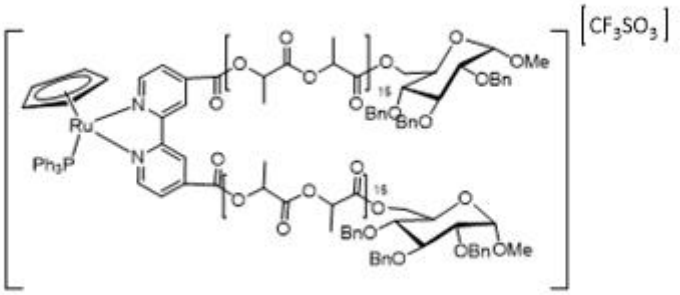



Figure 1.9-Structure of RuPMC

Other compounds from this family were also recently tested, PMC78 and PMC85 (Fig.1.10). The results obtained show that these ruthenium-based compounds present lower $IC_{50}$ than cisplatin in MDA-MB-231 breast cancer derived cells.[40] PMC78 and PMC85 were also tested in MCF7 breast cancer derived cell line and in A2780 ovarian cancer derived cell line. Both compounds show low values of $IC_{50}$ comparable to those of cisplatin.[40]

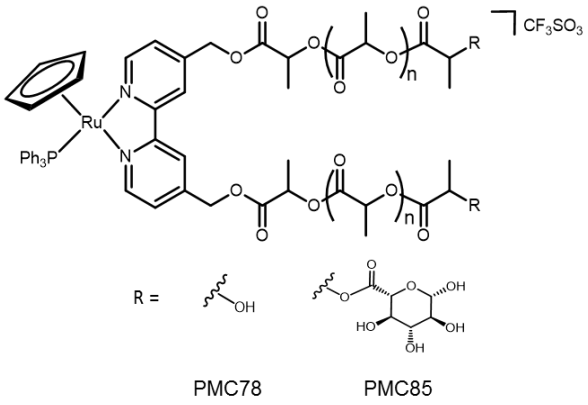



Figure 1.10-Structure of PMC78 and PMC85

The low molecular weight parental complex of PMC78 and PMC85 has been also synthesized and its activity against cancer cells determined.[40] PMC79, $[Ru^{II}(\eta^5\text{-}C_5H_5)(bipy\text{-}CH_2OH)(PPh_3)][CF_3SO_3]$ (Fig.1.11), was tested in MDA-MB-231 breast cancer derived cells and

the results obtained show that this ruthenium-based compound presents eight-fold lower $IC_{50}$ when compared to cisplatin. For MCF7 breast cancer derived cell line and in A2780 ovarian cancer derived cell line, this compound revealed also low $IC_{50}$ values comparable to cisplatin, as observed for the polymer-ruthenium conjugates.[40]

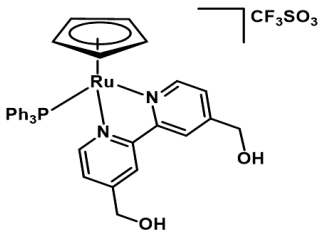



*Figure 1.11*- Structure of structure $[Ru^{II}(\eta^5\text{-}C_5H_5)(N, N)(PPh_3)][CF_3SO_3]$(PMC79)

The anticancer effects of the three compounds, PMC79, PMC78 and PMC85 was also determined in the colorectal cancer derived cell lines, revealing low $IC_{50}$ values of the same order of magnitude as those obtained for the MDA-MB-231 breast cell line.[41]

To evaluate the mechanism of cells death, possible targets and antimetastatic potential for these compounds several studies were undertaken. Briefly, the compounds induced hypertrophy of mitochondria and cell death by apoptosis.[41] A clear reduction in the formation of colonies was also observed, as well as a significant decrease of the migratory ability of cancer cells in comparison with the controls.[41] These results suggest that these compounds have antimetastatic potential.[41]

Since PMC85 has glucose terminal groups, the expression of GLUT1 after 48 h of exposure to the compound was also studied.[41] The results showed that all of the three ruthenium-based compounds, reduced GLUT1 expression of MDA-MB-231, specially the glucose derivative PMC85.[41] Moreover, cell fractioning assays showed that PMC78 and PMC85 are mostly accumulated in the cytoskeleton of cancer cells, while PMC79 is manly accumulated in the membranes.[41] Treatment of the cancer cells with the different ruthenium based compounds induce strong changes in the cytoskeleton of the cells, indicating that this organelle might be a cellular target.[41]

All these results suggest that PMC78, PMC79 and PMC85 have a higher potency anticancer activity, relatively to the platinum-derived agent, cisplatin.

## 1.3 Context and Objectives of this Project

The development of pharmacologic agents that can block ABC transporter proteins raises the possibility of circumventing active drug efflux as a mechanism of chemo resistance, a strategy that has been reported successful in some diseases.[18]

Being cancer one of the leading causes of death worldwide, the search for new anticancer drugs is a subject of utmost importance. Ruthenium is an appealing candidate to be used in anticancer drugs and several of its complexes have already shown anticancer properties. In particular, the 'Ru-Cp' family of compounds, which is being developed at the Organometallic Chemistry Laboratory from *Faculdade de Ciências da Universidade de Lisboa,* has shown a wide range anticancer activity with different mechanisms of action and cellular targets than the typical platinum-based drugs, making the research in this topic a very relevant subject.

Therefore, this work aims to combine the solid knowledge and the experience of two research groups, *i.e.* the "Drug Resistance and Membrane Proteins team" specialized in ABC proteins and "Bioinorganic Chemistry and Drug Development" Group, specialized in the development of new organometallic anticancer agents, to reach a final goal: finding the role of ABC proteins in the mechanism of action of promising ruthenium anticancer agents.

To achieve this goal, seven compounds bearing the same $[Ru(\eta^5\text{-}CpR')(2,2'\text{-bipyridine-}R)(PPh_3)]^+$ (Fig.1.12) core have been selected. The work has the following general objectives:

i) Assessment of the cytotoxicity of the compounds under study by the MTT assay;
ii) Inhibition assays by cytometry techniques on cells that overexpress ABC transporters, in order to evaluate the ability of compounds to block ABC pumps;
iii) Ruthenium quantification in the overexpressed cells by mass cytometry technique (CyTOF) in order to quantify the ruthenium abundance inside the cells;
iv) Based on the results from i)-iii), synthesis and characterization of a new ruthenium compound that might enhance the previously results;
v) Evaluation of the biological activity of the new compound and compare to the others tested.

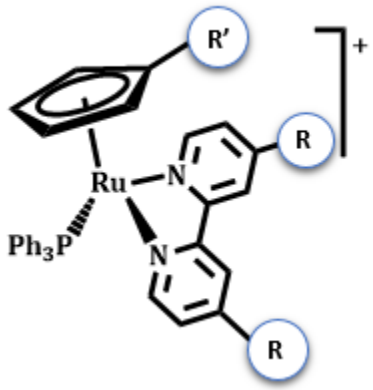



Figure 1. 12-General structure of $[Ru(\eta^5\text{-}CpR')(2,2\text{-bipyridine-}R)(PPh_3)]^+$

## 2. Biological Evaluation

### 2.1 Compounds under study

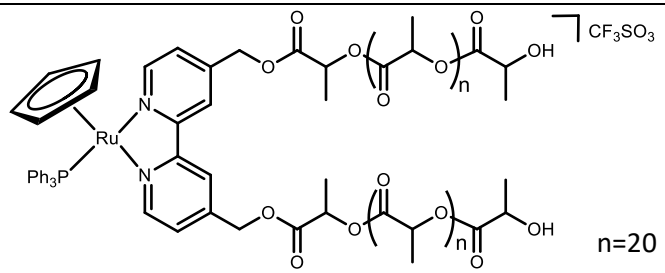
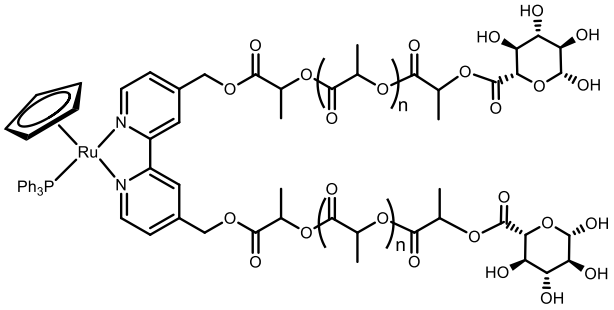
In this work, seven new ruthenium compounds, previously synthetized in our research group at FCUL were used. All these compounds share the "Ru-cyclopentadienyl bipyridine" core in their structure and the same phosphane ligand (triphenylphosphane). The compounds were judiciously selected in order to infer about the importance of substituents on the bipyridine and on the $\eta^5$-Cp over the activity, selectivity and inhibition potential on ABC transporters. According to the research evaluation previously made by our group, it was observed that this type of structure displayed interesting results in terms of cytotoxicity for cancer cell lines, thus understanding the role of the ABC transporters on their activity is of upmost importance. Table 2.1 shows the compounds selected, and summarizes some relevant features in the frame of this thesis. All compounds showed excellent activities towards the cell lines tested with $IC_{50}$ values in the low micromolar range. PMC78 and PMC85 are polymer-ruthenium conjugates and both have shown important features as anticancer agents. Previous studies have also shown that resistant cells (A2780cisR) treated with PMC78 and PMC85, accumulate similar levels of ruthenium than the sensitive cells (A2780), which could avoid drug resistance. PMC79, the low molecular weight parent compound of PMC78 and PMC85 has also revealed good results for cytotoxicity assays, but a different mechanism of action seems to be operating. In this case, an important difference in the Ru accumulation levels between A2780 and A2780cisR was observed, which could mean that PMC79 is more efficient for the sensitive cancer cell line that the resistant one. This suggests that PMC79 might be subject to some resistance mechanisms that need to be further explored.

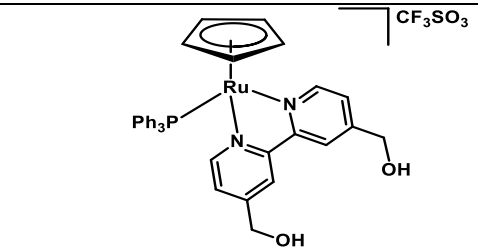
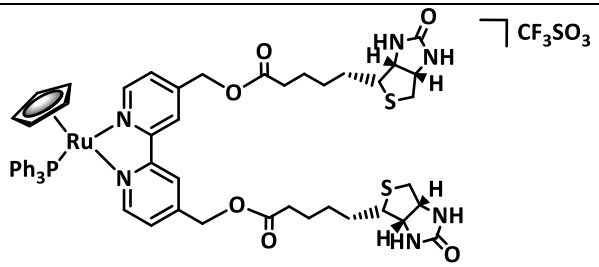
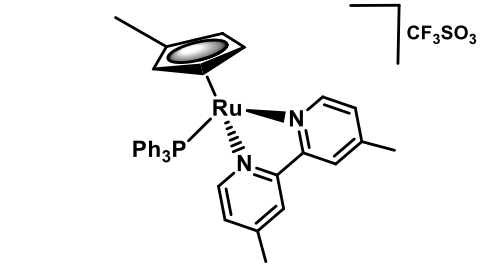
Complex LCR134 has two biotin molecules linked to the bipyridine. Biotin, commonly known as vitamin H or B7, is essential for our organism. There are several bibliographic studies reporting cell membrane transporters uptaking biotin into cancer cells.[42] One of those studies reported the role of the ABC importers[ii]. For example, *Walker and Altman*[43] reported that Gram-negative *E.coli*, *Salmonella enterica serovar Typhimurium* and *Pseudomonas aeruginosa* can import 10-31 amino acid peptides, once conjugated with biotin. Despite that, there is no study reporting the biological activity of an anticancer compound linked to biotin on the activity of the ABC transporters to be studied in this project (exporters proteins). Thus, LCR134 seems like a good model to be studied.

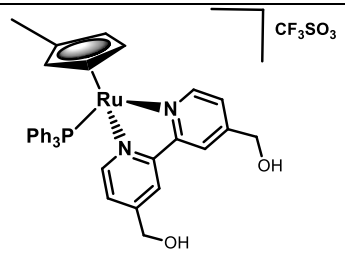
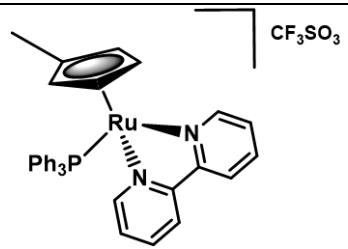
For the sub-family {Ru($\eta^5$-metilcyclopentadienyl)} (compounds LCR136, RT11 and RT12) we aim to find any correlation between the biological activity that might be related to the different substituents on the bipyridine ligand. All the compounds show good stability at 24 h in the cellular media DMEM (containing up to 5 % DMSO), allowing their study.

[ii] Other type of ABC transporters, that instead of export can import substrates into the cell.

Table 2.1-Structure, name, molecular weight, $IC_{50}$ against A2780 ovarian cells at 72 h and *24h at 37 ºC, and stability of all the compounds under study for biological evaluation.

| Compound | Reference | Molecular Weight, g/mol | $IC_{50}$ µM | Stability | Obs. |
|---|---|---|---|---|---|
|  n=20 | PMC78 | 4206 | 3.4 ± 1.3 | Stable in DMEM (variation at 24 h < 2 %) | - |
|  n=20 | PMC85 | 5580 | 2.2 ± 0.85 | Stable in DMEM (variation at 24 h < 2 %) | - |

| | | | | | |
|---|---|---|---|---|---|
|  | PMC79 | 793.75 | 3.9 ± 1.3 | Stable in DMEM<br>(variation at 24 h < 4 %) | - |
|  | LCR134 | 1248.36 | - | - | $IC_{50}$ calculated in this project. |
|  | RT11 | 839.95 | *1.67 ± 0.27 | Stable in DMEM<br>(variation at 24 h < 10 %) | - |

| | | | | | |
|---|---|---|---|---|---|
|   | RT12 | 871.95 | *2.26 ± 0.60 | Stable in DMEM (variation at 24 h < 5 %) | - |
|   | LCR136 | 811.90 | *2.06 ± 0.6 | Stable in DMEM (variation at 24 h < 1 %) | - |

## 2.2 General Information

In this work HEK293 (human embryonic kidney cell line), NIH3T3 (embryo mouse fibroblast cell line) and 2008C (Ovarian cancer cell line) cells were used to test the seven organometallic ruthenium compounds. The HEK293 cells were either wild-type (WT, transformed with an empty vector) and the same transfected with a plasmid containing a gene coding for the transporters proteins: ABCG2, MRP1, MRP2. NIH3T3 cells were used equally to test the P-gp.

Substrates that can be transported out of the cell by the ABC transporters were used as positive and negative controls, either in a specific way or in a non-specific way. Reference inhibitors (compounds that can block ABC pumps) were used. The substrates used were: calcein AM for the cells with MRP1 and MRP2 transporters, mitoxantrone for ABCG2 transporters and Rhodamine 123 for P-gp transporters.[44,45,46] Concerning the control inhibitors, verapamil was used for MRP1, cyclosporine A for MRP2, Ko143 for ABCG2 and GF120918 was used for P-gp. All these compounds were chosen because, according to the literature, these are the most potent and appropriate for this type of transporters, in the presence of the substrates used.[8,22,23,29]

## 2.3 Cellular Viability

In the search for new drugs to be applied in therapy, the $IC_{50}$ (half maximal cytotoxic concentration) values of the tested compounds should ideally be low. Therefore, the $IC_{50}$ values of new compounds are one of the first parameters assayed *in vitro*.[47]

Cell survival was studied using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) colorimetric assay for the ovarian cancer cell line 2008C (for those compounds we had less information up to date) and in HEK293 WT and overexpressed with ABCG2, MRP1 and MRP2, and in NIH3T3 WT and overexpressed with P-gp.

As observed from Table 2.2 all the complexes tested in the 2008C cancer cell line, which is considered a sensitive cell line to chemotherapy[48], show high cytotoxicity with $IC_{50}$ values in the low micromolar range at 48 h incubation.

Table 2.2-$IC_{50}$ (μM) for ruthenium complexes with a range between 2,5-100 μM, at 48 h incubation expressed as a mean ± SD, in ovarian cancer cell line, 2008C.

| | **$IC_{50}$ (μM)** |
|---|---|
| LCR134 | 4.5 ± 0.08 |
| LCR136 | 2.1 ± 0.1 |
| RT11 | 1.1 ± 0.07 |
| RT12 | 4.1 ± 0.2 |

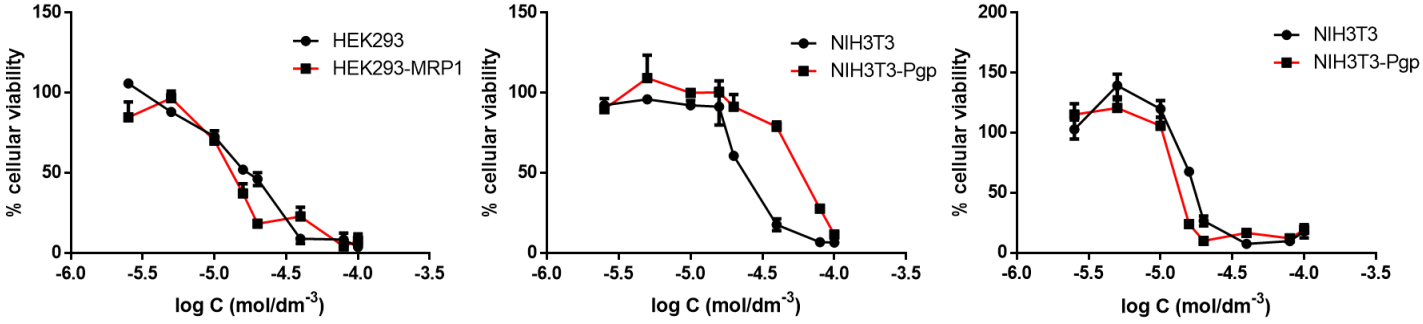
Concerning the HEK293 and NIH 3T3 cells, the $IC_{50}$ values were obtained after 48 h incubation in the presence of the complexes within concentrations range 1-100 μM. From the results obtained (Table 2.3), we can conclude that all the compounds show activities towards the cell lines tested with $IC_{50}$ values in the low micromolar range and, consequently, they are all cytotoxic for the cell lines mentioned.

Table 2.3-*In vitro* cytotoxicity activity of ruthenium complexes in the cell lines HEK293 and NIH3T WT and overexpress ABC transporters at 48 h, 37 °C, measured as the half cytotoxicity concentration ($IC_{50}$). The WT cells (HEK293 and NIH3T3) are shown on the left side.*Experiments that need to be repeated.

| $IC_{50}$ (μM) | | | | |
|---|---|---|---|---|
| | NIH3T3 / P-gp | HEK293 / MRP1 | HEK293 / MRP2 | HEK293 / ABCG2 |
| PMC78 | 27.0 ± 2.1 / 18.0 ± 1.3 | 16.8 ± 0.5 / 12.9 ± 0.5 | 19.6 ± 0.6 / 18.9 ± 0.5 | 19.4 ± 1.4 / 21.4 ± 1.6 |
| PMC79 | 28.1 ± 1.2 / 67.8 ± 3.5 | 4.8 ± 0.1 / 10.0 ± 0.4 | 1.6 ± 2.2 / 4.9 ± 0.9 | 6.7 ± 0.2 / 19.1 ± 0.6 |
| PMC85 | 4.3 ± 0.2 / 2.8 ± 0.1 | 11.3 ± 0.4 / 7.4 ± 0.5 | 7.4 ± 0.2 / 7.0 ± 0.3 | 5 ± 2.8 / 3.7 ± 1.9 |
| LCR134 | 7.3 ± 0.2 / 7.3 ± 0.3 | 1.6 ± 0.9 / 1.7 ± 1.2 | 5.7 ± 2.3 / 3.0 ± 1.2 | 5.6 ± 2.9 / 1.1 ± 0.4 |
| LCR136 | 1.8 ± 0.7 / 44.0 ± 2.4 | 3.9 ± 2.4 / 3.3 ± 2.1 | 8.5 ± 3.8 / 2.4 ± 1.0 | 5.5 ± 2.9 / 6.2 ± 0.5 |
| RT11 | 1.2 ± 0.4 / 5.4 ± 1.7 | 3.1 ± 1.6 / 3.4 ± 2.2 | 1.9 ± 0.7 / 0.8 ± 0.4 | 3.9 ± 1.9 / 3.9 ± 1.9 |
| RT12 | 2.3 ± 1.1 / 20.7 ± 1.9 | 5.1 ± 0.1 / 15.0 ± 0.4 | *12.8 ± 0.9 / 13.8 ± 1.4 | 6.3 ± 0.3/ 16.4 ± 0.4 |

Some differences between the wild-type (WT) cell lines and those overexpressed with ABC transporters can be observed. In the case of higher cytotoxicity for wild-type cells, the compounds can easily kill cells that are not overexpressing the pumps. Some examples of this behavior are: PMC79 for all the ABC pumps, RT12 for NIH3T3/NIH3T3-P-gp, HEK293/HEK293-MRP1 and HEK293/HEK293-ABCG2 and LCR136 for NIH3T3/NIH3T3-P-gp. Contrarily, some compounds can easily kill cells overexpressing ABC transporters, such as PMC78 for HEK293/HEK293-MRP1 and NIH3T3/NIH3T3-P-gp, PMC85 for HEK293/HEK293-MRP1, LCR134 for HEK293/HEK293-ABCG2, LCR136 for HEK293/HEK293-MRP2 and RT11 for MRP2.

A closer look into the $IC_{50}$ curves allow us to conclude that the compound PMC78 (Fig.2.1 A) does not seem to have any effect as substrate or inhibitor on the MRP1 pumps. For all the tested concentrations, the compound kills WT and MRP1-overexpressing cells.




A) PMC78 B) PMC79 C) PMC78

Figure 2.1-Cellular viability for the compounds A) PMC78, B) PMC79 and C) PMC78. Concentration range between 2.5-100µM.

On the other hand, compound PMC79 seems to act as substrate for the cells NIH3T3/NIH3T3-P-gp (Fig.2.1 B), HEK293/HEK293-ABCG2 and HEK293/HEK293-MRP1 (Annexes) and MRP2, since the compound seems to kill more WT cells than those overexpressed for all the tested concentrations. The same behaviour is observed for RT12 in NIH3T3/NIH3T3-P-gp, HEK293/HEK293-MRP1 and HEK293/ HEK293-ABCG2 cells (Annexes). These results corroborate the $IC_{50}$ results already presented for these cell lines, where in all the cases, a higher cytotoxicity was observed for wild type cell lines in relation to the overexpressed cell lines.

Besides the effect observed for some compounds acting as substrates, some compounds seem to act as inhibitors and block the pumps. This is the case of PMC78 for NIH3T3-P-gp (Fig.2.1 C), PMC85 for HEK293-MRP1, LCR136 for HEK293-MRP2 (Annexes), RT11 for HEK293-MRP2 and LCR134 for HEK293-ABCG2. These compounds seem to be selective inhibitors according to Table 2.3 and Annexes. In all cases, the compounds have the capacity to kill more efficiently overexpressed cells which is an indirect observation of the inhibitory capacity for ABC pumps. However, this finding needs to be confirmed by some additional experiments.

## 2.4 Study of the compounds' inhibitory properties

One way to understand the role of organometallic compounds towards ABC transporters is to identify effective and selective inhibitors and use them as chemical tools to investigate the effect on drug pharmacokinetics and efflux. Using flow cytometry, the quantification of the intracellular accumulation of the metal in the cell lines under study is possible. This technique can be used in efflux pumps using fluorescent reference substrates. The intracellular fluorescence due to the compounds' accumulation allows the quantification of the molecules inside the cell. So, indirectly, these results allow to understand if the compounds can inhibit the ABC pumps.

Thus, for the flow cytometry assays several solutions were prepared as showed in Figure 2.2. As described, using a reference inhibitor (in blue) and a reference substrate (in light green) one can compare the tested compounds (dark green) and see how much the inhibition activity is. As expected, the solutions containing only DMSO and DMEM (in red), do not have any intracellular fluorescence.

For the solutions with the substrate there is a slightest fluorescence that correspond to 0 % inhibition band and for the inhibitor solution a 100 % inhibition band is observed.

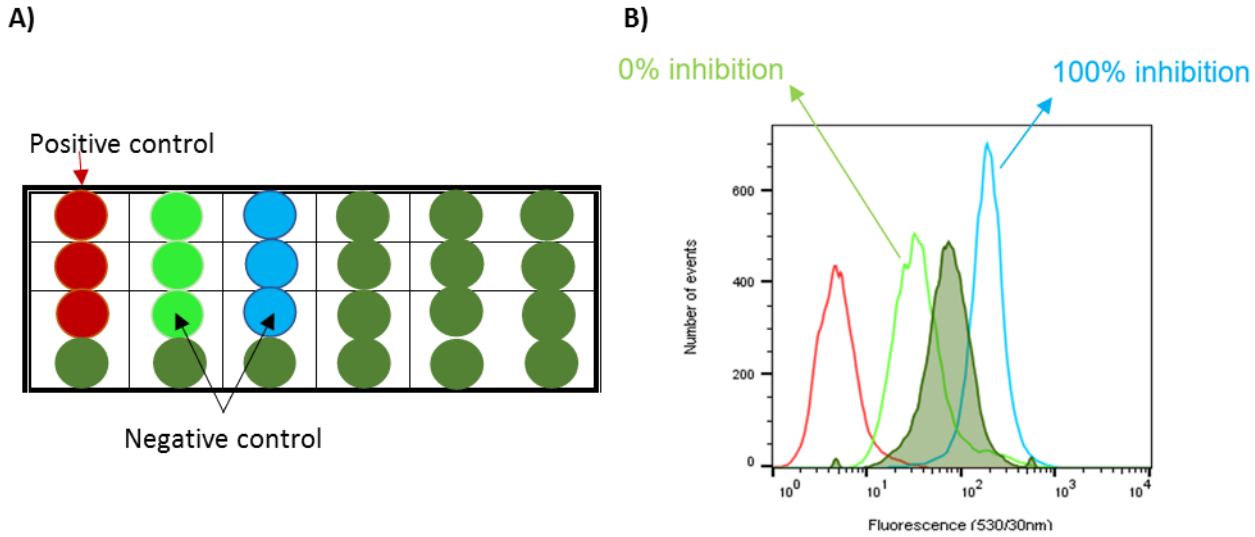



Figure 2.2- A) 24 wells plate used for cytometry study. ● 500µL DMSO + DMEM; ● 250µL DMSO + 250 µL reference substrate; ● 250µL reference substrate + 250 µL reference inhibitor; ● 250µL reference Substrate + 250 µL Compound. The blue, red and green triplicates are the control; B) Example of the cells population with an example inhibitor compound (filled pick with dark green colour), against a reference inhibitor (blue), a reference substrate (light green) and the cell line with DMEM+DMDO (red) using flow cytometry technique.

Although one looks for specificity, this is difficult to achieve in the case of multidrug ABC transporters which are characterized by substrates and inhibitors overlapping. Therefore, it is crucial to find compounds with a high percentage of inhibition and selective for each pump. Only inhibition activities of 50 % or more were considered to account as good inhibitors as it can be seen in Table 2.4, the best inhibitory activity was obtained for LCR134 in NIH3T3-P-gp, PMC78 in NIH3T3-P-gp, LCR136 in HEK293-MRP2, and RT11 in HEK293-MRP1 and -MRP2. Some negative values are also observable, suggesting an efflux enhancement effect, also verified on the cellular viability test for PMC79 and RT12.

Table 2.4- Percentage of inhibition for all ruthenium complexes at 20 µM in HEK293 WT cells and those overexpressing ABCG2, MRP1 and MRP2, NIH 3T3 WT and overexpressing P-gp. The concentration used for the reference substrates was: 5 µM of mitoxantrone for ABCG2, 0.5 µM of rhodamine 123 for P-gp, 0.2 µM of calcein AM for for MRP1 and MRP2. The reference inhibitors, Ko143, GF120918, verapamil and cyclosporine A, were used at 1, 5, 35 and 25 µM, respectively.

| | HEK293-ABCG2 | NIH3T3-P-gp | HEK293-MRP1 | HEK293-MRP2 |
|---|---|---|---|---|
| PMC78 | -0.7 % | 71% | 3% | -19% |
| PMC79 | 3.9 | -4% | 20% | -5% |
| PMC85 | -0.7% | -0.6% | 3% | -17% |
| LCR134 | -0.1% | 159% | 7% | 7% |

| | | | | |
|---|---|---|---|---|
| LCR136 | 14% | 9% | 42% | 57% |
| RT11 | 16% | 24% | 77% | 59% |
| RT12 | 4% | -16% | -0.1% | 35% |

As shown in Fig.2.3 among the pool of the most efficient compounds were LCR134, PMC78 and LCR136 that displayed higher selectivity towards the reference inhibitors (GF120918, verapamil and cyclosporine A), blocking in 159 % and 71 % P-gp activity at 20 µM, respectively, and 57 % of MRP2. The same compounds do not show significant inhibition activity for the others cell lines, so we can consider these three compounds as selective inhibitors. In the case of compound RT11 some inhibition activity at 20 µM was observed for HEK293-MRP2 and -MRP1 (59 % and 77 %, respectively).

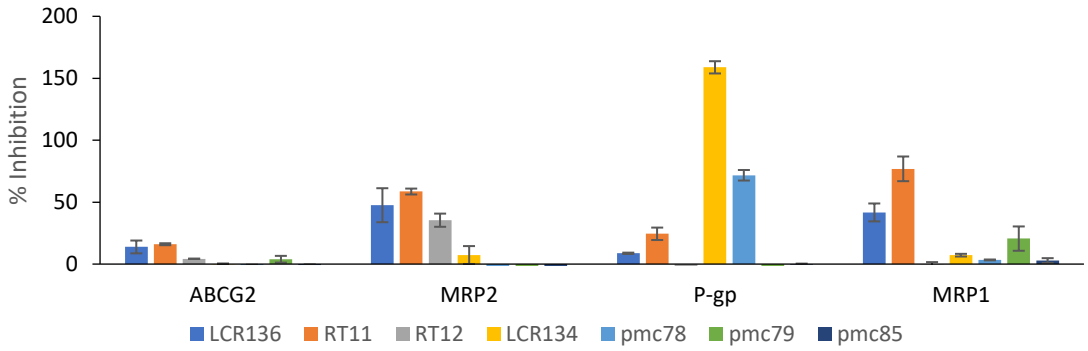



Figure 2.3-Inhibition comparison of the compounds at 20 µM in HEK293 overexpressing ABCG2, MRP1 and MRP2 and MRP2 and NIH3T3 overexpressing P-gp, in the same conditions of table 2.4.

## 2.5 Mass cytometry (CyTOF)

With the aim of understanding if the compounds can be internalized in cancer cells, a mass cytometry technique has been used. For this purpose, the flow cytometry was coupled with a microplate reader and an autosampler. In this technique (Fig.2.4), every cell is stained with a stable isotope tag and injected into a mass cytometer. Cells are then atomized and ionized in a high temperature Inductively Coupled Plasma (ICP) and the atomic composition of each cell (including metal tags) is then measured by time of flight mass spectrometry (TOF-MS), generating distinct mass spectra of each cell.[49]

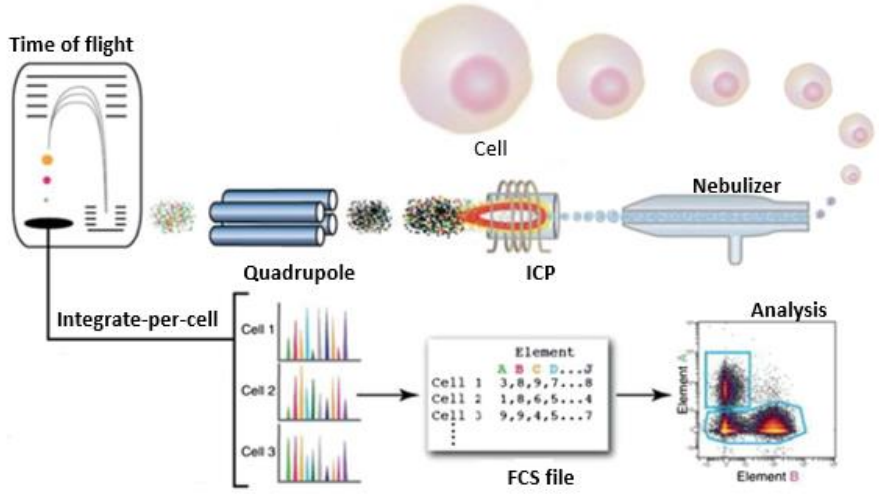



Figure 2.4- Mass cytometry technique. Adapted from [49].

Due to time constrains, only the compounds RT11, RT12 and LCR136 were analysed for the HEK293 and NIH3T3 cells (WT and with ABC transporters). All the compounds were analysed in the ovarian cancer cell line (2008C).

The compounds LCR136 and RT11 have the highest ruthenium cell internalization for HEK293 and HEK293-ABCG2 (Fig.2.5). This happens for both WT and with ABC transporters cell lines. The low Ru content in the case of RT12 seems in accordance with the lower $IC_{50}$ values obtained for this compound.

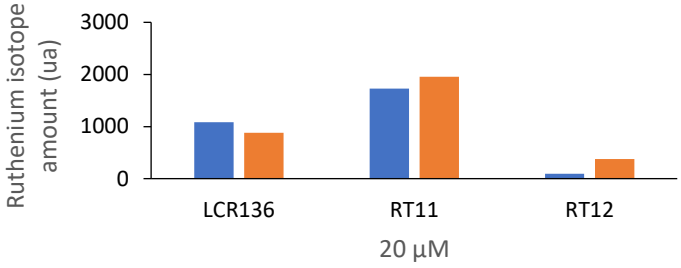



Figure 2.5-Determination of ruthenium complexes at 20 µM internalization in HEK293 WT (blue) and HEK 293 ABCG2 (orange) cells line, by CyTOF.

Finally, the abundance of ruthenium inside the cell for all the compounds in the 2008C cell line was quantified. It was already known that the compounds with high molecular weight (PMC78 and PMC85) had a good internalization for others cancer cell lines[40]. Fig.2.6 shows that, the compound that displayed the lowest internalization in the conditions tested was PMC79. Surprisingly, when incubated with A2780 ovarian cell line for 15 min at 20 µM[40], PMC79 had the highest internalization abundance when compared with PMC78 and PMC85. However, for the resistant cancer cell line A2780CisR, the Ru accumulation for PMC79 was drastically reduced, while it was maintained for PMC78 and PMC85. Due to these interesting results, it might be relevant to analyze the internalization of these compounds

in 2008C resistant cell line (2008-MRP1) to compare with the previously results. Moreover, it would be interesting to perform the internalization assays at different incubation times to verify if PMC79 accumulation is cumulative or if it is a dynamic process, i.e., if the compound is internalized and effluxed.

Unlike the internalization for HEK293 and NIH3T3, for 2008C cancer cell line, the compounds with the highest internalization in 2008C were RT11 and RT12. The compound RT12 has the same effect previously discussed for PMC79 in A2780 cell line. This compound has a high accumulation in the 2008C cells but a lower accumulation for overexpressed cells, which could mean that RT12 is more active in sensitive cells than in resistant ones, in agreement with the previously results. As for PMC79, will be necessary corroborate these conclusions with some analyses to 2008C resistance cell line.

To sum up, the compound RT12 seems to behave similarly to PMC79. Both compounds are more cytotoxic for WT cells than for ABC pump overexpressing cells and none of them is a good inhibitor for the cell lines tested. In structural terms we can conclude that the -$CH_2OH$ group on the bipyridine ligand has some effect on the efflux of these compounds through their interaction with ABC transporters.

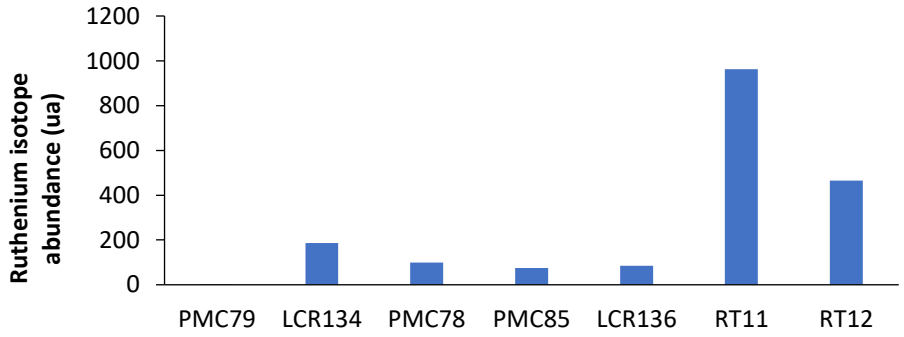



Figure 2.6- Quantification of ruthenium by CyTOF for the complexes after 30 min incubation at 20µM in ovarian cancer cell line, 2008C.

## 2.6 Molecular Docking

The molecular docking approach can be used to model the interaction between a small molecule and a protein, which allows to characterize the behavior of small molecules in the binding site of target proteins, as well as to elucidate fundamental biochemical processes. The docking process involves two basic steps: prediction of the ligand conformation as well as its position and orientation within these sites (usually referred to as pose) and assessment of the binding affinity.[50]

For this work, a molecular docking for the protein P-gp and the complex LCR134 (Fig.2.7) was carried out. This compound was chosen due to its good ability to block P-gp pumps.

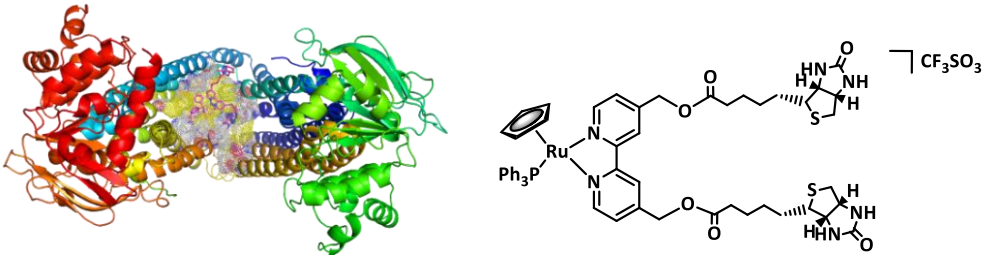



Figure 2.7 - Molecular docking of the protein P-gp with the compound LCR134 insert in one of her pocket (left side) and the structure of LCR134 compound (right side).

The affinity results obtained were found to be between -13.5 and -12.9 kcal/mol, suggesting that LCR134 may have a high affinity for this protein. Additionally, according to the literature, the molecular docking for the reference substrate (rhodamine 123), used in NIH3T3-P-gp, gives an affinity of -8.5 kcal/mol[51]. As seen in Fig.2.8 Rhodamine 123 and LCR134 have binding sites close to each other and potentially exhibit some competition between them.

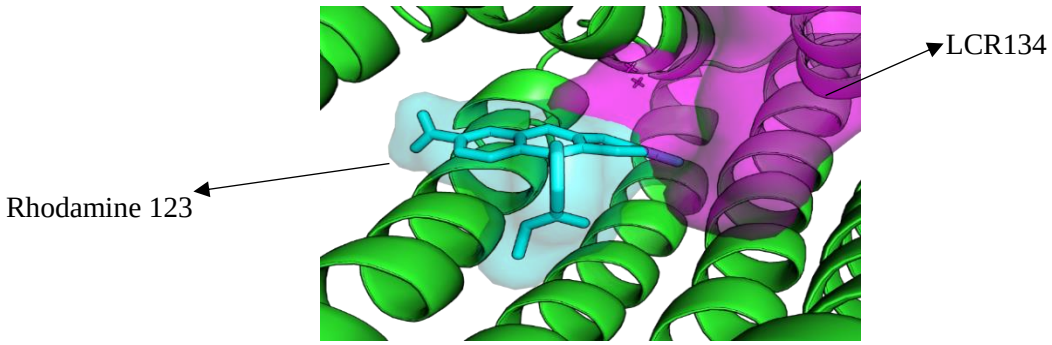



Figure 2.8- Molecular docking of LCR134 and P-gp reference substrate, rhodamine 123.

## 2.7 Conclusions and Perspectives

One of the problems with cancer disease is the fact that cells can create resistance to the drugs. This multidrug resistance can be caused by efflux transporters. One of the most common mechanisms of MDR is the overexpression of ABC transporters by the cells, which mediate the efflux of anticancer drugs to limit the effective use of chemotherapeutic drugs. Therefore, to solve the problem of multidrug resistance in chemotherapy, attempts to find efficient inhibitors of these transporters have been made. This work aimed to evaluate the biological activity of organometallic compounds on ABC transporters, trying to understand the role of these proteins in their mechanism of action.

Seven new organometallic complexes containing the same "Ru-cyclopentadienyl bipyridine" core were chosen and four types of ABC efflux transporters tested (P-gp encoded by ABCB1, MRP1 encoded by ABCC1, MRP2 encoded by ABCC2 and BCRP encoded by ABCG2). In this frame, HEK293 and NIH3T3 cell lines and also an ovarian cancer cell line (2008C) were used.

All the compounds were cytotoxic against the cell lines under study (HEK293, NIH3T3 and 2008C). The polymer-ruthenium conjugates PMC78 and PMC85 were more cytotoxic against the cells with transporters than wild type, namely for P-gp and MRP1, respectively. PMC78 was proven to be a good inhibitor for P-gp pump. Both polymer-ruthenium conjugates have shown similar amounts of ruthenium internalization for the cancer cell line 2008C. Altogether, these results could indicate that the use of a polylactide polymer can potentiate the anticancer action of the non-polymeric compounds (like the parent compound PMC79).

Another compound for which the mechanism of action could be related to its ability as ABC transporter inhibitor is LCR134 over P-gp pumps. LCR134 is a recent compound, synthetized by our Organometallic Chemistry Laboratory, bearing a 2,2'-bipyridine-4,4'-dibiotin ester ligand. Even if the viability assays did not give any relevant information on LCR134, by flow cytometry it was clear that this compound shows a very good percentage of inhibition over P-gp. A possible competition with the known substrate Rhodamine 123 for the active site of P-gp was verified through molecular docking, although experimental validation is needed. Overall, these results suggest that this compound is a potential candidate for blocking P-gp pumps. In addition, LCR134 has also a good internalization in 2008C cells that could indicate that the use of a 2,2'-bipyridine-4,4'-dibiotin ester ligand can potentiate the anticancer action (by comparison with PMC79).

Concerning the compounds LCR136 and RT11, belonging to the $\eta^5$-MeCp sub-family, they are both more cytotoxic for cells overexpressing MRP2 than for WT. They are also selective inhibitors for MRP and have a good internalization for 2008C. Interestingly, both compounds have a lower $IC_{50}$ for the cancer cell line, which could indicate that these two compounds can be active in both resistant and sensitive cell lines. In this work, the amount of ruthenium internalization into the cell has been measured for this family on the ABC overexpressed cells. It was observed that the compounds with highest inhibitory power from the $\eta^5$-MeCp family presented the highest internalization for all ABC transporters studied, suggesting a correlation between internalization and activity.

PMC79 is the low molecular weight parental compound of PMC78 and PMC85. The compound RT12 belongs to the $\eta^5$-MeCp sub-family, being the methyl group at the cyclopentadienyl ring the only structural difference between RT12 and PMC79. In terms of biological activity they are very similar. Both compounds are more cytotoxic for WT cells than the overexpressed ones and they do not seem to have any effect on resistant cells. Do to their similar structure, we can conclude that the $-CH_2OH$ group

on the bipyridine ligand has an important role for the efflux, turning these compounds into substrates. In this frame, it would be interesting to perform some internalization assays to verify if both compounds are internalized and effluxed in a dynamic process.

Finally, with the aim of enhancing the biological activity a new Ru-cyclopentadienyl bipyridine complex was synthesized, having into account the biological results on the ABC transporters. Given the enormous potential of biotin-mediated approaches for drug delivery[42,43], and the good cytotoxic and inhibition results observed for the $\eta^5$-Me-cyclopentadienil family *versus* cyclopentadienil, a compound bearing a bypyridine-biotin ligand and a $\eta^5$-MeCp group was envisaged. All the synthesis and characterization of the new compound are shown and discussed in the following chapter.

## 3. Synthesis and Characterization of a new ruthenium organometallic compound

### 3.1 Synthesis description

A new ruthenium complex with the structure $[Ru(\eta^5\text{-}(Me\text{-}C_5H_4)(PPh_3)(bipy\text{-}biot)][CF_3SO_3]$ **Ru2**, where bipy-biotin is 2,2'-bipyridine-4,4'-dibiotin ester (**L1**), was synthesized by adding silver trifluoromethanesulfonate to $[Ru(Me\text{-}C_5H_4)(PPh_3)_2Cl]$ (**Ru1**) in a stirred methanol solution. The ligand **L1** was added to this brown mixture and the solution was heated to reflux temperature for a period of 6 h (Fig.3.1). Then, the product was filtrated and the solvent evaporated. The compound was twice recrystallized from dichloromethane/*n*-hexane mixture giving red crystals in 69 % yield.

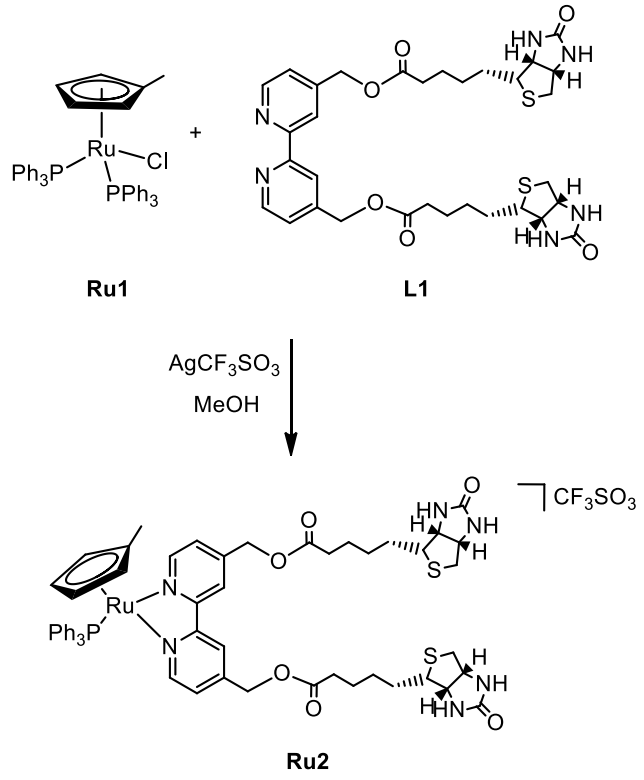



Figure 3.1- Reaction scheme of **Ru2** synthesis using **Ru1** and **L1**

The change of the solution color was a good indicative of the compound's formation enabling an easy follow-up of the reaction. The starting material **Ru1** was orange, the ligand **L1** was white and the final complex **Ru2** was red. **Ru2** is soluble in polar solvents as dimethyl sulfoxide (DMSO) and methanol and in apolar solvents such as dichloromethane.

The ligand and the new compounds were characterized by NMR ($^{1}H$, $^{31}P$, $^{13}C$, dimensional experiments), FTIR, UV-Vis and their purity was determined by elemental analyses.

### 3.2 IR Spectroscopy

Even if the synthesis of **L1** was not new, a throughout characterization was done. The solid state FT-IR spectra (KBr pellets) presented the NH stretch (3385 $cm^{-1}$), the C-N stretch (1142 and 1319 $cm^{-1}$), the ester C-O stretch (1732 $cm^{-1}$) and the NH-C-O-NH group (1705 $cm^{-1}$) from the biotin fragment. Also, the binding of the biotin to the 4,4'- bishydroxymethyl-2,2'-bipyridine is confirmed by the disappearance of the alcohol signal (3069 $cm^{-1}$)[52] and the appearance of the ester signal in **L1** (1732 $cm^{-1}$).

The FT-IR spectrum of complex **Ru2** present the characteristic bands for the methylcyclopentadienyl ring along with the aromatic rings of the phosphane and the bipy-biotin with the presence of C-C and C=C stretch (~3048 $cm^{-1}$, 1435 and 1475 $cm^{-1}$ respectively) and by the other characteristic bands from **L1**, such as, the amino aromatic band at 1150 $cm^{-1}$ from the bipyridine ring and by the NH stretch (3450 $cm^{-1}$), the C-N stretch (1275 and 1100 $cm^{-1}$), the C-S stretch (700 and 650 $cm^{-1}$) and the ester C=O stretch (1740 $cm^{-1}$) from the biotin fragment and the NH-C=O-NH group (1695 $cm^{-1}$). The presence of counter-ion $CF_3SO_3^-$ (~1250 $cm^{-1}$) confirms the proposed cationic nature of complex **Ru2**. **L1** coordination to the ruthenium centre in **Ru2** is confirmed by the difference on the vibration of the ester C=O stretch between **Ru2** and **L1** ($\Delta\upsilon$ = 8 $cm^{-1}$), showing a donation of electrons from the ligand to the metallic centre.

### 3.3 NMR Spectroscopy

The NMR characterization was made in DMSO-d6. The results from $^{1}$H-NMR characterization including chemical deviation ($\delta$), multiplicity and integration, for the new complex (**Ru2**) are in Materials and Methods Chapter. The attribution of **Ru2** was accomplished using 2D-NMR (cosy, HMBC, HSQC), $^{31}$P and $^{13}$C-NMR experiments. For **Ru1** and **L1** only $^{1}$H and $^{31}$P spectra will be presented since these compounds are already known.[53,54,55,56.]

Complex **Ru2** presents NMR spectra consistent with the proposed structure (Fig.3.2).

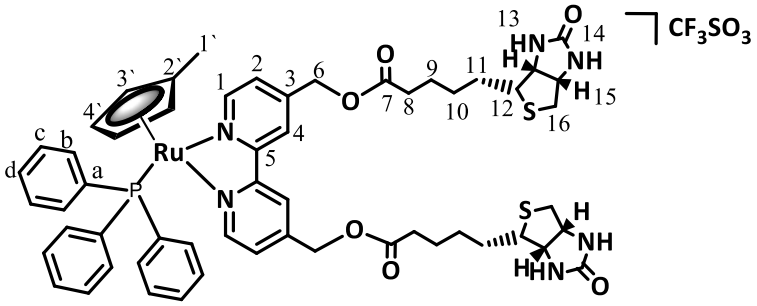



Figure 3.2- Structure of complex **Ru2** numbered for NMR purposes.

The displacement of the $\eta^5$-coordinated $\eta^5$-MeCp ring signals allow to confirm that the synthesis was successful and coherent with a cationic compound. The H1′ signal from $\eta^5$-MeCp suffered a shielding in relation to **Ru1** (Tab.3.1 and Fig.3.3), while the protons H3′ and H4′ of the ring are deshielded. These different behaviors on the Cp proton signals might be explained by observation of the X-ray structure

of similar structures from the Organometallic Research Group database, where there is evidence that methyl group is in the shielding cone of the bipyridine (Annexes).

Table 3.1- Table of selected $^{1}$H-NMR signals for complexes **Ru1** and **Ru2** in DMSO-d$^{6}$. The protons number are according to the present in figure 3.2.

| **Compound** | **Me-Cp /ppm** | | | **$PPh_3$/ppm** | | |
|---|---|---|---|---|---|---|
| | **H3'** | **H4'** | **H1'** | **Hb** | **Hc** | **Hd** |
| **Ru1** | 3.87 | 3.24 | 1.79 | ~7.26 | 7.17 | ~7.26 |
| **Ru2** | 4.70 | 4.59 | 1.57 | 6.94 | 7.28 | 7.36 |
| **Δ / ppm*** | 0.83 | 1.35 | -0.22 | -0.32 | 0.11 | 0.1 |

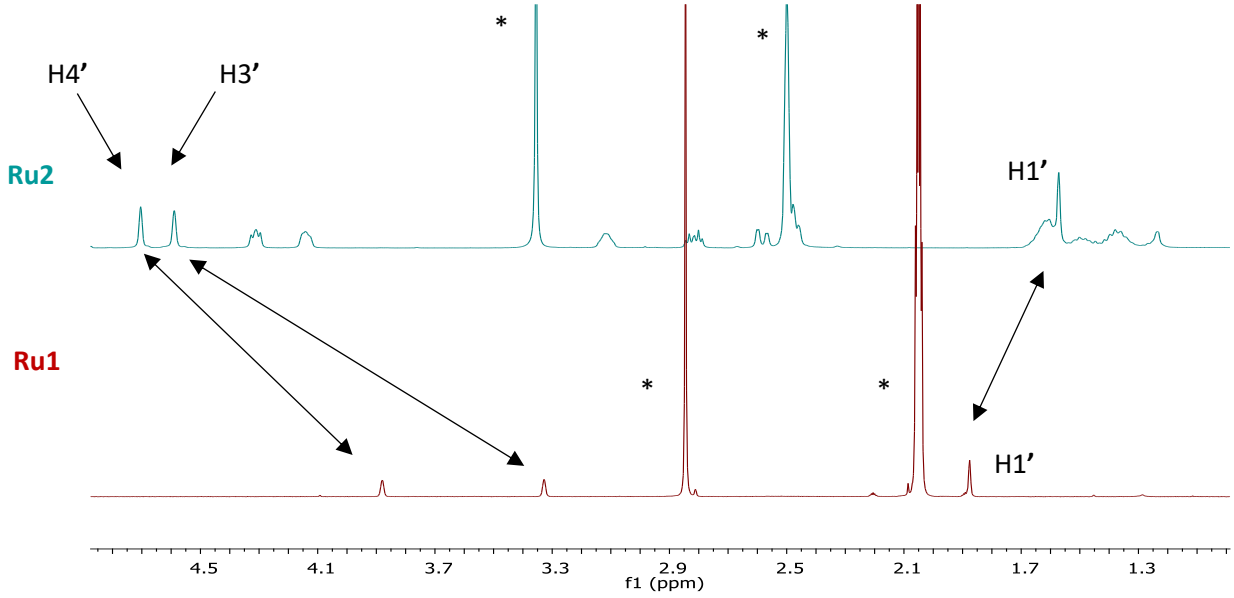



Figure 3.3- Comparison between $^{1}$H-NMR spectres of initial complex **Ru1** (red) and new complex **Ru2** (blue), in DMSO-d$^{6}$ in the region of the MeCp signals (H1',H3' and H4'). The signals that are not identified here belong to **L1** signals, which are all identified in Material and Methods chapter and Annexes. *-solvents signals.

In terms of the phosphane signals, a shielding in Hb ($\Delta\delta$ = -0.32 ppm) in **Ru2** is observed due to the retrodonation of the metal to the pohsphane. A unique sharp singlet resonance corresponding to the coordinated triphenylphosphane co-ligand was found in the $^{31}$P-NMR ($\delta$ = 52.0 for **Ru2**) corroborating $^{1}$H spectra.

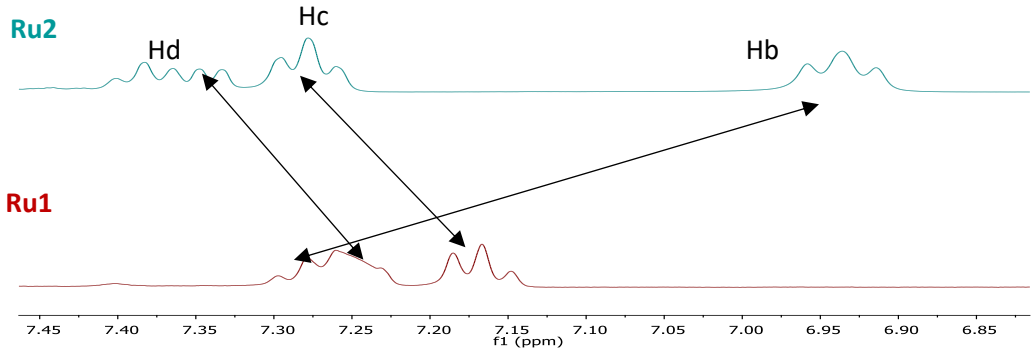



Figure 3.4- Comparison between $^{1}$H-NMR spectres of initial complex **Ru1** (red) and new complex **Ru2** (blue), in DMSO-d$^{6}$, for phosphane signals.

The coordination of **L1** to the metal centre can be confirmed by the deshielding of H1, adjacent to the nitrogen of the bipyridine ring, and by the shielding on the H4 proton (Table 3.2). This effect has been already observed for related compounds, where the bipyridine is substituted at the *para*-position (relatively to the nitrogen).[57] Besides the $^{1}$H and $^{31}$P-NMR spectra already mentioned, the $^{13}$C-NMR spectra shows the same general effect observed for the protons in **Ru2** (Material and Methods Chapter, Annexes).

Table 3.2- Table of selected $^{1}$H-NMR signals for the ligand **L1** and the complex **Ru2** in DMSO-d$^{6}$. The protons numbers are according to figure 20.

| **Compound** | **H1** | **H2** | **H4** | **H6** | **H8** | **H12** |
|---|---|---|---|---|---|---|
| **L1** | 8.69 | 7.43 | 8.37 | 5.25 | 2.45 | 3.07 |
| **Ru2** | 9.30 | 7.56 | 8.07 | 5.19 | 2.57 | 3.12 |
| **Δ / ppm** | +0.61 | +0.13 | -0.3 | -0.06 | +0.12 | +0.05 |

In order to assess the effect of biotin in the electronic behaviour of the bipyridine, and on the overall complex, **Ru2** NMR signals were compared with [Ru($\eta^5$-MeCp)(2,2'-bipyridine-4,4'-bishydroxylmethyl)($PPh_3$)]$^{+}$ (**RT12**; Fig.3.5). It is interesting to observe that H6 is clearly deshilded ($\Delta\delta$ = +0.58 ppm) in **Ru2** due to the change from an alcohol to an ester (Fig.3.6).

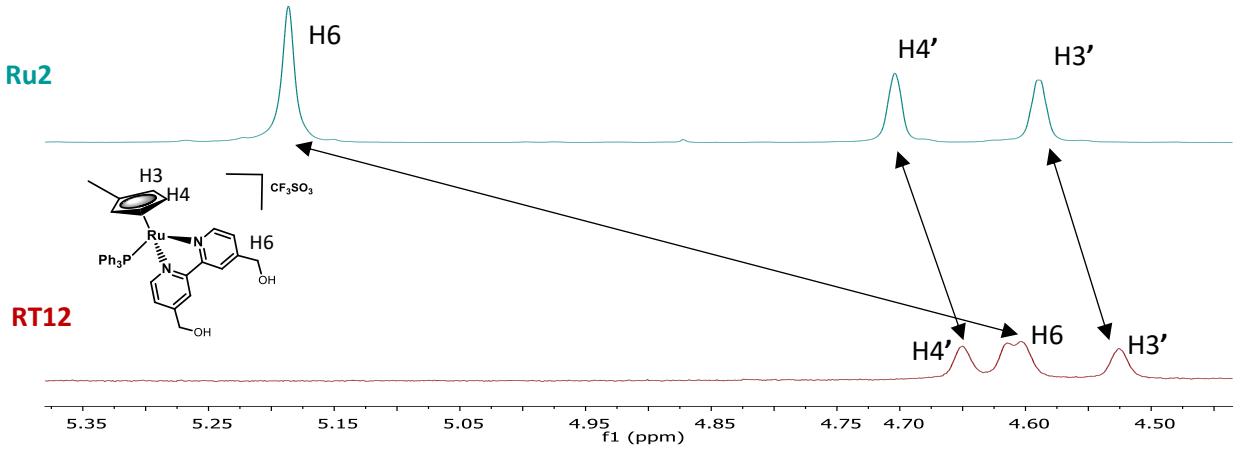



Figure 3.5- Comparison between $^{1}$H-NMR spectra of the new complex **Ru2** (top) and the complex RT12 (bottom) in DMSO-$d^6$ for the protons H6 and H4, H3 of methylcyclopentadienyl.

### 3.4 UV-Vis Spectroscopy

Optical absorption data were recorded with concentrations between 1.0 x $10^{-3}$ M to 1.0 x $10^{-5}$ M for the complexes and the ligand. The solvents were chosen considering their polarities (dichloromethane = 3.1 and DMSO = 7.2)[58] and solubility of the compounds. All spectra were recorded within the range allowed by the solvents used. Table 3.3 shows the values of wavelength and molar absorptivity for **Ru1** and **Ru2**.

As expected, there is an obviously alteration of the bands between the complex **Ru1** and **Ru2** due to the substitution of a phosphane and chloride ligands (weak field ligand) for a bipyridine (strong field ligand), which increase the energy between d orbitals causing the change of complex colour, in this case from orange (**Ru1**) to red (**Ru2**).

For both complexes, an intense absorption band at ca. 240 nm for **Ru1** and 250 nm for **Ru2** is observed, attributed to the organometallic fragment {Ru($\eta^5$-MeCp)($PPh_3$)}$^+$. An absorption band (or shoulder) at ~294 nm is also observable and corresponds to $\pi \rightarrow \pi^*$ electronic transitions of the coordinated ligands.

Table 3.3- Optical spectra data for complexes **Ru1** and **Ru2** in DMSO and dichloromethane solutions at room temperature. Sh-shoulder. The values from Ru1 are from a master thesis of our Organometallic Chemistry group[59].

| | Solvent | Wavelength (nm) | Molar Absorptivity/ε ($M^{-1}$ $cm^{-1}$) |
|---|---|---|---|
| **Ru1** | DMSO | 293<br>371 | Sh<br>2530 |

| | | | |
|---|---|---|---|
| | | 426 | Sh |
| | Dichloromethane | 240 | Sh |
| | | 297 | Sh |
| | | 366 | 2375 |
| | | 448 | Sh |
| **Ru2** | DMSO | 294 | 18000 |
| | | 345 | Sh |
| | | 428 | 2990 |
| | | 489 | Sh |
| | Dichloromethane | 249 | Sh |
| | | 294 | 17700 |
| | | 337 | 5450 |
| | | 434 | 3360 |
| | | 489 | Sh |

In figure 3.6, are presented comparisons between the **L1** and both complexes in DMSO.

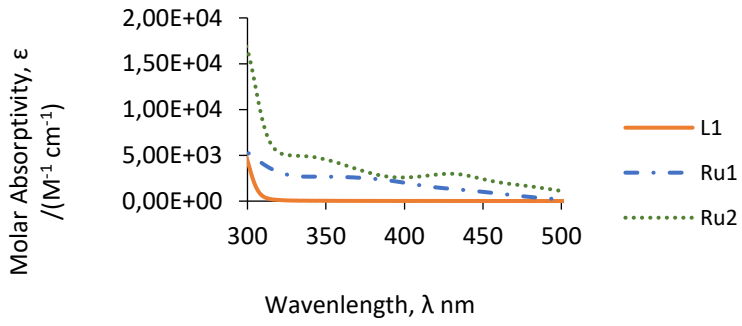



Figure 3.6- Electronic spectra of the complexes **Ru1**, **Ru2** and the ligand **L1** in DMSO.

In order to assign the other bands missing in the visible region, the solvatochromic response of these complexes in solvents of different polarities was evaluated. (Fig.3.7)

The aim of this study was to evaluate the behaviour in the UV-Vis region according to the solvent used. These alterations in the spectra are due to the neighbourhood modifications of the species. The time that it takes for the species to stay in the excited state is not enough for the solvate sphere reorganization. This effect is called solvatocroism (position, intensity and shape alteration of the band

with different solvents polarity). Due to this effect it is crucial to do the UV-Vis analyses with different polarity solvents, in order to visualize the charge transference bands, the only bands that suffer alteration with the effect.

At 345 nm in DMSO and 337 nm in dichloromethane a band, possibly to corresponding to intraligand charge transference (ILCT), is observed.

Two bands in DMSO solvent spectra are observed (at 428 nm and 489 nm), in relation to dichloromethane band (Fig.3.7), which reveals a clear hypsochromic shift (change of spectral band position to a shorter wavelength position where there is an increase of solvent polarity). In this frame, there is an evidence of the effect of charge transference (CT) in both cases. According with the NMR results these bands could be a ligand to metal charge (LMCT) transference due to the shielding for bipyridine ligand and a metal to ligand charge transference (MLCT) due to the donation of metal to the phosphane co-ligand. However, it will necessary to do further experiments to corroborate these hypotheses, like to confirm with density functional theory.

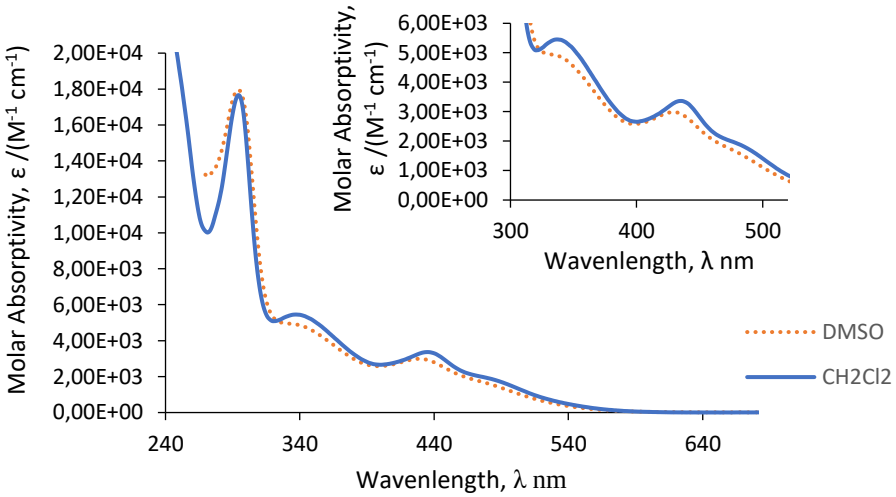



Figure 3.7- Electronic spectra of the complex **Ru2** in DMSO and dichloromethane.

### 3.5 Stability Studies by UV-Vis

To obtain an efficient drug for all the type of diseases treatment it is crucial that the drug could be stable until it reaches its target. In this frame, the stability evaluation in biological medium (cells growing medium) is very important, since this compound will be tested as anticancer agent in cells culture.

With this aim, the stability of **Ru2** was studied through UV-Vis spectroscopy, using DMEM solution (Dulbecco's modified Eagle´s medium) with 2 % DMSO. This experiment was performed during approximately 24 hours with consecutives measurements along the first 6 hours.

As shown in the graph below (Figure 3.8), **Ru2** is stable in biological medium within the 24 h period of the study since the variations observed were lower than 5%. This result showed that is possible to continue the study in biological assays in cell lines.

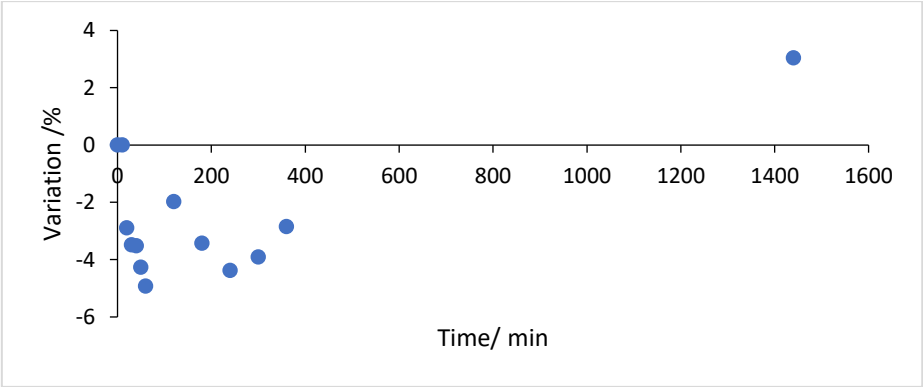



Figure 3.8- Variation plot from t = 0 h to t = 24h for the stability study for the complex **Ru2.** The stability area is around ±5%.

### 3.6 Partition Coefficient (logP) determination

According to the International Union of Pure and Applied Chemistry (IUPAC)[60] lipophilicity represents the affinity of a molecule or a moiety, for a lipophilic environment. The importance of hydrophobicity/lipophobicity of the compounds for medicinal purposes is a key feature in the development of new drugs since it affects, for example, their tissue permeability. It is commonly measured through its distribution in a biphasic system (e.g. partition coefficient (log P) in octanol-water). In this frame, the *n*-octanol/water partition coefficient was determined using the shake-flask method[61], at room temperature, using UV-Vis spectroscopy to determine the concentrations of the compound in each phase. Formula (3.1) was used to calculate the log P value.

$$Log\ P_{O/w} = log \frac{[Solute]_{n-Octanol}}{[Solute]_{Water}} \quad (3.1)$$

Firstly, a calibration curve was prepared accordingly to the protocol described in Method and Material Chapter (Figure 3.9).

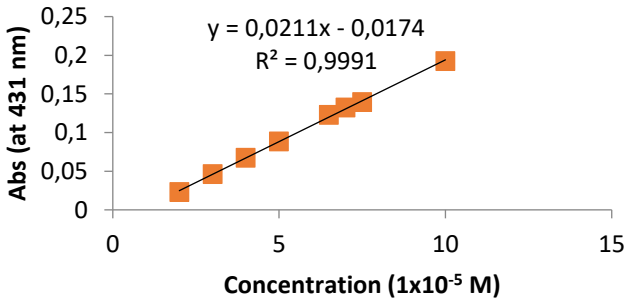



Figure 3.9- Calibration curve in octanol obtained by diluted solutions from a $10^{-4}$ M stock solution in octanol, at room temperature.

From the analysis of the spectra in octanol it was verified that **Ru2** has a lipophilic character ($logP_{o/w}$= 1.6), as predictable by the known lipid solubility of others "Ru($\eta^5$-MeCp)" compounds (data from our organometallic chemistry group).

### 3.7- Biological Evaluation

As it was discussed in chapter 2, the biotin fragment could be responsible for the ability of LCR134 in blocking P-gp pumps (by comparison with the other compounds). The sub-family $\eta^5$-MeCp, also presented very interesting results, especially on the inhibition activity of MRP2 pumps.

Thus, **Ru2** was tested on the cell lines HEK293 WT and overexpressing ABCG2, MRP1, MRP2 and NIH3T3 WT and overexpressing P-gp and on the cancer cell line 2008C, in order to assess the effect of these two groups on the activity of the compound. These assays were performed by Laurent Chanteloup from "Drug Resistance and Membrane Proteins team", using the same experimental conditions used in Chapter 2.

Interestingly, **Ru2** is much less cytotoxic for NIH3T3 WT and NIH3T3-P-gp ($IC_{50}$ = 29 ± 6.8 and 62 ± 33 µM, respectively) and for 2008C (I\$C_{50}$ = 28.3 ± 17 µM) than the compounds used to rationalize the synthesis of this compound. Analysing the graphs at different concentrations, one can observe that **Ru2** is a substrate over P-gp (Fig. 3.10); for all the other pumps this compound is not cytotoxic. These results indicate that adding the biotin and $\eta^5$-Me-Cp fragments in a single structure seems to change the ability of the compound to act as inhibitor of P-gp and MRP2 becoming a substrate for P-gp instead. Additional molecular docking studies, might help to elucidate these experimental findings.

Still, this compound shows cytotoxic activity for the 2008C cancer cell line and its mechanism of action should be unrevealed. To be able to draw more conclusions, it is important to carry out more tests to confirm these results in the near future.

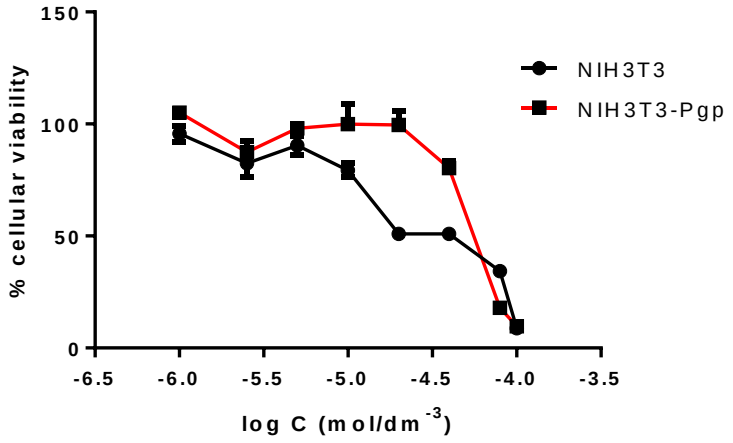



Figure 3.10- Cellular viability for Ru2. Concentration range between 1-100 µM.

### 3.6 Conclusions and Perspectives

With the aim of optimizing the chemical structures for enhancing the biological activity, a new Ru-cyclopentadienyl bipyridine complex, [Ru($\eta^5$-(Me-$C_5H_4$)($PPh_3$)(bipy-biot)][$CF_3SO_3$] **(Ru2)**, was synthesized. The rationale for the synthesis of this compounds took into consideration the features of the seven related compounds studied in Chapter 2 such as the enormous potential of biotin-mediated approaches for drug delivery[42,43] (LCR134), and the good cytotoxic and inhibition results of the Me-Cyclopentadienil family (LCR136, RT11).

The structure of new compound was undoubtedly confirmed by several spectroscopic techniques (FTIR, NMR and UV-Vis) and the complex purity was assessed by elemental analysis. According to all the techniques studied one could observe the electronic flow through the complex that identifies the 'Ru($\eta^5$-MeCp)(bipy-biotin)' as donor groups, reflecting an overall π-backdonation towards the coordinated triphenylphosphane.

The partition coefficient was determined and revealed that **Ru2** is lipophilic ($logP_{o/w}$= 1.6). The lipophilicity is an important property due to the fact that facilitates the entrance of the drug through the lipid bilayer of the cells.

Finally, **Ru2** revealed adequate stability in cellular medium to carry on the biological studies.

Interestingly, this compound seems to suffer some interaction between the $\eta^5$-MeCp and the biotin groups that cancel the inhibitory effect of each fragment. In this case **Ru2** is much less cytotoxic for NIH3T3-P-gp and 2008C, acts as substrate for P-gp pump and is not cytotoxic for all the other pumps.

Looking ahead, it would be interesting to synthetize compounds adding the polylactide polymer and the biotin, or change the phosphane group to see if could have some influence on the inhibitory potential.

## 4. Final Conclusion

Statistics report 8.8 million of the deaths worldwide by cancer in 2015[2]. Despite all the efforts in the last years and all the developments in this subject, cancer is still one of the major problems of our society. A major concern regarding chemotherapy, one of the first lines of treatment options in cancer therapy, is the rise of drug resistant phenotypes that considerably limit the efficiency of such drugs. One of the most common mechanisms of MDR is the overexpression of adenosine triphosphate (ATP)-binding cassette (ABC) superfamily of transporters, which mediate the efflux of anticancer drugs to limit the effective use of chemotherapeutic drugs.

With the aim to contribute for the study of the role of ABC transporters on the mode of action of ruthenium organometallic compounds, and perhaps to the development of compounds with inhibitory activity for these pumps, this work has presented a correlation between the structure of seven ruthenium compounds and their biological activity for ABC pumps.

Thus, within this work we have shown that depending on small changes on the compounds' structure, the biological results can be completely different. Between the compounds studied, four of them (PMC78, LCR134, LCR136, RT11) act as selective inhibitors of different ABC pumps. This is an unprecedented and very important result. As far as we are aware, this type of behaviour for ruthenium compounds has never been described, which might constitute and important landmark on our studies.

According to our studies, it was verified that the use of a polylactide polymer (PMC78 and PMC85) can potentiate the anticancer action of the non-polymeric compounds. Therefore, based on the good results that both compounds had, it could be interesting to carry on the research of this type of molecules for cancer therapy of resistant cancers.

Most of the cancer cells have an overexpression of several receptors, due to their abnormal growing and energy need. It is already known that the use of biomolecules-drug conjugates, which receptors are overexpressed in cancer cells, could increase the selectivity of such drugs facilitating their entrance on malignant cells. LCR134, bearing biotin in its structure, showed very interesting results in the cells that overexpress P-gp. As verified by flow cytometry this compound is a very good inhibitor of P-gp pumps and can perhaps compete for it active centre with the P-gp substrate. Altogether, these results suggest that compounds with biomolecules should be a good approach on the multidrug resistance research.

The last two compounds with inhibitory activity for ABC transporters were RT11 and LCR136. These two compounds have as common characteristic the $\eta^5$-MeCp. We aimed to find any correlation between the biological activity that might be related to the different substituents on the bipyridine ligand. Indeed, in contrast to RT11 (bearing 4,4'-methyl-2,2'-bipyridine) and LCR136 (bearing 2,2'-bipyridine), RT12 bearing a $-CH_2OH$ group on the bipyridine did not show any inhibition on any ABC transporter. Interestingly, the presence of the $-CH_2OH$ group on the structure of PMC79 (bearing Cp instead of MeCp) also seems to induce an efflux on the ABC pumps. Thus, perhaps this chemical group should be avoided if one is envisaging ABC pumps inhibitors. However, these compounds seem to be good candidates for sensitive cells.

Based on the overall results a new compound was successfully synthesized and completely characterized. Although our aim was to try to merge some of the features of the seven compounds initially studied, in order to have a more potent inhibitor, the opposite happened. The new compound **Ru2** did not show any effect for ABC transporters tested, indeed it was found to be a substrate for the

P-gp pumps. These results are very interesting in terms of structure-activity relations and should be further explored.

Looking ahead, there are some assays that need to be repeated in order to have statistical significance. In addition, it could be interesting to continue the tests on these promising compounds, such as assays to determine the mechanism of cell death, to quantify the ruthenium accumulation at different times, to use a large range of cancer cells while comparing with cisplatin, verify which organelles are affected by the compounds and which compounds are the faster to reach their target. As a next stage it could be interesting to monitor *in vivo* the effectiveness of the tested inhibitors. Another interesting approach could be the synergic use of the P-gp inhibitor LCR134 and the MRP2 inhibitor RT11 or LCR136.

This project unrevealed important relations between small structural changes in $[Ru(\eta^5\text{-CpR'})(2,2'\text{-bipyridine-R})(PPh_3)]^+$ and their ability to act as inhibitors of ABC pumps.

As final conclusion, we can say that the aim of this project was successfully achieved by revealing a new and important area of study that has been neglected so far: the role of ABC transporters on the mechanism of action of organometallic compounds in order to fight multidrug resistant cancers.

# 5. Material and Methods

## 5.1 Biological Evaluation

### 5.1.1 Reagents and Cell lines

Substrates (Calcein AM, Rhodamine 123, Mitoxantrone) and Inhibitors (Ko143, GF120918, verapamil, cyclosporine A), Dulbecco's Phosphate Buffered Saline (DPBS), paraformaldehyde (PFA), Phosphate Buffered Saline (PBS) and Trypsin were all obtained from Sigma-Aldrich. Cisplatin was obtained from Selleckem, MaxPar water, Cell-ID Intercalator-Ir in DVS sciences. Dulbecco's modified Eagles's medium (DMEM) and Roswell Park Memorial Institute 1640 Medium (RPMI) were obtained from Gibco.

Cell lines

The HEK293 (Human embryonic kidney cell), NIH3T3 (embryo mouse fibroblast) and 2008C (Ovarian cancer cells control). The HEK293 cells were either wild-type (WT, transformed with an empty vector) and the same transfected with a plasmid containing a gene coding for the transporters proteins: ABCG2, MRP1, MRP2. NIH3T3 cells were used equally to test the P-gp.

Cell culture

All cell lines were grown at 37 °C in 5% $CO_2$.

The HEK 293 and NIH 3T3 cells were cultured in Dulbecco's modified Eagles's medium (DMEM high glucose) (PAA, GE Healthcare Life Sciences, Velizy-Villacoublay, France) supplemented with 10 % fetal bovine serum (FBS, PAA, GE Healthcare Life sciences, Velizy-Villacoublay, France), 1% penicillin / streptomycin (PAA, GE Healthcare Life sciences, Velizy-Villacoublay, France), with selection for the MRP1, MRP2, ABCG2 for HEK293 and P-gp-transfected cell line for NIH 3T3.

The 2008C cell line was cultured in RPMI 1640 medium with HEPES and L-glutamine (PAA, GE Healthcare Life Sciences, Velizy-Villacoublay, France) with 10% FBS and 1% penicillin.

To continue the culture, is necessary to wait until at least 80 % confluence was reached, by which time cells were subcultured: old medium was removed and cells were washed with phosphate buffer saline (PBS) 1x and then incubated with trypsin 0.05% (v/v). Trypsin was inactivated by adding fresh complete culture media to the culture flask. Cells were then suspended and transferred into new, sterile, culture flasks, or seeded in sterile test plates for the different assays.

All cells were manipulated under aseptic conditions in a flow chamber.

Ruthenium compounds under study

All the compounds were dissolved in DMSO and divided in aliquots of 10 µL each. After, they were store at -20 ºC.

### 5.1.2 Measurements

Was used Flow cytometry technique, cellular viability test, Mass Cytometry technique and finally was also carry out molecular docking for P-gp protein. Flow cytometry and cytotoxicity experiments were run in duplicates or triplicates, with positive and negative controls, and then analysed statistically using specific software and analysis tools. GraphPad was used for cellular viability test, FlowJo V10 for the analysis of flow-cytometry data and mass cytometry, and Excel 2013 package for the interpretation and graphical representation of data.

MTT test

Cell viability was evaluated by using a colorimetric assay based on the reduction of tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide] to insoluble purple formazan crystals, by mitochondrial succinate dehydrogenase in metabolic active cells. Absorbance was measured in a microplate reader at 570 nm, and this was corrected with the absorbance measured at 690 nm.

Flow Cytometry

This technique is based in the light diffusion and measurement of the fluorescence in one cell, to be able to do his characterization. The cells are marked with a intra or extracellular fluorescence probe. One liquid permits the passage of just one cell each time in front of the laser, giving us the wavelength of this cell. The excited fluorophore will go reissue the photons to a given wavelength (emission λ), where is capture by photo multiplicator. The optical signal is traduced in a electric signal giving us all the analysis we need. With the light diffraction of the blue laser we obtain Forward Scatter (FSC), this parameter is proportional to the size of the cell, the reflection of the blue light gives us the parameter Side Scatter (SSC) which is proportional to granularity. To distinguish between dead and live cells we use these two parameters. This technique can be used in efflux pumps using fluorescents substrates, the intracellular fluorescence allows us to understand the amount of molecules inside the cell and how many were transported.

Mass cytometry

In this technique, every cell is stained with a stable isotope and injected into a mass cytometer. Cells are then atomized and ionized in a high temperature of Inductively Coupled Plasma (ICP). The mass to charge ratio of an ion in each cell is then measured by time of flight mass spectrometry (TOF-MS), generating distinct mass spectra of each cell. The mass cytometer is capable of measuring heavy elements naturally present or introduced into a cell, such as ruthenium. In TOF analysis, all ions are accelerated through an electric field of a known strength, and the time it takes for these ions to reach the detector over a known distance is measured. The heavier the ion, the longer it takes to get to the detector. We used for this technique the DVS Sciences Inc., Markham, ON, Canada machine. To analyse the data we used FCS format in FlowJo v10.0.7 software.

Cells Counting

This technique allows us to know the cell viability, the number of dead cells, the cells diameter and the cells concentration in the trypan blue solution. We used the Cellometer Auto T4 (Nexcelom Bioscience).

### 5.1.3 Procedures

MTT assay

Cells were plated in 96-well sterile plates at a density to ensure exponential growth of untreated control samples throughout the experiment, $10^4$ cells per well with 200 µL of medium. For 24 h cells were allowed to settle followed by the addition of dilution series of the test compounds. Ligands and complexes were solubilized in DMSO/DMEM, with a maximum of 0.5 % of DMSO per concentration, in a range of 0 to 200 µM. After continuous exposure to the compounds for 48 h, at 37 °C with 5% of $CO_2$, the media were removed and cells were incubated with MTT solution in PBS (0.5 mg/mL) at same conditions. After 4h, the yellow solution was carefully removed and the purple formazan crystals formed inside the cells were dissolved with DMSO through shaking. Incubate again for 1h under stirring. The cellular viability was evaluated by measurement of the absorbance at 570 nm by using a plate spectrophotometer, and this was corrected with the absorbance measured at 690 nm.

Flow Cytometry

Cells were seeded at a density of $10^5$ cells/well into 24-well culture plates. After a 24-hour incubation period, they were exposed to different concentrations of compounds and substrates for 30 minutes at 37°C37˚C 5% CO2. After treatment, the cells were then washed with phosphate buffer saline (PBS) and undetached with trypsine from the plates. After was neutralized with PBS, resuspend and transferred to cytometer tubes. Was keep in the ice until the analysis (max 2h) in FACSCalibur cytometer (BD Biosciences, San Jose, CA, USA) and BD LSR-II system.

Mass cytometry

Were seeded in 6-well plates for 48 h to reach a density of ~ $10^6$ cells / mL. Cells were treated for 15 min with 20 µM compounds and then washed with PBS, trypsinize for 5 minutes, neutralized with medium, resuspended and transferred to Eppendorf microcentrifuge tubes. Centrifuged at 300 x g during 5 minutes, discarded supernatant and treated with cisplatin 5 µM during 5 minutes. Centrifuged again, washed with DPBS and fixed overnight in 1 mL of 4% (PFA). The next day, the supernatant was discarded and we added 0.25 µM Iridium to label the DNA for 45 minutes. Then, we carefully washed the cells for discarding traces of metals with MaxPar water and DPBS. After one last centrifuged the cells were then analysed using a cyTOF mass cytometer (DVS Sciences Inc.).

Cells Counting

We added 30 µL of cells to a 30 µL of trypan blue solution, then we took 20 µL of that solution and we transferred for a counting chamber. Introduce this chamber in Cellolmeter Auto T4 and we read the measure.

## 5.2 Chemistry

### 5.2.1 Solvents and Reagents

Most of the reagents used (Table 5.1) for the organometallic synthesis were purchased from Sigma-Aldrich and used directly without any further purification. The solvents used in the synthesis were dried and distilled under dinitrogen atmosphere following the literature procedures and are presented in the following table.

Table 5.1- Drying processes of different solvents used.

| Solvent | Boiling Point (°C) | Pre-drying | Reflux-Destilation |
|---|---|---|---|
| Dichloromethane | 40 | $CaCl_2$ | $CaH_2$ |
| *n*-Hexan | 68.7 | Sodium wire | Sodium wire |
| Methanol | 64.7 | $CaSO_4$ | - |
| Acetone | 56.0 | $CaSO_4$ | $CaH_2$ |

### 5.2.2 Measurements

Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR spectra were recorded on an AC Bruker of 300MHz (samples from Chapter 3) or on a Brucker Avance of 400 MHz spectrometer, at the respective probe temperature. The deuterated solvent DMSO-d6 (99%), were purchased from CIL and were used directly. Approximately 3 and 30 mg of sample were directly dissolved into the NMR tube in 0.5 mL of solvent for 1H and 13C NMR, respectively. The chemical shifts given in parts-per-million (ppm) were calibrated using the residual resonances of the deuterated solvent. For characterization: s = singlet, d = doublet, t = triplet, m = multiplet.

Ultraviolet-visible spectroscopy (UV-vis)

The electronic UV-vis spectra were recorded at room temperature on a Jasco V-660 spectrometer at air in 1 cm path quartz cell, in dichloromethane or dimethyl sulfoxide (used directly without purification). The scanning was at air and room temperature. The concentration used for the spectrum was in range $10^{-3}$-$10^{-5}$.

Fourier transform Infrared spectroscopy (FTIR)

IR spectra were recorded in a Shimadzu IRAffinity-1 FTIR spectrophotometer with KBr pellets at air and room temperature. For characterization: s = strong, m = medium and w = weak.

Elemental analysis

Elemental analysis were obtained at Laboratório de Análises, Instituto Superior Técnico, using a Fisons Instruments EA1108 system. Data acquisition, integration and handling were performed using a PC with the software package EAGER-200 (Carlo Erba Instruments).

Stability studies by UV-Vis

The stability studies were realized using Jasco V-660 spectrometer at air in 1 cm path quartz cell. The experiment was along approximately 6 hours and after was made the last mediation at 24h. The solvents used were DMSO (5%) and DMEM. The solutions were prepared at air and room temperature.

Octanol-Water partition coefficient logP

Was performed using a Jasco V-660 spectrometer at air in 1 cm path quartz cell. The solvents used were octanol and water. In the shake-flask method, the compound was added, dissolved in octanol and water. The distribution of solute measured by UV-Vis. Dilutions from a main solution $10^{-4}$ M, were prepared. The solutions were prepared at air and room temperature. The concentration used for the spectrum was in range $10^{-4}$ - $2\times10^{-5}$ M.

### 5.2.3 Procedures

The synthesis of **Ru1** ([Ru(Me-$C_5H_5$)($PPh_3$)$_2$Cl]) was done following a modified literature procedure[56] and the Ph.D. thesis of Leonor Côrte-Real.[58] The ligand **L1** synthesis was based in a modified literature procedure.[53,54,55]

This synthesis was made under nitrogen atmosphere and using *Schlenk* techniques. All solvents used were dried and freshly distilled under nitrogen prior to use. $^{1}$H , $^{13}$C and $^{31}$P chemical shifts (s = singlet; d = duplet; t = triplet; m = multiplet; comp = complex) are reported in parts per million (ppm).

**Ru1- [Ru (Me-$C_5H_4$)($PPh_3$)$_2$Cl]**

The complex **Ru1** was synthesized by addition of freshly distilled methylcyclopentadiene (5 mL) and triphenylphosphane (2.90 g;11 mmol) to a stirred ethanolic solution (40 mL) of ruthenium trichloride (0.50 g; 2.4 mmol), following a modified literature procedure.[56] The dark brown solution obtained was then heated to reflux temperature for a period of 8 h. After that time, the solution got lighter in color and an orange precipitate was formed. Isolation of analytical pure, this neutral complex was achieved twice by wash with water (2×20mL), ethanol(2×20mL), and a mixture of ethanol and petroleum ether (50:50 (%v/v), 2x20 mL). Was recrystallized from dichloromethane/*n*-hexane mixture giving dark orange/red crystals in 61 % yield.

**$^{1}$H NMR -** [DMSO, δ/ppm [(assignment, multiplicity)]: 7.28 (m, 12H, H-$PPh_3$), 7.26 (m, 6H, $H_{para}$ $PPh_3$), 7.17 (t, 12H, $J_{HH}$ = 8, H-$PPh_3$), 3.87 (s, 2H, $H_3$), 3.24 (s, 2H, $H_4$), 1.79 (s, 3H, $H_1$).

**$^{31}$P NMR –** [DMSO, δ/ppm [(assignment, multiplicity)]: 39.97 [s, $PPh_3$].

**L1- 2,2'-bipyridine-4,4'-dibiotin ester**

The synthesis was performed by addition of 4,4´-dihydroxymethyl-2,2´-bipyridine (0.15 g; 0.69 mmol), 5-[(3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d] imidazol-4-yl]pentanoic acid (biotin) (0.42 g; 0.017 mmol) and 4-dimethylaminopyridine (DMAP; 0.085 g; 0.69 mmol) in dimethylformamide (DMF; 10mL) to a stirred solution and with ice/water bath. Then EDC was added (0.33 g; 0.017 mmol), to the colourless solution obtained and continue to stir for 30 minutes with the ice/water bath. After the 30 minutes, the bath was removed and the solutions stayed in stirring all night at room temperature. On the other day an incolor solution was obtained. This solution was evaporated under vacuum and washed with ethyl ether. After was filtrated under vacuum and washed with water and ethyl ether, until to have white crystals in 60 % yield.

**$^{1}$H NMR –** [DMSO-d$^{6}$ δ/ppm [(assignment, multiplicity), 400 MHz]: 8.68 (d, 2H, $^{3}J_{HH}$=4.8, H1), 8.37 (s, 2H, H4), 7.43 (d, 2H, $^{3}J_{HH}$=4.8, H2), 6.45 (s, 2H, NH1), 6.37 (s, 2H, NH2), 5.25 (s, 4H, H6), 4.29 (t, 4H, $^{3}J_{HH}$=6,8, H13), 4.11 (t, 4H, $^{3}J_{HH}$=4, H15), 3.08 (m, 2H, H12), 2.79 (dd, 4H, $^{3}J_{HH}$=5.2, $^{3}J_{HH}$=5.2, H16), 2.57 (s, 4H, H16), 2.44 (d, 4H, $^{3}J_{HH}$=7.2, H8), 1.63-1.33(m,12H, H9, H10, H11) .

**FTIR -** [KBr, cm$^{-1}$]: 3385.1-3238.5 cm$^{-1}$ (NH stretch), 3082.3-2862.4 cm$^{-1}$ (C-H stretch aromatic), 1732.1 cm$^{-1}$ (C=O stretch ester), 1141.9 cm$^{-1}$ (C-N stretch), 1705 cm$^{-1}$ (NH-C=O-NH).

**UV-Vis-** [DMSO, $\lambda_{max}$/nm (ε/M$^{-1}$cm$^{-1}$)]: 286 (9495).

**Elemental Analysis -** Cal for $C_{32}H_{40}N_6O_6S_2$ (668.83): C57.47, H6.03, N12.57, S9.59. Found: C57.01, H6.19, N12.47, S9.34.

**Ru2 - [Ru(η$^{5}$-(Me-C$_5$H$_4$)(PPh$_3$)(bipy-biot)][CF$_3$SO$_3$]**

The new ruthenium complex (**Ru2**) was obtained by adding the silver trifluoromethanesulfonate (1.5 eq) to neutral compound (1.7g; 0.23 mmol) in a stirred methanol solution (40 mL) during one hour. Then, the ligand **L1** (1.1 eq) was added to this brown mixture and after this solution was heated to reflux temperature for a period of 6 h. Then was filtrated by cannula technique and the solvent was evaporated. Was also twice recrystallized from dichloromethane/*n*-hexane mixture giving red crystals in 69 % yield.

**$^{1}$H NMR -** [DMSO-d$^{6}$ δ/ppm [(assignment, multiplicity), 400 MHz]: 9.29 (dd, 2H, $^{3}J_{HH}$=2.4, $^{3}J_{HH}$=2.4, H1), 8.07 (37 (s, 2H, H4), 7.56 (m,2H, H2), 7.36 (m, 3H, Hd-PPh3), 7.27 (t, 3H, $^{3}J_{HH}$=7.2, Hc-PPh3), 6.93 (t, 3H, $^{3}J_{HH}$=8.8, Hb-PPh3), 6.48 (s, 2H, NH1), 6.42 (s, 2H, NH2), 5.19 (s, 4H, H6), 4.70 (s, 1H, H3-Cp), 4.59 (s, 2H, H4-Cp), 4.31 (s, 4H, H15), 4.14 (m, 4H, H13), 3.12 (s, 2H, H12), 2.81 (m, 4H, H16), 2.58 (dd, 4H, $^{3}J_{HH}$=2, $^{3}J_{HH}$=2.8, H16), 2.57 (m, 4H, H8), 1.57 (s, 3H, H1-Cp), 1.60-1.24 (m, 12H, H9, H10, H11).

**$^{31}$P NMR –** [$CDCl_3$, δ/ppm [(assignment, multiplicity)]: 51.96 [s, $PPh_3$].

**$^{13}$C NMR -** [DMSO-d$^{6}$ δ/ppm [(assignment, multiplicity), 100 MHz]: 172.64 (C7), 162.77 (C14), 155.36 (C1), 154.75 (C5),132.50 (d, $^{2}J_{CP}$=11, Cb-PPh3), 131.12 (C3, $^{1}J_{CP}$=41), 130.91 (Ca-PPh3), 129.98 (Cd-PPh3), 128.49 (d, $^{3}J_{CP}$ =9, Cc-PPh3), 123.60 (C2), 121.35 (C4), 102.34 (C2.Cp), 75.92 (d, C3-Cp), 75.70 (C4-Cp), 63.16 (C6), 61.07 (C15), 59.21 (C13), 55.52 (d,C12), 40.9 (d,C16), 33.13 (C8), 28.05-22.11 (C9,C10,C11) 11.31 (C1-Cp).

**FTIR-**[KBr, cm$^{-1}$]: 3048 cm$^{-1}$ ($\upsilon_{CH}$ aromatics), 1435 cm$^{-1}$ (C-C stretch), 1475 cm$^{-1}$ (C=C stretch), 1250 cm$^{-1}$ (counter-ion), 1150 cm$^{-1}$ (amino aromatic), 3450 cm$^{-1}$ (NH stretch), 1740 cm$^{-1}$ (C=O stretch

ester), 1275 $cm^{-1}$ and 1100 $cm^{-1}$ (C-N stretch), 700 $cm^{-1}$ and 650 $cm^{-1}$ (C-S stretch), 1695 $cm^{-1}$ (NH-C=O-NH).

**UV-Vis -** [DMSO, $\lambda_{max}$/nm ($\varepsilon/M^{-1}cm^{-1}$)]: 250 (Sh), 294 (18000), 345 (Sh), 428 (2990), 489 (Sh).

[$CH_2Cl_2$, $\lambda_{max}$/nm ($\varepsilon/M^{-1}cm^{-1}$)]: 249 (Sh), 294 (17700), 337 (5450), 434 (3360), 489 (Sh).

**Elemental Analysis -** calc. For $C_62H_{82}F_3N_6O_9PRuS_3$ (1340.58): C52.85, H5.17, N6.43, S7.36. Found: C52.7, H4.7, N6.1, S7.0.

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# 7. Annexes

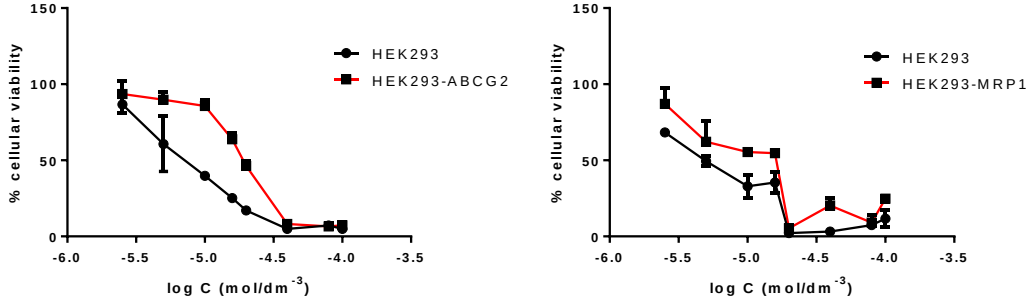


Figure 7.1- Cellular viability for the compound PMC79 in HEK293 cell lines. Concentration range between 2,5-100µM.

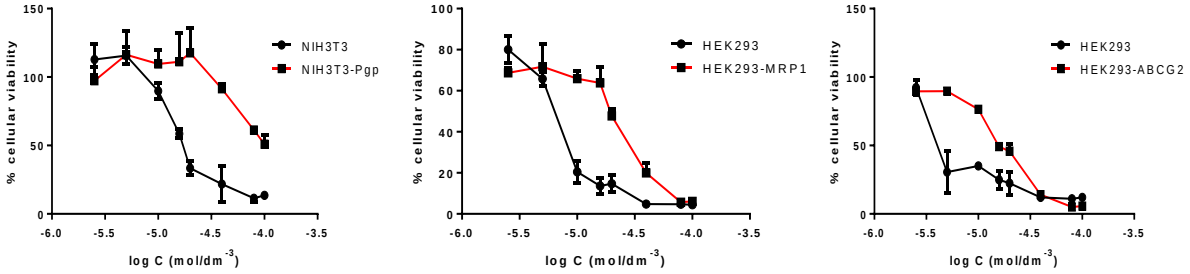


Figure 7.2- Cellular viability for the compound RT12 in HEK293 and NIH3T3 cell lines. Concentration range between 2,5-100µM.

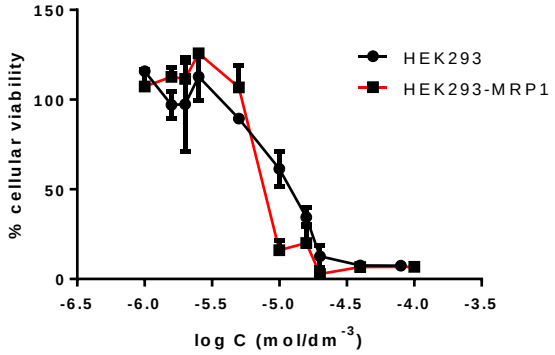


Figure 7.3- Cellular viability for the compound PMC85 in HEK293 cell lines. Concentration range between 1-100µM.

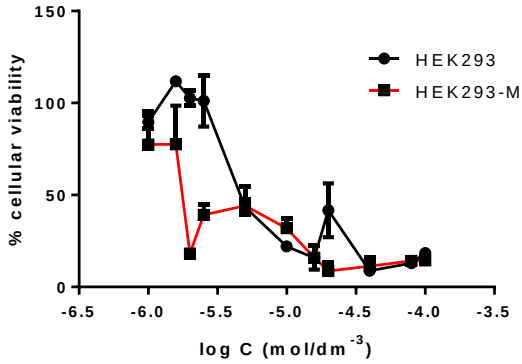


Figure 7.4- Cellular viability for the compound LCR136 in HEK293 cell lines. Concentration range between 1-100µM.

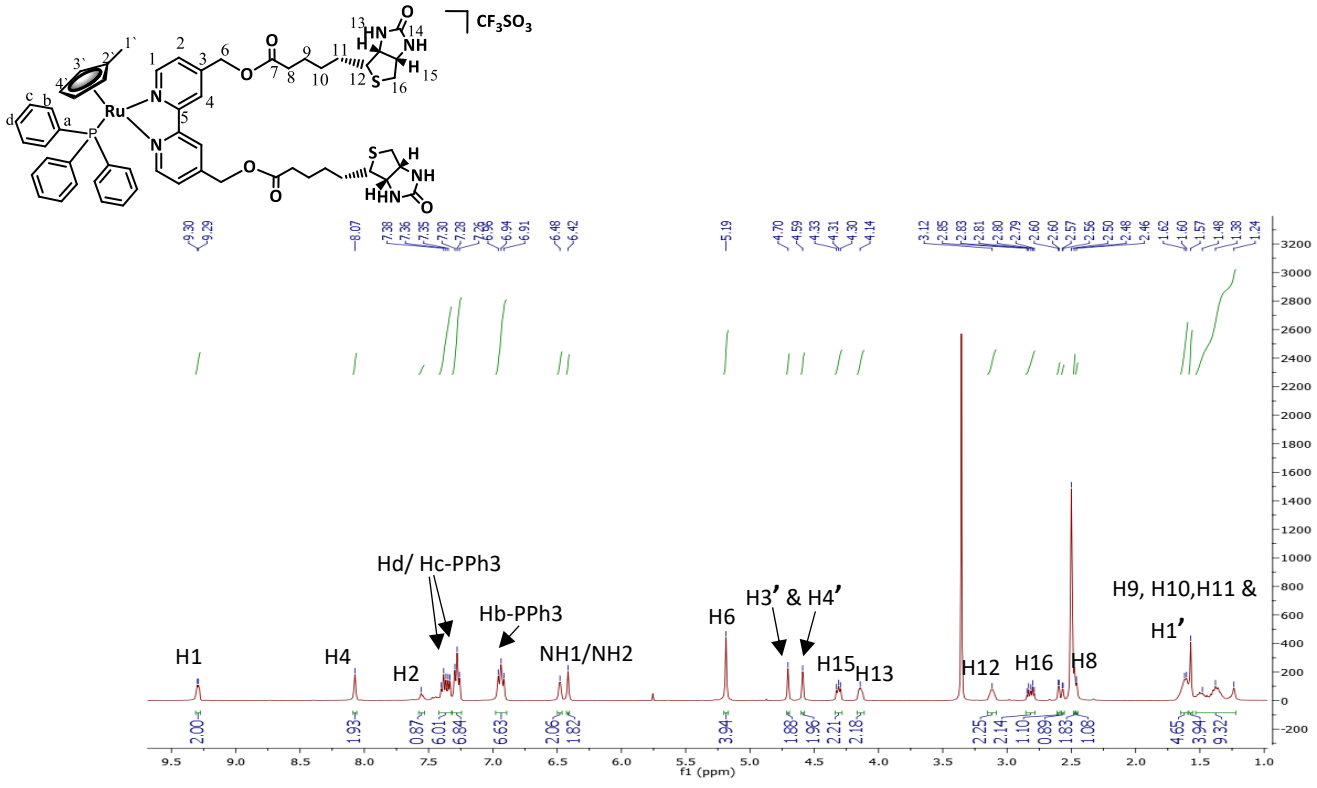


Figure 7.5-$^{1}$H-NMR for the complex **Ru2** in DMSO-d$^{6}$.

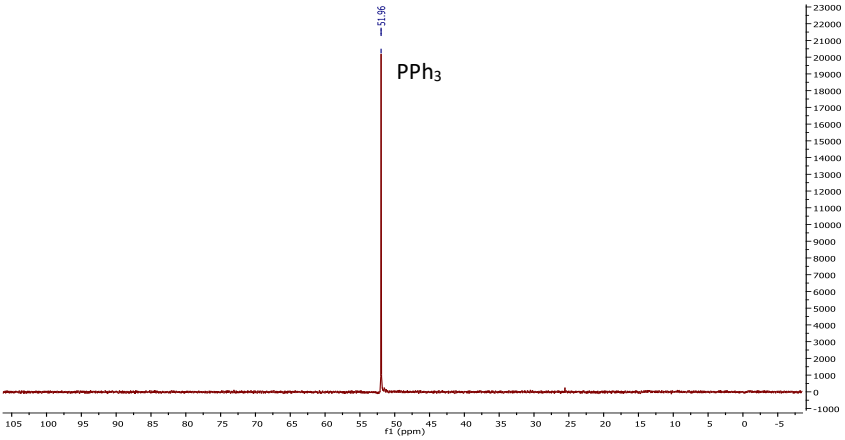


Figure 7.6-$^{31}$P-NMR for the complex **Ru2**.

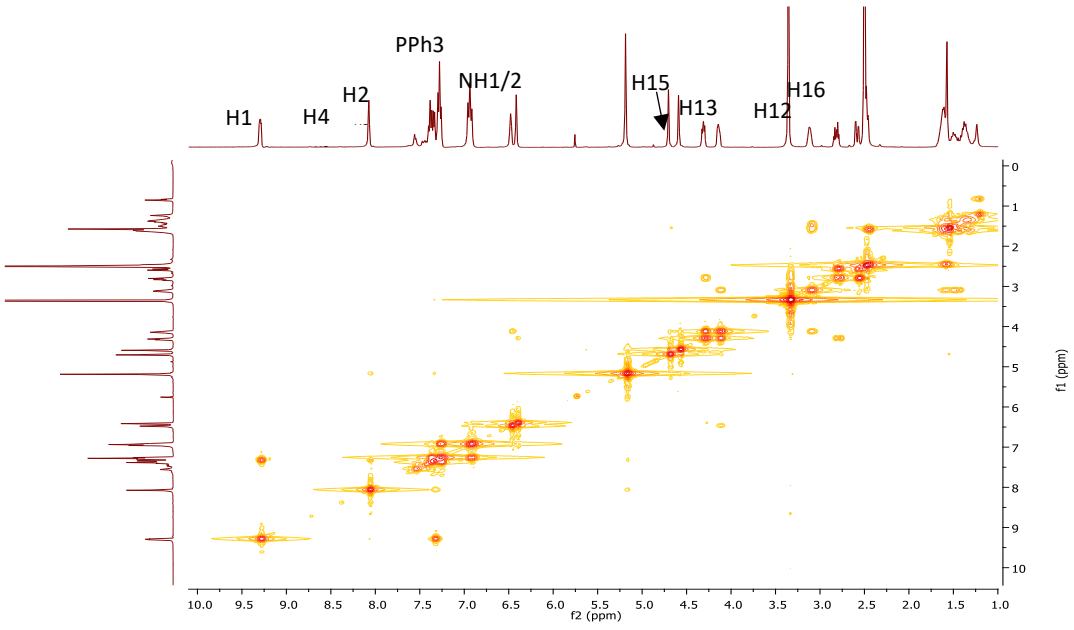


Figure 7. 7- 2-D NMR experience (cosy) for the complex **Ru2**.

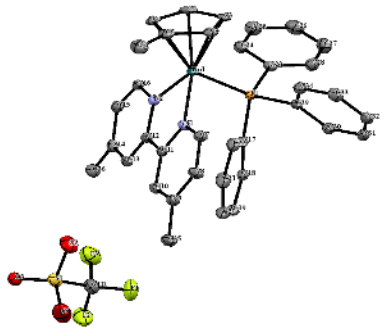

Fig. 7.8 -Molecular structure of RT11