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Organometallic compounds of tin and ruthenium – Applications in Medicinal Chemistry

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Ana Soraya LIMA BARBOSA

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Membros da comissão julgadora da defesa de tese da doutoranda **Ana Soraya Lima Barbosa** intitulada: "***Organometallic compounds of tin and ruthenium – Applications in medicinal chemistry***", apresentada ao Programa de Pós-Graduação em Química e Biotecnologia da Universidade Federal de Alagoas no dia 29 de abril de 2016, às 09h, no Auditório da FEAC, Faculdade de Economia, Administração e Contabilidade da Universidade Federal de Alagoas.

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« Mets ta joie dans le Seigneur : il comblera les désirs de ton coeur. » (Psaume 36, 4)

“Põe tuas delícias no Senhor, e os desejos do teu coração ele atenderá.” (Salmo 36, 4)

RESUMO

A Química Inorgânica Medicinal é um belo e complexo campo de pesquisa que pode, entre outras coisas, conduzir à descoberta de novos medicamentos e também à compreensão do mecanismo de ação dos compostos à base de metal. Neste contexto, o objetivo desta tese teve uma dupla motivação: (i) encontrar novos compostos organoestânicos que pudessem ser aplicadas como antimicrobianos; e (ii) contribuir para a elucidação do mecanismo de ação anticâncer de compostos à base de rutênio. Em relação à pesquisa de antimicrobianos, embora alguns novos medicamentos estejam em desenvolvimento, eles não cobrem adequadamente as crescentes necessidades médicas, além do que essas novas alternativas são derivadas principalmente das classes de antibióticos em uso e, portanto, propenso à resistência bacteriana por mecanismos existentes. Desse modo, são urgentemente necessárias novas classes de antimicrobianos. Neste sentido, foi desenvolvido no grupo de pesquisa "Grupo de Catálise e Reatividade Química" compostos de estanho derivado de três ácidos graxos bioativos (ácidos undecilênico, ricinoleico e caprílico) e descobrimos que eles possuem muito alta atividade contra algumas cepas de bactérias e leveduras, chegando à escala nM, e sendo até quatro mil vezes mais potente contra *Staphylococcus aureus* do que contra células de mamíferos. Para os compostos de rutênio, por sua vez, sabe-se que muitos deles exibem promissora atividade anticâncer, e que dois deles entraram ensaios clínicos: NAMI-A e KP1019, ambos compostos derivados do rutênio(III). Visto que estes complexos de Ru(III) dependem da sua redução *in vivo* para exercer a atividade antitumoral, compostos com Ru(II) parecem ser protótipos mais interessantes para aplicação como agentes anticâncer. Portanto, o "*Laboratoire de Synthèses Métallo Induites*" (atualmente "*Laboratoire de Chimie et Systémique Organo-Métalliques*") tem desenvolvido compostos que podem ser considerados candidatos interessantes a fármacos anticancerígenos. Neste trabalho, foi confirmado que o mecanismo de ação do **RDC11** é indubitavelmente diferente daqueles observados para a cisplatina ou outros compostos de rutênio, devido à sua elevada estabilidade frente a reações de substituição (na ausência de aquecimento ou de luz). Conseguimos ainda vetorizar com sucesso **RDCs** com uma proteína de afinidade chamada *Affitin*, utilizando a química "click". Ao trabalhar com complexos com diferentes valores de potencial redox, provamos a importância dessa propriedade para transferência de elétrons para a proteína utilizada, culminando com sua precipitação significativa no caso do complexo **NCN-Ru-N_3**. Desta maneira, a partir desta descoberta casual (*serendipity*), fomos capazes de adquirir conhecimentos importantes sobre um possível mecanismo de ação anticâncer deste tipo de composto.

Palavras-chave: Química Inorgânica Medicinal, organoestânicos, ácido graxo, antimicrobianos, compostos derivados de rutênio, agentes anticâncer, Affitin.

ABSTRACT

Medicinal Inorganic Chemistry is a complex and beautiful field of research that might, among other things, lead to discovery of new drugs and also to understanding of the mechanism of action of metal-based compounds. In this context, the aim of this thesis had a dual motivation: (i) to find new organotin compounds which could be applied in the antimicrobial domain; and (ii) to make a contribution to elucidating the mechanism of anticancer activity of ruthenium-based compounds. Related to antimicrobial research, although some new drugs are actually in development, they do not adequately cover growing medical needs and also these drugs are mostly derivatives of older classes already in use and therefore prone to existing bacterial resistance mechanisms, being new antimicrobial classes urgently needed. In this sense, we developed in the research group "*Grupo de Catálise e Reatividade Química*" tin compounds derived from three bioactive fatty acids (undecylenic, ricinoleic and caprylic acids) and we found that they show very high activity against some strains of bacteria and yeast, even in nM range, being up to four thousand times more potent against *Staphylococcus aureus* than against mammalian cells. For ruthenium compounds, in turn, it is known that many of them display promising anticancer activity, and two of them have entered clinical trials: NAMI-A and KP1019, both ruthenium(III)-derived compounds. Since Ru(III) complexes depending on the *in vivo* reduction to exerce the anticancer activity, Ru(II)-derived compounds seems to be more interesting anticancer prototypes. Therefore, the "*Laboratoire de Synthèses Métallo Induites*" (actually "*Laboratoire de Chimie et Systémique Organo-Métalliques*") has developed compounds that can be considered interesting anticancer drugs candidates. In this work, we have confirmed that the mode of action of **RDC11** is undoubtedly different from cisplatin or other ruthenium compounds, because of its high stability toward substitution reactions (in the absence of heat or light). We also successfully vectorized **RDCs** with an affinity protein called Affitin by click chemistry. By working with complexes with different redox potential values, we proved the importance of this property to electron transfer to the affitin, culminating with a significant protein precipitation in the case of **NCN-Ru-N_3** complex. Therefore, from this serendipitous discovery, we were able to gain important knowledge about a possible mechanism of action of this kind of molecules.

Keywords: Medicinal Inorganic Chemistry, organotin, fatty acid, antimicrobial agent, ruthenium-based compounds, anticancer agent, Affitin.

RÉSUMÉ

La Chimie Inorganique Médicinale est un domaine complexe et beau de la recherche, et qui pourrait mener à la découverte de nouveaux médicaments et aussi à la comprehension du mécanisme d'action des composés métalliques. Dans ce contexte, l'objectif de cette thèse a eu une double motivation : (i) trouver de nouveaux composés organostanniques qui pourraient être appliqués dans le domaine antimicrobien; et (ii) apporter une contribution à l'élucidation du mécanisme d'activité anticancéreuse de composés à base de ruthénium. En ce qui concerne la recherche antimicrobienne, bien que certains nouveaux médicaments sont mis au point, ils ne couvrent pas adéquatement l'augmentation des besoins médicaux, en plus ces nouvelles alternatives comprenent principalement classes d'antibiotiques déjà en utilisation et donc susceptibles à des mécanismes de résistance existants. Ainsi, il est nécessaire de toute urgence de nouvelles classes d'antibiotiques. En ce sens, il a été développé dans le groupe de recherche "Grupo de Catálise e Reatividade Química" composés d'étain provenant de trois acides gras bioactive (acide undécylénique, ricinoléique et caprylique) et il a été constaté qu'ils ont une activité très élevée contre certaines souches de bactéries et de levures, atteindre la plage des nM et avec un maximum de quatre mille fois plus puissant contre *Staphylococcus aureus* que contre des cellules de mammifères. Pour les composés de ruthénium, à son tour, il est connu que beaucoup d'entre eux présentent une activité anticancéreuse prometteuse, et dont deux ont été soumis à des essais cliniques: NAMI-A et KP1019, deux composés dérivés du ruthénium (III). Etant donné que ces complexes de Ru (III) dépendent d'une réduction *in vivo* pour exercer une activité anti-tumorale, les composés de Ru (II) semblent être des prototypes les plus intéressants pour être utilisés comme agents anti-cancéreux. Par conséquent, le "Laboratoire de Synthèses métallo Induites" (actuellement nommé "Laboratoire de Chimie et Systémique organo-métalliques") a mis au point des composés qui peuvent être considérés comme des candidats intéressants comme des médicaments anticancéreux. Dans ce travail, il a été confirmé que le mécanisme d'action de RDC11 est sans doute différent de ceux observés pour Cisplatine ou d'autres composés dérivés du ruthénium, en raison de sa grande stabilité dans les réactions de substitution (en l'absence de chauffage ou de la lumière). Enfin, pendant la vectorisation des composés dérivés du ruthénium avec la protéine d'affinité appellée *Affitine* nous avons pu acquérir des connaissances importantes sur un éventuel mécanisme d'action de ce type de molécules. Il se pourrait en effet que ces composés qui ont un potentiel redox très abaissé par rapport aux composés correspondants puissent provoquer la polymérisation de protéine par transfert d'électron entre le complexe du ruthénium et les protéines, provoquant sa précipitation. Ainsi, ce résultat inattendu nous oriente vers une nouvelle piste du mécanisme d'action des anticancéreux cyclométallés du ruthénium.

Mots-clés: Chimie Inorganique Médicinale, organostaniques, acides gras, antimicrobien, composés dérivés du ruthénium, agents anti-cancéreux, Affitine.

LIST OF ABBREVIATIONS

3LL	Lewis lung carcinoma cell line
AIDS	Acquired Immune Deficiency Syndrome
AKT	Serine / threonine kinase
ANOVA	Analysis of variancy
B16F10	Subcutaneous melanoma cell line
BCG	Bacillus Calmette-Guerin
ATCC	American Type Cell Collection
BHI	Brain-heart infusion broth
CFU	Colony forming units
CHOP	Acronym for a chemotherapy regimen used in the treatment of non-Hodgkin lymphoma. Consists of: cyclophosphamide, doxorubicin hydrochloride, vincristine sulfate and prednisone.
CLSI	Clinical and Laboratory Standards Institute
COLO 205	colon cancer cell line
CuAAC	Copper-catalyzed azide-alkyne cycloaddition
DMEM	Dulbecco's Modified Eagle's *medium*
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
EEF1AI	Translation elongation factor that controls proper enzyme folding
ER	Endoplasmic reticulum
ESI-MS	Electrospray ionisation mass spectrometry
FBS	Fetal bovine serum
FDA	Food and Drugs Administration
FFA	Free fatty acid
HBSS	Hank's balanced salt solution.
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-1	Hypoxia-inducible factor 1
IDH	The isocitrate dehydrogenase
IND	Investigational new drug
IUPAC	International Union of Pure and Applied Chemistry
J774	Murine macrophages cell line
LC_{50}	Concentration required to induce 50% death of cells

MIC	Minimal Inhibitory Concentration
MALME 3M	Melanoma cell line
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaAsc	Sodium ascorbate
NCCLS	National Committee for Clinical Laboratory Standards
NCI	National Cancer Institute
NCI60	60 human tumour cell lines anticancer screen carried out by the NCI
NDA	New drug application
NSCLC NCI-H522	Non-small-cell lung carcinoma cell line
OB-fold	Oligonucleotide/oligosaccharide-binding-fold proteins
OVCAR-3	Ovarian cancer cell line
PDUFA	Prescription Drug User Fee Act
PHD2	Cancer cell oxygen sensor
RDC	Ruthenium-derived compounds
RDC-Affitin	Ruthenium-derived compounds conjugated to an Affitin
Sac7d	DNA-binding protein 7d
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
S.E.M	Standard error of the mean
SK-MEL-2	Melanoma cell line
SK-MEL-5	Melanoma cell line
Terpy	2,6-bis(2-pyridyl)pyridine
THPTA	Tris(3-hydroxypropyltriazolylmethyl)amine
TTC	Triphenyltetrazolium chloride
U87	Human glioblastoma cell line
UAC-62	Melanoma cell line
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

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General Introduction

General Introduction

i. Medicinal Chemistry*

For better understanding the medicinal chemistry, it is important to know some concepts related to this field of research. Firstly, as recommended by IUPAC (1998), *medicinal chemistry is a chemistry-based discipline, also involving aspects of biological, medical and pharmaceutical sciences. It is concerned with the invention, discovery, design, identification and preparation of biologically active compounds, the study of their metabolism, the interpretation of their mode of action at the molecular level and the construction of structure-activity relationships*. A drug is thus any substance presented for treating, curing or preventing disease in human beings or in animals, and may also be used for making a medical diagnosis or for restoring, correcting, or modifying physiological functions.[1]

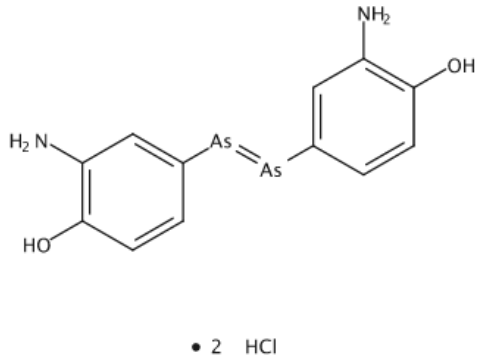
The process of identifying active new chemical entities, which by subsequent modification may be transformed into a clinically useful drug, is called lead discovery. Lead generation, in turn, is the term applied to strategies developed to identify compounds which possess a desired but non-optimized biological activity; and lead optimization is the synthetic modification of a biologically active compound, to fulfill all stereoelectronic, physicochemical, pharmacokinetic and toxicologic requirements for clinical usefulness. [1]

It is often assumed even by the most studied historians that the history of medicine began with the Greeks, and that before the time of Hippocrates there was little knowledge if any that can be called a science of medicine. But the Papyrus Ebers is the longest and the most famous document relating to the most ancient practice of medicine and, although written about 1500 b.C., it is really a collection of bits and pieces of folklore, much of it five and some of it probably twenty centuries oldier.[2]

The advent of modern medicinal chemistry is generally attributed to Paul Ehrlich's discovery of salvarsan (Figure 1), following screening of a large number of compounds. Salvarsan is a potent antibiotic that was originally used to treat syphilis. Over many years the structure of salvarsan was refined to improve its pharmacological properties, e.g. water solubility, and these latter organo-arsenic compounds were only phased out of clinical use after the discovery of penicillins, that were safer drugs and that are still in use nowadays.[3]

* This section was mostly extracted from Lipsky, M. S.; Sharp, L. K. *J. Am. Board. Fam. Med.* **2001**, *14*, 362–367, that explain the steps to register a drug on FDA.

Figure 1. Chemical structure of salvarsan



Drug development can generally be divided into phases. As an example, in United States of America the steps are as following [4]:

- The first step, a **preclinical phase**, is to find a promising agent, which involves taking advantage of the advances made in understanding a disease, pharmacology, computer science, and chemistry. Breaking down a disease process into its components can provide clues for targeting drug development. Numerous compounds might be synthesized and tested before a promising agent emerges. Computer modeling often helps select what compounds might be the most promising.

The next step before attempting a clinical trial in humans is to test the drug in living animals, usually rodents. The FDA (Food and Drugs Administration) requires that certain animal tests be conducted before humans are exposed to a new molecular entity. The objectives of early *in vivo* testing are to demonstrate the safety of the proposed medication. For example, tests should prove that the compound does not cause chromosomal damage and is not toxic at the doses that would most likely be effective. The results of these tests are used to support the investigational new drug (IND) application that is filed with the FDA. The IND application includes chemical and manufacturing data, animal test results, including pharmacology and safety data, the rationale for testing a new compound in humans, strategies for protection of human volunteers, and a plan for clinical testing. If the FDA is satisfied with the documentation, the stage is set for phase 1 clinical trials.

- **Phase 1 studies** focus on the safety and pharmacology of a compound. During this stage low doses of a compound are administered to a small group of healthy volunteers who are closely supervised. In cases of severe or life-threatening illnesses, volunteers with the disease may be used. Generally, 20 to 100 volunteers are enrolled in a phase 1 trial. These studies usually start with very low doses, which are gradually increased. On average, about two thirds of phase 1 compounds will be found safe enough to progress to phase 2.

- **Phase 2 studies** examine the effectiveness of a compound. To avoid unnecessarily exposing a human volunteer to a potentially harmful substance, studies are based on an analysis of the fewest volunteers needed to provide sufficient statistical power to determine efficacy. Typically, phase 2 studies involve 100 to 300 patients who suffer from the condition the new drug is intended to treat. During phase 2 studies, researchers seek to determine the effective dose, the method of delivery (e.g., oral or intravenous), and the dosing interval, as well as to reconfirm product safety. Patients in this stage are monitored carefully and assessed continuously. A substantial number of these drug trials are discontinued during phase 2 studies. Some drugs turn out to be ineffective, while others have safety problems or intolerable side effects.

- **Phase 3 trials** are the final step before seeking FDA approval. During phase 3, researchers try to confirm previous findings in a larger population. These studies usually last from 2 to 10 years and involve thousands of patients across multiple sites. These studies are used to demonstrate further safety and effectiveness and to determine the best dosage. Despite the intense scrutiny a product receives before undergoing expensive and extensive phase 3 testing, approximately 10% of medications fail in phase 3 trials.

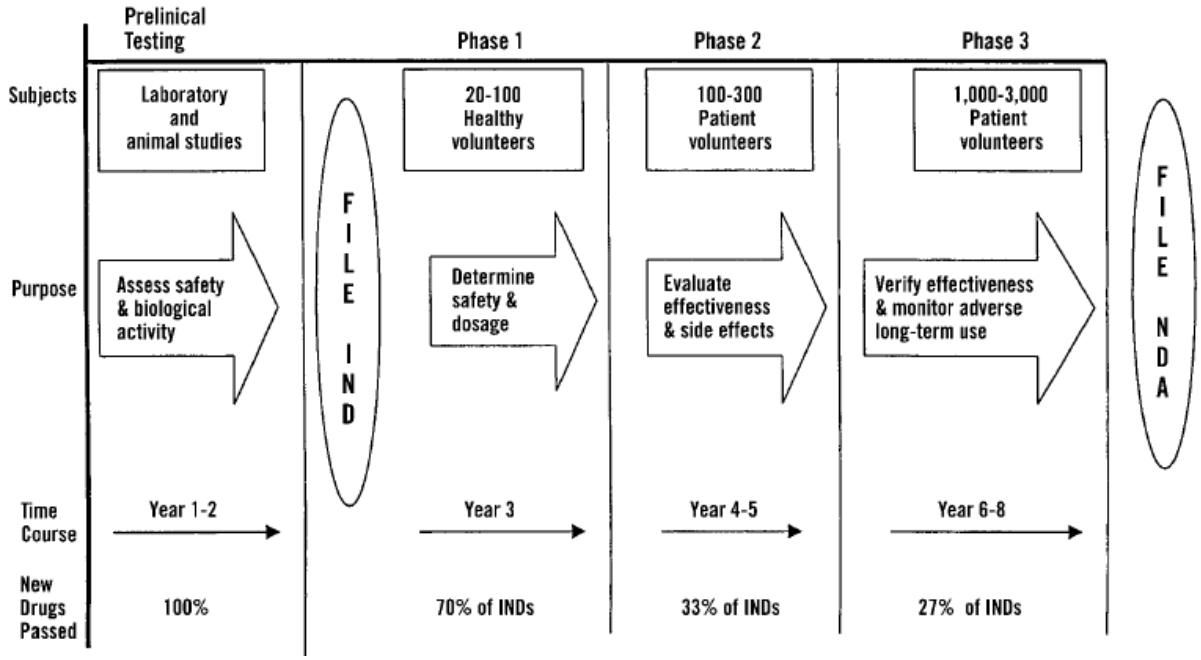
If a drug survives the clinical trials, an NDA (new drug application) is submitted to the FDA. An NDA contains all the preclinical and clinical information obtained during the testing phase. The application contains information on the chemical makeup and manufacturing process, pharmacology and toxicity of the compound, human pharmacokinetics, results of the clinical trials, and proposed labeling. An NDA can include experience with the medication from outside the United States as well as external studies related to the drug.[4]

After receiving an NDA, the FDA completes an independent review and makes its recommendations. The Prescription Drug User Fee Act of 1992 (PDUFA) was designed to help shorten the review time. This act allowed the agency to collect user fees from pharmaceutical companies as financial support to enhance the review process. The 1992 act specifies that the FDA reviews a standard drug application within 12 months and a priority application within 6 months. Application for drugs similar to those on the market are considered standard, whereas priority applications represent drugs offering important advances in addition to existing treatments. If during the review the FDA staff feels there is a need for additional information or corrections, they will make a written request to the applicant. During the review process it is not unusual for the FDA to interact with the applicant staff. [4]

Once the review is complete, the NDA might be approved or rejected. If the drug is not approved, the applicant is given the reasons why and what information could be provided to make the application acceptable. Sometimes the FDA makes a tentative approval recommendation, requesting that a minor deficiency or labeling issue be corrected before final approval. Once a drug is approved, it can be marketed. [4]

Shortly, the first is the preclinical phase, which usually takes 3 to 4 years to complete. If successful the preclinical phase, it is followed by an application to the FDA as an investigational new drug (IND). After an IND is approved, the next steps are clinical phases 1, 2, and 3, which require approximately 1, 2, and 3 years, respectively, for completion. Importantly, throughout this process the FDA and investigators leading the trials communicate with each other so that such issues as safety are monitored. The manufacturer then files a new drug application (NDA) with the FDA for approval. This application can either be approved or rejected, or the FDA might request further study before making a decision. Following acceptance, the FDA can also request that the manufacturer conduct additional postmarketing studies. Overall, this entire process, on average, takes between 8 to 12 years. Figure 2 summarizes the drug approval process. [4]

Figure 2. Overview of drug development process and review [4]



Where: IND - investigational new drug; and NDA - new drug application.

It is not surprising that from conception to market most compounds face an uphill battle to become an approved drug. For approximately every 5,000 to 10,000 compounds that enter preclinical testing, only one is approved for marketing. [4]

Some approvals contain conditions that must be met after initial marketing, such as conducting additional clinical studies. For example, the FDA might request a postmarketing, or phase 4, study to examine the risks and benefits of the new drug in a different population or to conduct special monitoring in a high-risk population. Alternatively, a phase 4 study might be initiated by the sponsor to assess such issues as the longer term effects of drug exposure, to optimize the dose for marketing, to evaluate the effects in pediatric patients, or to examine the effectiveness of the drug for additional indications. Postmarketing surveillance is important, because even the most well-designed phase 3 studies might not uncover every problem that could become apparent once a product is widely used. Furthermore, the new product might be more widely used by groups that might not have been well studied in the clinical trials, such as elderly patients. The manufacturer must report adverse drug reactions at quarterly intervals for the first 3 years after approval, including a special report for any serious and unexpected adverse reactions. [4]

So, as was illustrated above, medicinal chemistry is a very important and complex field of research that is responsible for all steps involved in the discovery and development of new medicines from idea to market.

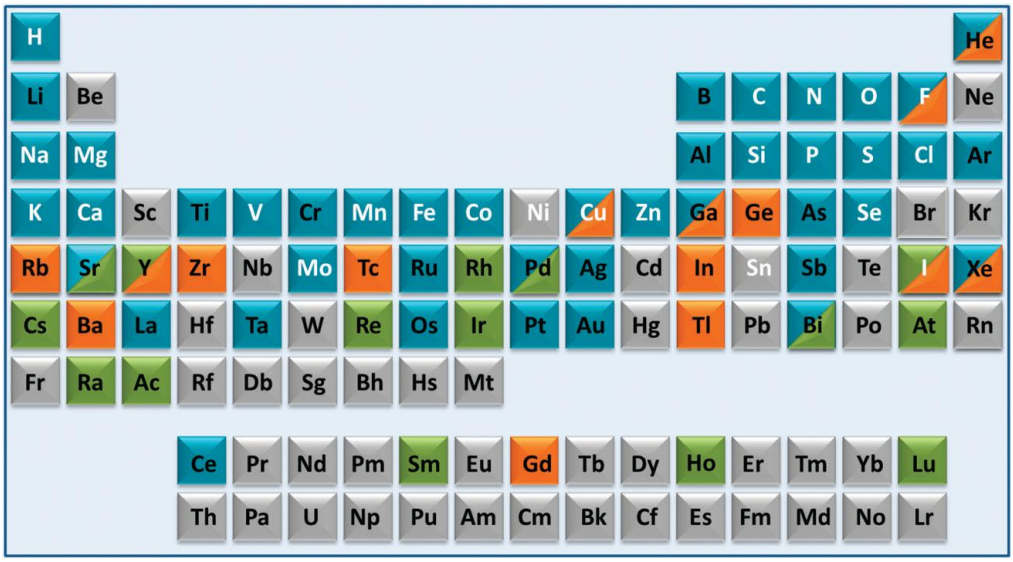
ii. Medicinal Inorganic Chemistry

About 24 elements are essential for man (see Figure 3). In principle, we should consider the roles of all essential elements in therapy (control of homeostasis), but this is currently hampered in many cases (e.g. Si, V, Ni, Sn) because their natural biochemistry is poorly understood and we do not know how to diagnose conditions associated with their metabolism. As is evident from the examples of Pt, Gd and Tc, inorganic medicinal chemistry can make use of non-essential as well as essential elements for the design of drugs and diagnostic agents (Figure 3).[5]

The application of metal-based compounds in biology and medicine is nothing new – indeed, nature has been using organometallic systems to sustain life for a rather long time. The organometallic chemistry of cobalamine, better known as vitamin B_{12} (Figure 4), and derivatives has been investigated for decades, along with that of a variety of enzymes and

cofactors containing metal-carbon bonds. The structure of vitamin B_{12} was determined by Dorothy Hodgkin, for which she was awarded the Nobel Prize in Chemistry in 1964.[6]

Figure 3. A medical periodic table [5]

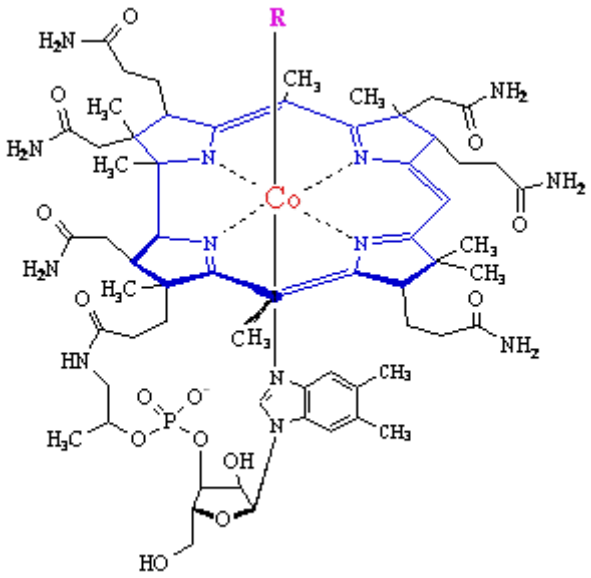


Where: essential elements for man (symbols in white font); medical radioisotopes (green fill); elements currently used in therapy (blue fill) or diagnosis (orange fill). The entries (limited to 2 fill colours, illustrative and not comprehensive) are mainly restricted to elements/compounds which are clinically approved or on current clinical trials (e.g. as listed on http://www.clinicaltrials.gov/). Some entries for implants are included (e.g. Ti, Ta).

The core of the vitamin B_{12} molecule is a corrin ring with various attached side groups. The ring consists of 4 pyrrole subunits, joined on opposite sides by a $C–CH_3$ methylene link, on one side by a C–H methylene link, and with the two of the pyrroles joined directly. It is thus like a porphyrin, but with one of the bridging methylene groups removed. The nitrogen of each pyrolle is coordinated to the central cobalt atom.[7]

It is worth noting that metal-based medicines have been used since the early days of civilization. The medical use of gold can be traced back to 2500 b.C. in China. The ancient Egyptians used copper to sterilize water, and the Greek physician Hippocrates used mercury in 400 b.C. The seventeenth century Swiss alchemist and physician Paracelsus pioneered the use of minerals in medicine using antimony, arsenic and mercury salts such as mercurous chloride as a diuretic. An English contemporary, the herbalist Nicholas Culpepper, advocated a gold elixir *aurum potabile* for ailments caused by a decrease in the vital spirits including melancholy, fainting, fevers and falling sickness.[8] The Ebers Papyrus (from 1500 b.C.) also presents iron compounds to treat anemia, that is actually still in use.[2]

Figure 4. Vitamin B_{12} [7]



Vitamin B_{12} is the only known essential biomolecule with a stable metal-carbon bond, that is, it is an organometallic compound. The cobalt can link to: (1) a methyl group - as in methylcobalamin; (2) a 5'-deoxyadenosine at the the 5' positon - as in adenosylcobalamin (coenzyme B_{12}); and (3) a cyanide group - as in vitamin B_{12} - as supplied from drug companies (R= 1, 2 ou 3).

For many centuries the use of metallodrugs has been driven by empiricism. Whilst random screening is still a useful weapon in drug discovery, these days it should be guided largely by rational design.[5]

Metal-based pharmaceuticals offer unprecedented versatility in medicinal chemistry because of the different building blocks from which they can be constructed, the variety of available interactions (H-bond, π-stacking, coordinative bond, spatial recognition), the combination of rigidity around the metal and flexibility in the ligands, the kinetics of ligand substitution when coordinative bonds with biomolecules are formed and because of their redox properties.[9]

The term “bioorganometallic chemistry” was introduced in 1985 by Gérard Jaouen who defined the subject as the study of biomolecules or biologically active molecules that contain at least one carbon directly bound to a metal or metalloid.[10] Nevertheless, synthetic organometallics have tended to be designed for applications in industrial fields such as catalysis rather than in the biological domain. This focus has largely resulted from the sensitivity of certain organometallics to water and oxygen and from concerns over the toxicity of many metals, in particular the heavier transition metals which have such a rich organometallic chemistry. However, many organometallic compounds are entirely stable to

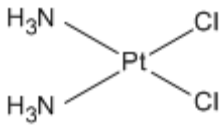
water and air, and while many do show some toxicity to certain organisms, this property may be desirable for certain therapeutic applications.[6,9]

Therefore, it is not surprising that with no fundamental obstacles to their application under physiological conditions and the vast range of geometries, reactivities, and tunable physicochemical features such as polarity, charge, and lipophilicity available to the imaginative organometallic chemist, there are now numerous applications of organometallic complexes in biology and medicine. Moreover, there is perhaps no greater challenge than delineating the mechanism by which an organometallic compound exerts a particular effect, medicinal or otherwise, in an environment as complicated as a cell or more complex organisms, including rodents and even humans.[6]

Metal-based pharmaceuticals can be categorized into seven classes, those in which: (1) the entire inert complex is active, (2) the entire reactive complex is active, (3) a fragment of the complex is active, (4) the metal ion or one of its biotransformation products is active, (5) the metal is a radiation enhancer, (6) the metal is radioactive, and (7) one or more of the ligands is responsible for the biological activity. Each of these classes provides different opportunities and faces different challenges on the road to clinical registration.[11]

As we could see, over the past several centuries various metal-based compounds were applied in medicine. However, medicinal inorganic chemistry, as a discipline, started to be developed after the serendipitous discovery of the anti-tumor activity of cisplatin.

Figure 5. Cisplatin



The anti-proliferative properties of platinum coordination complexes were first observed in 1965 by Barnett Rosenberg at Michigan State University. Whereas the credit for this discovery appropriately goes to the Rosenberg group, it is not widely known that the molecule of cisplatin was first synthesised in 1844 in Turin by Michele Peyrone, a young chemist who was pursuing research in the area of medical chemistry. The compound was known as the “Peyrone's chloride” and it was not until 1892 that, in Zurich, Alfred Werner elucidated the sterical configuration of the molecule.[12]

After Rosenberg's observation, cisplatin was one of the four compounds initially tested, and found to be active, in the murine sarcoma 180 model. The antitumor activity of

cisplatin was independently confirmed by other laboratories and in other tumor models, in the U.S.A. and in the U.K. Following the completion of toxicology studies in rodents, dogs and monkeys, the first cancer patient received cisplatin treatment in April 1971 at the Wadley Institute of Molecular Medicine in Dallas, Texas. The US National Cancer Institute (NCI) also became interested in cisplatin and sponsored the clinical development of the compound, beginning with a phase I trial initiated in June 1971 by the Southwest Cancer Chemotherapy Study Group at Henry Ford Hospital in Detroit, Michigan and MD Anderson Hospital in Houston, Texas, U.S.A. The first evidence of substantial single-agent antitumour effect became available in 1974, both in testicular and in ovarian cancer, with reports of objective responses in 3/7 and 7/19 patients, respectively. The excitement of these results, however, was tempered by the observation of high incidence of organ toxicity, renal in particular. Only in 1976 did investigators from Memorial Sloan-Kettering in New York and from Roswell Park Memorial Institute in Buffalo, New York report on the possibility to circumvent the nephrotoxicity of cisplatin via high-volume fluid hydration and forced diuresis, thus allowing the safe administration of high doses of the drug (1-3 mg/kg).[12]

The success of the clinical applications of this platinum complex has stimulated considerable interest in searching for new metal complexes as modern therapeutics, diagnostic and radiopharmaceutical agents, for example, silver(I) complexes commonly used as anti-microbial agents, bismuth(III) complexes for anti-ulcer treatments, gold(I) complexes as anti-arthritic agents, gadolinium(III), manganese(II) and iron(III) complexes as magnetic resonance imaging contrast agents, technetium (^{99}Tc) and scandium (^{47}Sc) as radiopharmaceutical agents.[13]

To develop new medicines, medicinal inorganic chemists will confront the same barriers as other medicinal chemists, but will also have to overcome the distrust of metals that is borne out of the ignorance that still exists in most areas of pharmaceutical industry and may be exacerbated by the heightened concerns over toxicity. However, the situation also provides many opportunities for medicinal inorganic chemistry.[11]

The field of Medicinal Inorganic Chemistry encompasses a wide range of topics, including: (1) the development of metal-based reagents as therapeutic and diagnostic agents for human disease; (2) the investigation of the role of metal ions, metalloproteins, and metal ion homeostasis in health and disease; (3) the identification of the role of metal ions in pathogenic protein misfolding; (4) the design of metal chelators for use in treating metal ion overload, exposure to toxic metal ions, or inhibition of metalloproteins; and (5) the

elucidation of the general functions of both endogenous and exogenous metal ions in living systems at the molecular level.[14]

Organometallic complexes provide versatile platforms for drug design. Metal-carbon bonds can exert major electronic and steric effects, which in turn can be used to control their biological activity. In particular such complexes offer the possibility of novel mechanisms of action compared to purely organic drugs and have the potential for combating drug resistance as well as treating currently intractable conditions.[9]

It is evident from many papers that collaborations are essential to drive the multidisciplinary field of medicinal inorganic chemistry forward. Not surprisingly, many synthetic organometallic chemists are collaborating with biologists and clinicians in order to find new innovative tools to illuminate biological processes, to identify and diagnose diseases, and ultimately to cure diseases such as cancers.[6]

iii. Concluding remarks and aim of the present thesis

In this general introduction, I tried to highlight the fact that medicinal chemistry is a complex and beautiful field of research, and especially that medicinal inorganic chemistry is a very important field that might lead to the discovery of new drugs. In this context, the aim of this thesis had a dual motivation: (i) to find new organotin compounds which could be applied in the antimicrobial domain; and (ii) to make a contribution to elucidating the mechanism of anticancer activity of ruthenium-based compounds.

In the part I of this thesis, I will talk about the medicinal application of organotin compounds, mainly the antimicrobial properties of new tin-based complexes derived from fatty acids. These compounds were tested *in vitro* against some strains of microorganisms, as Gram-positive and Gram-negative bacteria, and a yeast (**Chapter I.1**). This part of the work was developed in Maceió (Brazil), in the laboratories of the Grupo de Catálise e Reatividade Química (GCaR), under the direction of Prof. Dr. Mario R. MENEGHETTI.

The part II is about cytotoxic ruthenium-derived complexes synthesized in the Laboratoire de Synthèses Métallo-Induittes (actually Laboratoire de Chimie et Systhémique Organo-Métalliques) during the last 12 years.[15] In order to acquire the knowhow on the coordination chemistry of the cycloruthenated compounds I first contributed to the elucidation of the lability of MeCN ligands, as this ligand was a key component (besides the cyclometalated ligands) of the ruthenium containing compounds studied at Strasbourg (**Chapter II.2**). The second project I was involved in, was to develop new Affitin-vectorized

ruthenium complexes (**RDC-Affitin** conjugates), that were expected to greatly enhance their anticancer selectivity by attaching a ruthenium derivative to an affinity protein called Affitin via a well-known “click reaction” between an azido and a terminal alkyne groups (**Chapter II.3**). Through this study it was hoped to gain important knowledge about the mechanism of action of these metal-based complexes. This part of the thesis was developed in Strasbourg (France), under the direction of Dr. Michel PFEFFER.

I hope that this thesis motivating other pharmacists and chemists to use their skills and knowhow to solve problems in biology and medicine and to improve the knowledge about Medicinal Inorganic Chemistry.

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Part I:

Organotin compounds in Medicinal Chemistry

Chapter I.1:

*Antimicrobial activity of organotin compounds derived from fatty acids**

I.1.1 – Introduction

I.1.1.1 – Infectious diseases and antimicrobial therapy

The nature and extension of infectious diseases depends on the virulence (or pathogenicity) of the microorganism and the immune response of the host. Indeed, the infection in the microbiological sense is not synonymous with infection in the clinical sense, and infectious disease occurs only when there is tissue damage or a change in host physiology. It is worth remembering that cooperation between these organisms and humans is the rule, the disease is the exception. [1]

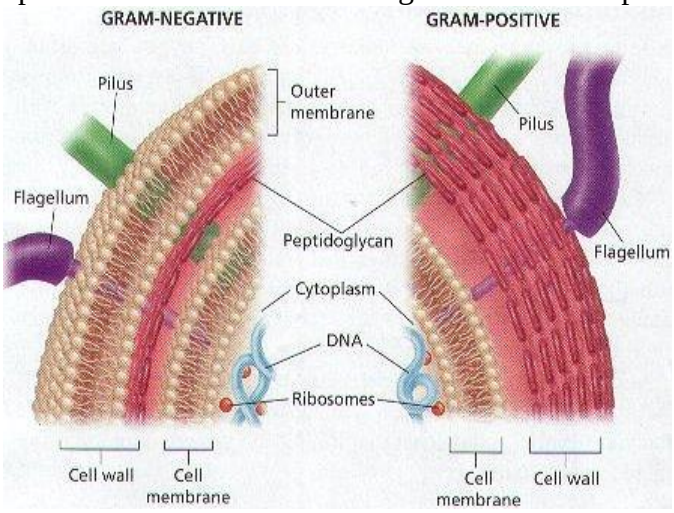
Usually, bacteria can be differenciated into two fundamental varieties of cells according to the Gram stain response as Gram-negative and Gram-positive. Differences between these two groups lie mainly in its permeability properties and surface components, such as the presence of an outer membrane in Gram-negative bacteria and a thick layer of peptidoglycan in Gram-positive bacteria (see Figure 1). [2]

Gram-negative bacteria have a cell wall composed of several layers, which differ in their chemical composition and, consequently, is more complex than the Gram-positive bacteria cell wall that, although thicker, shows predominantly one type of macromolecule. This thick wall of Gram-positive bacteria prevents the passage of hydrophobic compounds due to the presence of sugars and amino acids. Contrarily, the outer membrane of Gram-negative bacteria (with hydrophobic lipoprotein characteristics) makes these bacteria to folow mechanisms that allow the entry of hydrophilic compounds like sugars, amino acids and

* We are grateful to Prof. Dr. Maria Lysete de Assis Bastos (Universidade Federal de Alagoas) for her laboratorial support for the performing of the antimicrobial assays and Prof. Dr. Magna Suzana Alexandre-Moreira and the student Amanda Evelyn (Universidade Federal de Alagoas) for determining the cytotoxicity of our compounds against mammalian cells.

certain ions: this membrane has special channels called porins, which allow diffusion of these compounds. Moreover, outer membrane constitutes a further barrier to entry, for example, of antibacterial agents. [2]

Figure 1. Comparative structure of Gram-negative and Gram-positive bacteria



Source: <http://www.americanaquariumproducts.com/images/graphics/bacteria.jpg>

Unlike bacteria (prokaryotic organisms), fungi, plants and animals (eukaryotic organisms) present individualized nucleus and a rich membrane system and have diverged from a common ancestor about a billion years ago. However, when comparing fungi to plants and animals, we observe that fungi, in most cases, are potentially capable of unlimited growth, unlike the others. [3]

Two fungal cell structures are especially important in medicine: cell wall, which consists mainly of chitin; and cell membrane containing ergosterol and zimosterol, in contrast with human cell membranes, which contain cholesterol. [4]

According to Brown *et al.*, candidiasis is one of the ten most significant invasive fungal infections and *Candida albicans* is the most common responsible species, being an opportunistic invasive mycose. Indeed, *Candida* species are the fourth most common cause of hospital-acquired bloodstream infections and an estimate of the annual global incidence of *Candida* bloodstream infections is about 400,000 cases, with most patients in economically developed regions of the world and with very high mortality rates even in comparison to the most aggressive types of bacterial and viral sepsis. [5,6]

Opportunistic infections are present when usually non-pathogenic microorganisms produce disease in immunocompromised hosts. Elderly and individuals with AIDS (Acquired Immune Deficiency Syndrome) are often susceptible to this type of infection; however, the

progress in medicine, such as cancer chemotherapy and immunosuppression for organ transplantation has also created a new class of patients susceptible to normally innocuous organisms. [1]

I.1.1.2 – Antimicrobial resistance

Antimicrobial agents are substances that cause death or inhibiting the growth of microorganisms. The main purpose of its use is to prevent or treat an infection, reducing or eliminating pathogens and, if possible, preserving the normal microbiota. [7]

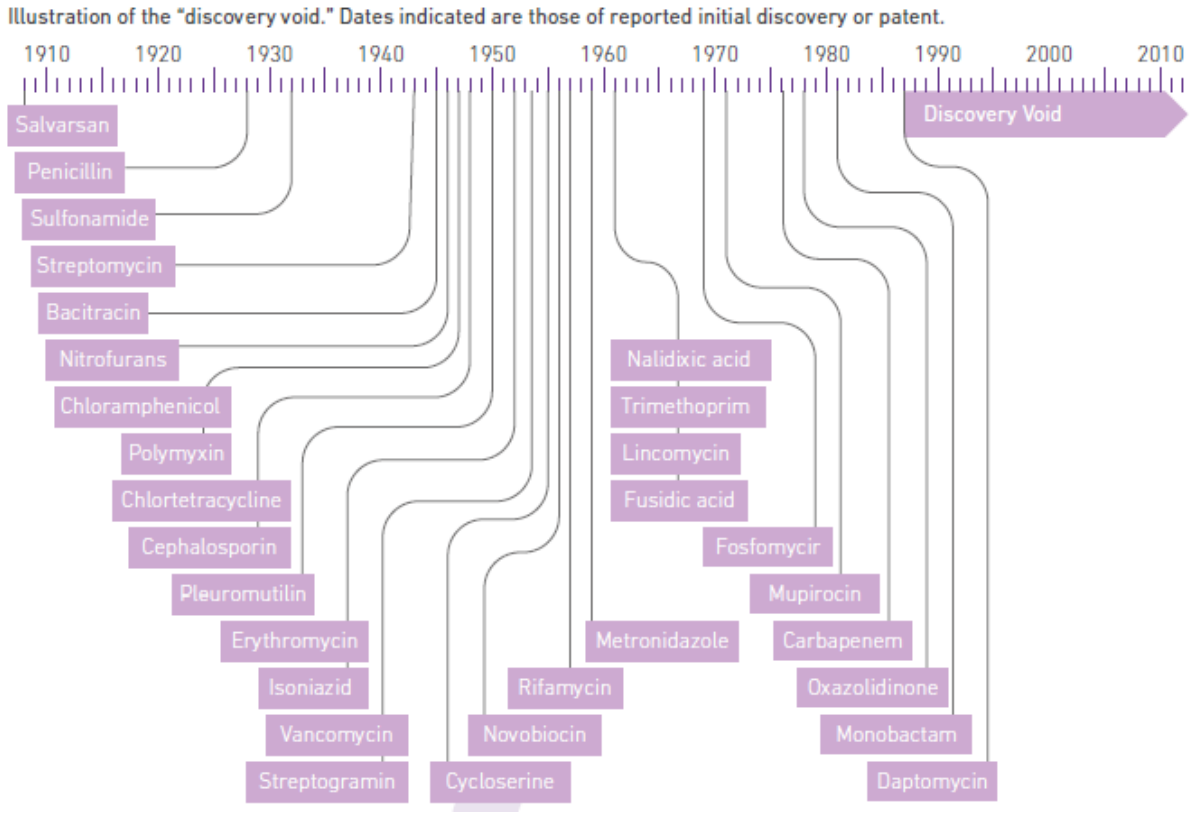
When the first antibiotics were introduced in the 1940s, they were hailed as "wonder drugs", the miracles of modern medicine, since generalized infections that killed millions of people each year could at the time be cured. Thus, it resulted in profound benefits for human society and the human condition had a dramatic improvement, with significant increase in life expectancy. [8]

Microbial resistance is defined as the ability of microorganisms to interrupt the activity of an antimicrobial agent. It is a natural evolutionary phenomenon because when a microorganism is exposed to an antimicrobial agent, the more sensitive organisms succumb and those resistant survive and can pass their resistance to offspring. As a result, conventional treatments become ineffective, infections persist and may be transmitted to others, imposing huge costs to individuals and society. [9]

Alexander Fleming (discoverer of penicillin), in his Nobel Prize speech in 1945, already warned that bacteria could become resistant to these remarkable drugs. Indeed, the development of each new antibacterial drug has been followed by the detection of resistance to it. [6] Therefore, antimicrobial multidrug resistance is expanding all over the world, especially in healthcare-associated infections in large highly specialized hospitals. [10] From 1940 to 1990, the repeated and successful response to emerging resistance was to discover new antibacterials, but actually this strategy has failed, with resistance accumulating faster than new antibiotics have been developed [11] (see Figure 2).

According to Chopra, [12] although some new drugs are in development they do not adequately cover growing medical needs and also these drugs are mostly derivatives of older classes already in use and therefore prone to existing bacterial resistance mechanisms. Thus, new antimicrobial classes are urgently needed.

Figure 2. Dates of discovery of distinct classes of antibacterial drugs



Source: WHO, 2014. [6]

Thereby, to control microbial resistance it is important to know that the process requires a multidisciplinary approach, in which all medical staff must attend effectively and promote the reduction of inappropriate requirements, either in simple precautions to prevent infection, such as with hands and environment sanitization, or continuous monitoring to identify the appearance of unknown mechanisms of resistance. [13] And also reserve the treatment with this class of substances for situations where the use is proven beneficial, minimizing the use in cases of dubious benefit, because apart from these drugs inducing the development of resistance, they may have adverse effects and represent costs for the individual and for society. [7]

About environment sanitization, a panel of scientists from various European countries discussed "The Role of Surface Disinfection in Infection Prevention" and concluded that contaminated surfaces have high importance in the spread of hospital pathogens and that it is unquestioned that environmental disinfection is necessary in certain risk areas and in outbreak situations as part of a set of strategies. Indeed, the purpose of routine or targeted disinfection of inanimate surfaces prevents subsequent infection transmission. [14]

It is important to comment that the effectiveness of a disinfectant depends overall on factors inherent to the product (such as concentration, formulation, water solubility and pH), those inherent to the application (with or without mechanical action, for example), and also those factors inherent to the microorganism. It also must be considered, for instance, the concentration exponent (relationship between dilution and activity of a biocide), as well as the bioavailability of the substance and its stability. [14]

In view of the arguments presented above, new alternatives for controlling microbial infections should be developed to circumvent the problem of microbial resistance.

I.1.1.3 – Examples of application of organotin compounds in medicine

Many inorganic elements, generally present at low concentrations, are essential for all living beings. It is known that certain alterations in the metabolism of these trace elements are the cause of various diseases and physiological disorders. [15] Tin (Sn) is a metal that is among these essential elements for human and animals body composition, and the deficiency in this compound is related to growth retardation. [16] The average value of concentration of tin present in composition of the human body is 0.03 g (reference: adult male of 70 kg). [16]

The first organotin compound produced in laboratory, diethyltin diiodide (Et_2SnI_2), was obtained by Frankland in 1849. However, the applications of these organotin compounds were exploited industrially only one century after its discovery. [17] Their applications are varied, but result primarily from two basic characteristics: firstly, the high affinity from this metal for Lewis donors, such as oxygen, nitrogen, phosphorus or sulfur atoms; and secondly, the biocide properties of organotin against bacteria, fungi, insects, mollusks and small animals. [18]

Among the various metals that have been studied for biological applications, complex of tin(IV) has attracted attention as new therapeutic agents. A large number of references present different organotin complexes as bioactive entities, for example as antimicrobial, [19] antitumor, [20] leishmanicide, [21] trypanocidal, [22] and anti-inflammatory [23] agents.

Organotin compounds can also be applied as a wood preservative, anti-fouling additives for ships, rodents repellents, among other examples. [24]

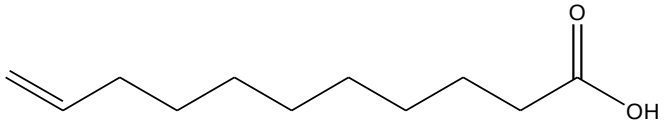
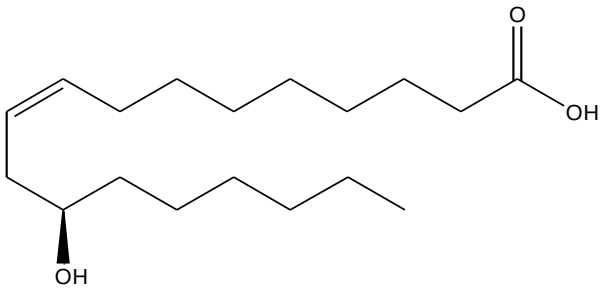
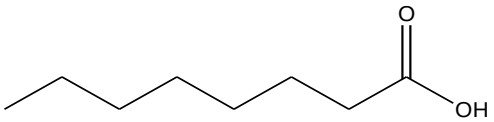
Given the importance of researcher new drugs for the treatment of microbial infections, the development of new methodological strategies is critical for obtaining new prototypes of drugs. Complexation of bioactive molecules to metals such as tin, may be an interesting option for the discovery of new alternatives for antimicrobial therapy.

I.1.1.4 – Fatty acids in medicine

Several fatty acids present important biological activity, most of them related to defense against pathogenic or opportunistic microorganisms, acting mainly through the inhibition of growth or direct killing these microorganisms. And although the mechanism of action of antimicrobial fatty acids is not fully understood, some theories have been proposed by Desbois & Smith in a review article [25] to explain this activity: *"the prime target of free fatty acid (FFA) action is the cell membrane, where FFAs disrupt the electron transport chain and oxidative phosphorylation. Besides interfering with cellular energy production, FFA action may also result from the inhibition of enzyme activity, impairment of nutrient uptake, generation of peroxidation and auto-oxidation degradation products or direct lysis of bacterial cells. Their broad spectrum of activity, non-specific mode of action and safety makes them attractive as antibacterial agents for various applications in medicine, agriculture and food preservation, especially where the use of conventional antibiotics is undesirable or prohibited."*

In this work, we adopted three fatty acids as bioactive ligands to form organotin complexes: caprylic (8C), undecylenic (11C) and ricinoleic (18C) acids (see Table 1).

Table 1. Chemical structures of adopted fatty acids ligands

Compound	Chemical structure
Undecylenic acid (UndH)	
Ricinoleic acid (RicH)	
Caprylic acid (CapriH)	

Undecylenic acid is a terminal unsaturated fatty acid that presents antifungal, antibacterial and antiviral activity. Although rarely found in nature, it can be naturally found in tears and sweat. [26] It is used as a topical antifungal agent for the treatment of

dermatomycoses as well as aphthae [27] and denture stomatitis. [27,28] This acid is primarily a fungistatic agent, while a fungicidal activity can be observed with prolonged exposure to high concentrations. [29]

McLain *et al.* [28] propose the inhibition of morphogenesis of *Candida albicans* as possible antifungal mechanism of action for undecylenic acid since this compound was found to inhibit conversion of yeast to the hyphal form and it is possibly related to problems in fatty acid biosynthesis. And undecylenic acid, as medium-chain fatty acids, can also disrupt the regulation of cytoplasmic pH by carrying protons across the plasma membrane and interfere with the alkalinization of the cytoplasm which accompanies germ tube formation.

Regarding antiviral activity, undecylenic acid has been demonstrated *in vitro* and topical *in vivo* activity against herpes simplex virus both in animals and in humans. [30, 31]

Ricinoleic acid, an unsaturated 18-carbon fatty acid, derives from castor oil and is primarily known for its laxative properties, but may present pro-anti-inflammatory or anti-inflammatory actions through, respectively, acute or repeated topical application which resemble those described for capsaicin and its vanilloid analogues with the advantage of not having the poignant and painful effect of capsaicin. Moreover, part of the chemical structure of the ricinoleic acid has been used for the development of novel compounds similar to capsaicin. [32]

This fatty acid is used in dressings as a chemical debriding and facilitator of the healing process and also in the treatment of infected wounds as an adjunct to systemic antibiotic therapy. It also shows cytolytic activity, acting as a solvent for chitin (an important component of microorganism cell membrane). [33]

Caprylic acid, in turn, is a saturated fatty acid containing eight carbons being present in the lipid portion of the milk and palm oils, coconut and babassu. It is reported especially as antifungal, antibacterial, anti-protozoal, anti-helmintic and larvicidal agent [25, 34, 35] and it is considered a reliable substance by FDA (Food and Drug Administration, USA) to be added to foods as a preservative. [36]

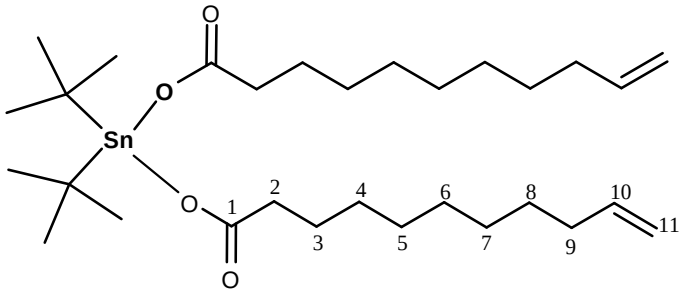
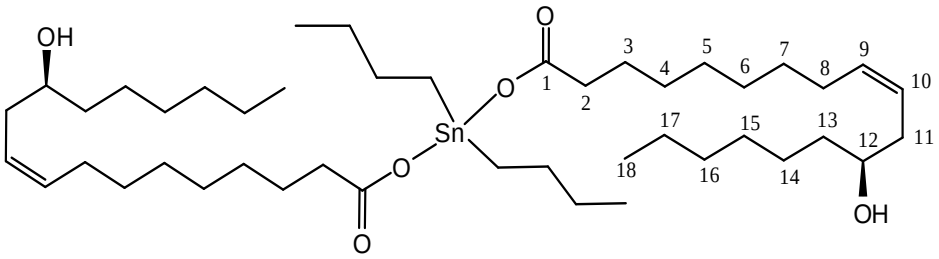
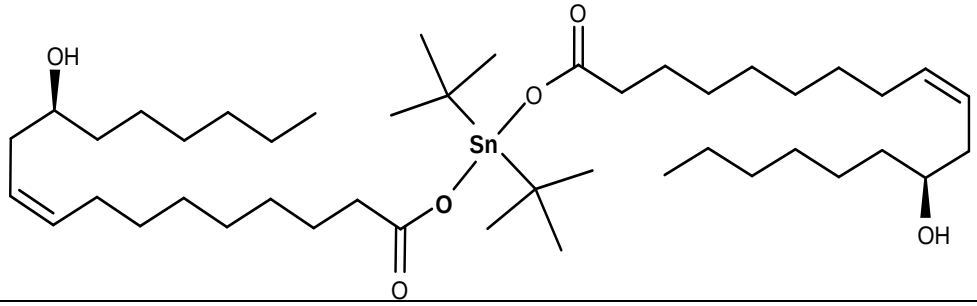
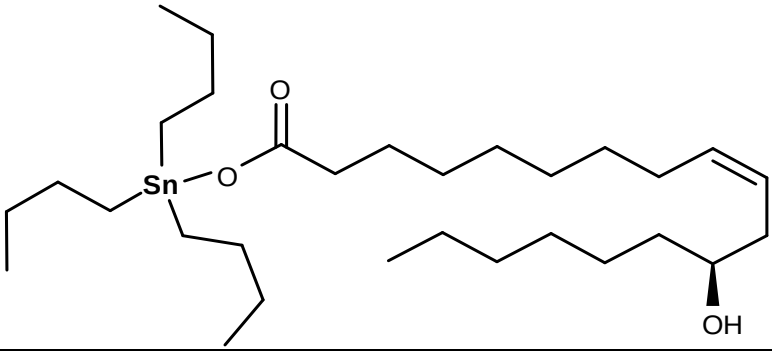
Marounek *et al*. [37] in a study of the antibacterial activity of fatty acids containing between 2 and 18 carbons, concluded that caprylic acid was the most effective in inhibiting glucose metabolism in *Escherichia coli*, and consequently its development.

Therefore, the purpose of this work was to prepare new organotin derivatives of bioactive fatty acids and carry out the assessment of their antimicrobial activity aiming to extend its range of action by the metal-ligand synergistic effect.

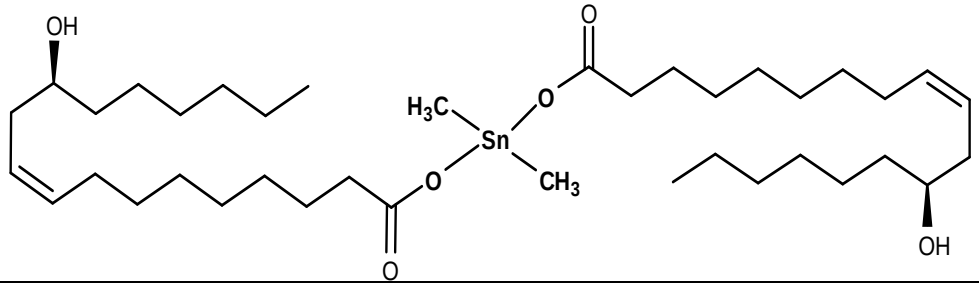
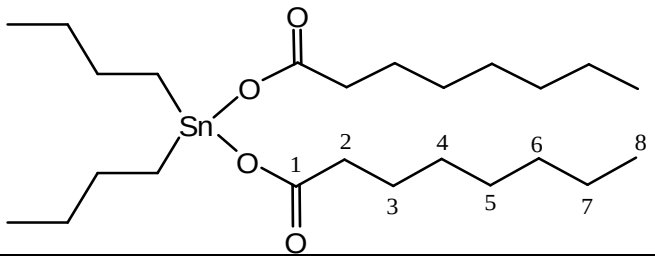
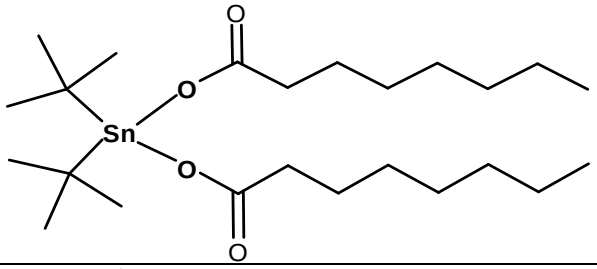
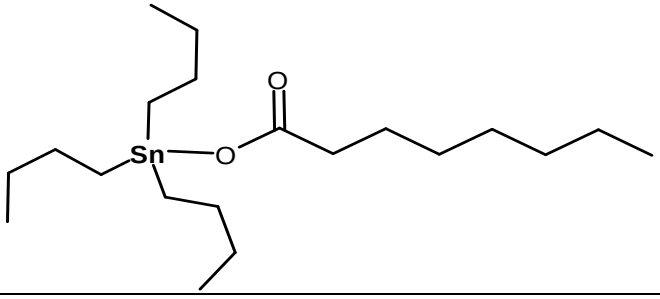
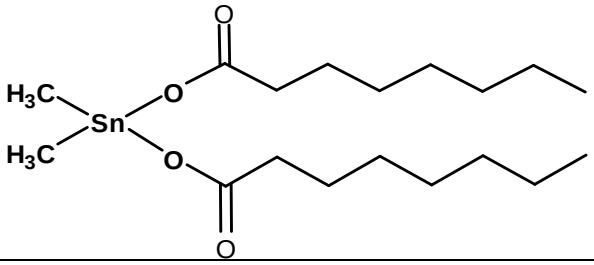
I.1.2 – Organotin complexes derived from fatty acids

Organotin compounds represented by the formula R_nSnL_{4-n} have a variety of biological effects, depending on the number **n**, the type of **R** group (usually, alkyl or aryl groups), and the **L** ligand (usually, carboxylates, alcohoxylates, and halides) bonded to the metal ion. [38] For this work, we synthesized a serie of organotin complexes derived from undecylenic, ricinoleic and caprylic acids as **L** ligand. Nine complexes were obtained: ***t*-Bu_2SnUnd_2**, ***n*-Bu_2SnRic_2**, ***t*-Bu_2SnRic_2**, ***n*-Bu_3SnRic**, **Me_2SnRic_2**, ***n*-$Bu_2SnCapri_2$**, ***t*-$Bu_2SnCapri_2$**, ***n*-$Bu_3SnCapri$** and **$Me_2SnCapri_2$** (Table 2).

Table 2. Chemical structure of organotin complexes synthesized

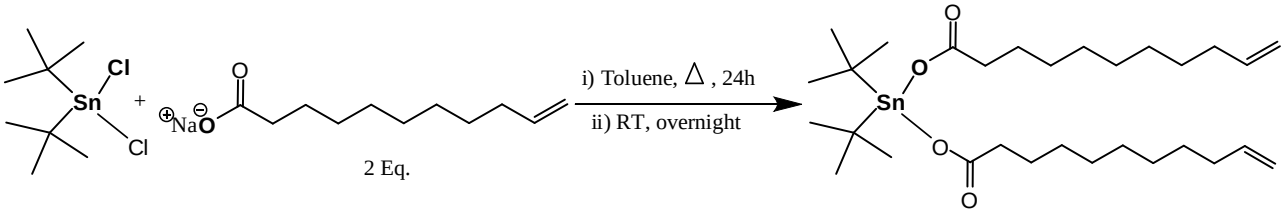
Compound	Structural formula
***t*-Bu_2SnUnd_2**	
***n*-Bu_2SnRic_2**	
***t*-Bu_2SnRic_2**	
***n*-Bu_3SnRic**	

(To be continued)

Me_2SnRic_2	
***n*-$Bu_2SnCapri_2$**	
***t*-$Bu_2SnCapri_2$**	
***n*-$Bu_3SnCapri$**	
$Me_2SnCapri_2$	

The synthesis were done simply and effectively, according to the methodology described by Muhammad *et al.*, [39] by the reaction between the carboxylate and the related organotin chloride complex (Figure 3).

Figure 3. General reaction conditions employed for the synthesis of $\mathbf{R_nSn(OOCR')_{4-n}}$ (n= 1 to 3). Example: $\mathbf{\textit{t}\text{-}Bu_2SnUnd_2}$

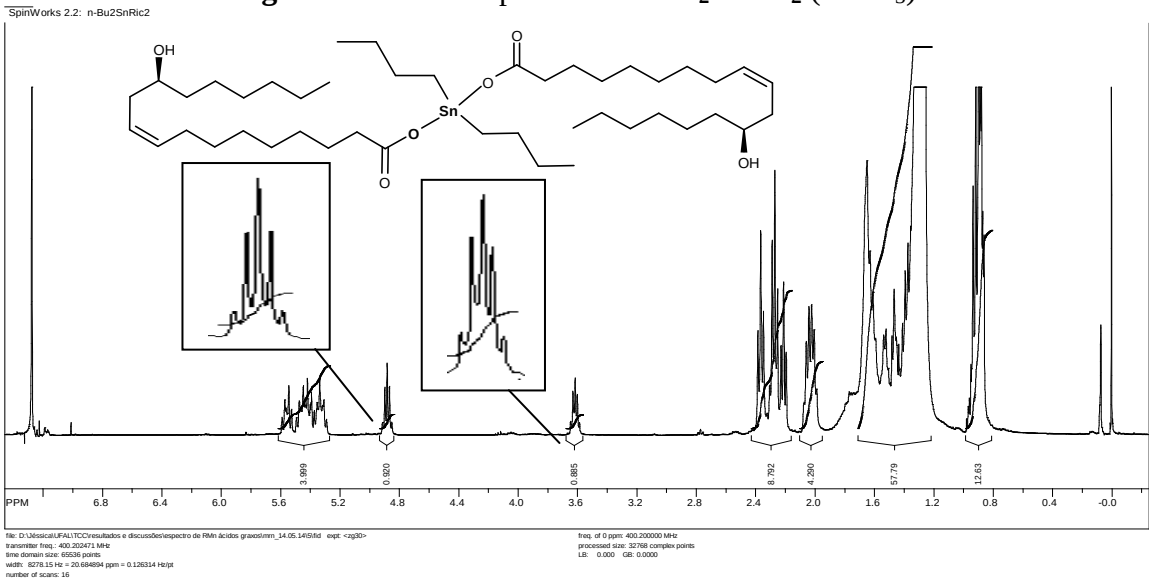


Sodium salts of undecylenic, ricinoleic and caprylic acids are typically water soluble solids of low melting point. The resulting complexes were, in turn, colorless to yellow oils, miscible in ether, chloroform and dimethyl sulfoxide.

By analyzing the ^{1}H NMR spectra, we observe that the signals for the undecylenic, ricinoleic and caprylic fragments can still be detected in the related complex, and we also observe the presence of corresponding signals relating to the respective organic **R** chains. In almost all spectra it is possible to see the coupling satellites $^2J_{\text{H-}^{119}\text{Sn}}$ e $^2J_{\text{H-}^{117}\text{Sn}}$ ($\delta \sim 0{,}1$). [40]

Despite signals related to both the **L** and **R** ligands, the ^{1}H NMR spectra of the compounds derived from ricinoleic acid, in particular, are more complex to interpret. We can attribute this, in part, to the purity of ricinoleic acid used as reagent (technical grade, approximately 80%) and also to the two positional isomers (relating to two different positions of the hydroxyl group). The presence of these two isomers is quite noticeable when we analyze the ^{1}H NMR spectrum, which reveals two quintuplets with chemical shifts at 4.87 and 3.62 ppm (Figure 4), related to the hydrogen bonded to the chiral carbon having the hydroxyl group. If only one isomer were present, only one signal (quintuplet) should be observed.

Figure 4. ^{1}H NMR spectrum - $\mathbf{\textit{n}\text{-}Bu_2SnRic_2}$ ($CDCl_3$)



The synthesized complexes were also characterized by IR spectroscopy in the medium infrared region to confirm the presence of the expected bands and their vibration frequencies are shown in the Table 3.

Table 3. Vibration Frequency (cm^{-1}) of the main chemical bonds of organotin complexes and their free fatty acids adopted as ligands

Compound	Main frequencies / cm^{-1} – Assignment				
	$C=O_{ac.}$	**C=O**	**COO ν as/s**	**Sn-C ν as/s**	**Sn-O**
UndH	1704	-	-	-	-
UndNa	-	-	1557/ 1444, 1418	-	-
***t*-Bu_2SnUnd_2**	-	1737	1602/1387	640/555	532
RicH	1709	-	-	-	-
RicNa	-	1731	1557/1423	-	-
***n*-Bu_2SnRic_2**	-	1723	1598/1378	667/611	529
***t*-Bu_2SnRic_2**	-	1731	1602/1367	665/566	534
***n*-Bu_3SnRic**	-	1732	1618/1376	670/609	530
Me_2SnRic_2	-	1731	1557/1378	623/581	543
CapriH	1704	-	-	-	-
CarpriNa	-	-	1559/1415	-	-
***n*-$Bu_2SnCapri_2$**	-	1734	1596/1378	670/631	530
***t*-$Bu_2SnCapri_2$**	-	1737	1602/1383	668/563	532
***n*-$Bu_3SnCapri$**	-	-	1545/1376	667/609	532
$Me_2SnCapri_2$	-	1714	1556/1404	640/571	532

The values found for the Sn-C and Sn-O bonds are close to those obtained by Nath *et al.* [41] for tributyltin tryptophanyl glycinate (674 and 604 cm^{-1} for the ν/s Sn-C, and 517 cm^{-1} for Sn-O).

Therefore, the analysis of these data coroborates the formation of all complexes we intended to synthesize.

I.1.3 – Antimicrobial activity

Antibacterial and antifungal activities of the tin(IV) complexes were obtained by using the broth microdilution method, as described in the experimental part. The minimum

inhibitory concentrations (MIC) were determined for all complexes and their ligands in order to quantitatively evaluate the effects of the complexation on their *in vitro* activity.

Ostrosky *et al.* [42] affirm that the broth microdilution method has been widely used mainly because of its sensitivity and minimal amounts of reagents, which enables a larger number of replicas, increasing the reliability of the results. According to Alves *et al.*, [43] this method is the best option to determine the antimicrobial activity when compared to other methodologies.

For comparison, we also retested some complexes derived from undecylenic acid (***n*-Bu_2SnUnd_2**, ***n*-Bu_3SnUnd** and **Me_2SnUnd_2**) that were synthesized during my Master course. [44]

To interpret the results, compounds were considered to be active only with MIC values lower than 128 µg/mL, since the *Clinical and Laboratory Standards Institute* (CLSI) recommends concentrations from 128 to 0.25 µg/mL to identify the minimal inhibitory concentration for most antimicrobial compounds. [45]

Our antimicrobial results are presented below.

I.1.3.1 – Antifungal activity

The minimum inhibitory concentrations of the fatty acids and their derived complexes against the yeast *Candida albicans* are shown in Table 4.

According to the criteria adopted to classify the tested substances as active compounds (MIC < 128 µg/mL), we observed that the undecylenic acid and all its derived complexes were active against *C. albicans*. This fact was expected, since this acid is pharmacologically classified as an antifungal agent. [29] Among compounds derived from caprylic acid, only the *n*-butyl complexes were considered active, while among complexes derived from ricinoleic acid, only the triorganotin presented antifungal activity.

The micromolar (µM) scale is the best way to compare results of biological activity, because it takes into account the quantity of compound that is present in the media, thus, the results do not change depending on their molar relative mass. Indeed, some of these compounds present the same MIC expressed in µg/mL but we observed, for example, that approximately 3 times less material were necessary to show the highest antifungal activity when we compared the molar concentration of ***n*-Bu_2SnUnd_2** or ***t*-Bu_2SnUnd_2** to UndH, and even more when we compared the molar concentration of ***t*-Bu_2SnRic_2** or **$Me_2SnCapri_2$** to CapriH (Table 4).

Table 4. Antifungal activity against *Candida albicans* (ATCC 10231)

Compound	MIC	
	µg/mL	**µM**
UndH	62.5[a]	339
***n*-Bu_2SnUnd_2**	**62.5**[a]	**104**
***t*-Bu_2SnUnd_2**	**62.5**[a]	**104**
***n*-Bu_3SnUnd**	**0.5**[a]	**1.1**
Me_2SnUnd_2	**125**[a]	**243**
RicH	> 500	> 1,675
***n*-Bu_2SnRic_2**	**> 500**	**> 604**
***t*-Bu_2SnRic_2**	**500**	**604**
***n*-Bu_3SnRic**	**0.25**[a]	**0.4**
Me_2SnRic_2	**> 500**	**> 672**
CapriH	500	3,467
***n*-$Bu_2SnCapri_2$**	**125**[a]	**241**
***t*-$Bu_2SnCapri_2$**	**250**	**481**
***n*-$Bu_3SnCapri$**	**0.25**[a]	**0.6**
$Me_2SnCapri_2$	**500**	**1,149**
Fluconazole	1*	3.3
Amphotericin B	0.125*	0.1

[a] Active samples / *Ref. Guo *et al*., 2009. [46]

We observed in all cases that the complexation improved the antifungal activity, since the minimal inhibitory concentration was higher for free fatty acids than for their derived complexes. In the case of triorganotin complexes, it was a critical improvement related to the acid form of their ligands, with MIC being in submicromolar range. Indeed, their MIC were lower than that of fluconazole (3.3 µM) and comparable to that of amphotericin B (0.1 µM), regardless of acid ligand.

Note that there was no significant difference in the activity against *C. albicans* between branched or straight butyl group chains, and their presence contributes more to antifungal activity than methyl group.

Vieira [47] reported that some organotin compounds decreased lipid levels in *C. albicans*, suggesting the interference of these compounds in this cell component. Indeed, morphological evaluations by electronic and transmission scanning microscopy revealed the

presence of several cell damage, including leakage of cytoplasmic material. According to this study, changes in membrane permeability could induce pore formation, probably by interaction of organotin with steroid compounds present in the cell envelope and possibly by a solubilization of lipids. Thus, these results suggests that the mechanism of action of organotin complex in *C. albicans* cells is similar to that of polyene compounds, such as nystatin, which are prescribed for the treatment of infections caused by this yeast.

I.1.3.2 – Antibacterial activity

The minimum inhibitory concentrations of the fatty acids and their organotin complexes counterpartes against bacteria selected for this study are presented in the Table 5. For this work we also tested the antibacterial activity of some organotin chlorides and *n*-$Bu_2Sn(OAc)_2$ for comparison.

Following the criteria used to classify the samples as active compounds (MIC < 128 µg/mL), we conclude that:

• The active compounds against *S. aureus* were: ***n*-Bu_2SnUnd_2**, ***t*-Bu_2SnUnd_2**, ***n*-Bu_3SnUnd**, ***n*-Bu_2SnRic_2**, ***t*-Bu_2SnRic_2**, ***n*-Bu_3SnRic**, ***n*-$Bu_2SnCapri_2$**, ***t*-$Bu_2SnCapri_2$**, ***n*-$Bu_3SnCapri$**, **$Me_2SnCapri_2$**, all chloride complexes and *n*-$Bu_2Sn(OAc)_2$.

• The active compounds against *E. coli* were: ***n*-Bu_2SnUnd_2**, ***t*-Bu_2SnUnd_2**, ***n*-Bu_3SnUnd**, ***t*-Bu_2SnRic_2**, ***n*-Bu_3SnRic**, *n*-$Bu_2Sn(OAc)_2$, and also all complexes derived from caprylic acid and all chloride compounds.

• The active compounds against *P. aeruginosa* were: UndH, ***t*-Bu_2SnUnd_2**, ***n*-Bu_3SnUnd**, ***n*-Bu_3SnRic**, ***n*-$Bu_3SnCapri$**, *n*-$Bu_2Sn(OAc)_2$ and all chloride complexes.

In general, the complexation of these fatty acids with organotin moieties promoted a critical increase in their antibacterial activity, corroborating our expectation. Note that triorganotin compounds were much more active against *S. aureus* than their acid ligands and their diorganotin related complexes. They show, indeed, MIC values in nM range (from 5 to 60 nM), and were even more potent than ampicillin (400 nM) and levofloxacin (800 nM). They must therefore be further investigated as new antibacterial agent candidates.

The low or absent activity of organotin complexes against Gram-negative bacteria and the general high activity against Gram-positive bacteria are consistent with literature data for this kind of compound. [48] Indeed, gram-negative bacterial membranes have a strong permeability barrier to many types of antibiotics, thus they are currently resistant to many substances that are active against Gram-positive organisms.

Table 5- Antibacterial activity against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*

Compound	MIC					
	Staphylococcus aureus (ATCC 25923)		*Escherichia coli* (ATCC 25922)		*Pseudomonas aeruginosa* (ATCC 27853)	
	µg/mL	**Mm**	**µg/mL**	**µM**	**µg/mL**	**µM**
UndH	500	2,713	> 500	> 2,723	125[a]	678
***n*-Bu_2SnUnd_2**	**3.9**[a]	**6.5**	**31.2**[a]	**52**	**250**	**417**
***t*-Bu_2SnUnd_2**	**15.6**[a]	**26**	**31.2**[a]	**52**	**62.5**[a]	**104**
***n*-Bu_3SnUnd**	**0.03**[a]	**0.06**	**31.2**[a]	**65.9**	**125**[a]	**264**
Me_2SnUnd_2	**250**	**485**	**500**	**970**	**250**	**485**
RicH	250	838	> 500	> 1,675	> 500	> 1,675
***n*-Bu_2SnRic_2**	**31.2**[a]	**37.7**	**> 500**	**> 604**	**> 500**	**> 604**
***t*-Bu_2SnRic_2**	**15.6**[a]	**18.8**	**31.2**[a]	**37.7**	**250**	**302**
***n*-Bu_3SnRic**	**0.008**[a]	**0.011**	**31.2**[a]	**44.5**	**62.5**[a]	**89.1**
Me_2SnRic_2	**250**	**336**	**> 500**	**> 672**	**> 500**	**> 672**
CapriH	> 500	> 3.467	> 500	> 3,467	> 500	> 3,467
***n*-$Bu_2SnCapri_2$**	**7.8**[a]	**15**	**31.2**[a]	**60.1**	**> 500**	**> 953**
***t*-$Bu_2SnCapri_2$**	**7.8**[a]	**15**	**15.6**[a]	**30**	**250**	**481**
***n*-$Bu_3SnCapri$**	**0.002**[a]	**0.005**	**15.6**[a]	**39.7**	**31.2**[a]	**79.4**
$Me_2SnCapri_2$	**125**[a]	**287**	**250**	**574**	**> 500**	**> 1,149**
n-Bu_2SnCl_2	1.95[a]	6.4	≤ 7.8[a]	≤ 26	≤ 7.8[a]	≤ 26
t-Bu_2SnCl_2	0.98[a]	3.2	≤ 3.9[a]	≤ 12.8	≤ 7.8[a]	≤ 26
n-Bu_3SnCl	≤ 0.24[a]	≤ 0.7	7.8[a]	24	≤ 3.9[a]	≤ 12
Me_2SnCl_2	125[a]	569	125[a]	569	62.5[a]	285
n-$Bu_2Sn(OAc)_2$	15.6[a]	44.4	15.6[a]	44.4	≤ 7.8[a]	≤ 22
Ampicilin	0.13*	0.4	2*	5.7	128*	366
Levofloxacin	0.3[#]	0.8	0.3[#]	0.8	0.15[#]	0.4

[a] Active samples / *Ref. Şahin *et al*., 2013. [49] / [#]Ref. Lei *et al*., 2008. [50]

According to Pruchnik *et al*., [51] triorganotin are the most biologically active organotin compounds. In the trialkyl series, we observe the following properties: (1) trimetyltin compounds have a high toxicity to insects and mammals; (2) triethyltins are most toxic to mammals; (3) tripropyltins are very active against Gram-negative bacteria; and (4) the tributyltins are active against Gram-positive bacteria.

Looking our results closely, as for antifungal activity, the presence of butyl groups contribute more to the antibacterial activity than methyl group in all studied cases of this work. Against *S. aureus* there was no significant difference in activity between branched or straight butyl group substituted compounds. Against Gram-negative bacterias (*E. coli* and *P. aeruginosa*), in turn, *t*-Bu-Sn-derived compounds were more active than their related complexes with straight butyl chains.

Comparing complexes with **R**= *n*-butyl and **n**= 2, the following rank of activity was observed, according to the **L** group:

• *S. aureus*: Cl^- = Und^- > $Capri^-$ > Ric^- > OAc^-

• *E. coli*: Cl^- > OAc^- > Und^- > $Capri^-$ >> Ric^-

• *P. aeruginosa*: Cl^- and OAc^- >> Und^- > Ric^- >> $Capri^-$

According to these results, we can state that there is no an absolute general trend regarding to the increase in the ligand carbon chain or to the electron withdrawing character of the ligand **L** against Gram-positive bacteria. Against Gram-negative bacterias, on the other hand, we observe higher activities for complexes with higher electron withdrawing character and small volume **L** ligands.

Regarding to organotin chloride compounds, we observe that they present high antibacterial activity, but unfortunately they present very high toxicity (see **section I.1.4**), which have impelled us to propose structural modifications such as coordination with bioactive ligands aiming to modulate their toxicity whilst maintaining their bioactivity.

Although there are only little available informations about tin compounds effects on microbial processes, it is known that some bacterial membrane functions can be inhibited by organotin complexes, including effects on energy generation, solute transport, substrate retention and oxidation. [19]

I.1.4 – Cytotoxic studies against mammalian cells

The cytotoxic profile of undecylenic, ricinoleic and caprylic acids, organotin chlorides and their derived compounds against mammalian cells was investigated by the cell viability assay using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), called MTT assay. [52]

In this study murine macrophages cell line J774 was treated with these compounds at different concentrations (0.1–100 μM) and the half maximal lethal concentration (LC_{50} =

concentration required to induce 50% death of cells) was determined as illustrated in Table 6. [53]

Table 6. Cytotoxicity against macrophages (J774 cell line) in 100, 10 and 1 μM concentrations in MTT assay

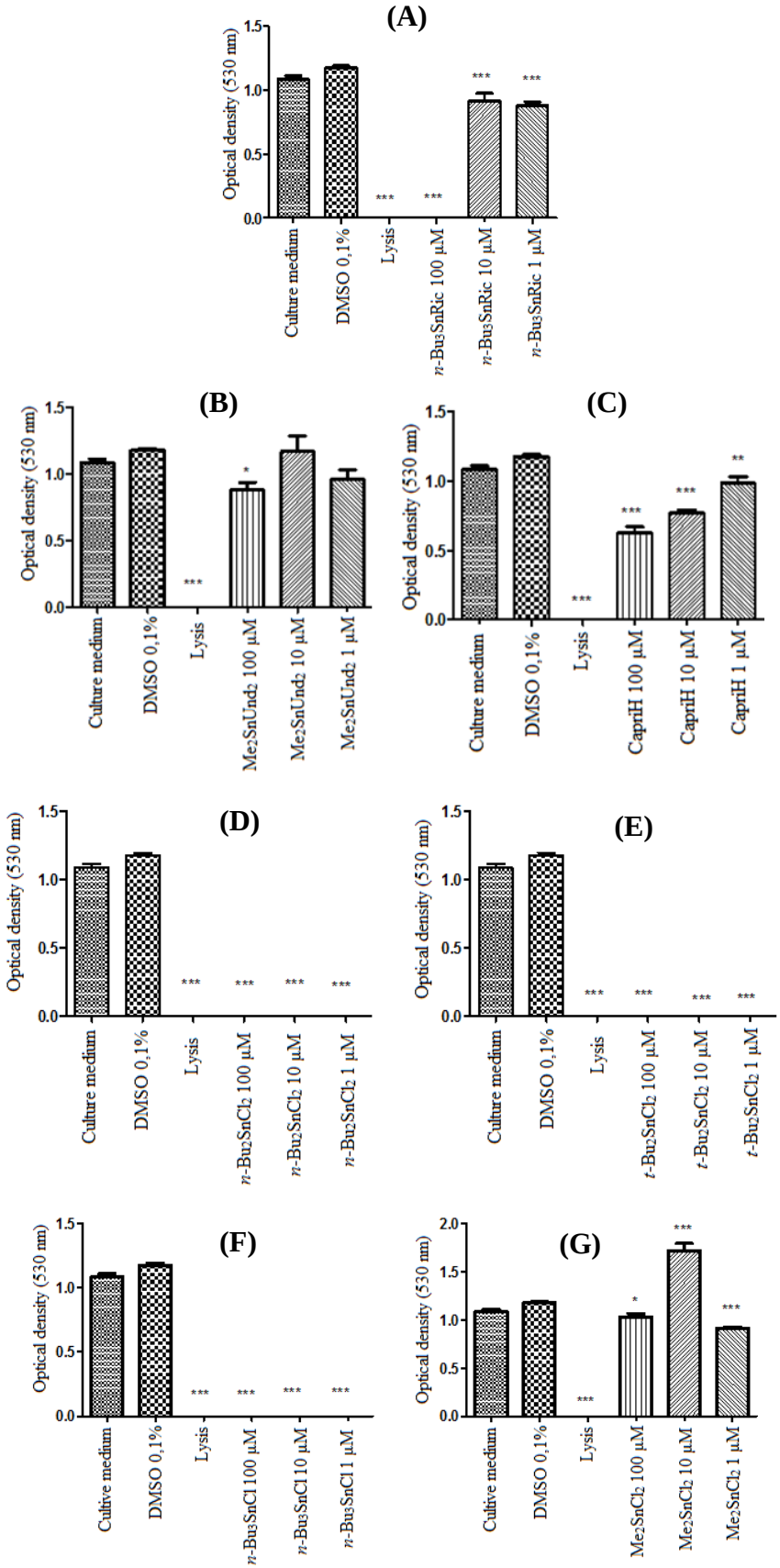
Compound	LC_{50} (μM ± S.E.M.)[a]	Maximal Cytotoxicity (% ± S.E.M)[b]
UndH	41.3 ± 7	100 ± 0***
***n*-Bu_2SnUnd_2**	30 ± 0	100 ± 0***
***t*-Bu_2SnUnd_2**	2.5 ± 1.5	100 ± 0***
***n*-Bu_3SnUnd**	2.3 ± 0.3	100 ± 0***
Me_2SnUnd_2	**> 100**	**NT**
RicH	79 ± 0	46.7 ± 6.3***
***n*-Bu_2SnRic_2**	3.9 ± 1.5	100 ± 0***
***t*-Bu_2SnRic_2**	4.1 ± 0.6	100 ± 0***
***n*-Bu_3SnRic**	44 ± 12.5	100 ± 0***
Me_2SnRic_2	1.2 ± 0	68.4 ± 12
CapriH	**> 100**	**NT**
***n*-$Bu_2SnCapri_2$**	< 1	95.4 ± 7.9***
***t*-$Bu_2SnCapri_2$**	< 1	100 ± 0***
***n*-$Bu_3SnCapri$**	< 1	100 ± 0***
$Me_2SnCapri_2$	13 ± 4.2	66.6 ± 13.4*
n-Bu_2SnCl_2	< 1	100 ± 0***
t-Bu_2SnCl_2	< 1	100 ± 0***
n-Bu_3SnCl	< 1	100 ± 0^{***}
Me_2SnCl_2	**> 100**	**NT**

NT = Not toxic

[a] LC_{50} (concentration required to induce 50% death of cells) calculated by toxic concentration-response curves. [b] Mean ± standard error of the mean (Mean ± S.E.M.) from maximal cytotoxicity of triplicates of a representative experiment. The maximum effect values were considered significant when compared to DMSO group. Values were considered significant when *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

As rule, the larger the LD_{50} value, the less toxic the chemical is. Thus, according to results, only **Me_2SnUnd_2**, CapriH and Me_2SnCl_2 showed no cytotoxic activity to mammalian cell with LC_{50} > 100 μM (Table 6 and Figure 5).

Figure 5. Effect of ***n*-Bu$_3$SnRic** (A), **Me$_2$SnUnd$_2$** (B), CapriH (C), ***n*-Bu$_2$SnCl$_2$** (D), ***t*-Bu$_2$SnCl$_2$** (E), ***n*-Bu$_3$SnCl** (F), **Me$_2$SnCl$_2$** (G) against macrophages (J774 cell line) in cell viability assay (MTT) after 48 h



According to Pruchnik *et al.*, [54] many studies suggest that the toxicity of organotin complexes is mainly due to their action on the membrane. They present an extra property: a great capacity to cross the lipid bilayer.

We may explain the toxicity showed by UndH and RicH, since Lima *et al.* [55] affirm that fatty acids can induce apoptosis and necrosis to J774 cells with cytotoxicity not strictly related to carbon-chain length and the number of double bonds in the molecule. The mechanisms involve changes in mitochondrial transmembrane potential and intracellular neutral lipid accumulation. Nevertheless, fatty acids in plasma are bound to serum albumin, which decreases their availability and thus the toxicity to the cells.

Despite the high toxicity of most organotin compounds, our triorganotin compounds have showed minimal inhibitory concentration values in the nM range against *S. aureus*, being thus up to four thousand times more potent against this microorganism than against macrophage J774 (Table 7). And perhaps the presence of fatty carboxylates as ligands may support the linkage of our compounds to serum albumin, decreasing consequently their toxic effects.

Table 7. Comparison between MIC against *S. aureus* and LC_{50}

Compound	**MIC (µM)** *S. aureus*	**LC_{50} (µM ± S.E.M.)** Macrophage (J774 cell line)	**Comparison**
***n*-Bu_3SnUnd**	**0.06**	2.3 ± 0.3	MIC almost **40 times** lower than LC_{50}
***n*-Bu_3SnRic**	**0.011**	44 ± 12.5	MIC **4,000 times** lower than LC_{50}

I.1.5 – Conclusion

The complexation of bioactive fatty acids (undecylenic, ricinoleic and caprylic acids) with organotin moieties led, in most cases, to compounds with a high antimicrobial activity. Despite the high toxicity of most organotin compounds, our triorganotin complexes have showed minimal inhibitory concentration values in nM range against *S. aureus*, being up to four thousand times more potent against this microorganism than against mammalian cells. It reassures us of being on the right path to overcoming challenges to develop new alternatives in antimicrobial field.

I.1.6 – Experimental part

I.1.6.1 – General remarks

Experiments were carried out under an argon atmosphere, using a vacuum line. Diethyl ether and toluene were distilled over sodium/benzophenone under an argon atmosphere immediately prior to use. The other starting materials were purchased from Sigma−Aldrich and Vetec and used as received, without further purification. All formulated microbiological media were obtained from HiMedia Laboratories PVT. Ltd.

Undecylenic [44] and ricinoleic [56] sodium salts were synthesized following already reported procedures. ***n*-Bu_2SnUnd_2**, ***n*-Bu_3SnUnd** and **Me_2SnUnd_2** were synthesized according to methodology described in my Master work. [44]

The NMR spectra were obtained at room temperature on Bruker spectrometers. ^1H NMR spectra were recorded at 400.13 MHz (Model Avance 400). The chemical shifts are referenced to the residual solvent peak. Chemical shifts (δ) and coupling constants (*J*) are expressed in units of ppm and Hz, respectively. (Multiplicity: s = singulet, d = doublet, t = triplet, q = quadruplet, h = heptuplet, m = multiplet).

Infrared spectra were recorded on a instrument FT-IR Varian 660-IR with an ATR apparatus.

I.1.6.2 – Synthesis protocols

CapriH: Sodium caprylate was prepared by reacting equimolar amounts of caprylic acid in methanol solution with an aqueous solution of sodium bicarbonate. The mixture was stirred for 2 hours at room temperature and the solvent removed in a rotary evaporator and the residue was dried under vacuum. **Yield:** 99%.

NMR ^1H (400.13 MHz, D_2O): 2,01 (t, 2H, $C\underline{H_2}COO$, *J* =7,05 Hz); 1,37 (qt, 2H, $C\underline{H_2}CH_2COO$, *J* = 7,13 Hz); 1,11 [m, 8H, $(C\underline{H_2})_4CH_3$]; 0,69 [t, 3H, $(CH_2)_4C\underline{H_3}$, *J* =7,05 Hz].

IR (FTR) [cm^{-1}]: 2952 e 2870 (ν_{as}/ν_s CH_3); 2922 e 2853 (ν_{as}/ν_s CH_2); 1559 e 1415 (ν_{as}/ν_s COO); 1444 (δ_{as} CH_3 e δ_s CH_2); 1383 (δ_s CH_3) 720 (ρ CH_2).

***t*-Bu_2SnUnd_2, *n*-Bu_2SnRic_2, *t*-Bu_2SnRic_2, Me_2SnRic_2, *n*-$Bu_2SnCapri_2$, *t*-$Bu_2SnCapri_2$,** and **$Me_2SnCapri_2$:** In a round bottom flask, 1 equiv. of tin chloride (*n*-Bu_2SnCl_2, *t*-Bu_2SnCl_2 or Me_2SnCl_2) was solubilized in toluene. Under stirring, 2 equiv. of sodium salt (UndNa, RicNa or CapriNa) was added. After 24 h under reflux, reaction mixture was let reacting at room

temperature overnight. Diethyl ether was then added, followed by filtration. The solution was transferred into a rotary evaporator to remove the solvents and the residue was finally dried under vacuum. All products are colorless to light yellow oils.

***t*-Bu_2SnUnd_2 Data. Yield:** 64%.

^{1}H NMR (400.13 MHz, $CDCl_3$): 5,81 (m, 2H, =C<u>H</u>); 4,96 (m, 4H, =C<u>H$_2$</u>); 2,37 (t, 4H, C<u>H$_2$</u>COO, *J* =7,57 Hz); 2,08 (q, 4H, C<u>H$_2$</u>CH=, *J* = 7,13 Hz); 1,65 [qt, 4H, C<u>H$_2$</u>CH_2COO, *J* = 7,48 Hz); 1,31 [m, 38H, Sn-C$(CH_3)_3$ + $(C\underline{H_2})_5$].

IR (FTR) [cm^{-1}]: 3075 (*ν* =CH); 2974 e 2883 (ν_{as}/ν_s CH_3); 2925 e 2853 (ν_{as}/ν_s CH_2); 1737 (*ν* C=O); 1638 (*ν* C=C); 1602 e 1367 (ν_{as}/ν_s COO); 1460 (δ_{as} CH_3 e δ_s CH_2); 1387 (δ_s CH_3); 993 e 908 (γ =CH); 723 (ρ CH_2); 640 e 555 (ν_{as}/ν_s Sn-C); 532 (*ν* Sn-O).

***n*-Bu_2SnRic_2 Data. Yield:** 78%.

^{1}H NMR (400.13 MHz, $CDCl_3$): 5,45 (m, 4H, C<u>H</u>=C<u>H</u>); 4,88 (qt, 1H, C<u>H</u>-OH); 3,61 (qt, 1H, C<u>H</u>-OH); 2,29 (m, 8H, C<u>H$_2$</u>CH=CHC<u>H$_2$</u>); 2,03 (br sig, 4H, C<u>H$_2$</u>COO); 1,52 [m, 52H, Sn(C<u>H$_2$</u>)$_3$ + $(C\underline{H_2})_5CH_2$COO + $(C\underline{H_2})_5CH_3$]; 0,90 [m, 12H, $(CH_2)_5C\underline{H_3}$ + $Sn(CH_2)_3C\underline{H_3}$].

IR (FTR) [cm^{-1}]: 3011 (*ν* =CH); 2957 e 2870 (ν_{as}/ν_s CH_3); 2925 e 2853 (ν_{as}/ν_s CH_2); 1723 (*ν* C=O); 1598 e 1378 (ν_{as}/ν_s COO); 1461 (δ_{as} CH_3 e δ_s CH_2); 667 e 611 (ν_{as}/ν_s Sn-C); 529 (*ν* Sn-O).

***t*-Bu_2SnRic_2 Data. Yield:** 79%.

^{1}H NMR (400.13 MHz, $CDCl_3$): 5,44 (m, 4H, C<u>H</u>=C<u>H</u>); 4,88 (qt, 1H, C<u>H</u>-OH); 3,62 (qt, 1H, C<u>H</u>-OH); 2,33 (m, 8H, C<u>H$_2$</u>CH=CHC<u>H$_2$</u>); 2,03 (br sig, 4H, C<u>H$_2$</u>COO); 1,44 [m, 58H, Sn-C$(C\underline{H_3})_3$ + $(C\underline{H_2})_5CH_2$COO + $(C\underline{H_2})_5CH_3$]; 0,88 [br sig, 6H, $(CH_2)_5C\underline{H_3}$].

IR (FTR) [cm^{-1}]: 3008 (*ν* =CH); 2952 e 2867 (ν_{as}/ν_s CH_3); 2925 e 2853 (ν_{as}/ν_s CH_2); 1731 (*ν* C=O); 1602 e 1367 (ν_{as}/ν_s COO); 1458 (δ_{as} CH_3 e δ_s CH_2); 727 (ρ CH_2); 665 e 566 (ν_{as}/ν_s Sn-C); 534 (*ν* Sn-O).

Me_2SnRic_2 Data. Yield: 71%.

^{1}H NMR (400.13 MHz, $CDCl_3$): 5,43 (m, 4H, C<u>H</u>=C<u>H</u>); 4,88 (qt, 1H, C<u>H</u>-OH); 3,62 (qt, 1H, C<u>H</u>-OH); 2,29 (m, 8H, C<u>H$_2$</u>CH=CHC<u>H$_2$</u>); 2,03 (br sig, 4H, C<u>H$_2$</u>COO); 1,43 [m, 40H, $(C\underline{H_2})_5CH_2COO$ + $(C\underline{H_2})_5CH_3$]; 1,01 (br sig, 6H, $SnC\underline{H_3}$); 0,88 [br sig, 6H, $(CH_2)_5C\underline{H_3}$].

IR (FTR) [cm^{-1}]: 3008 (*ν* =CH); 2923 e 2852 (ν_{as}/ν_s CH_2); 1731 (*ν* C=O); 1557 e 1378 (ν_{as}/ν_s COO); 1457 (δ_{as} CH_3 e δ_s CH_2); 724 (ρ CH_2); 623 e 581 (ν_{as}/ν_s Sn-C); 543 (*ν* Sn-O).

***n*-$Bu_2SnCapri_2$ Data. Yield:** 70%.

^{1}H NMR (400.13 MHz, $CDCl_3$): 2,35 (t,4H, C<u>H$_2$</u>COO, *J* =7,66 Hz); 1,65 [m, 12H, $C\underline{H_2}CH_2COO$ + $Sn(C\underline{H_2})_2$]; 1,31 [m, 20H, $(C\underline{H_2})_4CH_3$ + $Sn(CH_2)_2C\underline{H_2}$]; 0,88 [m, 12H, $(CH_2)_4C\underline{H_3}$ + $Sn(CH_2)_3C\underline{H_3}$].

IR (FTR) [cm^{-1}]: 2954 e 2869 (ν_{as}/ν_s CH_3); 2923 e 2855 (ν_{as}/ν_s CH_2); 1734 (*ν* C=O); 1596 e 1378 (ν_{as}/ν_s COO); 1458 (δ_{as} CH_3 e δ_s CH_2); 722 (ρ CH_2); 670 e 631 (ν_{as}/ν_s Sn-C); 530 (*ν* Sn-O).

***t*-$Bu_2SnCapri_2$ Data. Yield:** 72%.

^{1}H NMR (400.13 MHz, $CDCl_3$): 2,37 (br sig, 4H, $C\underline{H}_2COO$); 1,65 (br sig, 4H, $C\underline{H}_2CH_2COO$); 1,31 [m, 34H, $(C\underline{H}_2)_4CH_3$ + Sn-$C(C\underline{H}_3)_3$]; 0,87 [br sig, 6H, $(CH_2)_4C\underline{H}_3$].

IR (FTR) [cm^{-1}]: 2956 e 2870 (ν_{as}/ν_s CH_3); 2926 e 2853 (ν_{as}/ν_s CH_2); 1737 (ν C=O); 1602 e 1367 (ν_{as}/ν_s COO); 1458 (δ_{as} CH_3 e δ_s CH_2); 1383 (δ_s CH_3); 724 (ρ CH_2); 668 e 563 (ν_{as}/ν_s Sn-C); 532 (ν Sn-O).

$Me_2SnCapri_2$ Data. Yield: 69%.

^{1}H NMR (400.13 MHz, $CDCl_3$): 2,34 (br sig, 4H, $C\underline{H}_2COO$); 1,63 (br sig, 4H, $C\underline{H}_2CH_2COO$); 1,29 [m, 16H, $(C\underline{H}_2)_4CH_3$]; 0,96 [m, 12H, $(CH_2)_4C\underline{H}_3$ + $SnC\underline{H}_3$].

IR (FTR) [cm^{-1}]: 2954 e 2869 (ν_{as}/ν_s CH_3); 2925 e 2855 (ν_{as}/ν_s CH_2); 1714 (ν C=O); 1556 e 1404 (ν_{as}/ν_s COO); 1458 (δ_{as} CH_3 e δ_s CH_2); 1378 (δ_s CH_3); 724 (ρ CH_2); 640 e 571 (ν_{as}/ν_s Sn-C); 532 (ν Sn-O).

***n*-Bu_3SnRic** and ***n*-$Bu_3SnCapri$:** In a round bottom flask containing 1 equiv. of *n*-Bu_3SnCl in toluene, we added 1 equiv. of sodium salt (RicNa or CapriNa), under stirring. After 24 h under reflux, reaction mixture was let reacting at room temperature overnight. Diethyl ether was then added, followed by filtration.. The solution was transferred into a rotary evaporator to remove the solvents and the residue was finally dried under vacuum.

***n*-Bu_3SnRic Data. Yield:** 75%. As a colorless to light yellow oil.

^{1}H NMR (400.13 MHz, $CDCl_3$): 5,42 (m, 2H, $C\underline{H}$=$C\underline{H}$); 4,87 (qt, 1 H, $C\underline{H}$-OH); 3,60 (qt, 1H, $C\underline{H}$-OH); 2,26 (m, 4H, $C\underline{H}_2CH$=CH$C\underline{H}_2$); 2,01 (br sig, 2H, $C\underline{H}_2COO$); 1,33 [m, 38H, $Sn(C\underline{H}_2)_3$ + $(C\underline{H}_2)_5CH_2COO$ + $(C\underline{H}_2)_5CH_3$]; 0,85 [br sig, 12H, $(CH_2)_5C\underline{H}_3$ + $Sn(CH_2)_3C\underline{H}_3$].

IR (FTR) [cm^{-1}]: 3007 (ν =CH); 2952 e 2867 (ν_{as}/ν_s CH_3); 2923 e 2852 (ν_{as}/ν_s CH_2); 1732 (ν C=O); 1618 e 1376 (ν_{as}/ν_s COO); 1458 (δ_{as} CH_3 e δ_s CH_2); 724 (ρ CH_2); 670 e 609 (ν_{as}/ν_s Sn-C); 530 (ν Sn-O).

***n*-$Bu_3SnCapri$ Data. Yield:** 73%. As a white solid.

^{1}H NMR (400.13 MHz, $CDCl_3$): 2,30 (t, 2H, $C\underline{H}_2COO$, J =7,35 Hz); 1,63 [m, 8H, $C\underline{H}_2CH_2COO$ + $SnC\underline{H}_2$]; 1,30 [m, 20H, $(C\underline{H}_2)_4CH_3$ + $SnCH_2(C\underline{H}_2)_2$]; 0,91 [m, 12H, $(CH_2)_4C\underline{H}_3$ + $Sn(CH_2)_3C\underline{H}_3$].

IR (FTR) [cm^{-1}]: 2954 e 2870 (ν_{as}/ν_s CH_3); 2920 e 2853 (ν_{as}/ν_s CH_2); 1545 e 1404 (ν_{as}/ν_s COO); 1457 (δ_{as} CH_3 e δ_s CH_2); 1376 (δ_s CH_3); 725 (ρ CH_2); 667 e 609 (ν_{as}/ν_s Sn-C); 532 (ν Sn-O).

I.1.6.3 – Antimicrobial assays

The microorganisms used in this study were *S. aureus* ATCC 25923, *C. albicans* ATCC 10231, *P. aeruginosa* ATCC 27853, and *E. coli* ATCC 25922. These standardized and distributed by American Type Cell Collection (ATCC, Manassas/VA/USA).

The MIC for each compound was determined using the microdilution method.

In test tubes containing approximately 5 mL of the BHI (brain-heart infusion broth) one disc or about 10 mg of microorganism was activated. This material was placed into bacteriological incubator for 24 hours at 35 °C. After this period it was put on Mueller-Hinton agar plate (for bacteria) or Sabouraud Dextrose Agar plate (for *C. albicans*). It was incubated for 24 hours at 35 °C. The inoculum was made from a suspension of each microorganism in phosphate buffered saline.

Antifungal assay: The test for determining MIC against *C. albicans* was adapted from document M27-A3 (CLSI, 2008). [57] 0.05 g of each sample was solubilized in DMSO to a final volume of 1 ml. It was added 100 µL of this solution to a vial containing 4.9 mL of the BHI culture medium, resulting in a solution of 1,000 µg/ml of sample and 2% of DMSO. In a 96-well microtiter plate, we did a serial dilution with resulting concentrations from 500 µg/mL to 0.01 µg/mL, in duplicate for each tested compound. Then we added a suspension containing *C. albicans*, obtaining an inoculum with density of 1-5x10^3 CFU/mL. We adopted DMSO 2%, culture medium and microbial inoculum as negative control; culture medium and inoculum as growth control; and wells containing only the culture medium (without inoculum) as sterility control. Plates were incubated at 35 °C during 24 h and the MIC was determined by the lowest concentration of compound to prevent microbial growth, i.e., the well with the lowest concentration of tested sample in which no turbidity was observed.

Antibacterial assay: The test for determining MIC against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* was adapted from document M7-A9 (CLSI, 2012). [58] 0.05 g of each sample was solubilized in DMSO to a final volume of 1 ml. It was added 100 µL of this solution to a vial containing 4.9 mL of the Mueller-Hinton broth culture medium, resulting in a solution of 1,000 µg/ml of sample and 2% of DMSO. In a 96-well microtiter plate, we did a serial dilution with resulting concentrations from 500 µg/mL to 0.01 µg/mL, in duplicate for each tested compound. For samples whose MIC was less than 0.01 µg/mL, the test was repeated with lower concentrations doing more dilutions. Then we added a bacterial suspension, obtaining an inoculum with density of 5 x 10^5 CFU/mL. We adopted DMSO 2%, culture medium and bacterial inoculum as negative control; culture medium and inoculum as growth control; and wells containing only the culture medium (without inoculum) as sterility control. Plates were incubated at 35 °C during 18 h. After that, a 2,3,5-triphenyl-tetrazolium chloride (TTC) solution and plates were reincubated for more 3 h. MIC

was determined by the lowest concentration of compound to prevent bacterial growth, i.e., the well with the lowest concentration of tested sample in which no red color was observed.

I.1.6.4 – Cytotoxicity against mammalian cells

To evaluate the cytotoxic activity against the J774 cell line, the macrophages were plated in 96-well vessels at 2×10^5 cells per well in complete culture medium 10% FBS (fetal bovine serum) at 37 °C. After 1 h wells were washed with warm HBSS (Hank's balanced salt solution) to remove non-adherent cells, leaving approximately 10^5 adherent macrophages. All cultures were done in DMEM (Dulbecco's Modified Eagle's medium) complete supplemented with 10% FBS. The compounds and pentamidine were added at serial concentrations (0.1–100 μM). The cells were also cultured with medium free from compounds or vehicle (basal growth control) or in media with DMSO 0.1% (vehicle control). Positive control (dead cells) was obtained by cellular lysis with 1% of Triton 100X in DMEM complete. After 48 h, the cytotoxicity was evaluated by the MTT assay. [52] Data obtained from experiments were expressed as the mean ± standard error of the mean (Mean ± S.E.M.) and statistical differences between the treated and the vehicle groups of experiments were evaluated by ANOVA and Dunnett hoc tests. LC_{50} (concentration required to give 50% death of cells) was calculated by linear regression analysis from the Kc values at employed concentrations. [53]

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Part II:

Ruthenium compounds in Medicinal Chemistry

Chapter II.1:
Introduction

II.1.1 – Generalities about cancer and antineoplastic therapy

According to World Health Organization (WHO), *"cancer is a generic term for a large group of diseases that can affect any part of the body."* Its defining feature is *"the rapid creation of abnormal cells that grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs"* (metastasis). [1]

There are several main types of cancer classified according to their origin tissues: [2]

- **Carcinoma** is a cancer that begins in the skin or in tissues that line or cover internal organs;
- **Sarcoma** is a cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue;
- **Leukemia** is a cancer that starts in blood-forming tissue, such as the bone marrow, and causes large numbers of abnormal blood cells to be produced and enter the blood;
- **Lymphoma** and **multiple myeloma** are cancers that begin in the cells of the immune system;
- **Central nervous system cancers** are cancers that begin in the tissues of the brain and spinal cord.

Cancer can be caused by external factors, such as chemicals, radiation and viruses, as well by internal factors, such as hormones, immune conditions and genetic mutations, that may act together or in sequence to initiate or promote carcinogenesis. [3] Indeed, it is known that environmental factors can play a key role in cancer formation and progression, but the relationship between factor and cause can be challenging to unravel. [4]

It is important to emphasize that not all tumors are cancer. The word "tumor" corresponds to increased volume noted in any part of the body. When the tumor occurs by growth of cells, it is called neoplasia - which can be benign or malignant. Unlike cancer that is malignant neoplasm, benign tumors have their growth in an organized manner, usually slow, and features well clear limits. The lipoma and leiomyoma are examples of benign tumors. [3]

Evidences based on paleontological findings of tumors in animals, cancer has been present on Earth even before the appearance of man. [5, 6] Old Egyptian documents, the Edwin Smith Papyrus and the Ebers Papyrus, describe cancer as a grave incurable disease related to "the curse of the gods". [5] The first description of human cancer can be found in the Edwin Smith Papyrus dated 3000 BC that illustrated a case of breast cancer. [7]

Di Lonardo *et al.* [7] elaborated a table with a timeline of theories and achievements on cancer. Some of these informations are showed in the Table 1.

Table 1. Timeline of some theories and achievements on cancer [7]

When?	What happened?
Palaeolithic	Tumors in prehistoric animals.
3000 BC	Edwin Smith Papyrus is the earliest written description of cancer.
1500 BC	Ebers Papyrus describes cancers of the skin, uterus, stomach, and rectum.
400 BC	**Hippocrates proposes the humoral theory: "cancer caused by a surplus of black bile in the body".**
25 BC- 20	Aulus Celsus describes several varieties of cancers in the book, *De Medicina*. He points out that breast cancers cause death by spreading to distant organs, and recommends surgical therapy.
23-79	Plinius describes remedies for cancer in the book, *Materia Medica*.
81-138	Aretaeus gives the first description and treatment of cancer of the uterus.
100-200	**Claudius Galen (130-200) implements Hippocrates' concepts. He proposes that thick black bile causes incurable cancer, and thin yellow bile causes curable cancer.** Galen uses the Greek word *oncos* (swelling) to describe tumors.
500-1300	Aetius (Constantinople 527-565) introduces treatment of breast cancer by amputation of the whole breast. Avenzoar (Cordova, Spain 1070-1162) describes the oesophagus and rectal cancers and introduces hysterectomy for the removal of uterine tumors.
1500s	**Paracelsus hypothesises that cancer is caused by accumulation of harmful substances in the bloodstream.** **A. Pare, a French surgeon, hypothesises the cause of cancer as being attributed to an irregular diet that induced an accumulation of feculent material in the blood.**
1600s	**Physicists propose cancer caused by coagulation and fermentation of blood or lymph.**
1700s	**Possible causes of cancer include chronic inflammation, injuries, traumas and familial predispositions. Autopsies highlight cancer as an "organ lesion".** 1713, B. Ramazzini notices the virtual absence of cervical cancer and high

(To be continued...)

	incidence of breast cancer among nuns. The scientist associates the different tumor incidences to lifestyle, namely sexual abstinence. This is the start of "cancer epidemiology". 1760, G. B. Morgagni suggests that cancer is correlated to pathological lesions of a particular organ.
1800s	The use of microscopes and the implementation of histological techniques permits the analysis of anomalous cell nuclei in tumors. **1838, J. Muller hypothesises that cancer is made up of cells.** **1860, R. Virchow proposes cancer as disease of cells.** **1889, S. Paget proposes the theory of "seed and soil", to explain metastasis formation.** **Several scientists propose that cancer is caused by "germs", and metastasis was explained as parasites attacking consecutive organs.**
1900s	**1914, T. Boveri hypothesises: "tumor growth is based on …a particular, incorrect chromosome".** 1915, K. Yamagiwa and K. Ichikawa are able to induce cancer in rabbits by applying coal tar to the skin of these laboratory animals. 1964, A. Epstein, B. Achong and Y. Barr identify the first human cancer virus, the Epstein-Barr Virus (EBV). **1964, H. Temin proposes the theory of provirus hypothesis.** **1969, R. Huebner and G. Todaro propose the virogene/oncogene hypothesis.** 1973, J. Rowley demonstrates chromosome abnormalities in cancer patient. 1979, A. Levine, D. Lane and L. Old individually discover p53 anti-oncogene. 1980, discovery of Human T-lymphotropic virus 1 (HTLV I), the first human retrovirus. 1986, US Department of Energy announces Human Genome Initiative. 1992, Low Resolution Linkage Map of entire human genome published. 1998, Celera Genomics Corporation launches private initiative to sequence genome. 1999, Public genome project completes sequence of first chromosome, chromosome 22.
XXI century	2001, Human genome draft version finished (3200 Mb). 2002, Presentation of human genome. 2008, first whole cancer genome sequenced from cytogenetically normal Acute Myeloid Leukemia (AML), compared with normal somatic skin cell from same individual. 2010, first whole genome of a cancer cell line sequenced. 2010, whole genome sequencing of melanoma tumor and normal pair identifies UV-induced mutation. 2010, first publication of whole genomes from primary and metastatic tumor from one breast cancer patient. 2010, 70 whole cancer genomes or exomes sequenced.

As presented in the Table 1, several theories were elaborated to explain the cancer (in bold). Actually, a widely accepted concept is that cancer is a clonal disease. However, the fundamental question about what is the cell that originates tumors has always challenged cancer researchers. Indeed, the cell of origin of many cancers is still largely unknown, and the exact initial incident that leads to the cascade of events culminating in malignant transformation is yet to be identified. In a review published in *Science* in 1976, "The clonal evolution of tumor cell populations", Peter C. Nowell [8] proposed that tumors originate from a single cell and that "*an induced change provides it with a selective growth advantage.*" Subsequently, "*acquired genetic lability permits stepwise selection of variant sublines and underlies tumor progression*." This groundbreaking theory is now known as the cancer clonal evolution model. [9]

According to Greaves, [10] *"cancer is, in its essence, a Darwinian dilemma. This should inform our attempts to control it."* Indeed, "*our understanding of cancer is being transformed by exploring clonal diversity, drug resistance, and causation within an evolutionary framework. The therapeutic resilience of advanced cancer is a consequence of its character as a complex, dynamic, and adaptive ecosystem engendering robustness, underpinned by genetic diversity and epigenetic plasticity. The risk of mutation-driven escape by self-renewing cells is intrinsic to multicellularity but is countered by multiple restraints, facilitating increasing complexity and longevity of species.*"

There are many types of cancer treatment and the types choosed for each patient depend on the type of cancer he has and how advanced it is. The main types of cancer treatment and their definitions according to National Cancer Institute of United States are presented below: [11]

- Surgery: A procedure to remove or repair a part of the body or to find out whether disease is present;

- Radiation Therapy: The use of high-energy radiation from x-rays, gamma rays, neutrons, protons, and other sources to kill cancer cells and shrink tumors. Radiation may come from a machine outside the body (external-beam radiation therapy), or it may come from radioactive material placed in the body near cancer cells (internal radiation therapy or brachytherapy). Systemic radiation therapy uses a radioactive substance, such as a radiolabeled monoclonal antibody, that travels in the blood to tissues throughout the body. Also called irradiation and radiotherapy;

- Chemotherapy: Treatment with drugs that kill cancer cells;

- **Immunotherapy:** A type of biological therapy that uses substances to stimulate or suppress the immune system to help the body fight cancer, infection, and other diseases. Some types of immunotherapy only target certain cells of the immune system. Others affect the immune system in a general way. Types of immunotherapy include cytokines, vaccines, bacillus Calmette-Guerin (BCG), and some monoclonal antibodies;
- **Targeted Therapy:** A type of treatment that uses drugs or other substances to identify and attack specific types of cancer cells with less harm to normal cells. Some targeted therapies block the action of certain enzymes, proteins, or other molecules involved in the growth and spread of cancer cells. Other types of targeted therapies help the immune system kill cancer cells or deliver toxic substances directly to cancer cells and kill them. Targeted therapy may have fewer side effects than other types of cancer treatment. Most targeted therapies are either small molecule drugs or monoclonal antibodies;
- **Hormone Therapy:** Treatment that adds, blocks, or removes hormones. For certain conditions (such as diabetes or menopause), hormones are given to adjust low hormone levels. To slow or stop the growth of certain cancers (such as prostate and breast cancer), synthetic hormones or other drugs may be given to block the body's natural hormones. Sometimes surgery is needed to remove the gland that makes a certain hormone. Also called endocrine therapy, hormonal therapy, and hormone treatment;
- **Stem Cell Transplant:** A method of replacing immature blood-forming cells in the bone marrow that have been destroyed by drugs, radiation, or disease. Stem cells are injected into the patient and make healthy blood cells. A stem cell transplant may be autologous (using a patient's own stem cells that were saved before treatment), allogeneic (using stem cells donated by someone who is not an identical twin), or syngeneic (using stem cells donated by an identical twin);
- **Precision Medicine:** A form of medicine that uses information about a person's genes, proteins, and environment to prevent, diagnose, and treat disease. In cancer, precision medicine uses specific information about a person's tumor to help diagnose, plan treatment, find out how well treatment is working, or make a prognosis. Examples of precision medicine include using targeted therapies to treat specific types of cancer cells, such as HER2-positive breast cancer cells, or using tumor marker testing to help diagnose cancer. Also called personalized medicine.
- **Phototherapy:** Also called light therapy, is the treatment of disease with certain types of light. Phototherapy can use lasers, LED, fluorescent lamps, and ultraviolet or infrared radiation. The light may activate photo-activating compounds to exercise their function.

Some people with cancer will have only one treatment. But most people have a combination of treatments, such as surgery with chemotherapy and/or radiation therapy. [11]

According to WHO, [1] cancer can have severe health consequences, and is a leading cause of death, mainly because of the metastasis process. However, more than 30% of cancer deaths could be prevented by modifying or avoiding key risk factors, especially tobacco and heavy alcohol use. The most common types of cancer in men are lung, prostate, colorectal, stomach, and liver cancer, while breast, colorectal, lung, uterine cervix, and stomach cancer are the most common among women. Early detection, accurate diagnosis, and effective treatment, including pain relief and palliative care, help increase cancer survival rates and reduce suffering.

Most of usual chemotherapeutic agents (mainly discovered as a result of screening compounds for cytotoxic potency *in vitro* against murine and/or human cancer cells or *in vivo* against rodent tumour models) cause cell death by directly inhibiting the synthesis of DNA or interfering with its function. These agents are commonly targeted at the process of cell division, since cancer cells commonly replicate faster than normal cells. However, they are responsible for significant toxicity because of their non specific action. The most notable examples of adverse effects include bone marrow suppression, alopecia, mucositis, nausea and vomiting. These compounds also only influence the ability of the cells to divide, having little effect on other aspects of tumour progression such as tissue invasion, metastases or progressive loss of differentiation. [12]

To overcoming the high toxicity of anticancer agents, an interesting alternative used in many cancers treatment is the combinations of cytotoxic agents, improving the effectiveness when compared with single agents. Possible explanations for this include: (1) exposure to agents with different mechanisms of action and nonoverlapping toxicities; (2) reduction in the development of drug resistance; (3) the ability to use combinations of drugs that may be synergistic. [12]

Targeted therapy is also a good alternative that may have fewer side effects than other types of cancer treatment. More details about this kind of treatment will be presented in the **Chapter II.3**.

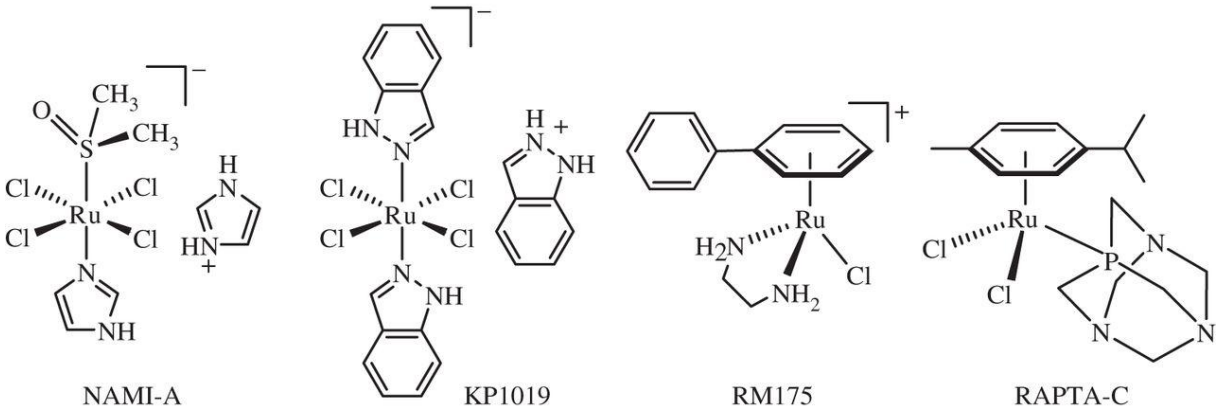
II.1.2 – Ruthenium compounds in medicine

Many ruthenium-based compounds display promising anticancer activity, and two ruthenium(III) complexes have entered clinical trials: NAMI-A, that is the *trans*-

$[RuCl_4(DMSO)(Im)]ImH$ (where Im = imidazole) and KP1019, the *trans*-$[RuCl_4(Ind)_2]IndH$ (where Ind = indazole). Their chemical structures are presented in Figure 1. KP1019 shows good activity against primary tumors, [13] although the structurally similar NAMI-A is more active against metastatic cells than against primary tumors. [14] Since it is believed that ruthenium(III) complexes act as prodrugs, depending on the *in vivo* reduction to the more reactive ruthenium(II) species to exerce the anticancer activity, [15] ruthenium(II) compounds seems to be more interesting prototypes in the anticancer therapy application. [16] Once in cells the Ru(III) may be activated by reduction to Ru(II), although Ru(IV) is also accessible under biological conditions. [17]

Both, the indazole complex KP1019 (under commercial clinical development as the sodium salt NKP-1339 and IT-139) and the imidazole complex NAMI (new anti-tumour metastasis inhibitor) A, are octahedral Ru(III) complexes that have completed phase I clinical trials. Ruthenium(III) from these complexes may be delivered to tumour cells by the Fe(III) transport protein serum transferrin, receptors which are over-expressed on cancer cells. KP1019 induces apoptosis via the intrinsic mitochondrial pathway. Organometallic Ru(II) arene complexes also exhibit promising anti-cancer activity and Ru(III) EDTA complexes have been investigated as NO scavengers for treatment of septic shock. [17]

Figure 1. Chemical structure of some anticancer ruthenium-based complexes [17]



Several works have studied the anticancer potential of half-sandwich Ru(II) arene complexes of the type $[(\eta^6\text{-arene})Ru(YZ)(X)]$, where YZ is a bidentate chelating ligand and X is a good leaving group (*e.g.* Cl, Figure 1). These halfsandwich "piano-stool" complexes offer much scope for design, with the potential to vary in each of the building blocks (arene, chelated ligand YZ and monodentate ligand X) to enable modifications of thermodynamic and kinetic parameters. [16]

As we could see previously in the **General Introduction**, the metallic compound cisplatin has been used for many years to treat various human cancers. Among the huge variety of organometallic compounds that have been tested, it appeared already 35 years ago, that compounds where a genuine covalent carbon-metal σ-bond is present might be of special interest in this research area. [18] This is indeed the case of cyclometalated compounds in which the carbon-metal bond is stabilized by an intramolecular coordination bond. [19]

In fact, cycloruthenated 2-phenylpyridine derivatives [20,21] are very interesting organometallic synthons, as many independent studies have shown them to be key ligands in different organometallic molecules that display properties for several applications such as electronic relays for redox enzymes, [22,23,24,25,26,27] as photosensitizers for photovoltaic applications [28,29] or for their *in vitro* and *in vivo* cytotoxicities that might be useful for cancer treatments. [30,31,32,33,34,35]

In order to improve activity and reduce side effects in anticancer agents our laboratory has developed for several years ruhenium-derived compounds (**RDC**s) that contain a ruthenium(II) atom covalently linked to carbon and nitrogen atoms and that present very important *in vitro* and *in vivo* antitumoral activity. [30,31,32,33,35]

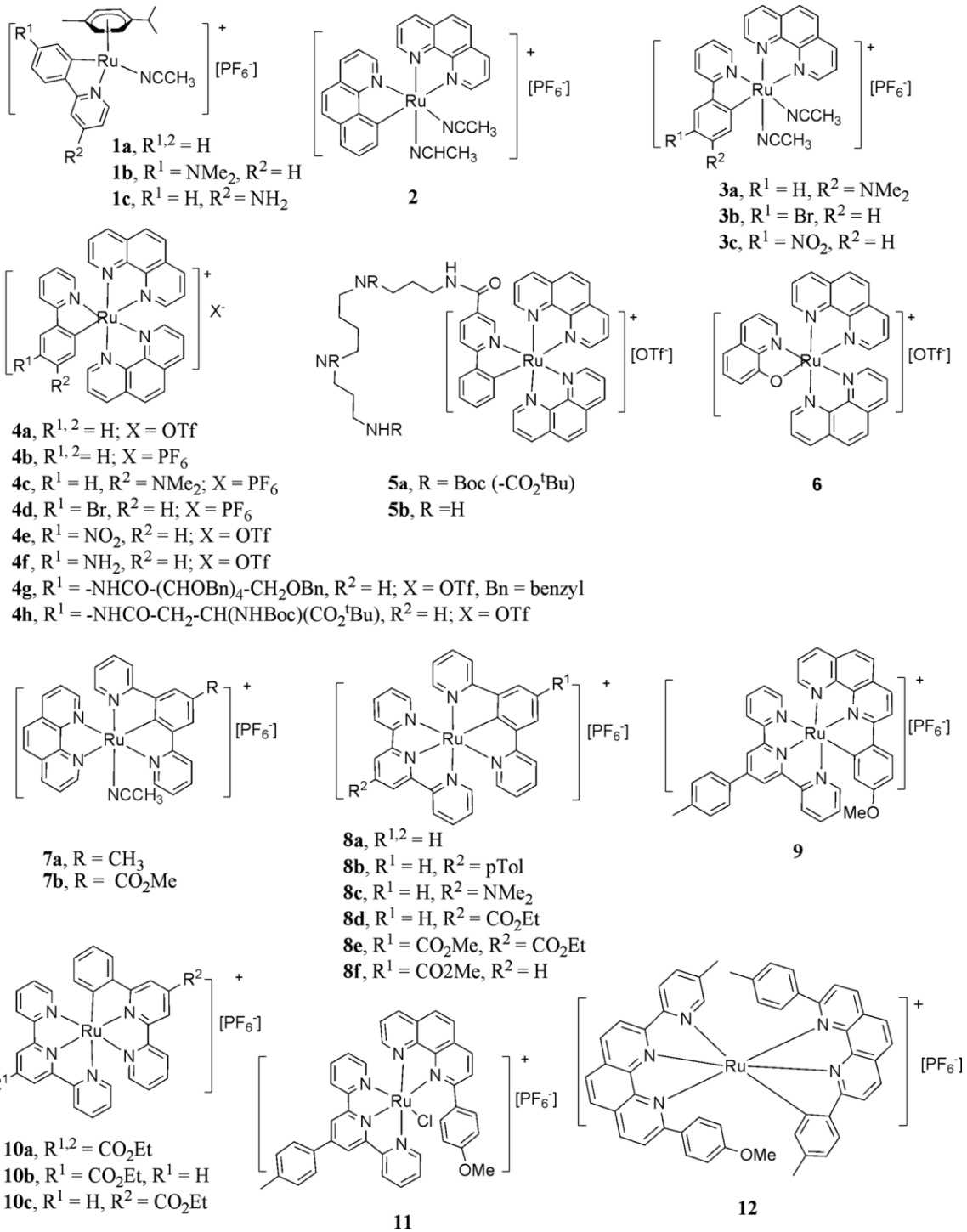
Compounds based on ruthenium were chosen because of their structural similariry with existing compounds that displayed interesting cytotoxicities against tumor cells. Today, however, we are still lacking enough information about the mechanism of action of these compounds so that we can hardly increase their selectivity toward cancer cells. They were also chosen because, after having extensively studied their reactivity, we had good evidence of their apparent inertness toward ligand exchange processes. [19]

II.1.2.1 – RDC: First and second generation

Commonly, the existing antitumor compounds are usually built up with a ligand weakly coordinated to the metal. Our laboratory, contrary, has synthesized and tested [31,33] ruthenacycles in which one ligand is strongly bound to the metal by a carbon-ruthenium σ bond and that is stabilized by a nitrogen-ruthenium intramolecular bond (Figure 2).

In the purpose to study cycloruthenated derivatives to anticancer application, we have defined three classes of compounds. The first class (**1–3** and **7**) consists in cycloruthenated 2-(Ar-H)Py derivatives, which have several coordination sites available on the Ru atom, by substitution of *a priori* weakly bound ligands on Ru such as one η^6-arene or one or two acetonitrile ligands.

Figure 2. First and second generation of **RDCs** [31]



The second class are those compounds in which three bidentate chelates (one monoanionic N^C chelate and two neutral N^N chelates) are found on the ruthenium atom (**4** and **5**), the coordination on Ru by external ligands being in principle impossible or less likely than with the compounds of the previous class. The third class of compounds (classified as a second generation complexes) derive from pincer ligands in which N^C^N or N^N^C

terdentate monoanionic chelates are present in conjunction to either neutral terdentate chelates such as terpy, for instance **8–12**, or the neutral bidentate phen ligand and one acetonitrile, **7**. The library of the compounds used in this study is shown in Figure 2.

All but two compounds, namely **6** and **11**, have a Ru–C bond which is part of a ruthenated heterocyclic unit in their structure.

II.1.2.2 – Importance of the redox properties of the ruthenium compounds

The characteristic of the redox enzymes involved in the cellular metabolism is that they shift back and forth between an oxidized and a reduced state and that their activity strongly depends on the electron exchanges between them, the substrates and the cofactors that provide and/or regenerate their active state. Using this information, our hypothesis is that we can design compounds with specific electron exchange properties (which is related to their redox properties) that would interfere with these electronic exchanges in the metabolic pathway and that would therefore show anticancer activity, displaying mechanisms of action that differ from that of more classical platinum derivatives.

Previous results of the laboratories involved in their development have indeed shown several important properties of these compounds: (1) *in vitro*, these compounds alter the activity of redox enzymes such as the glucose oxidase and peroxidase; [22,23,25,26] (2) in cells, these compounds regulate the activity of critical pathways involved in the cellular metabolism responsible for cancer cell survival; [32] (3) although these compounds are organometallic compounds like cisplatin or oxaliplatin, they do not require p53 activity or interaction with DNA for their cytotoxicity and they are efficient towards cancer cells that are resistant to platinum-derived compounds, including patient primary tumors (colon and ovarian); [32,30] (4) *in vivo*, these compounds reduce tumor size (≈45%) with a reduced toxicity toward healthy tissues when compared to gold standards such as cisplatin; (5) functional studies and a small-scale preliminary affinity chromatography purification using a matrix coupled with **RDC** followed by a mass spectrometry analysis have led to the identification of 3 pertinent targets for **RDC** in cancer cells: i) EEF1AI, a translation elongation factor that controls proper enzyme folding and whose deregulation leads to the accumulation of unfolded proteins and activation of the UPR/CHOP pathway, [36] ii) IDH, the isocitrate dehydrogenase that leads to the production of alpha ketoglutarate and HIF-1 regulation and this is mutated in several cancers, [37] and iii) PHD2, a HIF-1 hydroxylase that controls HIF-1 degradation by the proteasome pathway. [38] Interaction between **RDC** and these enzymes might explain the

activation of the UPR/CHOP pathway and the inhibition of the HIF-1 and mTOR pathways we have observed in cancer cells treated with **RDC**.

II.1.2.3 – *In vitro* and *in vivo* studies performed on RDC11*

The *in vivo* results we have accumulated so far on one of the first generation compound, **RDC11** (Figure 3), have indeed convinced us that further developments of this class of compounds are indeed worthwhile.

Figure 3. RDC11

As demonstrated by studies carried out by the National Cancer Institute (NCI), **RDC11** seems to be a good drug candidate, given that it is cytotoxic against several cell lines with different activity potentials. It is particularly active against one ovarian cancer (OVCAR-3), several melanomas (UAC-62, SK-MEL-5, SK-MEL-2, MALME 3M), one colon cancer (COLO 205) and also against one non-small-cell lung carcinoma (NSCLC NCI-H522). [32]

RDC11's *in vivo* activity was also evaluated on syngeneic tumour models B16F10 (subcutaneous melanoma) and 3LL (Lewis lung carcinoma) and also on xenograft models U87 (human glioblastoma). In all of those models, both **RDC11** and cisplatin demonstrated similar tumour growth inhibition of up to 40% compared with untreated mice. [32]

Other two criteria were also adopted to analyse the toxicity of **RDC11** in mice: the acute and chronic toxicity. In one single dose experiment, the lethal dose LD_{50} of **RDC11** is comparable to those of cisplatin. To assess the chronic toxicity, mice were regularly treated with equimolar doses of **RDC11** or cisplatin. After three weeks, cisplatin-treated mice had lost about 20% of their body weight in contrast to **RDC11**-treated mice which maintained their body mass. Moreover, no significant changes were observed on several blood markers

* This section was mostly based on the references: (a) Meng, X.; Leyva, M. L.; Jenny, M.; Gross, I.; Benosman, S.; Fricker, B.; Harlepp, S.; Hebraud, P.; Boos, A.; Wlosik, P.; Bischoff, P.; Sirlin, C.; Pfeffer, M.; Loeffler, J. P.; Gaiddon, C. *Cancer Res.* **2009**, *69*, 5458−5466; and (b) Boff, B. *Synthesis, physicochemical and biological evaluation studies of ruthenium(II) and osmium(III) anticancer organometallic complexes.* **2012.** PhD Thesis (Chemistry) – Strasbourg, Université de Strasbourg.

from **RDC11**-treated mice, except a drop in creatine enzyme concentration. Futhermore, after a chronic treatment, liver and kidneys from cisplatin-treated mice lost mass, further demonstrating that toxicities had arisen. Similarly after three weeks of treatment, cisplatin significantly reduces the speed conduction of sensory nerves (which is a classical toxicity unleashed by cisplatin in clinic), whereas **RDC11** has a modest effect on the nervous system. [32]

The NCI has carried out a 60 human tumour cell lines anticancer screen (NCI60) as an *in vitro* drug-discovery tool intended to supplant the use of transplantable animal tumours. In this screening, nine distinct tomour types are represented: leukemia, colon, lungs, central nervous system, renal, melanoma, ovarian, breast and prostate. Results from NCI demosnstrated that **RDC34** displays high cytotoxic properties in practically all tested cell lines. However, previous *in vivo* studies studies showed this compound is not well accepted in animal models because of its important chronic and acute toxicity. [39]

Therefore, our laboratory has long concentrated its research activity in determining the properties of organometallic compounds derived from the cyclometallation reaction. The compounds obtained by this procedure have been shown to display many fascinating properties in several area of chemistry, physics or biology. Indeed, these compounds have been shown to display *in vitro* and *in vivo* cytotoxic properties against several tumor cell lines at a significative enough level so that they can be considered as interesting anticancer drugs candidates. And that is why we choosed to continue studying this type of complexes, aiming to acquire new knowledge in this field of research.

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Chapter II.2:

*Further insight into the lability of MeCN ligands of cytotoxic cycloruthenated compounds**

II.2.1 – Isomerization of RDC11

As we have seen above (**section II.1.2.1**), our laboratory has developed several ruthenium-based compounds, some of them with important antitumoral activity. For a better control of the behavior of these compounds *in vitro* and hopefully *in vivo*, we decided to continue studying their chemical reactivity.

We recently discovered [1] that the compound that was named **RDC11** ([Ru(2-C_6H_4-2′-py-κC,N)(Phen, *trans*-C)(MeCN)$_2$]PF_6 (**1**) (where 2-C_6H_4-2′-Py-κC,N = cyclometalated 2,2′-phenylpyridine = PhPy, Phen = phenanthroline), with which our group have concentrated most of its biological studies so far, and which has long been believed to be thermally stable and nonreactive (as no substitution reaction of the MeCN ligands have been observed), could in fact be isomerized either thermally or photochemically (see Figure 1).

Figure 1. RDC11 (1) and its isomer **RDC11-*cis* (2)**

* The present chapter presents our latest results recently published at *Inorganic Chemistry*: Inorg. Chem. 2015, *54*, 7617−7626. DOI: 10.1021/acs.inorgchem.5b01236.

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Indeed, UV or visible light irradiation and/or thermal treatment of **1**, obtained *via* the addition of Phen to [Ru(2-C_6H_4-2′-Py-κC,N)$(MeCN)_4$]PF_6, had a dramatic consequence upon its stereochemistry as it was isomerized to a new specie **RDC11-*cis*** (**2**). In this new compound, one of the nitrogen atoms of Phen had moved from a position *trans* to C to a position *cis* to the same atom, with the freed position *trans* to C being occupied by an acetonitrile ligand.

As is known, isomers of a given compound can be defined as substances having the same stoichiometric composition, the same relative molecular mass, and the same chemical formula but different structures, which generally present distinct chemical and physical properties.[2] Indeed, we have discovered that the MeCN ligands of the new species could be readily substituted by N^N chelates, thanks to the large *trans* effect of the C atom of the cycloruthenated PhPy ligand. Thus we observed that in **RDC11**'s case the MeCN ligand is a poor leaving group when coordinated *trans* to an imine ligand but, contrariwise, it is a good leaving group when it is coordinated *trans* to a ligand that has a strong *trans* effect such as a phenyl group (as observed in **RDC11-*cis***).[1]

It has long been known that in square planar transition metal complexes certain ligands direct substitution reactions preferentially to a position *trans* to themselves. Both thermodynamics and kinetics provide the basis for this tendency. Ligands that form strong σ bonds, such as hydride and alkyl, or π acceptor ligands, such as CN^-, CO, and PR_3, which also bind strongly to the metal, tend to weaken the metal-ligand bond *trans* to the first ligand. In the ground state, this is a thermodynamic property called the *trans influence*. But the tendency of certain ligands to direct incoming groups to the *trans* position can also occur with reactions under kinetic control: this case is known as the *kinetic trans effect*, whereby the influence of the ligand *trans* to the incoming one is felt due to the difference in energy between the ground state and the transition state in rate-determining step.[3]

The *trans* effect is somewhat different in octahedral complexes due to the smaller *s* character of each bond and possibly due to steric effects caused by the relatively greater crowding found in octahedral *versus* square planar environments. In general, however, it appears that *trans* effects are related to the ability of the ligand to stabilize the transition state during rate-limiting dissociation.[3]

The isomerization property of **RDC11** is very interesting because it led us to propose new ways to obtain *tris*-heteroleptic compounds such as [Ru(PhPy)(Phen)(N^N)]-PF_6, in which one N atom of the incoming N^N ligand is always and exclusively *trans* to C.

Following the discovery of the new compound namely **RDC11-*cis***, we have embarked on a research aimed at satisfying our curiosity about the chemistry of **RDC11**, its stereoisomer and of the compounds derived from the latter.

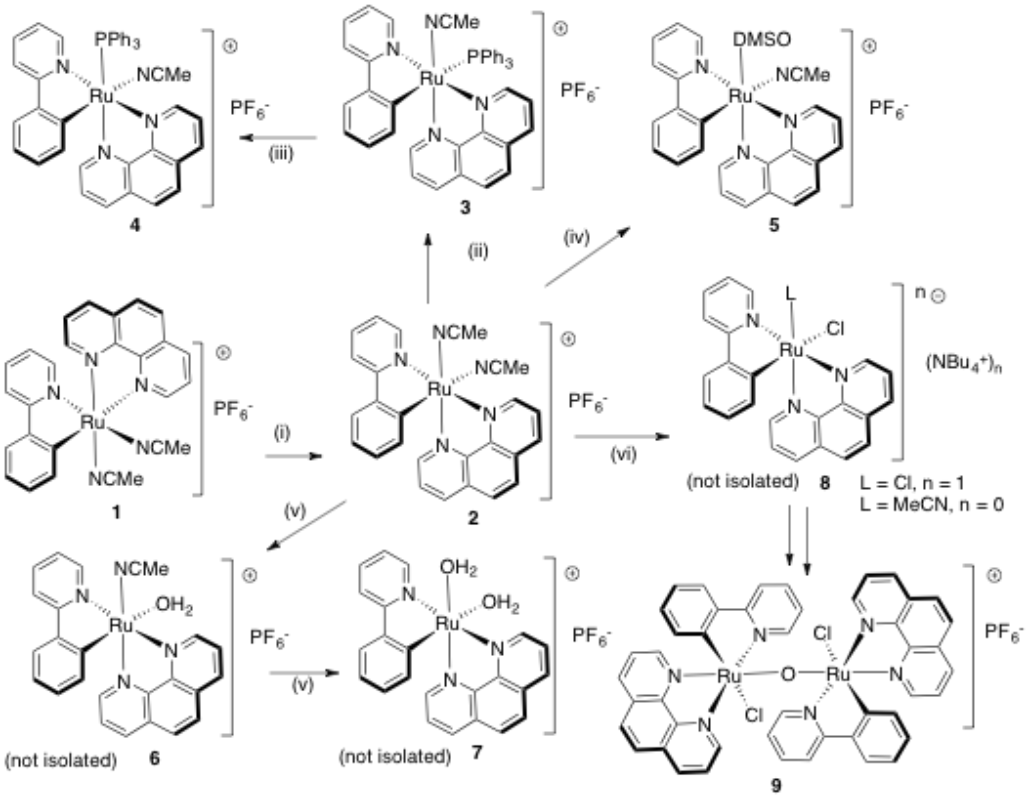
II.2.2 – Substituction reactions of MeCN in RDC11-*cis* by PPh_3, DMSO, H_2O, and Cl^-

We first checked that no reaction occurred between **1** and any of the nucleophiles used in this work when the reactions were performed in darkness or at room temperature. However, as the isomerization of **1** may already proceed at room temperature (although very slowly), tiny amounts of the isomerized **2** could be observed if the reaction was performed during more than 48 h. If the reaction was performed at higher temperature and during a long enough time, the isomerization of **1** to **2** was observed in good yields, as the isomerization reaction occurred almost quantitatively and the corresponding amounts of substitution products were produced. These results led us to conclude that the obtained products are, in most cases, the result of the substitution of the MeCN on the isomerized product **2**, and not of a direct substitution of one MeCN on **1**.

It is important to emphasize that the presence of coordinating solvents such as MeCN or acetone during the reaction is essential because, as it was previously shown,[1] the decomposition of the isomerized product **2**, is fast, e.g., in pure dichloromethane.

We thus treated **2** with excess PPh_3 in refluxing MeOH/MeCN and, after 1 h, we observed the quantitative formation of **3**, together with minute amounts of **4**. The **3:4** ratio decreased with time, and it was soon clear that **3** was the compound obtained under kinetic control, whereas **4** was the compound formed under thermodynamic control of the substitution reaction of one MeCN by PPh_3. Indeed, after 24 h at the reflux temperature of the solvent, **4** appeared to be the major compound of the reaction. The ^{1}H NMR spectrum of **3** and **4** displayed characteristic features that were assigned to their structures: for instance, for **3**, the signal of the proton *ortho* to the C atom covalently bound to Ru (5.81 ppm) showed a $^4J_{PH}$ coupling constant of 3.6 Hz, whereas no such $^4J_{PH}$ coupling was visible for the corresponding proton of **4** that resonated at 5.84 ppm. In addition, the ^{31}P NMR spectra revealed that the NMR signals of ^{31}P of PPh_3 were found at 24.46 and 52.64 ppm for **3** and **4**, respectively. These data are a strong indication that the PPh_3 was bound to Ru *trans* to the ruthenated phenyl group of PhPy in **3** and *trans* to one N of the Phen ligand (*i.e.*, *cis* to C) in **4**.

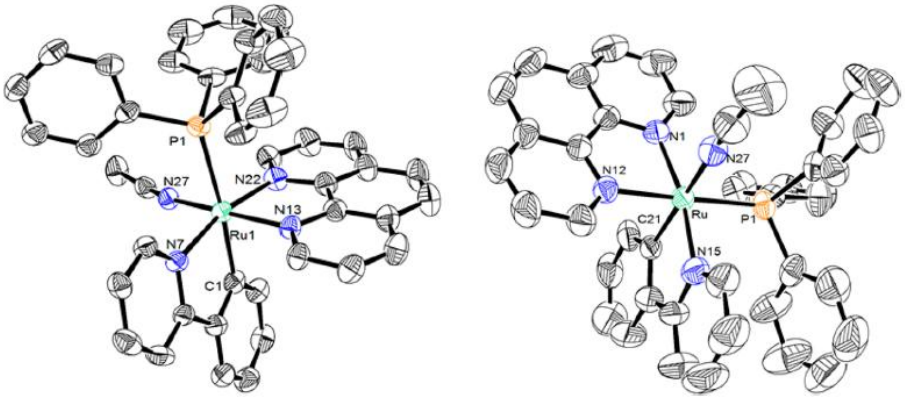
Figure 2. Reaction conditions [a] (Complexes **1–9**)



(i) *hv* in acetone/MeCN (9:1) (2 h), Δ in CH_2Cl_2/MeCN (9:1) (72 h) or in $CHCl_2CH_3$/MeCN (9:1) (48 h); (ii) PPh_3 (2 equiv) in acetone, RT, 2 h; (iii) MeOH/MeCN (6:1), reflux, 48 h; (iv) DMSO (2 equiv), CH_2Cl_2/MeCN (6:1), reflux, 12 h; (v) 3 min in MeCN/H_2O (1:9); 30 min in MeCN/H_2O (1:9); (vi) NBu_4Cl (5 equiv) in CH_2Cl_2/MeCN (6:1), 10 min.

We could grow crystals suitable for X-ray diffraction studies of both compounds. The perspective views of **3** and **4** are given in Figure 3. The structures that were obtained fully confirmed our assignments based on the NMR spectra. Moreover, the Ru−P bond distances were indeed characteristic of PPh_3 *trans* to a phenyl group (2.4309(13) Å) and *trans* to a pyridine ligand (2.325(12) Å) for **3** and **4**, respectively, this being consistent (see above) with the well-known larger *trans* influence of a phenyl *versus* that of a pyridine ligand.[4]

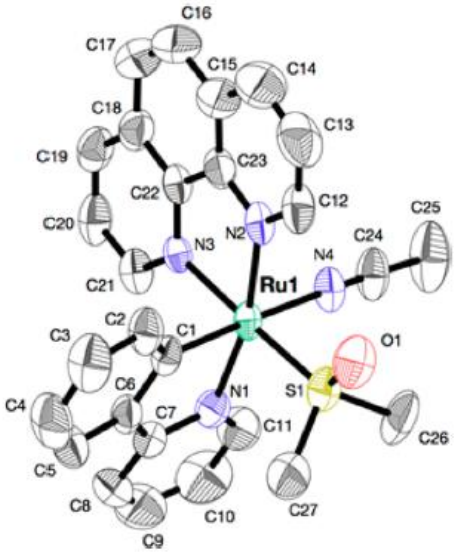
Figure 3. Perspective views of compounds **3** and **4**. Ellipsoids are drawn at a probability level of 50%. (H and PF_6^- omitted for the sake of clarity)



When we added 2 equiv. of DMSO in a CH_2Cl_2/MeCN solution of **2** under a strict absence of oxygen (*i.e.*, in a glovebox), the formation of a new compound **5** was observed, whose ^{1}H NMR spectrum revealed the presence of diastereotopic methyl groups of DMSO (3.05 and 2.13 ppm) coordinated on a chiral ruthenium center. In addition, the aromatic region of the spectrum showed resonances at chemical shifts significantly different from those of **2**, for example, one of the *ortho* to N protons of the phenanthroline ligand resonated at lower field (10.5 *vs* 9.5 ppm).

The crystal structure, which is depicted in Figure 4, indicated that the DMSO is indeed coordinated to Ru *via* its S atom and that this ligand is coordinated *trans* to one of the N atoms of the phenanthroline ligand. This result seemed to indicate that the substitution of the MeCN *trans* to the Phen ligand was observed prior to the substitution of the MeCN *trans* to C. However, we cannot rule out the fact that this compound could arise from a too fast isomerization of an intermediate compound that would have the DMSO ligand *trans* to C.

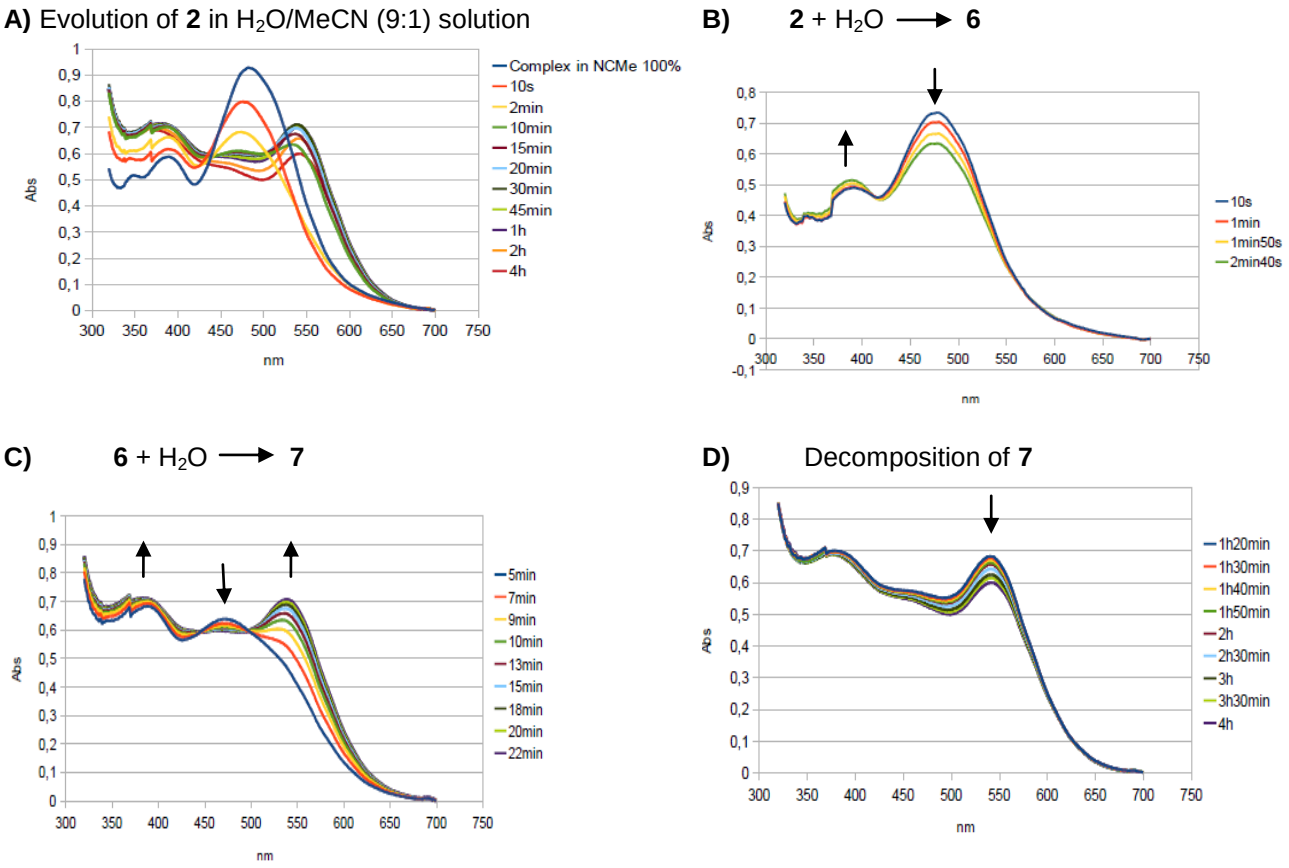
Figure 4. Perspective view of compound **5**. Ellipsoids are drawn at a probability level of 50%. (H and PF_6^- omitted for the sake of clarity)



We also observed a fast reaction in darkness between **2** and water when studying solutions of **2** in MeCN/H_2O *via* ultraviolet−visible light (UV-vis) spectroscopy, provided however that the amount of water was large enough, with respect to the amount of MeCN. Indeed, whereas no reaction seemed to occur in MeCN/H_2O (1:1) even after 4 h, a different behavior was observed when the amount of water was superior to that of acetonitrile. For instance, when we examined the UV-vis spectrum of **2** in a solution containing 9 equiv. of

water and 1 equiv. of MeCN, (MeCN/H_2O = 1:9), we could observe that three different reactions occurred successively, as shown on Figure 5.

Figure 5. Electronic spectra of **2** in H_2O/MeCN (9:1) solution (10^{-4} M): (A) evolution from 10 s to 4 h, (B) evolution from 10 s to 160 s (isosbestic point at 412 nm), (C) evolution from 5 min to 22 min (isosbestic points: 455 and 498 nm), and (D) evolution after 80 min

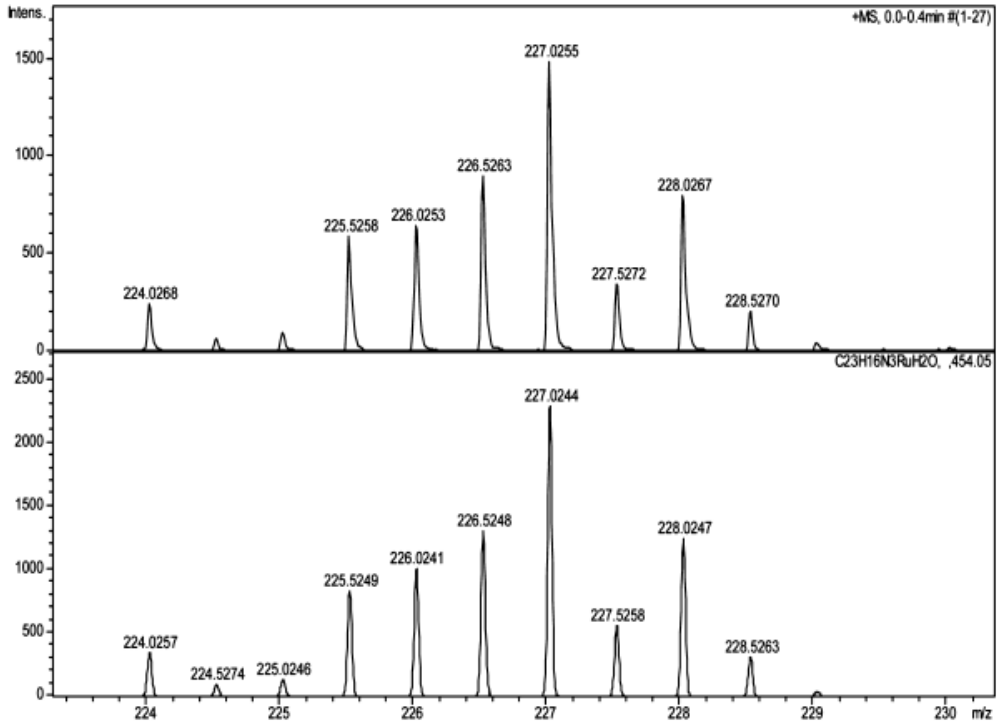


Whereas **1** had a strong absorption at 480 nm, which was shown to be a MLCT transition,[5] **2** had a maximum absorption at 484 nm. We saw a rapid evolution (within < 3 min) of the spectrum of **2** in this H_2O/MeCN solution with an isosbestic point, this pointing to the existence of only two species during this evolution. We suggest that one molecule of water substituted one MeCN ligand (probably the MeCN *trans* to the C atom, affording **6**) during this first process.

A second reaction took place within a time interval of 20 min during which a new absorption arose at 546 nm with the occurrence of two isosbestic points. This result suggests that the second MeCN ligand was substituted by water, leading to **7**. The third reaction occurred after 80 min and the obtained spectra pointed to decomposition of the products, no isosbestic points being visible during the evolution. We could check the coordination of water

at the Ru atom when dissolving **2** in a water/MeCN solution by running an electrospray-MS spectrum of **2** dissolved in these solvents. Indeed, we could detect (see Figure 6) a signal at *m/z* = 227.02 ($C_{23}H_{18}N_3ORu$) with an isotope pattern consistent with the simulation that corresponded to a dication of [**2** − 2MeCN + H_2O], *i.e.*, [Ru(III)(2-C_6H_4-2′-py-κC,N)(phen)(H_2O)]$^{2+}$. Another signal was observed at 453.04 with a correct isotope pattern (the precision was 10 ppm) that we assigned to the monocation of [**2** − 2MeCN + OH] in which again the Ru(II) has been oxidized. These results strengthen the hypothesis of the formation of aquo derivatives, however the Ru atom has probably been oxidized to Ru(III) prior or during the ionization of the compound. This behavior is fully in line with the lowering of the redox potential ($E^0_{Ru(II)/Ru(III)}$) that occurs when an oxygen containing ligand has substituted a nitrogen containing ligand at the ruthenium centre.[7]

Figure 6. ESI-MS spectrum of **2** in a H_2O/MeCN solution showing the peak for [Ru(2-C_6H_4-2′-Py-κC,N)(Phen)(H_2O)]$^{2+}$. The upper trace shows the experimental spectrum, and the lower trace shows a simulated one

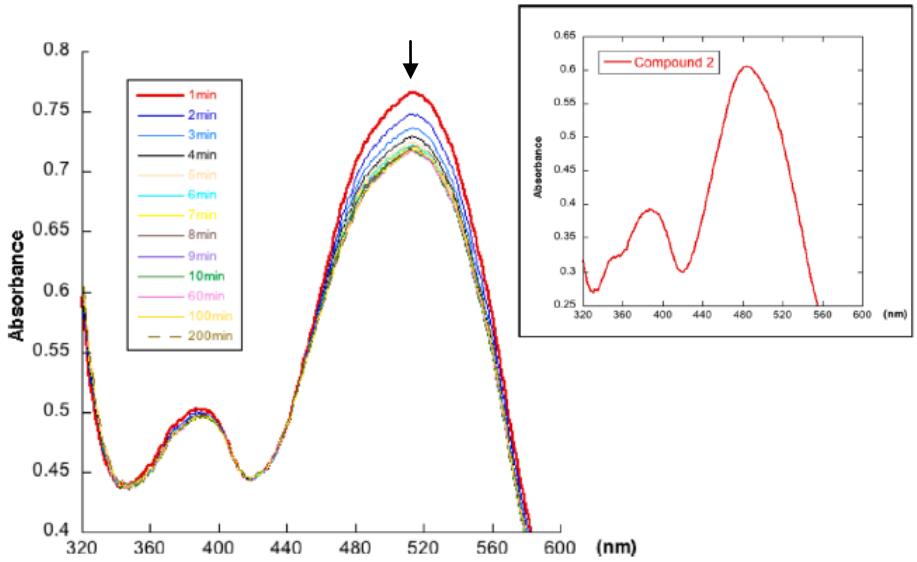


These results contradicts the conclusions made by Turro *et al.* in a recent paper whose objective was to study the substitution of the MeCN ligands of **1** under visible light.[6] However our results are also partly consistent with those of this paper. In fact these competitors believed that the isomerisation of the MeCN ligand could occur directly on **1** because they did not know that **1** is indeed isomerized under their reaction conditions. Thus, in this work,[6] the substitution process in water only took place after at least 90 min and the

first substitution reaction was over after 180 min. This can be rationalized by the fact that the first substitution only took place after the isomerization of **1** to **2** has occurred; in other words, the water ligand did not substitute a MeCN ligand as long as this ligand was *trans* to a pyridine or a phenanthroline ligand contrary to what was claimed by Turro *et al*. The authors also saw isosbestic points for their reaction: the two species that are thus present in their reaction medium were likely to be **1** and **6** (since **2** should not be present, because it led to **6** within < 3 min). The second substitution could then take place according to our present results.

Another reaction studied was the treatment of **2** with an excess of Cl^- (from Bu_4NCl) in CH_2Cl_2/MeCN (6:1) in a glovebox. We observed the immediate formation of a new purple species that we could only analyze by UV-vis and NMR spectroscopy, because our efforts to get crystals of the products of this reaction failed. The UV-vis spectrum showed a new absorption (as compared to **2**) at 514 nm (see Figure 7).

Figure 7. Behavior of **2** in a MeCN/CH_2Cl_2 solution in the presence of 5 equiv of Bu_4NCl. Inset shows the spectrum of **2** in the same solvent mixture (no change with time)



The ^1H NMR spectrum of this new species in CD_2Cl_2 displayed two signals, at 2.32 ppm and at 1.97 ppm, *i.e.*, typical of MeCN coordinated to Ru *trans* to N of PhPy and of noncoordinated MeCN, respectively. A few changes in the aromatic region relevant to a different coordination around the Ru center were also identified (see the Experimental part in the end of this chapter). We have unfortunately no data that could allow us to decide unambiguously whether the new compound (**8**) was a pure neutral compound with a Cl ligand *trans* to C of PhPy, or if it was a mixture of the latter and an anionic compound in which both

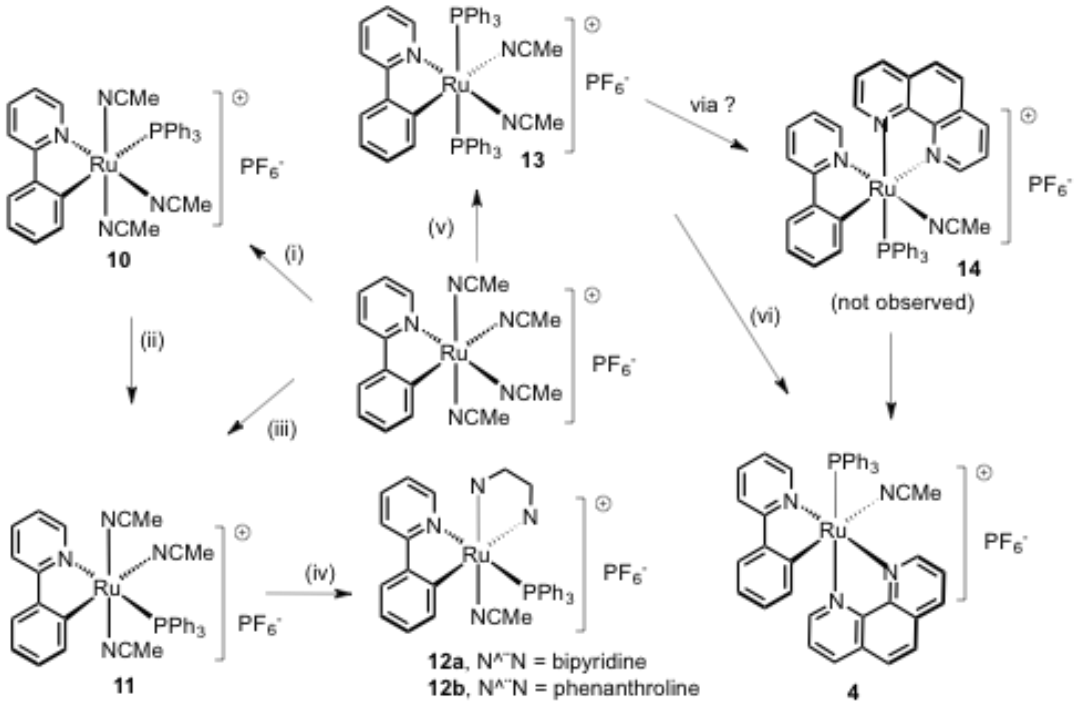
MeCN have been substituted by Cl^-. Contrary to what we observed for the reaction with water, these latter results are remarkably different from those previously reported by Turro *et al.* [6] for the substitution reaction of MeCN by chloride anion in the presence of visible light. The authors reported the formation of [Ru(PhPy)(Phen)(MeCN)Cl] based on the observation of two isosbestic points and the appearance of a strong absorption at ~ 560 nm that we did not observe. Since this reaction was very fast (it seemed to be completed within 120 s), it is not possible that the isomerization of **1** to **2** had taken place as it requires at least 1−2 h to be identifiable. Therefore, it is likely that, in this specific reaction, the isomerization of **1** to **2** was not a prerequisite for the substitution of MeCN by Cl^- to take place and that a reaction product, different from ours (derived from **1** with a Cl *trans* to a phenanthroline ligand), might have been obtained by Turro and co-workers.[6]

Solutions of **8** in CH_2Cl_2 decomposed in air, probably by following a process that we have encountered earlier. We have previously found [7] that irradiation of a methanol solution of **1** led to an intermediate that (i) we did not characterize and (ii) was reacted with Cl^-, forming, after oxidation in air, a dinuclear species (**9**) having a bridging oxo unit between two Ru atoms, each of them linked to a Cl^- ion, an orthometalated PhPy, and a phenanthroline ligand. Based on the results that we have now obtained in the present study, it is reasonable to assume that the uncharacterized intermediate is a bis-methanol adduct analogue to **7** as the treatment of **1** by light should have isomerized **1** and the substitution of the MeCN ligand by MeOH should have occurred according to what we have seen in the present work. Therefore, it is very likely that the decomposition of **8** in air might lead to a compound similar to **9**.

II.2.3 – Occurrence of an antisymbiotic effect *trans* to C on a phosphine derivative

Intrigued by the facile isomerization of **1** upon thermal or photochemical conditions,[8] we decided to investigate the behavior of the compound **10** (see Figure 8) that we described recently.[9] Following the same path previously described for compound **3** that isomerized to **4** after several hours at higher temperatures, the coordination of PPh_3 *trans* to C is likely to be kinetically unstable, because of an expected antisymbiotic effect that should exist in these compounds at the position *trans* to C. The concept of antisymbiosis, which was first proposed by Pearson,[10] predicted that *when a nucleophilic ligand such as a PPh_3 is coordinated to a metal of the second row* trans *to a soft atom such as a C from a phenyl unit, this ligand should be destabilized in such a way that it should isomerize to a position* trans *to a less nucleophilic atom.*

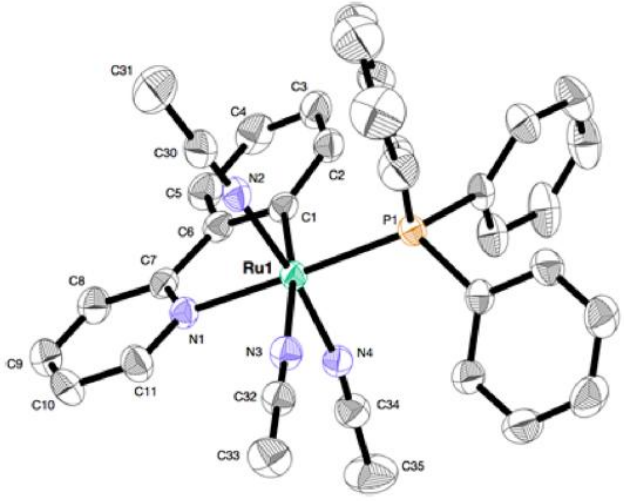
Figure 8. Reaction conditions [a] (Compounds **10**–**14**)



[a] (i) PPh_3 (1 equiv) in MeOH/MeCN (6:1), RT, 4 h; (ii) MeCN, reflux, 24 h; (iii) PPh_3 (1 equiv) in MeOH/MeCN (6:1), reflux, 24 h; (iv) 2,2′-bipyridine or phenanthroline in MeCN, reflux, 45 h; (v) PPh_3 (2 equiv) in MeCN reflux, 24 h; and (vi) phenanthroline (2 equiv), MeCN, reflux 4 h.

We thus treated **10** under thermal conditions, *i.e.*, under refluxing acetonitrile and found that, as expected, the compound totally isomerized after 24 h. The new structure of **11** was ascertained by a crystal structure determination, which unambiguously showed that PPh_3 was now located *trans* to N as the Ru−P distance (2.3102(7) Å) was significantly lower than in **10** (2.458(1) Å), as expected for a phosphine *trans* to a pyridine *versus* a phenyl ligand, respectively (see Figure 9).[4]

Figure 9. Perspective view of compound **11**. Ellipsoids are drawn at a probability level of 50%. (H and PF_6^- omitted for the sake of clarity)



On the other hand, the MeCN *trans* to C (Ru−N = 2.134(2) Å) is close to those of closely related compounds [9] in which this ligand was *trans* to the same C atom. This destabilization of a phosphine *trans* to a C−metal bond has been shown to also take place at the position *trans* to C in cyclopalladated complexes [11] and it recently led Vicente *et al.* to elaborate on the concept of transphobia for soft ligands coordinated *trans* to the C atoms of cyclopalladated compounds.[12]

Interestingly, a similar behavior has been observed in a cycloplatinated 2-dimethylbenzylamine compound in which the PPh_3 ligand was first coordinated *trans* to C of the cyclometalated ligand and then rapidly isomerized to the corresponding isomer in which the phosphine was *trans* to N.[13] The authors proposed that the tendency for isomerization of a ligand *trans* to C, which has a strong *trans* effect, is related to its own kinetic *trans* effect and its capacity of π back-bonding. Thus, a phosphine should be more destabilized *trans* to C, compared to MeCN, because of both its larger kinetic *trans* effect and its important π-acceptor capacities.

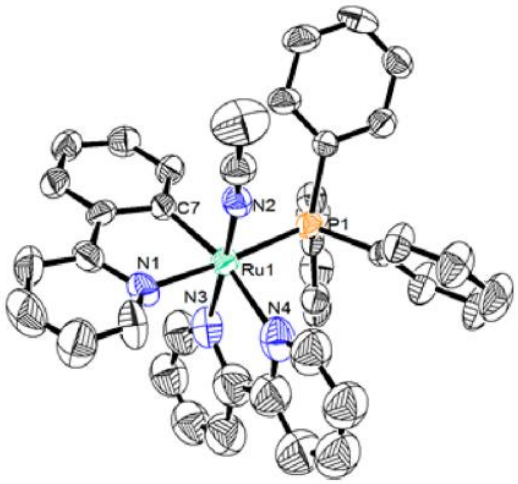
The new compound (**11**) with an acetonitrile ligand *trans* to C was, as expected, reactive toward 2,2′-bipyridine or phenanthroline, leading to **12a** (see Figure 10) and **12b** respectively. Note that this latter compound could not be obtained by treating **1** with PPh_3. Earlier, we also described the synthesis of compound **13**, by adding 2 equiv of PPh_3 on the starting compound $[Ru(PhPy)(MeCN)_4]PF_6$. In **13**, the MeCN *trans* to C was again susceptible to be substituted by a phenanthroline ligand to afford **14**. However, this did not occur, as we observed the formation of **4** only. Thus, as for the reaction of **2** with DMSO, the phen ligand was probably too destabilized at the position *trans* to C, because the reaction had to be performed at the refluxing temperature of MeCN to be effective. Again, we cannot rule out the formation of the transient **14**, which should quickly isomerize, placing the phen *trans* to N and not *trans* to C under thermodynamic control of the reaction.

Data regarding the bond distances and bond angles for selected complexes are given in Table 1.

Table 1. Bond distances and bond angles for selected crystal structures

complex **3**		complex **4**		complex **5**		complex **11**		complex **12a**	
Bond Distances (Å)									
C1−Ru	2.061(5)	C21−Ru	2.042(4)	N1−Ru	2.076(2)	N1−Ru	2.124(2)	C7−Ru	2.053(8)
N7−Ru	2.103(4)	N15−Ru	2.070(4)	N2−Ru	2.088(2)	C1−Ru	2.055(2)	N1−Ru	2.078(6)
N13−Ru	2.063(3)	N1−Ru	2.099(4)	N3−Ru	2.106(2)	N2−Ru	2.006(2)	N2−Ru	2.013(7)
N22−Ru	2.051(4)	N12−Ru	2.112(4)	N4−Ru	2.135(2)	N3−Ru	2.134(2)	N3−Ru	2.051(8)
N27−Ru	2.028(4)	N27−Ru	2.117(4)	C1−Ru	2.022(2)	N4−Ru	2.026(2)	N4−Ru	2.191(2)
P1−Ru	2.4309(13)	P1−Ru	2.325(12)	S1−Ru	2.2421(8)	P1−Ru	2.3102(7)	P1−Ru	2.308(2)
Bond Angles (deg)									
C1−Ru−N7	79.2(2)	N1−Ru−N12	78.5(1)	C1−Ru−N1	80.26(11)	C1−Ru−N1	79.55(7)	N3−Ru−C7	89.2(3)
N13−Ru−N22	80.4(1)	C21−Ru−N15	79.8(2)	C1−Ru−S1	88.54(8)	N4−Ru−N2	172.6(1)	N3−Ru−N4	81.6(4)
C1−Ru−P1	174.9(1)	N12−Ru−P1	178.7(1)	N3−Ru−S1	178.38(7)	N1−Ru−P1	177.5(1)	C7−Ru−P1	96.9(2)

Figure 10. Perspective view of compound **12a**. Ellipsoids are drawn at a probability level of 50%. (H and PF_6^- omitted for clarity)



II.2.4 – Conclusion

This study has allowed us to confirm the huge *trans* effect displayed by a σ-bonded C atom linked to a Ru(II) center. Indeed, it may not only displace a MeCN or a PR_3 ligand but also (although less readily) a chelating ligand. This behavior must be taken into account when studying the behavior of these compounds in biological media. The displacements of ligands that we have studied here led, in most cases, to compounds thermodynamically stable with a MeCN ligand *trans* to a C atom (see **2**, **4**, **5**, or **11**). It may well be anticipated that this position should now be easily occupied by any more or less strongly coordinating ligands that are found in biological media (see the reaction of **2** with water). Indeed, it has already been shown [6] that the *in situ*-formed **2** by irradiating **1** with visible light led to significantly improved cytotoxicity, with respect to **1**, and that the new photoproduct **2** showed much higher affinity for DNA than its precursor.

II.2.5 – Experimental part

II.2.5.1 – General Remarks

Experiments were carried out under an argon atmosphere, using a vacuum line. Diethyl ether and pentane were distilled over sodium/benzophenone, dichloromethane, and acetonitrile over calcium hydride and methanol and ethanol over magnesium under an argon atmosphere immediately before use. Chromatography columns were carried out on aluminum oxide (aluminum oxide 90, standardized, Merck). The other starting materials were purchased

from Sigma−Aldrich, Alfa Aesar, or Strem Chemicals and used as received, without further purification.

Ruthenium complexes listed hereafter were synthesized following already reported procedures: [Ru(2-C_6H_4-2′-py-κC,N)$(NCMe)_4$]PF_6,[14] [Ru(2-C_6H_4-2′-py-κC,N)(phen, *trans*-C)$(NCMe)_2$]PF_6 (**1**),[7] [Ru(2-C_6H_4-2′-py-κC,N)(phen, *cis*-C)$(NCMe)_2$]PF_6 (**2**),[1] [Ru(2-C_6H_4-2′-py-κC,N)$(PPh_3$, *trans*-C)$(NCMe)_3$]PF_6 (**10**),[9] and [*trans*-Ru(2-C_6H_4-2′-py-κC,N)-$(PPh_3)_2(NCMe)_2$]PF_6 (**13**).[9]

The NMR spectra were obtained at room temperature on Bruker or JEOL spectrometers. ^{1}H NMR spectra were recorded at 300.13 MHz (Model AC-300), 300.53 MHz (Model GX300), 400.13 MHz (Model AM-400), or 500.13 MHz (Bruker, Model Avance I 500). The chemical shifts are referenced to the residual solvent peak. $^{13}C\{^1H\}$ NMR spectra were recorded at 75.48 MHz (Model AC-300), 75.56 MHz (Model GX300), or 100.62 MHz (Model AC-400) and referenced to $SiMe_4$. ^{31}P NMR spectra were recorded at 161.98 MHz (Model AC-400) or at 121.5 MHz (Model GX300) and referenced to H_3PO_4. The NMR assignments were supported by COSY spectra for ^{1}H NMR. Chemical shifts (δ) and coupling constants (*J*) are expressed in units of ppm and Hz, respectively. (Multiplicity: s = singulet, d = doublet, t = triplet, q = quadruplet, h = heptuplet, m = multiplet)

Electronic absorption spectra were recorded using an UVIKON XL spectrophotometer from Bio-Tek Instruments with quartz cells of 1 cm wide.

Mass spectra (FAB^+) were obtained using a JEOL Model JMS-SX102A instrument with *m*-nitrobenzyl alcohol as a matrix. ESI-MS measurements were performed on a Bruker Daltonics microTOF spectrometer equipped with an orthogonal electrospray (ESI) interface.

Infrared spectra were recorded on a Nicolet FTIR Magna 750 instrument in KBr disks or on a Bruker-Alpha ATR apparatus.

II.2.5.2 – Synthesis protocols

[Ru(2-C_6H_4-2′-py-κC,N)(phen)(PPh_3, *trans*-C)-(NCMe)]PF_6 (3)

A solution of 100 mg (0.15 mmol) of 2 and 80 mg (0.3 mmol) of PPh_3 in 15 mL of acetone was stirred at room temperature for 2 h. The mixture was evaporated to dryness under vacuum, washed with Et_2O to remove excess of PPh_3 and the residue purified through Al_2O_3 using dichloromethane as eluent. Crystallization from dichloromethane/diethyl ether gave red crystals (53 mg, 40%) suitable for X-ray analysis, which were washed with diethyl ether and dried under vacuum.

^{1}H NMR (400.13 MHz, CD_3CN): 9.02 (d, 1H, ^{3}J = 5.3 Hz), 8.97 (d, 1H, ^{3}J = 5.3 Hz), 8.31 (d, 1H, ^{3}J = 8 Hz), 8.17 (d, 1H, ^{3}J = 8 Hz), 8.09 (d, 1H, ^{3}J = 8 Hz), 7.95 and 7.91 (AB pattern, 2H, ^{3}J = 8.8 Hz), 7.88−7.83 (m, 2H), 7.78 (d, 1H, ^{3}J = 8 Hz), 7.68−7.56 (m, 3H), 7.38 (m, 2H), 7.28 (m, 4H), 7.19 (m, 2H), 7.13 (m, 3H), 7.03 (td, 1H, ^{3}J = 6.8, ^{4}J = 1.8 Hz), 6.95 (m, 3H), 6.80 (td, 1H, ^{3}J = 7.6, ^{4}J = 1.1 Hz), 6.54 (td, 1H, ^{3}J = 7.3, ^{4}J = 1.4 Hz), 5.81 (ddd, 1H, ^{3}J = 7.3, ^{4}J = 1, $^{4}J_{PH}$ = 3.6 Hz), 2.18 (d, 3H, $^{5}J_{PH}$ = 3.5 Hz).
^{13}C{^{1}H} NMR (100.62 MHz, acetone-d_6): 186.64, 185.63, 168.55, 156.72, 156.61, 152.27, 151.15, 159.81, 147.52, 147.06, 145.34, 136.93, 133.69, 132.97, 132.82, 132.66, 132.32, 131.84 129.32, 128.42, 128.31, 127.47, 127.15, 125.61, 124.73, 121.97, 119.34, 3.54.
^{31}P{^{1}H} NMR (121.5 MHz, acetone-d_6): 24.46 (s, PPh_3), −144.21 (h, J_{P-F} = 705.97 Hz, PF_6).
IR (FTR) [cm^{-1}]: 2257 (m, *ν*CN), 833 cm−1 (s, *ν*PF_6^-).
M/S FAB$^+$: 698 [(M+H) − MeCN], 436 [(M+H) − (MeCN + PPh_3)], 282 [(M+H) − (MeCN + PPh_3 + phpy)]$^+$.
Anal. Calculated for $C_{43}H_{34}N_4F_6P_2Ru{\cdot}0.2CH_2Cl_2$: C, 57.60; H, 3.85; N, 6.22. *Found* C, 57.68; H, 3.50; N, 6.26.

Ru(2-C_6H_4-2′-py-κC,N)(phen)(PPh_3)(NCMe, *trans*-C)]PF_6 (4)

A solution of 200 mg (0.20 mmol) of [*trans*-Ru(Phpy)-(PPh_3)$_2$(NCMe)$_2$]PF_6 (**13**) and 72 mg (0.40 mmol) of 1,10-phenanthroline in 15 mL of acetonitrile was heated to reflux for 4 h. The mixture was evaporated to dryness under vacuum and the residue purified through Al_2O_3 using dichloromethane as eluent. Crystallization from dichloromethane/diethyl ether gave red crystals (120 mg, 68%) suitable for X-ray analysis, which were washed with diethyl ether and dried under vacuum.

^{1}H NMR (300.53 MHz, CD_3CN): 9.24 (dd, 1H, ^{3}J = 5.2 Hz, ^{4}J = 1.1 Hz), 8.67 (dd, 1H, ^{3}J = 5.2 Hz, ^{4}J = 1.1 Hz), 8.51 (dd, 1H, ^{3}J = 8.1 Hz, ^{4}J = 1.1 Hz), 8.38 (dd, 1H, ^{3}J = 8.1 Hz, ^{4}J = 1.2 Hz), 8.12 (d, 1H, ^{3}J = 8.9 Hz), 8.04 (d, 1H, ^{3}J = 8.9 Hz), 7.64−7.47 (m, 6H), 7.33−7.25 (m, 3H), 7.20−7.15 (m, 12H), 6.94 (td, 1H, ^{3}J = 5.7 Hz, ^{4}J = 1.7 Hz), 6.66 (td, 1H, ^{3}J = 7.4 Hz, ^{4}J = 1.1 Hz), 6.35 (td, 1H, ^{3}J = 7.4 Hz, ^{4}J = 1.1 Hz), 5.84 (d, 1H, ^{3}J = 7.4 Hz), 2.08 (s, 3H, NCMe).
^{31}P{^{1}H} NMR (121.5 MHz, CD_3CN): 52.64 (s, PPh_3), −144.02 (h, J_{P-F} = 706.87 Hz, PF_6).
IR KBr [cm^{-1}]: 2271 (m, *ν*NCMe), 840 (s, *ν*PF_6)
M/S [FAB$^+$, *m/z* (%)]: 739 (3) [M+H]$^+$, 698 (100) [M+H− NCMe]$^+$, 518 (3) [M+H − NCMe − phen]$^+$, 436 (65) [M+H − NCMe − PPh_3]$^+$, 256 (6) [M+H − NCMe − PPh_3 − phen]$^+$.
Anal. Calculated for $C_{43}H_{34}F_6N_4P_2Ru$: C, 58.88; H, 3.88; N, 6.34. *Found*: C, 59.09; H, 3.63; N, 6.33.

[Ru(2-C_6H_4-2′-py-κC,N)(phen)(DMSO)(NCMe, *trans*-C)]PF_6 (5)

A solution of **2** (100 mg, 0.15 mmol) and DMSO (0.022 mL, 0.30 mmol) in a mixture of dry CH_2Cl_2/acetonitrile 6:1 (15 mL) was refluxed for 12 h under vigorous stirring. After reduction of the volume of the orange/red solution, the solution was immediately filtered through alumina, using a 90:10 CH_2Cl_2/NCMe mixture as eluent. The dark red fraction was collected and evaporated to dryness under vacuum. Flash chromatography in CH_2Cl_2/MeCN (9:1) allowed the elimination of the remaining **2**. Crystallization from dichloromethane/pentane gave orange microcrystals (0.086 mg, 81%), which were found to be suitable for X-ray analysis.

^{1}H NMR (500.13 MHz, CD_2Cl_2): 10.51 (dd, *J* = 5.3, 1.4 Hz, 1H), 9.08 (dt, *J* = 5.5, 1.2 Hz, 1H), 8.50 (dd, *J* = 8.1, 1.4 Hz, 1H), 8.33 (dd, *J* = 8.1, 1.4 Hz, 1H), 8.06 (d, *J* = 8.8 Hz, 1H), 8.03−7.86 (m, 4H), 7.65 (dd, *J* = 7.8,

1.3 Hz, 1H), 7.60 (dd, *J* = 5.2, 1.4 Hz, 1H), 7.53 (ddd, *J* = 6.4, 5.3, 2.3 Hz, 2H), 6.81 (td, *J* = 7.5, 1.3 Hz, 1H), 6.59 (td, *J* = 7.4, 1.3 Hz, 1H), 6.29 (dd, *J* = 7.6, 1.3 Hz, 1H), 3.44 (q, Et_2O), 3.05 (s, 3H), 2.13 (s, 3H), 2.08 (s, 3H), 1.16 (t, Et_2O).
^{13}C NMR (126 MHz, CD_2Cl_2): 181.27, 167.06, 155.44, 152.39, 148.94, 147.92, 145.98, 145.27, 137.52, 136.08, 135.98, 135.02, 130.29, 129.73, 128.62, 127.75, 126.83, 125.22, 124.71, 124.18, 123.58, 121.76, 121.51, 119.55, 46.74, 44.02, 3.44.
HRMS (ESI, *m/z*): Calculated for $C_{27}H_{25}N_4ORuS$ (M): 555.0793. *Found:* 555.0751.
Anal. Calculated for $C_{27}H_{25}F_6N_4OPRuS \cdot 0.5Et_2O$: C, 47.28; H, 4.10; N, 7.61. *Found:* C, 46.91; H, 4.48; N, 7.35.

Ru(2-C_6H_4-2′-py-κC,N)(phen)(NCMe)Cl and/or [Ru(2-C_6H_4-2′-py-κC,N)(phen)Cl_2]Bu_4N (8)

A solution of **2** (10 mg, 0.015 mmol) and NBu_4Cl (77 mg, 0.075 mmol) in a mixture of dry CH_2Cl_2/CH_3CN 6:1 (15 mL) was stirred for ca. 10 min at room temperature, affording a dark purple solution. No purification of these compounds could be performed, because of their instability.

^{1}H NMR (400.13 MHz, CD_2Cl_2): 10.04 (d, *J* = 5.3 Hz, 1H), 9.63 (d, *J* = 5.0 Hz, 1H), 8.28−8.21 (m, 1H), 7.92 (m, 4H), 7.85−7.73 (m, 3H), 7.63 (d, *J* = 7.7 Hz, 1H), 7.33−7.18 (m, 2H), 6.63 (t, *J* = 7.4 Hz, 1H), 6.44 (t, *J* = 7.3 Hz, 1H), 6.19 (d, *J* = 7.8 Hz, 1H), 3.26−3.16 (m, NBu_4), 2.32 (s, 3H), 1.71−1.58 (m, NBu_4), 1.43 (h, *J* = 7.4 Hz, NBu_4), 1.97 (s, 3H), 1.02 (t, *J* = 7.3 Hz, NBu_4).

[Ru(2-C_6H_4-2′-py-κC,N)$(NCMe)_3$(PPh_3, *cis*-C)]PF_6 (11)

A solution of [Ru(2-C_6H_4-2′-py-κC,N)$(NCMe)_4$]PF_6 (692 mg, 1.23 mmol) and PPh_3 (323 mg, 1.23 mmol) in MeOH/CH_3CN (6:1) (50 mL) was refluxed for 24 h under vigorous stirring. After reduction of the volume of the orange/red solution, the solution was filtered through alumina using a 90:10 CH_2Cl_2/MeCN mixture as eluent. The yellow orange fraction was collected and evaporated to dryness under vacuum. Flash chromatography in CH_2Cl_2/MeCN (9:1) allowed the elimination of the remaining [Ru(2-C_6H_4-2′-py-κC,N)-$(NCMe)_4$]PF_6. Crystallization from dichloromethane/Et_2O/pentane gave orange microcrystals (450 mg, 47%), which were found to be suitable for X-ray analysis.

^{1}H NMR (400.13 MHz, CD_3CN): 9.03 (m, 1H), 8.07 (dd, 1H, *J* = 8.2, 1.2 Hz), 7.93 (ddd, 1H, *J* = 8.2, 7.4, 1.6 Hz), 7.66 (dd, 1H, *J* = 7.8, 1.4 Hz), 7.67−7.60 (m, 6H), 7.51 (d, 1H, *J* = 7.6 Hz), 7.48−7.37 (m, 9H), 7.35−7.30 (m, 1H), 6.89 (t, 1H, *J* = 7.6 Hz), 6.68 (td, 1H, *J* = 7.4, 1.4 Hz), 1.96 (s, 3H), 1.62 (s, 6H).
^{31}P NMR (161.98 MHz, CD_3CN): 57.01 (s, PPh_3), −144.7 (h, J_{P-F} = 685 Hz, PF_6).
Anal. Calculated for $C_{35}H_{32}F_6N_4P_2Ru$: C, 53.51; H, 4.11; N, 7.13. *Found:* C, 53.29; H, 4.38; N, 7.12.

[Ru(2-C_6H_4-2′-py-κC,N)(bipy)(PPh_3, *cis*-C)(NCMe)]PF_6 (12a) and [Ru(2-C_6H_4-2′-py-κC,N)(phen)(PPh_3, *cis*-C)(NCMe)] PF_6 (12b)

A solution of 200 mg (0.25 mmol) of **11** and 80 mg (0.50 mmol) of 2,2’-bipyridine (for **12a**) or 91 mg (0.50 mmol) of 1,10-phenanthroline (for **12b**) in 15 mL of acetonitrile was heated to reflux for 45 h. The mixture was evaporated to dryness under vacuum and the

residue purified through alumina using dichloromethane as eluent. Crystallization from dichloromethane/diethyl ether gave red microcrystals, which were washed with diethyl ether and dried under vacuum. Recrystallization from dichloromethane−acetonitrile/diethyl ether gave crystal of **12a** suitable for X-ray analysis.

12a NMR Data. Yield: 39% (84 mg).
^{1}H NMR (300.53 MHz, CD_3CN): 8.86 (d, 1H, ^{3}J = 5.2 Hz), 8.46 (d, 1H, ^{3}J = 8.2 Hz), 8.40 (d, 1H, ^{3}J = 8.9 Hz), 8.10 (d, 1H, ^{3}J = 8.2 Hz), 8.04 (td, 1H, ^{3}J = 7.2 Hz, ^{4}J = 1.4 Hz), 7.97 (td, 1H, ^{3}J = 7.2 Hz, ^{4}J = 1.4 Hz), 7.92 (dd, 1H, ^{3}J = 8.0, Hz, ^{4}J = 1.4 Hz), 7.82−7.73 (m, 2H), 7.56 (d, 1H, ^{3}J = 5.8), 7.43 (td, 1H, ^{3}J = 7.2 Hz, ^{4}J = 1.1 Hz), 7.32−7.19 (m, 15H), 7.02 (td, 1H, ^{3}J = 7.2 Hz, ^{4}J = 1.1 Hz), 6.97 (m, 4H), 2.37 (s, 3H).
^{31}P{^{1}H} NMR (121.5 MHz, CD_3CN): 55.04 (s, PPh_3), −144.02 (h, J_{P-F} = 706.87 Hz, PF_6).
IR KBr [cm^{-1}]: 843 (s, PF_6), 2268 (m, NCMe).
M/S [FAB^{+}, *m/z* (%)]: 715 (10) $[M+H]^{+}$, 674 (59) $[M+H-NCMe]^{+}$, 518 (100) $[M+H-NCMe-bpy]^{+}$, 412 (38) $[M+H-NCMe-PPh_3]^{+}$, 256 (13) $[M+H-NCMe-PPh_3-bpy]^{+}$.
Anal. Calculated for $C_{41}H_{34}F_6N_4P_2Ru\cdot0.25CH_2Cl_2$: C, 56.24; H, 3.95; N, 6.36. *Found:* C, 56.13; H, 3.77; N 6.51.

12b NMR Data. Yield: 62% (137 mg).
^{1}H NMR (300.53 MHz, CD_3CN): 9.03 (dd, 1H, ^{3}J = 5.22 Hz, ^{4}J = 1.1 Hz), 8.47 (dd, 1H, ^{3}J = 8.2 Hz, ^{4}J = 1.4 Hz), 8.18 (dd, 1H, ^{3}J = 8.0 Hz, ^{4}J = 1.4 Hz), 8.05 (d, 1H, ^{3}J = 8.8 Hz), 7.97 (d, 1H, ^{3}J = 8.8 Hz), 7.95 (m, 2H), 7.86 (dd, 1H, ^{3}J = 7.7 Hz, ^{4}J = 1.4 Hz), 7.72−7.66 (m, 2H), 7.58 (td, 1H, ^{3}J = 7.4 Hz, ^{4}J = 1.7 Hz), 7.27−7.21 (m, 3H), 7.12−7.75 (m, 15H), 6.92 (td, 1H, ^{3}J = 7.4 Hz, ^{4}J = 1.4 Hz), 6.66 (t, 1H, ^{3}J = 6.6 Hz), 2.21 (s, 3H).
^{31}P{^{1}H} NMR (121.5 MHz, CD_3CN): 55.30 (s, PPh_3), −143.63 (h, J_{P-F} = 706.87 Hz, PF_6).
IR KBr [cm^{-1}]: 842 (s, PF_6), 2263 (m, NCMe).
M/S [FAB^{+}, *m/z* (%)]: 739 (21) $[M+H]^{+\cdot}$ 698 (100) $[M+H-NCMe]^{+}$, 518 (4) $[M+H-NCMe-phen]^{+}$, 436 (71) $[M+H-NCMe-PPh_3]^{+}$, 256 (6) $[M+H-NCMe-PPh_3-phen]^{+}$.
Anal. Calculated for $C_{43}H_{34}F_6N_4P_2Ru\cdot0.4CH_2Cl_2$: C, 56.80; H, 3.82; N, 6.10. *Found:* C, 56.33; H, 3.72; N, 6.69.

II.2.5.3 – Crystal structure determinations

Compounds 5 and 11

Acquisition and processing parameters are displayed in Table 2. Reflections were collected with a Nonius Kappa CCD system and with an APEX diffractometer equipped with an Oxford Cryosystem liquid N_2 device, using Mo Kα radiation (λ = 0.71073 Å). The crystal detector distance was 38 mm. The cell parameters were determined (APEX2 software [15] from reflections taken from three sets of 12 frames, each at 10 s exposures. The structures were solved by direct methods using the program SHELXS-97.[16] The refinement and all further calculations were carried out using SHELXL-97.[17] The crystal structures acquired with the Nonius Kappa CCD were solved using SIR-97[18] and refined with SHELXL-97. The refinement and all further calculations were carried out using SHELXL-97. The H atoms were included in calculated positions and treated as riding atoms using SHELXL default parameters. The non-H atoms were refined anisotropically, using weighted full-matrix

leastsquares on F^2. A semiempirical absorption correction was applied using SADABS in APEX2.

Table 2. Details for the X-ray crystal structure determinations

	3	4	5	11	12a
chemical formula	$C_{45}H_{39}F_6N_4O_{0.5}P_2Ru$	$C_{45}H_{39}F_6N_4O_{0.5}P_2Ru$	$C_{31}H_{55}F_6N_4O_2RuSP$	$C_{36}H_{34}Cl_2N_4PRuF_6P$	$C_{41}H_{34}F_6N_4P_2Ru$
formula mass	920.81	920.81	773.73	870.58	959.73
crystal system	monoclinic	triclinic	triclinic	monoclinic	orthorhombic
a (Å)	14.807(2)	10.5548(7)	9.5091(4)	8.9301(3)	17.9446(15)
b (Å)	14.232(2)	13.0455(8)	12.5594(5)	18.1083(6)	18.7673(15)
c (Å)	21.532(3)	15.4633(10)	14.4211(4)	25.6637(8)	24.048(2)
α (°)	90	85.8168(13)	97.045(2)	90	90
β (°)	106.085(4)	86.5947(13)	104.120(2)	97.5720(10)	90
γ (°)	90	72.1131(13)	96.500(2)	90	90
unit cell volume (Å^3)	4360.1(11)	2019.3(2)	1639.21(11)	4113.9(2)	8098.6(12)
temperature (K)	150(2)	298(2)	173(2)	173	298(2)
space group	$P\,2_1/c$	$P\bar{1}$	$P\bar{1}$	$P\,2_1/c$	$Pbca$
No. of formula units per unit cell, Z	4	2	2	4	8
No. of reflections measured	49 323	20 305	14 376	39 712	65 183
No. of independent reflections	9553 [R(int) = 0.0695]	9223 [R(int) = 0.0354]	7468 [R(int) = 0.059]	13 142 [R(int) = 0.0354]	7456 [R(int) = 0.165]
final R_1 values ($I > 2\sigma(I)$)	0.0663	0.0487	0.0582	0.0403	0.0758
final $wR(F^2)$ values ($I > 2\sigma(I)$)	0.1177	0.1278	0.1510	0.0943	0.1874
final R_1 values (all data)	0.1093	0.0568	0.0687	0.0490	0.1462
final $wR(F^2)$ values (all data)	0.1360	0.1333	0.1649	0.0912	0.2101

Compounds 3, 4, and 12a

X-ray intensities were measured on a Bruker Smart Apex diffractometer (**4, 12a**) and a Bruker D8 Venture κ-geometry diffractometer (**3**) with a sealed tube and a microfocus X-ray source (λ = 0.71073 Å), respectively. The intensities were integrated using SAINT.[19] Absorption correction and scaling was performed with SADABS.[20] The structures were solved with Direct Methods using the program SHELXS-97.[21] Least-squares refinement was performed with SHELXL-2014.[22] against the F^2 values of all reflections. Non-hydrogen atoms were refined with anisotropic displacement parameters. All hydrogen atoms were located in difference Fourier maps. H atoms were positioned geometrically and refined using a riding model with isotropic displacement parameters tied to the parent C atoms. The three compounds display some type of disorder, affecting the cations, anions, and/or solvent included, which were modeled with SIMU and DELU restraints on anisotropic displacement parameters and SADI and SAME geometrical restraints. In addition, for compounds **3** and **12a**, the interstitial solvent is highly disordered and could not be modeled successfully; its contribution was removed from the refinement, using the SQUEEZE routine in the PLATON program.[22]

We are grateful to Lydia Brelot and Corinne Bailly (Université de Strasbourg) for determining the crystal structures of compounds **5** and **11** and Dr. C. Perret (Université de Strasbourg) for the analysis of **2** in a H_2O/MeCN solution by electrospray.

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Chapter II.3:

*Affitin-vectorized ruthenium complexes for cancer therapy – Contributions to determination of their mechanism of action**

II.3.1 – Targeted cancer therapy

Since the past century a scientific and technological revolution in cancer therapy has taken place. Despite major advances in this field, cancer still takes away many lives. [1]

The targeted therapy represents a new generation of cancer drugs designed to interfere with a specific molecular target that presents a critical role in tumour growth or progression. [2] According to NCI Dictionary of Cancer Terms, targeted therapy is "*a type of treatment that uses drugs or other substances to identify and attack specific types of cancer cells with less harm to normal cells.*" [3] Thus, it allows killing tumor cells with minimal side effects and it is in accordance with the idea of "magic bullet" conceived by Paul Ehrlich, that envisioned selective delivery of active agents to the affected cells. [1,4]

The identification of appropriate targets is based on a detailed understanding of the molecular changes underlying cancer, [2] and the tumor targeting efficiency depends on several factors such as high concentration of targeted receptor, enzyme, protein or other molecules in tumor and tumor-related tissues; specificity and avidity of ligand binding to the target; transposal of physiological barriers; and also extracellular and intracellular degradation. [4]

* The results presented in this chapter are fruit of cooperation between the *Laboratoire de Chimie et Systhèmique Organo-Métalliques* (Université de Strasbourg) and *Affilogic*, a privately-owned biotechnology company specialized in the class of affinity proteins namely *Affitin*. We are also grateful to Dr. Myriam Seemann (Université de Strasbourg) and their laboratory staff for their collaboration in the synthesis of the **RDC-Affitin** conjugates and to Dr. Hélène Nierengarten and Dr. Cécile Perret (Université de Strasbourg) for all mass-spectrometry analysis and interpretation.

II.3.1.1 – Considerations about possible anticancer targets[†]

Cellular metabolism ensures cell viability and growth through the production of the energy source (ATP) that is necessary to generate simple molecules, such as sugars and aminoacids, and complex macromolecules such as DNA, proteins, phospholipids… For aerobic organisms, the energy metabolism is largely dependent on the presence of oxygen, favoring the activity of the oxidative metabolism in state of the less efficient oxygen-independent glycolytic metabolism. However, in the recent years multiple studies have highlighted that transformed cells inside the tumors have a different metabolism compared to healthy cells. Indeed, tumors are often poorly irrigated by blood vessels and have therefore a reduced cancer cell oxygenation. To survive, cancer cells shift from the oxidative metabolism toward the glycolytic metabolism, a shift also known as the Warburg effect. [5] Several molecular mechanisms ensure this shift, namely the HIF-1/VEGF, the mTOR and the UPR pathways that respond to reduction in oxygen, glucose, and more generally nutrients. [6,7,8,9] In this respect, the UPR, HIF-1/VEGF and the mTOR pathways have been considered as targets for anticancer drugs, and small molecules (Rapamycin for example) or humanized monoclonal antibodies (Avastin for example) have been developed to target some of their actors.

Recently, some enzymes of the cellular metabolism have also been identified as pertinent anticancer targets. [10] For example, dhfr, which is involved in the folate metabolism, is inhibited by methotrexate used in the treatment of various cancers. Therefore, along the UPR, HIF-1 and mTOR pathways, the redox enzymes represent interesting anticancer targets of the energy metabolism that could contribute to diversify the therapeutic options.

Although these new targeted therapies have already contributed to progresses in cancer treatment, clinical data indicate several limitations, such as their ability to target only a subset of cancers with particular molecular signatures, the development of resistance mechanisms (Ex: rapamycin treatment activate AKT, through a feedback loop which eventually enhances tumor growth), the existence of side effects or the high production cost for monoclonal antibody-based therapies. To overcome these limitations, clinicians use combinatory treatments in which the new molecules are combined with more traditional cytotoxic therapies. In addition, the intra-tumor heterogeneity, the vast diversity of the molecular characteristics of tumors and their constant adaptation to drug treatment, challenge

[†] This section is based on research project for my PhD thesis, elaborated by Dr. Michel Pfeffer (Strasbourg, 26th of March 2013).

the scientists to develop new drugs that will target novel molecules, widening the possibilities for combinatory treatments.

Vectorization of substances that already present good anticancer activity is a good alternative to minimize their toxic effects by targeting selectively cancer affected tissues. We adopted this proposal by using an affinity protein namely Affitin to vectorize ruthenium complexes aiming to direct them to cancer cells.

II.3.2 – Affitin: Origin and properties[‡]

During the last twenty years, a class of proteins called "scaffold proteins" has emerged as an alternative to classic antibodies for deriving artificial affinity reagents. In contrast to antibodies, non-immunoglobulin scaffolds are small single-domain proteins that require no post-translational modification and often lack disulfide bonds. As a useful reagent for biotechnological applications, a scaffold protein needs to be as stable as possible to ensure longer lifetimes, since insufficient stability is a limiting factor in the application of natural proteins for therapeutic or biotechnological uses. [11,12]

Affitins (commercially called Nanofitins®) are a class of artificial proteins that binds selected polypeptides with high specificity and affinity. They are derived from DNA-binding protein 7d (Sac7d) family and homologous oligonucleotide/oligosaccharide-binding-fold (OB-fold) proteins and, as it was discovered in extreme natural conditions (pH 2 and 85°C) in Yellowstone National Park geysers, affitin scaffold extends the scope of the properties of its derived binders with high stability to wide ranges of pH, temperature and chemicals, keeping its high specificity and affinity. [13,14]

Indeed, affitins are chemically and thermally stable (from pH 0 up to 12, toward detergents and chaotropic agents, and up to 90 °C). [15] Specificity is mediated by variations of specific amino acid residues located at conserved positions on the binding face, which result in a potential binding area of around 1200 $Å^2$. [16] With their small size and low structural complexity, affitins occupy an intermediate position between peptides and proteins. [17]

Extremophilic proteins are found in various Archaea such as *Sulfolobus*, *Acidianus*, and *Metallospharea* genera, [17] and particularly Sac7d protein is a very important genome protector from thermal denaturation since it binds to DNA (without any particular sequence preference) and increases its melting temperature by approximately 40 °C. [18]

[‡] This section was based both in references and in informations available in the website <http://www.affilogic.com >

Indeed, Sac7d is a highly stable archaeal protein (66 amino acids, 7 kDa) that has a well characterized OB-fold comprising a five-stranded β-barrel capped by an α-helix with no disulfide bridge. Proteins containing this structural motif specifically recognize a wide range of substrates such as oligosaccharides, oligonucleotides, proteins, and metal ions. [19,16]

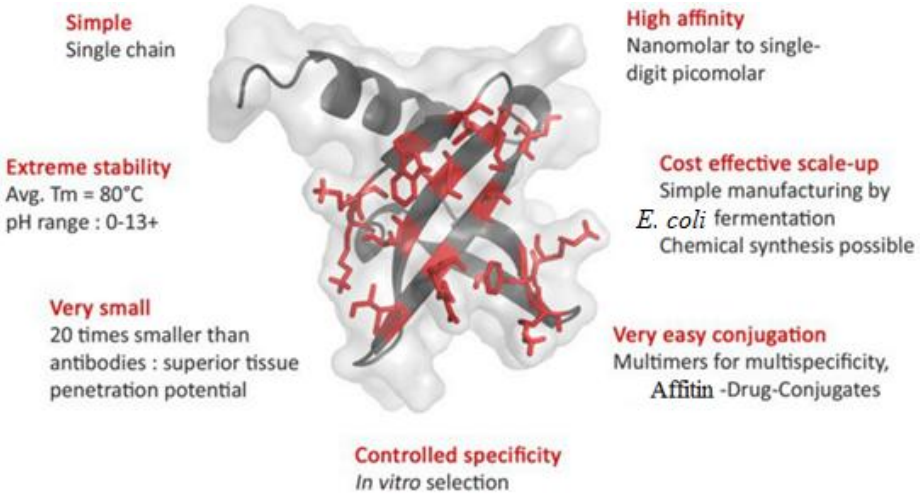
Affitin-based drugs aim at overcoming antibodies limitations and outperforming current targeted therapies. Indeed, affitins are potent antibody-mimetics, showing high affinity and specificity for biomolecules and also demonstrate many small molecule-like attributes such as an extreme robustness, around 20 times smaller size than a monoclonal antibody, and better tissue penetration. [20] Thus, intrinsic properties of this class of affinity proteins can be applied by other routes than injectable, such as topical administration (skin and cornea), up to oral route. Moreover, they can be designed for acting either as targeted inhibitors or as vectors for specific transport. [21]

Furthermore, affitins can be obtained chemically, however they are specially overproduced in *Escherichia coli* with yields from several dozen to several hundreds of milligrams per liter of culture, which result in low cost of production. [15]

Interestingly, they can also be easily conjugated to other moieties (small molecules, biologics, nanoparticles) by genetic fusion [14] or, as we present in this work, by click chemistry.

Thus, we conjugated ruthenium complexes to an affitin (**RDC-Affitin**) by click chemistry and the results will be presented further below.

Figure 1. Summary of mainly advantages of Affitin



Adapted from <http://www.affilogic.com/advantages>. Acess. Feb 2016.

II.3.3 – Background for vectorization of ruthenacycles for anticancer application[§]

Our laboratory's recent projects in the area of cancer research are aimed at modifying our organometallic molecules in such a way that they can be bound to a protein, a peptide or a peptoid that will be selectively recognized by a specific function of tumor cells with the hope that the modified carriers will modify the viability of the the tumor cell. Indeed, development of compounds with selective affinity for cellular targets related to energy metabolism, such as redox enzymes, can result in increased efficiency against cancer cells *in vitro* and *in vivo*.

The characteristic of the redox enzymes involved in the cellular metabolism is that they shift back and forth between an oxidized and a reduced state and that their activity strongly depends on the electron exchanges between them, the substrates and the cofactors that provide and/or regenerate their active state. Using this information, our hypothesis is that we can design compounds with specific electron exchange properties (which is related to their redox properties) that would interfere with these electronic exchanges in the metabolic pathway and that would therefore show anticancer activity.

To do that, we have chosen to develop compounds that contain specific ruthenium metal cores and whose specificity is that the metal is linked to one ligand via a C-M covalent bond that is intramolecularly stabilized by a coordination N-M bond (this structure is called a metallocyclic unit). This specific bonding mode via both a covalent C-M and a coordination N-M bond to a ligand, altogether provide particularly interesting redox properties to the molecule by shrinking the HOMO-LUMO gap of the organometallic molecule, as compared to related molecules that do not display the same metallocyclic unit, especially those that are lacking the covalent C-M bond.

Previous results of the laboratories involved in their development have shown several important anticancer properties of these compounds:

1) *In vitro*, these compounds alter the activity of redox enzymes such as the glucose oxidase and the horse-radish peroxidase. [22,23,24,25] This alteration of redox enzyme activity requires the proximity of *c.a.* 20 Å between the redox center of the enzyme and the ruthenium compound.

2) In cells, these compounds regulate the activity of pathways involved in the cellular metabolism such as the UPR/CHOP, the HIF-1 and the mTOR pathways, three critical pathways for cancer cell survival. [26]

[§] This section is mostly based on research project for my PhD thesis, elaborated by Dr. Michel Pfeffer (Strasbourg, 26th of March 2013).

3) Although these compounds are organometallic compounds like cisplatin or oxaliplatin, they do not require p53 activity or interaction with DNA for their cytotoxicity and they are efficient towards cancer cells that are resistant to platinum-derived compounds, including patient primary tumors (colon and ovarian). [26,27]

4) *In vivo*, these compounds reduce tumor size (≈45%) with a reduced toxicity toward healthy tissues when compared to gold standards such as cisplatin.

5) Functional studies and a small-scale preliminary affinity chromatography purification using a matrix coupled with **RDC** followed by a mass spectrometry analysis have led to the identification of 3 pertinent targets for **RDC** in cancer cells: i) EEF1AI, a translation elongation factor that controls proper enzyme folding and whose deregulation leads to the accumulation of unfolded proteins and activation of the UPR/CHOP pathway, [28] ii) IDH, the isocitrate dehydrogenase that leads to the production of alpha ketoglutarate and HIF-1 regulation and this is mutated in several cancers, [29] and iii) PHD2, a HIF-1 hydroxylase that controls HIF-1 degradation by the proteasome pathway. [6] Interaction between **RDC** and these enzymes might explain the activation of the UPR/CHOP pathway and the inhibition of the HIF-1 and mTOR pathways we have observed in cancer cells treated with **RDC**.

It has been shown that a chimerical molecule between a ruthenium compound and an antagonist peptoids of the VEGF receptor **2** is a much stronger VEGF inhibitor than the peptoids alone. [30] The principle is that the peptoids binds to the VEGF receptor and that the ruthenium compounds uses its redox properties to irreversibly inactivate the VEGF receptor. The advantage of this strategy is that it allows the directing of the metal core activity toward a cellular target that has been specifically chosen. However, the limitation of the metal compound used in this published study for *in vivo* anticancer activity is the necessity of activating the ruthenium core (N-Metal-N) by light. This limits the use of this type of compounds for several cancers that cannot be reached by natural light or optic fibers. In contrast, the redox potential of our compounds (with a metal core of C-Metal-N bound type) would not require light or irradiation to be able to inactivate the proteins. This particular redox potential of our compounds is documented [31] as well as their repercussion on protein activity. [32] Thus, the absence of light irradiation requirement will allow the production of compounds that would have less limitation for *in vivo* activity.

II.3.4 – Development of innovative RDC-Affitin conjugates

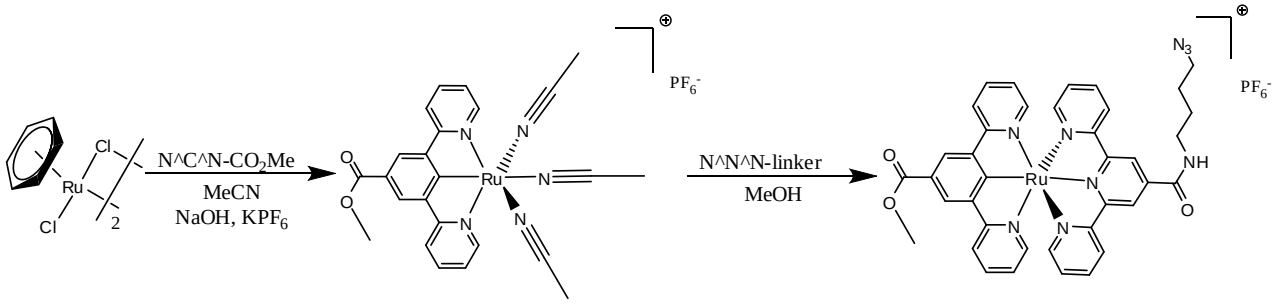
In this work, we developed innovative **RDC-Affitin** conjugates, *i.e.* an affitin bound to ruthenium complexes. They may combine the electron transfer properties of the organometallic compounds to the high stability and selectivity properties of the affitin.

This work was thought to give us two types of results: the first expected result is the production of new anticancer compounds which are more specific and more active for the selected target cell, reducing toxic effects against healthy cells. The second type of result is a more fundamental level: in fact, through this work, we wanted to improve our understanding about the mechanism of action of organometallic ruthenium complexes as anticancer agents.

II.3.4.1 – Synthesis of ruthenacycle-linker

In a first step, we worked with a ruthenium complex whose structure was based on the second generation of **RDC** (see **Chapter II.1**) formed by a monoanionic pincer N^C^N ligand and by a neutral N^N^N ligand derived from terpyridine, functionalized by a spacer arm containing a terminal azide (-N_3) function. **[Ru(N^C^N-CO_2Me)(N^N^N-linker)]PF_6** was obtained according to reactions showed in Figure 2.

Figure 2. Synthesis of **[Ru(N^C^N-CO_2Me)(N^N^N-linker)]PF_6**

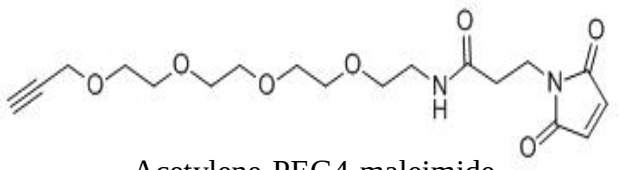


In order to improve water solubility, we exchanged the PF_6^- counter ion for Cl^- using an Amberlite column, following the procedure described by Diaz *et al.* [33] In contrast to the precursor, **[Ru(N^C^N-CO_2Me)(N^N^N-linker)]Cl,** namely **NCN-Ru-N_3** for simplify, was well soluble in water. It was a very important step, since we have planned to carry out the synthesis of **RDC-Affitin** under mild conditions for protein (*i.e.* in aqueous medium, at room temperature and with a pH close to that found in physiological conditions).

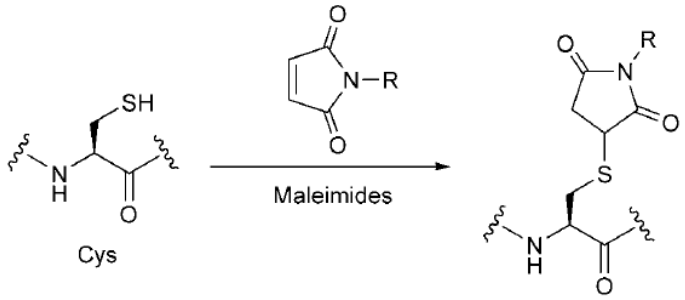
II.3.4.2 – Synthesis of the first RDC-Affitin: NCN-Ru-Affitin

The affitin supplied by our partner *Affilogic* was a model affitin that contain a terminal alkyne obtained by reaction between the related affitin and acetylene-PEG4-maleimide. The binding reaction of this compound to the protein occured on a cysteine added to the terminal sequence of the affitin by thiol-maleimide Michael addition reaction (Figure 3). [34,35]

Figure 3. Chemical structure of acetylene-PEG4-maleimide (A) and general reaction of cysteine with maleimides (B) [34]

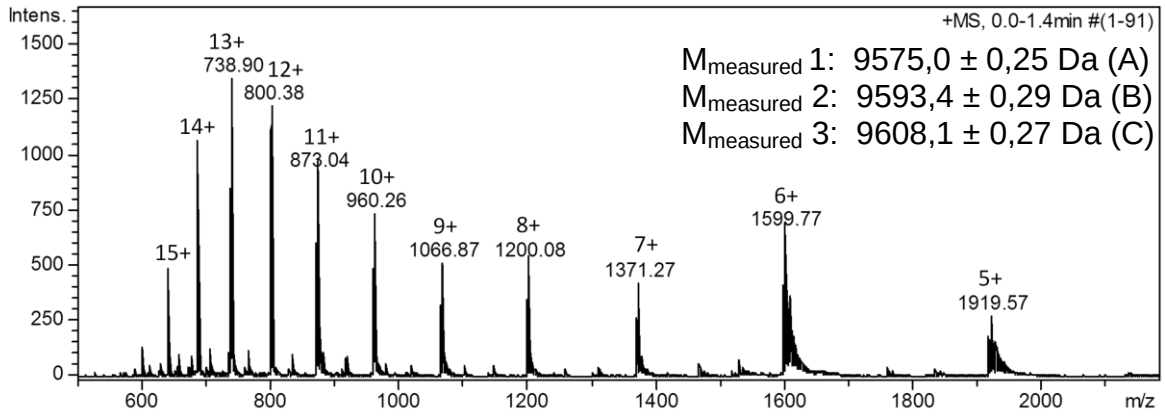


Acetylene-PEG4-maleimide



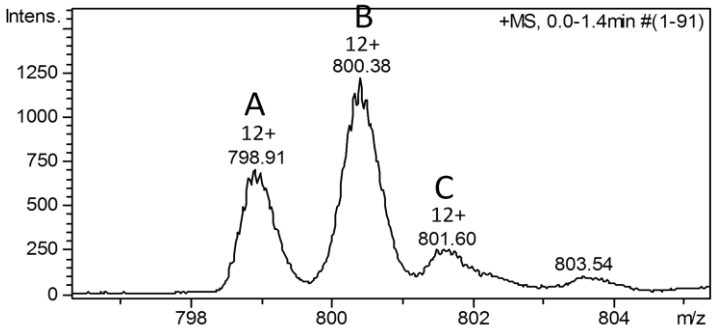
We present in Figure 4 the ESI-MS spectrum of Affitin after reaction with acetylene-PEG4-maleimide.

Figure 4. ESI-MS spectrum of Affitin after reaction with acetylene-PEG4-maleimide



Buffer: 20 mM $AcONH_4$, dilution in CH_3CN/H_2O (50:50) +1% HCOOH, final protein concentration: ~10 µM.

To be continued...



Relative abundances:

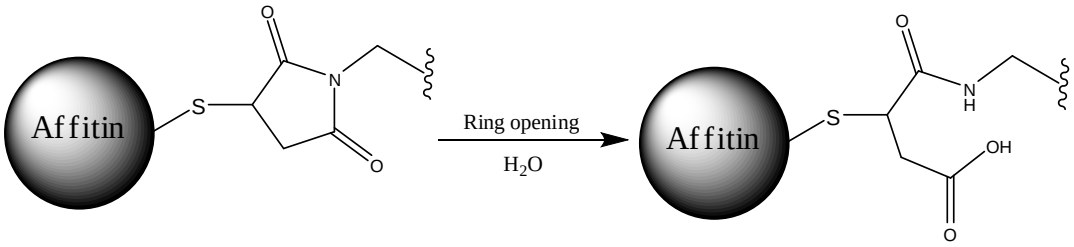
B (9592 Da): 100%

A (9574 Da): 61%

C (9607 Da): 14%

Despite the presence of expected product (measured mass of 9574.0 Daltons), we can also observe "adducts" M+18 and M+33. For the adduct M+33, we suppose that it is related to oxidation of N-terminal methionines, since they are largely exposed to solvent and thus very susceptible to be oxidized. For the adduct M+18, in turn, a possible and very probable explanation is that it is related to ring opening and insertion of a water molecule (Figure 5).

Figure 5. Possible explication for adduct M+18

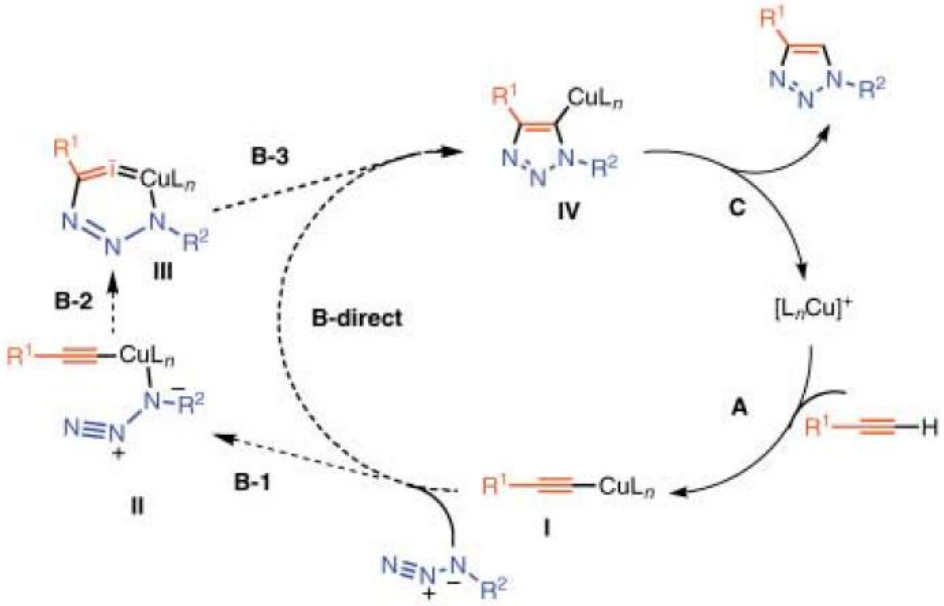


Bioconjugation presents significant challenges for any ligation methodology, since biomolecules are commonly more fragile and also because we work with very low concentrations. [36] We choose to carry out the reaction of our ruthenium complex with affitin by copper-catalyzed azide-alkyne cycloaddition (**CuAAC**) click reaction following the "*Protocol for CuAAC Coupling of 'Cargo-N_3' to 'Biomolecule-CCH'"* described by Hong *et al.* [36b]

According to Kolb, Finn, and Sharpless, [37] a click reaction *"must be modular, wide in scope, give very high yields, generate only inoffensive byproducts that can be removed by nonchromatographic methods, and be stereospecific (but not necessarily enantioselective). The required process characteristics include simple reaction conditions (ideally, the process should be insensitive to oxygen and water), readily available starting materials and reagents, the use of no solvent or a solvent that is benign (such as water) or easily removed, and simple product isolation.*"

The most commonly studied reaction of CuAAC reaction family is the Cu(I) catalysed Huisgen 1,3-dipolar cycloaddition of azide and terminal alkyne functionalities, which is the most widely recognized example of click chemistry. [38,39] It is a relatively recent reaction (discovered in 2002) that has been rapidly adopted for countless applications, due mainly to the selective reactivity of azides and alkynes with each other. [36] The mechanistic proposal for the catalytic cycle is shown in Figure 6.

Figure 6. Proposed catalytic cycle for the Cu(I)-catalyzed ligation [39]



Description: The catalytic cycle begins unexceptionally with formation of the copper(I) acetylide **I.** Extensive density functional theory calculations offer compelling evidence which strongly disfavors – by about 12-15 kcal – the concerted [2+3] cycloaddition (B-direct) and points to a stepwise, annealing sequence (B-1 → B-2 → B-3, hence the term "ligation"), which proceeds via the intriguing six-membered copper-containing intermediate **III**. The process exhibits broad scope and provides 1,4-disubstituted 1,2,3-triazole products in excellent yields and near perfect regioselectivity. [39]

For bioconjugation of proteins by CuAAC, the concentration of Cu(I) in the reaction media, and also some *in situ* reducing agents such as sodium ascorbate and their byproducts that can produce detrimental effects to protein are some critical points that have to be regarded. [36] In this sense, Hong *et al.* summaryze the "*key elements for the use of the optimized bioconjugation procedure*":

a) Sodium ascorbate is the preferred reducing agent for most applications, due to its convenience and effectiveness at generating the catalytically active Cu(I) oxidation state.

b) Copper concentrations should generally be between 50 and 100 µM. The lower limit is necessary to achieve a sufficient concentration of the proper catalytic complex which incorporates more than one metal center, and more than 100 µM Cu is usually not necessary

to achieve high rates. Some adjustments must be made when there are competitive copper binders in solution or on the substrates such as the presence of a hexahistidine sequence fused to the protein chain (that corresponds to our case with the affitin supplied by Affilogic).

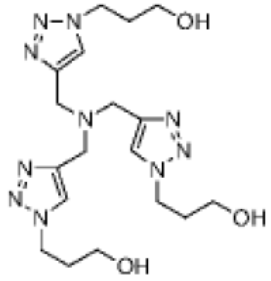
c) Some accelerating ligands, such as THPTA ([tris(3-hydroxypropyltriazolylmethyl)amine], Figure 7) have to be added to the reaction mixture with the purpose to intercept and quickly reduce reactive oxygen species generated by the ascorbate-driven reduction of dissolved O_2 without compromising the CuAAC reaction rate very much. And at least five equivalents of THPTA relative to Cu should be employed.

d) Aminoguanidine is a useful additive to intercept byproducts of ascorbate oxidation that can covalently modify or crosslink proteins.

e) Compatible buffers include phosphate, carbonate, or HEPES in the pH 6.5–8.0 range. Tris buffer should be avoided as the tris(hydroxymethyl)aminomethane molecule is a competitive and inhibitory ligand for Cu; sodium chloride (as in phosphate-buffered saline) up to 0.5 M can be used.

f) Ascorbate should not be added to copper-containing solutions in the absence of the accelerating ligand. As a matter of routine, we first mixed $CuSO_4$ with the ligand, add this mixture to a solution of the azide and alkyne substrates, and then initiated the CuAAC reaction by the addition of sodium ascorbate to the desired concentration.

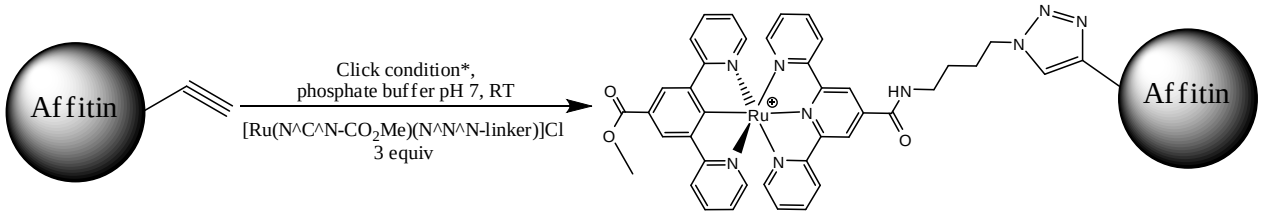
Figure 7. Chemical structure of the accelerating ligand THPTA



Therefore, we carried out the reaction of our ruthenium complex with affitin by click reaction as presented in Figure 8.

Despite the care to add all the reagents and additives in the sequence proposed by the protocol autors, [36b] we observed no significant formation of the coupling product in the time proposed although we have observed a high precipitation of our affitin during the reaction. We increased the reaction time to obtain more coupling product in order to obtain a high quality mass spectrum.

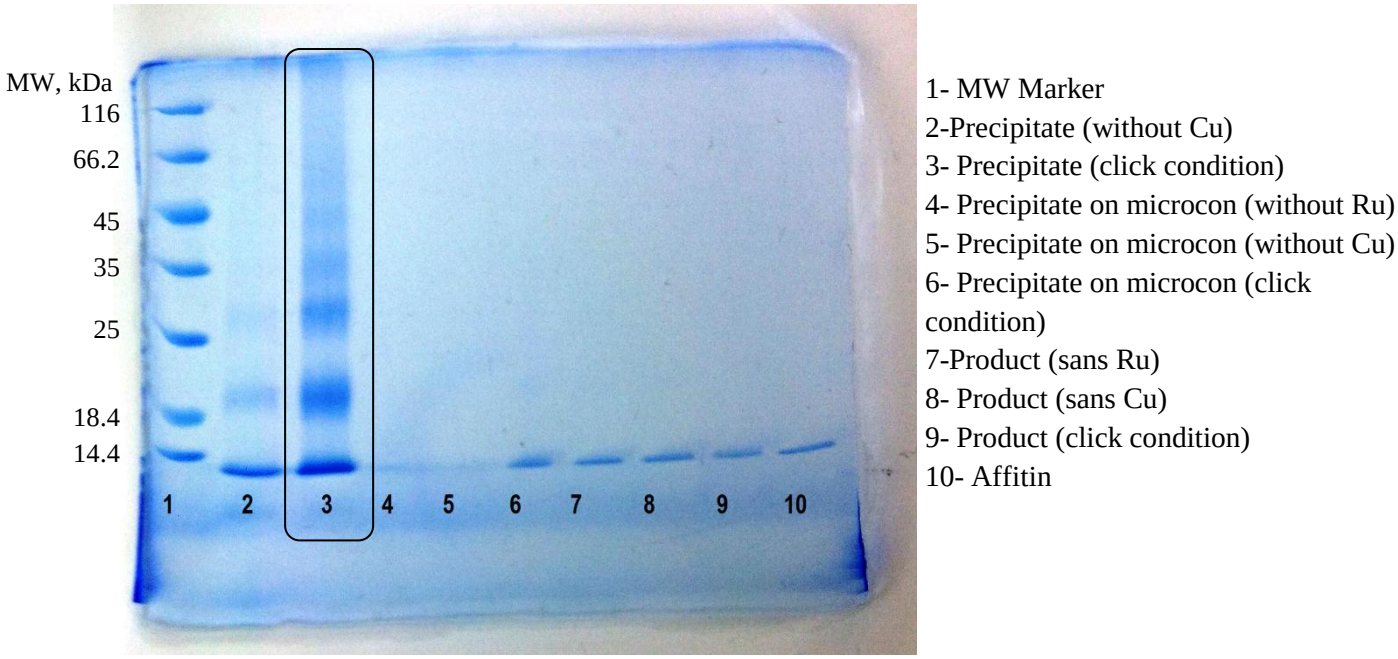
Figure 8. Synthesis of **NCN-Ru-Affitin**



* Click condition: $CuSO_4$, NaAsc, THPTA, Aminoguanidine

In the SDS-gel we could not differenciate the affitin to the coupling product to confirm its formation, because their molar mass are very close to each other. However, we noticed an interesting behavior of this precipitate: we suspected the occurrence of the polymerization of the protein, since we saw, besides the mass expected for the affitin, some compounds with molar mass bands quite related to dimers, trimers and tetramers of affitin (see Figure 9, number 3).

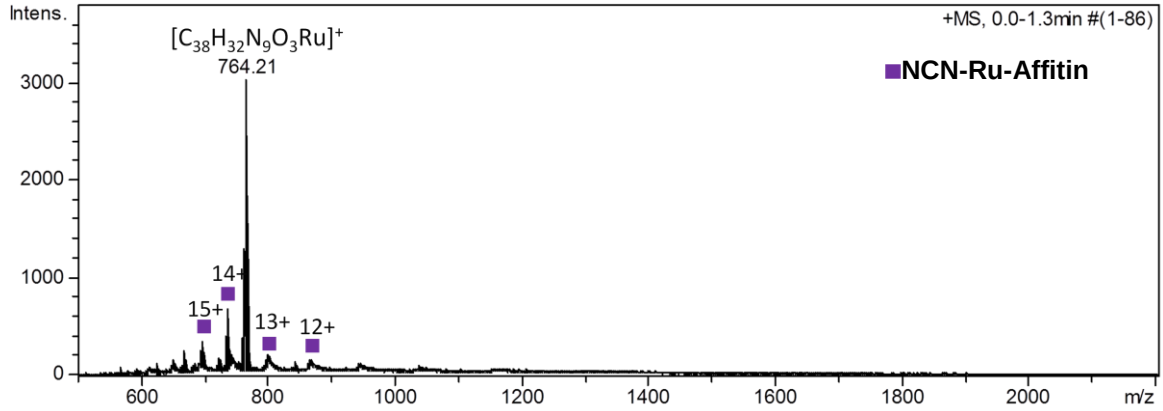
Figure 9. SDS-PAGE of affitin and their coupling products in different conditions



We obtained the ESI-MS spectrum of the precipitate in denaturing conditions (Figure 10) and we observed the coupling product **NCN-Ru-Affitin** and the free Ru complex. As we could not solubilize the protein precipitate to obtain the mass spectrum in native conditions, we could not unambiguously establish if it is related to polymers or simply to aggregates of proteins. However, we were convinced that we observed a polymerization of our affitin. In order to rationalise this phenomenon, we suspect the existence of a redox process with amino

acid residues that would generate radical species (especially after the formation of the coupling product, as we will demonstrate below), which was based upon the ability of our ruthenium complexes to promote elctron transfer reactions with oxido-reductase enzymes (see above).

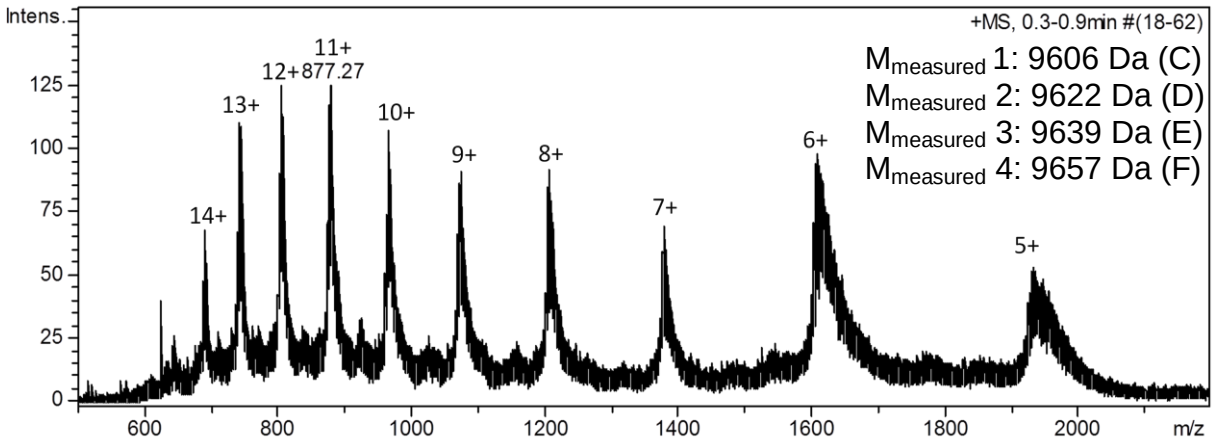
Figure 10. ESI-MS spectrum of the precipitate of the synthesis of **NCN-Ru-Affitin** (RT, 20h)



ESI spectrum under denaturing conditions. Solubilization of the precipitate in formic acid, then diluted 200 times in water / acetonitrile (50:50) + 1% formic acid.

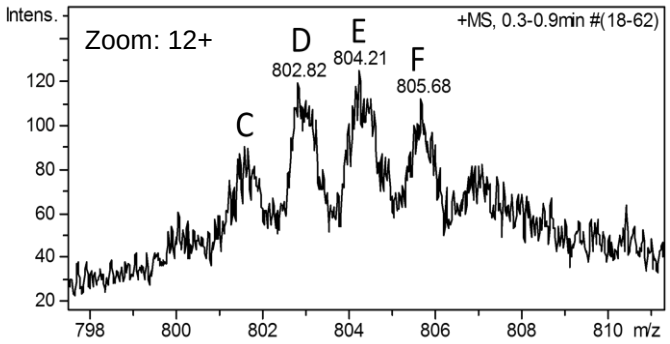
To confirm the importance of the formation of the coupling product to the precipitation, we did two control reactions: a) with all reagents and additives, except **NCN-Ru-N_3** (Figure 11); b) with all reagents and additives, except Cu (Figure 12).

Figure 11. ESI-MS spectrum of Affitin after contact with all click reagents and additives, except **NCN-Ru-N_3** (RT, 20h)



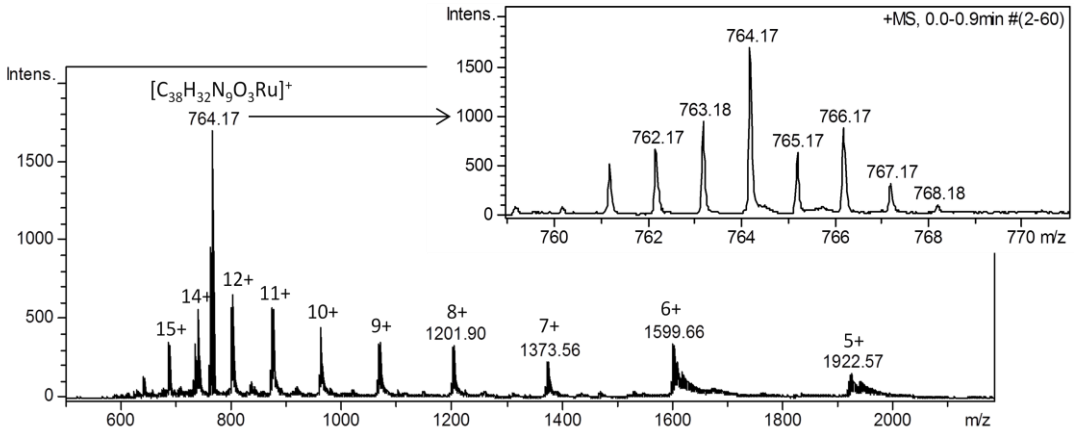
Buffer: 20 mM $AcONH_4$, dilution in CH_3CN/H_2O (50:50) +1% HCOOH, final protein concentration: ~10 µM.

To be continued...

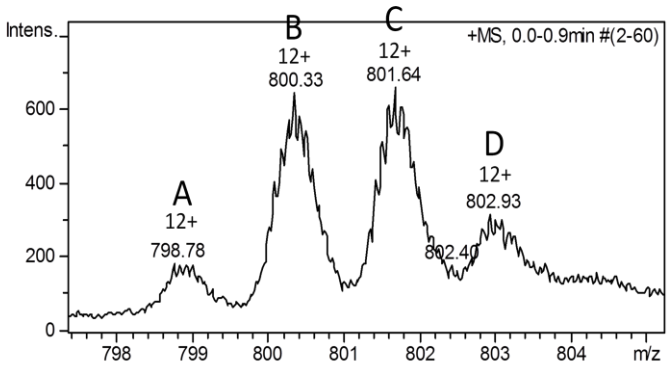


For the reaction with all click reagents and additives except **NCN-Ru-N_3**, we observe products with higher molecular mass than that obtained for free affitin (measured mass of 9574 Da). It is probably due to oxidation of some amino acids present in the protein chain or also due to adducts with sodium.

Figure 12. ESI-MS spectrum of Affitin after contact with all reagents and additives, except **Cu** (RT, 20h)



Buffer: 20 mM $AcONH_4$, dilution in CH_3CN/H_2O (50:50) +1% HCOOH, final protein concentration: ~10 µM.



<u>Relative abundances</u> :

C (9605,9 ± 0,15 Da): **100%**
B (9590,9 ± 0,11 Da): **77%**
D (9623,6 ± 0,31 Da): **43%**
A (9571,2 ± 0,17 Da): **26%**

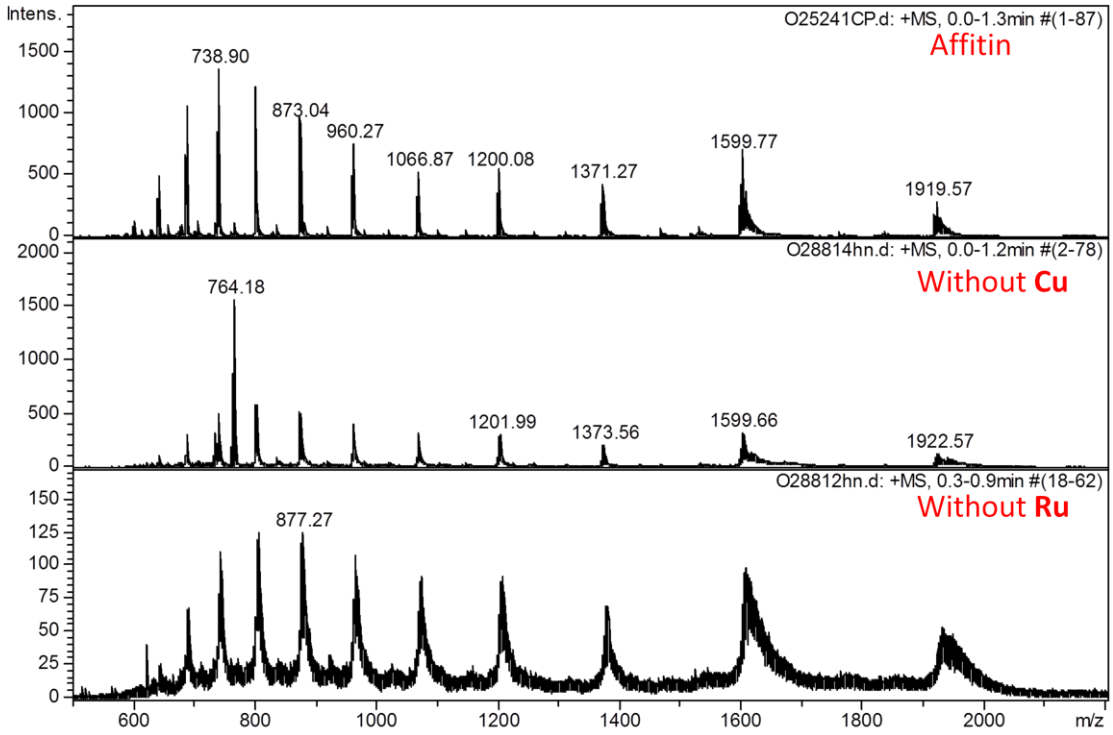
For the reaction with all reagents and additives except the copper catalyst, we observe free affitin and free Ru complex, since obviously no click reaction can occurs without Cu. We

also noticed precipitation of our protein, however in very lower quantity when compared with reaction under click condition.

Therefore, we can conclude from these experiments that the precipitation of our affitin is mainly due to the existence of a yet unknown process (probably redox) that involves the protein and the **NCN-Ru-N_3** complex, since even without click reaction conditions we could also notice protein precipitation in the presence of that complex. It is important to comment that it was especially after the formation of the coupling product that we observed a more significant precipitation of our affitin.

In the Figure 13 we can see a comparison between the effects of the presence of Cu or Ru on the affitin spectrum.

Figure 13. ESI-MS: Comparison between the control reactions

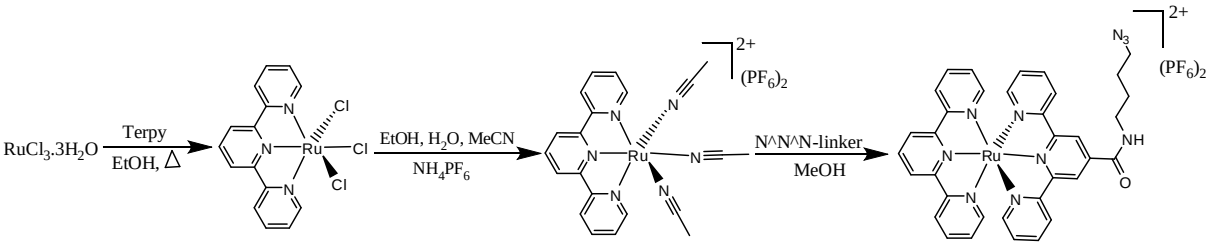


II.3.4.3 – Overcoming challenges: Synthesis of NNN-Ru-Affitin

With the aim to overcome the occurence of protein precipitation observed in presence of **NCN-Ru-N_3** and to obtain a high quality spectrum of a Ru-Affitin conjugate, we decided to synthesize the **NNN-Ru-N_3-Affitin**. Since ruthenium complexes that present only N-Ru bounds show much higher redox potential, they should not promote an electron transfer to our affitin and, thus, we should not observe precipitate in our reactional media.

Therefore, after many attempts by different methods we finally obtained the new complex **[Ru(N^N^N)(N^N^N-linker)]PF_6** (Figure 14).

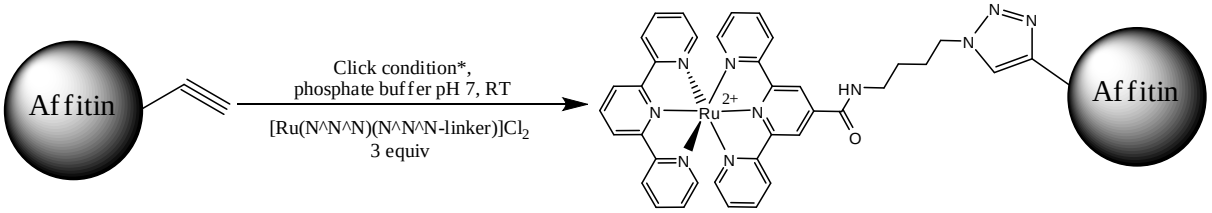
Figure 14. Synthesis of **[Ru(N^N^N)(N^N^N-linker)]PF_6**



We exchanged the PF_6^- counter ion for Cl^- using an Amberlite column, following the same procedure adopted for our organometallic ruthenium compound, [33], and we obtained the **[Ru(N^N^N)(N^N^N-linker)]Cl_2** (namely **NNN-Ru-N_3** for simplify) as a water soluble complex.

Thus, we carried out the reaction of our new ruthenium complex with affitin by click reaction as presented in Figure 15. [36b]

Figure 15. Synthesis of **NNN-Ru-Affitin**

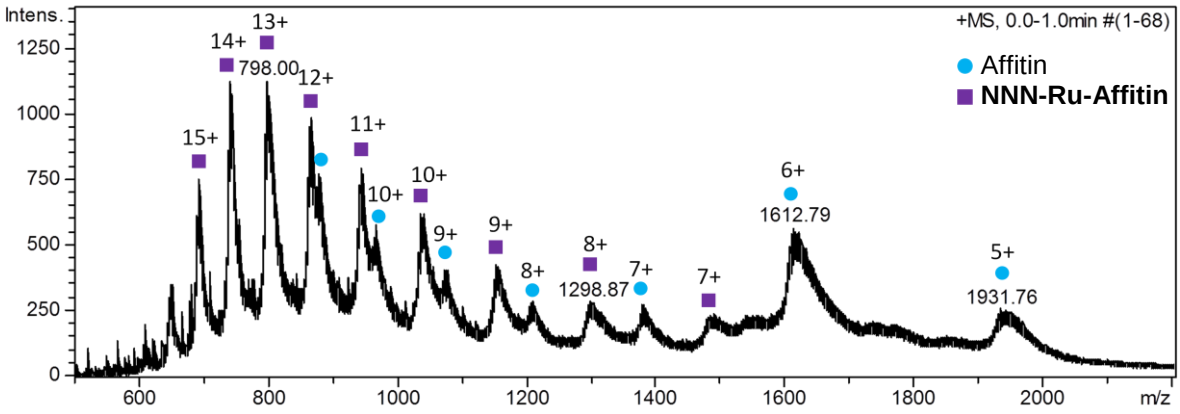


* Click condition: $CuSO_4$, NaAsc, THPTA, Aminoguanidine

As expected, we did not obseve protein precipitation in the click reaction with the **N^N^N-Ru-N_3** complex. The ESI-MS spectrum of the **NNN-Ru-Affitin** conjugate is shown in the Figure 16.

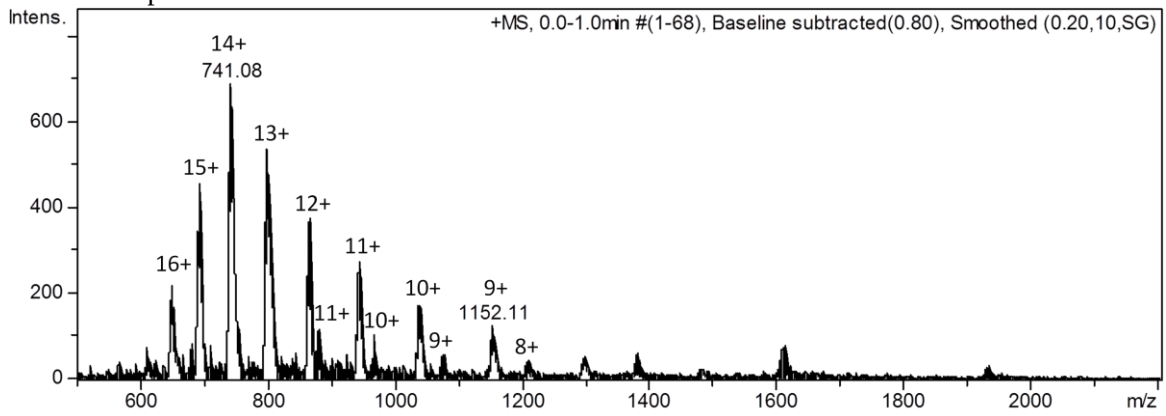
From this result, we could conclude that we successfully obtained the **NNN-Ru-Affitin** coupling product, that this is really a specific reaction (since we observed the 1:1 Ru/affitin proportion), and also that we have a robust complex (it remains attached to the protein and the terpyridine ligands remains coordinated to the metal core).

Figure 16. ESI-MS spectrum of the **NNN-Ru-Affitin** compound (RT, 20h)



Buffer: 20 mM $AcONH_4$, dilution in CH_3CN/H_2O (50:50) +1% HCOOH, final protein concentration: 50 µM.

After spectrum treatment:



Mass (approximated):

$M_{measured}$ 1: 9607 Da
$M_{measured}$ 2: 9623 Da
$M_{measured}$ 3: 9639 Da
$M_{measured}$ 4: 9656 Da

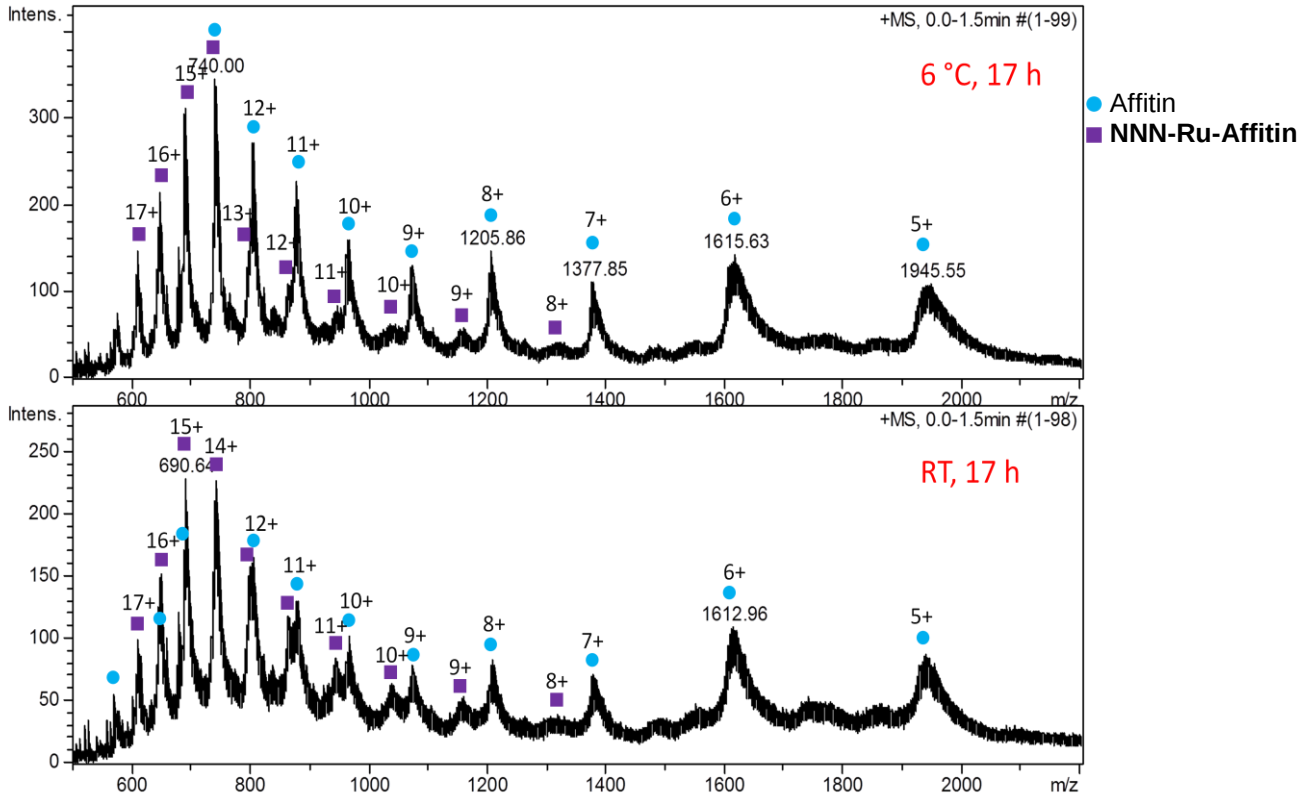
Affitin

$M_{measured}$ 5: 10327Da
$M_{measured}$ 6: 10344Da
$M_{measured}$ 7: 10361Da
$M_{measured}$ 8: 10376Da
$M_{measured}$ 9: 10392Da

NNN-Ru-Affitin

We observed the influence of the temperature in this click reaction (Figure 17). Despite the predominance of free affitin in both cases, we could observe, as expected, that the proportion between free affitin and the **NNN-Ru-Affitin** compound is lower at higher tested temperature.

Figure 17. ESI-MS: Comparison of temperature influence for **NNN-Ru-Affitin** conjugation



We also made a kinetic study with 3 different time reactions to monitor the profile of formation of the **NNN-Ru-Affitin** coupling product. The results are presented in the Figure 18. From this study, we observed that after 17 h of reaction we had a predominance of free affitin, and, as expected, it decreased with the increase of time reaction. Thus, after 70 h of reaction, we observed a much lower quantity of free affitin, with the predominance of the **NNN-Ru-Affitin** conjugate.

After many attempts, we finally obtained the mass spectrum of **NCN-Ru-Affitin** in solution after reaction in the cold chamber (6 °C) for 17 h (Figure 19). Although the spectrum quality was very low, we could detect the presence of free affitin, of the **NCN-Ru-Affitin** coupling product and we also observed the presence of free **NCN-Ru-N_3** complex, even after many buffer wash cycles, as also was noted in the reaction without the copper catalyst (Figure 11, section **II.3.4.2**). It can signify that the organometallic ruthenium complex remains attached to the affitin by an unknown way in addition to the triazole-ring.

Figure 18. ESI-MS: Kinetic comparison for **NNN-Ru-Affitin** conjugation

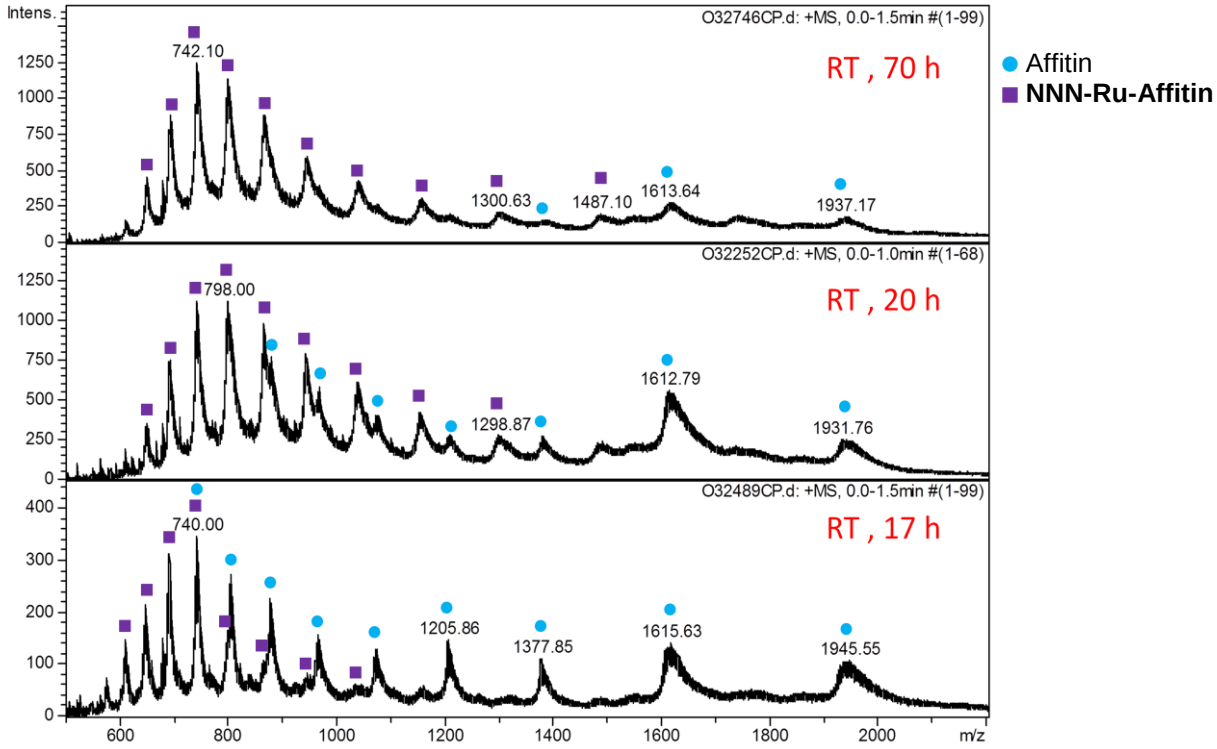
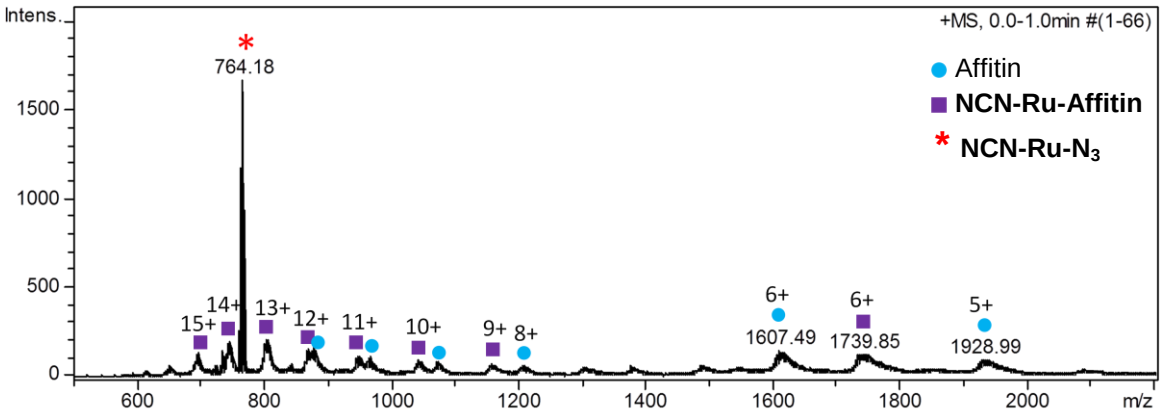
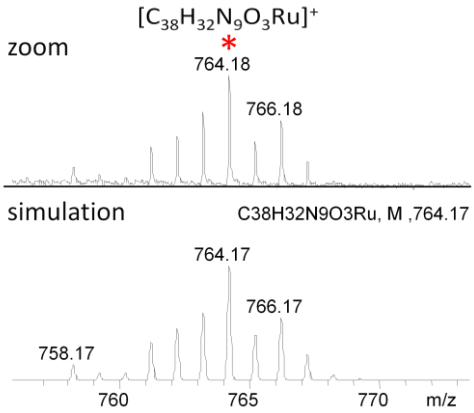


Figure 19. ESI-MS spectrum of the **NCN-Ru-Affitin** compound (6 °C, 17h)



Buffer: 20 mM $AcONH_4$, dilution in CH_3CN/H_2O (50:50) +1% HCOOH, final protein concentration: 15 µM.



Therefore, to conclude this work about affitin-vectorized ruthenium compounds, we obtained the UV-vis spectrum of our products (Figures 20-22). They also confirm that we successfully obtained the expected coupling products.

Figure 20. UV-vis spectrum of Affitin (30 µM)

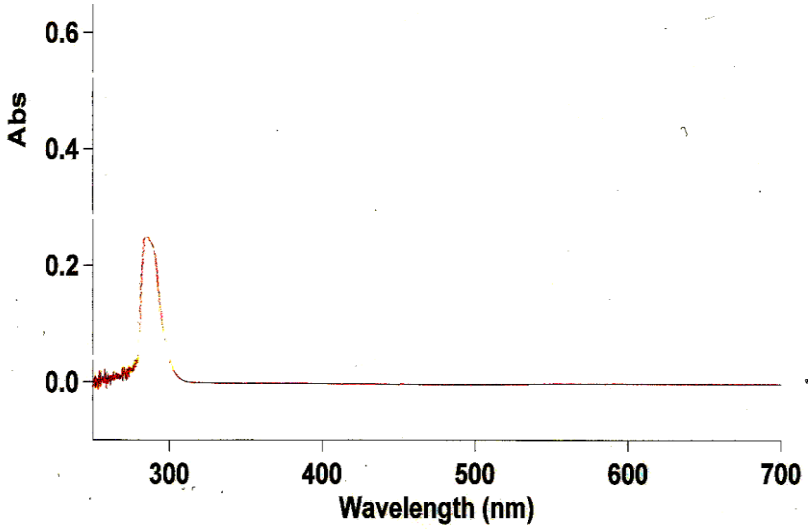


Figure 21. UV-vis spectrum of **NCN-Ru-Affitin** and **NCN-Ru-N_3** (6 µM)

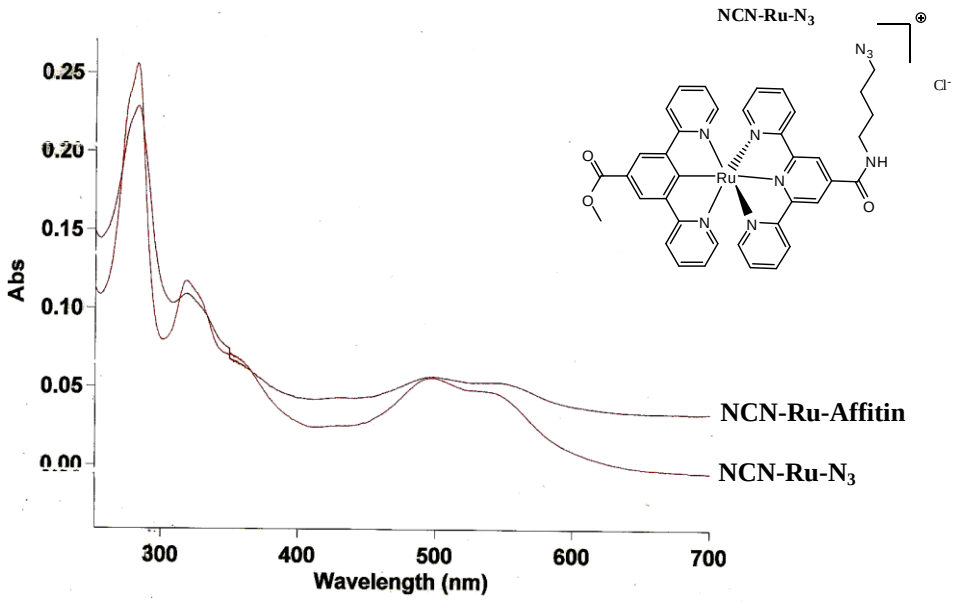
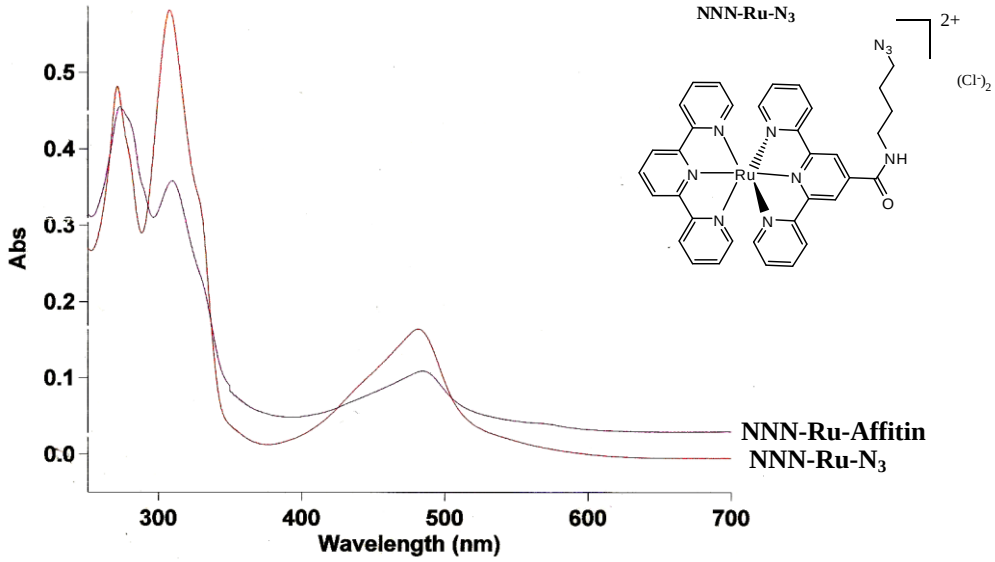


Figure 22. UV-vis spectrum of **NNN-Ru-Affitin** and **NNN-Ru-N_3** (13.6 µM)



Since the coupling protocol was validated, the next step, in further studies, would be to vectorize our **RDCs** using affitins with different selectivities to target metabolism enzymes of cancer cells, and after that, to test the anticancer activity of these coupling products *in vitro* and *in vivo*. However this will only be possible if we can avoid the polymerization of the coupled protein and hence if we have indeed a complete control on the polymerization process.

II.3.5 – Considerations about protein precipitation and mechanism of action of ruthenium compounds as anticancer agents

The difference in behavior of two structurally very similar compounds, but differing in particular for their redox properties is very instructive. Indeed, the **NNN-Ru-N_3** complex has a redox potential $E°_{RuII / RuIII}$ much higher than the compound **NCN-Ru-N_3**, and may not lead to the same electronic transfer process.

It is already known that the redox properties of ruthenium complexes play an important role in anti-tumor activity. Indeed, molecules that have a redox potential in the range between 0.4 and 0.6 V present the lowest IC_{50} values against cancer cells, and consequently the highest anticancer activity. In spite of this, their mechanism of action at the molecular level is still unknown. [40]

As we commented previously (section **II.3.3**), the characteristic of the redox enzymes involved in the cellular metabolism is that they shift back and forth between an oxidized and a reduced state and that their activity strongly depends on the electron exchanges between them, the substrates and the cofactors that provide and/or regenerate their active state. Knowing that, we believe that the **NCN-Ru-N_3** complex presents specific electron exchange properties that promote an electron transfer to our affitin and, thus, have a great probability that it can interfere on electronic exchanges in the metabolic enzymes.

As well, previous studies about the signaling pathways of anticancer **RDCs** led to interesting results, highlighting that several molecular mechanisms are induced by this kind of compounds, such as activation of p53 and p73 (in different intensities and kinetics to that observed for cisplatin), expression of p21 and Bax, induction of caspase-3, and nuclear fragmentation. [27]

Some of these cell effects, such as induction of caspase-3 and expression of Bax, are related to endoplasmic reticulum (ER) stress (*i.e.*, accumulation and aggregation of unfolded proteins in the ER). [41] The ER is primarily recognized as the site of synthesis and folding of secreted, membrane-bound, and some organelle-targeted proteins. As a consequence of this specialist environment, the ER is highly sensitive to stresses that perturb cellular energy levels, the redox state or Ca^{2+} concentration. [42]

Indeed, many disturbances, including those of cellular redox regulation, cause accumulation of unfolded proteins in the ER, initiating an evolutionarily conserved response, named unfolded protein response (UPR). [43] It might culminate with cell death if protein aggregation is persistent, since these protein aggregates are toxic to the cell. [42]

Therefore, if besides interfering on electronic exchanges in the redox enzymes, such as the glucose oxidase and the peroxidase, ruthenium compounds could promote the protein aggregation in cancer cells (similarly to that we saw in this work for affitin), they could also trigger the cell death *via* the UPR pathway. It might satisfactorily explain their molecular mechanism of action as anticancer agents, since our biologist partners have alredy observed the activation of the UPR/CHOP pathway in cancer cells treated with **RDC**.

II.3.6 – Conclusion

In this work, we successfully synthesized new affitin-vectorized ruthenium compounds by click reaction, adopting a satisfactory methodology and using robust

complexes. Another curious result was that, apparently, the organometallic ruthenium complex remains attached to the affitin by an unknown way in addition to the triazole-ring.

By working with complexes with different redox potential values, we proved the importance of this property to electron transfer to the affitin, culminating with protein aggregation in the case of **NCN-Ru-N_3** complex.

What seemed to be a dead end in a first look (the unexpected polymerization of the affitin when linked to the NCN-Ru derivative), proved in fact to be an experience of serendipity, as we found extremely interesting and original results that might finaly become, in the future, one possible mechanism of action at the molecular level of our anticancer **RDCs** against cancer cells. Indeed, we believe that, besides interfering on electronic exchanges in the redox enzymes, ruthenium compounds might promote the protein aggregation in cancer cells (similarly to that we saw in this work for affitin), inducing the cell death *via* the UPR pathway, as we have previously observed in cancer cells treated with **RDC**. However it is obviously necessary to further confirm the preliminary results we have described in this chapter by in depth studies to aquire the necessary insight in the polymerisation reactions induced by the **NCN-Ru** compounds as compared to the behaviour of the **NNN-Ru** compounds.

II.3.7 – Experimental part

II.3.7.1 – General Remarks

Experiments were carried out under an argon atmosphere, using a vacuum line. Diethyl ether and pentane were distilled over metallic sodium, dichloromethane, and acetonitrile over calcium hydride and methanol over magnesium under an argon atmosphere immediately before use. Chromatography columns were carried out on aluminum oxide (aluminum oxide 90, standardized, Merck). The other starting materials were purchased from Sigma−Aldrich, Alfa Aesar, or Strem Chemicals and used as received, without further purification. The Affitin was supplied by our partner *Affilogic*.

The methyl-3,5-di(2-pyridyl)benzoate (N^C^N-CO_2Me) ligand was synthesized according to reported procedures. [44,45,46] 4’-ethoxycarbonyl-2,2’:6’,2’’-terpyridine (N^N^N-CO_2Et) and 4’-methoxycarbonyl-2,2’:6’,2’’-terpyridine (N^N^N-CO_2Me) were synthesized following the method described by Wadman *et al.*, [47] using methanol instead of ethanol for final esterification in the second case.

Ruthenium complexes listed hereafter were synthesized following reported procedures: Di-μ-chloro-bis[cloro(η^6-benzene)ruthenium(II)] [(η^6-bz)$RuCl_2$]$_2$,[48] [Ru(N^C^N-CO_2Me)$(MeCN)_3$]PF_6, [49] [Ru(N^N^N)Cl_3], [50] and [Ru(N^N^N)$(MeCN)_3$]$(PF_6)_2$. [51]

Anion exchange was performed over Amberlite column following previous described method. [33]

The accelerating ligand, THPTA (tris(3-hydroxypropyltriazolylmethyl)amine), needed for the click reaction, was synthesized according to Hong *et al.* [36b]

The NMR spectra were obtained at room temperature on Bruker spectrometers. ^{1}H NMR spectra were recorded at 300.13 MHz (Model AC-300). The chemical shifts are referenced to the residual solvent peak. Chemical shifts (δ) and coupling constants (*J*) are expressed in units of ppm and Hz, respectively. (Multiplicity: s = singulet, d = doublet, t = triplet, q = quadruplet, h = heptuplet, m = multiplet)

Electronic absorption spectra were recorded using an UVIKON XL spectrophotometer from Bio-Tek Instruments with quartz cells of 1 cm wide.

ESI-MS measurements were performed on a Bruker Daltonics microTOF spectrometer equipped with an orthogonal electrospray (ESI) interface, using the following settings: nitrogen to 0.4 bar as nebulization gas; nitrogen at 4 L/min and 180 °C as desolvation gas; and -4500 V for capillary tension.

II.2.5.2 – Synthesis protocols

N^N^N-$CONH(CH_2)_4$-N_3 (N^N^N-linker)

The N^N^N-linker compound was synthesized according to the method described in the Irene Mustieles Marín's master thesis, [52] with some adaptations.

To obtain the **amide** form:

1st Step

To a heated mixture of N^N^N-CO_2Me, 4'-ethoxycarbonyl-2,2'-terpyridine, (670 mg, 1.87 mmol) and 7 mL of absolute ethanol was added, dropwise, 7 mL of sodium hydroxide solution (1N). The mixture was stirred under reflux during 2 hours. The ethanol was removed under reduced pressure and the mixture was treated with a solution of HCl (0.5N) to decrease the pH till 4-5. The solid appeared was filtered and dried under vacuum (502 mg, 98%).

^{1}H NMR (300MHz, DMSO-d6): δ 8.87 (s, 2H), 8.76 (d, 2H, ^{3}J 4.2), 8.65 (d, 2H, ^{3}J 7.9), 7.04 (td, 2H, ^{3}J 7.7, ^{4}J 1.6), 7.54 (ddd, 2H, ^{3}J 7.7, 4.6, ^{4}J 0.9).

2nd Step

N^N^N-CO_2H (502 mg, 1.81 mmol), was dissolved in 20 mL of thionyl chloride and the mixture was let stirring under Ar at reflux overnight. Solvent was removed under vacuum leading to a pale yellow solid (we suppose the totally conversion, 535 mg, 1.81 mmol). ^{1}H NMR analysis was not possible due to the presence of water in the deuterated solvents which promotes the inverse reaction, we suppose the totally conversion (535 mg).

3rd Step

To a solution of N^N^N-COCl (535 mg, 1.81 mmol) in dichloromethane (15 mL) were added a solution of 4-(4-azidobutan-1-amine) (220 mg, 1.9 mmol), 0.6 mL of triethylamine (384 mg, 3.8 mmol) in 15 mL of distillated dichloromethane. After stirring for 2 hours at 40ºC, the mixture was let stirring overnight at room temperature. 15 mL of ammonium hydroxide solution (1N) were added and the organic layer was washed with more ammonium solution (2 x 15 mL). Organic layer was dried over Na_2SO_4. Dichloromethane was removed under reduced pressure leading to a pale lavender solid (397 mg, 60%).

^{1}H NMR (300.13 MHz, $CDCl_3$): 8.77 (s, 2H), 8.71 (d, 2H, ^{3}J 4.8), 8.60 (d, 2H, ^{3}J 7.9), 7.86 (td, 2H, ^{3}J 7.9, ^{4}J 1.8), 7.36 (dd, 2H, ^{3}J 7.7, 4.8), 1.72 (m, 8H).

[Ru(N^C^N-CO_2Me)(N^N^N-CONH$(CH_2)_4$-N_3)]PF_6 ([NCN-Ru-N_3]PF_6)

A mixture of [Ru(N^C^N-CO_2Me)$(MeCN)_3$]PF_6 (69.2 mg, 0.105 mmol) and the ligand N^N^N-linker (58.9 mg, 0.157 mmol) in 15 mL of dry methanol was let stirring under reflux and argon overnight. The product was dried under reduced pressure and then it was purified over alumina column using dichloromethane and after dichloromethane/acetonitrile (80:20) as eluent. The complex was precipitate in dichloromethane/pentane solvent combination leading to a dark purple solid (81 mg, 84%).

^{1}H NMR (300.13 MHz, CD_3CN): δ 9.09 (s, 2H), 8.86 (s, 2H), 8.53 (d, 2H, ^{3}J 8.1), 8.26 (d, 2H, ^{3}J 8.1), 7.74 (td, 2H, ^{3}J 8.6, 6.4, ^{4}J 1.5), 7.65 (td, 2H, ^{3}J 8.6, 6.4, ^{4}J 1.5), 7.13 (d, 2H, ^{3}J 5.7), 7.05 (d, 2H, ^{3}J 5.5), 7.96 (td, 2H, ^{3}J 7.9, 5.7), 6.70 (td, 2H, ^{3}J 7.7, 4.6, ^{4}J 1.3), 6.15 (s, 1H), 4.05 (s, 3H), 3.62 (m, 2H), 3.46 (t, 2H), 1.83 (m, 4H).
ESI-MS: $[C_{38}H_{32}N_9O_3Ru]^+$ = 764.17.

Prior to bioconjugation, Ru(II) complex was exchanged with chloride salt to increase solubility and yield.

[Ru(N^N^N-CO_2Me)(N^N^N-CONH$(CH_2)_4$-N_3)]$(PF_6)_2$ ([N^N^N-Ru-N_3]$[PF_6]_2$)

The ligand N^N^N-linker (23.2 mg, 0.062 mmol) was added to a suspension of [Ru(terpy)$(NCMe)_3$]$(PF_6)_2$ (43 mg, 0.056 mmol) in 5 mL of methanol (under argon

atmosphere) and the mixture was refluxed for 48h under ilumination of an office luminaire. The product was firstly purified over celite (small column), and the solvent was evaporated. Then the product was solubilized in acetonitrile and it was purified over alumina column using acetronitrile as eluent. Acetonitrile was evaporated, the final product was washed with pentane, solubilized in dichloromethane and precipitated with pentane. After vacuum, we obtained 35 mg of a dark orange product (Yield: 63%).

^{1}H NMR (300.13 MHz, CD_3CN): δ 9.05 (s, 2H), 8.76 (d, 2H, ^{3}J 8.4), 8.58 (d, 2H, ^{3}J 8.4, B3), 8.49 (d, 2H, ^{3}J 8.4), 8.44 (t, 1H, ^{3}J 8.4), 7.98–7.88 (m, 5H), 7.37 (d, 2H, ^{3}J 5.4), 7.31 (d, 2H, ^{3}J 5.1), 7.19 (dt, 4H, ^{3}J 15.5, 6.4).
ESI-MS: $[C_{35}H_{30}F_6N_{10}OPRu]^+ = 853.14$; $[C_{35}H_{30}N_{10}ORu]^{2+} = 305.56$

Prior to bioconjugation, Ru(II) complex was exchanged with chloride salt to increase solubility and yield.

RDC-Ru-Affitin bioconjugate (NCN-Ru-Affitin and NNN-Ru-Affitin)

By adapting the protocol described by Hong *et al.* [36b] to our reagents, we added the in an Eppendorf tube:

- 54.2 µL of a solution of Affitin 221 µM in phosphate buffer pH 7 (final concentration: 120 µM);
- 5 µL of a solution 100 mM of aminoguanidine in water (final concentration: 5 mM);
- 3.6 µL of a solution 10 mM of Ru complex (**NNN-Ru-N_3** in water or **NCN-Ru-N_3** in water/acetonitrile, 1:1) resulting in a final concentration of 360 µM); and
- 31.7 µL of phosphate buffer pH 7 to complete the final volume of 100 µL.

To this solution was added a pre-mixed solution containing:

- 1 µL of a solution 20 mM of $CuSO_4$ in water (final concentration: 200 µM); and
- 2 µL of a solution 50 mM of THPTA in water (final concentration: 1 mM).

Then, for start the click reaction, 2 µL of a solution 50 mM of THPTA in water was added (final concentration: 1 mM).

The Eppendorf tube was put under argon during the first 15 min and it was let reacting during 20 h at room temperature. In the case of the organometallic complexe, the reaction tube was centrifuged to separe the precipitate, and the soluble protein (supernatant) was washed with phosphate buffer and concentrated using Microcon or Ultracon devices (3 kDa, Millipore), at 10 °C, at least for 4 cycles from 500 µL to 50 µL, followed by the exchange from the phosphate buffer to the ammonium acetate 20 mM (at least for 4 cycles from 500 µL to 50 µL) to obtain the ESI-mass spectrum in denaturing condition (dilution in CH_3CN/H_2O (50:50) +1% HCOOH).

ESI-MS (NNN-Ru-Affitin): $M_{measured}$ 1: 10327 Da; $M_{measured}$ 2: 10344 Da; $M_{measured}$ 3: 10361 Da; $M_{measured}$ 4: 10376 Da; $M_{measured}$ 5: 10392 Da. E*xpected mass:* 10283 Da.

Unfortunately, we could not obtain a high quality mass spectre of **NCN-Ru-Affitin** to determine its exact mass.

The protein concentrations were tested following the Bradford method, [53] using a standard range of bovine serum albumin. The products were also analysed by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) [54] with 12% of acrylamide, using the unstained protein molecular weight marker #06U-0511 (Euromedex).

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General Conclusion

The aim of this thesis was to study different approaches in the field of Medicinal Inorganic Chemistry, by developing new bioactive compounds of tin and ruthenium, and also by contributing with the discovery of original clues about the mechanism of action of ruthenium-based compounds against cancer cells.

Thus, we developed tin compounds derived from three bioactive fatty acids (undecylenic, ricinoleic and caprylic acids) and we found that they show very high activity against some strains of bacteria and yeast. Moreover, we observed that organotin chlorides adopted as source of organotin moieties also presented antibacterial activity, but, as known, most of them were very toxic against mammalian cells. Also most of our complexes were much more active than the antimicrobial fatty acids used as ligands, even in nM ranges, being up to four thousand times more potent against *S. aureus* than against mammalian cells (J774 macrophages). These results allowed us to believe to being on the right path to overcoming challenges to develop new alternatives in antimicrobial field by using the approaches provided by the Medicinal Inorganic Chemistry.

For ruthenium compounds, in turn, we have confirmed that the mode of action of **RDC11** is undoubtedly different from cisplatin or other ruthenium compounds, because of its high stability toward substitution reactions (in the absence of heat or light). Therefore, we can conclude that its DNA interactions, for example, are necessarily different from that observed for cisplatin and very probably that other mechanism of action playing an important role in its anticancer activity should exist.

Finally, whilst vectorizing **RDCs** with Affitin, we were able to gain important knowledge about a possible mechanism of action of this kind of molecules. Indeed, we successfully obtained affitin-vectorized **RDCs** by using the recent but well-known copper-catalyzed azide-alkyne cycloaddition (CuAAC) click reaction between an azido and a terminal alkyne groups. However, when we reacted our **NCN-Ru-N_3** with affitin we observed a significant unpredicted protein precipitation. Therefore, from this serendipitous discovery, we could propose that, besides interfering on electronic exchanges in the redox enzymes, ruthenium compounds might also promote protein aggregations in cancer cells, inducing the cell death *via* the UPR pathway, for example, as we have previously observed in cancer cells treated with **RDC**. However this hypothesis should be confirmed by further in depth studies, by which we must get more insight in the exact role of the ruthenium compounds upon the observed protein polymerisation.

In this way, I really hope that this thesis will be motivating other pharmacists and chemists to use their skills to improve the knowledge about Medicinal Inorganic Chemistry,

especially developing new drugs and searching to understand the mechanism of action at molecular level of metal-based compounds.

Résumé de la thèse en Français

Composés organométalliques de l'étain et du ruthénium – Applications en Chimie Médicinale

Introduction Générale

Pour une meilleure compréhension de la Chimie Médicinale, il est important de connaître quelques concepts liés à ce domaine de recherche. Tout d'abord, comme recommandé par l'IUPAC (1998), *la chimie médicinale est une discipline de la chimie, impliquant également les aspects des sciences biologiques, médicales et pharmaceutiques. Ce domaine de recherche se concentre sur l'invention, la découverte, la conception, l'identification et la préparation de composés biologiquement actifs, l'étude de leur métabolisme, l'interprétation de leur mode d'action au niveau moléculaire et la construction de relations structure-activité. Un médicament est donc toute substance présentée pour traiter, guérir ou prévenir la maladie chez les êtres humains ou chez les animaux, et peut également être utilisé pour établir un diagnostic médical ou de restaurer, corriger ou modifier des fonctions physiologiques.* [1]

Plusieurs étapes sont nécéssaires pour le developement de nouveaux composés pour l'application médicinale, entre elles, la phase préclinique qui, en cas de succès, est suivie des phases cliniques 1, 2 et 3. Dans l'ensemble, tout le processus prend, en moyenne, entre 8 à 12 ans. Il n'est donc pas surprenant que dès la conception jusqu'au marché la plupart des composés subissent des tests très élaborés pour devenir un médicament approuvé. Pour environ tous les 5.000 à 10.000 composés qui entrent dans les essais précliniques, un seul, en moyenne, est approuvé pour la commercialisation. [2]

Ainsi, la chimie médicinale est un domaine très important et complexe de la recherche qui est responsable de toutes les étapes impliquées dans la découverte et le développement de nouveaux médicaments à partir de l'idée jusqu'au marché.

L'application des composés à base de métaux en biologie et médecine n'a rien de nouveau - en effet, la nature a utilisé des systèmes organométalliques pour maintenir la vie pour un temps assez long. Par example la chimie organométallique de cobalamine, mieux connu sous le nom de vitamine B_{12} (dont la structure a été déterminée par Dorothy Hodgkin, prix Nobel de chimie en 1964), et de ses dérivés a été étudiée pendant des décennies, ainsi que des differents enzymes et cofacteurs contenant des liaisons métal-carbone. [3]

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Les produits pharmaceutiques à base de métaux offrent une polyvalence sans précédent dans la chimie médicinale en raison des différentes unités structurales à partir desquels ils peuvent être construits, la variété des interactions disponibles, la combinaison de la rigidité autour du métal et la flexibilité des ligands, la cinétique de substitution des ligands ainsi qu'en raison de leurs propriétés oxydo-réductrices. [4]

Il est intéressant de noter que les médicaments à base de métaux ont été utilisés depuis les débuts de la civilisation : l'utilisation médicale de l'or remonte à 2500 av. J.-C. en Chine, alors que les anciens Egyptiens utilisaient le cuivre pour stériliser l'eau ; le Papyrus de Ebers (écrit vers 1500 av. J.-C.) présente également des composés de fer pour le traitement de l'anémie, métal qui est encore en usage actuellement ; [3] le médecin grec Hippocrate a utilisé le mercure dès 400 av. J.-C ; et Paracelse, alchimiste et médecin suisse du XVIIe siècle, a lui aussi employé des minéraux dans la médecine en utilisant des sels d'antimoine, d'arsenic et du mercure. [5]

Au cours des derniers siècles divers composés à base de métaux ont été appliqués en médecine. Cependant, la chimie inorganique médicinale, en tant que discipline, a commencé à se développer après la découverte fortuite de l'activité anti-tumorale du Cisplatine. Le succès des applications cliniques de ce composé de platine a stimulé l'intérêt considérable dans la recherche de nouveaux complexes métalliques comme agents thérapeutiques modernes, agents diagnostiques et radiopharmaceutiques. [6]

Pour développer de nouveaux médicaments, les chimistes inorganiques médicinaux seront confrontés aux mêmes obstacles que les autres chimistes médicinaux, mais ils devront aussi surmonter la méfiance à l'égard des métaux qui est en grande partie due à l'ignorance qui existe encore dans la plupart des domaines de l'industrie pharmaceutique et qui peut être exacerbée par une préoccupations accrue sur la toxicité. Toutefois, ces composés offrent également de nombreuses possibilités pour la chimie inorganique médicinale. [7]

Dans cette introduction générale, on a essayé de mettre en évidence le fait que la chimie médicinale est un domaine complexe et beau de la recherche, et en particulier que la chimie inorganique médicinale est un domaine très important qui pourrait mener à la découverte de nouveaux médicaments. Dans ce contexte, l'objectif de cette thèse a eu une double motivation : (i) trouver de nouveaux composés organostanniques qui pourraient être

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appliqués dans le domaine antimicrobien; et (ii) apporter une contribution à l'élucidation du mécanisme d'activité anticancéreuse de composés à base de ruthénium.

Dans la partie I de cette thèse, nous abordons l'application médicale des composés organostanniques, principalement les propriétés antimicrobiennes de nouveaux complexes à base d'étain dérivés d'acides gras. Ces composés ont été testés *in vitro* contre certaines souches de micro-organismes, comme les bactéries Gram-positives et Gram-négatives, et une levure (chapitre I.1). Cette partie du travail a été dévéloppé à Maceió (Brésil), dans le laboratoire du Grupo de Catálise e Reatividade Química (GCaR), sous la direction du Prof. Dr. Mario R. MENEGHETTI.

La partie II de cette thèse trait sur les complexes cytotoxiques dérivés du ruthénium synthétisés dans le Laboratoire de Synthèses Métallo-Induittes (actuellement nommé Laboratoire de Chimie et Systhémique Organo-Métalliques) au cours des 12 dernières années. [8] Dans le but d'acquérir le savoir-faire sur la chimie de coordination de ces composés cycloruthenés, j'ai contribué à l'élucidation de la labilité des ligands MeCN, un ligand clé d'un des composés développés à Strasbourg, le RDC11 (chapitre II.2). En France, j'ai participé aussi à un deuxième projet qui avait comme ambition de développer de nouveaux complexes de ruthénium vectorisés avec une protéine d'affinité appellée *Affitin* (les conjugués RDC-*Affitin*). Cela devait se faire grâce à une "réaction clique" entre un azido et un alcyne terminal, les composés obtenus devaient améliorer grandement leur sélectivité anticancéreux (chapitre II.3). Grâce à cette étude, on espérait acquérir des connaissances importantes sur le mécanisme d'action de ces complexes à base du Ru. Cette partie de la thèse a été développée à Strasbourg (France), sous la direction du Dr. Michel PFEFFER.

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Partie I – Composés de l'étain en Chimie Médicinale

Chapitre I.1- L'activité antimicrobienne des composés organostanniques dérivés d'acides gras [9]

Les agents antimicrobiens sont des substances qui provoquent la mort ou inhibent la croissance des micro-organismes. Le but principal de son utilisation est de prévenir ou traiter une infection, réduire ou éliminer les agents pathogènes et cela, si possible, en conservant la flore microbienne normale. [10]

La résistance microbienne est définie comme la capacité des micro-organismes pour interrompre l'activité d'un agent antimicrobien. C'est un phénomène naturel d'évolution parce que quand un micro-organisme est exposé à un agent antimicrobien, les organismes les plus sensibles succombent et ceux qui sont résistants survivent et peuvent transmettre leur résistance à leur descendance. En conséquence, les traitements conventionnels deviennent inefficaces, les infections persistent et peuvent être transmises à d'autres, et imposent des coûts énormes pour les individus et la société. [11]

Selon Chopra, [12] bien que certains nouveaux médicaments sont en développement, ils ne couvrent pas suffisamment les besoins médicaux en croissance et de plus, ces médicaments sont principalement dérivés des classes en usage depuis longtemps, ils sont donc sujets à des mécanismes de résistance bactérienne existants. Ainsi, de nouvelles classes d'antimicrobiens sont nécessaires d'urgence.

Compte tenu des arguments présentés ci-dessus, de nouvelles alternatives pour lutter contre les infections microbiennes doivent être développées pour contourner le problème de la résistance microbienne.

De nombreux éléments inorganiques, généralement présents à des concentrations faibles, sont essentiels pour tous les êtres vivants. Il est connu que certaines altérations dans le métabolisme de ces oligo-éléments sont la cause de diverses maladies et troubles physiologiques. [13] L'étain (Sn) est un métal qui est parmi ces éléments essentiels pour la

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[9] Nous sommes reconnaissants au Prof. Dr. Maria Lysete de Assis Bastos (Universidade Federal de Alagoas) pour son soutien laboratorial pour l'exécution des tests antimicrobiens et Prof. Dr. Magna Suzana Alexandre-Moreira et l'étudiant Amanda Evelyn (Universidade Federal de Alagoas) pour déterminer la cytotoxicité des composés contre les cellules de mammifères.

[10] Machado, A.; Guimarães, J. F.; Barros, E. Principais grupos farmacológicos: conceitos e propriedades. In: *Antimicrobianos: consulta rápida*. Barros, E. *et al.*, 2008. Porto Alegre: Artmed, Chapter 2, pp. 25-55.

[11] WHO – World Health Organization. *10 facts on antimicrobial resistance.* 2012. <http://www.who.int/features/factfiles/antimicrobial_resistance/en/index.html>. Acessé en fev. 2016.

[12] Chopra, I. *J Antimicrob Chemother*. **2013**, *68*, 496–505.

[13] Baran, E. J. *Cad. Tem. Quím. Nova Esc.* **2005**, *6*, 7-12.

composition corporelle des animaux et de l'homme, et le déficit dans ce composé est lié à un retard de croissance. [14] La valeur moyenne de la concentration de l'étain présente dans la composition du corps humain est de 0,03 g (référence : mâle adulte de 70 kg). [14]

Parmi les différents complexes à base de métaux qui ont été étudiés pour des applications biologiques, les complexes d'étain(IV) ont attiré l'attention en tant que nouveaux agents thérapeutiques. Un grand nombre de références présentent les complexes organo-étain comme des entités bioactives différentes, par exemple comme des agent antimicrobiens, [15] antitumoraux, [16] leishmanicides, [17] trypanocides, [18] et anti-inflammatoires. [19]

Étant donné l'importance des recherches de nouveaux médicaments pour le traitement des infections microbiennes, le développement de nouvelles stratégies méthodologiques est critique pour l'obtention de nouveaux prototypes de médicaments. La complexation des molécules bioactives à des métaux tels que l'étain peut être une solution intéressante pour la découverte de nouvelles alternatives pour la thérapie antimicrobienne.

Les composés organostanniques représentés par la formule R_nSnL_{4-n} ont une variété d'effets biologiques, en fonction du nombre **n**, du type de groupe **R** (habituellement des groupes alkyle ou aryle), et du ligand **L** (habituellement des carboxylates, des alcohoxylates et des halogénures) liés à l'ion métallique. [20]

Pour ce travail, nous avons synthétisé une série de complexes organostanniques dérivés d'acides gras bioactifs, en utilisant les acides caprylique (8C), undécylénique (11C) et ricinoléique (18C) comme ligand **L**. Neuf complexes ont été obtenus : ***t*-Bu_2SnUnd_2**, ***n*-Bu_2SnRic_2**, ***t*-Bu_2SnRic_2**, ***n*-Bu_3SnRic**, **Me_2SnRic_2**, ***n*-$Bu_2SnCapri_2$**, ***t*-$Bu_2SnCapri_2$**, ***n*-$Bu_3SnCapri$** et **$Me_2SnCapri_2$**.

La synthèse a été réalisée de manière simple et efficace, selon la méthodologie décrite par Mohammed *et al*., [21] par la réaction entre le groupe carboxyle et le complexe de chlorure de organostannique correspondent.

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[14] Benite, A. M. C.; Machado, S. P.; Barreiro, E. J. *Quím. Nova*. **2007**, *30*, 2062-2067.
[15] Baul, T. S. B. *Appl. Organomet. Chem.* **2008**, *22*, 195-204.
[16] Hadjikakou, S. K.; Hadjiliades, N. *Coord. Chem. Rev.* **2009**, *253*, 235-249.
[17] Khan, M. I.; Baloch, M. K.; Ashfaq, M.; Gul, S. *J. Braz. Chem. Soc.* **2009**, *20*, 341-347.
[18] Shuaibu, M. N.; Kanbara, H.; Yanagi, T.; Ichinose, A.; Ameh, D. A.; Bonire, J. J.; Nok, A. J. *Parasitol. Res.* **2004**, *92*, 65-73.
[19] Nath, M.; Singh, H.; Kumar, P.; Kumar, A.; Song, X.; Eng, G. *Appl. Organomet. Chem.* **2009**, *23*, 347-358.
[20] Katsoulakou, E.; Tiliakos, M.; Papaefstathiou, G.; Terzis, A.; Raptopoulou, C.; Geromichalos, G.; Papazisis, K.; Papi, R.; Pantazaki, A.; Kyriakidis, D.; Cordopatis, P.; Manessi-Zoupa, E. *J. Inorg. Biochem.* **2008**, *102*, 1397-1405.
[21] Muhammad, N.; Shah, A.; Zia-ur-Rehman; Shuja, S.; Ali, S.; Qureshi, R.; Meetsma, A.; Tahir, M. N. *j. Organomet. Chem.* **2009**, *694*, 3431-3437.

L'activité antibactérienne et antifongique des complexes dérivés de l'étain(IV) a été obtenue en utilisant la méthode de microdilution. Les concentrations minimales inhibitrices (CMI) ont été déterminées pour tous les complexes et leurs ligands, afin d'évaluer quantitativement les effets de la complexation sur leur activité *in vitro*.

A titre de comparaison, nous avons également retesté certains complexes dérivés de l'acide undécylénique (***n*-Bu_2SnUnd_2**, ***n*-Bu_3SnUnd** et **Me_2SnUnd_2**) qui ont été synthétisés au cours de mon cours de Master. [22]

Selon les critères retenus pour classer les substances testées en tant que composés actifs (CMI < 128 µg/mL), on a observé que l'acide undécylénique et tous ses complexes dérivés étaient actifs contre *C. albicans*. Ce fait était attendu, étant donné que dans le point de vue pharmacologique cet acide est classé comme un agent antifongique. [23] Parmi les composés dérivés de l'acide caprylique, seuls les complexes avec *n*-butyle ont été considérés comme actifs, alors que parmi les complexes dérivés de l'acide ricinoléique, seul le triorganostannique a présenté une activité antifongique.

L'échelle molaire (M) est la meilleure façon de comparer les résultats de l'activité biologique, car elle prend en compte la quantité de composé qui est présent dans le milieu de culture, par conséquent, les résultats ne changent pas en fonction de leur masse molaire relative. En effet, certains de ces composés présentent la même CMI exprimée en µg/mL, mais nous avons observé, par exemple, que environ 3 fois moins de matériel a été nécessaire pour montrer l'activité antifongique élevée lorsque nous avons comparé la concentration molaire de ***n*-Bu_2SnUnd_2** ou ***t*-Bu_2SnUnd_2** à UndH, et plus encore lorsque on compare la concentration molaire de ***t*-Bu_2SnRic_2** ou **$Me_2SnCapri_2$** à CapriH.

Nous avons observé dans tous les cas que la complexation a amélioré l'activité antifongique, étant donné que la concentration minimale inhibitrice était plus élevée pour les acides gras libres que pour leurs complexes dérivés. Dans le cas des complexes triorganostanniques, il y a eu une amélioration critique par rapport à la forme acide de leurs ligands, avec CMI étant dans la gamme micromolaire. En effet, leur CMI était inférieure à celle du fluconazole (3,3 µM) et comparable à celle de l'amphotéricine B (0,1 µM), indépendamment du ligand acide.

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[22] Barbosa, A. S. L. *Síntese e avaliação da atividade antimicrobiana de complexos organometálicos de estanho(IV) com ligantes bioativos.* **2011**. Master dissertation (Chemistry) – Maceió, Universidade Federal de Alagoas.

[23] Bennett, J. E. Agentes antimicrobianos: agentes antifúngicos. In: *Goodman & Gilman. As bases farmacológicas da terapêutica.* **2006**. 11. ed. Porto Alegre: McGraw-Hill, Chapter 48, pp. 1103-1118.

Vieira [24] a rapporté que certains composés organiques de l'étain ont diminué les taux de lipides dans *C. albicans*, ce qui suggère l'intervention de ces composés dans ce composant cellulaire. D'après cette étude, les changements dans la perméabilité de la membrane peuvent induire une formation de pores, probablement par l'interaction d'organo-étain avec des composés steroides présents dans l'enveloppe cellulaire et, éventuellement, par une solubilisation des lipides. Ainsi, ces résultats suggèrent que le mécanisme d'action du complexe organo-étain en cellules de *C. albicans* est similaire à celle des composés polyènes, tels que la nystatine, qui sont prescrits pour le traitement des infections causées par cette levure.

Par rapport à l'activité contre les bactéries, en général, la complexation de ces acides gras avec des fractions organiques de l'étain a favorisé une augmentation critique de leur activité anti-bactérienne, ce qui corrobore nos attentes. Il est important de noter que les composés de triorganoétain sont beaucoup plus actifs contre *S. aureus* que leurs ligands acides et les complexes diorganoétain. Ils montrent, en effet, les valeurs de CMI dans la gamme nM (5-60 nM), et étaient encore plus puissant que l'ampicilline (400 nM) et la lévofloxacine (800 nM). Ils doivent donc être étudiés comme des nouveaux candidats d'agents antibactériens.

L'activité faible ou absente des complexes organostanniques contre les bactéries Gram-négatives et l'activité généralement élevée contre les bactéries Gram-positives sont conformes aux données de la littérature pour ce type de composé. [25] En effet, les membranes bactériennes Gram-négatives ont une forte barrière de perméabilité à de nombreux types d'antibiotiques, elles sont donc actuellement résistants à de nombreuses substances qui sont actives contre des organismes Gram-positifs.

Bien que il n'y a que peu d'informations disponibles concernant des effets des composés d'étain sur les processus microbiens, il est connu que certaines fonctions de la membrane bactérienne peut être inhibée par les complexes organiques de l'étain, y compris les effets sur la production d'énergie, le transport de solutés, le maintien du substrat et l'oxydation. [26]

Pour analyser le profil cytotoxique des acides undécylénique, ricinoléique et capryliques, les chlorures organo-étain et leurs composés dérivés contre des cellules de

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[24] Vieira, F. T. *Compostos organoestânicos com ação farmacológica.* **2008**. PhD Thesis (Chemistry) – Belo Horizonte, Universidade Federal de Minas Gerais.
[25] Gleeson, B.; Claffey, J.; Ertler, D.; Hogan, M.; Müller-Bunz, H.; Paradisi, F.; Wallis, D.; Tacke, M. *Polyhedron.* **2008**, *27*, 3619-3624.
[26] Baul, T. S. B. *Appl. Organomet. Chem.* **2008**, *22*, 195-204.

mammifères, nous les avons sousmis au test de viabilité cellulaire en utilisant le MTT (3-(4,5-diméthylthiazol-2-yl)-2,5-diphényltétrazolium). [27] Selon les résultats, seuls **Me_2SnUnd_2**, CapriH et Me_2SnCl_2 n'ont pas montré d'activité cytotoxique sur la cellule de mammifère à des concentration létale CL50 > 100 μM.

Selon Pruchnik *et al*., [28] de nombreuses études suggèrent que la toxicité des complexes organiques de l'étain est dû principalement à leur action sur la membrane et leur capacité à traverser la bicouche lipidique.

Ainsi, la complexation d'acides gras bioactifs (acides undécylénique, ricinoléique et caprylique) avec des groupements organiques de l'étain aboutissent dans la plupart des cas, à des composés avec une activité anti-microbienne élevée. En dépit de la forte toxicité de la plupart des composés organostanniques, les complexes de triorganoétain ont montré des valeurs de concentration minimale inhibitrice dans l'échelle nM contre *S. aureus*, soit jusqu'à quatre mille fois plus puissant contre ce micro-organisme par rapport aux cellules de mammifères. Ces résultats nous rassurent quant à la possibilité de surmonter les défis pour développer de nouvelles alternatives dans le domaine antimicrobien.

Partie II – Composés du ruthénium en Chimie Médicinale

Chapitre II.1- Introduction

De nombreux composés à base de ruthénium présentent une activité anticancéreuse prometteuse, et deux dérivés de ruthénium(III) sont testés dans les essais cliniques: NAMI-A, qui est la *trans*-$[RuCl_4(DMSO)(Im)]ImH$ (où Im = imidazole) et KP1019, la *trans*-$[RuCl_4(Ind)_2]IndH$ (où Ind = indazole). KP1019 a montré une bonne activité contre les tumeurs primaires, [29] alors que la structure similaire NAMI-A est plus active contre les cellules métastatiques que contre ces types de tumeurs. [30] Une fois dans les cellules, Ru(III) peut être activé par réduction en Ru(II), bien que le Ru(IV) est également accessible dans les conditions biologiques. [31] Il est maintenat admis que les composés de ruthénium(III) agissent en tant que précurseurs de médicaments, et qu'ils sont réduits *in vivo* en complexes de

[27] Hussain, R. F.; Nouri, A. M.; Oliver, R. T. A new approach for measurement of cytotoxicity using colorimetric assay, *J. Immunol. Method*. **1993**, *160*, 89-96.
[28] Pruchnik, H.; Teresa Kral, T.; Poradowski, D.; Pawlak, A.; Drynda, A.; Obminska-Mrukowicz, B.; Hof, M. Chemico-Biological Interactions. **2016**, *243*, 107-118.
[29] Jakupec, M. A.; Arion, V. B.; Kapitza, S.; Reisner, E.; Eichinger, A.; Pongratz, M.; Marian, B.; Graf von Keyserlingk, N.; Keppler, B. K. *Int. J. Clin. Pharmacol. Ther.* **2005**, *43*, 595–596.
[30] Rademaker-Lakhai, J. M.; van den Bongard, D.; Pluim, D.; Beijnen, J. H.; Schellens, J. H. *Clin. Cancer Res.* **2004**, *10*, 3717–3727.
[31] Chellan, P.; Sadler, P. J. *Phil. Trans. R. Soc. A* **2015**, *373*, 20140182.

ruthénium(II), l'espèce qui exerce l'activité anti-cancéreuse. Ainsi, les composés de ruthénium(II) semblent être des prototypes plus intéressants pour l'application dans la thérapie anticancéreuse. [32,33]

Les deux complexes, KP1019, complexe indazole (en cours de développement clinique commercial en tant que sel de sodium NKP-1339 et IT-139) et le complexe imidazole NAMI-A (inhibiteur de métastases anti-tumorale nouveau), sont des composés octaédriques de Ru(III) qui ont terminé la phase I des essais cliniques. Ces complexes peuvent être délivrés à des cellules tumorales par la transferrine sérique, la protéine de transport du Fe(III), ces récepteurs étant surexprimés dans les cellules cancéreuses. Les complexes organométalliques de Ru(II) avec des arènes présentent également une activité anti-cancéreuse prometteuse et des complexes de Ru(III) avec des ligands EDTA ont été étudiés comme piégeurs d'oxyde d'azote (NO) pour le traitement du choc septique. [31]

Afin d'améliorer l'activité et de réduire les effets secondaires chez les agents anti-cancéreux, le Laboratoire de Synthèses Métallo-Induittes a mis au point des composés dérivés du ruthénium (RDC) qui contient un atome de carbone lié de façon covalente au métal, ainsi qu'un ou deux atomes d'azote coordinées au Ru qui présentent des activité anti-tumorales remarquables *in vitro* et *in vivo*. [34,35,36,37,38]

Par conséquent, le laboratoire a longtemps concentré son activité de recherche pour déterminer les propriétés des composés organométalliques dérivés de la réaction de cyclométallation. Les composés obtenus par cette procédure ont montré de nombreuses propriétés fascinantes dans plusieurs domaines de la chimie, de la physique ou de la biologie. En effet, ces composés se sont avérés afficher *in vitro* et *in vivo* des propriétés cytotoxiques contre plusieurs lignées de cellules tumorales à un niveau suffisamment significatif pour qu'ils puissent être considérés comme des candidats intéressants pour devenir des médicaments anticancéreux. C'est pour toutes ces raisons que nous avons choisi de continuer à étudier ce

[32] Clarke, M. J.; Zhu, F.; Frasca, D.R. *Chem. Rev*. **1999**, *99*, 2511–2533.
[33] van Rijt, S. H.; Sadler, P. J. *Drug Discov. Today*, **2009**, *14*, 1089-1097.
[34] Gaiddon, C.; Jeannequin, P.; Bischoff, P.; Pfeffer, M.; Sirlin, C.; Loeffler, J. P. *J. Pharmacol. Exp. Ther*. **2005**, *315*, 1403−1411.
[35] Leyva, L.; Sirlin, C.; Rubio, L.; Franco, C.; Le Lagadec, R.; Spencer, J.; Bischoff, P.; Gaiddon, C.; Loeffler, J. P.; Pfeffer, M. *Eur. J. Inorg. Chem*. **2007**, 3055−3066.
[36] Meng, X.; Leyva, M. L.; Jenny, M.; Gross, I.; Benosman, S.; Fricker, B.; Harlepp, S.; Hebraud, P.; Boos, A.; Wlosik, P.; Bischoff, P.; Sirlin, C.; Pfeffer, M.; Loeffler, J. P.; Gaiddon, C. *Cancer Res*. **2009**, *69*, 5458−5466.
[37] Fetzer, L.; Boff, B.; Ali, M.; Xiangjun, M.; Collin, J.-P.; Sirlin, C.; Gaiddon, C.; Pfeffer, M. *Dalton Trans.* **2011**, *40*, 8869−8878.
[38] Vidimar, V.; Meng, X.; Klajner, M.; Licona, C.; Fetzer, L.; Harlepp, S.; Hébraud, p.; Sidhoum, M.; Sirlin, C.; Loeffler, J. P.; Mellitzer, G.; Sava, G.; Pfeffer, M.; Gaiddon, C. *Biochem. Pharmacol*. **2012**, *84*, 1428−1436.

type de complexes, dans le but d'acquérir de nouvelles connaissances dans ce domaine de la recherche.

Chapitre II.2- Un autre éclairage sur la labilité des ligands MeCN de composés cytotoxiques cycloruthénés [39]

Nous avons récemment découvert [40] que le composé qui a été nommé RDC11 ([Ru(2-C_6H_4-2′-py-κC,N)(Phen,*trans*-C)(MeCN)$_2$]PF_6 (où 2-C_6H_4-2′-Py-κC,N = 2,2'-phénylpyridine cyclométallée = PhPy ; Phen = phénanthroline), avec lequel notre groupe a concentré la plupart de ses études biologiques jusqu'à présent, et qu'on a longtemps considéré comme devant être thermiquement stable et non-réactif (comme aucune réaction de substitution des ligands MeCN n'ont été observées), pourrait en fait être isomérisé, soit par voie thermique, soit pas voie photochimique.

En effet, l'irradiation en UV ou en lumière visible et / ou le traitement thermique de RDC11 ont eu une conséquence dramatique sur sa stéréochimie puisqu'ils ont conduits à une nouvelle espèce, RDC11-*cis*. Dans ce nouveau composé, l'un des atomes d'azote du ligand bidentate Phen est déplacé d'une position *trans* par rapport à C, à une position *cis* par rapport au même atome, et où la position libérée *trans* à C a été occupé par un ligand acétonitrile (MeCN).

La propriété d'isomérisation du RDC11 est très intéressante car il nous a conduit à proposer de nouvelles façons d'obtenir des composés tris-hétéroleptique tels que [Ru(PhPy)(Phen)(N^N)]PF_6, dans lequel un atome N du ligand N^N est toujours et exclusivement *trans* à C.

Suite à la découverte du nouveau composé, à savoir RDC11-*cis,* nous avons entrepris une recherche visant à satisfaire notre curiosité sur la chimie du RDC11, son stéréoisomère et des composés dérivés de ce dernier.

Cette étude nous a permis de confirmer l'importance de l'effet *trans* affiché par un atome de carbon lié à un Ru. En effet, il peut non seulement déplacer un MeCN ou un ligand

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[39] Le présent chapitre présente nos derniers résultats publiés récemment au *Inorganic Chemistry*:
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[40] Boff, B.; Ali, M.; Alexandrova, L.; Espinosa-Jalapa, N. A.; Saavedra-Díaz, R. O.; Le Lagadec, R.; Pfeffer, M. *Organometallics* **2013**, *32*, 5092−5097.

PR_3, mais également (bien que moins facilement) un ligand chélatant. Ce comportement doit être pris en compte lors de l'étude du comportement de ces composés dans les milieux biologiques. Les déplacements de ligands que nous avons étudiés ici conduisent, dans la plupart des cas, à des composés thermodynamiquement stables avec un ligand MeCN *trans* à un atome de carbon. Il se pourrait cependant que cette position devrait maintenant être facilement occupée par des ligands qu'on trouve dans les milieux biologiques. En effet, il a déjà été montré [41] qu'en irradiant une solution de RDC11 avec une lumière visible, on observe une amélioration significative de sa cytotoxicité, de plus le nouveau photoproduit RDC11-*cis* formé *in situ* a montré une affinité beaucoup plus élevée pour l'ADN que son précurseur.

Chapitre II.3- Complexes de ruthénium vectorisée avec *Affitine* pour le traitement du cancer - Contributions à la détermination de leur mécanisme d'action [42]

La vectorisation de substances qui présentent déjà une bonne activité anticancéreuse est une bonne alternative pour réduire au minimum leurs effets toxiques en ciblant sélectivement les tissus cancéreux touchés. Nous avons adopté cette proposition en utilisant une protéine d'affinité nommée *Affitine* pour vectoriser les complexes de ruthénium visant à les diriger vers les cellules cancéreuses.

L'affitine (commercialement appelé Nanofitine®) est une classe de protéines artificielles qui lie les polypeptides avec une spécificité et une affinité sélectionnés. Ils sont dérivés des protéines de liaison de l'ADN (Sac7d) qui ont été découvert dans des conditions naturelles extrêmes (pH 2 et 85 ° C) dans les geysers du Parc National de Yellowstone, ainsi les *Affitines* dérivés portent des propriétés de ses liants avec une grande stabilité à de larges gammes de pH, de température et vis à vis des produits chimiques, en gardant sa haute spécificité et de l'affinité. [43,44]

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[41] (a) Sears, R. B.; Joyce, L. E.; Ojaimi, M.; Gallucci, J. C.; Thummel, R. P.; Turro, C. *J. Inorg. Biochem.* **2013**, *121*, 77−87. (b) Palmer, A. M.; Peña, B. R.; Sears, R. B.; Chen, O.; El Ojaimi, M.; Thummel, R. P.; Dunbar, K.; Turro, C. *Philos. Trans. R. Soc. A* **2013**, *371*, 20120135-1−20120135-10.

[42] Les résultats présentés dans ce chapitre sont les fruits de la coopération entre le Laboratoire de Chimie et Systhèmique Organo-Métalliques (Université de Strasbourg) et Affilogic, une société de biotechnologie privée spécialisée dans la classe des protéines d'affinité, à savoir Affitin. Nous sommes également reconnaissants au Dr. Myriam SEEMANN (Université de Strasbourg) et leur personnel pour leur collaboration dans la synthèse des conjugués RDC-Affitine et au Dr. Hélène NIERENGARTEN et le Dr. Cécile PERRET (Université de Strasbourg) pour tous les analyses par spectrométrie de masse et leurs interprétations.

[43] Mouratou, B.; Schaeffer, F.; Guilvout, I.; Tello-Manigne, D.; Pugsley, A. P.; Alzari, P. M.; Pecorari, F. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 17983-17988.

[44] Huet, S.; Gorre, H.; Perrocheau, A.; Picot, J.; Cinier, M. PLoS ONE. **2015**, *10*, e0142304.

En effet, ces protéines sont de puissants mimétiques des anticorps, montrant une forte affinité et une spécificité pour les biomolécules, et démontrent également de nombreux petits attributs de la molécule analogue à une telle extrême robustesse, environ 20 fois plus petite que la taille d'un anticorps monoclonal et une meilleure pénétration dans les tissus. [45] Ainsi, les propriétés intrinsèques de cette classe de protéines d'affinité peuvent être appliqués par d'autres voies que par voie parentérale, comme l'administration topique (peau et cornée), jusqu'à voie orale. De plus, ils peuvent être conçus pour agir soit comme inhibiteurs ciblés soit comme vecteurs de transport spécifique. [46]

Les affitines peuvent être modifiées de sorte qu'elles puissent présenter, par exemple, une affinité accrue pour les enzymes oxydo-réductases impliquées dans le métabolisme des cellules cancéreuses, ce qui aurait pour conséquence la possibilité de diriger les protéines modifiées par le ruthénium jusqu'à leurs probables sites d'action et ainsi d'augmenter significativement la sélectivité de nos molécules cytotoxiques. Ainsi, nous avons développé des conjugués innovants RDC-Affitin, à savoir une affitine lié à des complexes de ruthénium. Ils peuvent combiner les propriétés de transfert d'électrons des composés organométalliques aux hautes propriétés de stabilité et de sélectivité des affitines.

Ce travail a été élaboré pour nous donner deux types de résultats: le premier résultat escompté est la production de nouveaux composés anticancéreux qui sont plus spécifiques et plus actifs pour la cellule cible sélectionnée, avec la réduction des effets toxiques sur les cellules saines. Le second type de résultat se situe à un niveau plus fondamental: en effet, grâce à ce travail, nous voulions améliorer notre compréhension du mécanisme d'action des complexes de ruthénium organométalliques comme agents anticancéreux.

Nous avons donc préparé deux complexes (voir schémas 1) dont l'un est un composé monocationique dit cyclométallé contenant une liaison C-Ru, (Y = C, soit NCN-Ru-N_3) et l'autre est un composé analogue où Y = N (NNN-Ru-N_3) dicationique. Le premier composé est bel et bien cytotoxique, présentant un IC50 < 1μM vis à vis de cellules cancéreuses, alors que le deuxième ne l'est pas (IC50 > 100 μM). Le résultat du couplage de ces deux composés avec une nanofitine fonctionnalisée par un acétylénique terminal est très différent, puisqu'avec le deuxième composé on obtient le composé couplé présentant une masse moléculaire conforme à ce qu'on attend, alors que le premier composé évolue rapidement après couplage vers des oligomères de la nanofitine (la masse moléculaire des oligomères étant 2 à 4 fois celle attendue pour le produit de couplage).

[45] <http://www.affilogic.com >. Consulté en fev 2016.
[46] <http://www.affilogic.com/nanofitin-therapeutics>. Consulté en fev 2016.

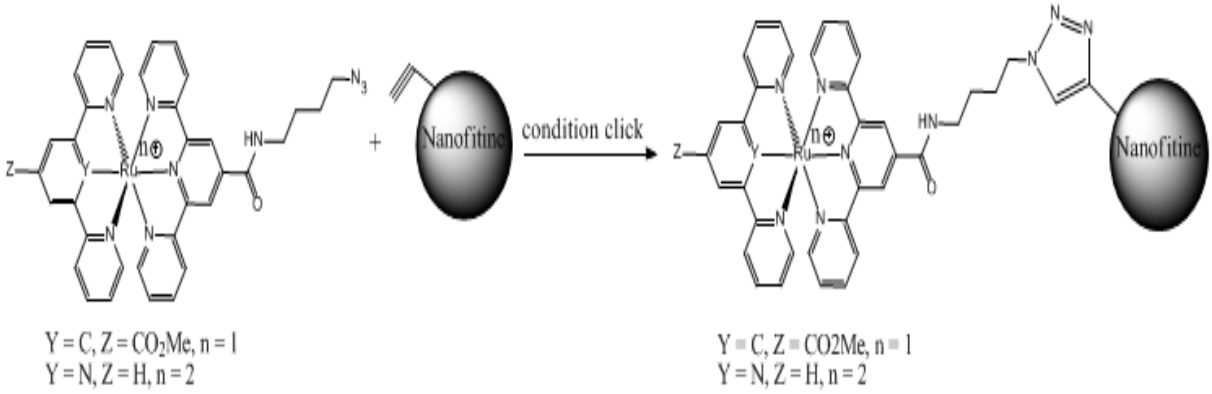

Schéma 1. Conditions de la réaction click : Nanofitine® (1 eq.), complexe de Ru (3 eq.), $CuSO_4$ (1,6 eq.), THPTA (8,3 eq.), ascorbate de sodium (20,8 eq.), aminoguanidine (41,6 eq.), dans tampon phosphate pH 7.

Ce résultat comporte un aspect négatif en cela qu'il bloque la possibilité d'utiliser cette réaction de couplage pour obtenir un anticancéreux transférable vers les cibles biologiques qu'on envisageait. Cependant, ce résultat inattendu nous oriente vers une nouvelle piste du mécanisme d'action des anticancéreux cyclométallés. Il se pourrait en effet que ces composés qui ont un potentiel redox très abaissé par rapport aux composés correspondants puissent provoquer la polymérisation de protéine par transfert d'électron entre le complexe du ruthénium et les protéines, provoquant ainsi un stress du réticulum des cellules cancéreuses. Ce stress du réticulum a déjà été mis en évidence par les biologistes qui travaillent sur le sujet et la suite des recherches qui vont être entreprises vont se pencher sur cet aspect particulier et inédit de l'élucitation du mécanisme d'action anticancéreux des composés du ruthénium.

Conclusion Générale

L'objectif de cette thèse a été d'étudier les différentes approches dans le domaine de la Chimie Inorganique Médicinale, en développant de nouveaux composés bioactifs de l'étain et du ruthénium, et aussi en contribuant à la découverte d'informations originales sur le mécanisme d'action des composés à base de ruthénium contre les cellules cancéreuses.

Ainsi, nous avons développé des composés d'étain provenant de trois acides gras bioactive (acides undécylénique, ricinoléique et caprylique) et nous avons constaté qu'ils montrent une activité très élevée contre certaines souches de bactéries et de levures. De plus, nous avons observé que les chlorures organostanniques adoptés en tant que source de fragments organostanniques ont également présenté une activité antibactérienne, mais, comme on le sait, la plupart d'entre eux étaient très toxiques contre les cellules de mammifères.

De plus, la plupart de nos complexes sont beaucoup plus actifs que les acides gras antimicrobiens utilisés en tant que ligands, même dans des gammes nM allant jusqu'à quatre

mille fois plus puissant contre *S. aureus* que contre des cellules de mammifères (macrophages J774). Ces résultats nous ont permis d'espérer être sur la bonne voie pour surmonter les défis pour développer de nouvelles alternatives dans le domaine antimicrobien en utilisant des approches prévues par le Chimie Inorganique Médicinale.

Pour les composés du ruthénium, à son tour, nous avons confirmé que le mode d'action du RDC11 est sans doute différent des composés de cisplatine ou d'autres composés du ruthénium déjà testé cliniquement, du fait de sa grande stabilité à l'égard des réactions de substitution (en l'absence de chaleur ou de la lumière). Par conséquent, nous pouvons conclure que ses interactions avec l'ADN, par exemple, sont nécessairement différentes de celle observée pour le cisplatine et que très probablement d'autres mécanismes d'action de son activité anticancéreuse devrait exister.

Enfin, pendant la vectorisation des composés dérivés du ruthénium avec la protéine d'affinité appellée *Affitine* nous avons pu acquérir des connaissances importantes sur un éventuel mécanisme d'action de ce type de molécules. En effet, nous avons obtenu avec succès des produits RDC-Affitine-vectorisés en utilisant la cycloaddition azide-alcyne (aussi appelée réaction click), une métodologie récente mais bien connu catalysée par le cuivre (CuAAC) entre un azido et un alcyne terminal. Toutefois, lorsque nous avons fait réagir notre NCN-Ru-N_3 avec l'affitine fonctionnalisée nous avons observé une précipitation non prévue des protéines oligomérisées. Par conséquent, à partir de cette découverte fortuite, nous pourrions proposer que, en plus d'interférer sur les échanges électroniques dans les enzymes redox, les composés de ruthénium peuvent également promouvoir des agrégations de protéines dans les cellules cancéreuses, ce qui induit la mort cellulaire par la voie de l'UPR, par exemple, comme nous l'avons observé précédemment dans les cellules cancéreuses traitées par le RDC. Cependant cette hypothèse doit être confirmée par d'autres études approfondies, par lequel nous devons obtenir plus de perspicacité dans le rôle exact des composés de ruthénium sur la polymérisation de la protéine observée.

De cette façon, j'espère vivement que cette thèse sera motivante pour les autres pharmaciens et chimistes afin de les inciter à utiliser leurs compétences pour améliorer les connaissances sur la Chimie Inorganique Médicinale, en particulier le développement de nouveaux médicaments et la recherche pour comprendre le mécanisme d'action au niveau moléculaire des composés à base de métaux.