UNIVERSITÀ DEGLI STUDI DI SALERNO

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Synthesis of novel ligands for the stabilization of organometallic complexes having potential antitumor activity

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Abstract

Synthesis of novel ligands for the stabilization of organometallic complexes having potential antitumor activity

The design of new metal complexes as anticancer agents has received considerable interest in recent years. Complexes of titanium (e.g.: titanocene dichloride), lanthanides complexes (e.g.: texaphyrrins lanthanide) and carbenic complexes of gold, silver and copper showed significant biological activity, and have progressed into clinical trials.

Thus, the target of this PhD project are the synthesis of new ligands and metal complexes. Firstly 5 cyclopentadienyl pro-ligands were synthesised: *6-phenylfulvene*, *6-(p-metoxyphenyl)-fulvene* e *6-(3',4'-dymethoxyphenyl)fulvene*, *6-(3',5')-dymethoxyphenyl)fulvene* and *6-(2',4')-dymethoxyphenyl)fulvene*. Then, the synthesis of 12 novel scandium, yttrium and neodymium complexes with these cyclopentadienyl ligands was carried out.

The complexes were tested on DU146 (Prostatic carcinoma) and MDA.MB213 (Breast cancer) to verify inhibition of cell-proliferation, using MTT test with standard procedures. All the complex showed a strong concentration-dependent ability of inhibiting the growth tumor cell, referring to antiblastic activity.

In the last years the synthesis of new carbenic ligands (*N-methyl-N'-[2-hydroxycyclopentan]-4,5-dychloroimydazole iodide*, *N-methyl-N'-[2-hydroxycyclopentan]-4,5-dyphenylimydazole iodide, N-methyl-N'-[(2-hydroxy-2-phenyl)ethyl]-imydazole iodide)* and 8 new complexes of gold, silver and copper was carried out.

The complexes were tested on MCF-7 (human mammary carcinoma expressing the estrogen receptor ERα/ER-positive), MDA-MB-231 (human mammary carcinoma not expressing the estrogen receptor/ER-negative), MCF-10 (breast glandular epithelium), using MTT test with standard

procedures. All the molecules showed a good inhibitory effect on the proliferation of two cancer cell lines. Many of them did not showed na any inhibitory effect on healthy cell. Instead, some compounds showed a only a mild effects only at very high concentrations.

Then, it was investigated if the antiproliferative activity of the complex AuL20 on MCF-7 was connected to the mechanisms of regulation of the cell cycle. Therefore, it was evaluated the level of expression of two proteins involved in the regulation of the cell cycle, p53 and p21 with *immunoblotting*, using β-actina as "loading control". Results showed a marked modulation of the expression of p21 and p53, confirming that AuL20 can stop cell proliferation between the G1 and S phase.

Chapter I

Introduction

Introduction

Biomedical inorganic chemistry has been increasingly developed in recent years becoming an important area of chemistry. The term "bioinorganic" includes a semantic contradiction that it seems to reflect a misconception going back to the beginning of modern science.

In 19th century chemistry was still divided into two groups: organic and inorganic chemistry. "Organic" chemistry included only substances isolated from organisms, while "inorganic" chemistry was referred to dead matter. In 1828 this distinction between inorganic and organic chemistry became meaningless when Friedrich Wöhler discovered the conversion of ammonium cyanate into urea. [1]

Nowadays organic chemistry is the chemistry of hydrocarbons and their derivatives. It consists of non-metallic hetero-elements such as N, O and S. "Biochemistry" becomes the new term to designate the chemistry of living organisms, independent from the origin of matter. For a long time biochemistry was concerned mainly with organic compounds. The improvement of trace analytical methods has also demonstrated the importance of some inorganic elements in biochemical processes and it has revealed a multitude of partially inorganic natural products such as:

- Metalloenzymes (ca.40% of the known enzyme), especially oxidoreductase (Fe, Cu, Mn, Mo, Ni, V) and hydrolase (e.g. phosphatases: Zn, Mg, Ca, Fe).
- Non enzymatic metalloproteins (e.g. hemoglobin: Fe)
- Low-molecular-weight natural products (e.g. chlorophyll: Mg)
- Coenzymes,(vitamin B_{12}: Co)
- Nucleic acids: (e.g. DNA n-(M+)n M= Na, K)
- Hormones (e.g thyroxine, triiodothyonine: I)

- Antibiotics (e.g. ionophores: valinomycin/K)
- Biominerals (e.g. bones, teeth, shells, coral, pearls: Ca. Si)

After the 1960 "bioinorganic" chemistry became an independent and highly interdisciplinary research area. This has been possible thanks to scientific contributions such as: the detection and characterization techniques in physics; the material and specific modifications based on site-directed mutagenesis from the biology; in the agricultural and nutritional sciences the effects of inorganic elements and their mutual interdependence; in pharmacology it comes from the interaction between drugs and endogenous or exogenous inorganic substances; from medicine the imaging and other diagnostic aids and chemotherapy; from the toxicology and environmental sciences the potential toxicity of inorganic compounds, depending on the concentration.[2]
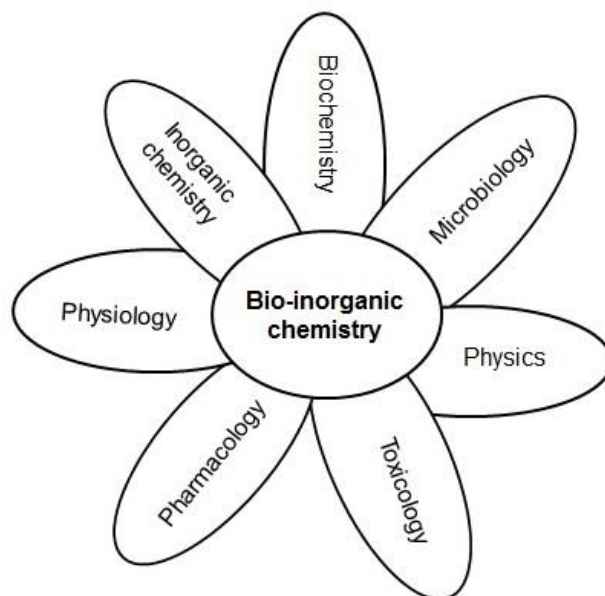
Figure 1.1. *Application of bioinorganic chemistry.*

The Bioinorganic Chemistry can be apply in many scientific fields such as:

- Industrial sector

- Anaerobic bacterial degradation in sewage plants or in sediments: Fe, Ni, Co;

- Biomining (bacterial leaching, ≈15% of the global copper production): Cu, Au, Fe, U.

- Environmental sector: agricultural trace elements problems (nitrogen fixation: Fe, Mo, V), pollution through metal species (Pb, Cd, Hg, As, Al, Cr), detoxification (e.g peroxidases(Fe, Mn, V)

- Biomedical sector: radiodiagnostics (single, SPECT, PET; radiotherapy, Tc, I, Ga, In, Re), (MRI, x-ray, Gd, Ba, I), chemioterapy (Pt, Au, Li, B, Bi, As), biominerals, "inorganic" nutrients and noxious food components, drug development.

The previous list could be expanded, but certainly the more innovative field of the "bioinorganic" chemistry is biomedicine. All diagnostic radiology analyses such as SPECT (Single Proton Emition Computed Tomography), PET (Positron Emission Tomography) and other imaging techniques such as MRI (Magnetic Resonance Imaging), x-ray, use inorganic ions. For example Gd(III) complexes can be safely inject as contrast agents in MRI. In fact contrast agents toxicity can be easily controlled by their ligand design because paramagnetic ions can be targeted to specific organs. Other contrast agent used in in radiotherapy are also Tc, I, Ga, In, Re.

In the chemotherapy medicinal inorganic chemistry offers the possibility to discover novel drugs with new mechanisms of action. After the success of cisplatin scientists have tried to synthesize new orally administrable metal complexes with reduced toxicity and active against resistant tumors.[2]

1.1 MRI Contrast Agents

Nowadays a new powerful analysis in clinical diagnosis is MRI (Magnetic resonance imaging).[3] Diseases can be detected from differences in ^{1}H NMR resonances (mainly of H_2O) between normal and abnormal tissues by administration of contrast agents. These molecules are generally external paramagnetic agents. In fact, metal ions can also characterize according to their behaviour in a magnetic field based on atomic number, mass, isotopic abundance and oxidation state. Instead the radioactive elements can be differ by their half-life and energy of isotopic decay.

Most contrast agents contain Gd(III), Mn(II), or Fe(III) ions which have a large number of unpaired electrons (7, 5, and 5, respectively, high spin) and long electron spin relaxation times.[4,5] Gadolinium, for example, is a mid-series lanthanide widely used in MRI contrast agents.[6] The electronic properties of Gd(III) is useful to enhance the clarity of MRI scans.

Generally non-physiological metals, such as the lanthanides, gallium, indium, technetium, and many others, hydrolyse, accumulate in tissues or are excreted too rapidly to be used in imaging in the absence of an appropriate ligand. A ligand is required not only to obviate accumulation of these metals but also to ensure a reasonable plasma half-life and enough time to obtain a high-contrast image. Gadolinium needs to be used with a ligand that has high thermodynamic stability and one vacant coordination position to bind the water molecule needed for MRI. The protein-binding moiety is chosen to target the chelated metal ion to the right tissue.[7]

Several Gd(III) complexes are now approved for clinical use and applied for the detection of abnormalities of the blood-brain barrier.[8]

Complexes containing DTPA (Magnevist) and DOTA ligands (Dotarem) are ionic, while BMA-DTPA (Omniscan) and HP-DOTA (Prohance) ones are neutral. Structures of Gd complexes approved for clinical uses are shown in figure 1.2.

Optimark™ (Mallinckrodt Inc., MO. USA)
Gadoverselamide
Gd-DTPA-BMEA)

Magnevist™ (Berlex, NJ, USA)
Gadopentetate dimeglumine
Gd-DTPA^{2-}

ProHance™ (Bracco Diagnostics NJ, USA)
Gadoteridol
Gd-HP-DO3A

Omniscan™ (GE Healthcare, NJ, USA)
Gadodiamide
Gd-DTPA-BMA

Dotarem™ (Guerbet, France)
Gadoterate meglumide
Gd-DOTA^{1-}

MultiHance™ (Bracco Diagnostics, NJ, USA)
Gadobenatedimeglumine
Gd-BOPTA^{2-}

Figure 1.2. *Structures of approved Gd(III) complexes.*

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Their low osmolarity decreases the pain of the injections. All these agents are extracellular, and they diffuse rapidly into the interstitial space. The Gd(III) center is nine-coordinate in each complex and it contains one bound H2O ligand. Water exchange on Gd(III) is dissociative and steric crowding at the H2O site enhances the exchange rate.

Magnevist does not enter in cells and is excreted almost exclusively by the kidney. Introduction of a benzyloxymethyl substituent on the a-C atom of a terminal acetate of DTPA as in BOPTA produces a Gd(III) complex Gadobenate (MultiHance). It enters hepatocytes and is excreted in bile.[9,10] The coordination sphere of Gd(III) in Gadobenate is almost identical to that in Magnevist (nine-coordinate, distorted tricapped trigonal prism), and both complexes have similar stabilities and relaxivities.[10]

Mangafodipir (figure 1.3), known also with the brand name of Teslascan or as mangafodipir trisodium [11] is a contrast agent delivered intravenously to enhance the contrast during the MRI. It has two parts: paramagnetic manganese (II) ion and the chelating agent fodipir (dipyridoxyl diphosphate, DPDP). The distorted octahedral complex is used in clinical diagnosis to enhance contrast in the liver and to detect hepatocellular carcinomas).[12] The relaxivity of this complex is about 35% higher than Mn complexes of DTPA and DOTA, which also does not contain directly coordinated water [13]
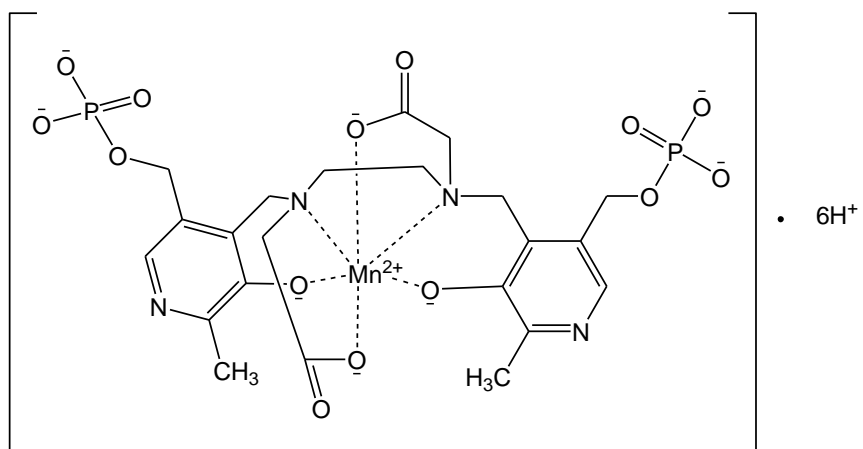
Figure 1.3. *Structures of Mangafodipir*

1.2   Radiopharmaceuticals

^{99m}Tc and ^{201}Tl, ^{111}In, ^{67}Ga, ^{51}Co, ^{51}Cr, ^{169}Yb are high intensity γ-ray emitters, ^{89}Sr, ^{153}Sm, and ^{186}Re are β-emitters. Several of these radionuclides centers are used for diagnostic imaging and for therapy[14]. Nuclear medicine has grown mainly to the availability of ^{99m}Tc radiopharmaceuticals; this isotope is used in over 80% of all diagnostic procedures.

The first generation of technetium radiopharmaceuticals was developed mainly thanks to these proprieties of the common complexes of ^{99m}Tc: absorption, distribution, metabolism and excretion. These studies led to ^{99m}Tc radiopharmaceuticals to examine the thyroid (^{99m}TcO$_4^-$), the liver (^{99m}Tc-colloids), the bone (^{99m}Tc-phosphonates) and the kidney (^{99m}Tc-DTPA). [15, 16]

The modern analytical tools, such as nuclear magnetic resonance spectroscopy, mass spectrometry and X-ray diffraction, helped researchers to understand the structure-activity relationships underlying the biological behavior of the ^{99m}Tc agents for their ability to determine the exact molecular structure of coordination compounds. Therefore, the precise design of new ligands and their ^{99m}Tc complexes led to discovery imaging agents for perfusion in the myocardium and brain. The cardiac imaging agents ^{99m}Tc-MIBI (sestamibi, Cardiolite®) and ^{99m}Tc-tetrofosmin (Mvoview®) and the brain imaging agents ^{99m}Tc-HMPAO (exametazime, Ceretec®) and ^{99m}Tc-EDC (bicisate, Neurolite®) are the results of the above strategy in the development of ^{99m}Tc complexes. [7]

Cardiolite® is used for myocardial perfusion imaging. Lipophilic cationic complexes imitate potassium ion and are taken up by the myocardium. Therefore the complex structure was designed[17] on the basis of this principle. The sequential metabolism of the six identical methoxy groups and of eight to hydroxyl groups in the liver leads to formation of ^{99m}Tc complexes with higher hydrophilicity which are not retained in myocardial tissues[18].

While Ceretec® is an approved cerebral perfusion imaging agent for evaluation of stroke. It is taken up by the brain and is transformed into more hydrophilic species which are retained in the brain.

Current designs of imaging agents are based on the careful selection of the suitable biomolecules to function as effective vectors. It concerns in vivo delivery of radioactivity to more specific biological targets such as receptors and transporters.

This strategy implies that the method of marking must not induce any distortion in the active site of the biomolecule. Thus, these agents have required the development of sophisticated marking methods that go beyond the technologies used previously.

The introduction of the bifunctional chelating agents (BFCA), the new chemistries such as the Tc-tricarbonyl, Tc-nitrido, Tc-HYNIC and mixed ligands complexes have helped to achieve this objective. [7]

Monoclonal antibodies (mAbs) conjugated with radionuclides are used clinically for diagnosis of colorectal and ovarian cancer such as ^{111}In satumomab pendetide, which contains the murine mAb B72.3 and is directed to TAG-72, an antigen expressed by many adenocarcinomas.[19] There are several other murine mAbs linked to ^{99m}Tc and ^{111}In in clinical trials.[20] Substantial progress has been made recently in the development of ^{99m}Tc-based receptor-specific radiopharmaceuticals.[21] Encapsulation in fullerenes may also provide a novel method for the delivery of radionuclides to target sites.[22, 23]

1.3 Superoxide Dismutase Mimics

Oxidative damage is an inevitable side effect of cellular metabolism and it is the cause of systemic aging and genome instabilities such as telomere shortening, mitochondrial mutation, and chromosomal pathologies. Reactive Oxygen Species (ROS) are also one of the major causes of degenerative diseases affecting the elderly. [7]

The free radical superoxide, O_2^-, is a product of activated leukocytes and endothelial cells. It is a mediator of ischemia-reperfusion, inflammatory and vascular diseases. It can react with NO to form damaging peroxynitrite, ONO_2^-.

The metalloenzyme superoxide dismutase (SOD) can destroy O_2^-:. Cu-SOD and Zn-SOD are found in the cytoplasm of eukaryotic cells, while Mn-SOD is located in mitochondria (figure 1.4)

$$M^{n+1} + O_2^- \longrightarrow M^{n+} + O_2 \quad (1)$$
$$M^{n+} + O_2^- + 2H^+ \longrightarrow M^{n+1} + H_2O_2 \quad (2)$$
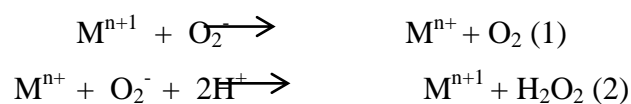
Figure1.4. *Superoxide dismutase reactions.*

MnSOD mimics have been designed for laboratory synthesis by computer-assisted modelling.

Intramolecular electron transfer requires Mn(II) in the active site of the enzyme. Mechanistic studies confirmed that during catalysis the ion can reduce binding-energy barrier and help to bind ligands in a correct geometry. A series of active Mn(II) macrocycle compounds have been designed and two are now in clinical trials.[24]

Also Catalase mimics appear to require Mn(III) in the "active site". A series of manganese complexes that are mimetic of both superoxide dismutase

and catalase have proven to be active in rodent models as synthetic catalytic scavengers of ROS (Figure).[25]

The use of these molecules in therapy is limited by their short plasma half-life because they endured clearance by the kidney and by their inability to penetrate cell membranes; in fact they have only an extracellular activity. Low molecular mass mimics of SOD are molecules with potential pharmaceutical interest.[26] For example, a variety of Mn and Fe-based on porphyrins and macrocyclic complexes exhibits SOD mimic activity.[27-30] Mn^{II} and Mn^{III} macrocycles appear particularly promising.[31, 32] For example, SC-52608 (figure 1.5) is able to scavenge superoxide and to protect effectively myocardium from injury of ischemia and reperfusion.[33]

SC-52608

X-40403

M-40401

SC-54417

MnCAM

SC-55858
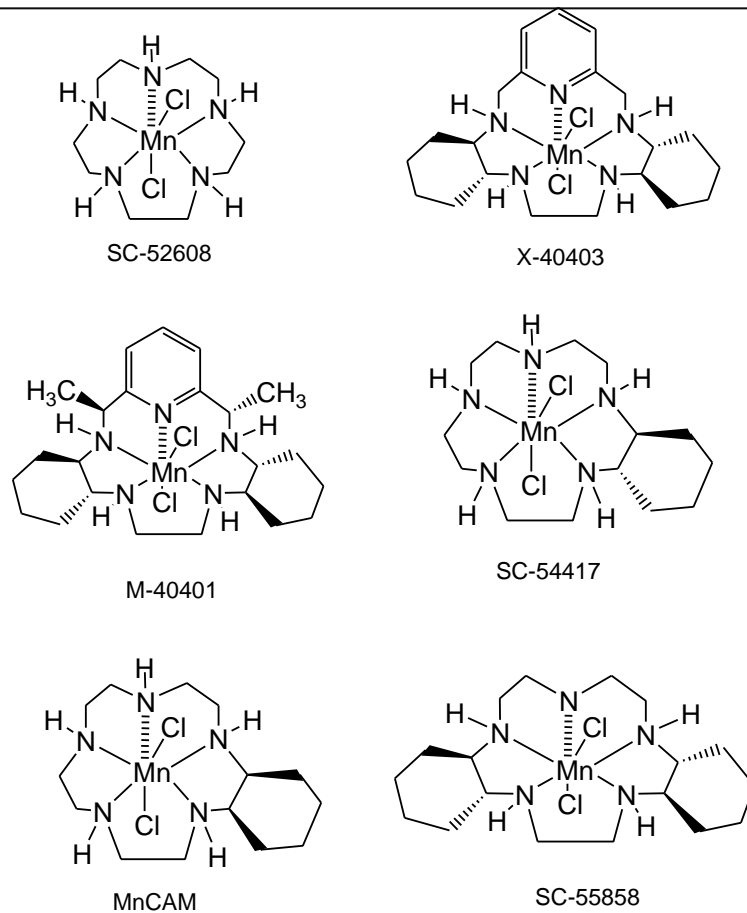
Figure 1.5. *Structures of manganese complexes tested as mimetics of superoxide dismutase.*

Instead, Manganese(III) 5,10,15,20-tetrakis(4-benzoic acid)-porphyrin (MnTBAP) can protect against neurodegeneration. It is studied for the treatment of brain diseases such as Parkinson's and Alzheimer's diseases.[34]

1.4 Platinum anticancer drugs

Chemotherapy is more widely used than surgery and radiotherapy in treatments for cancers.

Cisplatin (**23**, figure 1.6) or cis-diamminedichloroplatinum(II) (DDP) is considered the successful historical example of a metal-based drug since it was discovered by Rosenberg et al.[35] Platinum complexes are used in a lot of solid tumors such as lung tumor, colorectal tumor, ovarian tumor, head and neck cancer, genitourinary cancer and breast cancer [36, 37]

The glycolato complex (nedaplatin, **25**) and oxalato complex (oxaliplatin, *l*-OHP, **26** , which contains R,R-1,2- diaminocyclohexane, DACH) are approved for clinical use respectively in Japan and in France. Particularly they are used in multi-drugs chemotherapy for treatment of advanced lung, colorectal, and ovarian cancers.[38, 39]
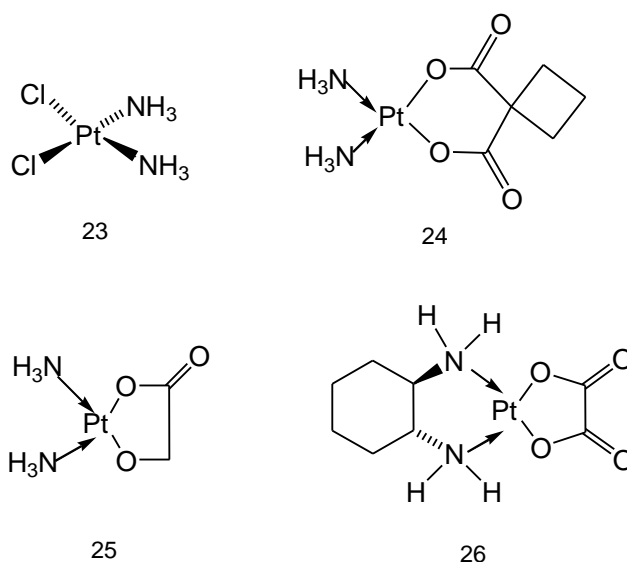
Figure 1.6. *Structures of platinum complexes clinically approved.*

Platinum derivatives are alkylating agents. Cisplatin by diffusion enters in the cells where it is converted into the active form. Scientists have not determinate the active species with great precision. They believe that the active species is the monohydrate even if the most common species is the dehydrate form. The main function of the cisplatin is to bind itself to the DNA. In fact, although cisplatin is able to interact with many types of vital proteins for DNA replication and cell division, the main target remains the DNA.

Cisplatin, Carboplatin and oxalilplatino enter in the cells thanks to an active conveyor of the CU^{2+} - the CTR1 (copper transporter 1) - causing the degradation at the same time. The extrusion of these compounds occurs by active transport mediated by the conveyors of copper, ATP7A and ATP7B, and by the MRP system1 (multidrug resistance protein 1).[40] Inside of the cells the binders chloride of cisplatin are offset by water molecules producing strongly reactive molecules. Guanine N7 is the electron-richest site on DNA. It represents the most important binding site in the nucleic acids. The complexes of platinum activated react with the nitrogen in position 7 of guanine on DNA, causing the formation of guanine-guanine crosslinks. 1,2-GpG and 1,2-ApG crosslinks are the major adducts of platinum drugs with DNA. These ones inhibit the processes of replication and transcription and lead to breakage of single and double-stranded. Moreover they determine coding errors (miscoding) which are recognized by proteins of cell cycle control determining apoptosis. The properties of these adducts have been extensively characterized and reviewed.[41]

Platinum complexes possess considerable toxicity and numerous side effects. One of the main effects is the nephrotoxicity. Cisplatin may in fact cause renal damages: tubular degeneration, necrosis and mineralization of the epithelial tubular cells. Another effect is ototoxicity that arises especially in children. Ototoxicity leads to loss of hearing capacities. The initial symptom

includes chiming (ringing in your ears). These effects should stop at the end of the treatment, but some patients lost irreversibly the use of hearing over the high-frequency range (> 4KHz).

Other toxic manifestations such as nausea, vomiting, decrease in red blood cells, tingling in the hands and feet are also common of other antitumor drugs. The neurotoxicity of the drug manifests itself particularly in high doses, while decreases in platelets, facial swelling, dyspnea (shortness of breath), muscle cramps, blurred vision and loss of appetite are less common. Nausea and vomiting occur normally about an hour after the administration of the drug and they can last for several hours.

In addition as other drugs that form adducts with DNA, platinum complexes are associated with the onset of acute myelocytic leukemia even after 4 years from the treatment.[42]

Despite the use of these compounds in many multi-drug treatments there is a large interest in developing novel compounds that exhibit innovative characteristics in respect to the existing ones. The research activity is on time for the development of novel compounds. In addition to determining a minor systemic toxicity they should ensure a marked antineoplastic activity even for the tumor lines that have proved resistant. In order to achieve these objectives we began to design and to study metal complexes which possess in their structures metal centres different from platinum.

Pt–NHC complexes have been highlighted as a promising and original platform for building new cytotoxic drugs of the cisplatin series. [43-48]

Mixed NHC–amine Pt(II) complexes were prepared through modular two-step sequence leading to trans-configured square planar species by Marinetti et al.[47]. Their efficiency against both cisplatin-sensitive (CEM and H460) and -resistant (A2780/DDP, CH1/DDP, and SK-OV-3) cell lines was demonstrated by in vitro experiment. All novel complexes exhibited cytotoxic

activities with IC_{50} at a micromolar range. IC_{50} of the measured complexes against CEM T leukemia cells was in the 0.6–2.7 mM range, generally lower than that of cisplatin (3.0 mM) under the same experimental conditions. Some of the complexes outperformed cisplatin against H460 lung cancer cells, too. This study afforded a potent and innovative new chemical platform for cytotoxic drugs of the Pt(II) series based on NHC ligands.[47]

1.5   Ruthenium complexes

The first metal that arouses the interest of the scientific community with regard to the design of complex innovative anticancer was the ruthenium. Scientists believe that the ruthenium and platinum, sharing the same group on the periodic table of the elements, have the same antitumor effect that is a direct interaction with the DNA comparable with cisplatin.

However, subsequent studies have demonstrated that complexes of ruthenium(III) have actually distinct properties from those of the platinum. These compounds tend to accumulate more and more in neoplastic cells exploiting a transport system mediated by transferrin. In this way they have a certain degree of specificity while it is absent in the complexes of platinum. In fact, the ruthenium is able to mimic the iron by binding to the protein transferrin. The cells in rapid division, including tumor cells, require a greater amount of iron. This process translates into an up-regulation of receptors for transferrin on the cell membrane and a greater uptake of these complexes from the blood. In addition, before reaching the tumor mass, complexes of ruthenium(III) are inactive and their bio-activation is performed by reduction of the metal centre to ruthenium(II), favoured by the low levels of oxygen and by the high acidity of the tumor environment.[49]

Currently, two complexes of ruthenium(III) *trans*-[RuCl$_4$(Im)(DMSO)]ImH (NAMI-A) and *trans*-[RuCl$_4$(Ind)$_2$]IndH (KP1019) are in clinical trial phase. Their structures are shown in figure 1.7.

Both complexes of Ru(III)$^+$ are formed by a heterocyclic counter-ion, heterocyclic ligands and binders chloride. Despite their structural similarities, these complexes differ greatly for their antitumor activity profile.
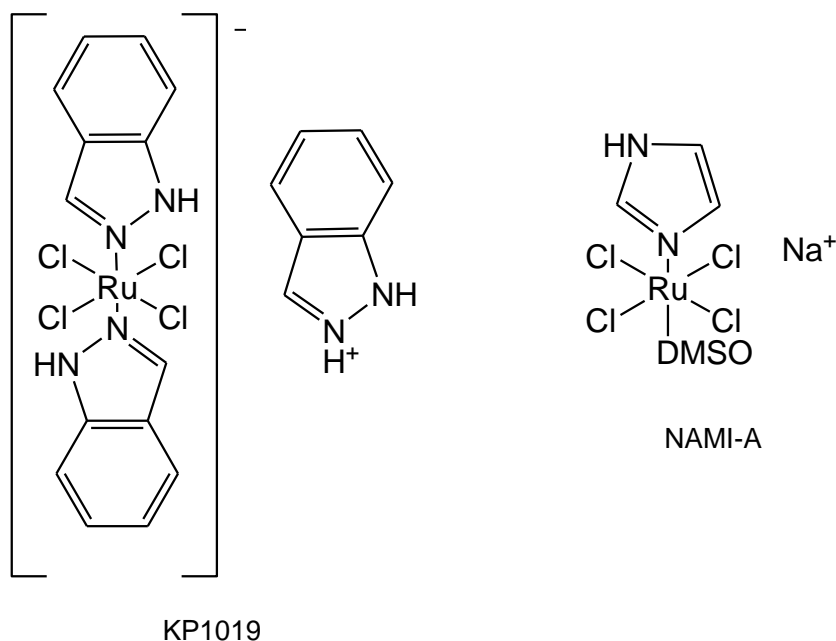
Figure 1.7. *Complexes of Ruthenium studied in clinical trials.*

Preclinical studies have shown that nami-A is able to inhibit the formation of metastases in different tumor animal models but it does not show cytotoxic direct effects. On the contrary KP1019 has shown antitumor effects directed against a wide range of xenografts tumor, through the induction of apoptosis.[50-55] The mechanisms of action proposed for the nami-A compound include: the interaction with the processes of regulation of the cell cycle (accumulation of cells in the G2/M phase); the alteration of the functions of endothelial cells required for the process of angiogenesis and the increase of the extracellular matrix around the blood vessels that irrigate the tumor and direct interaction with the nitrogenous bases of DNA. The mechanisms involved in the process of apoptosis induced by compound KP1019 include interference with the chain for the transport of the electrons, depolarization of the mitochondrial membrane and down-regulation of anti-apoptotic bcl-2

factor. The compound is also able to interact directly with the DNA, but this mechanism is not the main responsible of the antineoplastic effects of this compound. [56]

Thus, the activity of other Ru(II) complexes is currently being explored. For example, arenes are known to stabilize ruthenium in its oxidation state (2^+), therefore the potential of Ru(II)–(6-arene) complexes as anticancer agents is under investigation [55,57,58]. In literature we can found several studies about half-sandwich (6-arene)Ru(II) complexes with imidazole, sulfoxide, phosphane, chelating aminoacids, and diamine or diimine ligands. All these molecules was evaluated for cytotoxic activity. Both the size of the arene and the lability of the Ru–Cl bond have found to play a crucial role in determining the cytotoxicity of ruthenium(II) complexes of the type [(6-arene)RuCl(LL´)](PF6) with bidentate ligands LL´. Compounds with extended polycyclic arenes (e.g. tetrahydroanthracene) and LL´=ethylenediamine (en) are the most active of A2780 human ovarian cancer cells, whereas those with polar substituents on the arene such as $COOCH_3$ (an electron-withdrawing group) or with aromatic diimine ligands such as 2,20-bipyridine or 1,10- phenanthroline exhibit either poor or no activity.

1.6   Metallocene and Titanocene complexes

In recent years new anticancer drugs containing a metal different from platinum have been developed. The objectives of these research were the improving of the clinical efficacy, the reducing the toxicity and the overcoming of drug-resistance, all own limitation of platinum derivatives. Obviously the broadening of the spectrum of action of platinum complexes was a good reason for making research in this context.[59] The research has focused on transition metal complexes bearing as binders chlorides in cis or like labile ligands. Among the drugs the metallocene complexes of the type $(C_5H_5)_2MCl_2$ received many attentions because they represented a logical extension of cis-platinum derivatives [51] . These complexes are constituted by a metal core, formed by metals transition such as Ti, Nb, Mo, etc. The coordination sites of the metal are occupied by two cyclopentadienyl rings (C_5H_5 or Cp) and by two labile linking, generally chlorides (Cl). See figure 1.8
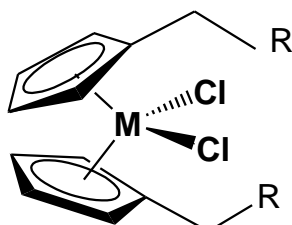
Figure 1.8. *General structure of metallocene complexes*

Köpf-Maier and coworkers[60] have investigated the antitumor activities of a whole series of metallocene dichloride complexes *in vivo*, after the variation of the transition metal. From this research, titanocene dichloride

(TDC) (figure 1.9) exhibited the most promising chemotherapeutic activity among all other metallocenes tested.[61.]

In fact, titanocene dichloride (Cp_2TiCl_2) showed medium antiproliferative activity *in vitro,* but promising results *in vivo.*[62,63]

Really titanocene dichloride was the first metal-based chemotherapeutics to reach Phase I clinical trials from the development of cisplatin. However it showed promising in these preliminary studies**,** it did not progressed beyond Phase II due to its low efficacy vs. toxicity ratio [64,65]. Unfortunately its efficiency in patients with metastatic renal cell carcinoma or metastatic breast cancer was too low to be pursued. [66,67]

The anticancer properties of cis-diethoxybis (1-phenylbutane-1,3-dionato) titanium(IV) [(bzac)2- $Ti(OEt)_2$, known as Budotitane were first reported in 1982.[68] It possesses activity towards animal tumors such as EAT and colon tumors.[69,70] Also the budotitane (figure 1.9), due to its good biological results in preliminary studies, reached Phase I of clinical trials, but it did not progress in Phase II due to formulation problems.[70] These difficulties have spurred the development of titanium complexes that display higher potency and hydrolytic stability.

Budotitane   Titanocene Dichloride
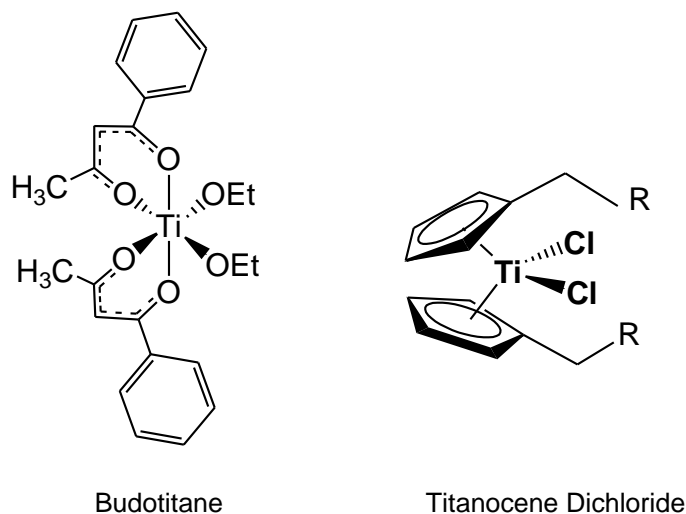
Figure 1.9. *Structure of Titanocene dichloride (TDC) and Budotitane.*

Titanocene dichloride does not bind with nucleotides and nitrogen bases, as the *cis*-platinum, but its mechanism and the biological action, as proposed by Sadler e co-workers on the basis of model studies, provides that titanium species could be complexed to phosphate and gives interaction with DNA. The titanium (IV) ion, by binding to specific sites of the iron (III), forms a strong complex with the transferrin, a protein of human plasma, and is thus supplied to tumor cells. [71]

The interesting results obtained with Cp_2TiCl_2 encouraged researches to develop new complexes of this metal which might have higher hydrolytic stability and higher cytotoxic activity.[11] In fact, in order to improve these properties of titanocene derivatives, polar side chains were attached to the Cp ligands. Titanocenes complexes, having polar substituents on cyclopentadienyl ring, such as alkoxy, amino or electron-withdrawing groups as carboxylic acid and esters, showed, in some cases, a very high activity in antitumoral tests.[72-76] Highly substituted *ansa*-titanocenes, containing a carbon-carbon bridge,

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reveal promising activity,[77-81] although, generally, higher activity was found in analogues where the two Cp units were not bound together. [82,83]

A lot of analogues of titanocene containing aromatic groups linked to the Cp have been synthesized.[84] One of the most interesting of this series, in the pharmacological field, is bis-[(*p*-methoxybenzyl)cyclopentadienyl]-titanium-dichloride (titanocene Y) shown in figure 1.10.

Its antiproliferative activity was studied in 36 human tumor cell lines[85] and in human tumors explanted.[86-89] The most significant results of these experiments *in-vitro* and *ex-vivo* had with the tumor cells of the prostate, cervix and kidney. Furthermore, titanocene Y was tested on breast cancer cell line MCF-7 revealing a promising medium-high cytotoxic activity with IC_{50} values of 76 M, quite close to those of the *cis*-platinum (37 M).[90]
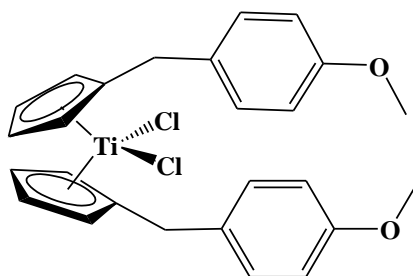
Figure 1.10. *Structure of Titanocene Y*

Furthermore, replacing the two titanocene Y chlorides with an oxalate group by a simple reaction of anion exchange, it is obtained a product that has a greater hydrolytic stability, due to the chelating effect of oxalate group and it shows an activity 13-fold higher than the titanocene Y *in-vitro* studies on the cell line LLC-PK.[91] It is worth noting that this effect was not observed in all titanocene derivatives studies.[92]

1.6.1   Metoxy-aryl and alkyl titanocenes

Recently our studies reported the synthesis and cytotoxic activity of some titanocene complexes that they have substituents on cyclopentadienyl rings able to intra-molecularly coordinate the titanium cation, stabilizing the active species. The complexes showed very interesting antiproliferative activity on several cancer cell lines.

In particular we replaced the methoxy-aryl substituent of cyclopentadienes of titanocene Y with ethenyl-methoxy in the way of having the presence of a strongly coordinating group, able to stabilize the cationic species responsible for cytotoxic activity. We also verified, by substitution of chlorine atoms with dimethylamide, oxalate, or amino groups, the influence of other ligands leaving on the activity of the complexes.[93]

Some of the synthesized compounds showed a good cytotoxicity, in particular the complex bis-ethenylmethoxyl-cyclopentadienyl-titanium dichloride (T_2) and ethenylmethoxyl-cyclopentadienyl titanium trichloride (T_1), see Fig. 1.11, on the cell line MCF-7 displayed IC_{50} values (84 and 57 µM, respectively) very close to those obtained with the cis-platinum and to that reported for the titanocene Y. Furthermore, the results of the hydrolysis of our titanocenes shown that the leaving groups (-Cl, -N(CH$_3$)$_2$, C_2O_4 or glycine) influencing the rate of hydrolysis of the ciclopentadienyl groups and thus the stability of the complexes. [93]

Therefore, the presence of substituents, aryl methoxy group on cyclopentadienyl ring in titanocene Y or ethenyl-methoxy group in titanocene T_2 or in the half-titanocene T_1, produces compounds having interesting cytotoxic activity (figure 1.11).
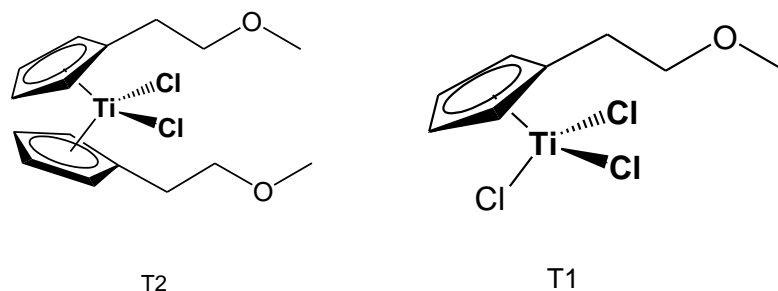
Figure 1.11. *Structures of bis-cyclopentadienyl-ethenylmethoxyl-titanium dichloride T₂ and cyclopentadienyl-ethenylmethoxyltitanium trichloride T₁*

Although generalizations regarding structure-activity relationships are not yet clear, we could hypothesize that the neutral nucleophilic substituents of cyclopentadienyl (aryl methoxy or ethenyl-methoxy group) could intramolecularly coordinate to the titanium cation, thus preventing decomposition reactions. On the other hand, this hypothesis was suggested for analogous complexes able to give polymerization of propene or styrene having microstructures strongly influenced by the possible coordination of neutral substituent of cyclopentadienyl at the metal center.[94-96]

As mentioned above, in the literature several examples of titanocene-complexes showing cytotoxic activity are reported, the cyclopentadienyl-ethenylmethoxyl-titanium trichloride represents the first example of half-titanocene complex having this interesting cytotoxic activity.

Therefore, a series of novel half-titanocenes compounds were synthesized and characterized (see figure 1.12) by nuclear magnetic resonance (NMR), mass spectroscopy and elemental analysis.
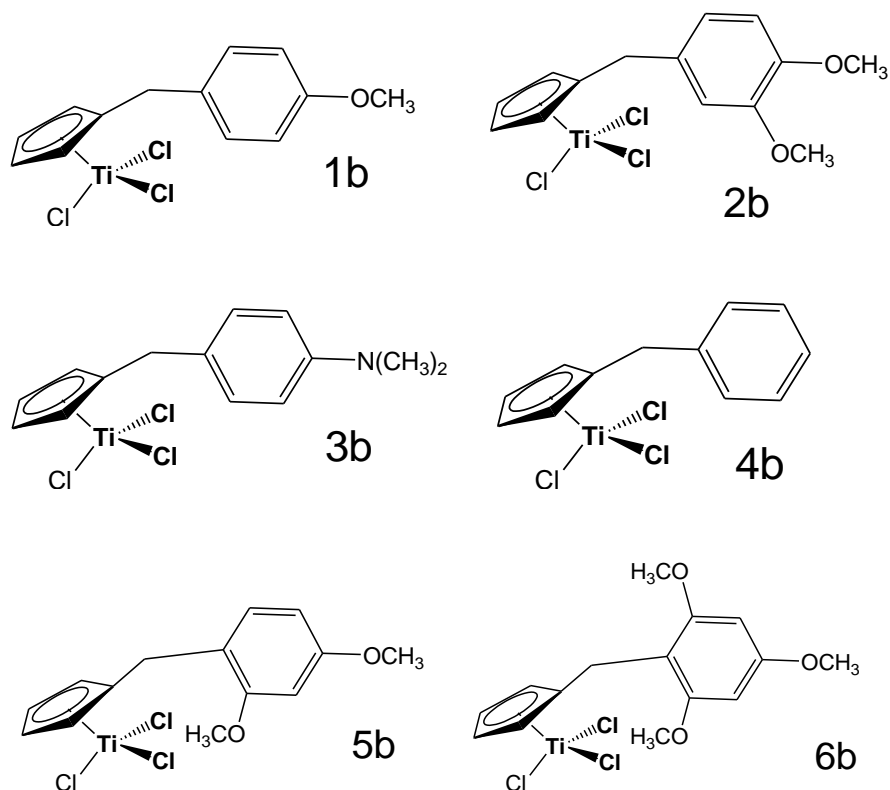
Figure 1.12. *Structures of synthesized half-titanocenes.*

These complexes had different substituents on the Cp ligands, able to stabilize the titanium cation by intramolecular coordination. Preliminary cytotoxic studies of these titanium based compounds evaluated their growth regulatory effects in MCF7 and SkBr3 breast cancer cells. Several agents showed a moderate or a high antitumor activity. For example, 4b showed the strongest antiproliferative activity against MCF7 breast cancer cells, whereas 5b showed a very interesting activity against SkBr3 breast cancer cells (see figure 1.13).

In order to understand if a different group can influence the intramolecular coordination and consequently the stability of the complexes

and if the ether group is really essential for cytotoxic activity, several new-titanocene complexes (see figure 1.14) were synthesized and characterized having as ligands the 2-cyclopentadienyl-ethoxy-benzene [Cp-CH$_2$CH$_2$-O-Ph] or the cyclopentadienyl-benzyl [Cp-CH$_2$-Ph]. Then their cytotoxic activities on human breast cancer cells MCF-7 and SkBr3 was evaluated.
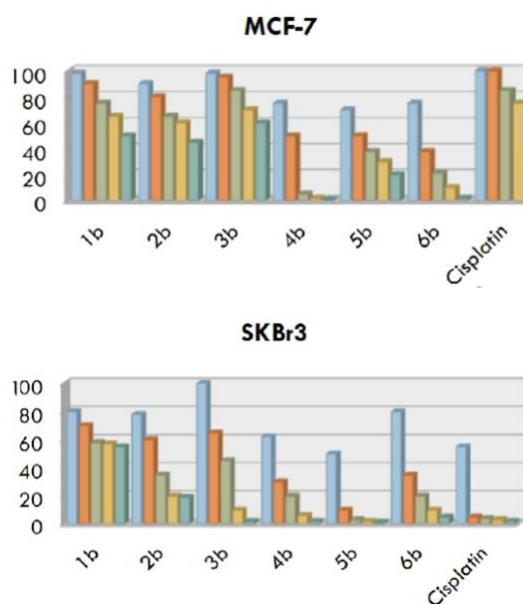
Figure 1.13. *Evaluation of growth responses to 10 μM of* **1b-6b** *in MCF7 (a) and SkBr3 (b) breast cancer cells, as determined by using the MTT assay.*

DC6

DC6-gly

DC6-oxalate

ES2

ES2-chelated
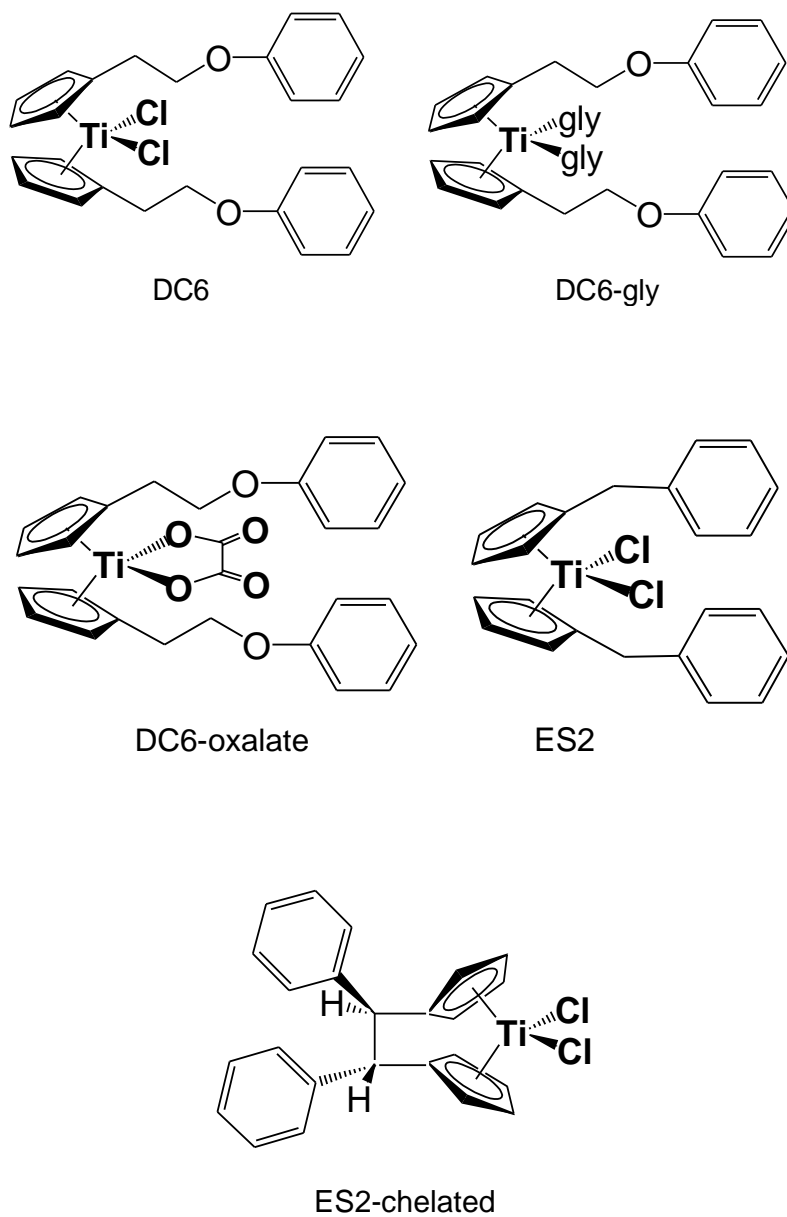
Figure 1.14: *Structures of synthesized complexes*

DC6 was synthesized to verify if a phenoxy group, instead of methoxy group present in T_2, could better stabilize the titanium cation. In fact this ligand may stabilize either with oxygen atom or with phenyl ring. DC6-oxalate and DC6-gly were synthesized to achieve different leaving groups, respect to chloride, on titanocene, which possibly could improve the activities of the complexes, by favorable pharmocokinetic properties.

ES2 was synthesized to prove if the ether group, which is present on titanocene Y, on T_1 and T_2, as well as on T_2, was really necessary to have a cytotoxic activity significantly higher than Cp_2TiCl_2, or if a more labile ligand, having higher capability to π-donation can produce a molecule as much as pharmacologically interesting. ES2-chelated could have the same properties of ES2, but this *ansa* derivative, having the two cyclopentadienyl rings covalently linked, should be less stable and possibly to have lower activity than ES2.

Preliminary cytotoxic studies of ES2 compound, in order to evaluate their growth regulatory effects in MCF7 and SkBr3 breast cancer cells were carried out. The results were very interesting because this complex showed strong antiproliferative activity on both cell lines (see figure 1.15).

Figure 1.15: *Evaluation of growth responses to 10 μM of* **ES-2** *in MCF7 (a) and SkBr3 (b) breast cancer cells, as determined by using the MTT assay.*
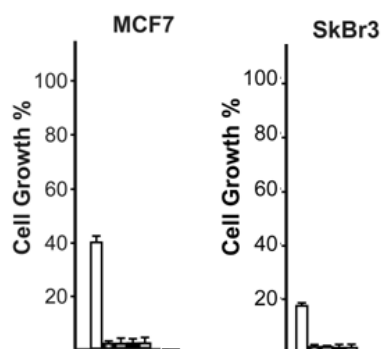
Several titanium complexes were synthesized and tested for their cytotoxic activity against cancer cell lines. Most of these compounds showed significant anti-proliferative effects compared to cisplatin

As indicated by these researches the presence of a coordinating group, such as phenyl or ether one, is necessary to stabilize the active species, delay hydrolysis and generate a cytotoxic complex; the ether groups are important to improve the hydrolysis stability but are not necessary to have a significant cytotoxic activity; a more labile ligand, as the benzyl group, having greater ability of π–donation, produce complexes with interesting cytotoxic properties; a lower activity was found in the analogues where the two Cp units were bound by a two-carbon bridge.

Half-titanocene showed lower cytotoxic activity compared to their titanocene analogues; the labile ligands (Cl) do not have a strong impact on cytotoxic activity; a methyl group on the linker between Cp ring and the phenyl group decreases the cytotoxic activity; a small steric hindrance all around the complex is favoured; the pro-ligands show interesting cytotoxic activity but are not related to complexes cytotoxicity; further studies are needed to understand the exact mechanism through which these compounds exercise their action.

Therefore, Ti(IV) complexes are good lead compounds for pharmaceutical applications.

1.7 NHC complexes in medicine.

The chemotherapy, flanked to surgery and radiotherapy, is used for the treatment of cancer. Moreover there are numerous metals (Ag, Au, Cu) with binders carbenic N-heterocyclic groups that were studied in the pharmacological field.

1.7.1 Ag-NHC complexes

Silver has been since antiquity used as antimicrobial agent, it was earlier employed in the purification of drinking water, wine and vinegar, and also Hippocrates remarked its therapeutic properties.[97] To our days the silver salts are still used for example to treat chronic ulcers, burns extended, to prevent conjunctivitis in the new-born and in so many other infections.[98]

The activity of silver against Gram-positive and Gram-negative bacteria, fungi and yeast is due to Ag^+ cations, which can interact with the cell membrane, interfere with the electron transport system of the cell and interact with thiol groups of the vital enzymes of bacteria. [99]

However the objective, in order to have a good therapeutic action antimicrobial, was to provide agents that released slowly and continuously the ion Ag^+ in the affected area. In fact, the already known salts of silver nitrate or sulfadiazine silver, for example, (Figure 1.16), while having an antibacterial topical action, expose the subject to a relapse precisely because of the rapid release of the Ag^+ and then have a rapid effect but not durable in combat infection.

Scientists tried therefore to synthesized stable complexes that could slowly release the ion in time. The choice of a suitable ligand, bonded to the metal, can make the difference.
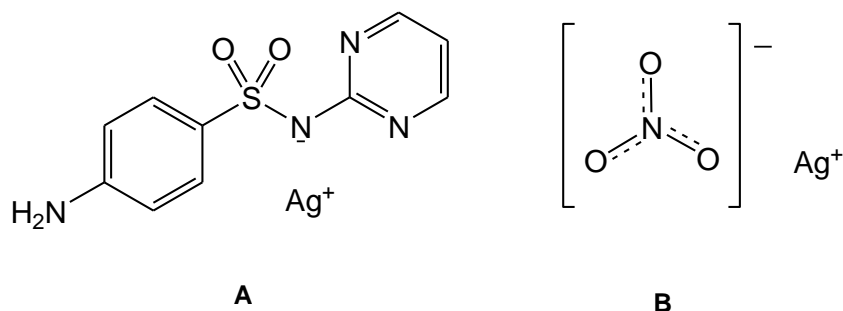
Figure 1.16. *Structures of Sulfadiazine (A) and silver nitrate (B).*

The use of the complexes Ag-NHC that show a bond C-Ag relatively strong to generate a more stable complex compared to previous silver-based drugs is the subject of numerous research.[100]

The Ag-NHC can be synthesized by three synthetic approaches. The first provides for the treatment of the NHC with appropriate "sources" of silver at the temperature of liquid nitrogen. The second consists in treating compounds of silver as Ag_2O, AgOAc or Ag_2CO_3 with the salt of the NHC at room temperature or warm. The last method envisages the transfer reaction of step in basic conditions between the silver salt and salt NHC. The second approach is the most common and used because it is more convenient too. [101]

The Ag-NHC were also tested to evaluate their antitumor properties and has been highlighted cell death by apoptosis without developing necrosis. For these complexes the suggested action mechanism provides the depolarization of the membrane potential of mitochondria with consequent and probable release of mitochondrial proteins that provide to the apoptosis. Complexes did not cause overproduction of oxygen reactive species (ROS) and activation of caspase-3, while instead occurred translocation of apoptosis inductor factor and caspase-12 in the nucleus. These events promoted the DNA fragmentation and finally cell death. Therefore, complexes were not genotoxic because they did not change the distribution of the cell cycle.[102]

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To further improve the release scientists encapsulated these silver complexes. This would also prevent the degradation at the level of the bloodstream in which there is present the chloride ion, which by interacting with Ag-NHC, it would cause the precipitation. For some complexes of Ag-NHC this encapsulation was made with poly (lactic-co-glycolic acid) combined with polyethylene glycol, and receptors of the folic acid were used to allow the delivery in a tumor site. Some of the various nanospheres prepared and loaded with complex Ag-NHC were then tested against MB157 (cell line breast cancer) and H460 (cell line of lung cancer), providing promising results.[103]

Good results are obtained with the saturated Ag-NHC and this has pushed to investigate the activity of unsaturated NHC and substituted in position 4 and 5 of the imidazole ring. In particular the effect of electron-attractor of chlorine atoms in position 4 and 5 determines a better stability of the relative complex Ag-NHC compared with a similar no-chlorinated.[104] This category of silver complexes derived from 4,5-dicloroimidazolo showed a certain degree of selectivity toward the ovarian cancer (OVCAR-3) and of the breast cancer (MB-157).

Scientists demonstrated in vivo activity of the complex 1, shown in figure 1.17, against a model of xenotransplantation ovarian cancer. Gautier Morel reported in the literature as the carbene N,N-diaryl-substituted, with its high lipophilicity, could be a good binder for metal complexes pharmacologically interesting indeed the cytotoxicity of the resultant complexes of silver was 40 times on MCF-7 and HL60 and 7 times on MCF-7R is higher than that of cisplatin.

The good obtained results pushed research in this direction and it was found that in order to obtain a high degree of accumulation in tumor cells, the aromatic rings of the metal complex must be substituted in position 2, 3 and 4

as follows: 2-F, 3-F, 4-F, 4-OH or 4-OCH$_3$. These substitutions have already been made on the complex 1,2-diariletilenediammino platinum(II) for which these complexes can be considered to be derivatives of the latter.[105]
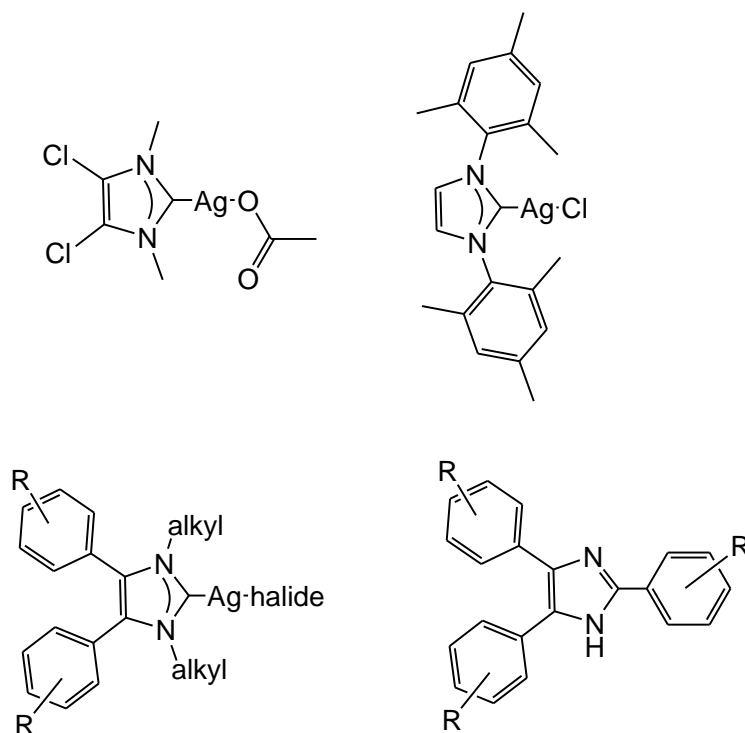
Figure 1.17. *Selected complexes of silver-NHC and 2,4,5 - triarylimidazoles.*

1.7.2   *Au-NHC complexes*

The gold was, and still is, used in various biomedical applications such as for example the treatment of rheumatoid arthritis, cancer and as antimicrobial agent.[106-118]

The two lead structures from which it started to develop new chemotherapeutic agents based on gold are the auranofin and its analogue Et$_3$PAuCl, shown in figure 1.18.
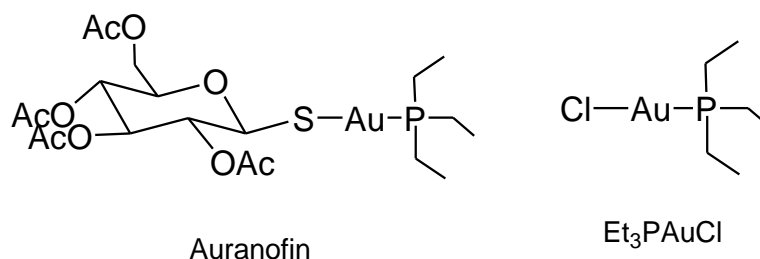
Figure 1.18. *Lead structures based on gold*.

Studies into structure-activity relationship highlighted the importance of fosfinic portion while replacing the carbohydrate or of the halide in the respective molecules does not induce loss of activity. In recent years, however, the discovery of the NHC was exploited also in the field of biology. They have proved to be effective alternatives to phosphine precursors[119], thanks to the capacity σ-donor comparable to that of the phosphine ligands and the ease with which they can be carried out the structural modifications to the nitrogen atoms .

As well as the auranofin and its analogue Et$_3$PAuCl induce apoptosis through inhibition of the enzyme thioredoxin reductase (TrxR mitochondrial), in the same way the Au-NHC cause the death of sun cancer cells.[120] This would be justified by assuming a certain affinity between the complexes based on Au and involved enzymes. The system thioredoxin/thioredoxin reductase (Trx/TrxR) is involved in the regulation of processes intracellular redox. The scheme of action general of TrxR is reported in figure 1.19.

Tioredoxine, depending on the redox potential of its substrate, catalyses the reversible reactions that involve the formation or breaking of the disulphide bonds.

Thioredoxin is involved both in the defence against oxidative stress that in apoptosis; in particular it has been postulated to the formation of the complex protein in reduced form and kinase apoptosis signal 1 (ASK1) as responsible for the inhibition of apoptosis.[121]

$$NADPH + H^+ \text{———} TrxR \longrightarrow NADP^+$$

Trx-S_2 Trx-$(SH)_2$

Protein-$(SH)_2$ Protein-S_2
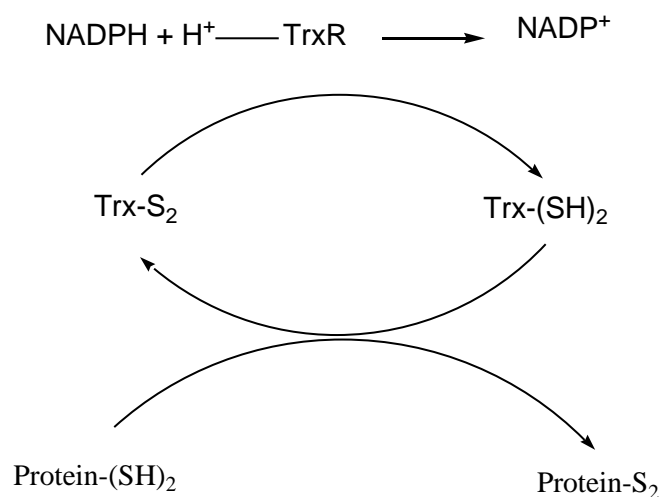
Figure 1.19. *Activities of reduttasica TrxR system/NADPH.*

The TrxR has in the N-terminal portion a peptide sequence of the type cysteine-AA-AA-cysteine and the C-terminal portion of a sequence of the type selenocysteine-AA-AA-cysteine (where AA are amino acids different from cysteine). The presence of the atom of selenium justifies the affinity of the gold atom of the complex for this enzyme and in more considering that cancer cells show an increased metabolism, you may also explain the over-expression of this enzyme in this type of cell. Consequently the selective inhibition of this

type of enzyme is one of the parameters on which it bases for developing new chemotherapeutic drugs based on gold.[122] The inactivation of TrxR, due to the formation of a covalent bond with the selenium atom of the enzyme[123], determines an increase of thioredoxin in oxidized form that induces cell death as shown in figure 1.20.
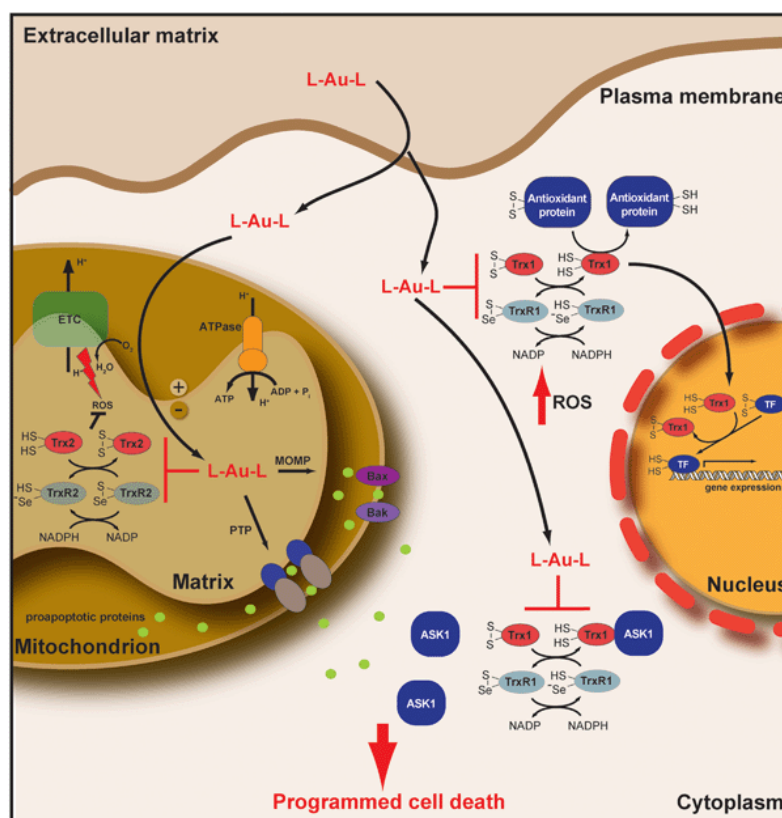
Figure 1.20. *The molecular mechanisms involved in the induction of the apoptotic process caused by the complex of gold.*

The lack of thioredoxin in reduced form involves less inhibition of ASK1 kinase for which there is the activation of the cascade of mitogen-activated protein kinase (MAPK), between which the c-Jun N-terminal kinases (JNK) and p38, which stimulates the apoptotic process. Added to this there is

the lesser capacity of TrxR to maintain in the reduced state proteins that play important roles in the cell as the Perossiredossina (Prx), favoring the accumulation of reactive oxygen species (ROS), in particular of hydrogen peroxide, and thus generating a strong oxidative stress. The oxidating environment created further contributes to the oxidation of thioredoxin and stimulates therefore indirectly the activation of ASK1 and the apoptotic process.[124]

However considering the great structural variety of binders it is not possible to define an univocal mechanism of action. In fact the gold compounds can trigger the apoptosis by various processes: mitochondrial damage that provides for the inhibition of thioredoxin reductase (TrxR) direct DNA damage, modification of the cell cycle, inhibition of the proteasome, modulation of specific kinase and other cellular processes.

An important factor for the targeting of malignant cells is represented by the possibility to adjust the lipophilicity of relevant complexes. In fact it has been shown an antimitocondrial effect which is manifested with swelling of these organelles, much faster because the greater the lipophilicity.[125]

The lipophilic cation delocalized (DLCs) can move quickly the lipid bilayer and focus on mitochondria guided by the increase of the membrane potential generated in turn from the respiratory chain. These DLCs is mainly concentrated in the mitochondria of cancer cells precisely as a result of the high potential of the membrane that is a characteristic of many tumor cells. Therefore the modulation of the lipophilicity might be an idea to generate a certain degree of selectivity toward the cancer cell than normal.

Furthermore the selectivity toward the tumor cell is an important parameter. In fact tumor cell shows an increased activity of TrxR, responsible of evasion of apoptosis. The inhibition of this enzyme can induce the cell apoptosis. The TrxR is considered an important target for this category of

drugs. Many compounds proved to be potent inhibitors of the purified TrxR enzyme, but few could inhibit it the cell.[126]

1.7.3   NHC-Cu complexes

The coordination compounds of Cu(I, II) have been investigated as potential antitumor agents only in the last few decades, particularly after the discovery of cisplatin, although copper has a long history of medical application.[127-132]

The complex Cu-NHC have been little studied from a biological point of view. The copper however, being important for the correct functionality of numerous enzymes and proteins and being involved in a series of physiological processes, should be less toxic than the other not essential metals.[133,134]

Recently a series of copper complexes was tested with regard to their own cytotoxicity and some have shown good antitumor activity both in vitro and in vivo. It has been demonstrated that the copper compounds may act through different mechanisms than those of cisplatin and the DNA would not represent the biological target main or however would not be the only molecular target for this class of molecules. In addition to the binding to DNA, the intercalation and the cleaving activity, these compounds can create damage cellular oxidative thanks to similar activities that show with superoxide dismutase and ROS generation after reaction with oxygen and intracellular peroxide. These are all events intracellular molecular that trigger the death of tumor cells through an apoptotic mechanism. Furthermore, for these copper compounds, an adjustment of the proteins antiapoptotic (Bcl-2 and Bcl-XL) has been highlighted on the tumor cells . This leads to the death of cancer cells through a programed cell death mechanism known as apoptotic paraptosi that

can be exploited for lead to death even those tumor cells with defects in the normal process of apoptosis as, for example, cells cisplatin resistant.[102]

The effect of complexes of copper-NHC reported in figure 1.21 was evaluated on the cell line MCF-7 (breast cancer).

All candidates, except the complex **14** that from a structural point of view is the most taken up, showed IC_{50} values lower than the micromolar and that of Cisplatin which was used as the reference drug (Table 1).
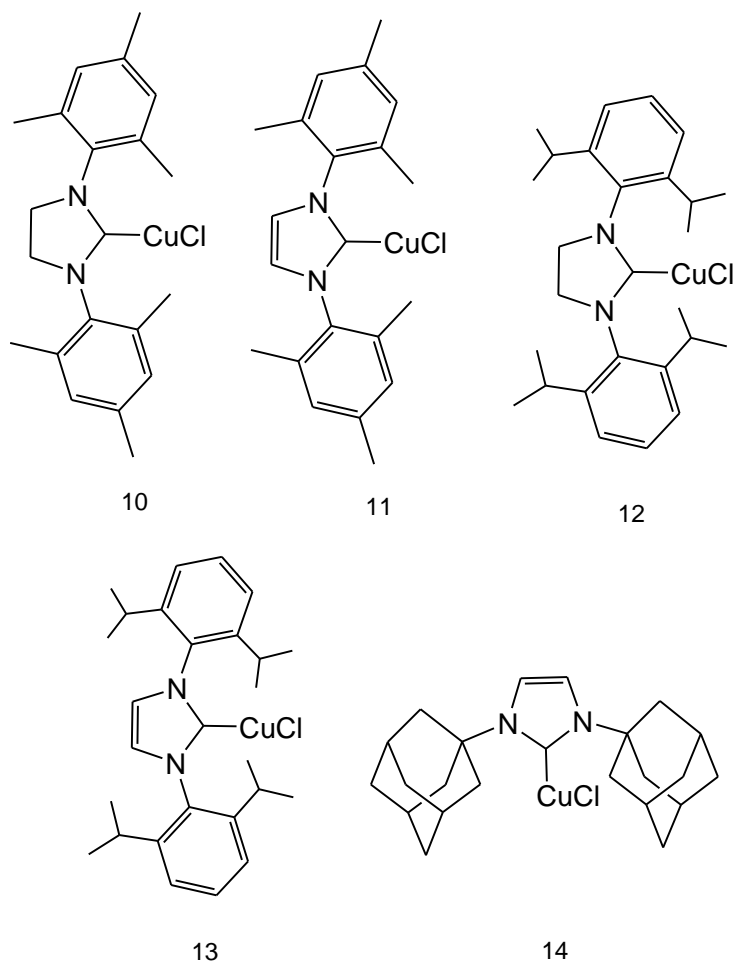
Figure 1.21. *Cu-NHC selected for biological studies*.

Table 1. *IC$_{50}$ of the complexes Cu-NHC on cell line MCF-7 (µM).*

Cisplatin	10	11	12	13	14
10,4µm	0,075µm	0.13µm	0,04µm	0,075µm	4,4µm

Due to greater activity the assembly 10 has been the object of further studies. In particular were evaluated the possible effects on cell cycle and it has been found a stop in step G1 at concentrations at least ten times lower than those of cisplatin (DDP).

Moreover, genotoxicity studies conducted using enveloped plasmids have demonstrated, as expected, that complexes of copper(I)-NHC, compared to those of palladium and silver, are the only ones capable of acting as nuclease.[104]

1.8 Mammary carcinoma

The mammary carcinoma is a malignant disease characterized by the uncontrolled proliferation of epithelial cells of the glandular tissue breast.

1.8.1 Epidemiology

Breast tumors represent, as a whole, the type of tumor most frequently diagnosed among women of all ages: 0-49 years (41%), 50-69 years (36%), ≥70 years (21%). It is the neoplasia more diagnosed in women in Italy (29% of all cancers diagnosed) and represents the first cause of oncological death.

Risk factors for the onset of breast carcinoma is associated with several risk factors:

- Age.
- Previous thoracic radiotherapy.
- Previous pathologies implants (dysplasia or cancer of the breast tissue).
- Menarche early and late menopause.
- First pregnancy at term in advanced age (> 30 years).
- Failure to breast-feeding.
- Lifestyle (obesity, poor physical activity, high consumption of alcohol, carbohydrates and saturated fats).
- Hormone replacement therapy.
- Familiarity and genetic factors: mutations of genes BRCA-1 and BRCA-2, p53, PTEN (disease of Cowden) and ATM (ataxia-telangiectasia).

Mutations of genes BRCA-1 (breast cancer susceptibility gene 1), located on chromosome 17, and BRCA-2 (breast cancer susceptibility gene 2), located on chromosome 13, are responsible of 2/3 of mammary carcinomas hereditary.[135]

The products of gene expression of BRCA1 and BRCA2 are two proteins involved in the reparative response mobile phone as a result of damage to the DNA. Mutations at the expense of these genes can determine an altered expression of these proteins or the expression of proteins with altered functionality, thus arranging the individual at the onset of neoplasias. The carcinomas related to mutations of genes BRCA-1 and BRCA-2 and familiar tend to occur at a younger age, with respect to the sporadic cases, although the increased risk related to the presence of these mutations persists throughout life.

1.8.2   TNM classification

The TNM system is the international system of classification of a tumor (Staging), which takes account of its evolution. This classification takes into account three parameters: the size of the original tumor (T), the involvement of the regional lymphonodes adjacent to the tumor (N) and the presence of metastases at distance (M).

Depending on the size of the original tumor has the classification

- T0: no evidence of the original tumor.
- T1: tumor up to 2 cm in the maximum size.
- T2: tumor comprised between 2 cm and 5 cm in the maximum size.
- T3: tumor more than 5 cm in the maximum size.
- T4: tumor of any size with direct extension to the chest wall and/or to the skin.

As regards the lymph nodes, you define N0 a condition in which the regional lymph nodes are not affected, and with a growing abbreviation N1 to N3 the progressive involvement of a large number of lymph node stations:

- N0: regional lymph nodes free of metastases.
- N1: metastases in axillary lymph nodes ipsilateral mobile.
- N2: metastases in axillary lymph nodes ipsilateral fixed.
- N3: metastases in lymph nodes subclavean or supraclavicular ipsilateral.

The presence of metastases at distance is openly by the indication M1, while M0 indicates their absence.[136]

1.8.3   Molecular Classification

The biological characterization and molecular of carcinoma of the breast has offered in the last years innovative elements as regards aspects of prognosis and treatment.

The application of molecular biology methods has made it possible to classify the breast carcinomas in five different subtypes, depending upon the expression by the cancer cells, of specific receptors:

- Luminal carcinomas: neoplasms characterized by the expression of Estrogen receptors (ER) and progesterone (PR), the absence of overexpression of the receptor HER2/neu and low proliferative index (reduced expression of the antigen Ki67).
- Luminal carcinomas B (HER2 negative): neoplasms characterized by the expression of hormone receptors, absence of overexpression of the receptor HER2/neu and intense proliferative activity (high expression of the antigen Ki67).

- Luminal carcinomas B (HER2 positive): neoplasms characterized by the expression of hormone receptors and by the overexpression of the receptor HER2/neu.

- Carcinomas HER2 positive (not luminal): neoplasms which overexpress the receptor HER2/neu and that do not express the hormone receptors.

- Carcinomas Basal-like: neoplasms characterized by failure of expression of hormone receptors and absence of overexpression of receptors HER2/neu.[137]

1.8.4   Therapy

Taking into account the fact that the mammary carcinoma can arise in different forms, the personalisation of therapy represents the biggest challenge . The choice of treatment must in fact take account of several factors which considerably influence the response to therapy, such as histological features, the molecular characteristics and the staging of the tumor. The main therapeutic strategies consist in loco-regional treatments, such as surgery and radiotherapy, and in systemic treatments of pharmacological type, such as chemotherapy, hormonal therapy and therapy with monoclonal antibodies (immunotherapy).

1.8.4.1 Surgery

In the field of surgery are distinguished destruction Surgery and conservative surgery. The destruction surgery comprises:
- Radical Mastectomy: removal in the block of the breast and of the pectoral muscles greater and lesser with complete axillary dissection.

- Modified radical mastectomy: preservation of the great pectoral muscle or both the pectoral muscles.

- Simple Mastectomy: removal of the entire breast with the areola complex-nipple, preserving the pectoral muscles and the axillary lymph nodes.

- Subcutaneous Mastectomy: removal of the mammary gland, preserving the areola and the nipple.

The interventions of conservative surgery include:

- Removal of tumor nodule with a small portion of mammary tissue surrounding healthy.

- Removal of the entire breast quadrant concerned by the neoplastic tissue. [138]

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1.8.4.2 Radiotherapy

Radiation therapy is a therapeutic strategy that consists in the use of radiation at high energy to destroy neoplastic cells and prevent its growth. The main purpose of the preoperative radiotherapy is to reduce the volume of the tumor and to prevent the seepage into the surrounding structures. In this way the radiotherapy may make operable injury otherwise unresectable not or may allow the surgeon to put in place measures less destructive. The postoperative radiotherapy, or adjuvant, is adopted instead after treatments of conservative surgery or in order to reduce the risk of relapse.

1.8.4.3 Chemotherapy

For treatment of tumors of the breast are available different chemotherapeutic drugs to be administered alone or in combination (poly-chemotherapy).

- Anthracyclines (doxorubicin and epirubicin): anticancer drugs belonging to the category of antibiotics cytotoxic. Their antineoplastic action is due to their intercalation between the nitrogenous bases of DNA and to the inhibition of topoisomerase type II. These mechanisms bring instability and DNA damage with consequent induction of the apoptotic process.

- Taxanes (Paclitaxel and Docetaxel): drugs that exert their anti-neoplastic action going to stabilize microtubules, upon binding with the subunits of β-tubulin, and preventing depolymerisation. The alteration of the dynamic equilibrium tubulin-microtubules is responsible for an altered functionality of the mitotic spindle, causing the block of cellular division.

- 5-Fluorouracil: drug belonging to the class of antimetabolites analogues of pyrimidines. The antineoplastic effect is due to a threefold mechanism of action. The fluorodeoxyuridine monophosphate (FdUMP), the ribonucleotide which is generated by enzymatic route from this analog of the uracil, inhibits thymidylate synthase, enzyme responsible for the synthesis of thymidine, interfering with the synthesis of DNA. In addition, the fluorouracil inhibits the enzyme uracil phosphatase by preventing the use of the uracil in RNA synthesis and, lastly, is incorporated, for a small fraction, in the RNA, producing an abnormal RNA.

- Methotrexate: drug that inhibits competitively reversible and the dihydrofolate reductase (DHFR), an enzyme which participates in the synthesis of the tetrahydrofolate, necessary for the synthesis of thymidine and purines. The result is the inhibition of the synthesis of DNA, RNA and, consequently , of proteins, With alteration of the functionality and cell death.

- Cyclophosphamide: a pro-drug inactive until the conversion by an hepatic oxidase P450-dependent in 4-idrossiciclofosfamide. The antineoplastic effect is due to the capacity alkylating agent of this active metabolite in respect of the nitrogenous bases of DNA, and in particular of guanine.

- Compounds of platinum (cisplatin, carboplatin): drugs capable of binding to the nitrogenous bases of nucleic acids, thus causing the formation of crosslinks (cross-link) between the two strands constituting the double helix of DNA. The result of this mechanism of action and the alteration of the structure of DNA and the loss of its functions, accompanied by the induction of apoptosis.

Combinations most commonly used in multi-drug chemotherapy clinical practice are: AC (doxorubicin and cyclophosphamide), CMF (cyclophosphamide, methotrexate and 5-fluorouracil), FEC (5-fluorouracil, epirubicin and cyclophosphamide), ACMF (doxorubicin and follow cyclophosphamide, methotrexate and 5- fluorouracil), and-CMF (epirubicin and follow cyclophosphamide, methotrexate and 5-fluorouracil), AT-CMF (doxorubicin/paclitaxel and follow cyclophosphamide, methotrexate and 5-fluorouracil), AC- paclitaxel or docetaxel (doxorubicin and cyclophosphamide and follow paclitaxel or docetaxel).

The side effects of chemotherapy depend on the fact that the cytotoxic action of these antineoplastic agents is not limited to cancer cells but also extends to healthy cells of tissues in active proliferation, such as cells of the oral mucosa and the intestinal cells of the bladder, embryonic cells and bone marrow precursors. The main side effects that occur during chemotherapy are nausea, vomiting, stomatitis, gastrointestinal disorders, alopecia and myelosuppression (reduction of the hematopoietic function of the bone marrow).[138]

1.8.4.4 Hormonal therapy

The hormonal therapy is indicated for patients with tumors hormone-responsive (ER+/PR+). It is based on pharmacological treatments adapted to interfere with the mechanisms that lead estrogens to stimulate the proliferation of cancer cells. The choice of the type of treatment to be adopted, in addition to the degree of progress of the disease depends on the fact that the woman is already entered or less in menopause. In women before menopause most of female sex hormones circulating is released into the blood by the ovaries. While in women after the menopause the ovaries no longer produce hormones, and estrogens circulating are produced by peripheral tissues of the organism (especially the adipose tissue, muscles and skin) starting from the androgens produced by the adrenal glands. The hormonal therapy can lead to various side effects, but smaller than those induced by chemotherapy fever, including: hot flushes and sweats, tiredness, articular pains, nausea, weight increase and "tumor flare", temporary exacerbation of the disease which may occur in less than a case on one hundred, in the early stages of care.

The main classes of drugs used in the hormonal therapy are represented by: Selective Estrogen Receptor Modulators or SERMS (selective estrogen

receptor modulator). This is drugs that act on the estrogen receptors exerting, depending on the fabric, a different activities and ensuring the ability to act as an agonist on certain functions or fabrics and as an antagonist on others. Belongs to this class tamoxifen, drug indicated in women in pre-menopausal women. Tamoxifen is a competitive antagonist of the receptor ER, which interrupts the stimulus to the proliferation of the cells by estrogens . Analogues of LHRH. Belonging to this class are the goserelin and tryptoreline , drugs that can be administered alone or in association with tamoxifen. Are indicated for women premenopausal, able to act more upstream of the other hormonal drugs because they block the production of the luteinizing hormone (LH), with which the pituitary stimulates tasks of the ovaries. Inhibitors of aromatase: to this class of drugs belong various substances, including anastrazole, exemestane and letrozole, capable of blocking the action of the aromatase enzyme, essential for estrogen synthesis starting from the androgens, produced by the adrenal cortex also in women.

1.8.4.5 Immunotherapy

Immunotherapy is a therapeutic approach that is based on the use of monoclonal antibodies directed against specific molecular targets and represents a pharmacological therapy targeted target (therapy) which allows to limit the side effects of chemotherapy fever. For the treatment of mammary carcinomas, monoclonal antibodies are commonly used Trastuzumab and bevacizumab. [138]

1.9 Prostate cancer

Prostate cancer is a malignant disease characterized by the uncontrolled proliferation gland cells, generating adenocarcinomas.
In addition to the adenocarcinoma, in the prostate can be also found sarcomas, small cell carcinomas and cells of transition carcinoma.[139]

1.9.1 Epidemiology

Prostate cancer is one of the more common tumors in the male population. It represents about 15% of all cancers diagnosed in man: in 2012, about 36.300 new cases were diagnosticated in Italy..[140]

One of the main risk factors for prostate cancer is the age: the possibility of becoming ill are very scarce before 40 years, but significantly increase after 50 years.Aabout two tumors on three are diagnosed in people with more than 65 years. Researchers have demonstrated that many (between 70% and 90%) men over 80 years have a cancer of the prostate, although in most cases the disease does not give signs.

Familiarity is another factor not to be overlooked. In fact, the risk of illness is double if there have any case in family. Also mutations in BRCA1 and BRCA2 genes, already involved in promoting the onset of tumors of the breast and ovary, or gene HPC1, can increase the risk of developing prostate cancer. The probability of illness could be also linked to high levels of hormones such as testosterone, which promotes the growth of prostatic cells, and hormone IGF1, similar to the insulin, but who works on the growth of cells and not on the metabolism of sugars.

1.9.2 Classification

Prostate cancer is classified by the grade of aggressiveness of the disease and the stadium, which indicates the status of the disease. Depending on the stage of the disease it is also proceeds to carry out examinations of staging as CT (Computed tomography or magnetic resonance. To check for the presence of any metastases to the skeleton often uses the bone scintigraphy. The pathologist that analyses the removed tissue with the biopsy assigns to the tumor the so-called degree of Gleason, i.e. a number between 1 and 5 that indicates how far the aspect of tumor glands is similar or different from that of normal glands.

More like are, the lower the degree of Gleason. Tumors with a degree of Gleason less than or equal to 6 are considered to be of low degree, those with 7 of intermediate degree, while those between 8 and 10 high-grade.

To define instead the stage to the tumor is typically use the TNM system. Description of the TNM systems is described in paragraphs above.

1.9.3 Therapy

Today there are many types of treatment for prostate cancer each of which presents the benefits and side effects specific. Only a careful analysis of the characteristics of the patient (age, life expectancy etc.) and of the disease (low, intermediate or high risk) will allow the specialist urologist will recommend the best strategy and custom and agreeing the therapy also according to the preferences of those who must be subject to treatment.[141]

In some cases, the treatment of cancer is called "wait" therapy. The Anglo-Saxons call watchful waiting, a "watchful waiting" that does not provide treatments, but only checks rather frequent (PSA, rectal exam, biopsy)

that allow you to control the evolution of the disease and to check for changes that deserve an intervention. This approach is used especially for elderly patients or man with other serious illnesses, or in the case of tumors of small dimensions and with low risk (micro outbreak in biopsy.

The active therapy, instead, often is a radical surgery. The radical prostatectomy - removal of the entire prostate gland and lymphonodes of the region close to the tumor. It is considered a curative intervention, if the disease is confined in the prostate. Thanks to the considerable improvements of surgical instruments, today the operation for the removal of the prostate can be carried out in a classic way (radical prostatectomy pubic back open), laparoscopically, or via laparoscopy robot-assisted.

For tumors in advanced stages, the scalpel alone is often unable to cure the illness and there is therefore the need to associate treatments such as radiotherapy or hormonal therapy. For the treatment of prostate cancer, treatments considered standard, it has been demonstrated that the radiotherapy external beam is effective in tumors of low risk, with results similar to those of the radical prostatectomy. Another radiotherapy technique that seems to offer similar results to previous in diseases of low risk is the brachytherapy, which consists in the insert in the prostate small "seeds" issuing radiation.

When prostate cancer is located in metastatic stage, unlike what happens in other tumors, chemotherapy is not the treatment of first choice and you prefer to hormonal therapy. This has the purpose of reducing the level of testosterone , male hormone that stimulates the growth of the tumor cells of the prostate, but brings with it side effects such as drop or annulment of sexual desire, impotence, flushing, weight increase, osteoporosis, loss of muscle mass and tiredness. Among the local therapies still being reviewed there are the cryotherapy (elimination of tumor cells with cold) and HIFU (focused ultrasound on tumor). There are also in the phase of experimentation in some

cases already very advanced, even vaccines which push the immune system to react against the tumor and destroy it, and drugs anti-angiogenic that block the formation of new blood vessels preventing cancer to receive the nourishment necessary to evolve and develop further.

1.10 References

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2 Chapter II

Synthesis of Group III metal complexes

2.1 Introduction

2.1.1 Group III metal complexes

Complexes of many metals in the periodic table have been studied up to now, including those of group III, which have shown a significant biological activity.[1]

Many of the pharmacological properties of the group III metals are due to their affinity for Ca^{2+} sites in biological molecules and to the formation of a stronger bond with water molecules. Moreover, they can also replace the metal ions Mg^{2+}, Mn^{2+} and Fe^{3+} in the biological processes. [1, 2]

The lanthanides can replace calcium in the protein, and so calcium dependent enzymes can be inhibited or be activated by lanthanides. It has been proposed that the stimulation or the inhibitory effect of the lanthanides may be a function of role of calcium in the native enzyme. If calcium plays a catalytic role, its substitution with a group III ion leads to the deactivation of the enzyme and the degree of inhibition depends on the ionic radius of metal. Instead, if calcium plays a structural role, the replacement with a M^{3+} ion gives, at least, the maintenance of the activity. [1, 2]

Accordingly, the mode of action of anticancer-active group III metals is related to their possible inhibition of ion function that is essential for cells cycle regulation and antitumor activity and significantly enhanced by complexation with various ligands.

In fact, lanthanide complexes with coumarins exhibited superior activity in various tumor models as compared to the corresponding inorganic salts. Coumarins are an important group of organic compounds that shows a wide variety of biological activity as antitumor and antiproliferative effects.[3-6]

Complexes of rare earth metal as cerium(III), lanthanum(III) and neodymium(III) having coumarines as ligand, were examined on different cell lines. [7]

Rogers and coworkers reported the cytotoxic activity of acenocumarol samarium(III) and gadolinium(III) complexes against melanoma B16 and fibrosarcoma L929 cell lines. [8]

Lanthanide complexes are the texaphyrins (figure 2.1), having macrocyclic monoanionic ligands containing five coordinating nitrogen atoms in the central core and they have progressed into clinical trials. [9,10]

1. Ln = Gd(III)
2. Ln = Lu(III)
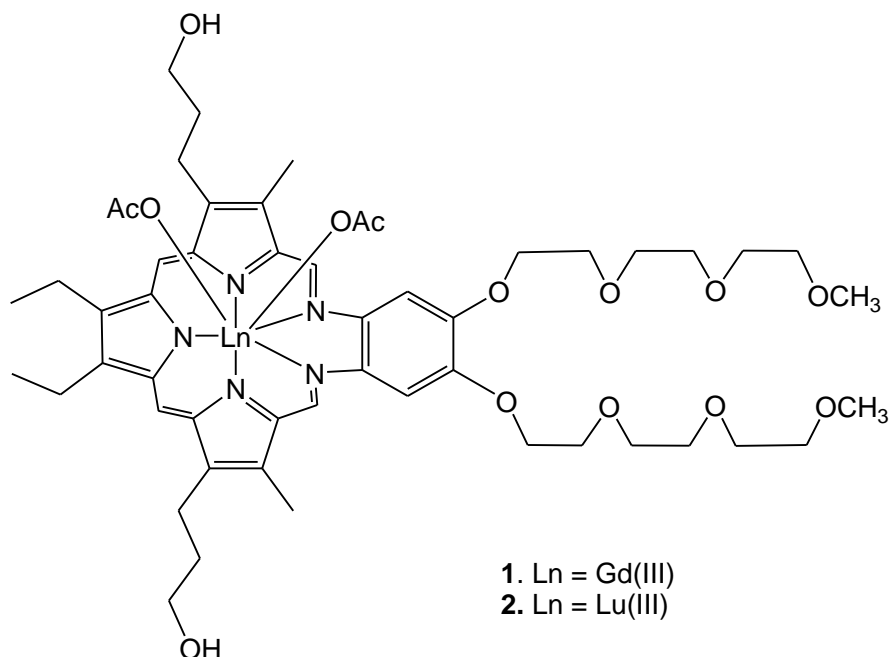
Figure 2.1. *Structures of texaphyrins complexes*

Furthermore, it has been reported that a series of cerium(III) complexes with bipyridyl or phenanthroline ligands showed an interesting

antiproliferative activity against tumoral cell lines (figure 2.2). Recently, the lanthanum complex of phenanthroline derivative (KP772) has shown to exert comparable activity to that of *cis*-platin and methotrexate against a wide range of *in vitro* tumor cell lines and a *in vivo* colon carcinoma xenograft model. [11,12]

Recently, it has been evaluated the cytotoxic activity of a significant number of new complexes of metals of group III and lanthanides with heterocyclic ligands,[13-19] and some of us have reported the synthesis and characterization of scandium(III), yttrium(III) and some lanthanide (III) complexes stabilized by tri-dentate monoanionic ligands which gave remarkable activity, and in some case were significantly more active than cis-platin (figure 2.3).
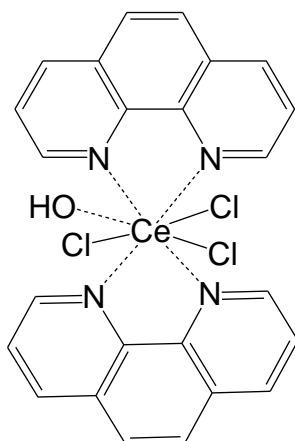
IC$_{50}$ (µm)			
41M	CH1	SKBr3	SW480
1.5 ± 0.2	1.2 ± 0.2	2.6 ± 0.4	2.7 ± 0.3

Figure 2.2. *Cytotoxicity of KP776 in Four Human Cancer Cell Lines. As determined by the MTT Assay Using 96-h Exposure.*

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ScCl$_2$(NNHO)(THF) = **1**

ScOTf$_2$(NNHO)(THF) = **4**

YCl$_2$(NNHO)(THF) = **5**

NdOTf$_2$(NNHO)(THF) = **7**

ScCl$_2$(NNO)(THF) = **2**

YCl$_2$(NNO)(THF) = **6**

SmOTi$_2$(NNO)(THF) = **8**

ScCl$_2$(NNCp)(THF) = **3**
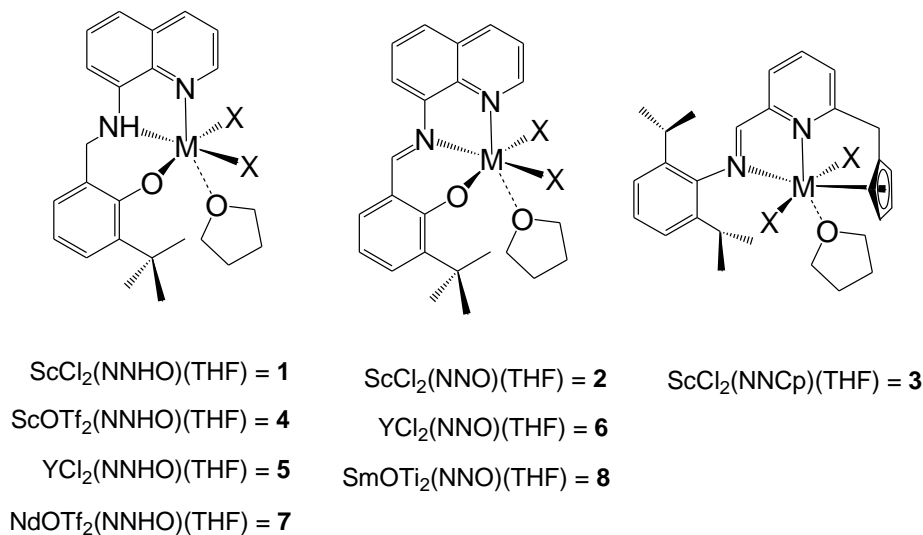
Figure 2.3. *Structures of scandium(III), yttrium(III) and some lanthanide (III) complexes stabilized by tri-dentate monoanionic ligands*

Recently, some of us have reported the synthesis and characterization of scandium(III), yttrium(III), samarium(III) and neodymium(III) chloro- or triflate-complexes with tridentate monoanionic quinoline-phenoxy-amine, quinoline-phenoxy-imine and *ansa*-monocyclopentadienyl-imino-pyridine ancillary ligands, and evaluated their cytotoxic activity on rat glioma (C6), murine fibroscarcoma (WHEI-164) and human embryonic kidney (HEK-293) cell lines.[20]

Moreover, we reported the synthesis and characterization of a series of group III and lanthanide [N,N,N]-heteroscorpionate complexes with η^3-NNN-coordination of the heteroscorpionate ligand N,N'-dicyclohexyl-2,2-bis-(3,5-dimethyl-pyrazol-1-yl)-acetamidinate (cybpamd), detailing on cytotoxic screening and further investigation of [Ln(OTf)$_2$(cybpamd)(THF)] {where Ln = Sc (**2**), Y (**3**), La (**4**), Nd (**5**), Sm (**6**), Dy (**7**), Yb (**8**); OTf = SO$_3$CF$_3$}.

Structures of these complexes are shown in figure 2.4. The complexes were tested on human epithelial lung adenocarcinoma (A549), human melanoma (A375), human epithelial cervix adenocarcinoma (HeLa), human embryonic kidney (HEK-293) and murine macrophage (J774.A1) cell lines. On murine macrophage (J774.A1) cell line all tested complexes, except that of scandium that shows a reasonable activity. On human epithelial cervix adenocarcinoma (HeLa) complexes **3**, **5** and **6** are significantly more active than cis-platinum, as well as complex **5** is more active on human embryonic kidney (HEK-293) cell line.

The continuing involvement of groups looking for absolute qualification in the field of the study of new potential anticancer agents suggests that it will be possible to further evaluate the potential of molecules already synthesized, as well as those that we are going to prepare, even on other cancer cell lines. Moreover, the study of the mechanism of action of these molecules provides valid indications also on the structural characteristics that these molecules must possess in order to best carry out their anticancer action. This will also suggest to the group that will be interested in the synthesis, the design and the development of new molecules to meet best the requirements needed to perform their pharmacological action.
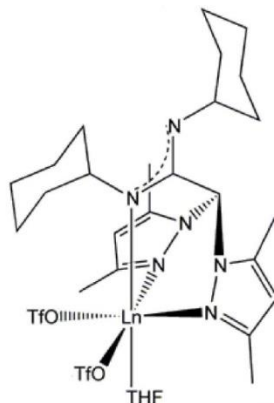
Ln = Sc (**2**), Y (**3**), La (**4**), Nd (**5**), Sm (**6**), Dy (**7**), Yb (**8**)

Figure 2.4. *Structures of lanthanide [N,N,N]-heteroscorpionate complexes with η^3-NNN-coordination of the heteroscorpionate ligand N,N'-dicyclohexyl-2,2-bis-(3,5-dimethyl-pyrazol-1-yl)-acetamidinate (cybpamd)*

Hence, the capability of this compound to elicit relevant repressive effects on cancer cell growth could be taken into account towards novel pharmacological approaches in cancer therapy. Further experiments are needed to clarify the molecular mechanisms involved in the growth responses of the compound tested as well as to evaluate it in vivo chemotherapeutic potential.

2.2 Aims of the project

The aim of this study was the synthesis and the characterization of ligands and related metal complexes by nuclear magnetic resonance (NMR) to evaluated their potential antitumor activity.

Starting from the literature, we synthesized new compounds with structural changes, adapted to investigate the role of the metal centre and of the substituents for antitumor activity. We proceeded on two fronts: modification of the metal centre and changes on the ligands.

Firstly group III metal complexes stabilized from cyclopentadienyl ligands were synthesized. The selected cp-ligands were yet able to stabilize the titanium cation by intramolecular or intermolecular coordination. Compounds were tested as anticancer agents, in particular for the topical application for the treatment of solid tumors, yet non-treatable, such as breast cancer triple negative (TN) and prostate cancer.

Hence, the stabilization of lanthanides with heterocyclic ligands having more than one donor atom was of great interest in the chemistry of coordination compounds as well as it can lead to compounds with noteworthy cytotoxic effects.

Thus, given the activities of many interesting complexes of group III with chelating ligands, we synthesized and tested on selected tumor cell lines, a series of complexes having the same ligands that used in the synthesis of titanium complexes. In fact, titanium and metals of group III should have different mechanisms of action and this could allow to have completely different specificity and selectivity of the two groups of complexes.

An interesting aspect that we intended to study,was related to significant differences in atomic radius and electronegativity for group III and rare earth metals, because it could provide some interesting specificity for

obtained compounds. In fact, these two parameters make it possible to achieve a wide variety of complexes with ligands capable of stabilize the metal ion by a coordination more or less strong, either acting as bidentate that as monodentate ligands. The latter type of coordination could make available a substituent still able to give additional interactions with target molecules.

2.2.1 Evaluation of the drug-membrane interactions

In the present study, the interaction of the antitumor agents CAM-2 and CAM-7 with biological membranes was investigated by using liposomes as cell membrane mimetic models.

The interaction of drugs with biological membranes, indeed, plays a key role in their pharmacological effects, since the site of action is often embedded within membranes[21] which act as physiological barriers in the absorption and distribution processes of therapeutic agents in the organism.

The drug's ability to interact with the membrane depends on its lipophilicity which influences the pharmacokinetic and pharmacodynamics profiles and can be correlated with the therapeutic and/or toxic effects. [22]

In the aim to evaluate the drug's hydrophilic/lipophilic equilibrium, a partition coefficient (Kp) between aqueous and lipid phases, which represents a significant parameter affecting the *in vivo* action of anticancer drugs, was determined by UV-Vis spectrophotometry employing liposomes made of egg phosphatidylcholine (EPC) as cell membrane mimetic models. Liposomes present, indeed, a membrane structure similar to the cellular one and EPC was chosen because natural plasma membranes contain a high amount of phosphatidylcholines. [23, 24]

2.3 Materials and Methods.

2.3.1   General procedure

All manipulations were carried out under oxygen- and moisture-free atmosphere, using Schleck techniques or working in an MBraun MB 200 glove-box.

2.3.2   Solvents

All the solvents were thoroughly deoxygenated and dehydrated under argon by refluxing over suitable drying agents, while NMR deuterated solvents (Euriso-Top products) were kept in the dark over molecular sieves.

2.3.3   Reagents

All chemicals were obtained from Aldrich chemical Co. and used without further purification. Cyclopentadiene was obtained by freshly cracked dicyclopentadiene.

2.3.4   Characterization techniques

^{1}H NMR, homodecoupled ^{1}H NMR, ^{1}H COSY and ^{13}C NMR spectra were recorded on Bruker Avance 250 Spectrometer operating at 250 MHz (^{1}H) and 62,5 MHz (^{13}C), on Bruker Avance 300 Spectrometer operating at 300 MHz (^{1}H) and 75 MHz (^{13}C) and on a Bruker Avance 400 Spectrometer operating at 400 MHz (^{1}H) and 100 MHz (^{13}C). Chemical shifts referred to tetramethylsilane used as internal standard.

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2.3.5 Cell lines

MDA.MB213-tumor cell line of human breast acquired from the ATCC (American Tissue Cell Cultures - ATCC® HTB-26™), triple negative for ER receptor.

DU145-human cell line of prostate tumor acquired from the ATCC (American Tissue Cell Cultures - ATCC® HTB-81™).

B16F10- tumor cell line of melanoma acquired from the ATCC (American Tissue Cell Cultures - ATCC® CRL-6475™)

2.3.6 Biological assay kit

MTT Cell Proliferation Assay Kit - purchased from TREVIGEN - Catalog # 4890-025-K.

2.3.7 Evaluation of the drug membrane interaction

2.3.7.1 Materials

Egg phosphatidylcholine (EPC) was obtained from Sigma (St. Louis, MO) and used as received.
Solutions were prepared with HEPES buffer (10 mM, I = 0.1 M, pH 7.4). The ionic strength was adjusted with NaCl. All solvents were reagent grade or HPLC-grade and provided by Carlo Erba reagents (Milan, Italy).

2.3.7.2 Liposomes preparation

Liposomes were prepared according to the thin film hydration method.
[25]

A solution of lipid (EPC) in chloroform was evaporated to dryness under a nitrogen stream. The resultant lipid film, in an amount that would provide a final lipid concentration of around 2 mM, was then left under vacuum overnight to remove all traces of the organic solvent. The resultant dried lipid film was hydrated with HEPES buffer and vortexed at room temperature in order to obtain multilamellar vesicles (MLVs). Lipid suspensions were equilibrated at 25°C for 30 min and further extruded 10 times through polycarbonate filters (Nucleopore, Pleasanton, CA) with a pore size of 100 nm, to form large unilamellar vesicles (LUVs, stock solution).

2.3.7.3. Determination of partition coefficient (Kp) by UV-Vis spectrophotometry

The partition coefficient (Kp) of CAM-2 and CAM-7 between liposomes and an aqueous phase was determined by UV-Vis spectrophotometry.

The EPC stock suspension was diluted to prepare a HEPES buffered LUVs suspension (EPC concentration 1000 μM) and contained a fixed concentration of drug (40 μM). The corresponding reference solution was prepared in the same conditions but in the absence of drug. All suspensions were incubated at 25°C in the dark for 2 h to allow drug partition equilibrium to be established. Then, the samples were placed in diafiltration tubes (Vivaspin 20 centrifugal concentrator, MWCO 3,000 Da) and centrifuged. The

UV-Vis absorption spectra of the aqueous phases were recorded with a Jasco V-530 UV/Vis spectrometer.

The corrected absorption spectra were obtained by subtracting each reference spectrum from the correspondent sample spectrum in order to eliminate the spectral interferences due to the light scattered by lipid vesicles.

Partition coefficients were then calculated by the following Equation (1):

$$K_p = \frac{n_m}{n_w} \qquad (1)$$

where n_m and n_w are the number of drug moles distributed on the lipid membrane phase and in the aqueous phase, respectively, while K_p is the partition coefficient.

2.3.8 Distillation of Cylopentadiene.

We make all the manipulation in nitrogen atmosphere. We prepare a 250 ml three-necked flask, on which we mounted a dropping funnel and a reflux condenser. On the consenser we put a head of Claisen, refreshed by current water and connected with a tailed tube where we collect the cylopentadiene distillate. We put the reaction flask in a heating hood on a plate of stirring. We put dicyclopentadiene in the dropping funnel and decalin in the reaction flask. As soon as the decalin begins to boil, reaching a temperature of 180°C, we leave dropping the dicyclopentadiene in the flask. At 180°C dicyclopentadiene turns in the monomer (undergoes back Diels-Alder). The monomer vapours converge in the head of Claisen and condensed into a collection tube cooled.

^{1}H NMR (300 MHz, CDCl$_3$): 3.00(t, 2H, C$_5$$H_6$,); 6.48(m, 2H, C$_5$$H_6$); 6.59(m, 2H, C$_5$$H_6$).

2.3.9   Synthesis of ligands

2.3.9.1 Synthesis of 6-phenylfulvene

The synthesis was carried out under nitrogen atmosphere. We added 3 ml of pyrrolidine to a solution of 5 ml of cyclopentadiene and 2,4 ml of benzaldehyde in 25 ml of dry methanol. After about 20 minutes from the addition, the color of the solution slowly turned from colorless to dark red. After 4,5 hours we followed the reaction by TLC., using pentane as eluent. When the TLC showed only one spot, we interrupted the reaction by adding 1.8 ml of acetic acid. The reaction mixture was, then, diluted with 20 ml of a mixture of diethyl ether and water (1:1). The resultant organic layer was separated and washed with 50 ml of diethyl ether for 3 times. We washed the combined organic extracts with a satured aqueous NaCl solution. The organic solution was dried over Na$_2$SO$_4$.

^{1}H NMR (300 MHz, CDCl$_3$): 6.70, 6.52, 6.36, 6.33 (m, 4H, C$_5$$H_4$,); 7.61-7.59-7.43-7.40-7.36 (m, 5H, C$_6$$H_5$); 7.24 (s, 1H, Ph-C$H$-Cp).

2.3.9.2 Synthesis of 4-methoxy-phenylfulvene

The reaction was carried out in inert nitrogen atmosphere. We mixed 30 ml of anhydrous methanol, 4.1 ml of cyclopentadiene and 2.4 ml of 4-methoxybenzaldehyde in a tailed flask. The reaction started after the addiction

of 2,5 ml of pyrrolidine. The colour of the solution turned immediately from transparent to red-orange. After about 20 minutes, we saw the formation of a red precipitate. We followed the reaction by TLC. We used as eluent a mixture of ethyl acetate and dichloromethane (7:3). When the TLC showed only one spot, we interrupted the reaction by adding 1.8 ml of acetic acid. We proceed to extraction, adding 10 ml of ethyl ether and 10 ml of distilled water, transferring the mixture in a separating funnel to separate the two phases. We washed the aqueous phase with 30 ml of ethyl ether for 3 times. We pooled and washed the organic phases with a concentrated solution of NaCl. We treated the organic phase with anhydrous Na_2SO_4 and finally, we filtered and dried it.

^{1}H NMR (300 MHz, $CDCl_3$): 6.70-6.64-6.45-6.30 (m, 4H, C$_5$$H_4$,); 7.53-7.50-6.71-6.88-6.85 (m, 4H, C$_6$$H_4$,); 3.75 (s, 3H, OC$H_3$,); 7.10 (s, 1H, Ph-C$H$-Cp).

2.3.9.3 Synthesis of 6-(3',4'-dimethoxyphenyl)fulvene

The synthesis was carried out in inert nitrogen atmosphere. We added 2,5 ml of pyrrolidine to a solution of 5 ml of cyclopentadiene and 2 g of 3,4-dimetoxy-benzaldehyde in 30 ml of anhydrous methanol. The color of the solution turned immediately from transparent to red-orange. After 20 hours a solid precipitated out the solution and acetic acid (1.8 ml) was added. We proceed to extraction, adding 10 ml of dichlorometane and 10 ml of distilled water, transferring the mixture in a separating funnel to separate the two phases. We washed the aqueous phase with 30 ml of dichloromethane for 3 times. We pooled and washed the organic phases with a concentrated solution of NaCl. We treated the organic phase is with anhydrous Na_2SO_4 and finally, we filtered and dried it.

¹H NMR (300 MHz, CDCl$_3$): 6.73-6.69-6.50-6.34 (m, 4H, C$_5$H$_4$); 6.93-6.90-6.79 (m, 3H, C$_6$H$_3$,); 3.94 (s, 6H, OCH_3); 7.19 (s, 1H, Ph-CH-Cp).

2.3.9.4 Synthesis of 6-(2',4'-dimethoxyphenyl)fulvene

The reaction was carried out in inert nitrogen atmosphere. We mixed 50 ml of anhydrous methanol, 5 ml of cyclopentadiene and 2 g 2,4-dimethoxy-benzaldehyde in a tailed flask. The reaction started after the addiction of 2,5 ml of pyrrolidine. The color of the solution turned immediately from transparent to red-orange. We refluxed the mixture for about 20 hours and then we interrupted the reaction by adding 1.8 ml of acetic acid. We proceed to extraction, adding 40 ml of dichloromethane and 40 ml of distilled water, transferring the mixture in a separating funnel to separate the two phases. We washed the aqueous phase with 50 ml of dichloromethane for 3 times. The combined organic phases were washed with a concentrated solution of NaCl. We dried the organic phase with anhydrous Na$_2$SO$_4$ and finally, we filtered and removed the solvent.

¹H NMR (300 MHz, CDCl$_3$): 6.68-6.63-6.48-6.29 (m, 4H, C$_5$H$_4$), 7.63-7.61-7.52 (m, 3H, C$_6$H$_3$), 3.87 (s, 6H, OCH_3), 6.65(s, 1H, Ph-CH-Cp).

2.3.9.5 Synthesis of 6-(3',5'-dimethoxyphenyl)fulvene

The synthesis was carried out in inert nitrogen atmosphere. We added 2,5 ml of pyrrolidine to a solution of 5 ml of cyclopentadiene and 2 g of 3,5-dimetoxy-benzaldehyde in 100 ml of dry methanol. The colour of the solution turned immediately from colourless to red. After 20 hours a solid precipitated out the solution and acetic acid (1.8 ml) was added. We proceed to extraction,

adding 10 ml of dichloromethane and 10 ml of distilled water, transferring the mixture in a separating funnel to separate the two phases. We washed the aqueous phase with 50 ml of dichloromethane for 3 times. We pooled and washed the organic phases with a concentrated solution of NaCl. We treated the organic phase is with anhydrous Na_2SO_4 and finally, we filtered and dried it.

^{1}H NMR (300 MHz, CDCl$_3$): 6.73-6.69-6.50-6.34 (m, 4H, C$_5$H$_4$, m), 6.93-6.90-6.79 (m. 3H. C$_6$H$_3$), 3.94 (s, 6H, OCH$_3$,); 7.19 (s, 1H, Ph-CH-Cp).

2.3.10 Synthesis of lithium salt

2.3.10.1 Reduction of 4-methoxy-phenylfulvene.

In inert atmosphere we picked up 6.5 ml of a solution of super-hydride and we transferred it to a 250 ml tailed flask. We removed the solvent (THF) to obtain a white solid and we dissolved it in 70 ml of anhydrous diethyl ether. We dissolved 1.0 g of 4-methoxyphenyl-fulvene in 30 ml of anhydrous diethyl ether and added it to the solution of the reductant. We left the reaction under a stream of nitrogen at room temperature, for one night until the formation of a suspension. In the morning we transferred the reaction mixture in a tailed tube where the lithium salt precipitated. We removed the solvent by siphoning and we washed the precipitate with 50 ml of anhydrous diethyl ether for three times. Finally, we dried and weighed the salt.

^{1}H-NMR (400 MHz, DMSO): 3.30 (s, 2H, Ph-CH$_2$-Cp), 3,67 (s, 3H, OCH$_3$), 5.13-5.14-5.15-5.16 (m, 4H, C$_5$H$_4$), 6.68-6.71-7.07-7.10 (d, 4H, C$_6$H$_4$).

2.3.10.2 Reduction of 6-(3',4'-dimethoxyphenyl)fulvene

In inert atmosphere we picked up 6.5 ml of a solution of super-hydride and we transferred it to a tailed flask 250 ml. We removed the solvent (THF) to obtain a white solid and we dissolved it in 70 ml of dry diethyl ether. We dissolved 1.0 g of 6-(3',4'-dimethoxyphenyl)fulvene in 30 ml of dry diethyl ether and added it to the solution of the reductant. We left the reaction under a stream of nitrogen at room temperature, for one night until the formation of a suspension. On the morning we transferred the reaction mixture in a tailed tube where the lithium salt precipitated. We removed the solvent by siphoning and we washed the precipitate with 50 ml of anhydrous diethyl ether for three times. Finally, we dried and weighed the salt.

^{1}H-NMR (300 MHz, CDCl$_3$): 3.63 (s, 2H, Ph-CH_2-Cp), 3.68 (s, 6H, OCH_3), 5.15 (s, 4H, C$_5H_4$), 6.70-6.81 (dd, 4H, C$_6H_4$).

2.3.11 Synthesis of yttrium cyclopentadienyl complexes

2.3.11.1 Synthesis of complexes [C$_5H_4$–CH$_2$–C$_6H_4$–OCH$_3$]YCl$_2$ [(4-methoxybenzyl)cyclopentadienyl]-yttrium-dichloride. (CAM 1)

We weighed in dry-box, in two separate balls, 200 mg of salt of lithium 4-methoxybenzyl-ciclopentedienile and 202 mg of YCl$_3$. We dissolved the ligand in 8 ml of anhydrous THF and moved the solution into the dropping funnel. We solubilized the metal halide in 32 ml dry THF and transferred it to the reaction flask. We immersed the flask in the thermostated bath at -78 °C, and then slowly we added the ligand in the solution containing the metal halide. We left it on stirring overnight and climbing slowly to room

temperature. We removed the solvent and we dissolved the resulting solid product in 40 ml of anhydrous dichloromethane. We filtered the mixture through celite to remove lithium chloride. We dried the complex and we carried it in dry box to recover and to weight it. The product was characterized by NMR analysis proton.

Yield: 42%

^{1}H-NMR (300 MHz, DMSO): 2.86 (s, 2H, CH_2), 3.33 (s, 3H, OCH_3), 5.99 (d, 1H, C$_5H_4$), 6.09 (s, 1H, C$_5H_4$), 6.24 (d, 1H, C$_5H_4$), 6.38 (m, 1H, C$_5H_4$), 6.84 (m, 2H, C$_6H_4$), 7.05 (m, 2H, C$_6H_4$).

^{13}C-NMR (300 MHz, DMSO): 54.97 (s, 1C, OCH_3), 41.78 (s, 1C, CH_2), 113.74-126.83-127.14-129.49-131.19-132.21-132.68-134-134.45-146.65-157.51 (s, 11C, C_5H_4/C_6H_4).

2.3.11.2 Synthesis of complex [Cp-CH$_2$-C$_6$H$_5$.OCH$_3$]$_2$YCl Bis-[(4-Methoxybenzyl)cyclopentadienyl]-yttrium-chloride.(CAM 2)

We weighed in dry-box, in two separate balls, 150 mg of salt of lithium 4-methoxybenzyl-ciclopentedienile and 80 mg of YCl$_3$. We dissolved the ligand with 50 ml of dry THF and moved the solution into the reaction flask. We solubilized the metal halide in 11 ml dry THF and transferred it to the dropping funnel. We immersed the flask in the thermostated bath at -78 °C, and then slowly we added the metal in the solution containing the ligands. We left it on stirring overnight and climbing slowly to room temperature. We removed the solvent and we dissolved the resulting solid product in 40 ml of anhydrous dichloromethane, We filtered the mixture through celite to remove lithium chloride. We dried the complex and we carried it in dry box to recover and to weight it. The product was characterized by NMR analysis proton.

Yield: 21%

¹H NMR (300 MHz, DMSO): 2.87 (d, 4H, CH_2), 3.60 (s, 4H, CH_2), 3.71 (s, 6H, OCH_3), 5.99 (s, 2H, C$_5H_4$), 6.09 (s, 2H, C$_5H_4$), 6.21 (s, 2H, C$_5H_4$), 6.38 (s, 2H, C$_5H_4$), 6.84 (m, 4H, C$_6H_4$), 7.10 (m, 4H, C$_6H_4$).

¹³C-NMR (300 MHz, DMSO): 54.30 (s, 2C, OCH_3), 40.87 (s, 2C, CH_2),   113.11-125.78-125.97-129.34-130.40-131.12-131.68-133.67-134.02-145.56-156.15 (s, 22C, C_5H_4/C_6H_4).

2.3.11.3 Synthesis of [C$_5$H$_4$–CH$_2$–C$_6$H$_3$(OCH$_3$)$_2$]YCl$_2$ [(3,4-dimethoxybenzyl)-cyclopentadienyl]-yttrium-dichloride. (CAM 5)

We weighed in dry-box, in two separate balls, 200 mg of salt of lithium 3,4-dimethoxybenzyl-ciclopentedienile and 176 mg of YCl$_3$. We dissolved the ligand in 8 ml of anhydrous THF and moved the solution into the dropping funnel. We solubilized the metal halide in 32 ml dry THF and transferred it to the reaction flask. We immersed the flask in the thermostated bath at -78 °C, and then slowly we added the ligand in the solution containing the metal halide. We left it on stirring overnight and climbing slowly to room temperature. We removed the solvent and we dissolved the resulting solid product in 40 ml of anhydrous dichloromethane. We filtered the mixture through celite to remove lithium chloride. We dried the complex and we carried it in dry box to recover and to weight it. The product was characterized by NMR analysis proton.

Yield: 46%

¹H-NMR (300 MHz, CDCl$_3$): 2.87 (s, 2H, CH_2), 3.70 (s, 6H, OCH_3), 6.01 (s, 1H, C$_5H_4$), 6.11 (s, 1H, C$_5H_4$), 6.24 (s, 1H, C$_5H_4$), 6.40 (s, 1H, C$_5H_4$), 6.67–6.86 (m, 3H, C$_6H_3$).

^{13}C-NMR (300 MHz, DMSO): 53.50 (s, 2C, OCH$_3$), 34.7 (s, 1C, CH$_2$), 110.37-111.45-113.32-118.89-120.78-130.67-135.87-147.54-148.34 (s, 11C, C_5H$_4$/C_6H$_3$).

2.3.11.4 Synthesis of complex [Cp-CH$_2$-C$_6$H$_5$-(OCH$_3$)$_2$]$_2$YCl Bis-[(3,4-dimethoxybenzyl)-cyclopentadienyl]-yttrium-chloride (CAM 6)

We weighed in dry-box, in two separate balls, 150 mg of salt of lithium 3,4-dimethoxybenzyl-ciclopentedienile and 66 mg of YCl$_3$. We dissolved the ligand with 6 ml of dry THF and moved the solution into the reaction flask. We solubilized the metal halide in 11 ml dry THF and transferred it to the dropping funnel. We immersed the flask in the thermostated bath at -78 °C, and then slowly we added the metal in the solution containing the ligands. We left it on stirring overnight and climbing slowly to room temperature. We removed the solvent and we dissolved the resulting solid product in 40 ml of anhydrous dichloromethane. We filtered the mixture through celite to remove lithium chloride. We dried the complex and we carried it in dry box to recover and to weight it. The product was characterized by NMR analysis proton.

Yield: 36%

^{1}H NMR (400 MHz, DMSO): 2.87 (d, 4H, CH_2$), 3.60 (d, 4H, C$H$_2$), 3.70 (s, 12H, OCH_3$), 5.98 (s, 2H, C$_5H$_4$), 6.11 (s, 2H, C$_5H$_4$), 6.23 (s, 2H, C$_5H$_4$), 6.39 (s, 2H, C$_5H$_4$), 6.86 (m, 4H, C$_6H$_4$), 7.12 (m, 4H, C$_6H$_4$).

^{13}C-NMR (300 MHz, DMSO): 54.87 (s, 4C, OCH$_3$), 35.79 (s, 2C, CH$_2$), 111.30-111.89-114.21-119.02-121.95-131.67-136.87-148,54-148.89 (s, 22C, C_5H$_4$/C_6H$_3$).

2.3.12 Synthesis of scandium cyclopentadienyl complexes

2.3.12.1 Synthesis of complex [Cp-CH$_2$-C$_6$H$_5$-OCH$_3$)]ScCl$_2$ [(4-methoxybenzyl)cyclopentadienyl]-scandium-dichloride.(CAM 3)

We weighed in dry-box, in two separate balls, 200 mg of salt of lithium 4-methoxybenzyl-ciclopentedienile and 157 mg of ScCl$_3$. We dissolved the ligand with 8 ml of anhydrous THF and moved the solution into the dropping funnel. We solubilized the metal halide in 32 ml of THF-dry and we transferred it to the reaction flask. We immersed the flask in the thermostated bath at -78 °C, and then slowly we added the ligand in the solution containing the metal halide. We left it on stirring overnight and climbing slowly to room temperature. We removed the solvent and we dissolved the resulting solid product in 50 ml of anhydrous dichloromethane. We filtered the mixture through celite to remove lithium chloride. We dried the complex and we carried it in dry box to recover and to weight it. The product was characterized by NMR analysis proton.

Yield: 95%

^{1}H NMR (300 MHz, CDCl$_3$): 2.80 (d, 2H, CH_2), 2.92 (d, 2H, CH_2), 3.60 (s, 3H, OCH_3), 5.98 (s, 1H, C$_5$$H_4$9, 6.09 (s, 1H, C$_5H_4$), 6.23 (d, 1H, C$_5$$H_4$), 6.37 (m, 1H, C$_5H_4$), 6.84 (m, 2H, C$_6$$H_4$), 7.05 (m, 2H, C$_6H_4$).

^{13}C-NMR (300 MHz, DMSO): 55.79 (s, 1C, OCH_3), 42.87 (s, 1C, CH_2),   113.34-125.65-128.15-129.97-131.65-132.67-132.96-134.56-134.78-147.03-158 (s, 11C, C$_5$H$_4$/C$_6$H$_4$).

2.3.12.2 Synthesis of complex [Cp-CH$_2$-C$_6$H$_5$-OCH$_3$]$_2$ScCl Bis-[(4-methoxybenzyl)cyclopentadienyl]-scandium-chloride.(CAM 4)

We weighed in dry-box, in two separate balls, 150 mg of salt of lithium 4-methoxybenzyl-ciclopentedienile and 59 mg of ScCl$_3$. We dissolved the ligand with 8 ml of anhydrous THF and moved the solution in the reaction flask. We solubilized the metal halide in 8 ml of THF-dry and we transferred into the dropping funnel. We immersed the flask in the thermostated bath at -78 °C, and then slowly we added the metal in the solution containing the ligand. We left it on stirring overnight and climbing slowly to room temperature. We removed the solvent and we dissolved the resulting solid product in 40 ml of anhydrous dichloromethane, We filtered the mixture through celite to remove lithium chloride. We dried the complex and we carried it in dry box to recover and to weight it. The product was characterized by NMR analysis proton.

Yield: 24%

^{1}H NMR (300 MHz DMSO): 2.86 (s, 4H, C*H*$_2$), 3.60 (s, 4H, C*H*$_2$), 3.71 (s, 6H, OC*H*$_3$), 5.76 (s, 2H, C$_5$H$_4$), 6.09 (s, 2H, C$_5$H$_4$), 6.23 (d, 2H, C$_5$H$_4$), 6.38 (s, 2H, C$_5$H$_4$), 7.08 (m, 4H, C$_6$H$_4$), 7.12 (m, 4H, C$_6$H$_4$).

^{13}C-NMR (300 MHz, DMSO): 53.99 (s, 2C, O*C*H$_3$), 40.12 (s, 2C, *C*H$_2$), 112.95-124.67-125.79-129.38-130.21-130.94-131.33-132.62-133.75-144.33-155.14 (s, 22C, *C*$_5$H$_4$/*C*$_6$H$_4$).

2.3.12.3 Synthesis of complex [C$_5$H$_4$–CH$_2$–C$_6$H$_4$–(OCH$_3$)$_2$]ScCl$_2$ [(3,4-dimethoxybenzyl)cyclopentadienyl]-scandium-dichloride.(CAM 7)

We weighed in dry-box, in two separate balls, 200 mg of salt of lithium 3,4-dimethoxybenzyl-ciclopentedienile and 157 mg of ScCl$_3$. We dissolved the

ligand in 8 ml of anhydrous THF and moved the solution into the dropping funnel. We solubilized the metal halide in 32 ml dry THF and transferred it to the reaction flask. We immersed the flask in the thermostated bath at -78 °C, and then slowly we added the ligand in the solution containing the metal halide. We left it on stirring overnight and climbing slowly to room temperature. We removed the solvent and we dissolved the resulting solid product in 40 ml of anhydrous dichloromethane. We filtered the mixture through celite to remove lithium chloride. We dried the complex and we carried it in dry box to recover and to weight it. The product was characterized by NMR analysis proton.

Yield: 33%

^{1}H NMR (300 MHz, CDCl$_3$): 3.44 (s, 2H, CH_2), 4.18 (s, 2H, CH_2), 4.39 (s, 6H, OCH_3), 6.52 (s, 1H, C$_5H_4$), 6.67 (s, 1H, C$_5H_4$), 6.80 (s, 1H, C$_5H_4$), 6.94 (s, 1H, C$_5H_4$), 7.26 (m, 2H, C$_6H_3$), 7.31 (m, 1H, C$_6H_3$).

^{13}C-NMR (300 MHz, DMSO): 54.80 (s, 2C, OCH_3), 33.99 (s, 1C, CH_2), 110.67-111.87-112.26-117.99-119.98-130.02-134.98-146.78-148.34 (s, 11C, C_5H$_4$/C_6H$_3$).

2.3.12.4 Synthesis of complex [Cp-CH$_2$-C$_6$H$_5$-(OCH$_3$)$_2$]$_2$ScCl Bis-[(3,4-dimethoxybenzyl)cyclopentadienyl]scandium-chloride.(CAM 8)

We weighed in dry-box, in two separate balls, 150 mg of salt of lithium 3,4-dimethoxybenzyl-ciclopentedienile and 59 mg of ScCl$_3$. We dissolved the ligand with 8 ml of anhydrous THF and moved the solution in the reaction flask. We solubilized the metal halide in 8 ml of THF-dry and we transferred into the dropping funnel. We immersed the flask in the thermostated bath at -78 °C, and then slowly we added the metal in the solution containing the ligand. We left it on stirring overnight and climbing slowly to room temperature. We removed the solvent and we dissolved the resulting solid

product in 40 ml of anhydrous dichloromethane, We filtered the mixture through celite to remove lithium chloride. We dried the complex and we carried it in dry box to recover and to weight it. The product was characterized by NMR analysis proton.

Yield: 37%

^{1}H NMR (400 MHz, DMSO): 2.89 (d, 4H, CH_2), 3.62 (d, 4H, CH_2), 3.72 (s, 12H, OCH_3), 6.01 (s, 2H, C_5H_4), 6.13 (s, 2H, C_5H_4), 6.25 (s, 2H, C_5H_4), 6.41 (s, 2H, C_5H_4), 6.88 (m, 4H, C_6H_4), 7.15 (m, 4H, C_6H_4).

^{13}C-NMR (300 MHz, DMSO): 55.01 (s, 4C, OCH_3), 36.33 (s, 2C, CH$_2$),   111.97-112.22-114.99-1120.42-122.13-132.67-137.24-148.94-149.19 (s, 22C, C_5H$_4$/C_6H$_3$).

2.3.13 Synthesis of neodymium cyclopentadienyl complexes

2.3.13.1 Synthesis of complexes [C$_5$H$_4$–CH$_2$–C$_6$H$_4$–OCH$_3$]NdCl$_2$. [(4-methoxybenzyl)cyclopentadienyl]-neodymium-dichloride.(CAM 9)

We weighed in dry-box, in two separate balls, 150 mg of salt of lithium 4-methoxybenzyl-ciclopentedienile and 195 mg of NdCl$_3$. We dissolved the ligand with 6 ml of anhydrous THF and moved the solution into the dropping funnel. We solubilized the metal halide in 24 ml of THF-dry and we transferred it to the reaction flask. We immersed the flask in the thermostated bath at -78 °C, and then slowly we added the ligand in the solution containing the metal halide. We left it on stirring overnight and climbing slowly to room temperature. We removed the solvent and we dissolved the resulting solid product in 50 ml of anhydrous dichloromethane. We filtered the mixture through celite to remove lithium chloride. We dried the complex and we

carried it in dry box to recover and to weight it. The product was characterized by NMR analysis proton.

Yield: 49%

^{1}H NMR (250 MHz. DMSO): 2.87 (d, 2H, CH_2), (d, 2H, CH_2), 3.71 (s, 3H, OCH_3), 5.99 (s, 1H, C$_5H_4$), 6.10 (s, 1H, C$_5H_4$), 6.23 (d, 1H, C$_5H_4$), 6.39 (m, 1H, C$_5H_4$), 6.85 (m, 2H, C$_6H_4$), 7.10 (m, 2H, C$_6H_4$).

^{13}C-NMR (300 MHz, DMSO): 53.89 (s, 1C, OCH_3), 40.67 (s, 1C, CH_2), 112.75-125.44-126.12-127.34-130.22-131.26-131.98-133.99-134.01-145.45-156.89 (s, 11C, C_5H$_4$/C_6H$_4$).

2.3.13.2 Synthesis of complex [Cp-CH$_2$-C$_6$H$_5$-OCH$_3$]$_2$NdCl Bis-[(4-methoxybenzyl)cyclopentadienyl]-neodymium-chloride.(CAM 10)

We weighed in dry-box, in two separate balls, 150 mg of salt of lithium 4-methoxybenzyl-ciclopentedienile and 97 mg of NdCl$_3$. We dissolved the ligand with 6 ml of anhydrous THF and moved the solution in the reaction flask. We solubilized the metal halide in 24 ml of THF-dry and we transferred into the dropping funnel. We immersed the flask in the thermostated bath at -78 °C, and then slowly we added the metal in the solution containing the ligand. We left it on stirring overnight and climbing slowly to room temperature. We removed the solvent and we dissolved the resulting solid product in 40 ml of anhydrous dichloromethane, We filtered the mixture through celite to remove lithium chloride. We dried the complex and we carried it in dry box to recover and to weight it. The product was characterized by NMR analysis proton.

Yield: 23%

¹H NMR (300 MHz, DMSO,): 2.86 (s, 4H, CH_2), 3.68 (d, 4H, CH_2), 3.80 (s, 6H, OCH_3), 6.14 (s, 2H, C_5H_4), 6.30 (s, 2H, C_5H_4), 6.41 (d, 2H, C_5H_4), 6.85 (s, 2H, C_5H_4), 7.10 (m, 4H, C_6H_4), 7.12 (m, 4H, C_6H_4).

¹³C-NMR (300 MHz, DMSO): 53.30 (s, 2C, OCH_3), 41.87 (s, 2C, CH_2),   112.11-124.78-124.97-128.34-129.40-130.12-130.68-132.67-133.02-144.56-155.15 (s, 22C, C_5H_4/C_6H_4).

2.3.13.3   Synthesis   of   [C_5H_4–CH_2–C_6H_3(OCH_3)$_2$]$NdCl_2$ [(3,4-dimethoxybenzyl)-cyclopentadienyl]-neodymium-dichloride. (CAM 11)

We weighed in dry-box, in two separate balls, 150 mg of salt of lithium 3,4-dimethoxybenzyl-ciclopentedienile and 169 mg of $NdCl_3$. We dissolved the ligand in 5 ml of anhydrous THF and moved the solution into the dropping funnel. We solubilized the metal halide in 10 ml dry THF and transferred it to the reaction flask. We immersed the flask in the thermostated bath at -78 °C, and then slowly we added the ligand in the solution containing the metal halide. We left it on stirring overnight and climbing slowly to room temperature. We removed the solvent and we dissolved the resulting solid product in 40 ml of anhydrous dichloromethane. We filtered the mixture through celite to remove lithium chloride. We dried the complex and we carried it in dry box to recover and to weight it. The product was characterized by NMR analysis proton.

Yield: 30%

¹H-NMR (250 MHz, CDCl$_3$): 2.87 (s, 2H, CH_2), 3.70 (s, 6H, OCH_3), 6.01 (s, 1H, C_5H_4), 6.16 (s, 1H, C_5H_4), 6.28 (s, 1H, C_5H_4), 6.42 (s, 1H, C_5H_4), 6.67-6.86 (m, 3H, C_6H_3).

^{13}**C-NMR** (300 MHz, DMSO): 54.50 (s, 2C, OCH_3), 35.77 (s, 1C, CH_2), 110.12-111.34-113.27-117.89-121.67-130.55-134.67-146.22-147.37 (s, 11C, C_5H_4/C_6H_3).

2.3.13.4 Synthesis of complex [Cp-CH$_2$-C$_6$H$_5$-(OCH$_3$)$_2$]$_2$NdCl Bis-[(3,4-dimethoxybenzyl)-cyclopentadienyl]-neodymium-chloride.(CAM 12))

We weighed in dry-box, in two separate balls, 150 mg of salt of lithium 3,4-dimethoxybenzyl-ciclopentedienile and 75 mg of NdCl$_3$. We dissolved the ligand with 6 ml of dry THF and moved the solution into the reaction flask. We solubilized the metal halide in 11 ml dry THF and transferred it to the dropping funnel. We immersed the flask in the thermostated bath at -78 °C, and then slowly we added the metal in the solution containing the ligands. We left it on stirring overnight and climbing slowly to room temperature. We removed the solvent and we dissolved the resulting solid product in 40 ml of anhydrous dichloromethane. We filtered the mixture through celite to remove lithium chloride. We dried the complex and we carried it in dry box to recover and to weight it. The product was characterized by NMR analysis proton.

Yield: 55%

^{1}**H NMR** (DMSO, 400 MHz): 2.87 (d, 4H, CH_2), 3.60 (d, 4H, CH_2), 3.70 (s, 12H, OCH_3), 5.98 (s, 2H, C_5H_4), 6.11 (s, 2H, C_5H_4), 6.23 (s, 2H, C_5H_4), 6.39 (s, 2H, C_5H_4), 6.86 (m, 4H, C_6H_4), 7.12 (m, 4H, C_6H_4).

^{13}**C-NMR** (300 MHz, DMSO): 55.87 (s, 4C, OCH_3), 36.79 (s, 2C, CH_2), 112.30-112.89-113.21-120.02-122.95-132.67-137.87-149.54-150.01 (s, 22C, C_5H_4/C_6H_3).

2.3.14 MTT assay.

We dissolved the compounds in a solution 100% concentrated of DMSO. From this we obtained the dilutions (5µm-10µm-20µm-50µm-100µm) in multiwell having plates 96 are deposited (plated) $2X10^4$ cells. After 24 hours are used the compounds that are the subject of study in contact with the cells to the pre-set concentrations, in order to determine the lethal dose 50, the dose to which we have the 50% inhibition of cell growth. After 48 hours of exposure to our compound, it is determined the effect of the growth inhibition by a colorimetric assay. The assay of MTT is a measurement of sensitivity of the cell proliferation based on the reduction of the tetrazolium salt, 3,[4,5-dimethylthiazol-2-]-2,5-diphenyl-bromide-tetrazolium (MTT). Changes in proliferative activity of the cell caused by our compounds, can be quantified with the MTT. The MTT is reduced to a insoluble formazan coloring, by mitochondrial enzymes associated with metabolic activity. The reduction of MTT is due primarily to glycolytic activity all inside the cell associated with the presence of NADH and NADPH.

2.4   Results and discussion

2.4.1   Synthesis of cyclopentadienyl ligands

The selected ciclopentadienyl ligands were yet able to stabilize the titanium cation by intramolecular or intermolecular coordination. In particular these compounds were substituted aromatic with methoxyl groups (inducers of lipophilicity).

Ligands were synthesized from *6-phenyl-fulvene, 6-(p-methoxyphenyl)-fulvene, 6-(3',4'-dimethoxyphenyl)fulvene, 6-(2',4'-dimethoxyphenyl)fulvene* and *6-(3',5'-dimethoxyphenyl)fulvene.* Structures of pro-ligands are shown in figure 2.5.

6-phenyl-fulvene   *6-(p-methoxyphenyl)-fulvene*

6-(3',4'-dimethoxyphenyl)fulvene

6-(2',4'-dimethoxyphenyl)fulvene   *6-(3',5'-dimethoxyphenyl)fulvene*
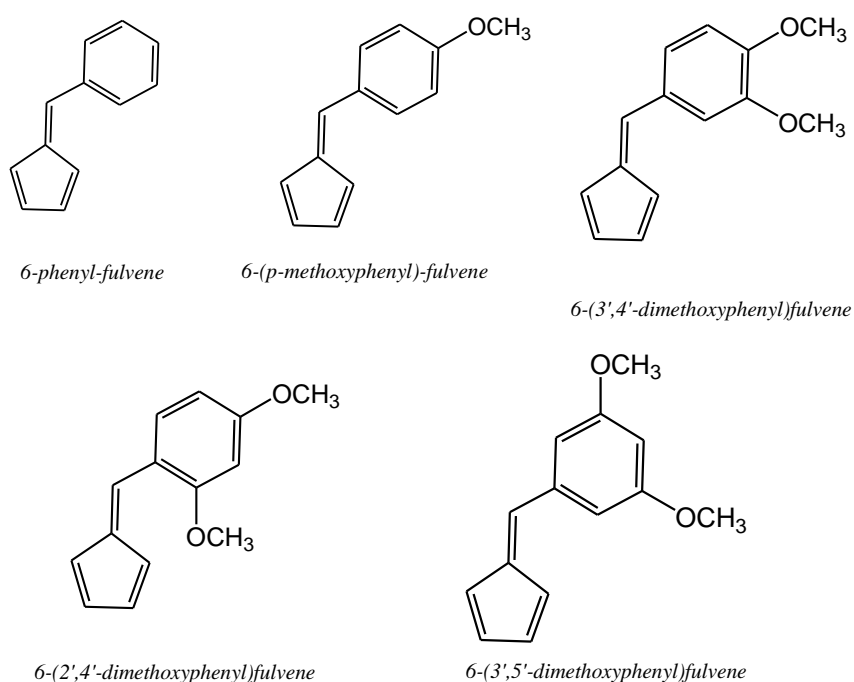
Figure 2.5. *Structures of synthesized pro-ligands*

Synthesis of cyclopentadienyl ligands was carried out in four steps in inert nitrogen atmosphere. (figure 2.6).

Step I

Cyclopentadiene was obtained by a retro diels-alder of dicyclopentadiene. The process exploited the different boiling point of the dimer and the monomer. Therefore, the dimer was dropped into a solution of boiling decalin (180°C). The high temperature broke the dimer and formed the vapors of the cyclopentadiene, which converge in the head of a Claisen of the distiller. Cyclopentadiene condensed into a collection tube cooled with ice.

Step II

The synthesis of pro-ligands fulvenes was carried out starting from suitable aromatic aldehyde.

Step III

Lithium salt of ligands was synthesized reacting an appropriate fulvene with LiBEt₃H (Super Hydride), in dry diethyl ether. Reaction was a nucleophilic addition of a hydride to the double bond 5-6 of fulvene, using $LiB(Et)_3H$ as a donor of reducing equivalents. We used a slight excess than the stoichiometric value of super-hydride. The choice of the $LiB(Et)_3H$ as reducing agent derived from its high selectivity. In fact, the double bond 5-6 of fulvene had a high polarity due to the inductive effect of the methoxyl group bonded to benzene. By this increase of the polarity, the reducing agent attacked the double bond 5-6 and not the dienic component of fulvene. We bought the super-hydride as 1 molar solution of THF, therefore, before use, we dried it from the solvent.
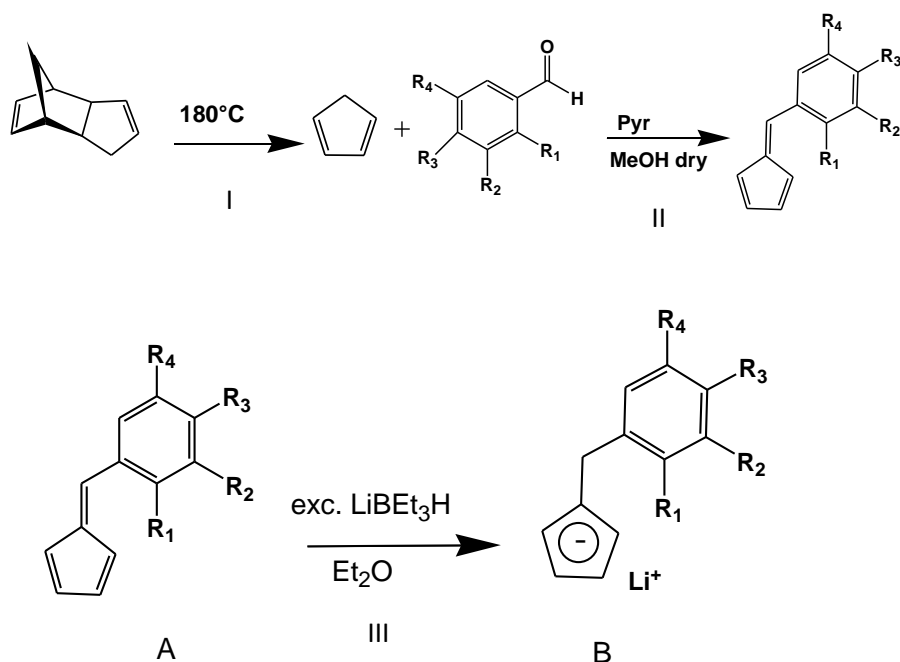
Figure 2.6. *Synthetic schemes of ligands.*
*A: 6-phenyl-fulvene: **R1=R2=R3=R4=H**; 6-(p-metoxyphenyl)-fulvene: **R1=R2=R4=H**;*
***R3=OCH₃**; 6-(3',4'-dimetoxyphenyl)fulvene: **R1=R4=H**; **R2=R3=OCH₃**; 6-(2',4'-dimetoxyphenyl)fulvene: **R2=R4=H**; **R1=R3=OCH₃**;6-(3',5'-dimetoxyphenyl)fulvene:*
R1=R3=H; R2=R4=OCH₃.
*B: 6-phenyl-cyclopentadienyl lithium intermediate: **R1=R2=R3=R4=H***
*6-(p-metoxyphenyl)cyclopentadienyl lithium intermediate: **R1=R2=R4=H**; **R3=OCH₃**; 6-(3',4'-dimetoxyphenyl)cyclopentadienyl lithium intermediate: **R1=R4=H**; **R2=R3=OCH₃**; 6-(2',4'-dimetoxyphenyl)cyclopentadienyl lithium intermediate: **R2=R4=H**; **R1=R3=OCH₃** ; 6-(3',5'-dimetoxyphenyl)cyclopentadienyl lithium intermediate : **R1=R3=H; R2=R4=OCH₃***

The interesting results obtained with several of the synthesized compounds convinced us to expand the library of these group III complexes, making additional changes to the substituents of the cyclopentadienyl ring.

Specifically, to complete the project, we designed the synthesis of cyclopentadienyl ligands binded with a pyridine, a 2,2'-bipyridine or a 1,10-phenanthroline suitably replaced, or substituted with other nitrogenated heterocyclic compounds such as carbazoles.

In the first case, we thought that the 2,2'-bipyridine or 1,10-phenanthroline linked to the cyclopentadienyl ring could chelate and coordinate strongly the metal cation, thereby increasing the stability and improving in the antitumor activity.[26]

Carbazoles with suitable functionalizations showed a significant cytotoxic activity on several cancer cell lines, therefore, in the second case, these groups, in association with the cyclopentadienyl derivatives of titanium, could hopefully have a synergistic effect in the antitumoral activity.[27]

The synthesis procedure followed were those reported in the literature for the ligands synthesized in previous years. The synthesis will be carried out following possibly one of the reaction schemes reported in figures 2.7 and 2.8.
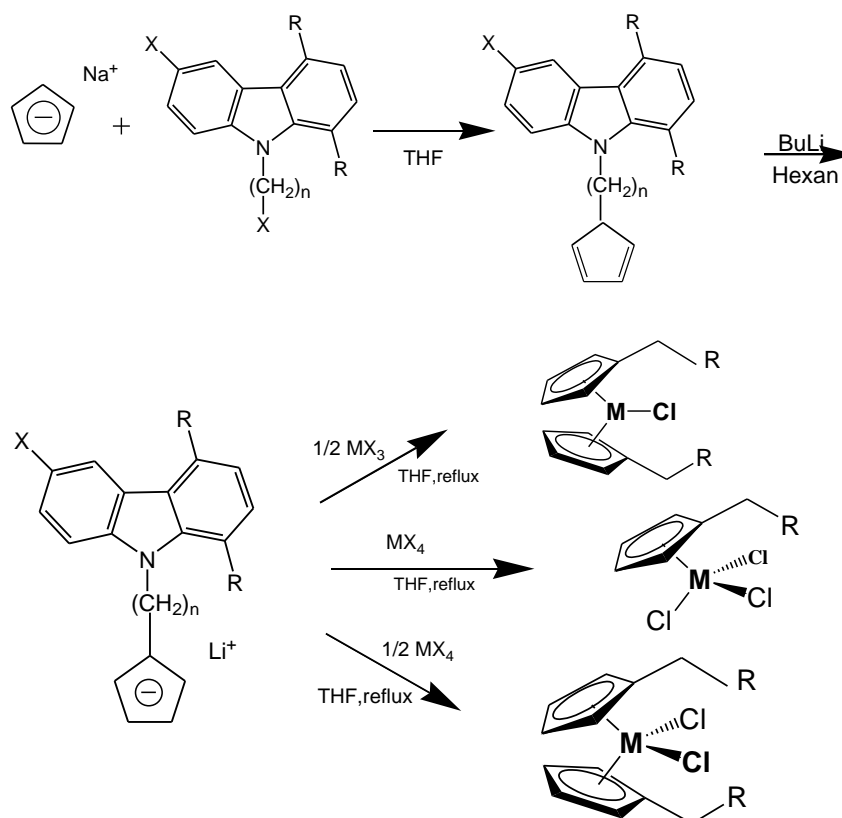
Figure 2.7. *Synthetic schemes of reaction of cyclopentadiene and carbazoles.*
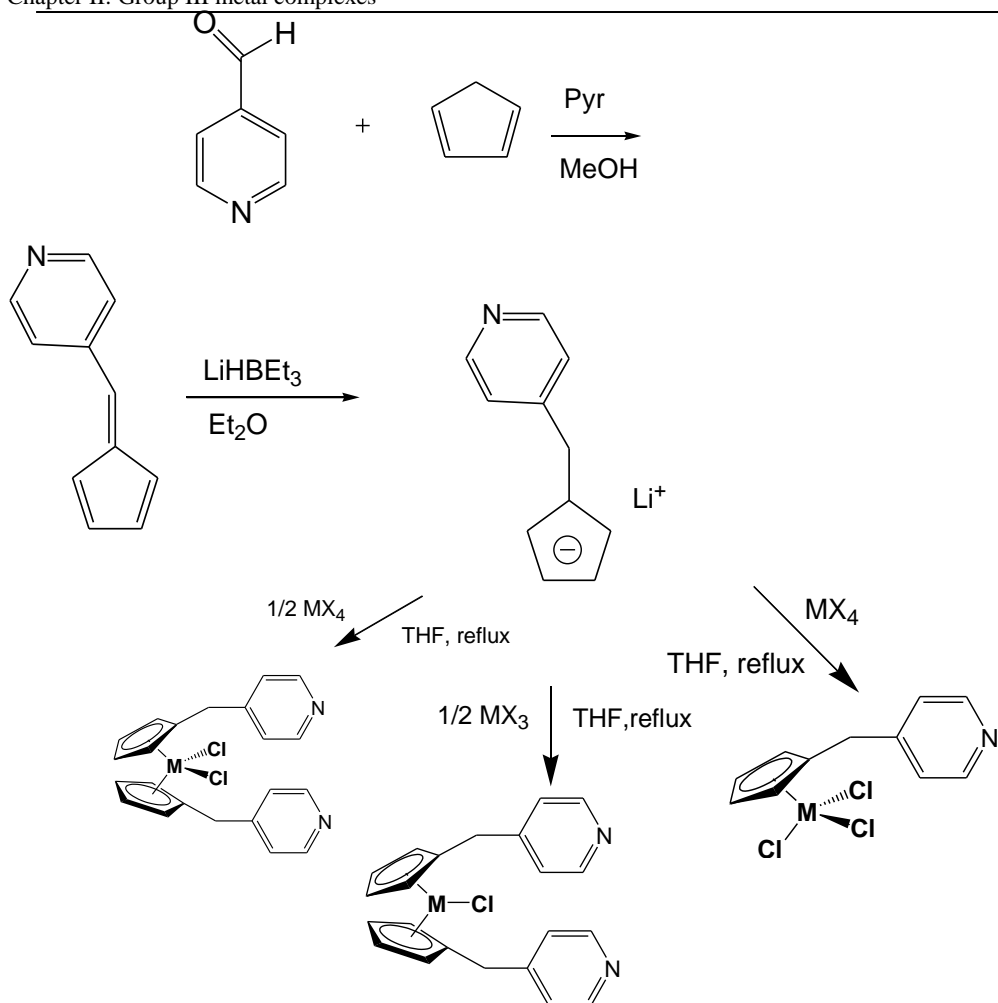
Figure 2.8. *Synthetic schemes of reaction of cyclopentadiene with 4-methoxy-pyridyncarboxaldehyde*

However, the reactions have not led to the synthesis of the designed pro-binders. The reactions between cyclopentadiene and carbazoles was disadvantaged due to steric hindrance of the carbazole and cyclopentadiene and the synthesis is not occurred. Instead, the reaction with 4-pyridin-carboxaldehyde, has led to an intermediate not isolatable (and therefore not

characterised) because of its poor stability in time. Probably the cause must be sought in the high reactivity of pyridine.

2.4.2   Synthesis of group III cyclopentadienyl metal complexes

The ligand synthesized had a various number of methoxyl group on aromatic ring linked to cyclopentadiene. New scandium(III), yttrium(III) and neodymium(III) complexes, both in the form of monosubstituted products both of disubstituted, were synthesized to evaluate the influence of these lipophilic group on the biological activity of the compounds. Structures of synthesized compounds are shown in figure 2.9.

The group III metals have significant differences in atomic radius and electronegativity. Moreover we tried to study the correlation between these two parameters and antitumor activity.
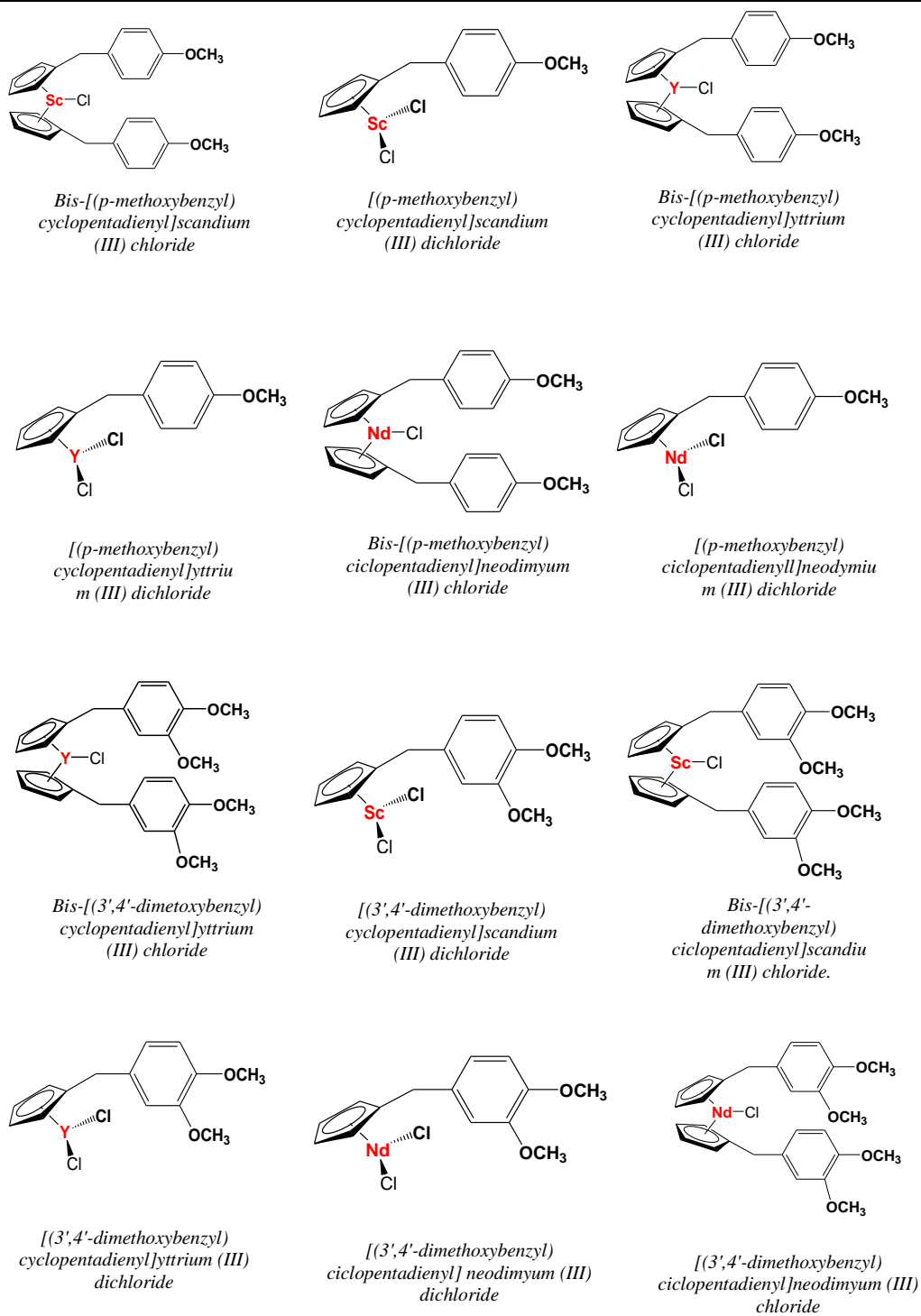
Synthesis was carried out in a only one step of reaction, in nitrogen inert atmosphere. The lithium salt of ligands was reacted with the suitable metal halide in THF dry at -78°C. (Figure 2.10)
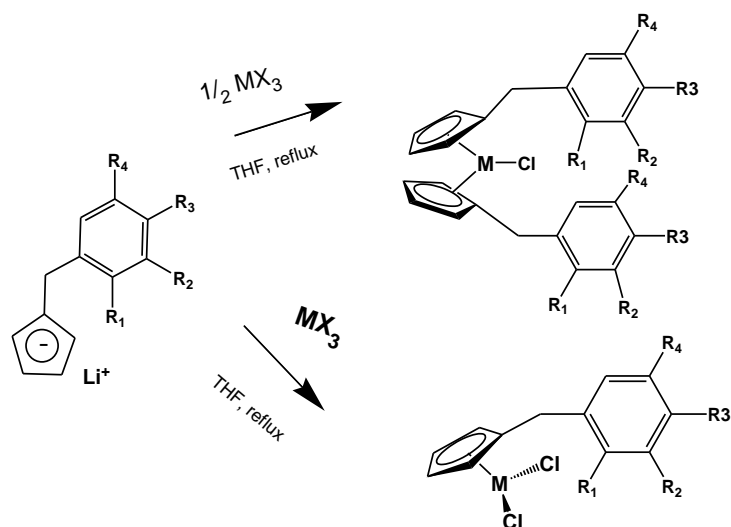
Figure 2.10. *Synthetic scheme of cyclopentadienyl complexes.*
6-phenyl-cyclopentadienyl lithium intermediate: **R1=R2=R3=R4=H**; *6-(p-metoxyphenyl)cyclopentadienyl lithium intermediate:* **R1=R2=R4=H; R3=OCH₃**; *6-(3',4'-dimetoxyphenyl)cyclopentadienyl lithium intermediate:* **R1=R4=H; R2=R3=OCH₃**; *6-(2',4'-dimetoxyphenyl)cyclopentadienyl lithium intermediate:* **R2=R4=H; R1=R3=OCH₃**; *6-(3',5'-dimetoxyphenyl)cyclopentadienyl lithium intermediate* **: R1=R3=H; R2=R4=OCH₃**

Bis-[(p-methoxybenzyl)
cyclopentadienyl]scandium
(III) chloride

[(p-methoxybenzyl)
cyclopentadienyl]scandium
(III) dichloride

Bis-[(p-methoxybenzyl)
cyclopentadienyl]yttrium
(III) chloride

[(p-methoxybenzyl)
cyclopentadienyl]yttriu
m (III) dichloride

Bis-[(p-methoxybenzyl)
ciclopentadienyl]neodimyum
(III) chloride

[(p-methoxybenzyl)
ciclopentadienyll]neodymiu
m (III) dichloride

Bis-[(3',4'-dimetoxybenzyl)
cyclopentadienyl]yttrium
(III) chloride

[(3',4'-dimethoxybenzyl)
cyclopentadienyl]scandium
(III) dichloride

Bis-[(3',4'-
dimethoxybenzyl)
ciclopentadienyl]scandiu
m (III) chloride.

[(3',4'-dimethoxybenzyl)
cyclopentadienyl]yttrium (III)
dichloride

[(3',4'-dimethoxybenzyl)
ciclopentadienyl] neodimyum (III)
dichloride

[(3',4'-dimethoxybenzyl)
ciclopentadienyl]neodimyum (III)
chloride

Figure 2.9. *Structures of synthesized group III metal complexes.*

2.4.3   Biological results

Complexes were tested on MDA.MB213, breast carcinome cell-lines, not expressing the estrogen receptor ERα (ER-negative) and DU145, prostatic carcinoma.

All complexes were tested by anchorage-dipendent assay (MTT assay). The yellow tetrazolium salt 3-[4,5-dimethylthiazol- 2yl]-2,5-diphenyl-tetrazolium bromide (MTT) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resultant intracellular purple formazan can be solubilized and quantitated by spectroscopic means. MTT Cell Proliferation Assay allows measurement of proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability.

All complexes were solved in DMSO, tested at various concentration. Cell viability was expressed as the percentage of proliferated cells compared to cells treated with the different compounds . The same cells were used as control.

CAM-2 showed concentration-dependent cell growth inhibition activity. At higher concentrations the inhibition of growth is stronger (about 100%), contrary to lower concentrations is lower (45%) The compound also showed an activity of DL50 at low concentrations (5μM). From the graphs we was be excluded that the activity of the compound can be added to that of the solvent. We can say finally that the compound object of our study shows a strong activity inhibition of cell growth, tumor refers to antiblastic activities.
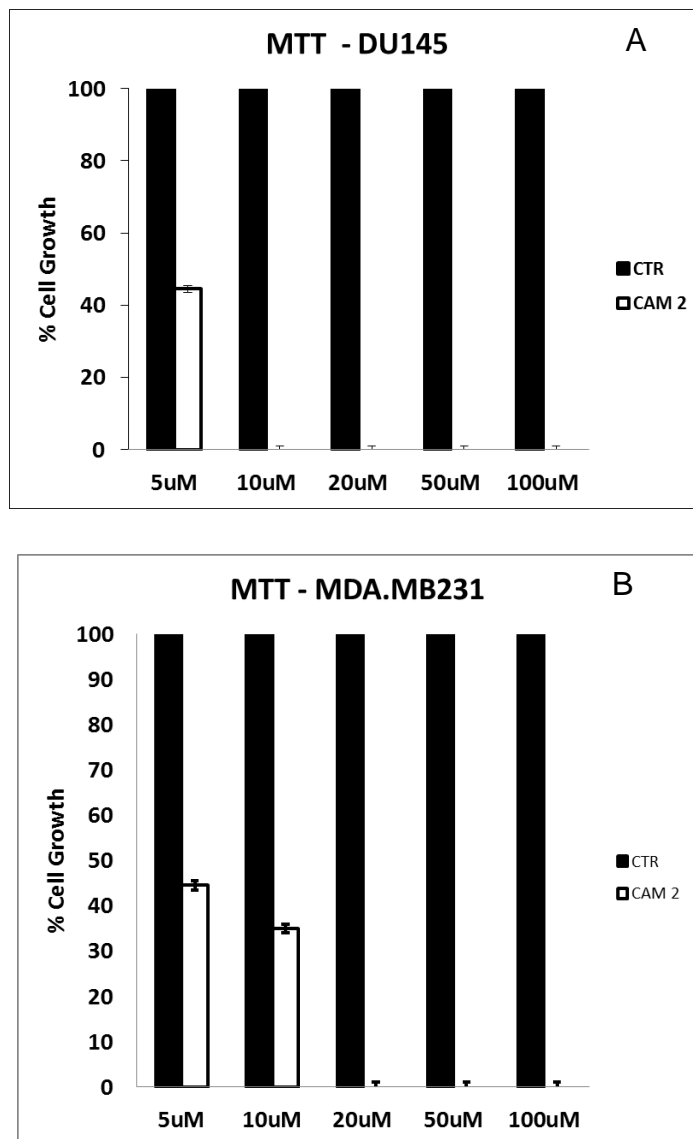
Figure 2.11. *Evaluation of the inhibitory activity of CAM 2 on cell-growth of cell-lines DU145 (A) and MDA.MB231 (B), determined by MTT assay.*
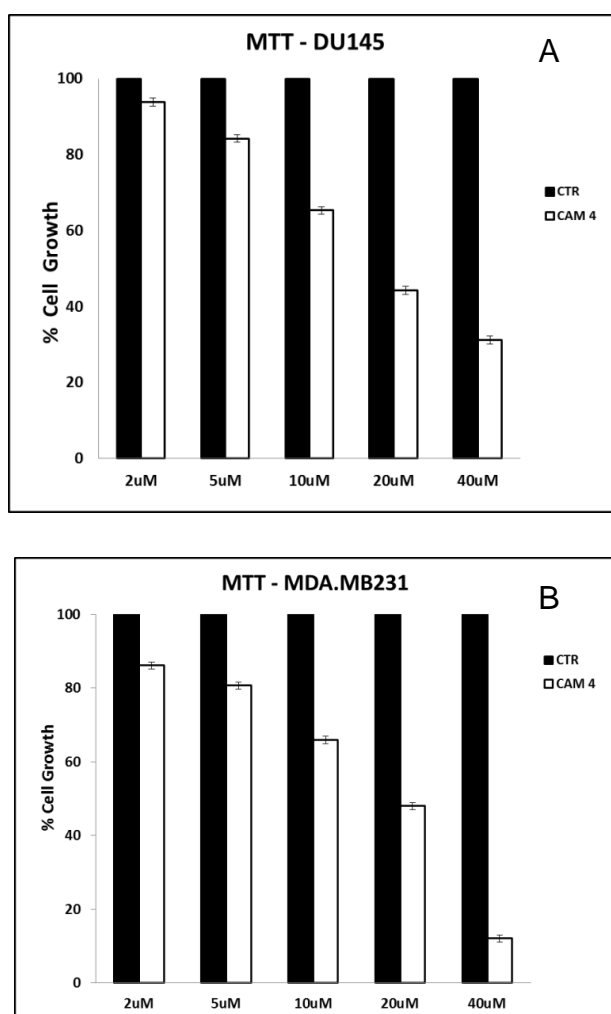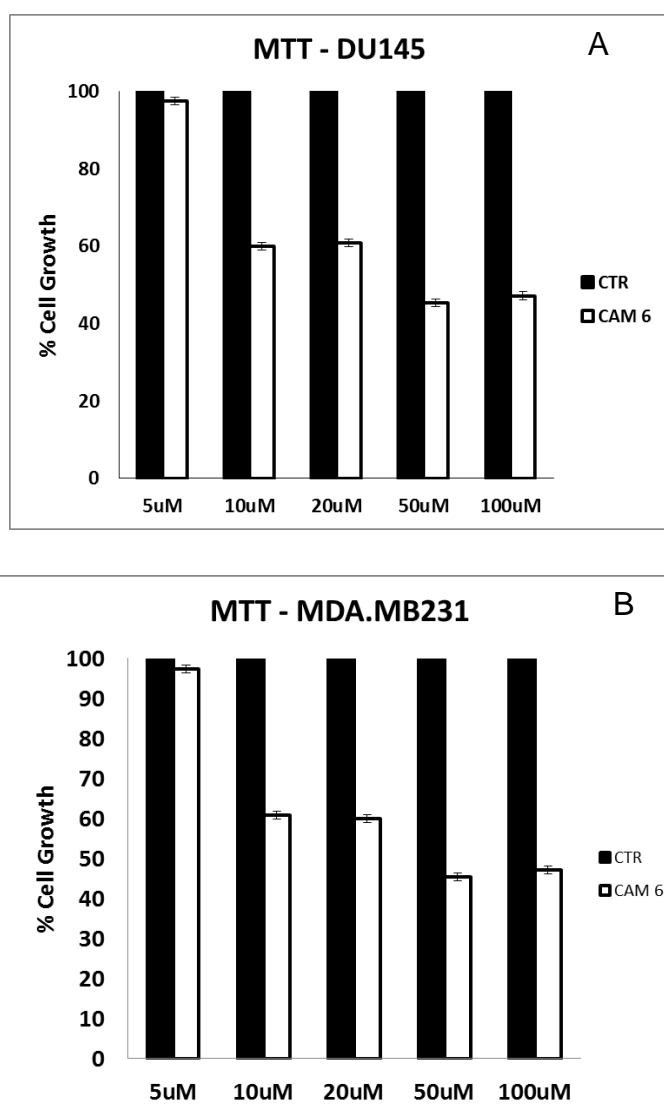
CAM-3 showed concentration-dependent cell growth inhibition activity. At higher concentrations the inhibition of growth is stronger (95%),

contrary to lower concentrations is lower (58%) The compound also shows an activity of DL50 at low concentrations (5-10 μM). From the graphs was be excluded that the activity of the compound can be added to that of the solvent. We can say finally that the compound object of our study shows a strong activity inhibition of cell growth, tumor refers to antiblastic activities.
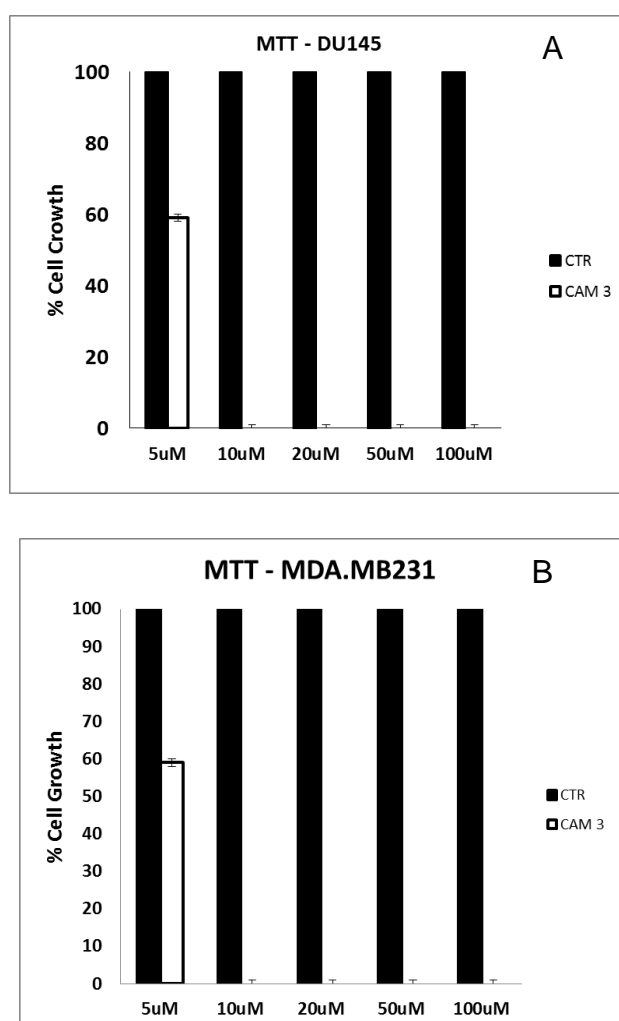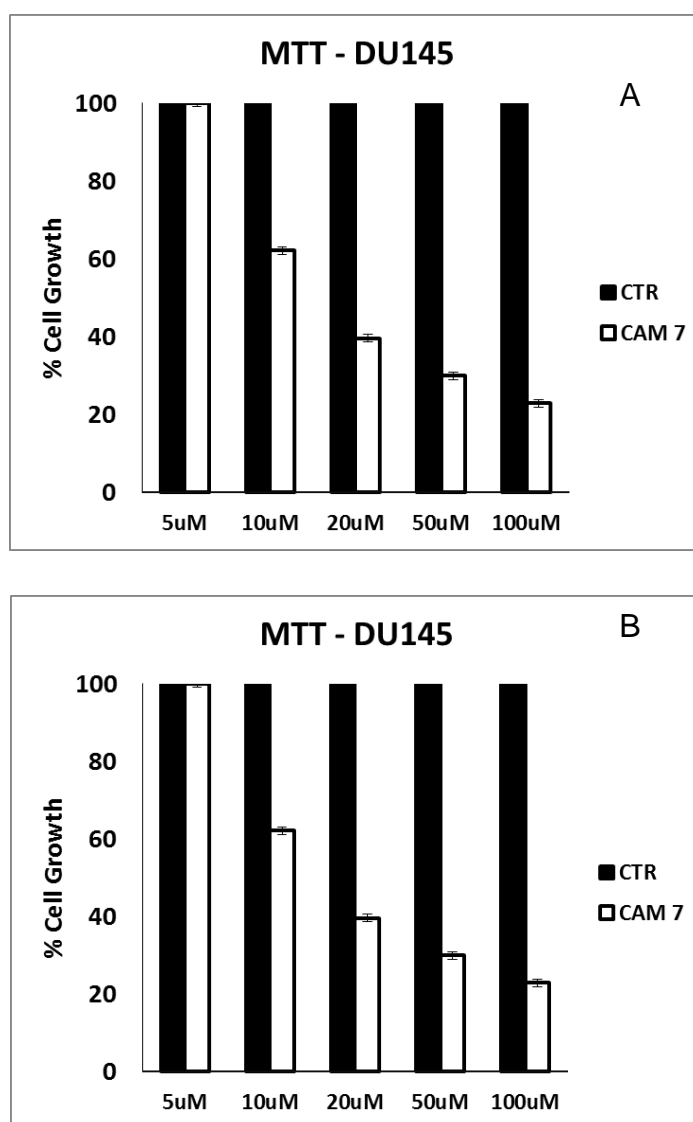

Figure 2.12. *Evaluation of the inhibitory activity of CAM 3 on cell-growth of cell-lines DU145 (A) and MDA.MB231 (B), determined by MTT assay.*

CAM-4 showed concentration-dependent cell growth inhibition activity. At higher concentrations the inhibition of growth is stronger (88%), contrary to lower concentrations is lower (14%) The compound also shows an activity of DL50 at low concentrations (20 μM). From the graphs was be excluded that the activity of the compound can be added to that of the solvent. We can say finally that the compound object of our study shows a strong activity inhibition of cell growth, tumor refers to antiblastic activities.

Figure 2.13. *Evaluation of the inhibitory activity of CAM 4 on cell-growth of cell-lines DU145 (A) and MDA.MB231 (B), determined by MTT assay.*

CAM-6 showed concentration-dependent cell growth inhibition activity. At higher concentrations the inhibition of growth is only of 50%, at lower concentrations is almost nothing (0/40%) The compound also shows an activity of DL50 at low concentrations (50/100 µM). From the graphs was be excluded that the activity of the compound can be added to that of the solvent.

Figure 2.14. *Evaluation of the inhibitory activity of CAM 6 on cell-growth of cell-lines DU145 (A) and MDA.MB231 (B), determined by MTT assay.*

CAM-7 showed concentration-dependent cell growth inhibition activity. At higher concentrations the inhibition of growth is stronger (80%), but at lower concentrations is very low (0/40%) The compound also shows an activity of DL50 at low concentrations (50/100 µM). From the graphs was be excluded that the activity of the compound can be added to that of the solvent.
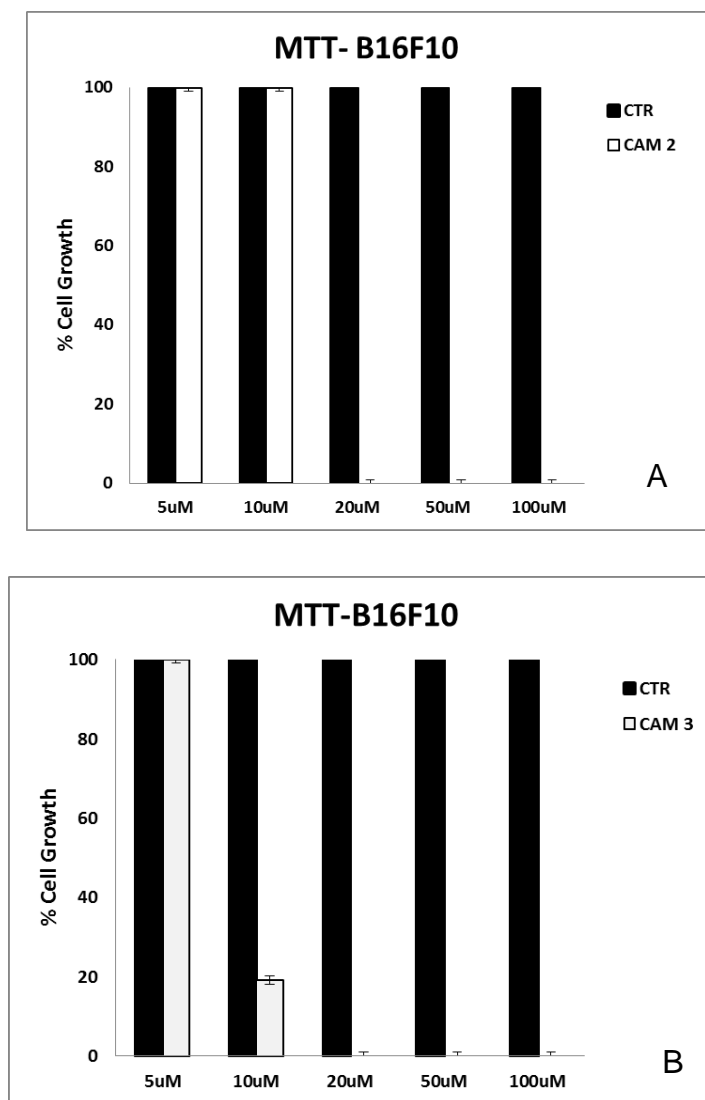

Figure 2.15. *Evaluation of the inhibitory activity of CAM 7 on cell-growth of cell-lines DU145 (A) and MDA.MB231 (B), determined by MTT assay.*

CAM-9 showed cell growth inhibition activity concentration-dependent. At higher concentrations the inhibition of growth is stronger (95-90%), contrary to lower concentrations is lower (12-32%) The compound also showed an activity of DL50 at low concentrations (10/5 µM). In this case the solvent was involved in the toxic activity on the cell line.
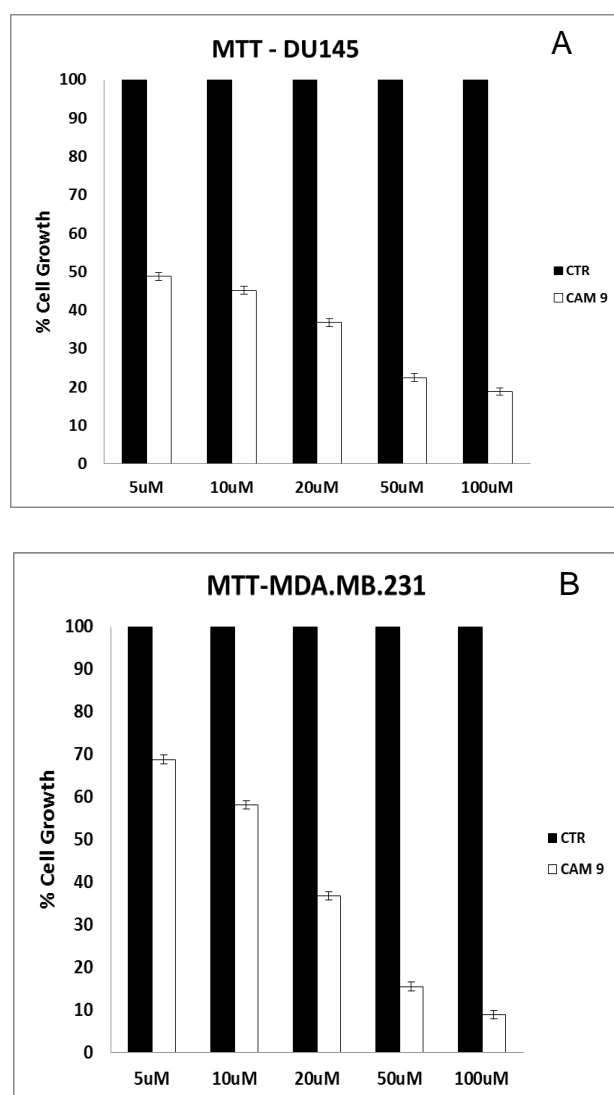
Figure 2.16. *Evaluation of the inhibitory activity of CAM 9 on cell-growth of cell-lines DU145 (A) and MDA.MB231 (B), determined by MTT assay.*

CAM-2, CAM-3 and CAM-4 lead a methoxyl group in the para position to the cyclopentadienyl ring. Instead, the aromatic ring of CAM-6 and CAM-7 leads two methoxyl group at the position 3 and 4. The first ones showed an antiproliferative activity better than the second compounds of the series. The biological activity of CAM-9 confirm the previous data. Indeed it has only one methoxyl group in position 4 of the aromatic rings and showed a good cell growth inhibitory activity at high concentration. Unfortunately for this experiment cannot be excluded the interference of the solvent in compound's activity.

By the assessment of the biological activity of the synthesized molecules, our compounds have a considerable effectiveness in the inhibition of the two considered solid tumors that depends on the chemical structure of the same. These compounds, therefore, represent a new class of molecules organo-metallic equipped with antiproliferative activity. Experiments performed on cell cultures of mammary tumors triple negative (MDA.MB213) and prostate cancers (DU145), establish that the inhibition of cell growth, obtained with some of the new synthesized molecules, can be referred to antiblastic activities, at concentrations employees with a lethal dose 50 (LD50) at low concentrations (5 µM)

The good results obtained with the cell lines described above, have pushed us to test the molecules also on cell line B16F10 of melanoma.

All complexes were solved in DMSO, tested at various concentration. Cell viability was expressed as the percentage of proliferated cells compared to cells treated with the different compounds . The same cells were used as control.

Figure 2.17. *Evaluation of the inhibitory activity of CAM 2 (A) and CAM 3 (B) on B16F10 (melanoma cell line) viability, determined by MTT assay.*
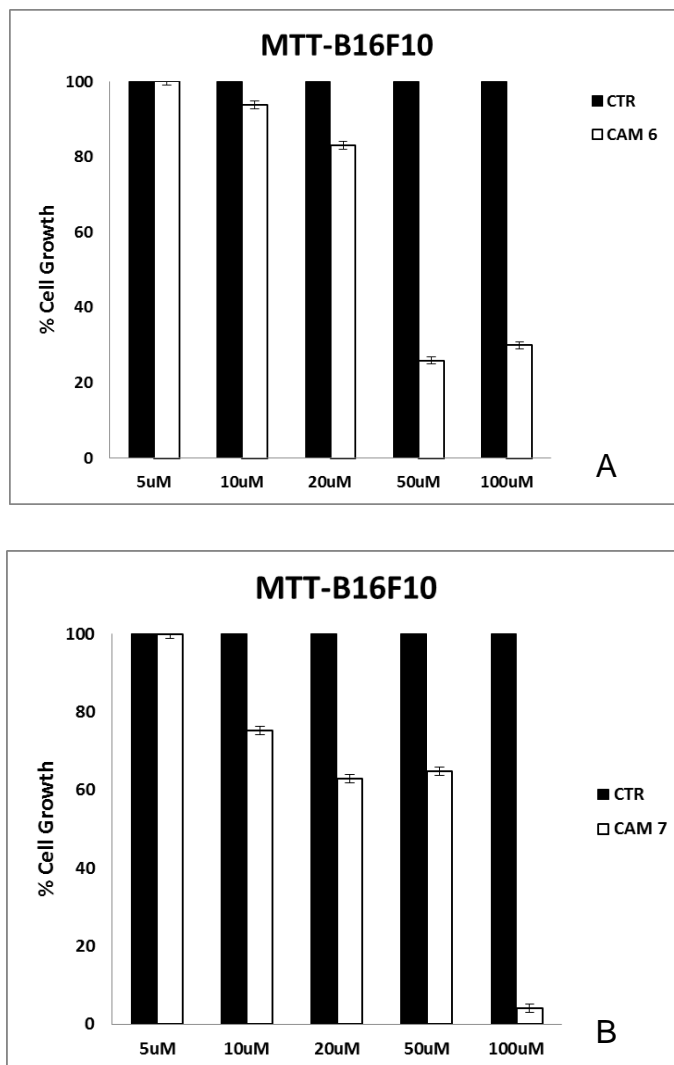
Figure 2.18. *Evaluation of the inhibitory activity of CAM 6 (A) and CAM 7(B) on B16F10 (melanoma cell line) viability, determined by MTT assay.*

Preliminary data confirmed the results of the previous experiments, as the complex CAM 2 and CAM 3 were the most active ones, while the complex

bearing two methoxyl groups in position 3 and 4 showed a profile of growth inhibition was poor.

2.4.4   *Evaluation of drug-membrane interaction*

The partition coefficient of CAM-2 and CAM-7 were determined by UV-Vis spectrophotometry using liposomes as cell membrane mimetic models.

The elimination of the light scattered by lipid vesicles is crucial in the aim to determine the partition coefficient of drugs. Therefore, in the present work the elimination of LUVs interference on the absorption spectra of the anticancer agents was obtained by subtracting each reference spectrum from the correspondent sample one and Equation (1) was used to calculate Kp of CAM-2 and CAM-7 from the data of the corrected absorption spectra. Kp values were 1700 and 1200 for CAM 2 and CAM 7 respectively.

The experimental results indicate that both the anticancer agents exhibit a significant membrane partition.

2.5 Conclusions

We have synthesized and characterized several ligands and group III metal complexes linked with cyclopentadienil ligands.

Complexes were tested on DU145 (prostatic carcinoma cell line), MDA.MB231 (breast carcinoma cell line, not expressing the estrogen receptor ERα) and B16F10 (melanoma cell line) to evaluate their cytotoxic potential activity.

As indicated by our results, the presence of a coordinating group, such as phenyl or ether one, is important to stabilize the active species and generate a cytotoxic complex.

CAM-2, CAM-3 and CAM-4 showed significant anti-proliferative effects. These three molecules which have a methoxyl group in para to the benzene ring, show the best biological activity and have been included in a patent application.

Further study are needed to understand the exact mechanism through which these compounds exercise their action.

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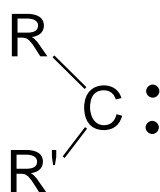
3 Chapter III

Synthesis of NHC-complexes

Introduction

3.2.1   Carbene

The carbene is a chemical species metastable with the general formula $R_2C:$, shown in figure 3.1.

Figure 3.1. *Chemical structure of carbene.*

It is widely used in organic chemistry and organometallic because of its reactivity. The properties of the carbon unsaturated bivalent atom depend on the nature and by the steric hindrance of the substituents R and R'.

Geuther and Hermann, in 1855, were the first scientists to assume the existence of that chemical species. They affirmed that by alkaline hydrolysis of the chloroform could form this product. [1] At that time the existence of the radicals was not yet known and it was only 3 years after that Gomberg obtained by reaction of the triphenylchloromethane with metallic zinc in benzene, shown in figure 3.2, the first free radical that characterized by means of elemental analysis and chemical reactivity. [2]
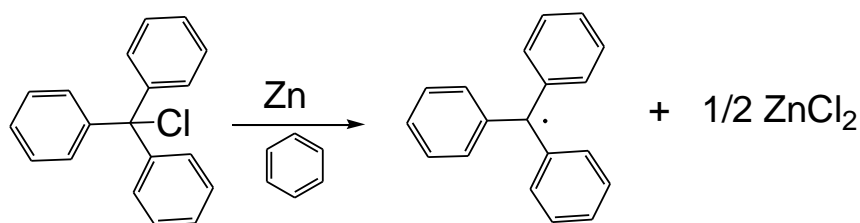
**Figure 3.2**. *The reaction which led to the formation of the first free radical.*

From that moment onwards the scientific community welcomed its discovery and further contributions were made by Staudinger and Kupfer which revealed the existence of carbenic intermediates studying the formation of derivatives of the methylene and of the diazomethane.

In the fifties the study on these new species was intensified and the growing interest toward the reactions, where were involved the carbenes, brought, a few years later, to the synthesis of derivatives of tropone by a reaction wherein the methylene is added to benzene derivatives (Figure 3.3).[3]
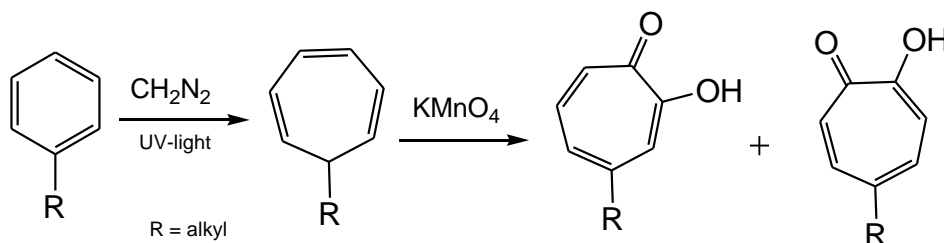
Figure 3.3. *The synthesis of derivatives of tropone by insertion of a methylene intermediate to substituted benzenes.*

Subsequent studies identified the geometry and properties of carbenes. Carbenes can exist in 2 electronic states (Figure 3.4).
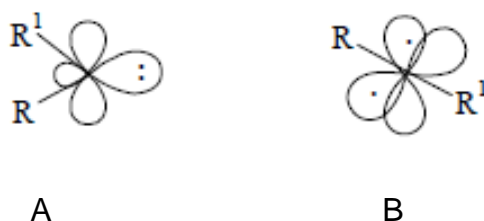
Figure 3.4. *Electronic states of carbenes: singlet (A), triplet (B).*

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In the singlet state, one of the three hybrid orbital sp^2 of the carbon atom carries a pair of electrons of non-bond that justifies the nucleophilic function, while the not occupied orbital p is responsible of the electrophilic characteristics. In the state of triplet, instead the carbon atom has two orbital p containing unpaired electrons and orbital 2 hybridized sp.[4]

In 1915, Chugaev, together with his collaborators, reported a study which showed the production of platinum complexes to bridge according to the reaction shown in figure 3.5:
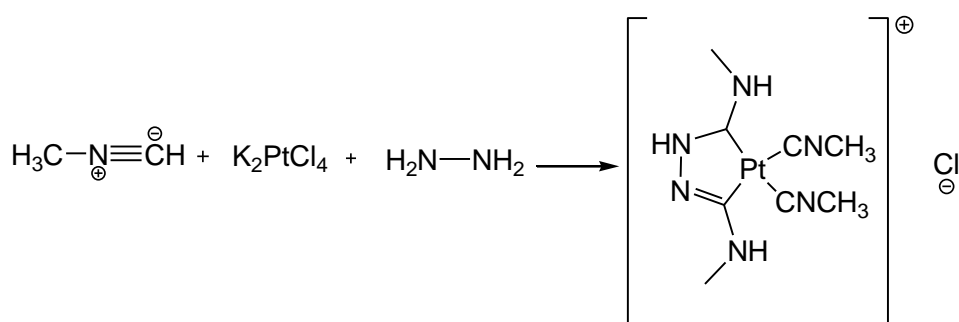
Figure 3.5. *The synthesis of the salt of Chugaev.*

This one was the first true synthesis of metal-carbene complex, but unfortunately there were still the spectroscopic techniques necessary to confirm this theory.

Only in 1964, while the carbenes were in vogue in organic chemistry, Fischer scored and marked in an unequivocal way the first tungsten-based metal-carbene complex (figure 3.6). [3]
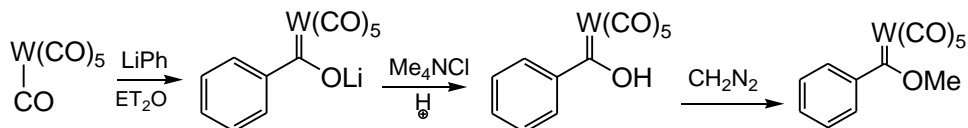
Figure 3.6. *The synthesis of the first complex metal-carbene*

The carbene metal complexes have general formula L_nMX_m where M is the metal that constitutes the center of reaction, L is the carbene, the ligand capable of influencing the electronic properties of the metal and, consequently, the possible catalytic activity of the complex and X is a not carbenic ligand. In most cases it can be a halide, a carboxylate or an alkoxide anion. A particular type of carbenichave is L_nM formula. In this complexes the metal is located in an oxidation state of zero.

The complexes between a carbene and a transition metal can be of 3 types as schematically shown in figure 3.7.
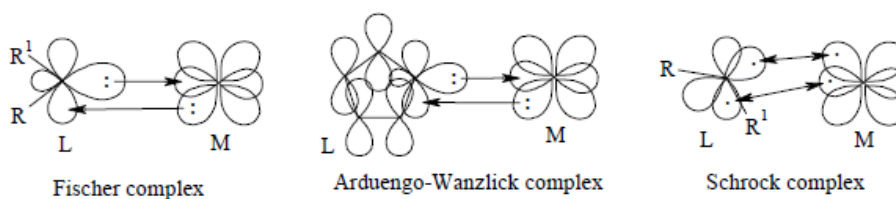
Figure 3.7. *Types of complex metal-carbene.*

In the complexes of Fischer it has the donation of the pair of electrons on the part of the binder to the metal and the donation of the pair of electrons of the metal to the binder which gives the complex a partial character of the double bond.

In the complexes of Schrock, the interaction C-M occurs between the unpaired electrons of the metal and the electrons of the carbon carbenic atom in the state of triplet with the formation of a bond that approximates the covalent bond.

In the complexes Arduengo-Wanzlick the orbital of the carbon carbonic atom is involved in aromatic conjugation and retrodonation is weakened. The interaction between carbene and metal approximates a single bond. [4]

The complexes Arduengo-Wanzlick, also known as carbenic N-heterocyclic complex, were used primarily for their catalytic activity but in recent years their use has also been extended in scope pharmacological.
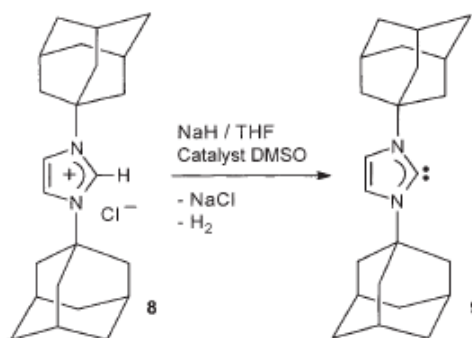
3.2.2 N-heterocyclic (NHC). Carbenes

N-heterocyclic carbene has the following chemical structures (figure 3.8):
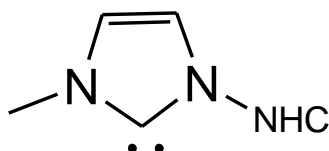
**Figure 3.8.** *Chemical structure of a N-heterocyclic carbene.*

The first stable N-heterocyclic (NHC) carbene was obtained from Arduengo[5] by the reaction shown in Figure 3.9:

**Figure 3.9**. *Synthesis of Arduengo of first carbene N-heterocyclic stable*

The imidazolium salt taken up, N,N-diadamantil imidazolium chloride was reacted with NaH and a catalytic amount of DMSO in a THF solution. The reaction by-products, such as H_2 and NaCl, was easily separated from the THF solution colourless. Arduengo obtained crystals. The X-ray analysis of these crystals showed unequivocally the structure of carbene 9 which falls between the imidozolin-2-unsaturated ilydene that probably represents the largest group of heterocyclic carbenes stable. However the discovery of Arduengo was only the starting point because in the next 15 years other NHC variously substituted were isolated and many of made metal complexes were proved to have good catalytic activity. The imidazolium salt (as for example the precursor 8) can be prepared by two synthetic strategies: the nucleophilic substitution to 2 nitrogen atoms or a multicomponent reaction. Imidazole is initially deprotonated on nitrogen. Subsequently an alkyl halide and/or aryl radical attacks the electrophilic centre previously generated and eventually the replacement occurs to load of the second nitrogen atom with another equivalent of halide. Usually this method is limited to the introduction of substituents primary alkyl, while a symmetrical substitution of both the

nitrogen atoms of the ring can be realized in a synthesis one-pot in the presence of a suitable base such as shown in reaction of figure 3.10:
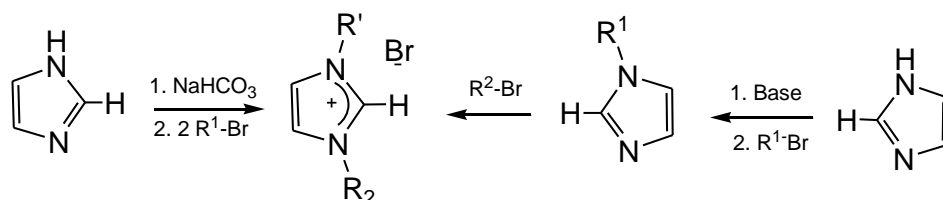
**Figure 3.10.** *Synthesis of imidazolium salts N,N-disubstituted symmetrical and asymmetrical.*

In the multicomponent reaction instead an amine, glyoxal and formaldehyde in the presence of a Bronsted acid reacts forming a salt of imidazolium N,N-disubstituted as shown in figure 3.11:
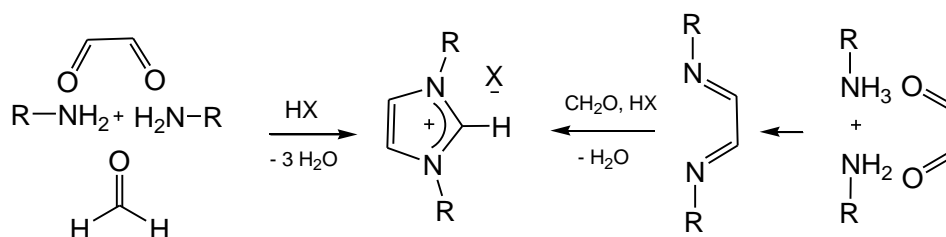
Figure 3.11. *Synthesis of a salt of imidazolium realized with a multicomponent reaction*

However the condensation product between the amine and the glyoxal can also be isolated before cyclization with formaldehyde which gives the possibility of synthesizing an NHC with bulky substituents on the nitrogen atoms.

A salt of imidazolium asymmetrically N,N-disubstituted can be obtained by combining a multicomponent cyclization with a N-alkylation as shown in figure 3.12.[6]
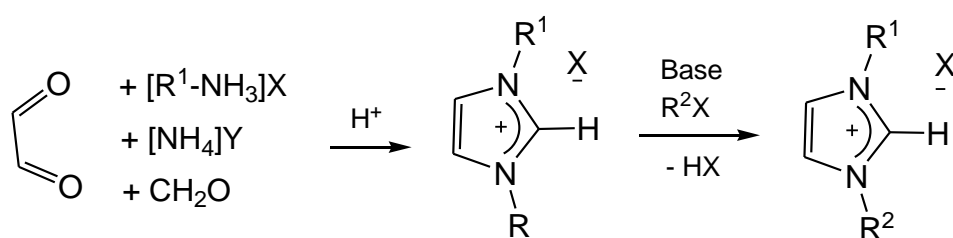
Figure 3.12. *Combination of multicomponent reaction and a N-alkylation to synthesize a salt of imidazolium asymmetrically N,N-disubstituted.*

3.3   Aim of the project

The researches on N-heterocyclic carbene groups (NHC) started with the synthesis of the first stable NHC by Arduengo: 1,3-bis(adamatil)-imidazol-2-ylidene. The ease of synthesis of these molecules and the possibility of coordinating the transition metals with consequent formation of metal complexes NHC, justify broad use both in the catalytic field that biological.

The objective of this project was to synthesize new silver, gold and copper complexes with binders NHC in order to test the potential pharmacological activity. The structures of these complexes were realized to evaluate the influence of increased lipophilicity on the pharmacological effect, as known from the literature.[8] Indeed, the lipophilic cation delocalized can pass through biological membranes more quickly and concentrate mainly in the mitochondria of cancer cells.

NHC-ligand lipophilicity can increase replacing the hydrogens in positions 4 and 5 of the imidazole ring, or functionalizing the nitrogen atoms with lipophilic substituents.

Objective of the work was the changes to the positions 4 and 5 of the imidazole ring. Both the iodine synthesized salts exhibit the same substituents at the nitrogen atoms or a cyclopentanol on the nitrogen atom 1 and a methyl on the nitrogen atom 3 while differ for the substituents in position 4 and 5. In particular the salt R3 binds two chlorine atoms, while R5 binds two phenyl groups.

In the last part of our work, instead, starting from the imidazole, it is also evaluated the effect of a different substituent on one of the two nitrogen atoms on the pharmacological activity. It presents in position 1 a side chain containing a phenyl group and a hydroxyl group and in position 3 there is once again the methyl.

Finally, antiproliferative activity of new synthetic complexes, in breast carcinoma cells was evaluated.

3.4 Materials and methods

3.4.1 General procedure

All manipulations were carried out under oxygen and moisture-free atmosphere, using Schleck techniques or working in an MBraun MB 200 glove-box.

3.4.2 Solvents

All the solvents were thoroughly deoxygenated and dehydrated under argon by refluxing over suitable drying agents, while NMR deuterated solvents (Euriso-Top products) were kept in the dark over molecular sieves.

3.4.3 Reagents

All chemicals were obtained from Aldrich chemical Co. and used without further purification.

3.4.4 Characterization techniques

^{1}H NMR, homodecoupled ^{1}H NMR, ^{1}H COSY and ^{13}C NMR spectra were recorded on Bruker Avance 250 Spectrometer operating at 250 MHz (^{1}H) and 62,5 MHz (^{13}C), on Bruker Avance 300 Spectrometer operating at 300 MHz (^{1}H) and 75 MHz (^{13}C) and on a Bruker Avance 400 Spectrometer operating at 400 MHz (^{1}H) and 100 MHz (^{13}C). Chemical shifts referred to tetramethylsilane used as internal standard.

Molecular weights were determined by ESI mass spectrometry. ESI-MS analysis in positive and negative ion mode, were made using a Finnigan LCQ ion trap instrument, manufactured by Thermo Finnigan (San Jose, CA, USA), equipped with the Excalibur software for processing the data acquired. The sample was dissolved in acetonitrile and injected directly into the electrospray source, using a syringe pump, which maintains constant flow at 5 µl /min. The temperature of the capillary was set at 220°C.

3.4.5   Synthesis of NHC-ligands

3.3.5.1   Synthesis of L20 salt N-methyl-N'-[(2-hydroxy-2-phenyl)ethyl]-imidazole iodire

We added 6 g of imidazole and 10.6 g of 1,2-epoxyethylbenxene in a 100 ml two-necked reaction flask. Mixture was stirred for 48 hours at 60°C. After this time, we added 12,5 g of methyl iodide and 20 ml of dry acetonitrile and we refluxed it for 5 hours. We cooled the reaction mixture to room temperature and we removed the solvent in a rotavapor to obtain a crude reaction product as a yellow oil. This one was dissolved in acetone and recovered by cold precipitation (-20°C) as a white powder.

Yield: 18,2%

^{1}H-NMR (300 MHz, DMSO): 9.10 (s, 1H, NC*H*N), 7.71-7.70 (d, 2H, C*H*=C*H*), 7.43-7.31 (m, 5H, aromatic *H*), 5.96 (d, 1H, O*H*), 4.95 (m, 1H, OC*H*), 4.43 (dd, 1H, NC*H*$_2$), 4.22 (m, 1H, NC*H*$_2$), 3.88 (s, 3H, NC*H*$_3$).

^{13}C-NMR (400 MHz, DMSO): 141.1 (N*C*HN), 136.9-128.3-127.7-125.9 (aromatic *C*),123.0 (*C*H=*C*H), 70.5 (O*C*H), 55.6 (N*C*H$_2$), 35.8 (NH*C*$_3$).

3.3.5.2 Synthesis of R3 salt N-methyl-N'-[2-hydroxycyclopentan]-4,5-dichloroimidazole iodire

We added 3 g of 4,5-dichloroimidazole and 3.70 g of 1,2-epoxycyclopentane in a 50 ml two-necked reaction flask. Mixture was stirred for 48 hours at 60°C. After this time, we added 6,2 g of methyl iodide and 20 ml of dry acetonitrile and we refluxed it for 24 hours. We cooled the reaction mixture to room temperature and we removed the solvent in a rotavapor to obtain a crude reaction product as a powdery yellow solid. This one was dissolved in acetone and recovered by cold precipitation (-20°C) as a white powder.

Yield: 74,4%

^{1}H-NMR (250 MHz, CD_2Cl_2): 9.59 (s, 1H, NC*H*N), 5.47 (d, 1H, O*H*), 4.45 (m, 1H, C*H*O), 4.27 (m, 1H, C*H*N), 3.82 (s, 3H, HC*H*$_3$).

^{13}C-NMR (300 MHz, DMSO): 135,3 (N*C*HN), 119.3-118.5 (*C=C*), 75.9 (O*C*H), 66.6 (N*C*H), 35.2 (NH*C*$_3$), 31.3-28.5-19.6 (*C*H$_2$*C*H$_2$*C*H$_2$).

ESI-MS (CH$_3$CN, m/z): 237.8 Dalton referred to $[C_9H_{14}Cl_2IN_2O]^+$.

3.3.5.3 Synthesis of R5 salt N-methyl-N'-[2-hydroxycyclopentan]-4,5-diphenylimidazole iodire

Step I

We added 2 g of 4,5-diphenylimidazole, 0,35 g of K and 8 ml di DMF in a 25 ml two-necked reaction flask. Mixture was stirred until the complete dissolution of the potassium at room temperature. The resulting yellow solution was stirred for 17 hours at 100 °C after the addition of 0,84 g of 1,2-epoxycyclopentane. After this time, we recovered the reaction product in three times. We added, firstly, a volume of water 10 times greater than the volume

of DMF. We filtered the mixture, recovering a yellow solution and a white precipitated, than dissolved in chloroform. We washed the chloroform solution firstly with a HCl 5N solution and then with water. We dried the resulting solution with magnesium sulphate and we removed the solvent by a rotavapor. We recovered the reaction product, for the first time, after cold-precipitations (-20°C) firstly from acetone and then from ethyl acetate.

The yellow solution was extracted with chloroform and dried with magnesium sulphate. In this way, we recovered a white solid, washed firstly with acetone and then with ethyl acetate to obtain the second fraction of reaction product.

Finally we neutralized the acid solution with a solution of NaOH at 40%. We extracted the neutral solution just obtained with chloroform, washed it with water and dried it with magnesium sulphate. We filtered and removed the solvent from the mixture, obtaining the third tranche of the reaction product.

Yield: 20%

^{1}H-NMR (250 MHz, CD_2Cl_2): 7.55 (s, 1H, NC*H*N), 7.35-7.12 (m, 10H, aromatic *H*), 4.35 (m, 1H, C*H*O), 3.89 (m, 1H, C*H*N), 1.48-2.02 (m, 6H, C*H*$_2$C*H*$_2$C*H*$_2$).

^{13}C-NMR (250 MHz, CD_2Cl_2): 137.2 (N*C*HN), 134.7-133.7-131.7-130.6-129.9-128.9-128.8-128.3 (aromatic *C*), 126.9-126.5 (*C*=*C*), 77.8 (*C*HO), 63.2 (*C*HN), 31-4-31.3-19.5 (*C*H$_2$*C*H$_2$*C*H$_2$).

ESI-MS (CH$_3$CN, m/z): 305.6 Dalton referred to $[C_{20}H_{21}N_2O]^+$; 221.6 Dalton referred to the fragmentation with loss of cyclopentanol $[C_{20}H_{21}N_2O]^+$.

Step II

We added 0,55 g of the product of the previous step, 2,58 g methyl iodide and 15 ml of acetonitrile in a 25 ml two-necked reaction flask. Mixture was stirred for 3,5 hours at 82 °C. We cooled the reaction mixture to room temperature

and we removed the solvent in a rotavapor to obtain a crude reaction product. This one was dissolved in acetone and recovered by cold precipitation (-20°C).

Yield: 52,6%

^{1}**H-NMR** (300 MHz, CD_2Cl_2): 9.98 (s, 1H, NC*H*N), 7.45-7.38 (m, 10H, aromatic *H*), 4,91 (m, 1H, C*H*O), 4.35 (d, 1H, O*H*), 4,25 (d, 1H, C*H*N), 3.88 (s, 3H, NCH$_3$), 1,87-1,84 (m, 6H, C*H$_2$*C*H$_2$*C*H$_2$*).

^{13}**C-NMR** (250 MHz, DMSO): 134,7 (N*C*HN),132-131.3-131.2-130.6-130-129-128.9 (aromatic *C*) 125.3- 125.1 (*C=C*), 76.9 (*C*HO), 64.9 (N*C*H), 34.8 (NH*C$_3$*), 31.4-30.6-19.4 (*C*H$_2$*C*H$_2$*C*H$_2$).

ESI-MS (CH_3CN, m/z): 319,7 Dalton referred to $[C_{21}H_{23}I_2N_2O]^+$, 320.7 Dalton referred to $[C_{21}H_{24}IN_2O]^+$

3.4.6   Synthesis of silver(I)-NHC complexes

3.3.6.1   Synthesis   of   AgR3   N-methyl-N'-[2-hydroxyciclopentan]-4,5-dichloroimidazol-2-ilydene silver(I) iodire

We added 2 g of R3 salt, 30 ml di dry CH_2Cl_2 and molecular sieves in a 50 ml two-necked reaction flask. Mixture was stirred until the complete dissolution of the salt at room temperature. The resulting solution was refluxed sheltered from light for 1,5 hours after the addition of 1,92 g of silver oxide. After this time, we filtered the solution in nitrogen atmosphere, and we removed the solvent in vacuum trap by trap. The resulting product was washed with dry hexane and recovered as a black powder.

Yield: 25,2%

^{1}**H-NMR** (ppm CD_2Cl_2) 4.86 (m, 1H, C*H*O), 4.68 (m, 1H, C*H*N), 3.83 (s, 3H, NC*H$_3$*), 2.26- 1.74 (m, 6H, C*H$_2$*C*H$_2$*C*H$_3$)

^{13}**C-NMR** (300 MHz CD$_2$Cl$_2$) 183.0 (NCHN), 118.6-117.3 (C=C), 76.7 (CHO), 68.5 (CHN), 39.0 (NCH$_3$), 33.0-29.8-20.7 (CH$_2$$CH_2$$CH_2$).

ESI-MS (CH$_3$CN, m/z): 577.3 Dalton referred to [C$_{18}$H$_{24}$AgCl$_4$N$_4$O]$^+$.

3.3.6.2 Synthesis of AgR5 N-methyl-N'-[2-hydroxyciclopentan]-4,5-diphenylimidazol-2-ilydene silver(I) iodire

We added 0,15 g of R5 salt, 25 ml di dry CH$_2$Cl$_2$ and molecular sieves in a 50 ml two-necked reaction flask. The resulting suspension was stirred and refluxed, sheltered from light, for 2 hours after the addition of 0,53 g of silver oxide. After this time, we filtered the solution in nitrogen atmosphere, and we removed the solvent in vacuum trap by trap. The resulting product was washed with dry hexane and recovered as a beige powder.

Yield: 25%

^{1}**H-NMR** (250 MHz, CD$_2$Cl$_2$): 7.34-7.25 (m, 10H, aromatic H), 5,12 (m, 1H, CHO), 4.26 (d, 1H, CHN), 3.78 (s, 3H, NCH_3), 1.49-2.24 (m, 6H, CH_2CH_2CH_2).

^{13}**C-NMR** (400 MHz, DMSO) 176.5-175.7 (NCHN), 133.3-132.9-132.8-132-131.6-130.9-129.4-129.2-128.9-128.8 (aromatic C), 127.7- 127.6-127.5 (CH=CH), 78.5-78.4 (CHO), 65.1-63.2 (CHN), 38.3-38.2 (NCH$_3$), 32.8-31.06- 20.3 (CH$_2$$CH_2$$CH_2$).

ESI-MS (CH$_3$CN, m/z): 765 Dalton referred to [C$_{42}$H$_{44}$AgN$_4$O$_2$]$^+$ with loss of a water molecule and addition of a potassium ion; pick at 319 referred to the fragmentation of monomer complexes that lost a phenyl group and acquired a potassium ion.

3.3.6.3 Synthesis of AgL20 N-methyl-N'-[2-hydroxy-phenyl)ethyl]-imidazole-2-ilydene silver(I) iodire

We added 0,5 g of L20 salt, 25 ml di dry CH_2Cl_2 and molecular sieves in a 100 ml two-necked reaction flask. 1,92 g of silver oxide were added to the resulting solution. This one was stirred and refluxed for 1,5 hours sheltered from light. After this time, we filtered the solution in nitrogen atmosphere, and we removed the solvent in vacuum trap by trap. The resulting product was washed with dry hexane and recovered as a beige powder.

Yield: 43,6%

^{1}H-NMR (300 MHz, CD_2Cl_2): 7.32-7.27 (m, 5H, aromatic H), 6.80-6.68 (d, 2H, C*H*=C*H*) 5,15 (m, 1H, C*H*O), 4.43 (m, 2H, C*H*$_2$N), 3.81 (s, 3H, NC*H*$_3$).

^{13}C-NMR (300 MHz $CDCl_3$) 141.3 (N*C*HN), 129.1-128.7-128-126.5 (aromatic *C*), 122.5-121.3 (*C*H=*C*H), 73.3(*C*HO), 59.1 (*C*H$_2$N), 38.9 (N*C*H$_3$).

ESI-MS (CH_3CN, m/z): 513 Dalton referred to $[C_{24}H_{29}AgN_4O_2]^+$.

3.4.7   Synthesis of gold(I)-NHC complexes

3.3.7.1   Synthesis of AuR3   N-methyl-N'-[2-hydroxyciclopentan]-4,5-dichloroimidazol-2-ilydene gold(I) iodire

We added 0,5 g of AgR3 complexes and 15 ml di dry CH_2Cl_2 in a 25 ml two-necked reaction flask. Mixture was stirred until the complete dissolution of the complex at room temperature. The resulting solution was refluxed overnight, sheltered from light and at room temperature after the addition of 0,16 g of chloro(dimethylsufide)of gold (I). After this time, we filtered the solution in nitrogen atmosphere, and we removed the solvent in

vacuum trap by trap. The resulting product was washed with dry hexane and recovered as a brown powder.

Yield: 77,2%

^{1}H-NMR (250 MHz, CD_2Cl_2): 5.08 (m, 1H, C*H*O), 4.90 (d, 1H, C*H*N), 3.88 (s, 3H, NC*H$_3$*), 2.29- 1.73 (m, 6H, C*H$_2$*C*H$_2$*C*H$_2$*)

^{13}C-NMR (250 MHz, CD_2Cl_2) 183.6 (N*C*HN), 119-117.8 (*C=C*), 76.8 (*C*HO), 71.3 (*C*HN), 38 (N*C*H$_3$), 32.9-29.6-21 (*C*H$_2$*C*H$_2$*C*H$_2$).

ESI-MS (CH$_3$CN, m/z): 667.4 Dalton referred to $[C_{18}H_{24}AuCl_4N_4O]^+$.

3.3.7.2 Synthesis of AuR5 N-methyl-N'-[2-hydroxyciclopentan]-4,5-diphenylimidazol-2-ilydene gold (I) iodire

We added 0,093 g of AgR5 complexes, 25 ml di dry CH_2Cl_2 in a 50 ml two-necked reaction flask. Mixture was stirred until the complete dissolution of the complex at room temperature The resulting solution was stirred and refluxed, sheltered from light, for 5 hours after the addition of 0,025 g of chloro(dimethylsulfide) of gold (I). After this time, we filtered the solution in nitrogen atmosphere, and we removed the solvent in vacuum trap by trap. The resulting product was washed with dry hexane and recovered as a beige powder.

Yield: 53,3%

^{1}H-NMR (400 MHz, CD_2Cl_2) 7.41-7.16 (m, aromatic *H*), 5.43-5.19 (m, 1H, C*H*O); 4.65 (d, 1H, O*H*); 4.30-4.18(m, 1H, C*H*N); 3.78 (s, 3H, NC*H$_3$*), 2.63-1.53 (m, 6H, C*H$_2$*C*H$_2$*C*H$_2$*).

^{13}C-NMR (400 MHz, DMSO) 166.3 (N*C*HN), 132.8-131.4-130.6-129.3 (aromatic *C*), 128.6-127.3 (*C=C*), 77.8 (*C*HO), 65.5 (*C*HO), 37.9 (N*C*H$_3$), 32.7-32.3-20.4 (*C*H$_2$*C*H$_2$*C*H$_2$).

3.3.7.3 Synthesis of AuL20 N-methyl-N'-[2-hydroxy-phenyl)ethyl]-imidazole-2-ilydene gold (I) iodire

We added 0,123 g of AgL20 salt, 20 ml di dry CH_2Cl_2 and molecular sieves in a 100 ml two-necked reaction flask. 1,92 g of silver oxide were added to the resulting solution. Mixture was stirred until the complete dissolution of the complex at room temperature The resulting solution was stirred and refluxed, sheltered from light, for 4 hours after the addition of 0,083 g of chloro(dimethylsulfide) of gold (I). After this time, we filtered the solution in nitrogen atmosphere, and we removed the solvent in vacuum trap by trap. The resulting product was washed with dry hexane and recovered as an orange powder.

Yield: 17,2%

^{1}H-NMR (300 MHz, CD_2Cl_2): 7.39-7.38 (m, 5H, aromatic H), 7.02-6.90 (d, 2H, C*H*=C*H*) 5,20 (m, 1H, C*H*O), 4.47 (m, 2H, C*H*$_2$N), 3.80 (s, 3H, NC*H*$_3$).

^{13}C-NMR (400 MHz, $CDCl_3$) 171.8 (N*C*HN), 140.9-129.5-128.9-126,4 (aromatic *C)*, 122.8-121.7 (*C*H=*C*H), 74.5(*C*HO), 58.6 (*C*H$_2$N), 38.7 (N*C*H$_3$).

ESI-MS (CH_3CN, m/z): 513 Dalton referred to $[C_{24}H_{29}AgN_4O_2]^{+}$.

3.4.8   Synthesis of copper(I)-NHC complexes

3.2.9.1   Synthesis   of   CuR3   N-methyl-N'-[2-hydroxyciclopentan]-4,5-dichloroimidazol-2-ilydene copper(I) iodire

We added 0,5 g of R3 salt and 25 ml di dry THF in a 100 ml two-necked reaction flask. Mixture was stirred for 1 hours at room temperature. We prepared copper chloride suspension in acetonitrile and then we added it to the R3 solution. The resulting suspension was refluxed for 24 hours. After this time, we filtered the solution in nitrogen atmosphere, and we removed the solvent in vacuum trap by trap. The resulting brown solid was dissolved in dry CH_2Cl_2 and stirred for 4 hours to precipitate R3 ligand and to recover the copper complex.

Yield: 13%

^{1}H-NMR (250 MHz, CD_2Cl_2): 4.70 (m, 1H, OH), 4.58 (m, 1H, CHO), 4.12 (m, 1H, CHN), 3.87 (s, 3H, NCH_3), 2.18-1.71 (m, 6H, CH_2CH_2CH_2).

^{13}C-NMR (300 MHz, DMSO): 149.0 (NCHN), 106.0-105.4 ($C=C$), 73.4(CHO), 61.3 (CHN), 32.0 (NCH$_3$), 31.6-19.4-13.6 (CH$_2$$CH_2$$CH_2$).

3.3.8.2 Synthesis of CuR5 N-methyl-N'-[2-hydroxyciclopentan]-4,5-diphenylimidazol-2-ilydene copper(I) iodire

We added 0,1 g of R5 salt, 0,041 g di lithio-bis(trimethyl)silamide and 15 ml di dry THF in a 100 ml two-necked reaction flask. Mixture was stirred for 1 hour. We prepared copper chloride suspension in acetonitrile and then we added it to the R3 solution. The resulting suspension was refluxed overnight. After this time, we filtered the solution in nitrogen atmosphere, and we removed the solvent in vacuum trap by trap. The resulting product was dissolved in dry CH_2Cl_2 and stirred for 4 hours at 4 °C to precipitate R5 ligand and to recover the copper complex.

Yield: 30%

^{1}H-NMR (250 MHz, CD_2Cl_2): 7.25-7.19 (m, 10 H, aromatic *H*), 5.15 (m, 1H, C*H*O), 3.79 (m, 1H, C*H*N), 3.66 (s, 3H, NC*H*$_3$), 2.25-1.73 (m, 6H, C*H*$_2$C*H*$_2$C*H*$_2$).

^{13}C-NMR (400 MHz, CD_2Cl_2): 174.2 (NCHN), 133.6-133.3-133.0-131.7-131.6-131.3-131.0-130.8 (aromatic *C*) 128.6-129.1 (*C*=*C*), 80.5(*C*HO), 68.3 (*C*HN), 38.6 (N*C*H$_3$), 34.3-33.5-21.1 (*C*H$_2$*C*H$_2$*C*H$_2$).

3.4.9   Biological procedures

3.3.9.1 Cell cultures

Cell lines used in these studies were:

- MCF-7: Human breast carcinoma cell line that expresses the estrogen receptor ERα (ER-positive. The cell line was maintained in culture in Dulbecco's Modified Eagle's Medium/Nutrient mixture F-12 HAM (DMEM/F12), with the addition of 5% Newborn calf serum (NCS) and 100 units/ml penicillin-streptomicina.

- MDA.MB-231: Human breast carcinoma cell line not expressing the estrogen receptor (ER-negative). The cell line was maintained in culture in Dulbecco's Modified Eagle's Medium/ Nutrient mixture F-12 HAM (DMEM/F12), with the addition of 5% Fetal Bovine Serum (FBS), 1% L-glutamine and 100 units/ml penicillin-streptomycin.

- MCF-10A: breast glandular healthy epithelium cell line. The cell line was maintained in culture in Dulbecco's Modified Eagle's Medium/ Nurient mixture F-12 HAM (DMEM/F12), with the addition of 5% horse serum (HS), growth factor of epidermis (EGF) 0,02 μg/ml hydrocortisone 0,5 μg/ml, cholera toxin 0,1 μg/ml insulin, 10 μg/ml and penicillin/streptomycin 100 units/ml.

Cells were maintained in culture at a temperature of 37°C in humidified atmosphere with 5% CO_2, inside an incubator for cells. Each experiment was performed using plates multi-well. Cells, after trypsinization, were plated in a defined quantity. The treatments were carried out in free of red phenol DMEM/F12 with 2% NCS.

3.3.9.2 Trypsinisation

The trypsinization was the technique used to remove cells adhered to the bottom of the plate of maintenance. We aspirated the culture medium from the plate and we washed the plate for three times with phosphate buffered saline (PBS) to remove the serum residues. Then we added tripsin, a protolithic enzyme able to remove cells from the substrate. The plate was left a few minutes in the incubator until the cells begin to detach. The cells were then suspended again in medium containing serum to neutralize the trypsin, counted and used appropriately.

3.3.9.3 Cell counting

Cell count is based on the exclusion of the dye Trypan Blue from viable cells, unlike the dead cells, possessing a cytoplasmic membrane integrates, does not allow its penetration in the cytoplasm. A share of the cell suspension obtained following trypsinization, suitably diluted in the colorant, and this dilution was loaded in a Burker chamber, to allow the count of the cells under a microscope. The number of cells/ml was calculated with the equation:

Average number of cells counted/square x dilution factor x factor of counts of the chamber (10^4).

3.3.9.4 Cell proliferation assays: MTT assay

The MTT-assay is a cell proliferation assay, which makes it possible to assess the viability of the cells in culture. It is based on the ability of viable cells to reduce the MTT (bromide of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) in corresponding insoluble formazan reaction, catalyzed by mitochondrial reductase. This assay of cell viability was performed on cells ($3x10^4$/well) plated in multiwell x24, following treatment with the compounds under examination. The culture medium contained in each well were added 100 µl of MTT solution 2mg/ml and the cells were allowed to incubate for 2 hours at 37°C. The metabolically active cells converted the yellow MTT into insoluble purple formazan. The medium was aspirated from each well, and the formazan crystals present inside the cells were solubilized with 500 µl of dimethyl sulfoxide (DMSO). The intensity of the coloration of the solution of formazan is directly proportional to the number of viable cells, and measured in a spectrophotometer at a wavelength of 570 nm.

3.3.9.5 Cell proliferation assays: Soft Agar Assay

Soft agar assay allows to evaluate the ability of the cells to form colonies in the absence of adhesion to the substrate (proliferation anchorage-independent) and its possible modulation following treatment with the compounds under examination. The cells (15000/well) were plated in 2 ml of Medium 5% cs-FBS to 0.35% Agarose (top layer) on the basis of medium to 0.7% agarose (bottom layer) on the bottom of the plate by twelve wells. After a day after sowing, was added to the cells, the treatment medium, and this was renewed every three days. After 2 weeks, 100 µl of MTT were added to each well, leaving the plate in an incubator at 37°C for 4 hours and subsequently

over-night at 4°C. The colonies of size greater than 50 μm were then counted under the microscope.

3.3.9.6 Preparation of total protein extracts

Cells were plated in multiwell x6 in complete culture medium and treated with the compounds under examination. At the end of the treatment period the medium was transferred into centrifuge tubes and centrifuged at 1500 rpm for 5 minutes. The cells present in the treatment means are then recovered as pellets. Cell lysis was carried out by adding to each well of the plate 100 μl of lysis buffer (HEPES 50 mM pH 7.5, NaCl 150 mM, $MgCl_2$ 1.5 mM, 1 mM EGTA, glycerol 10%, Triton-X-100 1%, PMS-F 1 mM, Aprotinin 0.2 mM Naortovanadato, 0.2 mM). The buffer of lysis, uniformly distributed on the plate, disrupted the cell membranes allowing the recovery of the whole protein cell content. The activity of the protease was inhibited by fenilmetansulfonil-fluoride (PMSF), aprotinin and Na-orthovanadate, protease inhibitors added to the buffer of lysis at the moment of use. After 5 minutes, the content of each well was used to resuspend the pellet previously obtained, and the suspension was then transferred to an eppendorf and kept on ice for 30 minutes, vortexing every 5 minutes. The content of each eppendorf was then centrifuged at 14000 rpm for 10 minutes at 4°C. The pellets was constituted by the cellular membranes, while the supernatant, the protein extract soluble, was transferred into new Eppendorf and stored at -80°C.

3.3.9.7 Determination of the protein concentration

The determination of the protein concentration of the cell extract was carried out by the spectrophotometric method of Bradford. This dosage was more advantageous than commonly used assays. It was characterized by a high sensitivity, a reduced presence of interference the dye used has an absorption relatively stable, requires only one reagent and has a reaction time of 5 minutes. The protein assay was based on the observation that the maximum of the absorption of an acidic solution of Coomassie Brilliant Blue G-250 moved from 465 nm to 595 nm when the dye, of an acidic nature, bindings to the basic residues of proteins. The dosage is executed building, first, a calibration straight line. We prepared different cuvettes with 1 ml of reagent of Bradford (diluted 1:5) added with known amounts of bovine serum albumin (BSA). The mixture was stirred and reacted for 5 minutes. Finally we read the absorbance at the spectrophotometer at a 595 nm. wavelength. After we prepared the calibration straight line, we proceeded to the analysis of unidentified samples, preparing various cuvettes, each with 1 ml of the diluted dye and 10 μl sample incognito, suitably diluted. The absorbance values at 595 nm of the protein samples were reported on the calibration line, obtaining in this way the respective concentration values.

3.3.9.8 Electrophoresis of proteins in denaturing conditions (SDS-PAGE)

25 μg of proteins from each sample, were separated by SDS-polyacrylamide gel electrophoresis. The percentage of acrylamide in the gel was chosen as a function of the molecular weight of the proteins that we wanted to study. The polyacrylamide gel was composed of two parts: the

stacking gel and the resolving gel. The first, placed in the upper part, represented the portion of the gel in which we created the wells of loading. Furthermore it allowed to "pack" all the loaded material so that it arrives uniformly on the front of the race. The second is the portion of the gel where the true and proper electrophoretic run that allows to separate the sample of interest according to its dimensions. Stacking Gel and resolving gels had same composition, but a different concentration of acrylamide, as reported in table 3.1.

Table 3.1 *The composition of the stacking gel and the resolving gel*

	Stacking gel	10%	12%	15%
H20	6,4 ml	4,9 ml	4,4 ml	3,43 ml
A.A./BIS A.A. (30/0.2)	1 ml	2,5 ml	3 ml	3,75 ml
TRIS/HCl 1,876 M*	2,5 ml	2,5 ml	2,5 ml	2,5 ml
SDS 10%	0,1 ml	0,1 ml	0,1 ml	0,1 ml
APS 10	0,2 ml	0,2 ml	0,2 ml	0,2 ml
TEMED	0,02 ml	0,02 ml	0,02 ml	0,02 ml

* TRIS/HCl at pH 6,8 for stacking gel, at pH 8,8 for resolving gel.

Before of electrophoretic separation, the samples were added to the Laemmli buffer (4% SDS, 20% glycerol, β-mercaptoethanol 10% bromophenol blue 0.004%, Tris-HCl 0.125%). Then the samples were brought to the same final volume by addition of the same buffer used for the extraction

of the proteins. Proteins contained in each sample were subjected to a treatment of thermal denaturation, at 100°C for 5 minutes. Simultaneously to the samples, we loaded in a well of the gel, a marker of molecular weights known (Precision Plus Protein™ to Blue Standards, Bio-Rad), essential for the identification of the protein of interest. The electrophoretic run was carried out in a vertical apparatus for electrophoresis in the presence of a buffer of stroke (Tris 25 mM glycine, 1.4% SDS, 0.1%, pH 8.3), 100 Volt for about 120 minutes. This operation allowed the separation of the proteins, negatively charged, depending on their molecular weight.

3.3.9.9 Immunoblotting

Proteins separated on a polyacrylamide gel are transferred by "electrotransfer" on a nitrocellulose membrane using a transfer apparatus in semi-dry (BIORAD). The gel and the membrane were immersed in the transfer buffer (20 mM Tris Glycine, 150mM, 0. 5% SDS and 20% methanol). We set up the transfer's apparatus as a sandwich (sponge, nitrocellulose membrane, gel, two sponges). The transfer chamber was constituted by two parallel electrodes and the nitrocellulose membrane was placed between the gel and the anode. We applied to the transfer chamber an electrical current (25 volts for 1 hour) perpendicular to the gel, and the proteins negatively charged will migrate from the gel toward the positive pole, being transferred on nitrocellulose membrane. The quality of the transfer can be verified through the reversible staining of the membrane with the Red Ponceau (solution at 1% of red Ponceau in 5% acetic acid). The membrane was decolorized by washings with TBST 1X (Tris buffer saline-Tween: Tween-20 0.1%, 10mM Tris/HCl and NaCl 100mM).

3.3.9.10 Immunological detection

The nitrocellulose membrane was subjected to stirring for 60 minutes at room temperature in blocking solution, formed by TBST 10X (Tris/HCl 100mM, 1 M NaCl and tween-20 1%) with the addition of milk powder to 5%. The function of blocking solution was to block all hydrophobic sites present on the membrane, responsible otherwise of aspecific interactions with the antibody. Subsequently the membrane was washed three times for five minutes with TBST 1X and is incubated overnight at 4°C with a solution of TBST 10X 5 % BSA containing the primary antibody specific for the protein of interest. The excess of antibody was subsequently removed by three washes in TBST 1X. The antigen-antibody complexes were detected after incubation for a hour at room temperature with a secondary antibody specific (in solution TBST 10X with 5% powdered milk), able to recognize the constant portion of the IgG molecule used as primary antibody. The secondary antibody is conjugated to horseradish peroxidase, an enzyme capable of catalysing, in basic conditions, the oxidation of a substrate luminescent chemo, Enhanced Chemiluminescence Substrate (ECL), in a product able to impress a photographic plate.

Primary antibodies used:

- antibody anti-p21, rabbit IgG (Santa Cruz Biotechnology), dilution 1:500;

- antibody anti-p53, mouse IgG (Santa Cruz Biotecnology), dilution 1:500;

- Anti-PARP, rabbit IgG (Santa Cruz Biotecnology), dilution 1:500;

- Anti-β-actin, mouse IgG (Santa Cruz Biotecnology), dilution 1:20000.

- Anti-GAPDH, rabbit IgG (Santa Cruz Biotecnology), dilution 1:10000.

Secondary antibodies used:

- Antibody biotinylated goat anti-rabbit IgG (Santa Cruz Biotechnology), dilution 1:7000;

- Antibody biotinylated goat anti-mouse IgG (Thermo Scientific), dilution 1:2000.

3.3.9.11 Stripping of the nitrocellulose membrane

After we have detected a certain protein on a nitrocellulose membrane of a Western blotting, we can reuse the same membrane for the analysis of another protein. Before we carried out a new analysis, we had to remove the antibodies bound to the membrane by a process called stripping. The membrane was washed for two times of 15 minutes with stripping buffer (glycine solution 0.2 M at pH 2.6), and then washed two times for 10 minutes with TBST 1X. The membrane was then stirred for 60 minutes at room temperature in blocking solution, and re-incubated with a new primary antibody.

3.3.9.12 DNA Extraction

The cells were plated on 100 mm plates. At the end of the treatment, the medium present in the plates was transferred into centrifuge tubes and

centrifuged at 2500 rpm for 5 minutes. The cells present in the treatment means were then recovered as pellets. The cells, adhering on the bottom of the plate, were detached using trypsin, then neutralized by the addition of medium enriched of serum. The cell suspension was transferred into tubes happy the pellet of centrifugal first, and the suspension was subjected again to centrifugation for 5 minutes at 2500 rpm. To the obtained pellet, represented by the cells, were added 100 μl of lysis buffer (Tris/HCl 50μM, EDTA 20nM, NP-40 1%), and this was allowed to act overnight at -80°C. SDS and Rnase-A were added to respectively denature the proteins and the RNA. Lysates were incubated at 56°C for 2 hours. In this conditions, Rnase-A enzyme could act and, at the same time, inhibit the activity of Dnase, possibly present in the sample. enzyme proteinase-K was subsequently added to degrade the proteins, and the samples were incubated for 2 hours at 37°C. 500 μl of cold absolute ethanol and 65μL of sodium acetate 3M were then added to each sample, to precipitate the DNA. After incubation at -80°C, overnight, we centrifuged the samples for 20 minutes at 12000 rpm at 4°C. The supernatant was removed and the pellet, represented by the DNA precipitate was washed with cold ethanol at 75% to solubilize the salts precipitated together with the DNA. After centrifugation at 12,000 rpm for 20 minutes at 4°C, the supernatant and the pellet were removed, and the DNA was resuspended in 50 μl of TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

3.3.9.13 DNA electrophoresis on agarose gel

The agarose gel electrophoresis was a technique that allows to booth DNA molecules according to their size, exploiting their different speed of migration through the agarose gel, subjected to an electric field. The agarose gel was

prepared at a concentration of 2% in TBE buffer (Tris-borate-EDTA) added with ethidium bromide, an intercalating agent that makes visible the DNA by fluorescence emission due to exposure to UV lamp. On agarose gel was loaded a marker, consisting of a mixture of DNA fragments having known molecular weight, and the different samples mixed with a loading buffer, containing thickening agents and dyes to facilitate loading of the samples and to monitor the progress of the electrophoretic run.

3.5 Results and Discussion

3.5.1 Synthesis of NHC ligands

3.4.1.1 Synthesis of L20 salt.

Synthesis of L20 salt N-methyl-N'-[(2-hydroxy-2-phenyl)ethyl]-imidazole iodire was carried out in inert nitrogen atmosphere as shown in figure 3.13. Imidazole was reacted with 1,2-epoxyethylbenzene. Mixture was stirred for 24 h at 60°C to obtain the monoalkylated product after the opening of epoxy-ring. In this way was synthesized the monoalkylating product by the opening of the epoxy ring. The was added the second alkylating agent (CH$_3$I) and acetonitrile and the mixture was refluxed for 3,5 h. This second reaction step, with which the alkylation of the second nitrogen atom, ended with cooling to room temperature the mixture, removal of the solvent under vacuum (in a rotavapor) and recovery as a precipitate, cold acetone, in racemic mixture of salt L20.
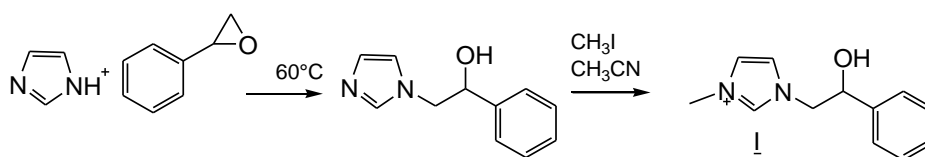
Figure 3.13. *Synthetic schemes of L20 salt.*

3.4.1.2   Synthesis of R3 salt.

Synthesis of R3 salt N-methyl-N'-[2-hydroxycyclopentan]-4,5-dichloroimydazole iodire was carried out in two steps in nitrogen atmosphere, as showed in figure 3.14.

4,5-dichloroimydazole was reacted with 1,2-epoxycyclopentane. The mixture of reaction was stirred for 48 h at 60°C. In this way the epoxy ring opened with formation of monoalkylated product, that was not isolated.
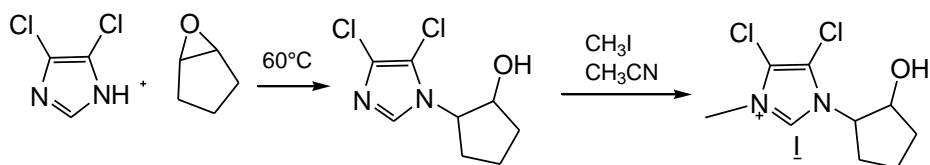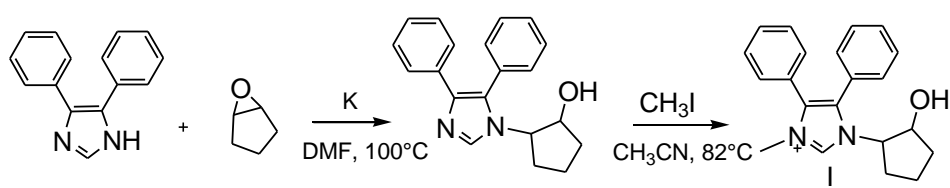

Figure 3.14. *Synthetic schemes of R3 salt.*

Then the second alkylating agent (CH_3I) and CH_3CN were added; the mixture was refluxed for 24 h. this second step of reaction, in which alkylation of second nitrogen atom was carried out, stopped with cooling to room temperature of mixture, removing of solvent (by rotavapor) and cold-precipitation from acetone as racemic mixture of R3 salt.

The salt was characterized by proton and carbon Nuclear Magnetic Resonance (^{1}H-NMR, ^{13}C-NMR) and by Mass Spectrometry

3.4.1.3   Synthesis of R5 salt

Synthesis of R5 salt *N-methyl-N'-[2-hydroxycyclopentan]-4,5-diphenylimydazole* iodire was carried out in two steps in nitrogen atmosphere. In the first step, shown in figure 3.15, the amine nitrogen was deprotonated by

potassium and the monoalkylated product was synthesized by the opening of the epoxy ring. The trans opening of the epoxy ring generated a molecule with 2 stereogenic centers; the product was obtained as a racemic mixture.

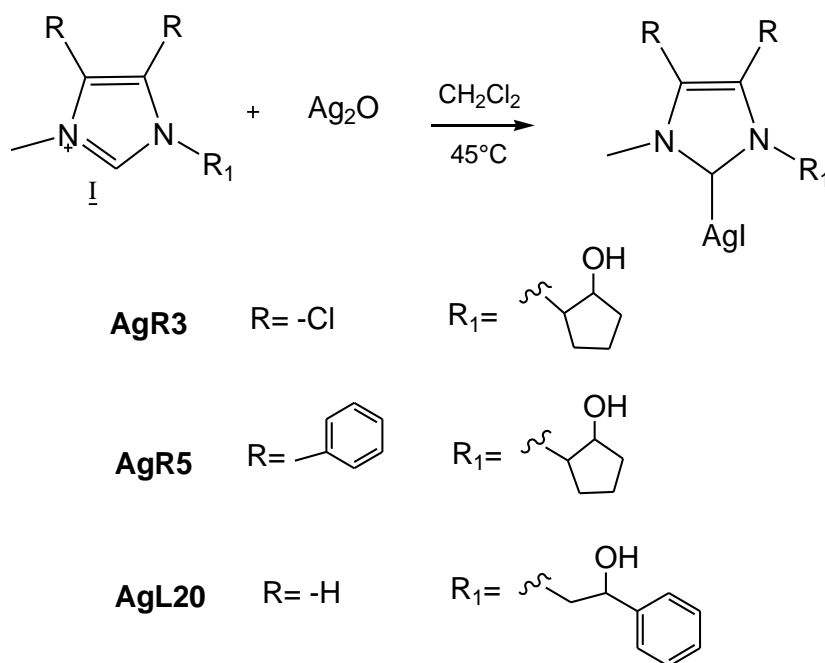**Figure 3.15** *Synthetic schemes of R5salt.*

Potassium was dissolved in a suspension of 4,5-diphenylimidazole in dimethylformamide (DMF) and then epoxide was added. Mixture was stirring for 17 h at 100 °C to alkylate one of the nitrogen atoms. For the work up, a quantity of water ten times greater than DMF was added and a white solid precipitated. The mixture was filtered, giving a yellow solution and a precipitate. This one was solved in chloroform. The solution was firstly washed with HCl 5N and then with water. The product was recovered as a white solid by cold-precipitation from ethyl acetate. Product was also recovered by yellow solution by extraction with chloroform. Then it was revovered by cold-precipitation from ethyl acetate.

The second step of reaction, shown in figure monoalkylated product (obtained in first step) was reacted with iodomethane (CH₃I) in acetonitrile (CH₃CN). Mixture was stirred and refluxed for 3,5 h in inert nitrogen atmosphere. The product was recovered by cold-precipitation from acetone (-20°C). Monoalkylated product (obtained in first step) was reacted with iodomethane (CH₃I) in acetonitrile (CH₃CN). Mixture was stirred and refluxed

for 3,5 h in inert nitrogen atmosphere. The product was recovered by cold-precipitation from acetone (-20°C).

3.5.2 Synthesis of Ag-NHC complexes.

Synthesis of Ag-NHC complexes AgR3 *N-methyl-N'-[2-hydroxycyclopentan]-4,5-dichloroimidazol-2-ylidene-iodire of silver (I)*, AgR5 *N-methyl-N'-[hydroxycyclopentan]-4,5-diphenylimidazol-2-ylidene-iodire of silver (I)* and *AgL20 N-methyl-N'-[(2-hydroxyphenyl)ethyl)]-imidazol-2-ylidene-iodire of silver (I)* was carried out as shown in figure 3.16 in inert atmosphere of nitrogen.

Figure 3.16. *Synthetic schemes of Ag-NHC complexes.*

The salt (R3, R5, L20) was reacted with silver oxide (Ag$_2$O) in dichloromethane (CH$_2$Cl$_2$) with molecular sieves. Reaction was refluxed and sheltered from light. In these conditions the silver oxide also acted as a base (tore the proton of carbon 2). In situ was generated the N-heterocyclic carbene that reacted with the metal creating the Ag-NHC complex. The water, formed as by-product, was subtracted from the molecular sieves, shifting the equilibrium of the reaction toward the right. The complexes Ag-R3, Ag-R5 and Ag-L20 were obtained as a racemic mixture.

All complexes were characterized by ^{1}H- and ^{13}C-NMR. The structure of the complexes was also confirmed by mass spectra. For Ag-R3 a peak at 577.3 Dalton, attributable to the molecular ion [C$_{18}$H$_{24}$AgCl$_4$N$_4$O$_2$]+, confirmed the structure. Ag-R5 mass-spectrum showed a peak at 765 Dalton, attributable to [C$_{42}$H$_{44}$AgN$_4$O$_2$]+ that loses a water molecule and buy a potassium ion, and a peak at 319.7 Dalton, attributable to a fragmentation in which the complex in the form of a monomer loses a phenyl and buy a potassium ion. For Ag-L20 was reported a peak 513 Dalton attributable to [C$_{24}$H$_{29}$AgN$_4$O$_2$]+. These mass values confirmed the literature datas., Mono-NHC Ag (I) complexes in solution seems to be in equilibrium with the dimeric species (figure 3.17).
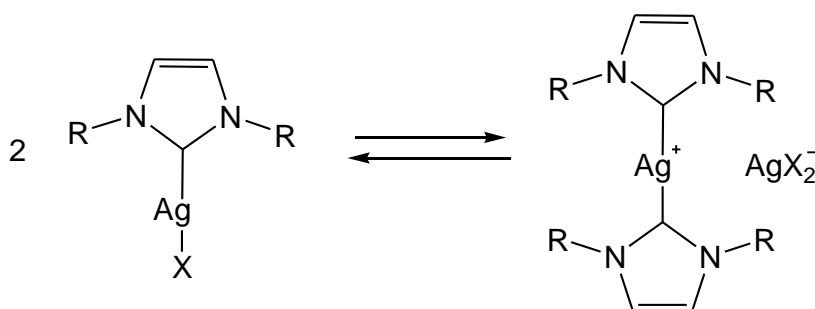
Figure 3.17. *Structures of mono Ag-NHC complxes in solution.*

3.5.3 Synthesis of Au(I)-NHC complexes.

Synthesis of Au-NHC complexes AuR3 *N-methyl-N'- [2-hydroxycyclopentan]-4,5-dichloroimidazol-2-ylidene-iodire of gold (I)*, AuR5 *N-methyl-N'-[2-hydroxycyclopentan]-4,5-diphenylimidazol-2-ylidene-iodire of gold (I)* and *AuL20 N-methyl-N'-[(2-hydroxy-2phenyl)ethyl]-imidazol-2-ylidene-iodire of gold (I)* were carried out as shown in figure 3.18 in an inert atmosphere of nitrogen. These complexes were obtained by transmetallation from the corresponding complex of silver-NHC. Ag-NHC was reacted with chloro(dimethylsulfide) of gold (I) $(CH_3)_2AuSCl$, in dichloromethane (CH_2Cl_2). Reaction was carried out at room temperature, sheltered from light. In these conditions it was realized the transmetalation reaction wherein the atom of silver is replaced by an atom of gold that bound to the carbenic carbon leading to the formation of the complex of Au-NHC as racemic mixture.
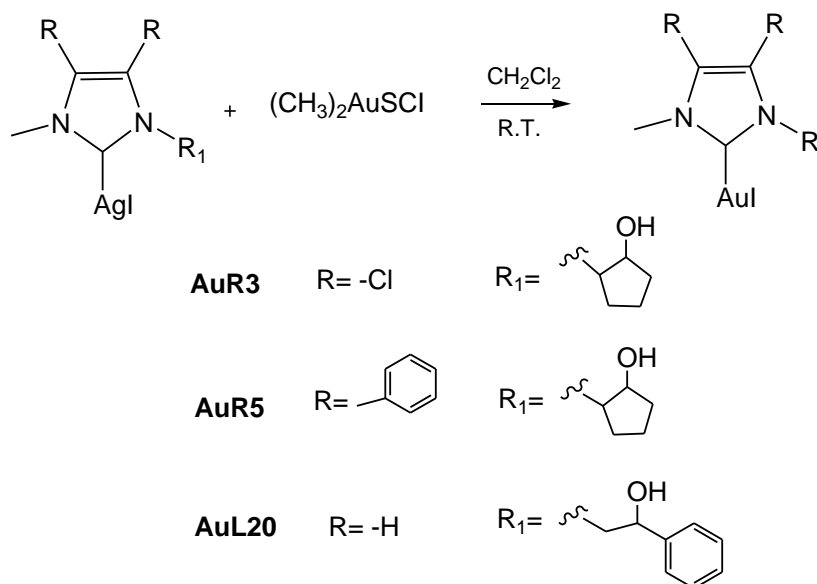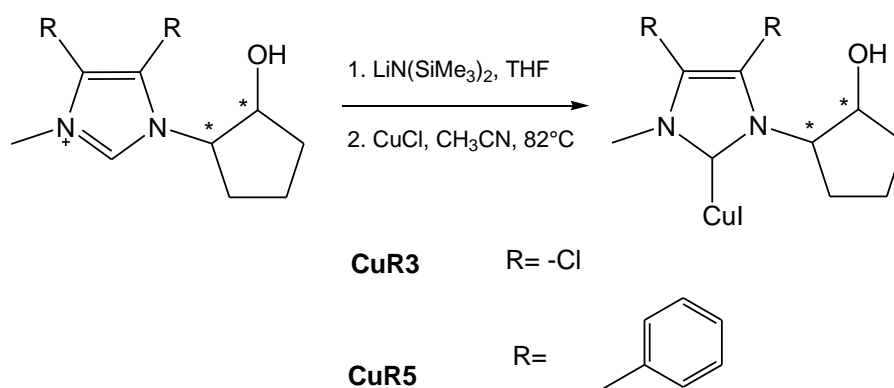

Figure 3.18. *Synthetic schemes of Au-NHC complexes.*

3.5.4 Syntesis of Cu(I)-NHC complexes.

Synthesis of Cu-NHCcomplexes **CuR3** *N-methyl-N'-[2hydroxycyclopentan]-4,5-dichloroimidazol-2-ylidene-iodire of copper(I)* e **CuR5** *N-methyl-N'-[2-hydroxycyclopentan]-4,5-diphenylimidazol-2-ylidene-iodire of copper(I).*

Synthesis was carried out as shown in figure 3.19 in nitrogen inert atmosphere. The salt (R3 or R5) was stirred with Lithium bis(trimethyl)sililamide in tetrahydrofuran (THF), until the complete dissolution of the salt. After 1 h, a suspension of copper chloride (CuCl) in acetonitrile was added in the mixture. Reaction refluxed overnight to synthesize a racemic mixture of Cu-NHC complexes. Copper complexes were unstable because of the rapid oxidation of the copper I to copper II in the presence of the oxygen atmospheric. Because of their insolubility, Cu-NHC complexes did not show any positive biological result. So, I did not synthesize CuL20.


**Figure 3.19.** *Synthetic schemes of Cu-NHC complexes*

3.5.5 Biological results

AuR3 e **AuL20** e **AgL20** was tested on MCF-7 (breast carcinome cell-line, expressing the estrogen receptor ERα, ER-positive), MDA-MB-231 (breast carcinome cell-line, not expressing the estrogen receptor ERα, ER-negative), MCF-10A (healthy glandular breast epithelium cell line).

Antiproliferative activity of AuR3 was evaluated by cell proliferation assay anchorage-dependent (MTT-assay) on MCF-7. The complex was diluted in DMSO and tested at different concentrations (0.10μm, 1μm, 10μm and 25 μm and 50 μm). Results are reported in the graph of figure 3.20.
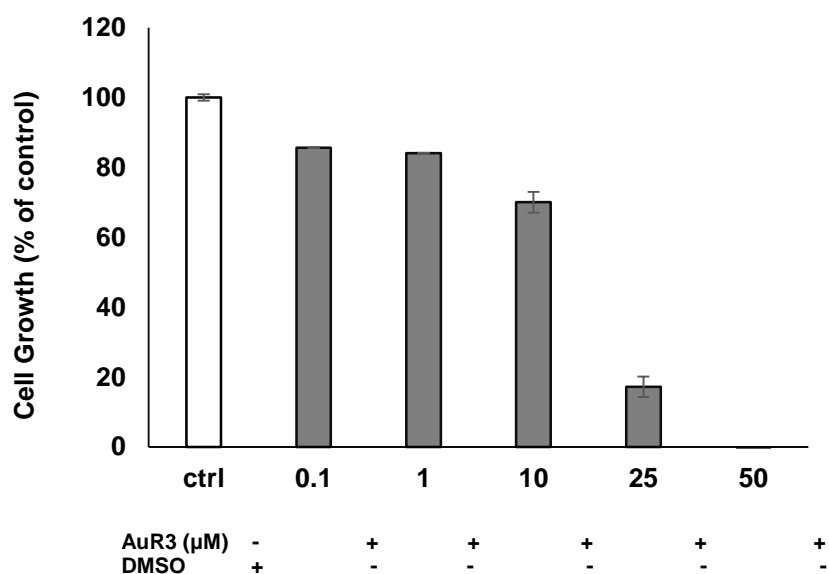
Figure 3.20. *Effect induced by the treatment with the complex of AuR3 at different concentrations on cell proliferation anchorage-dependent of the tumor cell line MCF-7.*

AuR3 showed a good inhibitory effect on the proliferation of this cell line. Was then evaluated the activity on MDA-MB-231 in the same conditions as the previous test and the results are shown in figure 3.12.
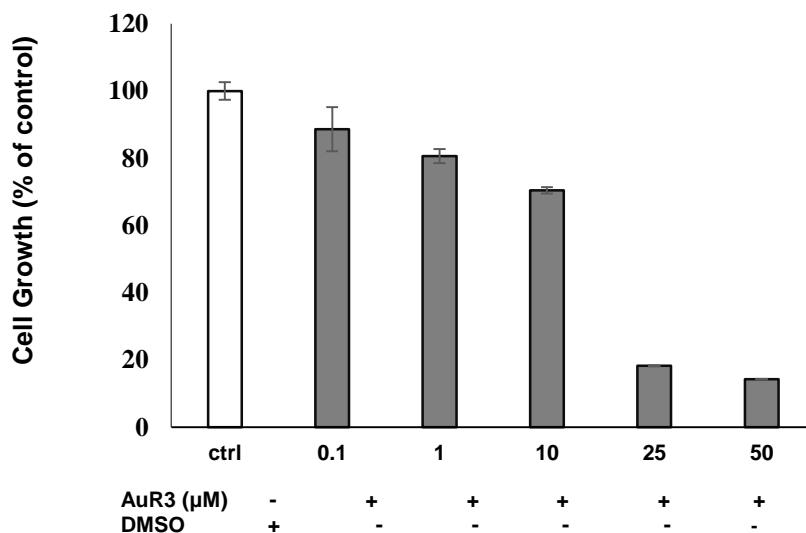
Figure 3.21. *Effects induced by the treatment with AuR3 on cellular proliferation anchorage-dependent of the tumor cell line MDA-MB-231.*

Also in this case the complex showed proliferative activity comparable to that observed with the previous cell line but at a concentration of 50 μM was more active on MCF-7.

Finally, to exclude a possible toxicity of healthy cells,AuR3 was tested on MCF-10A, using the same experimental conditions of the previous experiments. As we can bee from the graph of figure 3.22, it can be noted a mild cytotoxic effect only at a concentration equal to 50 μm.
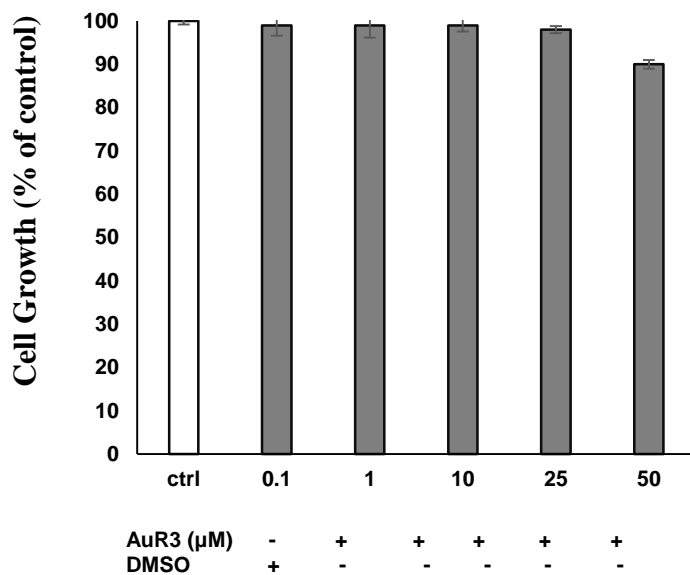
Figure 3.22. *Effects induced by the treatment with AuR3 on proliferation anchorage dependent cell line MCF-10A.*

The antiproliferative activity of AgL20 and AuL20 was evaluated by cell proliferation assay anchorage-dependent (MTT-assay) on MCF-7. The complexes were diluted in DMSO and tested at different concentrations (10nm, 100nm, 1μm, 5μm and 10 μm). The antiproliferative effect was evaluated after a treatment period of three days. The absorbance values, read at 590 nm, were related to that bed for a negative control represented by cells treated exclusively with DMSO. The results are reported in the graph of figure 3.23.
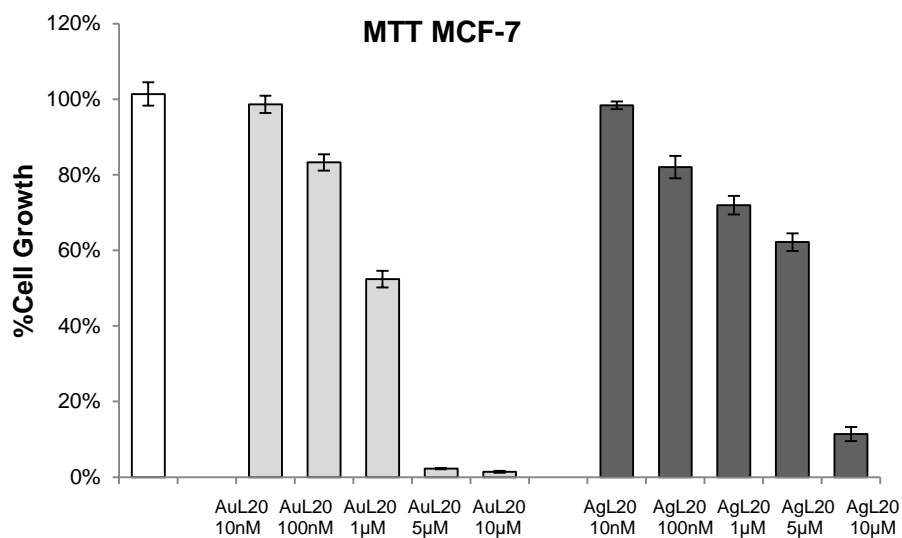
Figure 3.23. *Effect induced by the treatment with the complex AuL20 and AgL20 at different concentration on cell proliferation anchorage-dependent of the tumor cell line MCF-7.*

We observed an inhibitory effect on the proliferation of this cell line, dose-dependent and a marked antiproliferative effect already at low concentrations. Between the two complexes tested, the gold one demonstrated, at equal concentration, greater antiproliferative than AgL20.

From the results obtained, IC_{50} (concentration of the compound capable of inhibiting cellular proliferation of 50 % relative to the control) was calculated as the average of 5 experiments $\pm$ standard deviation. IC_{50} values are reported in table 3.2.

Table 3.2. *IC$_{50}$ values for AuL20 and AgL20 on tumor cells of the line MCF-7.*

| COMPLEXES	IC$_{50}$
AuL20	0,9±0,2Mm
AgL20	4±1,8µM

To confirm the antiproliferative activity observed with MTT-assay, was conducted a cell proliferation assay anchorage-independent (Soft Agar Assay) on the same cell line mammary MCF-7. The proliferation of the cells in the absence of a solid support mimics better conditions for the proliferation of tumor cells in the body, with respect to the proliferating in mono-layer, studied by the previous experiment of MTT assay. The ability of the cells of the line MCF-7 to form colonies, suspended in medium semi-solid, was evaluated after treatment of the cells with the two complexes, at concentrations 1 µm and 10 µM. The results of these experiments are reported in figure 3.24 and are expressed as the number of colonies of sizes greater than 50 µm, counted under the microscope. These experiments gave results in agreement with the MTT assay and confirmed the activity of the complexes; particularly active was the complex AuL20.
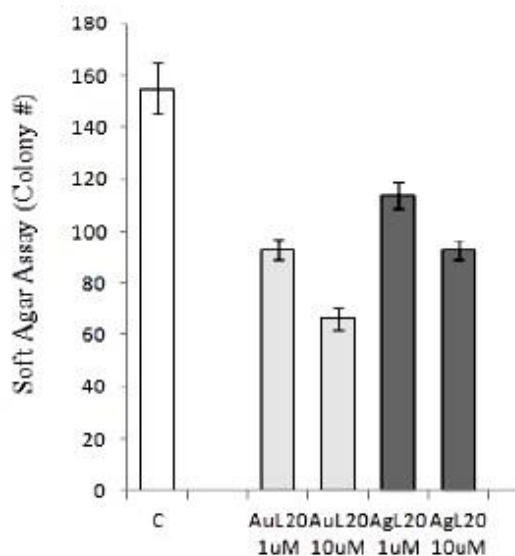
Figure 3.24. *Effects induced by the treatment with AuL20 and AgL20, on cellular proliferation anchorage-independent, of tumor cell line MCF-7.*

In order to evaluate the specificity of the antiproliferative effect only on tumor cell lines and therefore to exclude a possible toxicity on healthy cells was conducted a test of cell viability by MTT assay, on cell line MCF-10A. Cells were treated for three days with the two complexes, at the same concentrations used in the preliminary studies on MCF-7 and results, compared to the control, were reported in the graph of figure 3.25 from which emerges the absence of cytotoxic effects even at the highest concentrations assayed.
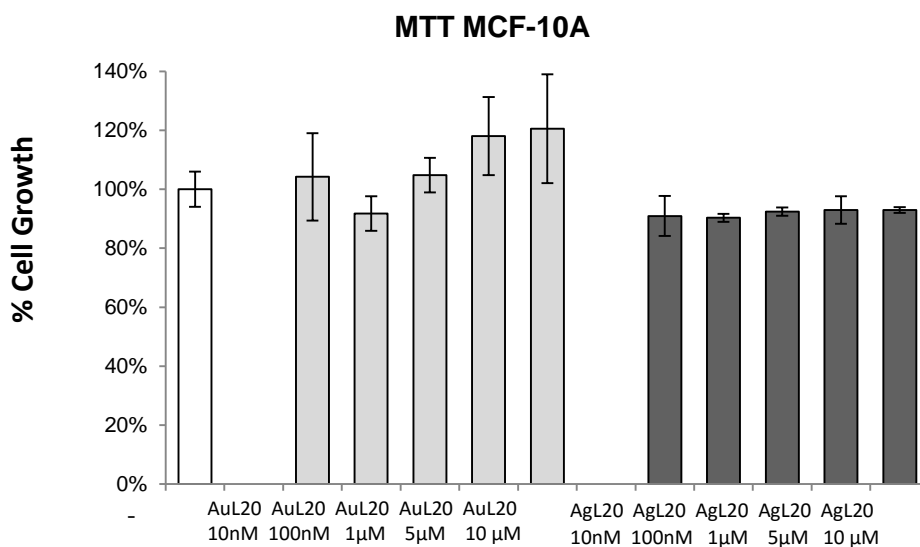
Figure 3.25. *Effects induced by the treatment with AuL20 and AgL20 on the proliferation anchorage dependent cell line MCF-10A*

Highlighted the absence of toxic effects on healthy cells, we decided to investigate if the antiproliferative activity exerted by AuL20 on MCF-7 was connected to the mechanisms of regulation of the cell cycle. Therefore, we evaluated the expression of two proteins involved in the regulation of the cell cycle, p53 and p21.

p53 is able to induce cell growth arrest, apoptosis and cellular senescence but in normal cells p53 is usually inactive, bound to the protein MDM2 that inhibits and promotes its degradation. Only after the cellular damage, such as for example a DNA damage, p53 is activated and stimulates the transcription of many genes including that which encodes for the protein p21. This proteins binds the complex cyclin-Cdk2 and cyclin-Cdk4 and it inhibits the activity, regulating the cell cycle progression at the level of the checkpoint between phases G1 and S.

Cells were treated with AuL20 (at concentrations of 100nM, 1μM and 5μM) for 24 hours. After this period were lysed was extracted the entire protein content which was then loaded on polyacrylamide gel allows for the electrophoretic separation of proteins. The expression of p21 and p53 was evaluated by immunoblotting. β-actin was used as "loading control" to confirm the loading of the same amount of protein for each sample. Results are shown in figure 3.26.
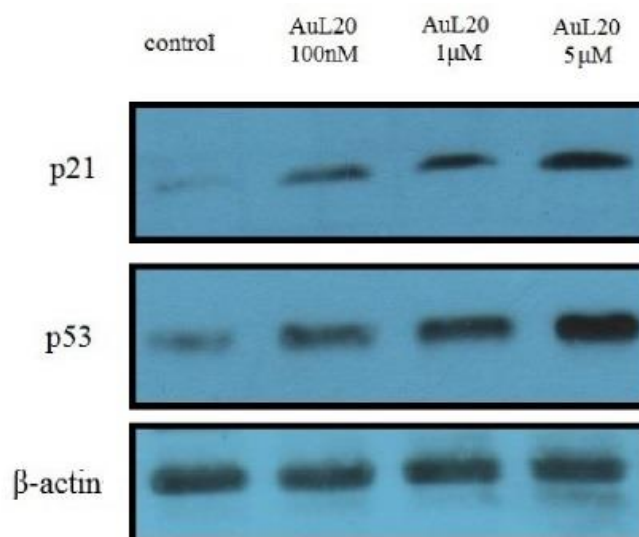
Figure 3.26. *The effects of treatment with AuL20 on the levels of expression of protein p53 and p21 in line mammary tumor MCF-7.*

Results showed a marked modulation of the expression of p21 and p53, confirming the involvement of these proteins in the antiproliferative activity of the complex AuL20 and the block of the proliferation of cells treated between the G1 phase and the step S.

AuL20 and AgL20 were tested on MDA.MB-231. Cells were treated for three days with the complexes, at concentrations of 10 nM, 100 nM, 1 μm, 5 μm and 10 μm. Cell proliferation was evaluated by MTT assay. The results, compared to the control, are shown in the graph of figure 3.27.
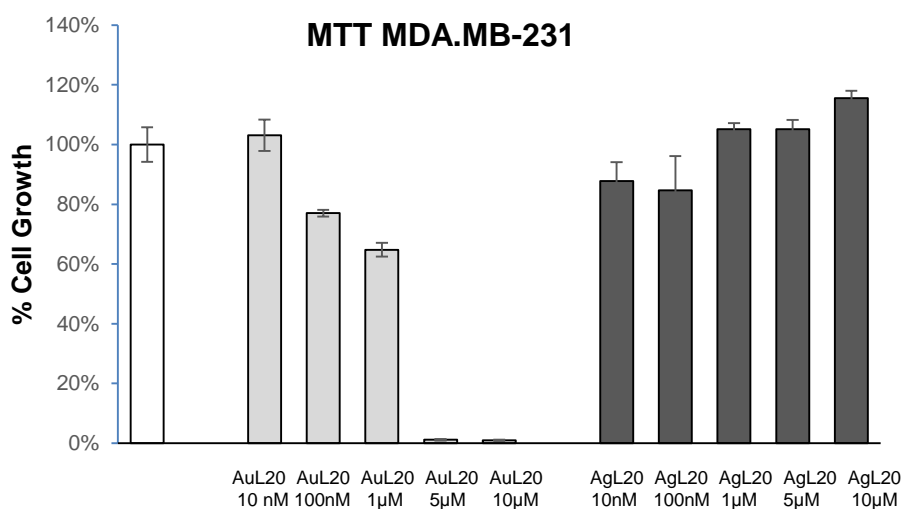

Figure 3.27. *Effects induced by the treatment with AuL20 and AgL20 on cellular proliferation anchorage-dependent of the tumor cell line MDA-MB-231.*

The complex AgL20 did not show any antiproliferative activity against cell line MDA-MB-231. We assumed for AgL20 a mechanism of action closely related to the expression of ER. Instead, AuL20 showed an intense antiproliferative activity also in breast carcinoma cells ER-negative. With the results obtained we calculated the value of IC50 on this cell line as the average of 5 experiments ± standard deviation (3.43 ± 1 μM). The marked antiproliferative activity of AuL20, also observed in the absence of estrogen receptor, suggested, for this compound, a mechanism of action independent of

ER expression. However by comparing the IC50 values obtained in cell lines MCF-7 and MDA-MB-231, it can be inferred that the presence of the estrogen receptor is partly related to the activity of the complex AuL20. The highest value of IC50, obtained in the tumor cell line ER-negative, is in fact indicate a lower activity of the complex.

The involvement of the estrogen receptor in the mechanism at the base of the antiproliferative action of the complex AuL20 was deepened by immunoblot experiments. Variation of expression of ERα was evaluated on MCF-7 after treatment of the cell line with the complex AuL20. The cells were treated for 46 hours at 100 nM, 1 μM and 5 μM concentrations. The results reported in figure 3.28, showed a marked negative modulation of the expression of the receptor.
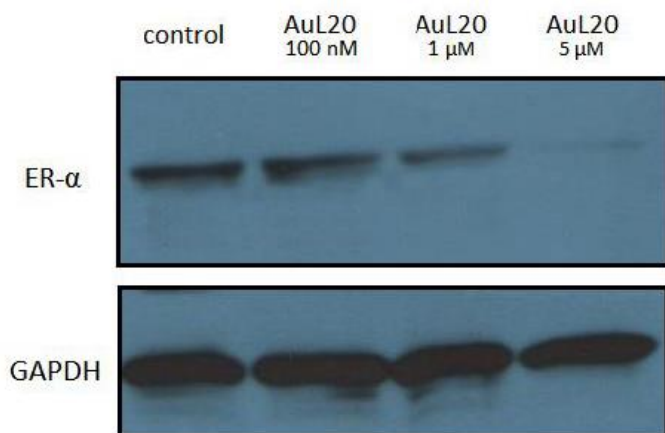
Figure 3.28. *The effects of treatment with AuL20 on the level of expression of the estrogenic receptor ERα.*

The reduction of the expression of this receptor was responsible for less stimulation of proliferation in the cell line ER-positive. This phenomenon justified the difference between the values of IC50 highlighted by previous experiments.

The latest experiments were focused on the study of the mechanism of cell death induced by complex AuL20. By immunoblot experiments we evaluated the presence of cleavage of the enzyme poly ADP-ribose polymerase (PARP), a phenomenon characteristic of the apoptotic process. The cleavage of PARP was highlighted after treatment of the cell line MCF-7 for 24 hours with the complex Aul20, at the highest concentration tested (5μm), as shown in figure 3.29.
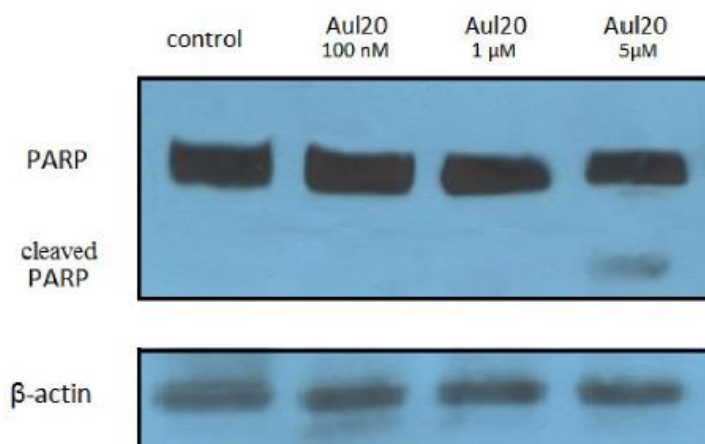

Figure 3.29. *Cleavage of the enzyme PARP in line mammary tumor MCF-7 after treatment with the complex AuL20.*

To confirm the apoptosis as the mechanism of cell death, it was decided to study a second key event in the apoptotic process, DNA fragmentation (DNA laddering). For this purpose the breast tumor cell line MCF-7 were treated for 24 hours at concentrations of 1 μM and 5 μM and, at

the end of the treatment period, the extracted DNA was subjected to electrophoresis separation on agarose gel. The result, shown in figure 3.30, has not revealed the DNA fragmentation.

In order to confirm or exclude definitively the induction of the apoptotic process on the part of the complex AuL20, are currently in progress new experiments to assess whether the phenomenon of fragmentation can be highlighted by prolonging the time of treatment beyond 24 hours.
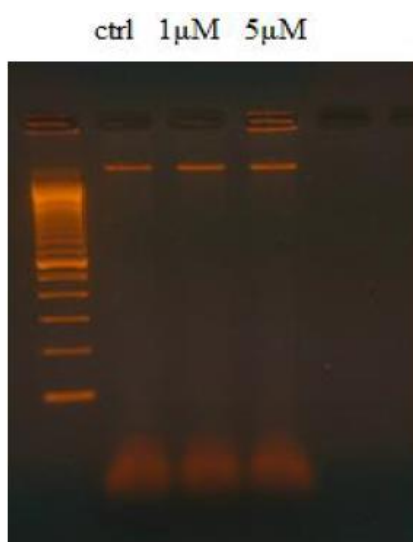
Figure 3.30. *DNA extracted from the line mammary tumor MCF-7 after treatment with AuL20.*

3.6 Conclusions

In this project various complexes NHC based on silver, copper and gold were synthesized with the purpose of evaluating the influence, that greater lipophilicity of the salt and the respective complexes NHC, could have on the pharmacological effect. The first two salts synthesized have the same substituents on the nitrogen atoms or a cyclopentanol on the nitrogen atom 1 and a methyl on the nitrogen atom 3 while differ for the substituents in position 4 and 5 of the imidazole ring. In particular the salt R3 binds two chlorine atoms while R5 binds two phenyl groups. The L20 salt was synthesized from the imidazole and it has a methyl group on the one nitrogen atom and a side chain containing a phenyl group and a hydroxyl group on the other nitrogen atom; in this case it is tried to understand what the different substituent on the nitrogen atom could influence the pharmacological effect. With these salts were synthesized complexes of silver and gold and copper which were then tested for their pharmacological activity.

The complex AgL20 was tested on MCF-7 by cell proliferation assay anchorage-dependent (MTT-assay) and showed a good activity, confirmed with the cell proliferation assay anchorage-independent (Soft Agar Assay), always performed on the same cell line mammary (MCF-7). To demonstrate the specificity of the antiproliferative effect only in relation to tumor cell, a MTT assay on MCF-10A was carried out. Finally, AgL20 was tested on MDA-MB-231 cellular line. In this case, it has shown no activity; therefore, we can assume a mechanism of action of AgL20 related to the expression of the estrogen receptor.

The complexes AuR3 and AuL20, instead, were tested on the same three cell lines used for AgL20. Both AuR3 that AuL20 showed antiproliferative activity on MCF-7 and MDA-MB-231 and no activity on

MCF-10A thus demonstrating the absence of toxicity on healthy cells and a selective antiproliferative action toward the tumor cells.

Biological studies AuL20 were intensified and oriented toward the search of the mechanism responsible for the antiproliferative activity. The cells of the line MCF-7, treated conAuL20 showed a negative modulation of the expression of two important proteins involved in the regulation of cell proliferation process, p53 and p21. The results obtained allow us to affirm that the treatment with the complex AuL20 determines the cell cycle arrest at the checkpoint G1/S. For AuL20 were also calculated values of IC_{50} on MCF-7 and MDA-MB-231. Whereas the highest value of IC_{50} obtained with the tumor cell line ER negative-it was possible to deduce that the presence of the estrogen receptor is related to the activity of the complex. Therefore, on the basis of the results obtained, the activity of AuL20, seems to be due to both mechanisms related to estrogen receptor that independent mechanisms from the ER expression. While the last two experiments were carried out to understand what was the mechanism of cell death induced by the complex of gold in examination. Results showed the presence of cleavage of the enzyme poly ADP-ribose polymerase (PARP), a phenomenon characteristic of the apoptotic process. For further confirmation, we investigated on the other phenomenon characteristic of apoptosis: the DNA fragmentation. After 24 hours of treatment, however, we did not highlight this event. In conclusion, from the preliminary data currently available, it can be stated that the carbenic complex of the silver and the gold(I) synthesized may be good antitumoral candidates.

3.7 References

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