STRUCTURE-ACTIVITY STUDIES, ROLE OF TRANSFERRIN, AND GENE EXPRESSION PROFILE OF THE CYTOTOXIC ACTIVITY OF Ti(IV) COMPLEXES IN COLON ADENOCARCINOMA HT29 CELLS

By

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Abstract

Ligand modification on titanocene dichloride has the potential to produce soluble and stable complexes. As part of our research efforts in the area of titanium-based antitumor agents, we have investigated the cytotoxic activity of $[Ti_4(maltolato)_8(\mu\text{-}O)_4]$, $(Cp\text{-}R)_2TiCl_2$ and $(Cp\text{-}R)CpTiCl_2$ ($R = CO_2CH_3$ and $CO_2CH_2CH_3$), and three water soluble titanocene-amino acid complexes—$[Cp_2Ti(aa)_2]Cl_2$ (aa = L-cysteine, L-methionine, and D-penicillamine)—on the human colon adenocarcinoma cell line, HT29. Both the capacity of these complexes to donate Ti(IV) to human apo-transferrin and their hydrolytic stability have been investigated to elucidate how changes in either the ancillary ligands or the functionalized cyclopentadienyl ligands affect their water properties, the titanium intake by human apo-transferrin, and their cytotoxicity in HT29 cells. The amino acid complexes transferred Ti(IV) to human apo-transferrin at a slower rate than titanocene dichloride but eventually loaded both the N- and C-lobes. The functionalized titanocenes, $(Cp\text{-}R)_2TiCl_2$ and $(Cp\text{-}R)CpTiCl_2$ ($R = CO_2CH_3$ and $CO_2CH_2CH_3$), undergo transference of Ti(IV) to human apo-transferrin at about the same rate as titanocene dichloride. Notably, the titanium-maltolato complex does not transferred Ti(VI) to human apo-transferrin at any time within the first seven days of its interaction, demonstrating the inert character of this species. Stability studies on these complexes showed that titanocene complexes decomposed at physiological pH while the $[Ti_4(maltolato)_8(\mu\text{-}O)_4]$ complex was stable

at this pH without any notable decomposition for a period of ten days. The antitumor activity of these complexes against colon cancer HT-29 cells was determined using an MTT cell viability assay at 72 and 96 hours. The titanocene-amino acid and the $(Cp\text{-}R)_2TiCl_2$/$(Cp\text{-}R)CpTiCl_2$ ($R = CO_2CH_3$) complexes were not biologically active when human transferrin was absent; they also were non-active when human transferrin was present at dose-equivalent concentrations. $(Cp\text{-}R)_2TiCl_2$ and $(Cp\text{-}R)CpTiCl_2$ ($R = CO_2 CH_2CH_3$) showed cytotoxic activity in HT29 cells comparable to that which is displayed by titanocene dichloride. The titanium-maltolato complex had higher levels of cytotoxic activity than any other titanocene complex investigated. Transferrin may be important in protecting the titanium center from hydrolysis, but this may be achieved by selecting ligands that could result in hydrolytically stable, yet active, complexes. The attack to nucleic acids such as DNA apparently are the main cause of the antiproliferative activity shown by titanium complexes in cancer cells, both organometallic as well as inorganic. The microarray data supported a direct DNA-damaging action for titanocene dichloride in HT29 cells, and the new titanium-maltolato complex is correlated with deregulation of genes involved in cell cycle arrest, apoptosis and autoimmune responses. This demonstrates that titanium drugs exert a true cytotoxic effect in HT29 colon adenocarcinoma cells, in which DNA damage is crucial.

Resumen

La sustitución o modificación de ligandos en dicloruro de titanoceno tiene el potencial de producir compuestos más solubles y estables. Como parte de los esfuerzos de investigación en el área de agentes antitumorales basados en el metal titanio, hemos investigado la actividad citotóxica de $[Ti_4(maltolato)_8(\mu\text{-}O)_4]$, $(Cp\text{-}R)_2TiCl_2$ y $(Cp\text{-}R)CpTiCl_2$ ($R = CO_2CH_3$ y $CO_2CH_2CH_3$), y tres complejos de titanoceno con aminoácidos – $[Cp_2Ti(aa)_2]Cl_2$ (aa = L-cisteína, L-metionina, y D-penicilamina) – en la línea celular de adenocarcinoma de colon humano, HT29. Tanto la capacidad de estos complejos de donar el Ti(IV) a la proteína transferrina como su estabilidad hidrolítica ha sido estudiada para investigar como los cambios en los ligandos ancilares o la funcionalización de los ligandos ciclopentadienilos afectan la solubilidad y estabilidad en agua, la capacidad de donar el titanio a la proteína transferina y la citotoxicidad en las células HT29. Los complejos de aminoácido transfirieron el Ti(IV) a la proteína transferina más lentamente que el dicloruro de titanoceno pero eventualmente saturaron ambos lóbulos de la proteína. Los titanocenos funcionalizados, $(Cp\text{-}R)_2TiCl_2$ y $(Cp\text{-}R)CpTiCl_2$ ($R = CO_2CH_3$ y $CO_2CH_2CH_3$), transfirieron el Ti(IV) a la proteína a una rapidez similar al dicloruro de titanoceno. Muy notable, el complejo de titanio y maltol no transfirió el centro metálico a la proteína en ningún momento durante siete días de interacción, demostrando la naturaleza inerte de la especie. Estudios de estabilidad en los complejos demostró que

los complejos de titanoceno se descomponen en pH fisiológico mientras que el complejo $[Ti_4(maltolato)_8(\mu\text{-}O)_4]$ fue estable a estos niveles de alcalinidad sin descomposición notable por un periodo de diez días. La actividad antitumoral de los complejos hacia las células de cáncer de colon HT29 fue determinada utilizando el ensayo de viabilidad celular basado en la reducción mitocondrial del tinte MTT en tiempos de 72 y 96 horas. Los complejos de aminoácido así como los complejos $(Cp\text{-}R)_2TiCl_2/(Cp\text{-}R)CpTiCl_2$ ($R = CO_2CH_3$) no fueron biológicamente activos en ausencia de transferina; tampoco fueron activos en presencia de transferina a concentraciones equivalentes a la dosis del complejo. Los compuestos $(Cp\text{-}R)_2TiCl_2$ y $(Cp\text{-}R)CpTiCl_2$ ($R = CO_2CH_2CH_3$) mostraron niveles de actividad citotóxica en las células HT29 comparables a los mostrados por el dicloruro de titanoceno. El complejo de maltol mostró niveles superiores de actividad citotóxica que todos los complejos de titanoceno estudiados. La transferina puede ser importante para proteger el centro metálico de hidrólisis, pero esto puede ser logrado seleccionando ligandos que resulten en complejos hidrolíticamente estables pero a su vez biológicamente activos. El ataque a ácidos nucleicos como el ADN es aparentemente la causa de la actividad antiproliferativa que demuestra el complejo de maltol en células cancerosas. El análisis de expresión es cónsono con el daño directo del dicloruro de titanoceno al ADN, y el nuevo complejo de maltol es correlacionado con desregulación de genes envueltos en arresto del ciclo celular, apoptosis y respuestas autoinmunes. Estos datos demuestran que las drogas de titanio ejercen un efecto citotóxico verdadero en las células de adenocarcinoma de colon HT29 en el que el daño al ADN es crucial.

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Dedicatory

To my wife Yajaira and my daughter Yadira; you both have touched my life in unique unimaginable ways, it's impossible to describe here.

To my parents, Héctor and Nirza, for your unconditional love and support.

"All substances are poisonous, there is none that is not a poison; the right dose differentiates a poison from a remedy."

– Paracelsus, 1538

"Cancer is an unfortunate natural consequence of being alive."

– Ramón L. Hernández-Castillo, 2008

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List of Symbols and Abbreviations

°C	Celsius temperature or centigrade scale
^{1}H-NMR	proton nuclear magnetic resonance
Å	angstrom (10^{-10} meter)
A2780	human ovarian cancer cell line
A2780cis	cisplatin-resistant human ovarian carcinoma cell line or A2780/CP
A549	carcinomic human alveolar basal epithelial cell line
ADP-ribose	Adenosine diphosphate ribose
Akt	PKB or Protein kinase B signaling pathway
ansa bridge	linking bridge between rings of a metallocene
A_{number}	absorbance at (number) wavelength in nanometers
apo-Tr	apo-transferrin
Arg	arginine aminoacid residue
ATCC	American Type Culture Collection
B16	mouse melanoma cell line
BF_4^-	tetrafluoroborate ion
bp	base pairs
BRCA1	breast cancer 1 gene
BRCA2	breast cancer 2 gene

budotitane	cis-diethoxybis(1-phenylbutane-1,3-dionato)titanium(IV)
bzac	1-phenylbutane-1,3-dionate
C1orf53	open reading frame 53 of chromosome one
C_2	180-degree proper rotation axis or point group
CD	cyclodextrin
CD28	molecule which provides co-stimulatory signals required for T-cell activation
CDADC1	cytidine and dCMP deaminase domain
cDNA	complementary DNA
CEA	carcinoembryonic antigen
CEACAM1	carcinoembryonic antigen-related cell adhesion molecule 1
CEACAM7	carcinoembryonic antigen-related cell adhesion molecule 7 or CGM2
CG	chorionic gonadotropin
CGB7	beta 7 subunit of chorionic gonadotropin gene
CGM2	CEA gene-family member 2 or CEACAM7
cis	type of geometric isomer
cis-DDP	cis-diamminedichloroplatinum(II) or cisplatin
C-lobe	C-terminal half of dimeric protein
CO_2	carbon dioxide
Colo	colon cancer cell line
colon 38	murine colon cancer cell line

Cp	cyclopentadienyl ion
Cp_2Fe	ferrocene
Cp_2Fe^+	ferrocenium ion
Cp_2TiCl_2	bis(cyclopentadienyl)Ti(IV) dichloride or titanocene
CTLA4	cytotoxic T-lymphocyte-associate protein 4 or CD152
Cy3 and Cy5	cyanine synthetic dye family belonging to polymethine groups
D-	type of stereoisomer
dCMP	deoxycytidine monophosphate
DLX1	distal-less homeo box 1
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dUMP	deoxyuridine monophosphate
E. coli	*Escherichia coli* (bacteria)
E2F	group of genes that codifies a family of transcription factors
EDTA	ethylenediamminetetraacetic acid
EHPG	ethylenebis[2-(o-hydroxyphenyl)glycine
E_{pa}	oxidation potential
EPI	epirubicin
ER	endoplasmic reticulum

Et	ethyl group
EtBr, EB	Ethidium bromide or 3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide
FBS	fetal bovine serum
FDA	United States Food and Drug Administration
FUT8	alpha (1,6) fucosyltransferase gene
Golgi	golgi complex or golgi apparatus (cellular organelle)
GOSR2	golgi SNAP receptor complex - involved in protein transport
GPCR	G-protein coupled receptor
GTPase	enzymes that can bind and hydrolyze guanosine triphosphate (GTP)
H1299	lung cancer cell line
H_2O	water, hydrogen oxide
HeLa	immortal cell line derived from human cervical cancer
HEPA	High efficiency particulate air
HL-60	human promyelocytic leukemia cell line
HNRPL	heterogeneous nuclear ribonucleoprotein L
HT29	human colon adenocarcinoma grade II cell line
HTB-129	human breast ductal carcinoma
IC_{50}	concentration of a drug that is required for 50% inhibition in vitro
ICP-AES	Inductively coupled plasma atomic emission spectroscopy
IL-2	interleukin 2

JNK	mitogen activated protein kinase
K562	human immortalized myelogenous leukemia cell line
KIF13A	kinesin family member 13A protein
k_{obs}	observed kinetic constant
KRT17	keratin 17 gene
KRT8L2	designation of a predicted gene - protein like keratin 8
L-	type of stereoisomer
LLC-PK	pig kidney carcinoma cell line
LMCT	ligand-to-metal charge transfer
LRRC14	leucine rich repeat - protein motif
LS2CR3	Trafficking protein kinesin binding 2 gene
M	Molarity
MCF-7	human breast adenocarcinoma cell line
MDA-MB-435-S-F	breast cancer cell line
MDA-MDA231	human breast adenocarcinoma cell line
MDM2	murine double minute 2 protein
Me	methyl group
μg	micrograms (10^{-6} grams)
min	minutes
MIT	motoxantrone

MKT-4	therapeutic formulation of titanocene dichloride
μL	microliter (10^{-6} liter)
MLCT	metal-to-ligand charge transfer
mM	millimolar (10^{-3} molar)
μM	micromolar (10^{-6} molar)
mRNA	messenger RNA
MS	mass spectrometry
MSI2	gene designation
MTBP	MDM2 binding protein
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mV	millivolts
NAC	N-acetylcysteine
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NCI-H209	human lung small cell carcinoma cell line
NF-AT	Nuclear factor of activated T-cells
NFKBIB	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
NFRKB	Nuclear factor related to kappa B binding protein or NF-κB
ng	nanogram (10^{-9} gram)
N-lobe	N-terminal half of dimeric protein

nm	nanometer (10^{-9} meter)
NMR	nuclear magnetic resonance
NSF	N-ethylmaleimide-sensitive factor - involved in protein transport
OVCAR-1	human ovarian cancer cell line
$p21^{ras}$	GTP-binding proteins product of the *ras* gene family
p38	mitogen activated protein kinase
p53	tumor protein 53 - involved in regulating cellular events
PARP2	poly (ADP-ribose) polymerase 2
pBR322	*E. coli* cloning vector, a plasmid
PBS	phosphate buffered saline
pH	measure of the activity of hidronium ions
POLR3D	DNA directed polymerase III polypeptide D
poly-A	polyadenylate
PPP3R1	protein phosphatase 3 or calcineurin
RAB	member of ras superfamily of proteins
RABGAP1L	RAB GTPase activating protein 1-like protein
Ras	Ras protein, Ras superfamily or Ras signaling pathway
Rb	retinoblastoma
RBBP1	retinoblastoma binding protein 1
RNA	ribonucleic acid

RNA pol II	RNA polymerase II
RNase	ribonuclease
ROS	reactive oxygen species, free radicals
SDS	sodium dodecyl sulfate
SEC22L2	vesicle trafficking protein
SEC22L3	vesicle trafficking protein
SEC24A	gene and protein designation - part of a protein complex which coats vesicles than mediate protein transport
SNAP	soluble NSF attachment proteins - involved in protein transport
SPG3A	spastic paraplegia 3A gene
$t_{1/2}$	half-life
TAF15	TATA box binding protein associated factor 15
TATA box	DNA sequence found in the promoter region of genes
TGF	Transforming growth factor
THRAP3	thyroid hormone receptor associated protein 3
Titanocene C	bis-(-dimethylamino-2(-methylpyrrolyl)methylcyclopentadienyl) titanium (IV) dichloride
Tr	transferrin
TRAK2	Trafficking protein kinesin binding 2
trans	type of geometric isomer
TRAP	Thyroid Hormone Receptor-associated Proteins
tRNA	transfer RNA

USP	Unites States Pharmacopoeia
UV-Vis	Ultraviolet-visible spectroscopy
V	volts
V79	chinese hamster lung cell line
w/o Tr	without additional transferrin present
w/Tr	with additional transferrin present
Wnt	Wingless and Integration signaling pathway
X	undefined ligand
ZNFnumber	Zinc finger protein

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Chapter I. Introduction

Cancer is the leading cause of death among U.S. population, followed by cardiovascular disease. It is estimated that more than 1.4 million people will be diagnosed, and around 565,000 will die from cancer in 2008. Among these deaths, around 40,000 women will die of breast cancer, the most common cancer and second cause of death by cancer in women, lung cancer being first. Also, almost 50,000 men and women will die of colon and rectum cancer, the third most common cancer, both in occurrence and cause of death, in men and women. The first and second causes of death by cancer in men are lung and prostate cancer, respectively.[1]

A cancer, or malignant neoplasm, is a group of diseases in which cells display three main traits: uncontrolled growth and division beyond what it is considered normal, invasion or intrusion on and destruction of adjacent tissue, and sometimes metastasis, which is spreading to other locations in the body. These three malignant properties make the difference between a cancer and what is usually called a benign tumor, which are those who do not invade or metastasize. Not all cancers form tumors. An example of this would be a leukemia, which is derived from blood-forming cells, also called hematopoietic cells.

As for leukemia, cancers are classified by the type of cell that resembles the cells in the tumor and, therefore, the tissue presumed to be the origin. Other categories are carcinoma, derived from epithelial cells, and sarcoma which are derived from

mesenchymal cells, among others. Carcinomas are of special interest because they account for more than eighty percent (80%) of the cancer-related deaths in the Western world. Carcinomas are classified as squamous cell carcinomas if they arise from epithelial cells that seal and protect the cavities or channels they line, or adenocarcinomas, when the cells secrete substances into these ducts or cavities.[2]

The first line of defense against cancer is prevention. Although we do not have the knowledge to prevent all cancers and there is no rule of thumb as to which lifestyles help in prevention of cancer, there is abundance of scientific and non-scientifically proven information about this subject. Most prevention comes from environmentally-related areas, including both the physical environment, by avoiding exposure to polluted natural resources, and a healthy lifestyle.

There is evidence that suggest a relationship of certain types of cancers with different ethnic groups,[3] but is has been attributed to lifestyle and not to genetic heredity. Most forms of cancer have no basis in heredity. There are, however, a number of recognized syndromes of cancer with a hereditary component, often a defective tumor suppressor allele. Examples of this are certain inherited mutations in the genes BRCA1 and BRCA2 that have been associated with an elevated risk of breast and ovarian cancer.

Cancer is not always preventable. Keeping the healthiest lifestyle possible and regular visits to your medical assistance provider is the best option. This will result in early detection of symptoms, if they occur, and treatment, if possible. Because cancer refers to a class of diseases, it is unlikely that there will ever be a single cure for

cancer. Cancer can be treated by surgery, chemotherapy, radiation therapy and immunotherapy, or a combination of these procedures. The choice will depend on the location of the tumor and the stage of the disease, as well as the general state of the patient. Sometimes, palliative procedures are performed to improve the quality of life of a patient with terminal cancer or life-threatening symptoms but complete removal of the cancer without damage to the rest of the body is the goal of curative treatment. In this regard, surgical excision of a solid tumor is sometimes followed or preceded by chemotherapy.

Chemotherapy is the treatment of cancer with drugs that can destroy cancer cells. Usually, these drugs affect rapidly dividing cells, like cells in a tumor, and are usually non-specific. The degree of specificity sometimes found with chemotherapy comes from the inability of many cancer cells to repair DNA damage, while normal cells generally can. Most regimes are given in combination to target as most cancer cells as possible. Since the rapidly growing cells will be present in different cell cycle stages, two or more drugs are given in a specific order to accomplish this goal.

In its more general form, chemotherapy can refer to antibiotics such as penicillin or to the organoarsenic complexes used to treat syphilis.[4] But in its oncological use, chemotherapy refers to the use of antineoplastic drugs to treat cancer. Chemotherapeutic drugs can be as different as taxanes, which enhance microtubule stability preventing chromosome separation, and alkylating-like platinum-based drugs, like cisplatin, which damage DNA mainly by formation of 1,2-intrastrand adducts and triggers apoptosis. An extensive list of anti-cancer agents can be found in the

Anatomical Therapeutic Chemical Classification System published yearly by the World Health Organization's Collaborating Centre for Drug Statistics Methodology.[5]

Cisplatin (1) is an example of a metal-based chemotherapeutic drug. The use of metal complexes for cancer chemotherapy started in 1965 with the discovery of the antiproliferative activity of cisplatin, as *cis*-diamminedichloroplatinum(II) is known.[6] Since then, thousands of metal-based complexes of platinum and other metals have been tested for antineoplastic activity. Three platinum complexes, namely cisplatin, carboplatin (2) and oxaliplatin (3) have received worldwide approval for cancer therapy. A fourth platinum complex, picoplatin (4), is in advanced clinical development (See Figure 1).

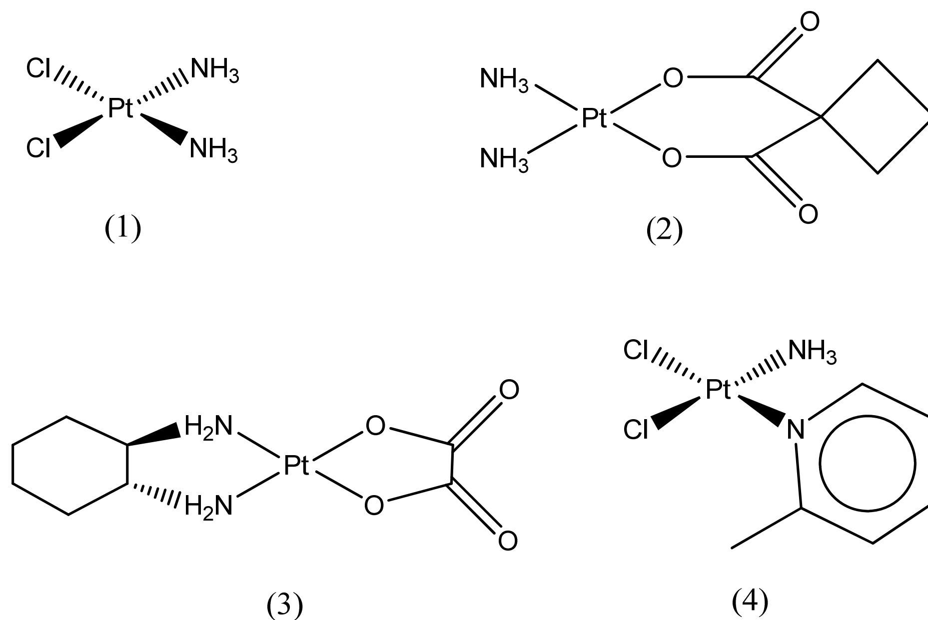


Figure 1. Structure of cisplatin (1), carboplatin (2), oxaliplatin (3) and picoplatin (4)

The major problems encountered with platinum-based chemotherapy are lack of specificity with toxicity as a consequence and, resistance, both acquired and intrinsic, as cells decrease the metal accumulation mechanisms and increase the deactivation and repair mechanisms. Consequently, other metals have been studied and active antineoplastic agents have been produced. Intense efforts have been made to develop new metal compounds that either display enhanced tumor specificity or are less prone to the development of resistance. Although various compounds have reached clinical trials, only one non-platinum metal compound, arsenic trioxide, has been approved for clinical use so far.[7] Arsenic trioxide is approved by FDA for the treatment of acute promyelocytic leukemia and is currently evaluated for the therapy of other cancers. An excellent review on the mechanism of action of arsenic-based antineoplastic drugs was published recently.[8]

Other drugs which are in preclinical or early phase of clinical development are based on metals such as titanium, iron, gold, cobalt, vanadium, rhodium, ruthenium, copper, bismuth, and the lanthanides.[7] For example, gold-based lipophilic cations are used because they target the mitochondrial membranes of certain cancer cells, because tumor cells have a high negative mitochondrial transmembrane potential in contrast to normal cells and therefore accumulate the metal complex more readily.[9] Ruthenium half-sandwich complexes show promising anticancer activity both *in vitro* and *in vivo*, including activity against cisplatin-resistant cell lines.[10] Lanthanides have been used to enhance the activity of other drugs, such as coumarin derivatives, although the mechanism by which this action is exerted is poorly understood.[11]

Organometallic complexes of titanium and iron, specifically metallocenes, are another class of metal complexes of special interest. Because of the stability of the ferrocenyl group, ferrocene (7) derivatives (See Figure 2) have found their way in many biological applications, such as conjugation with biomolecules.[12] Special interest have also been devoted to organometallic and inorganic titanium complexes, such as budotitane (5) and titanocene dichloride (6), for reasons that will be covered in subsequent sections.

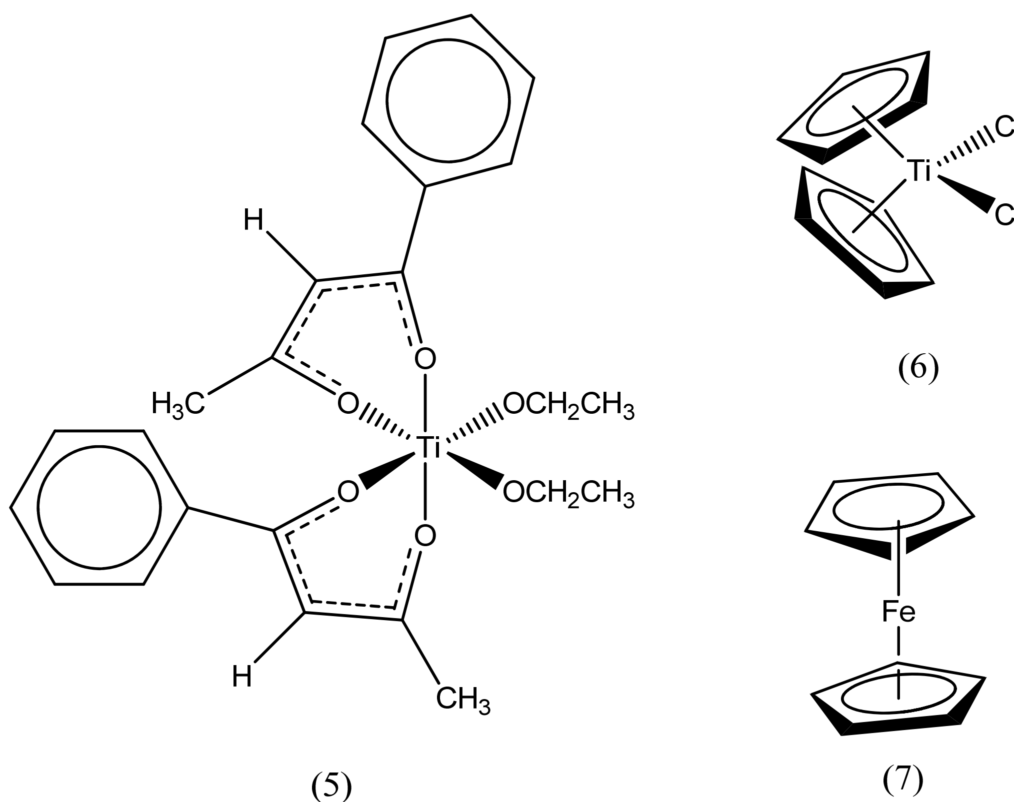


Figure 2. Structure of budotitane (5), titanocene dichloride (6) and ferrocene (7)

A. Budotitane

After platinum, the first metal complex tested in clinical trials as a potential antineoplastic drug was “budotitane”, as the compound *cis*-$[(CH_3CH_2O)_2(bzac)_2Ti(IV)]$, where bzac is 1-phenylbutane-1,3-dionate, is commonly known.[13] It was used against a wide variety of ascites, which are serous fluid abnormal accumulations, and solid tumors. Some analogous complexes were investigated and exhibited similar properties compared to this ethoxide complex. This type of complex was initially studied due to its structural similarity to active *cis*-platinum compounds, although biological applications were determinant in suggesting a different mechanism of action from that of platinum complexes.

Other metal complexes with β-diketonato ligands were studied with metals such as zirconium and hafnium, but the complexes containing titanium(IV) were the most active agents. The limited solubility and low water stability of these complexes was the main obstacle against clinical trials. These complexes hydrolyze extensively yielding oligomeric Ti(IV) species which precipitate in aqueous solutions. To circumvent this, these drugs were formulated in a mixture of water, a glycol and an emulsifier, which prevented the complex from hydrolysis,[14] but for regulatory aspects regarding the pharmacokinetics of the active species, the trials did not progress any further. Research in this area, on the other hand, has not been abandoned, but groups are scarce. The water properties of related complexes and their biological activity have been studied with interesting results and new patented formulations of budotitane for clinical evaluation.

In structural terms, the low ligand symmetry found in budotitane is considered an essential condition for antitumor activity. The β-diketone ligand type has been extensively studied in organometallic synthesis and catalysis, and it will not be reviewed here. Anyhow, the solution structure and isomer distributions of this type of complexes was studied theoretically and it was found that they correlate with physical data obtained by spectroscopic techniques such as NMR,[15] which point a predominant *cis-cis-cis* species, validating the dissymmetry as a condition for activity. It was not until recently that the crystal structure of budotitane and the dichloro derivative was resolved.[16] The budotitane molecules adopted a *cis-cis-trans* configuration, although it was not the major configuration occurring in solution, and the chloro derivative adopted a *cis-trans-cis* configuration. No molecule has been found in either solution or solid-state where the corresponding β-diketone ligands are in a *trans* square-planar-like configuration (see Figure 3).

At present, and to our knowledge, three research groups continue work in this area. Their main goal is to understand structure-activity relationships for this type of complexes and to overcome solubility considerations for their synthesis and for their biological applications. Efforts in budotitane-like dissymmetric complexes has been made and were reviewed recently.[17] Also, non-metallocene non-diketonato Ti(IV) complexes has been studied and found to exhibit cytotoxic activity against colon and ovarian cell lines.[18] Another reason that held back the promising development of budotitane-like complexes was the larger spectrum of antitumor activity of titanocene

dichloride, along with its somewhat better solubility in physiological medium. This is the subject of the next section.

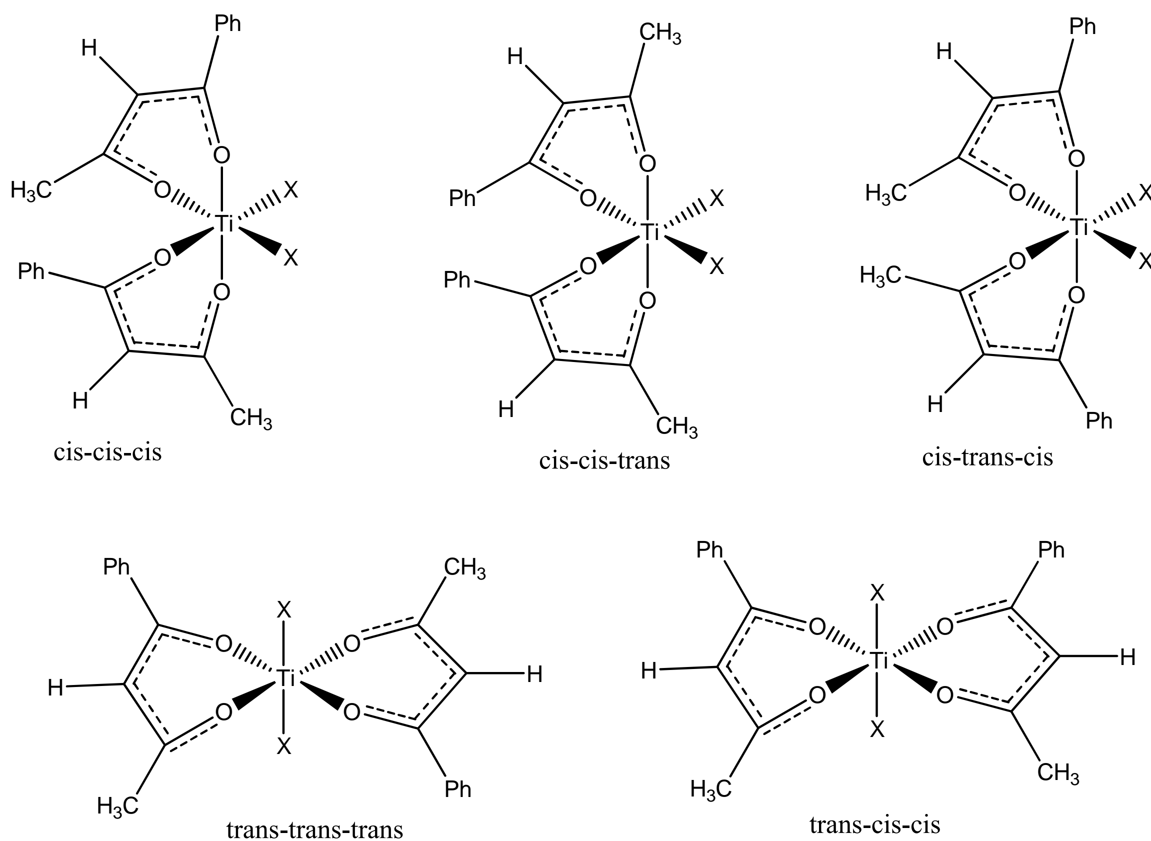


Figure 3. Possible isomers of asymmetrically substituted bis(β-diketonato) complexes of the type Ti(β-diketonato)$_2$X$_2$.

B. Titanocene dichloride

Soon after the discovery of the biological activity of budotitane, other titanium(IV) complex was reported to have antitumor activity. Titanocene dichloride, as the complex bis(cyclopentadienyl)titanium(IV) dichloride is known, was the first metallocene reported to have this property, back in 1979. Interestingly, it showed

strong activity against a wide variety of cancer cell lines, including Ehrlich ascites tumor, B16 melanoma, colon 38 carcinoma, and Lewis lung carcinoma, with less toxic effects than the well established cisplatin.[13] Titanocene dichloride was the most active complex from an extensive list of other metallocenes of vanadium, niobium and molybdenum, as well as ferrocenium ions and main-group analogues. These properties, along with limited or no toxicity to kidneys and other organs usually targeted by platinum drugs, led to clinical trials of titanocene dichloride.

Although titanocene dichloride is more stable than budotitane, it undergoes hydrolysis at physiological pH. The hydrolysis pattern is characterized by extensive hydrolysis of both the cyclopentadienyl (Cp) and chloride ligands, forming insoluble species which are difficult to characterize. At a lower pH, the system is more stable and a general scheme of ligand hydrolysis followed by proton loss from water molecules has been proposed.[19] To circumvent this problem, numerous modifications to the original parent compound have been performed. The following structure-activity relationships were determined initially: 1) highest activity is obtained with non-modified cyclopentadienyl ring ligands, and 2) chloride exchange can be performed without loss of antitumor activity.[20] These two relationships guided most research efforts in the early years of this area. Information was obtained at preclinical and clinical level for this and other derivatives of the parent drug.

Derivatives of titanocene dichloride can be categorized depending on the structural modification which leads to them. One type of modification is the manipulation of the cyclopentadienyl ligand. While original work suggested

modification of cyclopentadienyl ligands rendered less active compounds, this is now an active area of research as it has produced complexes with activities even higher than cisplatin.[21] Replacement of chloride ligands and the use of differently charged ligands to yield ionic titanocene complexes continue to be an extensive area of research. Leaving group exchange is exploited because of the capability of modulating solubility patterns, redox properties and substitution reactions, all of which are determinant for biological properties.

Other ionic and neutral titanocene complexes with aminoacids, thionucleobases and other biologically important ligands are sometimes developed with drug synergism in mind. Complexation of the titanium center with a small biologically active molecule could lead to a more potent drug. An example of this is the complexation of the titanocenyl moiety with thiophene ligands[22] which has been hypothesized to produce lesions by the titanocenyl fragment as well as intercalation by the decomplexed thiophene.

Finally, to circumvent water stability problems, efforts have been made to encapsulate the drug in liposomes and to characterize the penetration of the titanocene in cyclodextrin cavities. A new design in this area involves production of cyclodextrins bridged with the titanocene moiety, as to increase the specificity via the cyclodextrin binding sites. This complex has a higher activity against MCF-7 cells when compared to titanocene dichloride.[23]

One of the main modifications originally studied when attempting to establish activity patterns was the use of different metallic centers. Although titanocene

dichloride was at first the most active compound, research efforts with other metals can not be discarded on this basis. In fact, other metals have produced excellent antineoplastic agents, without water solubility or stability barriers, such as ferrocene and its derivatives.

C. Ferrocene

Ferrocene as a cytotoxic agent is known since the 1980's. The stability of the ferrocenyl group in aqueous aerobic media, the accessibility of a large variety of derivatives, and its favorable electrochemical properties have made it an excellent candidate compound for conjugation with biomolecules. This has resulted in applications which range from biosensors to bioelectronics.[12] Also, ferrocene linked to well-established drugs have produced excellent new versions, such as tamoxifen for breast cancer and chloroquine for malaria.

Ferrocene itself and its modifications lead to strong agents against different cancer types. Structural variations of ferrocene as a parent molecule, as in the case of ferrocenylalkyl azoles,[24] has produced complexes with antitumor activity comparable to cisplatin against Lewis lung carcinoma, and lower activity against other tumor types. Other complexes with protected phenol and thiophenol groups have produced complexes with their activity ranging from purely estrogenic to cytotoxic in estrogen-dependent and independent breast cancer cells.[25]

Through functionalization of one or both cyclopentadienyl rings, families of complexes are obtained and have been studied electrochemically to establish a

relationship between redox behavior and biochemical data,[26] which is not always possible. When working with redox-active ferrocene pyrazole conjugates, a relationship has been established as toxicity decreased with increased redox potential,[27] but more data is required to sustain this observation. Finally, cyclodextrin inclusion complexes of ferrocene derivatives have been studied in Ehrlich ascites tumor and found that encapsulation does not change toxicity *in vivo*.[26]

Summary:

Structure-activity relationships consist of the solubility and consequent biological availability of the drug, the possible substitution reactions and stability of the drug, the redox potential when present, along with the desired covalent and non-covalent interactions with the target biomolecules.

In the remaining chapters of this thesis we will review past efforts in the area of metal-based drugs within our research group and others (Chapter 2) which provided the rationale for the objectives pursued. We will describe all the experimental details of the studies performed (Chapter 3) and discuss the results obtained (Chapter 4). The last two chapters will present the main conclusions (Chapter 5) and future studies (Chapter 6).

Chapter II. Previous Work

For the past twenty-six years, titanocene dichloride (Cp_2TiCl_2) has attracted the attention of many researchers in the biomedical field. Lack of solubility and stability in aqueous media has been the main obstacles for this drug to be developed all the way through clinical trials. Few groups which continue working with titanium complexes as potential anticancer agents have made excellent contributions in areas such as synthesis, stability studies, formulation and transfer of the drug, interactions with biomolecules, as well as pre-clinical studies both in cells and animal models.

Our research group has made contributions in this area, mainly in the areas of synthesis, stability studies and interactions with biomolecules. In this chapter we will highlight these efforts and compare with results from other groups. The information will be presented in a stepwise manner depending on the modifications made to the parent drug. Stability studies as well as interactions with biologically important molecules will be presented as well as any toxicity data on cell lines.

A. Replacement of chloride with aminoacids and nucleobases

The first report from our group in this area corresponds to the characterization of titanocene complexes with thionucleobases.[28] The reasons for the development of this type of drug were varied. From the mechanistic point of view, since metallocenes were initially proposed to interact with nucleic acids in a way similar to cisplatin,

these complexes could be a model of the interaction of Cp_2TiCl_2 with DNA and RNA bases. On the other hand, to exploit the possibility of synergism, since thionucleobases have antitumor activity on their own, these complexes could release two active fragments inside the cell when hydrolyzed.

The complexes synthesized had the thionucleobases coordinating titanium in a bidentate fashion (See Figure 4). The Ti-Cp bond resulted stable in dried degassed dimethylsulfoxide (DMSO) but the solvent molecules slowly replaced the base. In DMSO/H_2O solutions, at low pH values, the Ti-Cp bond was more stable than the Ti-nucleobase bond. In general, the Ti-nucleobase interaction is more stable in DMSO/H_2O than in DMSO, and the chloride ligand did not appear to have a major effect on the decomposition of the complexes. No data on the cytotoxic activity of these complexes was published.

On the other hand, titanocene-aminoacid complexes were synthesized and their water properties studied.[29] The stability of the complexes regarding both Cp and aminoacid hydrolysis was studied by spectroscopy and electrochemistry, as well as the interaction studies with the protein transferrin. These complexes were synthesized for their expected increased solubility in water when compared to titanocene dichloride. Although improved solubility does not have a direct relationship with enhanced antitumor activity, it helps with stability as well as interaction studies.

Aminoacids coordinate Ti(IV) through their carboxylate groups in all the complexes studied (see Figure 4). These complexes had decreased stability regarding aminoacid loss compared to chloride loss in Cp_2TiCl_2. Cyclopentadienyl hydrolysis

was about the same for all the complexes. All the complexes showed a reversible one-electron redox process at low pH, while at physiological pH, all of them decompose at variable rates providing data which is not completely reliable.

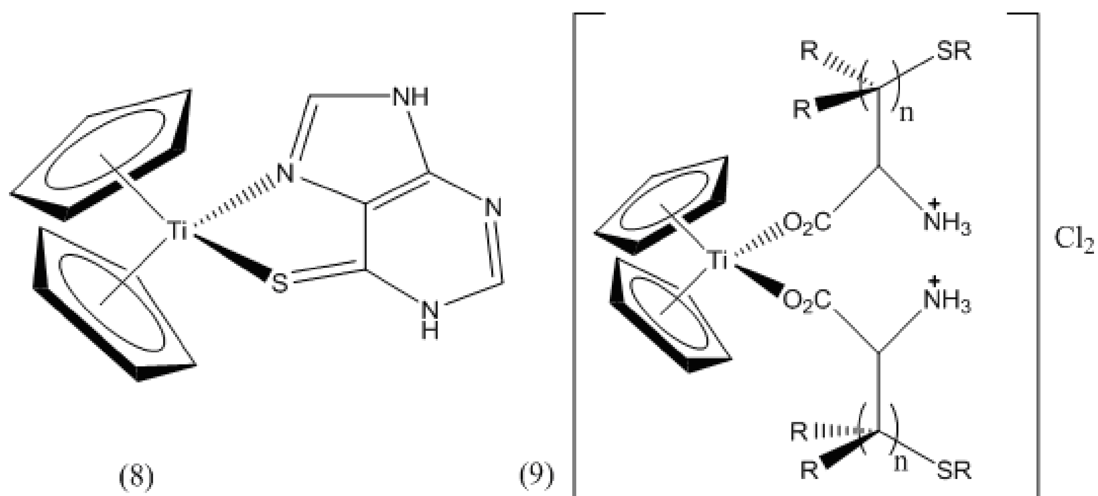


Figure 4. Example of titanocene-thionucleobase complex (8) and general structure of titanocene-aminoacid complexes (9).

Previous experimentation in this area was initially limited to substitutions of chlorides for other halides and pseudohalides, as well as hydrophilic ligands containing thiophenolate and carboxylate groups.[20] Planar ligands have been used with intercalative potential in mind,[22] but evidence involving nucleobases is limited to interaction studies involving DNA constituents.[30-32] There is a crystal structure reported which shows a monofunctional binding of purine-N9 to titanocene, which confirms the ability of nitrogen bases to coordinate titanium.[33]

Titanocene complexes have been synthesized with the following aminoacids: glycine, L-alanine and 2-methylalanine[34-35], DL-phenylalanine and its 4-fluoro derivative[36], and there are crystallographic reports of the titanocene complexes of N-methylglycine[37a] and L-methionine[37b]. There is also a report of a bis[η^5-pentamethylcyclopentadienyl)Ti(III)N,N-dimethylglycinato] complex as well as titanocene-malonato complexes.[38] All these complexes had aminoacids coordinated exclusively through the oxygen atoms of the carboxylate groups. There is no data on the cytotoxic activity of these complexes, except for the phenylalanine derivatives which were evaluated for inhibition of *E. coli* growth demonstrating up to 1000-fold activity when compared to Cp_2TiCl_2 and 10-fold when compared to the fluoro-substituted phenylalanine.[36]

B. Cyclopentadienyl functionalization

Since cyclopentadienyl modified complexes exhibited lower activity than the parent titanocene dichloride during the early development of this area, efforts in this area of research were mostly abandoned. The first recent research articles in this area (2000's) involved the carbomethoxy functionalization of cyclopentadienyl rings of titanocene dichloride,[39] and the enhanced anti-cancer activity of these derivatives in the small cell lung cancer NCI-H209. This approach was then abandoned since the extension of these and similar compounds containing butyl and phenyl ester substituents to a variety of other human tumor cell lines found little cytotoxic activity in general.[40]

The approach was then switched to the use of cyclic and acyclic alkylammonium substituents[40] which were water soluble and stable. The dicationic complexes (see Figure 5) showed promising activity since their potency was similar or better than the patented formulation of Cp_2TiCl_2 (MKT-4) in cisplatin-sensitive and cisplatin-resistant cell lines without the solubility problems. Other research group[41] reported a complementary study of six titanocene dichloride derivatives in different cancer cell lines, including cisplatin-resistant A2780cis ovarian cancer. Two of the complexes were published by both groups. This research area is very promising as new synthesized compounds have been found to be better than the patented formulation MKT-4, and one of them is better than cisplatin in HeLa cells.[21, 42] An interesting feature which needs further study is the varying degree of potency among structurally similar complexes.

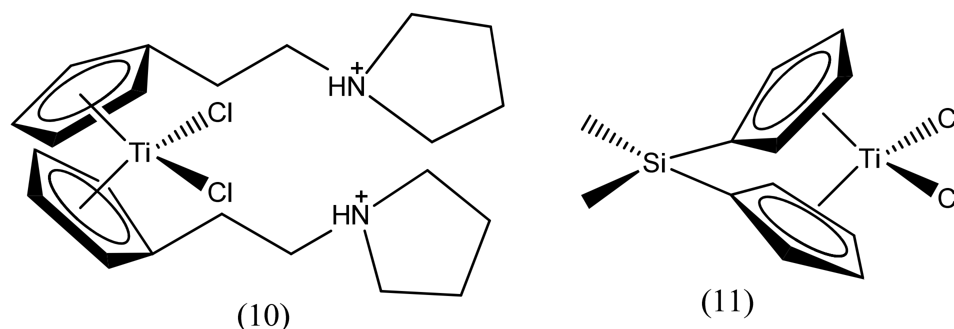


Figure 5. Examples of alkylammonium functionalized titanoneces (10) and silicon-ansa-bridged titanocene complexes (11)

Another type of cyclopentadienyl functionalization is the one involving a silicon-ansa-bridge between the cyclopentadienyl rings (see Figure 5), useful for olefin

polymerization reactions. An ansa bridge links together both rings of a metallocene. The resulting complexes showed modest activities in polymerization compared to the zirconium counterpart, and the researchers decided to test their cytotoxic potential in a variety of cell lines, finding activities in the sub-millimolar range.[43] Continued efforts from the research group have found that an alkenyl fragment in the cyclopentadienyl functionalization improves the activity in most of the cancer cells studied.[44]

Another research group in this area has produced cyclopentadienyl functionalized titanocene complexes of mainly three types. Cyclopentadienyl functionalized with substituted benzenes in ansa-titanocene complexes without a silicon bridge (see Figure 6) were prepared initially and demonstrated a cytotoxic effect against pig kidney carcinoma cells (LLC-PK) ranging from non-active to sub-millimolar range.[45-48] The same type of complex but without the ansa bridge (see Figure 6) and other complexes with substituted heteroaryl and benzene rings (see Figure 7) had a slightly improved cytotoxic activity against the same cell line with some of them falling in the upper micromolar range.[49-53]

Other type of compounds which resemble the electron-donor capabilities of the alkylammonium functionalized complexes discussed above, are the new dimethylamino-functionalized (see Figure 7) titanocene complexes.[54-57] One of this complexes, bis-(N,N-dimethylamino-2(N-methylpyrrolyl)methylcyclopentadienyl) titanium (IV) dichloride, also known as Titanocene C, is approximately 400 times more cytotoxic than titanocene dichloride *in vitro* and almost as cytotoxic as cisplatin in LLC-PK cells.

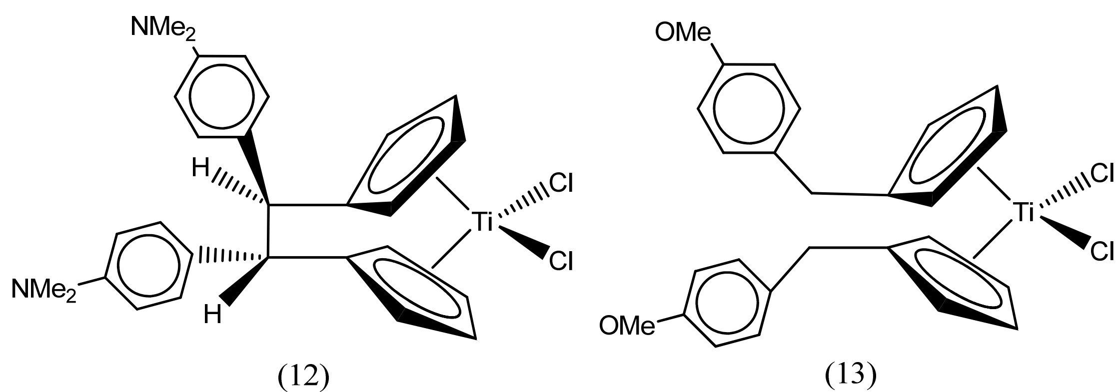


Figure 6. Examples of non-silicon-ansa-bridged titanocene complexes (12) and complexes with substituted benzenes as cyclopentadienyl functionalization (13).

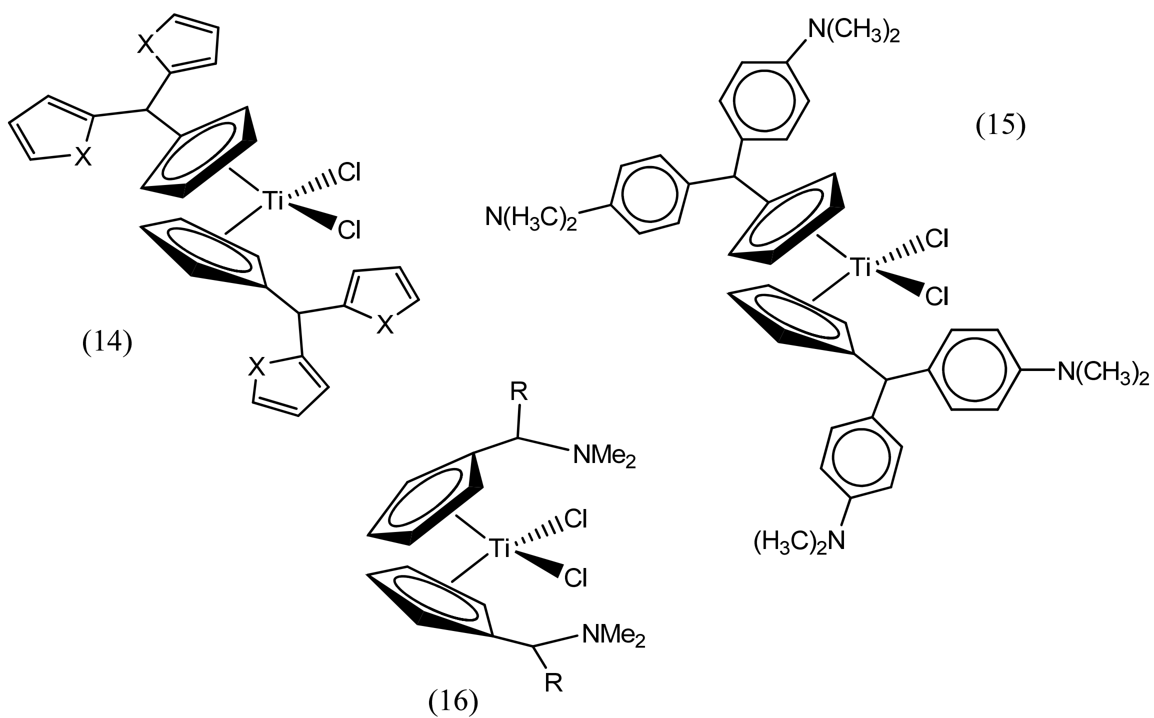


Figure 7. Examples of diheteroaryl (14), diarylmethyl (15) and dimethylamino-functionalized complexes (16)

Cyclopentadienyl functionalized titanocene complexes (See Figure 8) have been prepared with small polar electrowithdrawing groups. Carbomethoxy-functionalized and carboethoxy-functionalized titanocene complexes have been synthesized successfully and their titanium-donating capabilities to the protein transferrin were reported.[58] Their cytotoxic activity against HT-29 colon adenocarcinoma cells was also studied.

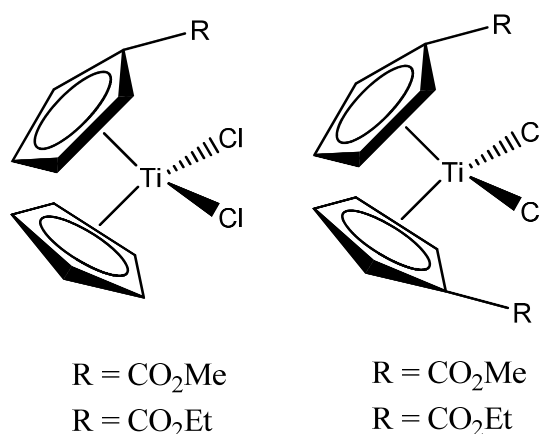


Figure 8. General structural formula of carboalkoxy-functionalized titanocene complexes.

C. Inorganic Ti(IV) complexes

Inorganic Ti(IV) complexes normally encountered in literature are used in catalytic or synthetic applications and little work is being performed as to develop them into antineoplastic agents. Budotitane-like complexes have been synthesized with 4-acyl-5-pyrazolonates,[17,59-60] and with 3-hydroxy-2-methyl-4-pyrone,[61] better known as maltol. It should be noted that the eight-membered alternating Ti-O ring

found in the complexes mentioned above and in budotitane, has been found in many other titanium derivatives.[17] Also, a stacking of aromatic rings in both structures and in budotitane has been shown in their crystal structures.[16,60-61]

There is some biological information on budotitane such as its ability to stay bound to DNA after long periods of time[14] but it is not as extensive as with other metal-based drugs, titanium and non-titanium, due to its limited water solubility. To overcome this limitation the complex has been encapsulated in a liposome. This has been done for budotitane,[17] and also for a related compound which has shown low toxicity and effective antitumor activity against a mouse mammary adenocarcinoma *in vivo* model.[59]

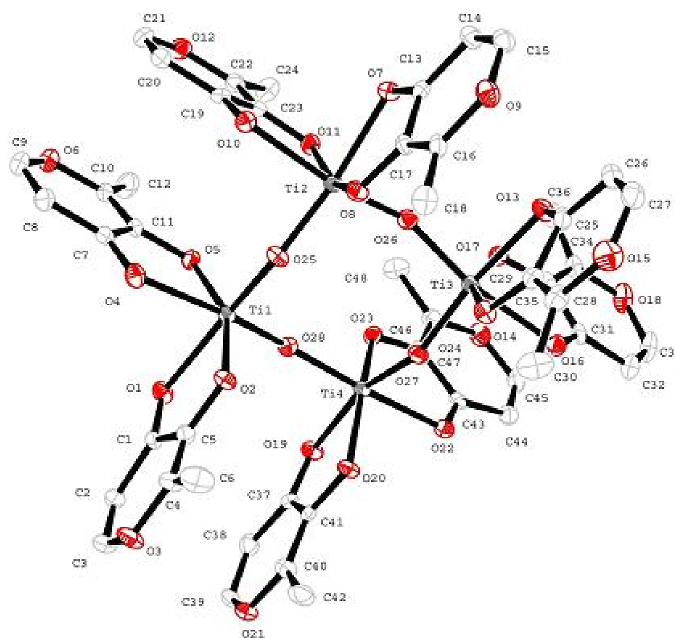


Figure 9. Structure of $[Ti_4(maltolato)_8(\mu\text{-}O)_4]$

The titanium-maltolato complex mentioned above has been found to exert a higher cytotoxic activity than titanocene dichloride on HT29 cells, in a transferrin-independent fashion.[62] This transferrin-independent behavior has been found for at least another non-titanocene Ti(IV) complex, in which cytotoxic assays performed in the presence of transferrin have not resulted in an increased toxicity on HT29 and OVCAR-1 cells.[18]

D. Ferrocene derivatives

Cyclopentadienyl-functionalized ferrocene derivatives have been developed extensively. One of the main research areas is the conjugation of ferrocene with other molecules,[12] particularly those that are drugs by themselves. Examples of these include organometallics analogs of tamoxifen used in both hormone-dependent as well as hormone-independent cancer cells.[63] Also, metallocene antimalarials such as ferroquine,[64] of which even ruthenocene derivatives have been prepared.[65]

Cyclopentadienyl functionalization in ferrocene is varied and derivatives have been obtained with good cytotoxic activity on some cell lines. Examples of these are purine derivatives with activity on leukemia cells in the low micromolar range,[66] benzoyl dipeptide ester derivatives with activity against highly invasive H1299 lung cancer cells,[67-68] and benzene carboxamide derivatives with activity against the hormone-independent MDA-MB-435-S-F breast cancer cell line in the upper micromolar range.[69] It should be noted that most of these compounds have a low redox

potential, which would make them better reactive oxygen species (ROS) producing complexes.

On the other hand, one article describing ferrocene containing peptide conjugates found that an enone-moiety (-CH=CH-CO-) of ferrocenylacrylic acid exhibited a remarkable antiproliferative effect against HL-60 leukemia cells. This effect was not improved when conjugated with the peptides, in contrast with ferrocene carboxylic acid, which improved solubility and antitumor effect upon conjugation.[70]

Our research group has produced cyclopentadienyl-functionalized ferrocenes with groups as carbomethoxy, carboethoxy, as well as alcohol and vinyl derivatives of ferrocene carboxylic acid. Their synthesis, characterization, electrochemical behavior as well as the solid-state X-ray structure of one derivative and their cytotoxic activity against HT29 (colon adenocarcinoma) and MCF-7 (breast adenocarcinoma) cell lines are the subject of a future publication.[71]

E. Other metals

1. Molybdocene derivatives

Similar to titanocene-thionucleobase complexes, molybdocene-thionucleobase complexes have also been synthesized.[72] These complexes had the same coordinating behavior of thionucleobases toward the molybdenum center. They are water soluble and a preliminary biological screening has shown that they have higher activity against HT29 cells than molybdocene chloride or thionucleobases.[73] Molybdocene-

aminoacid complexes were also synthesized and their cyclopentadienyl and aminoacid hydrolysis was studied.[74]

Unlike titanocene-aminoacid complexes, molybdocene dichloride forms highly water soluble, air-stable complexes with cysteine and glutathione via coordination of the deprotonated thiol groups.[75] Coordination to the imidazole nitrogen in histidine was observed, but no evidence for coordination with the amino or carboxylate groups in cysteine, histidine, alanine or lysine was detected. Molybdocene dichloride and other thiol derivatives have been found to enter cells but no correlation has been found with the activity in V79 Chinese hamster lung cells.[76] Other fluor-containing ligands have been used to produce molybdocene complexes and their structure and hydrolytic behavior have been studied, but no data on cancer cells was published.[77]

2. Vanadocene derivatives

Similar to titanocene-aminoacid complexes, vanadocene-aminoacid complexes have been synthesized.[78] Their water properties, electrochemical behavior and their interactions with transferrin and albumin have been studied. Preliminary screening have shown cytotoxic activity against HT29 cells.[79] Vanadocene complexes have been studied for their spermicidal activity,[80] but little work has been published on their cytotoxic activity. There is one report which describes an extensive family of vanadocene derivatives on human testicular cancer cells.[81]

F. Interactions with biomolecules

The interaction of titanocene dichloride and molybdocene dichloride with DNA has been studied by ICP-AES.[83] Also, molybdocene dichloride interactions with nitrogen bases, oligonucleotides and DNA have been studied by electrochemical analysis and NMR.[84-86] Most of the research work in this area has been targeted to the interactions of metallocene complexes with DNA or its constituents.[30-33, 87-89] The interactions of metallocenes with other biologically important molecules have been studied. Glutathione has been implicated in the mechanism of detoxification of metal-based drugs and this has been explored with metallocenes.[90] Also, the solution properties of vanadocene dichloride has been explored in phosphate buffered saline,[91] and both plasma and human blood.[92] Molybdocene dichloride interactions with DNA and oligonucleotides have been studied,[93-94] and a review on its bioorganometallic chemistry was published.[95]

In recent times, no biological molecule has attracted more interest regarding titanocene dichloride mechanism of action than the protein transferrin. The interaction of transferrin with titanium(IV) ions was published almost simultaneously by two groups.[96-97] The formation of a titanium(IV) complex with ethylenebis[2-(o-hydroxyphenyl)glycine, EHPG, a ligand used to model the active site of transferrin, was used as evidence of the possible mode of interaction of Ti(IV) with transferrin.[98] The uptake of Ti(IV)-transferrin by cells via the transferrin-receptor was then proof of the targeted delivery of Ti(IV) into cancer cells.[99] Titanium-45, a radioactive isotope of titanium, was then used to demonstrate that the complex remains intact *in vivo* and

there was an increased uptake of titanium by a tumor compared with non-target organs such as muscle.[100] Other studies have shown that, of the more than 40 metals known to bind transferrin, Ti(IV) binds more tightly than does Fe(III).[101]

The interaction of Ti(IV) complexes with transferrin has been accomplished with UV-Vis,[29,58,62] NMR,[102] and ICP-AES[103] spectroscopy. No relationship of the titanium(IV)-to-transferrin binding capabilities of titanocene complexes with their cytotoxic activity has been established.

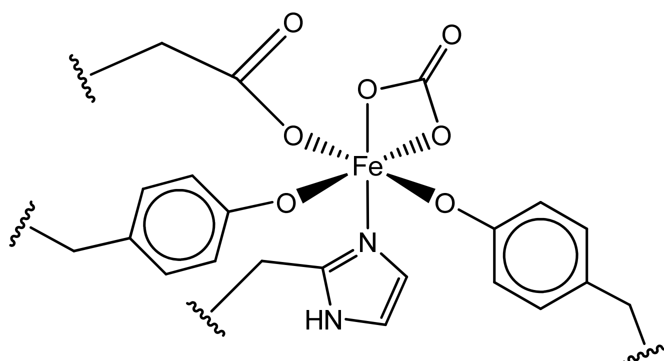


Figure 10. Transferrin binding site with Fe(III) ion bound to four aminoacid residues and carbonate anion.

G. Transport and delivery

Besides transport mediated by transferrin and the transferrin receptor, the use of liposomes has been explored to deliver Ti(IV) into the cells and to prevent its hydrolysis extensively has been the use of liposomes.[17,59] An alternate mechanism which has been explored is the use of cyclodextrins to form inclusion or covalently-linked metallocene complexes. Covalent modifications of titanocene with a β-

cyclodextrin dimer have been studied[104] and their activity against MCF-7 cells is higher than titanocene dichloride.[23] This has also been carried out with ferrocene derivatives with mixed results as the inclusion complexes with unmodified β-cyclodextrins has not improved activity but inclusion with modified CD has provided a little increase in the activity against Ehrlich ascites tumor cells.[26]

H. Biological activity at cellular level or against tumor models via expression profiling

It is known that titanium dioxide (TiO_2) nanoparticles have activity against cancer cells.[107] It is also known that titanium alloys are used to make osseointegrable devices because of their biocompatibility.[108] How do we know if these two "different" properties exerted by the same metal are related? Why have titanium complexes shown an antiproliferative effect on a variety of tumor cells and an unexpected proliferative effect[115] on others? To explore this and other issues, some researchers have changed their approaches and used microarray analysis to study gene expression profiles on a variety of conditions. By allowing simultaneous measurement of the expression of thousands of genes, more information on metabolic and signaling pathways on cells is available than with any other technique known to date. For example, microarray techniques have been used to study the translation regulation in osteoblasts exposed to both rutile and anatase, since anatase phase has been shown to have an enhanced ability to induce bonelike apatite when compared to the rutile phase.[109]

Microarray analysis has been used also to account for gene expression differences in different colon tumor cells. The HT29 colon adenocarcinoma cells have been used as a model for colon cancer in studying the inhibition of DNA methyltransferase,[110] the effect of the carcinoembryonic antigen on metastatic progression,[111] the transcriptional effect of tumor necrosis factor members,[112] the interferon-γ-induced sensitization to caspases,[113] and even the effect of aspirin in the prevention of colon cancer.[114]

Summary:

If we gather all the information presented here, the absence of toxicity information in cancer cell lines and the relationship it holds with the structure of the complexes is noteworthy. The natural evolution of this type of research must include an assessment of the ability of these drugs to fight cancer, since this feedback provides the researcher with tools to improve the drugs' potential as antineoplastic agents.

In terms of delivery, transferrin-mediated transport has been implicated in the ability of these complexes to exert their activity, but there is no study on the direct relationship between the titanium-donating capabilities of titanium complexes and their cytotoxic activity on cancer cells. Furthermore, although there is information on the ability of titanium drugs to interact with known cellular components, there is little information on the mechanism of action at the cellular level.

Objective

The proposed research was aimed at understanding structure-activity relationships of organometallic titanium(IV) complexes. Specifically, to elucidate how changes in the structure could affect the biological activity against HT29 colon adenocarcinoma cells via transferrin-mediated transport. The original hypothesis was that a complex should be more active if it is more soluble, water stable and is able to interact with cellular components such as metal-carrier proteins and DNA. In order to accomplish this goal, the following objectives were pursued:

1. To obtain cytotoxic activity data of inorganic and organometallic Ti(IV) complexes on HT29 colon adenocarcinoma cells.
2. To correlate cytotoxic data obtained with water solubility and stability studies previously published on the complexes, and to the ability of the complexes to donate the metal to the protein transferrin. To establish structure-activity relationships for the complexes studied.
3. To evaluate if the cytotoxic activity of the compounds when provided to the cells as the transferrin-titanium complex is enhanced or diminished.
4. To infer the possible mechanism of action of Ti(IV) drugs by generating an expression profile on a colon cancer cellular model.

5. To obtain cytotoxic activity data of cyclopentadienyl functionalized ferrocene complexes on HT29 and MCF-7 cells and establish structure-activity relationships.

The ultimate goal is to have a better knowledge of the parameters that govern the relationship between structure and activity and the mechanism of action of titanium(IV) drugs since this would facilitate the design of more effective antineoplastic drugs.

Chapter III. Experimental Section

A. Viability Assay

The colon adenocarcinoma cell line HT29 was purchased from American Type Culture Collection (ATCC HTB-38) and was maintained at 37°C and 95%Air/5%CO_2. Originally it was maintained in McCoy's 5A (ATCC) complete medium supplemented with 10% (v/v) triple-filtered fetal bovine serum (ATCC) and 1% (v/v) antibiotic/antimycotic (Sigma). This medium is supplemented by ATCC to contain 1.5mM L-glutamine and 2.2g/L sodium bicarbonate. All cell-related products were subsequently obtained from Sigma-Aldrich. The medium obtained from Sigma does not contain L-glutamine, but this was added from a concentrated stock to make the final growth medium used in the cell culture.

The breast adenocarcinoma cell line MCF-7 was provided by Dr. Jaime Matta at Ponce School of Medicine, Ponce, P.R., but is traceable to an original aliquot obtained from ATCC (HTB-22). This was maintained at 37°C and 95%Air/5%CO_2 in Eagle's Minimum Essential Media (Sigma) supplemented with 10% (v/v) heat inactivated FBS, 1% (v/v) antibiotic/antimycotic, 0.01mg/mL bovine insulin, non-essential aminoacids and L-glutamine. All supplements were obtained from Sigma-Aldrich.

Phosphate buffered saline (PBS) was prepared from Sigma-Aldrich products (sodium chloride, potassium chloride, sodium phosphate and potassium

phosphate) and double-distilled, deionized, autoclaved water. PBS was autoclaved and sterile-filtered through cellulose-acetate 0.2µm filters.

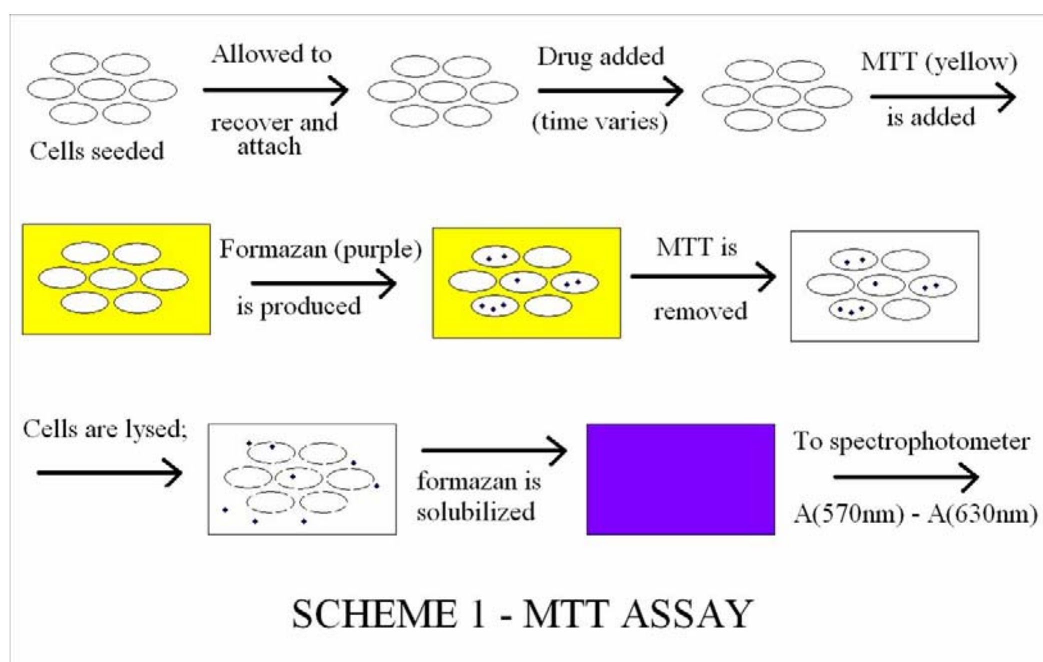
The reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) used for the cytotoxic assay was obtained from Sigma. Spectrophotometric grade 2-propanol was obtained from Fisher. The detergent Triton X-100 and the MTT Formazan were obtained from Aldrich. Cell-culture tested human apo-transferrin was obtained from Sigma and used without further purification. Anhydrous DMSO (Sigma), spectrophotometric grade methanol (Fisher) and absolute ethanol (Sigma) were also used for the optimization of the assay (for details see Appendix A). All MTT manipulations were performed in a dark room.

Titanium complexes (functionalized titanocene complexes, titanocene-aminoacid complexes, titanium-maltolato complex), and functionalized ferrocene complexes were provided from former and actual members of the research laboratory of Dr. Enrique Meléndez, University of Puerto Rico – Mayagüez.

Gamma-irradiated sterile plasticware, plates, flasks, microplates, centrifuge tubes, pipettes, pipet tips, filtration systems were obtained from varied sources. The incubator used was a Fisher water-jacketed CO_2 incubator and the laminar vertical flow hood was from Labconco. The carbon dioxide used in the incubator was United States Pharmacopoeia grade. All cell-culture techniques were performed under sterile conditions in a semi-restricted laboratory dedicated for this sensitive work, equipped with refrigerated centrifuges, refrigerators/freezers, water baths, pH meters, balances, among other materials. Air-conditioning system vents

are equipped with HEPA filters and an additional independent air-filtering system is constantly used. The laboratory is equipped with a UV light, which is used frequently to sterilize all exposed working areas.

Biological activity was determined using the MTT assay originally described by Mossman[116a], but using some modifications described by Denizot and Lang,[116b] after being optimized to our working conditions. Scheme 1 is a graphic representation of the assay. The assay is described in detail in the next paragraphs.



Scheme 1. Graphic representation of MTT assay

Asynchronously growing cells (HT29) were seeded at 1.0-1.5x10^4 cells per well in 96-well plates containing 100 μL of complete growth medium, and were

allowed to acclimate overnight. When working with MCF-7 cells, a higher initial cell density was used due to a longer doubling time compared with HT29 cells. The concentration range used for each complex was determined experimentally and details are presented in Appendix A.

For the assays in which transferrin was not present, various concentrations of the complexes (2–1400 μM; 10 data points evenly distributed) dissolved in 5%DMSO/95%Medium were added to the wells (eight wells per concentration; experiments performed in quadruplicate plates), and cells were incubated for an additional 70, 94, or 118 hours. For the assays in which transferrin was present, the original DMSO solution (into which the complexes had been dissolved) was slowly added to the corresponding amount of complete growth medium (supplemented so that it contained transferrin at a dose equivalent concentration to the final 5% DMSO/95% medium solution). Transferrin was diluted from a concentrated, spectrophotometrically determined, stock solution in PBS. A solution of the complex in the presence of transferrin was allowed to stand for the time required for the complete stripping of the Ti(IV) center and the subsequent uptake by transferrin. The process took ten minutes for Cp_2TiCl_2 and the functionalized cyclopentadienyl complexes and sixty minutes for amino acid complexes, which had been determined previously.[29,58] Although the $[Ti_4(\text{maltolato})_8(\mu\text{-}O)_4]$ complex was known not to transfer Ti(IV) to transferrin, it was also allowed to stand for one hour prior to dosing the plates.

After this time, MTT dissolved in complete growth medium was added to each well for a final concentration of 1.0 mg/mL and incubated for two additional hours for a final compound exposure times of 72, 96, or 120 hours, respectively. This high concentration of MTT and short exposure time was used to avoid exocytosis of formazan with subsequent loss during plate washing. After this, all the MTT containing medium was removed, and the cells were washed with cold PBS (optimum condition to avoid cell loss during washing) and were dissolved with 200 µL of a 10% (v/v) Triton X-100 solution in isopropyl alcohol.

The plates were incubated for one hour at 30°C and shaked in microplate reader for homogeneity. After complete dissolution of the formazan crystals, well absorbances were recorded in triplicates on a 340 ATTC Microplate Reader from SLT Lab Instruments at 570 nm with background subtraction at 630 nm, which was previously verified for optimum performance at the linear range under study. The concentration of compounds required to inhibit cell proliferation by 50% (IC_{50}) were calculated by the fitting data to a four-parameter logistic plot by means of the SigmaPlot software from the SPSS Company. All IC_{50} values reported here are the mean (±standard deviation) of four different plates for each compound.

B. Interaction with DNA or its constituents

The cancer cell's DNA is the proposed main target of titanium-based chemotherapeutic drugs. It could affect it directly, like it has been proven for platinum-based complexes, or it could affect it indirectly, by altering any other

biomolecule related to DNA stability, integrity or structural behavior. In an effort to elucidate if DNA is indeed implicated in the mechanism of action of the new titanium-maltolato complex, a series of experiments were carried out. These experiments are aimed as to provide preliminary data on the ability of the new drug to alter the DNA structure via covalent or intercalative interactions.

1. Agarose gel electrophoresis

Molecular biology grade agarose and Tris-acetate 50X buffer were obtained from Fisher. The gel electrophoresis casting system was from Bio-Rad Laboratories. A 50bp DNA ladder and a 100bp DNA ladder were obtained from Qiagen. Blue/Orange Loading Dye and Ethidium Bromide were obtained from Promega. The gels were photographed in a Amersham Biosciences Typhoon 9410 Variable Mode Imager.

Agarose (1.0 to 4.0%) dissolved in tris-acetate buffer was allowed to polymerize and suspended in a gel casting apparatus in Tris-acetate buffer as supporting electrolyte. Wells were large enough to accommodate up to 50uL of sample. Solutions of the 50bp and 100bp DNA ladders were allowed to react with a solution of the new titanium-maltolato complex for a period of 24 hours. In the case of the 50bp and 100bp DNA ladder, the solution of the complex was prepared so the tetramer was present at a 1:1 and 1:2 ratio, respectively, with DNA base pairs. Samples were flash-frozen after 2h, 5h, and 24h of incubation. Electrophoresis of all samples was performed under the same conditions (same gel), typically for 60 minutes at a rate of 5-6V/cm or up to the point in which the faster migrating dye had migrated

2/3 the length of the gel. After photographing the gel, densitometry analysis was performed to the bands present in the gel, as to compare samples treated with the new titanium-maltolato complex with control samples not treated but incubated for the same time.

2. Fluorescence Spectroscopy

Lyophilized Calf thymus DNA was obtained from Sigma and freshly-prepared solutions in RNase- and DNase-free water were used for the interactions with the new titanium-maltolato complex. A Perkin Elmer Lambda 2 UV-Vis Spectrophotometer was used to measure the concentration of the DNA solutions. A fluorometer model RF-5301 from Shimadzu company was used.

Prior to any addition of the titanium-maltolato complex to the DNA solution, the possible titanium-maltolato complex's fluorescence was verified and found to be non-existent in aqueous solution. A DNA-ethidium bromide complex was used as a standard solution of known fluorescence, and the titanium-maltolato complex was added in different complex/ethidium ratio so as to assess if the titanium-maltolato complex was able to intercalate strongly enough into DNA bases as to displace partially or totally the ethidium ion.

Originally a 6.6×10^{-4} M DNA solution was used. No fluorescence was observed. Ethidium bromide was added to obtain a final concentration of 6.31×10^{-7} M and the fluorescence spectrum was recorded. Titanium-maltolato complex was added to obtain a final concentration of 5.22×10^{-7} M (approximately 1:1 ratio with EtBr) and

fluorescence spectra were recorded every ten minutes for one hour. The experiment was repeated but with an ethidium bromide concentration of $2.52x10^{-6}$ M and the titanium-maltolato complex at $5.0x10^{-6}$ M (approximately 2:1 ratio with EtBr) and at $2.42x10^{-5}$ M (approximately 45:1 ratio with EtBr). All measurements were recorded at 28°C. No changes in the fluorescence spectrum of the DNA-ethidium interaction were observed at all ratios studied.

3. Proton nuclear magnetic resonance (^{1}H-NMR)

RNase- and DNase-free water was obtained from Fisher. Deuterated water (>99.9%D) and Tris-d_{11} (>99%D) were obtained from Fisher. The instrument used was a Bruker Avance DRX-500 NMR Spectrometer equipped with a 5mm BBO BB-^{1}H probe. Most experiments were performed with 256 or 512 scans, an acquisition time of 1.0 s and presaturation of the water signal.

Titanium-maltolato complex was dissolved in pure D_2O to a concentration of $1.05x10^{-4}$ M. The tetramer:monomer ratio was 3:1 and the pH of the resulting solution was 7.4 and was not adjusted. Different experiments were performed. The complex was allowed to stand for 14 hours at 37.0°C and no change in the monomer:tetramer ratio was observed. The titanium-maltolato complex was allowed to interact with DNA at 20°C for one hour and no change was observed in the monomer:tetramer ratio of the complexes' solution. The complex was also allowed to interact with DNA at 37.0°C for 14 hours and no change in the signals of the complex was observed. The

only observed change was when a solution of the complex allowed to interact with DNA for one hour at 37.0°C was cooled down to 25.0°C, in which the tetramer, but not the monomer, signals disappeared. When this solution was allowed to interact with DNA for 24 hours at 37°C and cooled down, no further change in the monomer signals was observed.

C. DNA Microarray analysis

Ethanol, 2-propanol and chloroform for molecular biology, and RNase- and DNase-free water were obtained from Fisher. Sodium citrate and guanidine hydrochloride, for molecular biology were obtained from Sigma-Aldrich. Trizol Reagent was obtained from Invitrogen. Sodium dodecyl sulfate was used from Bio-Rad Laboratories. RNeasy Mini Kit Plus was obtained from Qiagen. Zymo Research DNA-Free RNA Kit was obtained from Zymo Research Corporation.

Asynchronously growing HT29 cells were exposed to titanocene dichloride and the new titanium-maltol complex in concentrations related to the previously determined IC_{50} for both drugs. After the six hours of incubation with the titanium drug, RNA, DNA and protein were extracted from the cells. RNA was the main biological target of the extraction, but since RNA, DNA and proteins can be isolated from each other, DNA and proteins were also extracted and preserved for future use. The RNA extraction was made using a slightly modified Trizol protocol. Briefly, cells are homogenized with Trizol, samples are extracted with chloroform and RNA is subsequently purified from the upper aqueous phase. The interfase (DNA) and organic

phase (protein) were then separated by DNA precipitation with ethanol, followed by protein precipitation with 2-propanol. The DNA pellet was washed with an ethanolic solution of sodium citrate, and a water-ethanol mixture, before dissolving it in weak base. Then, the pH of the resulting solution was adjusted to 7.5 and EDTA was added. The protein pellet was washed in an ethanolic solution of guanidine hydrochloride, and absolute ethanol. Then, it was dissolved in an SDS solution.

Since RNA extracted using the Trizol protocol can sometimes contain traces of genomic DNA, samples were then purified using Zymo Research DNA-Free RNA Kit. Basically, RNA samples were allowed to react with RNase-free DNase I and then filtered using Spin Columns which bind RNA and non-RNA biological molecules are eluted first. After this procedure was performed, the RNA samples were then purified from small RNAs (<200 nt) to assure that mRNAs accounted for most of the RNA sample. This was performed with the RNeasy Plus Mini Kit from Qiagen. The RNA samples were analyzed for quantity, purity, integrity and stability prior to the microarray procedures.

RNA samples were shipped in dry ice to H.L. Moffit Cancer Center in Tampa, Florida for hybridization, amplification and DNA microarray analysis. This produced an expression profile of the cells in three different environments: untreated HT29 cells, HT29 cells treated with titanocene dichloride and HT29 cells treated with the new titanium-maltolato complex. DNA Microarray was performed with an Affymetrix GeneChip probe.

A typical microarray experiment can be divided in three main steps. The first one is the preparation of the microarray. Microarrays are available in two different formats: cDNA microarrays and oligonucleotide arrays. Oligonucleotide arrays are usually used to represent different organisms, although human oligonucleotide arrays are available for detecting mutations in specific genes, among others. These are synthesized in a predetermined spatial orientation using photolithography. cDNA microarrays are generated by printing a double-stranded cDNA onto a solid support. The second one is the preparation of the cDNA probes which are fluorescently labelled and their hybridization. The fluorescently labelled cDNA probes are produced from mRNA of excellent quality and labeling with fluorescent dyes. The most frequently used dyes are green (Cy3) for control samples and red (Cy5) for test samples. These are photosensitive and exposure to light should be minimized. The third one is the scanning of the microarray and the data analysis. A confocal laser is used to scan the glass slides (microarrays), which produces separate images of both Cy3- and Cy5- labeled probes, which are then used to calculate the relative level of expression of the genes. Detailed information on the statistical algorithms used for data analysis is found in the website of the Affymetrix company.[117]

Briefly, a DNA microarray consists of thousands of cells that each contains many copies of a unique oligonucleotide probe. In this context, cells refer to an independent point in the chip and not to living cells. Cells are paired together to form probe pairs. Within each pair, one contains oligonucleotides that match the target sequence exactly and the other contains a single base substitution in the middle of the

probe sequence. The expression of a gene is usually measured across 11 to 20 different probe pairs to arrive at its true expression. In order to determine the absence or presence of a particular transcript, the software calculates a discrimination value for each probe pair. Then the background is corrected before the signal intensity (quantity of transcript) of a particular cell is calculated.

After this is performed in a single microarray chip, comparison analyses can be made from two different chips. One chip is designated as the baseline and another chip is designated as the experiment. An additional value now can be calculated, which is the fold change. The fold change is the ratio of the signal from the control array and the experimental array for a specific transcript. This ratio provides the differences in gene expression in HT29 cells that can be correlated to the presence of the titanium chemotherapeutic drugs.

Chapter IV. Results and Discussion

A. Viability assay

1. Stability of titanium complexes

The syntheses of the titanocenes and titanium-maltolato complexes have been previously reported.[29,58,61] Along with the synthetic data for the titanocene-aminoacid complexes already published, kinetic studies under pseudo first-order conditions were performed to establish the stability of these species in an aqueous solution. The spectroscopic techniques used for this were UV-Vis and ^{1}H NMR spectroscopies. Table 1 and 2 present the rate constants and half-lives ($t_{1/2}$) for amino acid and cyclopentadienyl (Cp) loss.

The kinetic data showed, with regard to the ancillary ligands, that $[Cp_2Ti(L\text{-}methionine)_2]Cl_2$ and $[Cp_2Ti(L\text{-}cysteine)_2]Cl_2$ complexes are less stable than titanocene dichloride, having a half-life time of 8.26 and 61.9 minutes, respectively. By contrast, $[Cp_2Ti(D\text{-}penicillamine)_2]Cl_2$ was slightly more stable in water with respect to the amino acid loss, with a $t_{1/2}$ of 473 minutes. The Cp loss, when monitored by proton-NMR spectroscopy (Table 2), showed rate constants of about one order of magnitude larger than the k_{obs} for the amino acid loss. In this regard, Cp stability follows a pattern similar to that of amino acid stability, but the k_{obs} values fall within a narrower range. The L-methionine and L-cysteine complexes showed

slightly lower stability with respect to Cp loss, having $t_{1/2}$ values of 1.07 x 10^3 and 2.84 x 10^3 minutes, respectively, as compared to titanocene. On the other hand, the penicillamine complex had a higher level of stability with respect to Cp loss than titanocene dichloride. However, increasing the pH to 7.4 leads to the formation of a yellow solution, which eventually vanishes. A complete decomposition was observed as evidenced by the formation of a cloudy suspension within 5-10 minutes. Due to the formation of precipitates, we were not able to perform kinetic studies at physiological pH using NMR and UV-Vis spectroscopies.

Table 1. Observed rate constants for ancillary ligand (amino acid or chloride) loss at 25ºC, monitored by UV-Vis spectroscopy, pH 2.7-3.0

Complex	k_{obs} (min^{-1})	Half-life, $t_{1/2}$ (min)
Cp_2TiCl_2	2.24(4) x 10^{-3}	3.09(6) x 10^2
$[Cp_2Ti(L\text{-cysteine})_2]Cl_2$	1.120(9) x 10^{-2}	61.9(5)
$[Cp_2Ti(D\text{-penicillamine})_2]Cl_2$	1.47(2) x 10^{-3}	4.73(7) x 10^2
$[Cp_2Ti(L\text{-methionine})_2]Cl_2$	8.39(7) x 10^{-2}	8.26(7)

In marked contrast with titanocene complexes, $[Ti_4(maltolato)_8(\mu\text{-}O)_4]$ is remarkably stable in aqueous solution at physiological pH without any notable decomposition in a period of ten days. According to single crystal X-ray analysis, (Figure 9 is a drawing of the crystalline structure; bonding parameters are in Supplementary Material of reference 61), it was observed that $[Ti_4(maltolato)_8(\mu\text{-}O)_4]$ is a tetrameric tetranuclear species with all Ti(IV) metal centers coordinated by two

chelating maltolato and two bridging oxygens, in a pseudo-octahedral coordination geometry. Given that Ti(IV) is surrounded by six oxygens in the coordination sphere, this species is very stable in aqueous solution at high pH.

Table 2. Observed rate constants for cyclopentadienyl protonolysis at 25°C, monitored by ^{1}H NMR spectroscopy, pH 2.7-3.0

Complex	k_{obs} (min^{-1})	Half-life, $t_{1/2}$ (min)
Cp_2TiCl_2	$2.1(5) \times 10^{-4}$	$3.3(8) \times 10^{3}$
$[Cp_2Ti(L\text{-cysteine})_2]Cl_2$	$2.45(7) \times 10^{-4}$	$2.84(8) \times 10^{3}$
$[Cp_2Ti(D\text{-penicillamine})_2]Cl_2$	$1.1(1) \times 10^{-4}$	$6.6(7) \times 10^{3}$
$[Cp_2Ti(L\text{-methionine})_2]Cl_2$	$6.5(2) \times 10^{-4}$	$1.07(7) \times 10^{3}$

Interestingly, after its dissolution in water at a pH of 8, $[Ti_4(maltolato)_8(\mu\text{-}O)_4]$ dissociates to some extent to form *cis*-$Ti(maltolato)(OH)_2$ (monomer) in a ratio of 7:1 (tetramer:monomer). The identities of the monomeric species at low pH and tetrameric species at high pH have been characterized by ^{1}H NMR and MS spectroscopies and elemental analysis. As previously stated, the tetramer is the predominant species at high pH (> 7) while the monomeric species predominates at low pH (< 7), but none of the species degrade in aqueous solution at physiological pH.

The aqueous stability of titanocene dichloride with functionalized cyclopentadienyl [$(Cp\text{-}R)_2TiCl_2$ and $(Cp\text{-}R)CpTiCl_2$, R = CO_2CH_3 and $CO_2CH_2CH_3$] was not investigated due to their low solubility in water. Anyhow, their low solubility

in water will be discussed as well as the stability behavior of titanocene-aminoacid and titanium-maltolato complexes regarding their cytotoxic activity against HT29 cells.

2. Binding interactions of titanium complexes with the protein transferrin

A study was performed and published regarding Ti(IV) intake on iron-binding sites by human apo-transferrin.[29,58] The study was accomplished using electronic absorption (UV-Vis) spectroscopy that utilized a Tris buffer solution containing bicarbonate, which acted as a synergistic anion. The objective of this study was to understand how the ligands surrounding Ti(IV) affect human apo-transferrin intake. Titanocene dichloride was utilized in these studies as a reference.

Electronic absorption spectroscopy is commonly used to study metal binding to the specific iron-binding sites of transferrin.[29,58,97,99] The binding of metal ions to the phenolic groups of tyrosine residues located at the specific iron-binding sites of apo-transferrin produces two new absorption bands at 240 and 295 nm. These bands have been attributed to the deprotonation and subsequent coordination of the tyrosine residues to the metal ion. In addition, when Ti(IV) is loaded into apo-transferrin, it produces a band at 321 nm that is attributed to ligand-to-metal charge transfer (LMCT). Because our subject complexes have an absorption band about 243 nm, we used the band at 321 nm to monitor the binding interactions.

The titration of apo-transferrin (apo-Tr) with increasing amounts (aliquots) of Cp_2TiCl_2 yielded three absorption bands at 240, 295, and 321 nm in the UV-difference

spectrum and reached equilibrium in about five minutes. Increasing the titanium to transferrin ratio produces an increase in these bands and reaches a plateau at a 2:1 ratio. This demonstrates that the two metal-binding sites are being occupied by Ti(IV) and that both N- and C-lobes were loaded upon reaching saturation. The Ti(IV) products formed after the decomposition of the titanocene complexes in buffer solution at pH 7.4, did not have absorbance at 321 nm, which allowed for an accurate assessment of the titration profile.

In contrast, the $[Cp_2Ti(amino\ acid)_2]Cl_2$ complexes take longer to bind apo-transferrin, reaching equilibrium in 45-60 minutes. The competition of the amino acid ligands for Ti(IV) might explain the delay in releasing Ti(IV) to apo-transferrin. Consequently, the binding capabilities of these $[Cp_2Ti(amino\ acid)_2]Cl_2$ complexes to apo-Tr are lower than that of titanocene dichloride. Nevertheless, the titrations of apo-transferrin with the $[Cp_2Ti(amino\ acid)_2]Cl_2$ complexes produce the three characteristic bands at 240, 295, and 321 nm and reach a plateau at r = 2 ([Ti]/[Tr]), see Figure 11.

Likewise, the titrations of human apo-Tr with aliquots of the $(Cp\text{-}COOR)_2TiCl_2$ and $(Cp)(Cp\text{-}COOR)TiCl_2$ complexes (R = Me, Et) dissolved in DMSO/$NaCl_{(aq)}$ reached saturation at r = 2 ([Ti]/[Tr]), see Supplementary Material of reference 58. All these complexes reached equilibrium in about 2-10 minutes.

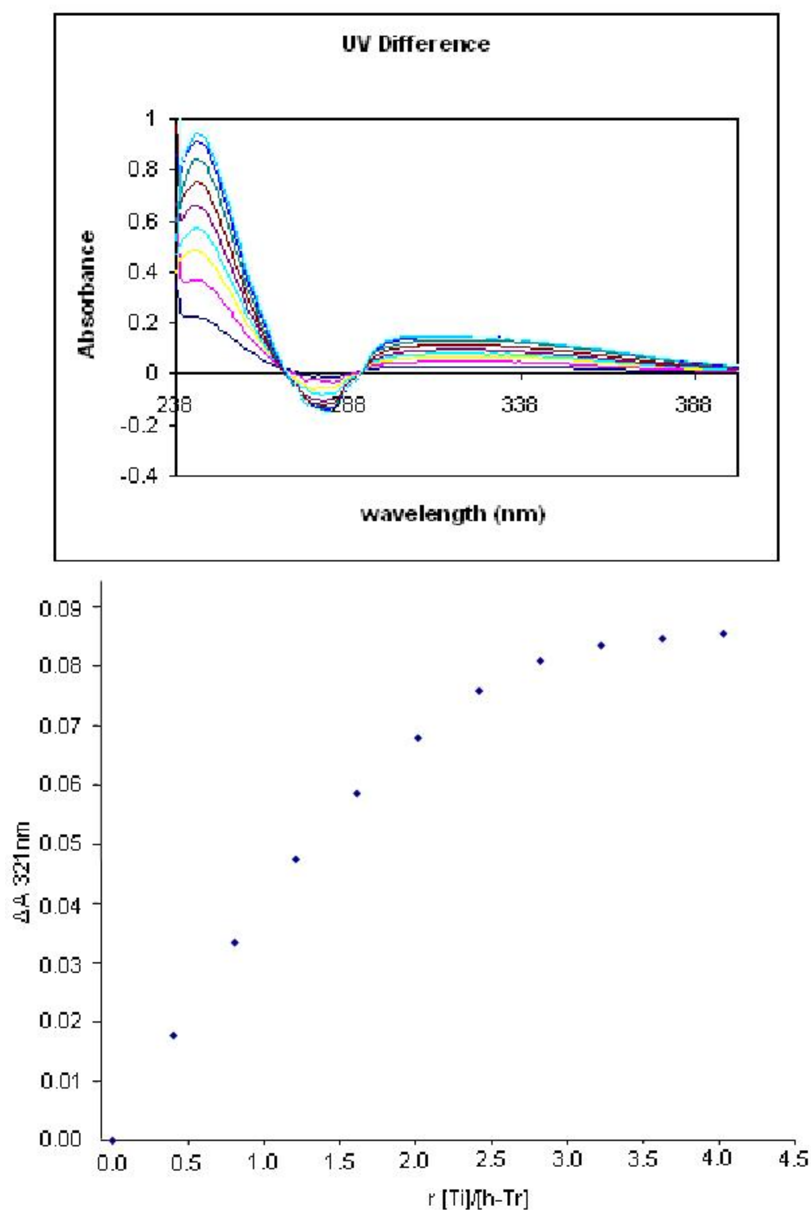


Figure 11. UV difference spectra (up) of 1.68 x 10^{-5} M apo-transferrin (in 25 mM $NaHCO_3$/10 mM NaCl/100 mM Tris buffer, pH 7.4) upon addition of increasing amounts of $[Cp_2Ti(L\text{-cysteine})_2]Cl_2$ (dissolved in 10 mM NaCl) (top) and a titration curve (down) of $[Cp_2Ti(L\text{-cysteine})_2]Cl_2$ (dissolved in 10 mM NaC1), with apo-transferrin monitoring the absorption at 321 nm (bottom).

The apo-Tr titrations with aliquots of $[Ti_4(maltolato)_8(\mu\text{-}O)_4]$ did not yield any of the characteristic absorption bands at 240, 295, or 321 nm in the UV difference spectra. At first, the transferrin was allowed to react for 60 minutes with the complex, since the time to reach equilibrium for Cp_2TiCl_2 and functionalized cyclopentadienyl titanocene dichlorides is about 2-10 minutes whereas for the titanocene-amino acid complex equilibrium is reached in 45-60 minutes. Since the $[Ti_4(maltolato)_8(\mu\text{-}O)_4]$ complex has a coordination sphere containing six oxygen ligands, it is not surprising that this complex is more stable than the titanocenes. Therefore, longer reaction times were used.

The interaction of transferrin with two molar equivalents of $[Ti_4(maltolato)_8(\mu\text{-}O)_4]$ was allowed to react for three days, recording a full UV-Vis spectrum every 10 minutes. The pH of the resulting solution was not altered after this period of time and remained at 7.4, but the reaction did not proceed, and the absorption bands at 240, 295, and 321 nm were not observed. After the transferrin was allowed to react with the complex for a seven-day period, the changes in the 321 nm band were less than 0.1% of the original absorbance value. Thus, the oxygen-rich octahedral coordination environment makes a robust Ti(IV) complex and the ligand-stripping process and the subsequent transfer of Ti(IV) to human apo-transferrin seems to be thermodynamically as well as kinetically unfavorable. The transferrin – donating capabilities of the titanium complexes will be discussed regarding the ability of transferrin to improve or diminish the activity of titanium complexes in HT29 cells.

3. Antiproliferative activity of titanium complexes

The cytotoxicities of the functionalized titanocene, the titanocene- amino acid, and the titanium-maltolato complexes on the human colon cancer cell line, HT-29, were measured using a slightly modified MTT assay.[116] Titanocene dichloride and the functionalized cyclopentadienyl complexes were tested in concentrations that ranged from 120-2500 μM at 72 hours.[58] Since titanocene dichloride has a longer intracellular activation period, it is usually tested at a time interval of 96 hours, as was done in our study. Table 3 summarizes the IC_{50} data for the titanocene complexes. There was an increase in the cytotoxic activity at 96 hrs both carboethoxy-functionalized cyclopentadienyl titanocenes (being higher for the (Cp-$COOCH_2CH_3$)(Cp)$TiCl_2$ complex), but the carbomethoxy-functionalized complexes remained non-active at concentrations below 0.9 mM. All titanocene-amino acid complexes were shown to be non-active at time intervals of 72 and 96 hours, at concentrations below 0.7 mM. These complexes are more soluble in water than the parent complex, titanocene dichloride, with comparable aqueous stability to Cp_2TiCl_2; in addition, they are able to donate both Ti(IV) ions to transferrin. Thus, it is unclear why they did not show biological activity against the HT-29 cell line.

The titanium-maltolato complex was evaluated at time intervals of 72, 96, and 120 hours to determine its optimal cytotoxic activity (Figure 12). The complex displays its highest level of activity at 96 hours, showing an IC_{50} value of 1.5×10^{-4} M, followed by 2.4×10^{-4} M at 120 hours, and 2.8×10^{-4} M at 72 hours (see Table 3). At

all three time intervals, $[Ti_4(maltolato)_8(\mu\text{-}O)_4]$ was the most active species of all the titanium complexes that were investigated in this study. This complex's activity against MCF-7 cells was also determined and it was found to be higher than that for titanocene dichloride. The IC_{50} values were $5.7(5) \times 10^{-4}$ M for titanocene dichloride and $1.6(1) \times 10^{-4}$ M for the maltolato complex. Details are presented in Appendix C.

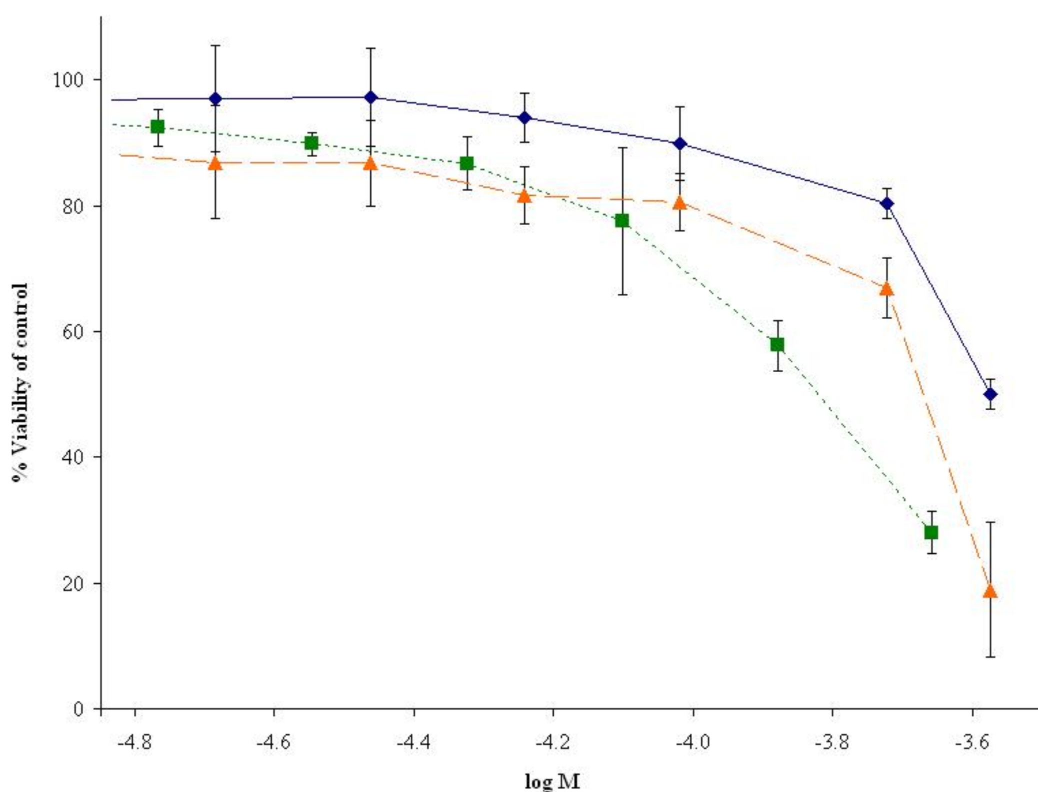


Figure 12. Dose-response curves for $[Ti_4(maltolato)_8(\mu\text{-}O)_4]$ against HT29 cells at drug exposure times of 72, 96, and 120 hours (diamonds, squares, and triangles), respectively.

Table 3. Cytotoxicities of the complexes studied in the HT-29 colon cancer cell line, as determined by the MTT assay. IC_{50} values are the average of four independent measurements. na = non-active at concentration range studied.

Complex	IC_{50} (μM)			
	72h	96h(w/o Tr)	96h(w/Tr)	120h
Cp_2TiCl_2	450(30)	420(30)	410(50)	-
$[Ti_4(maltolato)_8(\mu\text{-}O)_4]$	280(10)	150(10)	170(10)	240(10)
$(Cp\text{-}COOEt)_2TiCl_2$	580(40)	530(30)	-	-
$(Cp)(Cp\text{-}COOEt)TiCl_2$	630(20)	300(30)	-	-
$(Cp\text{-}COOMe)_2TiCl_2$	na	na	na	-
$(Cp)(Cp\text{-}COOMe)TiCl_2$	na	na	na	-
$[Cp_2Ti(L\text{-}cysteine)_2]Cl_2$	na	na	na	-
$[Cp_2Ti(L\text{-}methionine)_2]Cl_2$	na	na	na	-
$[Cp_2Ti(D\text{-}penicillamine)_2]Cl_2$	na	na	na	-

4. Relationship with hydrolytic stability

In the case of titanocene-aminoacid complexes as a series of complexes, it is impossible to assess the effect of the hydrolytic stability of these complexes upon their cytotoxic effect in HT29 cells, in terms of an order of activity since all of these complexes resulted non-active at the concentration range studied. This range was similar than the range for titanocene dichloride (see table 4), so it is safe to say that all

these complexes would have a higher IC_{50} value when compared to titanocene dichloride if they result active in a higher concentration range.

Table 4. Concentration range studied for titanocene-aminoacid complexes			
	concentration range studied (μM)		
Complex	72hours	96hours w/o Tr	96hours w/Tr
Cp_2TiCl_2	33-536	10-984	9-1406
$[Cp_2Ti(L\text{-cysteine})_2]Cl_2$	42-678	5-463	5-529
$[Cp_2Ti(L\text{-methionine})_2]Cl_2$	35-572	5-448	5-493
$[Cp_2Ti(D\text{-penicillamine})_2]Cl_2$	40-645	4-402	5-457

The calculated IC_{50} value for titanocene dichloride at 72 hours, 96 hours in absence of transferrin, and 96 hours in presence of transferrin were 450, 420 and 410μM, respectively. These values are inside the concentration range studied for all aminoacid complexes.

There is no clear correlation between the water solubility of these titanium-aminoacid complexes and their hydrolytic stability and cytotoxicity in HT29 cells. All these complexes were less stable than titanocene dichloride regarding ancillary ligand hydrolysis. The only exception is the D-penicillamine complex which is slightly more stable than titanocene dichloride, but also resulted non-active at the concentration range studied. Other titanocene-aminoacid complexes have been synthesized[34-37] but

no hydrolytic stability behavior has been published except sporadically use of phrases such as “stable in water for several hours”, “water soluble” and “stable enough that it was crystallized from water”. Use of hydrophilic ligands containing carboxylate or amino groups[20] or derivatives of thiophene[22] has resulted in complexes more active than titanocene dichloride both *in vitro* and *in vivo*. Anyhow, the available data is weakly supported as to correlate an increased activity pattern with increasing cyclopentadienyl hydrolysis.[22]

The aqueous stability of titanocene dichloride with functionalized cyclopentadienyl [$(Cp\text{-}R)_2TiCl_2$ and $(Cp\text{-}R)CpTiCl_2$, R = CO_2CH_3 and $CO_2CH_2CH_3$] can not be correlated with their cytotoxic activity. The stability studies were not possible due to their low solubility in water. Anyhow, from qualitative data, these complexes appear less stable than titanocene dichloride. They are moisture sensitive and show color changes upon standing in a moist environment.

There is an interesting activity pattern that was also observed with functionalized ferrocene complexes (section 4.A.7). The carbomethoxy functionalized complexes resulted non-active while the carboethoxy functionalized complexes resulted active although less active than titanocene dichloride or ferrocene. In fact, with ferrocene derivatives, the stepwise addition of carbomethoxy functionalized cyclopentadienyl ligands results in a decreased activity (higher IC_{50} value) for these complexes.

A prior report on the activity of carbomethoxy functionalized cyclopentadienyl complexes found an increased activity pattern for these complexes against the small

cell lung cancer cell lines NCI-H209. The highest activity was determined for the complex $(\eta^5\text{-}C_5H_4CO_2CH_3)_2TiCl_2$, followed by $(\eta^5\text{-}C_5H_5)(\eta^5\text{-}C_5H_4CO_2CH_3)TiCl_2$ and finally titanocene dichloride. The authors initially suggested[39] that this could be the result of an increased Lewis acidity of the titanium resulting in strong metal-DNA interactions, but an extension of the work with more hydrophobic esters and the use of other cell lines resulted in little cytotoxic activity[40,121] and was abandoned by the group. Unfortunately, there was no quantitative data published as to correlate the effect of these functionalizations with the activity or lack of activity.

Other functionalizations used for the cyclopentadienyl ligand in titanocene dichloride include alkylammonium groups. These can be either acyclic or cyclic and can be aromatic or aliphatic.[21,40-42] These functionalizations have resulted in more stable and more water-soluble complexes. Although no direct structure-activity relationship has been proposed by the authors since closely related complexes have resulted in different activity profiles, there is a general increase in the cytotoxic activity on H209 (human lung cancer) and A2780 (ovarian cancer), when compared to titanocene dichloride. There was no increased activity when using the cisplatin-resistant cell line A2780/CP.

The use of silicon-ansa-bridged substituted titanocenes in human myelogenous leukemia (K562) cells showed promising results with IC_{50} values in the mid micromolar range.[43] Because the complexes have limited solubility in water, dimethylsulfoxide was used in their evaluation. The lack of solubility of some complexes excluded them from cytotoxicity screening. The authors claim that use of

alkenyl substituents on the cyclopentadienyl rings result in an increase of the cytotoxicity in the cell lines tested, being K562 the most prominent, but careful observation of the data does not point to those conclusions.[44] Also, no direct relationships or conclusions were drawn regarding hydrolytic behavior and activity.

Non-silicon ansa-bridged and unbridged titanocenes with benzyl-substituted, aryl-substituted and heteroaryl-substituted cyclopentadienyl rings have resulted in a variety of complexes.[45-57] DMSO was usually used in testing of these complexes and the DMSO/medium solutions of the complexes was "applied within one hour of preparation to avoid interference with hydrolyzed compounds",[47-48] so we can infer the complexes are not hydrolytically stable. On the other hand, these complexes have cytotoxic activities that span a wide range of IC_{50} values in LLC-PK cells (pig kidney carcinoma). Use of methoxy substituents has improved the water solubility of phenyl-substituted complexes but cytotoxicity lies in the upper micromolar range.[48] The use of para-methoxy and para-methylammino substitutions have resulted in complexes with good solubility behavior and good cytotoxic activity.

Dimethylammino-substituted benzyl functionalized cyclopentadienyl complexes are the most promising compounds of this class.[54-57] The complexes have a good solubility profile (less than 0.7% DMSO is used in the formulations of the drug in the *in vitro* assays) and activity comparable to cisplatin in LLC-PK cells. Intramolecular stabilization of the Ti(IV) center through the amino groups is believed to be fundamental in the cytotoxic behavior of this type of complexes.

5. Relationship with transferrin presence

To study the role of transferrin in the cytotoxicity of these titanium complexes, we decided to retest both the titanocenes and the titanium-maltolato complexes, this time in a transferrin-enriched environment. The HT29 cell line was originally selected because of an increased expression of the transferrin receptor compared to other cancer cell lines. The aim of these studies was to determine whether the presence of transferrin in a dose-equivalent concentration could stabilize the Ti(IV) ion, deliver it to the cells, and, perhaps, increase the cytotoxic activity against the HT-29 cell line. The titanocene dichloride, the carbomethoxy-functionalized cyclopentadienyl titanocenes, $[Cp_2Ti(amino\ acid)_2]Cl_2$ and the titanium-maltolato complexes were added to the cells in the presence of transferrin in a concentration equivalent to that of the Ti(IV) complex. Both the carbomethoxy-functionalized and the amino acid complexes remained non-active at concentrations below 0.5 mM. This rules them out as potential chemotherapeutic agents, at least for the HT29 cell line. Both the titanocene dichloride and the titanium-maltolato complexes retained their IC_{50} value (See Figure 13), raising the issue of whether transferrin is a biologically favored route for Ti(IV) to exercise its toxic effect.

There is a report on the transferrin-independent cytotoxic activity of non-metallocene non-diketonato C_2-symmetrical titanium complexes on HT29 and OVCAR-1 cells.[18] The activity at 72hours of titanocene dichloride was reported to be enhanced in a transferrin-enriched environment. For HT29 cells, the IC_{50} values in absence and presence of transferrin are 710(±120)μM and 460(±40)μM and for the

OVCAR-1 cell line the values are 780(±90)μM and 520(±30)μM. The activity of the non-metallocene titanium(IV) complexes was reported not to improve. Supporting data shows that the activity did not improve, but also for one of the complexes it was slightly diminished in the protein-enriched environment. More detailed results are needed in order to fully compare these results. It should be noted that the authors report IC_{50} values similar to ours for titanocene dichloride on HT29 cells but with less precision. Also, if a transferrin-independent route of activity is proposed, neither an increase nor a decrease should be observed in the activity of the complexes toward the cell lines, and this is not the case for all the non-metallocene complexes reported.

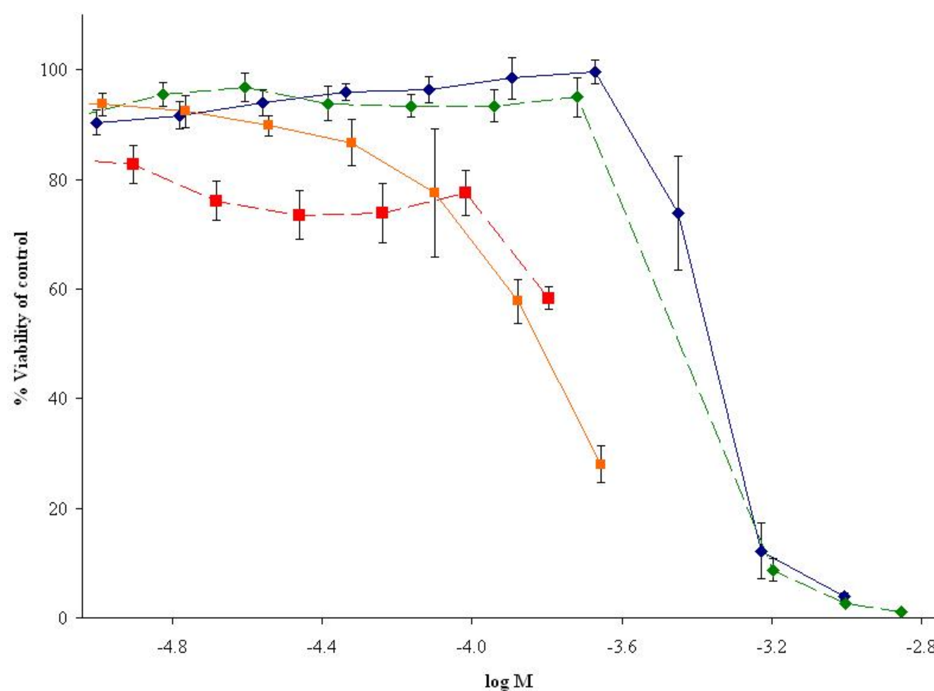


Figure 13. Dose-response curves for Cp_2TiCl_2 (diamonds) and $[Ti_4(maltolato)_8(\mu\text{-}O)_4]$ (squares) in HT29 cells at 96 hours of drug exposure, with (dashed lines) and without (solid line) transferrin.

While it cannot be generalized that transferrin is not a favorable route in Ti(IV)-based complexes for potential therapeutic use, it is highly probable that transferrin has only a stabilizing role in a hydrolytically hostile environment, such as plasma. In addition, the Ti(IV) that can enter the cell by a transferrin-mediated transport will be "naked" and still be prone to hydrolysis. The desired stabilizing role can be achieved by selecting the appropriate set of donors in polydentate ligands.

6. Relationship with structure and comparison of results with other cell lines

Original structure-activity relationships for titanocene-derived complexes stated that 1) the highest activity is obtained with non-modified cyclopentadienyl ring ligands, and 2) chloride exchange can be performed without loss of antitumor activity.[20] These relationships guided most efforts in the early years of this research area. There are reports[40,121] which clearly show that a specific modification through cyclopentadienyl functionalization renders compounds with little or no activity. Unfortunately, direct conclusions regarding structure-activity relationships have not been drawn since cytotoxic activity against the cell lines does not usually follow a clear pattern.[21,39-57]

The only direct structure-activity relationship which can be obtained from our data is the lowering in toxicity from carbomethoxy functionalization. It was apparent from titanocene derivatives, but was confirmed with functionalized ferrocenes (see section 4.A.7), in which the step-wise addition of carbomethoxy functionalization

lowers activity also in a step-wise manner. This behavior was confirmed for ferrocene derivatives in HT29 cells and also for MCF-7 cells.

The IC_{50} values in this study are comparable to previously reported IC_{50} values for Cp_2TiCl_2 in other cell lines, such as, for example, those of lung (H209, A549) or ovarian (A2780) cancer (IC_{50} ~10^{-4}-10^{-5} M).[39-41,45,48,54,118-119] On the other hand, our values were higher when compared to IC_{50} values of dimethylamino-functionalized titanocene complexes, bis-[(p-dimethyl-aminobenzyl)cyclopentadienyl titanium(IV) dichloride·HCl (IC_{50} 2.1 x 10^{-5} M), and bis-(N,N-dimethylamino-2(N-methylpyrrole)methyl-cyclopentadienyl titanium (IV) dichloride (IC_{50} 5.5 x 10^{-6} M), on LLC-pk pig kidney cancer cells.[49,54] Table X lists the above mentioned results along with reported IC_{50} values for cisplatin, titanocene dichloride and other titanocene derivatives. It is well-known that colon cancer cells are resistant to many cytotoxic drugs. Furthermore, it has been previously reported that titanocene dichloride is not very active in colon cancer cells.[13,118] Therefore, our results are within the expected IC_{50} values for this type of antineoplastic agents.

Our cytotoxic studies of the HT29 cancer cell line have shown that the titanocene-amino acid complexes and the carbomethoxy-functionalized titanocenes either have a lower or minimal activity compared to the titanocene dichloride and the titanium-maltolato complexes. In addition, transferrin is not a biologically favored route for these complexes to express their cytotoxic effect in HT29 cells.

Table 5. Reported IC_{50} values for other titanocene derivatives.

Complex	Cell line	IC50	Exposure time	Reference
heteroaryl substituted ansa-titanocenes	LLC-PK	low 10^{-4} M	48 hours	119
Cp-$COOC_2H_5$ functionalized Cp_2TiCl_2	HT29	low 10^{-4} M	96 hours	62
Titanium-maltolato complex	HT29, MCF-7	low 10^{-4} M	96 hours	62
Cp_2TiCl_2 with alkylammonium groups pendant to Cp	A549, H209, A2780	10^{-4} - 10^{-5} M	96 hours	40
Cp_2TiCl_2 with alkylammonium groups pendant to Cp	MCF-7, A2780	10^{-4} - 10^{-5} M	24, 144 hours	41
Silicon-bridged ansa titanocenes	HeLa, K562	10^{-4} - 10^{-5} M	72 hours	43, 44
aryl-substituted ansa titanocenes	LLC-PK, A2780	low 10^{-4} M	48 hours	45, 47
(para-substituted benzyl)-substituted titanocenes	LLC-PK	low 10^{-5} M	48 hours	49
dimethylamino-functionalized titanocenes	LLC-PK	10^{-4} - 10^{-6} M	48 hours	54
titanocene dichloride	HT29	710 μM	72 hours	18
cisplatin	HT29	50 μM	24 hours	176
cisplatin	MCF-7	71 μM	24 hours	176

7. Antiproliferative activity of ferrocene derivatives

The cytotoxicity of the functionalized ferrocene complexes on the HT-29 colon cancer cell line was measured using a slightly modified MTT assay as described in the

Chapter 3 of this thesis. Ferrocene was initially evaluated at time intervals of 72, 96 and 120 hours at concentrations that ranged from 10 – 1000 μM to determine its optimal activity (Figure 14). The complex displayed comparable activity at all three time intervals, with an IC_{50} value of 3.6×10^{-4} M. Since exposing the cells to ferrocene at longer periods of time did not improve the cytotoxic activity, all subsequent experiments were performed at a time interval of 72 hours.

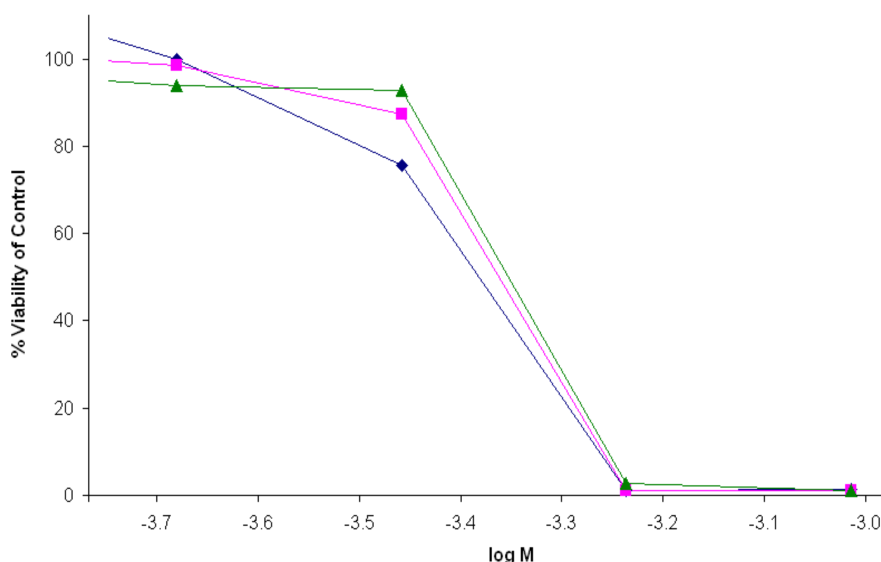


Figure 14. Cytotoxic activity of ferrocene against HT29 cells at time intervals of 72 (diamonds), 96 (squares) and 120hours (triangles). Error bars omitted for clarity.

Ferrocene and the carbomethoxy and carboethoxy functionalized complexes were tested in concentrations which ranged from 10-1200μM. Table 5 summarizes the results of the cytotoxicity experiments and Figure 15 depicts the cytotoxic curves from MTT assays showing the effect of the ferrocene complexes on the viability of HT-29 colon cancer cell line. The IC_{50} value represents the concentration of the ferrocene at which the cell viability is 50% of the control viability. It can be noted that

bis(carboethoxycyclopentadienyl)ferrocene showed activity comparable to ferrocene, with values of 3.7(1) and 3.6(3) $x10^{-4}M$, respectively. In contrast, (carboethoxycyclopentadienyl)-(carbomethoxycyclopentadienyl)-ferrocene and the bis(carbomethoxycyclopentadienyl)-ferrocene resulted less active than ferrocene, being less active the complex with both carbomethoxy functionalized cyclopentadienyls.

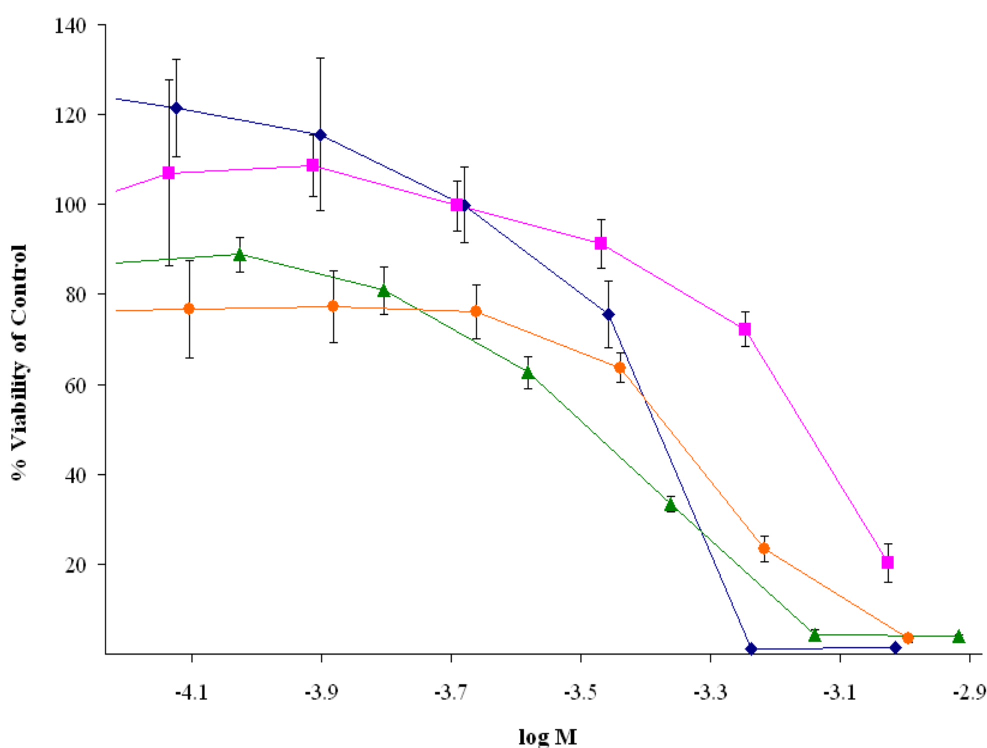


Figure 15. Cytotoxic activity of functionalized cyclopentadienyl ferrocenes against HT29 cells at 72hours drug exposure. Legend: Ferrocene - diamonds, Bis(carbomethoxy) - squares, Bis(carboethoxy) - triangles, (carbomethoxy)(carboethoxy) – circles.

The carbomethoxy functionalization has been shown previously to inactivate or lower the activity of titanocene complexes,[58] but apparently no numerical data was possible to account for this effect. The present results clearly show how the presence of the carbomethoxy substituent in the cyclopentadienyl ring lowers the activity of ferrocene in a step-wise manner, being the bis(carbomethoxy) functionalized complex the less active, followed by the (carbomethoxy)(carboethoxy)functionalized ferrocene. The bis(carboethoxy) functionalization does not improve the activity of the ferrocene complex neither it lowers it.

Table 6. Cytotoxicities of ferrocenes studied on HT-29 colon cancer cell line at 72 h, as determined by MTT assay and correlation with redox potential

Complex	IC_{50} (μM)	E_{pa} (mV) – oxidation potential at scan rates of 50 and 100 mV/s
Cp_2Fe	360(30)	779, 840
$(Cp\text{-}COOEt)_2Fe$	370(10)	1056, 1092
(Cp-COOMe)(Cp-COOEt)Fe	500(20)	1057, 1077
$(Cp\text{-}COOMe)_2Fe$	720(50)	1108, 1145
$(CpCOOCH_2CH_2OH)_2Fe$	370(20)	1073, 1115
$(CpCOOCH_2CH{=}CH_2)_2Fe$	180(10)	1014, 1027
$[Cp_2Fe]BF_4$	180(10)	NA

Other functionalized ferrocene complexes, varying the lipophilic character of the carboalkoxy groups, were also evaluated for cytotoxic activity against HT29 cells. A complex with a terminal alcohol group and a complex with a terminal allyl group were tested in concentrations that ranged from 13 to 1300 μM at a 72 hour time interval. The IC_{50} values for these complexes are also summarized in table 5 and figure 16 shows the cytotoxic curves for these complexes along with ferrocene for comparison. The complex with the terminal alcohol group shows an IC_{50} value of $3.7x10^{-4}$ M which is comparable to ferrocene, while the allyl complex shows higher cytotoxic activity than ferrocene at the time interval studied, with an IC_{50} value of $1.8x10^{-4}$ M.

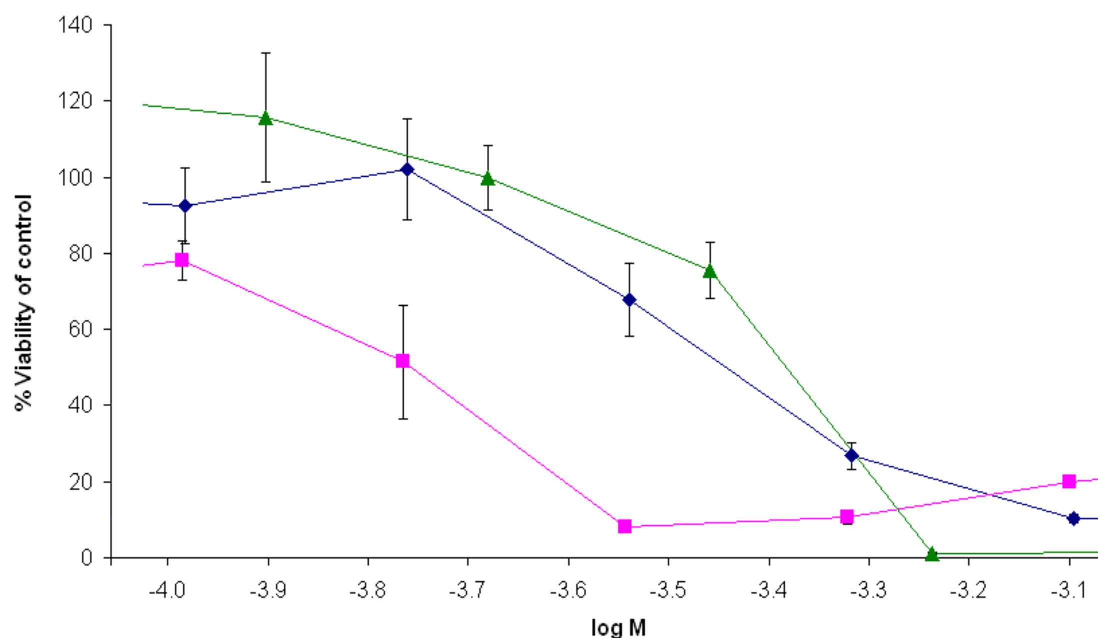


Figure 16. Cytotoxic activity of functionalized ferrocene complexes on HT29 colon adenocarcinoma cells at 72 hours. Complex with terminal alcohol (diamonds), complex with terminal allyl (squares) and ferrocene (triangles).

Finally, since redox chemistry is important for the activity of ferrocene complexes and ferrocenium ion has been implicated as an active species for this type of complexes, we decided to evaluate ferrocenium ion as well. Ferrocenium shows a higher activity compared to ferrocene, with IC_{50} values of $1.8x10^{-4}$ M and $3.6x10^{-4}$ M, respectively. Figure 17 depicts the curves from the MTT assays.

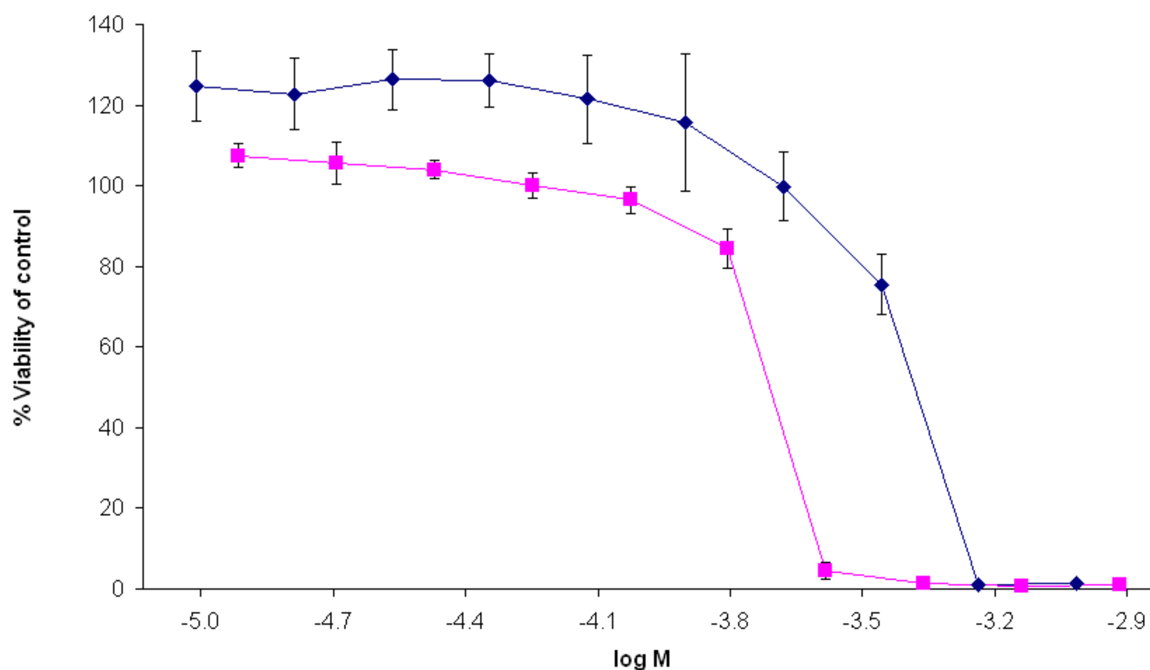


Figure 17. Cytotoxic activity of ferrocene (diamonds) and ferrocenium ion (squares) on HT29 colon adenocarcinoma cells at 72 hours.

The activity of ferrocene derivatives have been correlated with the redox potential of the Cp_2Fe/Cp_2Fe^+ couple. It is known that ferrocene can induce lymphocyte activation as part of its antitumor activity and it alters redox-sensitive

signaling such as activating p21^{ras} (GTPase). In fact, N-Acetylcysteine (NAC), a known free radical scavenger, modulates ferrocene-induced antitumor effect in B-16 melanoma-bearing mice.[122]

This redox behavior / activity pattern has been observed for ferrocene pyrazole conjugates in MCF-7 cells,[27] and for ortho- and meta- ferrocenyl benzoyl dipeptide esters.[67-68] It has been attributed to a better production of reactive oxygen species, which can damage DNA by oxidative stress and eventually lead to cell death.

The synthesized complexes were studied previously for their electrochemical properties in acetonitrile solutions with tetrabutylammonium hexafluorophosphate as supporting electrolyte in a BAS CV050W voltammetric analyzer of Bioanalytical Systems, Inc. As can be seen in table 5, there is no clear relationship between the redox potential of the complexes and the cytotoxic activity on HT29 cells. In fact, what has been observed is that complexes with a lower oxidation potential than ferrocene are more cytotoxic than the parent compound, but this behavior was not observed with this family of compounds. Ferrocene had the lowest oxidation potential but also the lowest cytotoxic activity.

The initial work performed by Köpf-Maier and coworkers demonstrated that ferrocenium possesses in vivo anticancer activity in breast cancer. Based on this precedent, we investigated our functionalized ferrocenes in MCF-7 breast cancer cell line (see table 6). Surprisingly, ferrocene showed to be less cytotoxic in breast cancer than in colon cancer. With regard to the functionalized ferrocenes, and similar to the results on HT-29 cell line, a pattern in the cytotoxicity as the functional pendant group

is changed is evident. First, in the functionalized ferrocenes with carboalkoxy groups, the incorporation of methyl ester groups on the Cp rings decrease the cytotoxic activity of the resulting complexes. Second, the increase in the lipophilic character on the carboalkoxy substituents such as in $Fe(C_5H_4CO_2CH_2CH{=}CH_2)_2$, where the ethyl group is substituted by an olefin, increases the cytotoxic activity. Such behavior has been reported previously.[123-124] Figure 18 depicts the curves from the MTT assays of selected complexes in MCF-7 cells.

Table 7. Cytotoxicities of ferrocenes studied on MCF-7 breast cancer cell line at 72 h, as determined by MTT assay (standard deviation in parenthesis).

Complex	IC_{50} (μM)
Cp_2Fe	1500(100)
$[Cp_2Fe]BF_4$	150(05)
$(Cp\text{-}COOEt)_2Fe$	250(20)
$(Cp\text{-}COOMe)_2Fe$	520(20)
(Cp-COOMe)(Cp-COOEt)Fe	320(30)
$(CpCOOCH_2CH_2OH)_2Fe$	340(30)
$(CpCOOCH_2CH{=}CH_2)_2Fe$	190(30)

A wide variety of functionalized ferrocenes have been synthesized by several research groups aimed to improve their anticancer activity as well as to understand the structure-activity relationship. Very active functionalized ferrocenes have been

synthesized containing estrogen receptor modulators as pendant groups.[63,125-126] Among them, 1,1'-bis-(4'hydroxyphenyl)-2-ferrocenyl-but-1-ene showed strong antiproliferative activity in both hormone-dependent (MCF-7) and hormone-independent (MDA-MDA231) breast cancer cells with IC_{50} = 0.7 and 0.6μM respectively. This complex demonstrated high activity due to estrogenic properties of the pendant group which serves as vector to carry ferrocene into breast cancer cells.[126] On the other hand, ferrocenyl carbohydrate conjugates have shown IC_{50} values between 87-468μM on HTB-129 human breast cancer cell line.[127] Our functionalized ferrocenes showed IC_{50} values similar to those of the ferrocenyl carbohydrates[127] and ferrocenium derivatives tested on MCF-7 cell line: ferrocenium carboxylic acid tetrafluoroborate (IC_{50} = 340μM), decamethylferrocene tetrafluoroborate (IC_{50} = 37μM), 1,1'-dimethylferrocenium tetrafluoroborate (IC_{50} = 320μM) and ferroceniumboronic acid tetrafluoroborate (IC_{50} = 317μM).[123] It should clearly be noticed that, as reported previously, the increase in the lipophilic character on the ferrocene or ferrocenium complex increases the cytotoxic activity as a result of increased cell membrane permeability.[123-124]

As a comparison only, the effect of membrane permeability and water solubility has been studied for peptide conjugates of ferrocene carboxylic acid in HL60 human leukemia cells.[70] The water solubility of both ferrocene carboxylic acid and ferrocenylacrylic acid is improved by conjugation but only ferrocene carboxylic acid shows a significant improvement by conjugation with oligoarginine. The IC_{50}

values of ferrocene carboxylic acid and its hexa and octaarginine conjugates are 694µM, 65µM and 85µM, respectively. In contrast, when ferrocenylacrylic acid is conjugated, its activity is decreased. The IC_{50} values for ferrocenylacrylic acid and its hexa and octaarginine conjugates are 9.4µM, 106µM and 39µM. Anyhow, comparing the free unconjugated complexes, it is clear that the acrylic moiety enhances significantly the activity of ferrocene compared to the carboxylic moiety.

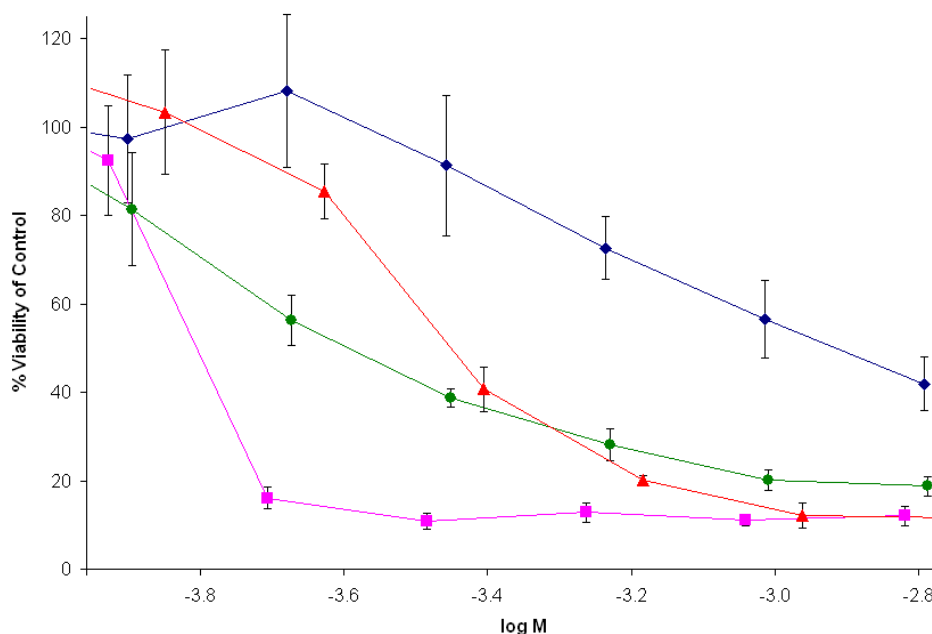


Figure 18. Ferrocene(diamonds), ferrocenium(squares), complex with terminal alcohol (triangles) and complex with terminal allyl (circles) activity on MCF7 cells as determined by MTT colorimetric assay.

For colon cancer, the cytotoxic data is more limited for a more meaningful comparison and assessment. On a recent review on the medicinal properties of ferrocenes, it was described a series of functionalized ferrocene conjugates anchored

to polyaspartimides (water soluble carrier polymers) and evaluated on Colo (colon) and HeLa (ovarian) cancer cell lines.[128] The IC_{50} values determined are in the 10^{-4} – 10^{-5} M range, analogous to some of our most active species. However, since the data came from different cancer cell lines, a direct comparison of the IC_{50} values would becomes less accurate.

Finally, what has become clear in this study and is in good agreement with previous report is that minor changes in the pendant group on the Cp ring may have notable impact in the cytotoxic activity of the resulting ferrocenes. It has been shown that increasing the lipophilic character of the functionalized ferrocenes apparently improves their biological activity, as a result of better transport of these species into the target place inside the cell.[123,124,129] This increased lipophilic character on the ferrocenes increases the cell uptake by improving cell membrane permeability and more ferrocene molecules become available inside the cell in their original form. It has been proposed previosly[122] that not only ferrocenium cation but also ferrocene is able to express oxidative stress. Ferrocene is able to generate hydrogen peroxide by autooxidation, and the immune stimulatory properties of ferrocene are postulated to be mediated by redox-sensitive signaling pathways, as described earlier. Since the usual low redox potential – high activity relationship was not found in our family of complexes, it is tantalizing to conclude that the higher activity found in the more lipophilic complexes comes from the potential increased cell uptake.

B. Interactions of new titanium-maltolato complex with DNA

Similar to platinum and other metals, the cells DNA is the proposed main target of titanium-based chemotherapeutic drugs. There is evidence about the ability of titanocene dichloride to coordinate nucleotides through nitrogen bases[32-33] and phosphate groups.[31-32] In fact, it has been shown that titanocene dichloride is able to disrupt the hydrogen bonding of DNA base pairs.[31] The new titanium-maltolato complex possess a high hydrolytic stability and water solubility at and above physiological pH, and it does not transfers the Ti(IV) center to the protein transferrin. On the other hand, this complex showed the highest cytotoxic activity among all titanium complexes studied, both in HT29 and MCF-7 cells. We decided to start a series of basic experiments in an effort of elucidating if DNA is indeed implicated in the mechanism of action of the new titanium-maltolato complex. These experiments are aimed as to provide preliminary data on the new drug's ability to alter the DNA structure via covalent or intercalative interactions

1. Agarose Gel electrophoresis

Agarose gel electrophoresis is usually used to assess changes in mobility of the plasmid pBR322 when exposed to metal compounds such as platinum(II)[130-131] and ruthenium[132] complexes. It is also used to study photocatalytic cleavage caused, for example, by ruthenium complexes,[133] and the "artificial" nuclease activity of Cu(II) complexes.[134] This is usually performed since this plasmid has two main forms: a supercoiled covalently closed form and a relaxed open circular form that is much more

compacted than the former one. Any drug which causes a single- or double-strand break in DNA, or interact with DNA via covalent modification or intercalation will have a direct effect on the electrophoretic mobility of these forms of DNA. Since our titanium-maltolato tetramer is a robust, hydrolytically stable, molecule with an apparently inert character, we decided to use the DNA standards. These are a set of relatively small molecules of DNA which will show varying degrees of tertiary structure, depending on their length. Although it was not aimed with this experiment to show the exact nature of the interaction, this varying degree of tertiary structure could allow assumptions on the type of lesion produced by the drug.

When ethidium bromide is used to stain DNA molecules in an agarose gel electrophoresis experiment, it is because of the ability of the intercalation complex to remain intact and run as a single molecule through the gel. With this in mind, we decided to test the ability of the titanium-maltolato complex to react with DNA strong enough as to run as a single molecule in an agarose gel. Standard DNA fragments of known size (DNA ladder standards) were used to interact with the titanium-maltolato complex at 37°C for periods of two, five and 24 hours. Samples were run side-by-side on a gel along with DNA ladder samples incubated with a blank which had no complex present but the dilution factor was accounted for. Figure 19 shows the gel photograph of a 100bp ladder incubated with the titanium-maltolato complex at different interaction times. Under this condition, the titanium-maltolato complex was in a 1:2 ratio with the DNA base pairs. Under these conditions, there are no marked

differences between treated and control samples and a densitometry analysis was not carried out on the resulting gel.

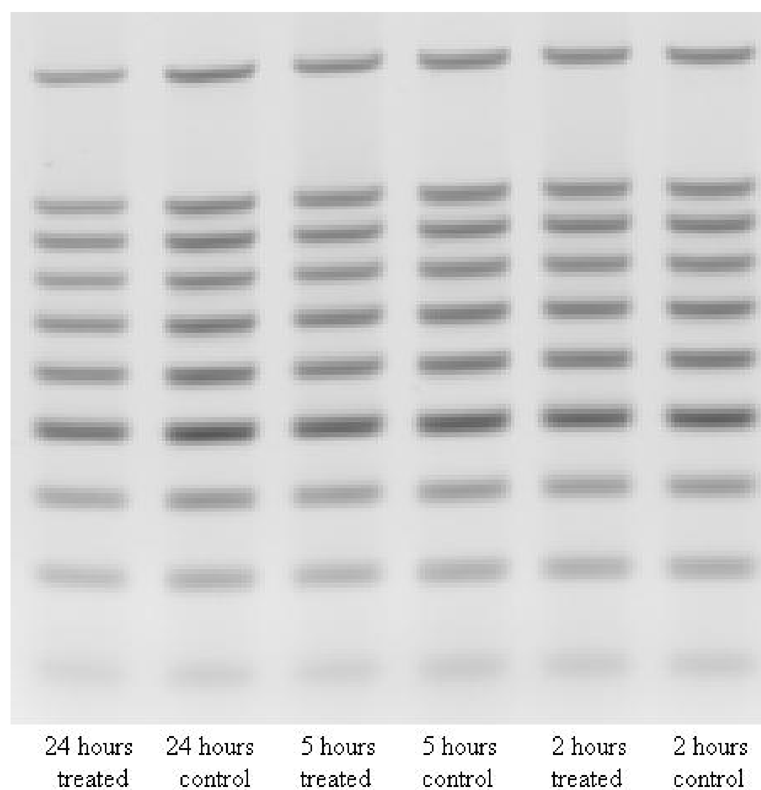


Figure 19. Gel electrophoresis photograph of 100bp DNA ladder incubated at 37°C with titanium-maltolato complex for two, five and 24 hours.

When the experiment was performed with the 50bp DNA ladder standard, there was a marked difference in the fastest migrating bands (See Figure 20). The lowest band which accounts for the faster migrating fragments clearly shows a marked difference when compared to controls. Also, the highest band which corresponds to the slowest moving high molecular size fragments shows an increasing smear-like behavior which is not present in control samples. A densitometry analysis was

performed on the three faster migrating bands on the gel. Table 7 shows a diminishing of the bands intensities of up to 45% when compared to control bands. Although theoretically the analysis can be done with all bands, this was not performed since as we move up through the gel, band separation gets smaller and the densitometry analysis becomes more difficult and less accurate.

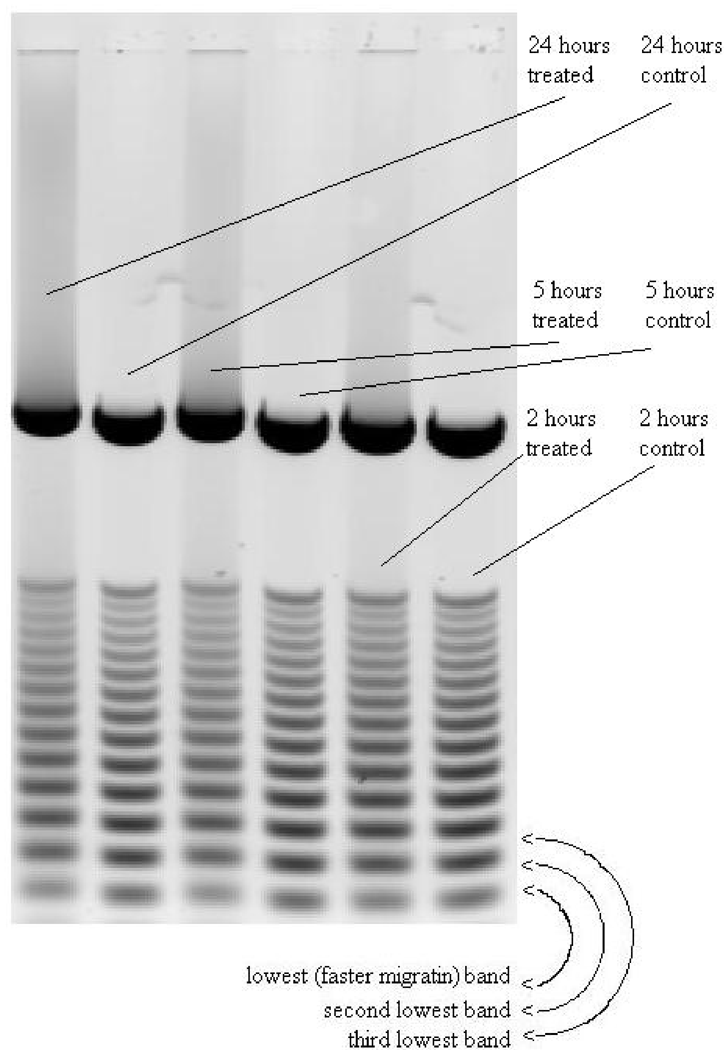


Figure 20. Gel electrophoresis photograph of 50bp DNA ladder incubated at 37°C with titanium-maltolato complex for two, five and 24 hours. Samples from right to left correspond to two hours control, two hours treated, five hours control, and so on.

Table 8. Densitometry analysis for 50bp DNA ladder fragments incubated with the titanium-maltolato complex.

Band row	Band column	Percent area occupied by band	Percent of control (only for treated samples)	Percent of 2h control (only for 5h and 24h controls)
Upper	farthest right	18.93		
		16.83	89	
		18.94		100
		13.2	70	
		18.3		97
	farthest left	13.8	75	
Middle	farthest right	19.61		
		16.05	82	
		19.49		99
		12.63	65	
		19.12		98
	farthest left	13.1	69	
Lower	farthest right	20.06		
		15.37	77	
		20.81		104
		11.2	54	
		20.42		102
	farthest left	12.15	60	

It is worth mentioning that no further decreased of the bands was observed after five hours of incubation with an actual increase for the 24hours bands. This is not attributable to loss of integrity of fragments due the long incubation period. The last column shows less than five percent is due to any consideration regarding control samples. No further study was performed as to attribute this to reagent-limiting,

kinetic-related or equilibrium-related considerations since addressing this issue was out of the scope of the present study.

This type of study is typically performed with the plasmid pBR322 because it is present in a covalently closed form and an open form and any interaction of a metal drug with the plasmid should be observable in the differences in migration of these two forms. For example, platinum complexes usually cause a relaxation of the covalently closed form and some folding of the open circular form, and are related to cross-links produced by the drugs, which is confirmed by other techniques such as circular dichroism and atomic force spectroscopy.[130-131] This technique is also used for ruthenium complexes.[132-133] Other type of compounds, such as copper complexes are known to be oxidative cleaving agents of DNA and pBR322 gel electrophoresis is used to assess their nuclease-like activity.[134] This plasmid could be used in the future as to evaluate the type of disruption created by titanocene dichloride or the new titanium-maltolato complex in DNA molecules.

Further studies should be performed as to define the exact nature of the possible interactions of this drug with biomolecules. Special consideration should be given not only to DNA but also to RNA molecules as possible targets. Anyhow, it is clear from the present study that the new titanium-maltolato complex is able to interact strongly with small fragments of double stranded DNA.

2. Fluorescence spectroscopy

It is not unusual to find reports in which intercalative potential of metal complexes is assessed with UV-Vis spectroscopy. For instance, ruthenium complexes with modified phenantroline ligands as intercalators have been shown to interact with DNA using UV-Vis spectroscopy, specifically monitoring MLCT bands.[133] But the main technique used to monitor intercalation is ethidium bromide displacement via Fluorescence spectroscopy. This technique is used widely when a metal complex or other molecule contains groups which could intercalate DNA bases. This has been the case for copper complexes of Neotrien, which is a crown-ether-like polyamine derived from phenantroline,[135] lanthanum complexes of Naringenin-derived ligands, which is a flavonoid,[136] ruthenium complexes[132,137] and even metal complexes of the antibiotic ciprofloxacin.[138] Since the new titanium-maltolato tetramer has stacked aromatic rings with a plane-to-plane separation of about 3.5Å, we decided to evaluate the complex's ability to interact with DNA via intercalation.

Metal complexes of phenantroline or other big planar molecules known to intercalate DNA are assessed using complex to EB ratios which are small (0.01:1) and then are increased gradually, usually up to a 1:1 ratio. Since the planar moiety of our titanium-maltolato complex is relatively small and tightly bound to the metallic center, we decided to start with a 1:1 complex to EB ratio and monitor the fluorescence for a period of one hour (Figure 21). Other ratios were used in which the complex was in a higher proportion and also monitored for one hour.

No quenching of ethidium bromide fluorescence was observed, even at a 45:1 complex-to-EB ratio, but considering the nature of EB intercalation of DNA bases and the small size of the rings of the titanium-maltolato tetramer, it is not surprising that the complex is not able to displace the ethidium cation under these conditions. For comparison of our results with other compounds, metal complexes of classical intercalators such as ruthenium complexes of bypiridine or phenantroline present in a 1:1 ratio with EB show a decrease in the fluorescence of EB of up to 40% its original intensity.[132] Macrocyclic cobalt, copper and zinc complexes derived from benzimidazole, which is able to intercalate DNA, promote a decrease in the fluorescence of ethidium bromide of up to 75% its original value.[134]

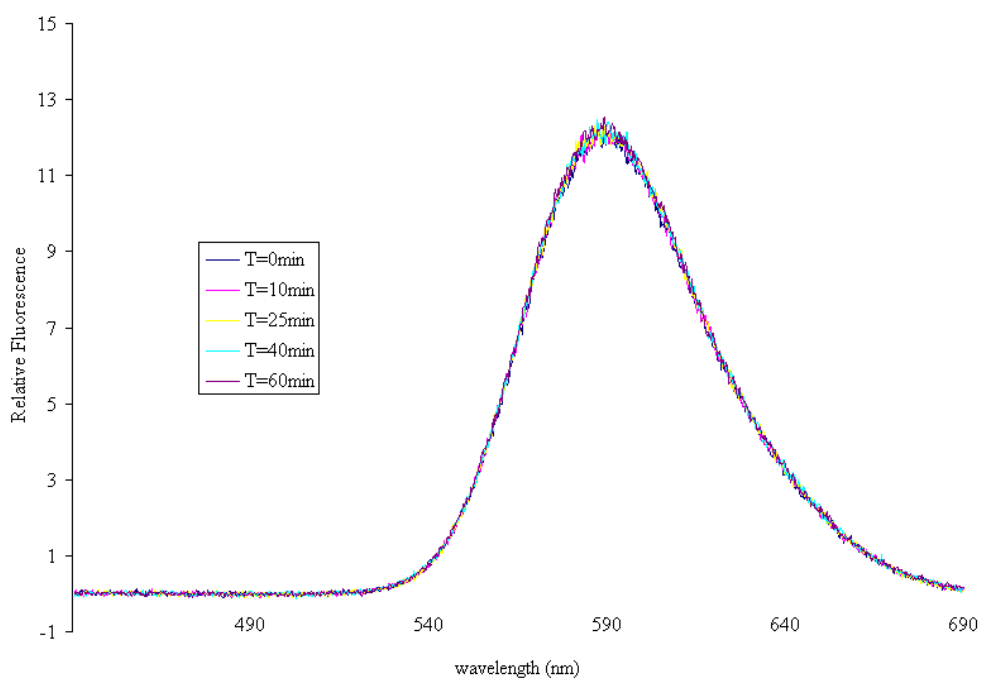


Figure 21. Fluorescence Overlay Spectra (λ_{ex} = 350nm) for ethidium bromide displacement using the titanium-maltolato complex (1:1 ratio) monitored for one hour.

Intercalation has been proposed for the observed antitumor effects of budotitane since planar stacking of adjacent phenyl rings was observed in the crystalline form of its dichloro derivative.[A16] Also, titanium complexes of 4-acyl-5-pyrazolonates, have shown this planar stacking of aromatic rings.[A60] Although is not clear if a partial intercalation of these rings into the DNA double helix is in effect responsible for the observed antitumor activity of these types of complexes, it can not be discarded based on assays such as ethidium bromide displacement. There are other techniques which provide information on partial intercalation of complexes to DNA. Thermal analysis of DNA (melting curves) is used to distinguish between non-intercalative and intercalative binding modes of ruthenium complexes.[132] Other techniques such as UV-Vis spectroscopy and viscosity measurements of DNA interactions with metal complexes are used to assess the degree of the intercalation of complexes of ruthenium[133] and other metals.[134] The accountability of the actual *in vitro* and *in vivo* species responsible for the observed biological activity of the new titanium-maltolato complex needs to be discovered with other types of studies.

3. ^{1}H-NMR Spectroscopy

Since the ^{1}H-NMR spectrum reported for the titanium-maltolato complex shows presence of both monomeric and tetrameric species in solution at physiological pH, it was envisioned that NMR spectroscopy is an excellent tool as to assess if one or other species is more favored to interact with DNA. Based on this, a series of basic experiments were designed, which were described in Chapter 3 of this thesis.

Briefly, since the ability of the titanium-maltolato complex to interact with DNA was going to be measured at 37.0°C, a stability study was initially performed to account for any change in the monomer/tetramer ratio due to long incubation at this temperature. No changes in the monomer/tetramer ratio (1:3) were observed upon incubation at 37.0°C for up to 14 hours.

Incubation with DNA at 25.0°C and 37.0°C showed no changes in the signals of the tetramer or monomer for up to 14 hours of incubation. This was surprising since gel electrophoresis showed interaction with DNA fragments as early as two hours. But since DNA used for gel electrophoresis was fragments of small size, tertiary structure beyond helicity could protect DNA double helix from interaction with this complex. This could also explain why in the gel electrophoresis study shorter fragments showed the most interaction and this was diminished as fragments increased in size.

The only change observed in this experiment was when a DNA-complex interaction was incubated for one hour at 37.0°C and allowed to cool to 25.0°C and the spectra recorded. Interestingly, the signals corresponding to the tetrameric species disappeared while signals corresponding to the monomer remained unchanged (See Figure 22). No further change in the signals of the monomer was observed up to 24 hours of incubation with DNA.

When ^{1}H-NMR is used to assess if partial intercalation of DNA base pairs can occur for a molecule, signals corresponding to aromatic protons are evaluated and usually found to shift slightly up-field due shielding caused by intercalation in the

electron-rich π-π stacking. This has been the case for the partial intercalation of aromatic residues of oligopeptides able to interact with nucleic acids.[139] Other studies such as the intercalation of norfloxacin, which is a quinolone, and caffeine with short

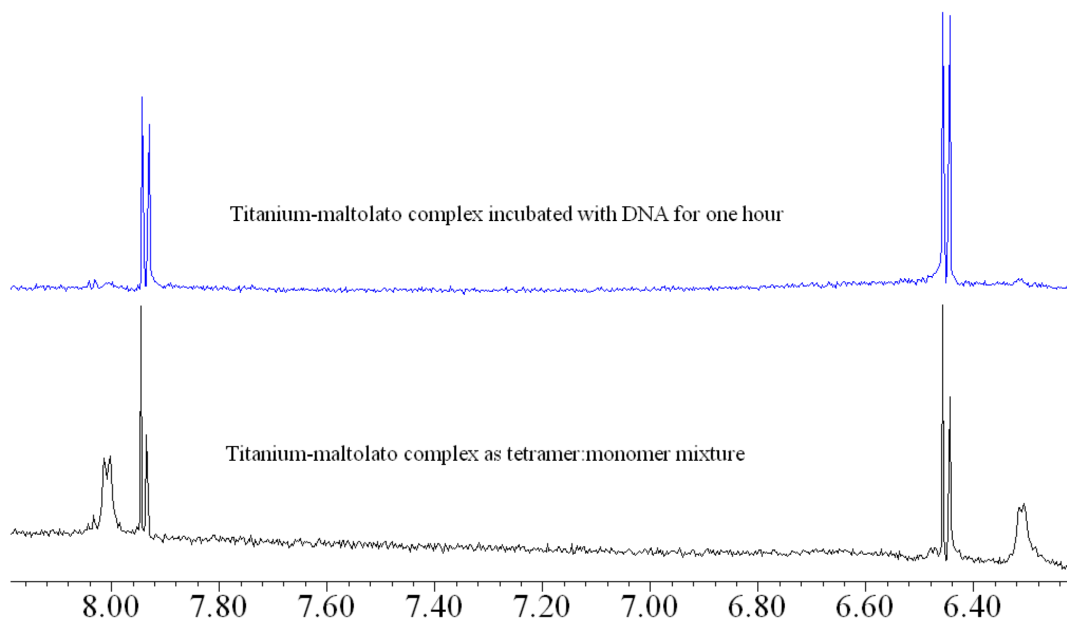


Figure 22. Spectra of titanium-maltolato complex in a 1:3 monomer/tetramer ratio (lower spectrum) and after one hour of incubation with DNA (upper).

self-complementary oligonucleotides,[140] the interaction of quercetin, which is a flavonoid, and G-quadruplex DNA,[141] and the complexation of novantrone, which is an anthracenedione, with the hairpin structure of a deoxyheptanucleotide[142] use not only the NMR resonances of the compound of interest but also the resonances of the

DNA molecules involved. Anyhow, complete disappearance of signals from the DNA molecules or the complex like the one observed in our study is not usual.

Our result could be explained if the interaction of the titanium-maltolato complex with long molecules of DNA is labile enough to be faster than the NMR time scale at 37.0°C, but DNA is able to "trap" the tetramer upon cooling with different DNA regions wrapping around the tetramer rings. The structure of DNA molecules is known to be dependent on temperature and heating could allow some unwinding, favoring a partial intercalation of the tetramer rings. This technique should be fully exploited in the future along with molecular modeling studies as to assess the ability of both the tetrameric and monomeric species to interact with DNA molecules.

Taken together all data allows us to conclude that this new titanium-maltolato complex is able to interact with DNA. If the tetrameric, the monomeric, or both species are responsible for the *in vitro* killing effect remains to be elucidated with other types of studies, if possible at all, since the tetrameric form of this complex is known to dissociate to some extent when dissolved depending on the pH of the resulting solution and controlling the ratio of this species is difficult.[61]

C. DNA Microarray Analysis

The DNA microarray analysis requires high-quality RNA samples as a starting material. Asynchronously growing HT29 cells were exposed to titanocene dichloride and the new titanium-maltol complex in concentrations related to the previously determined IC_{50} for both drugs. After the appropriate time of incubation with the

titanium drug, RNA, DNA and protein were extracted from the cells. The extraction procedure was also performed to untreated cells of the same passage number and under the same culture conditions.

Although DNA and protein preparations were obtained from the cells, the main target of the extraction was mRNA, which was analyzed for quantity, purity, integrity and stability prior to the microarray procedures. An Eppendorf BioPhotometer 6131 was used to read the samples absorbance to get an estimate of quantity and to calculate the A_{260}/A_{280} ratio as a parameter of the purity of the nucleic acid preparation. Table 8 presents these results. As can be obtained from the table, the RNA samples were concentrated enough as to save at least 3.8μg of RNA for this analysis.

Table 9. UV Spectrophotometric data of RNA samples as an assessment of quantity and purity.

Sample#	Treatment with…	Sample volume (μL)	[RNA] (ng/μL)	A_{260}/A_{280}
1	untreated	15	258	1.81
2	Titanocene dichloride	15	268	1.80
3	Titanium-maltolato complex	15	400	1.84

An agarose gel electrophoresis of the RNA samples was used to assess integrity and stability and to verify the samples were RNase-free. RNA samples which were incubated for 1hour at 37°C were run side-by-side along with samples not

incubated (see Figure 23) for this purpose and to show absence of nucleic acids other than RNA in the preparation.

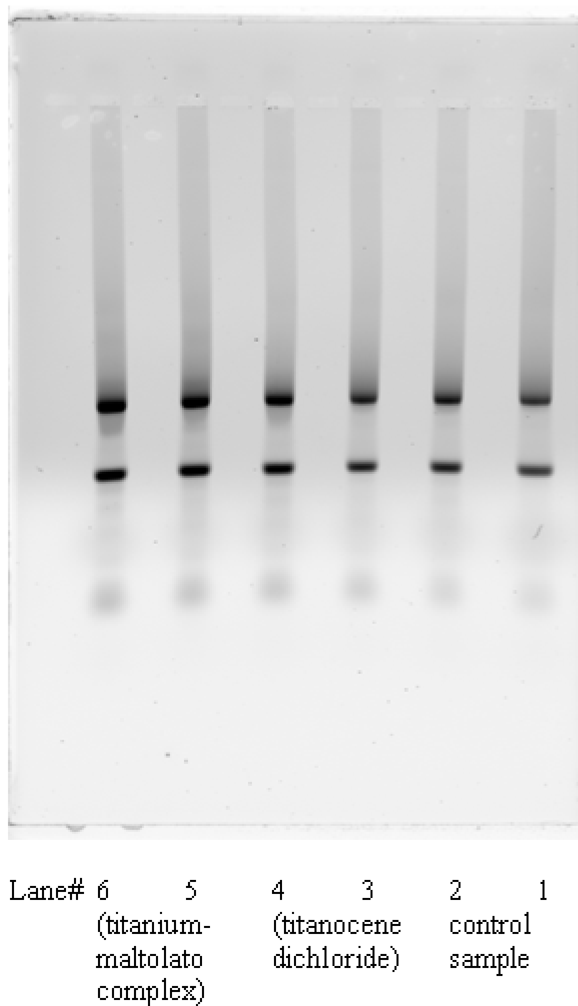


Figure 23. Agarose gel electrophoresis photograph of not-incubated RNA samples (lanes 1, 3 and 5) along with RNA samples incubated for one hour at 37°C (lanes 2, 4 and 6) for an assessment of integrity and stability.

It is known that titanocene dichloride have a longer intracellular activation period compared to other chemotherapeutic drugs, and this is the basis for cytotoxic

assays to be performed at a 96 hours interval instead of a 72 hours time interval. Since most cellular changes caused by a drug can be detected at some point between 30 minutes and six hours, we decided to perform the RNA extraction at a six-hour interval. Most cellular changes after this time correspond to secondary consequences of the early changes. For example, if the drug causes apoptosis, the changes observed in the microarray will be caused by the process of apoptosis. If the drug causes cell cycle arrest, the microarray will show the changes associated to this process. If the drug causes a developmental change, the vast majority of the gene expression differences will be a consequence of the differentiation of the cells and not due to drug treatment. The few changes that could be due the chronic exposure to the drug will be buried in a pool of changes due to the cellular response to the stress.

RNA samples were then subjected to the amplification procedure, followed by preparation of the fluorescently labeled cDNA probes and their hybridization. Lastly, the microarray is scanned and the images submitted to data analysis. It should be noted that the system used was the GeneChip® Human Genome U133 Plus 2.0 Array, which provides the comprehensive coverage of the transcribed human genome on a single array. It analyzes the expression level of over 47,000 transcripts and variants, including 38,500 well-characterized human genes. This array is comprised of more than 54,000 probe sets. Eleven pairs of oligonucleotide probes are used to measure the level of transcription of each sequence represented on this array. The array also has sequences corresponding to hybridization controls, poly-A controls, housekeeping/control genes and 100 probe sets of a normalization control set. This set

of genes serves as a tool to normalize and scale the data prior to performing data comparisons. This set of normalization genes is selected because it shows consistent levels of expression over a diverse set of tissues. The profile of this array can be found in the datasheet provided by the company if more information is desired.

A comparison analysis was performed on the arrays setting the expression profile of the control RNA sample as a baseline, and the expression profile of the treated samples as experiments. This was kindly preformed by Dr. Steven Enkemann, Senior Core Staff Scientist at the Microarray Core Facility of Moffitt Cancer Center in Tampa, Florida, to whom we are very grateful for his help.

Three different analyses of the data were performed. One analysis revealed the genes that seem to be changed specifically by titanocene dichloride (results on section 4.C.1). The second analysis shows the genes that appear to be changed specifically by the new titanium-maltol complex (section 4.C.2). Since it is difficult to set criteria that will select a change in one instance and a lack of change in another instance, these two analyses are inherently questionable because of the algorithm used to generate them. But since the results obtained are, to our knowledge, unique and unprecedented, we will still analyze them, although as preliminary and inconclusive data, due to limitations in sample size and statistical power.

The last analysis performed shows the genes that seem to be changed if either drug was used on the cells. The list of gene expression changes caused by both drugs is somewhat more reliable than the lists obtained for the individual drugs. Since we looked at the effect very early, where large changes were not expected, the quantity

and degree of changes (section 4.C.3) suggests that there is a significant effect to the cells at this early stage. Finally, it is important to point out that the fold-change value that is generated when a comparison analysis is performed is a rough estimate of the true difference in gene expression between the two experimental conditions analyzed. The fold-change is the difference in gene expression between the experimental conditions. An actual fold change should be defined from a series of experiments, not just one. Anyhow, these single measurements can be considered guides of the differences observed in the gene expression under two or more conditions. Since a two-fold change in real like can look like a 1.5 to 1.7-fold change in a microarray experiment, we used as a meaningful starting point genes which were up- or down-regulated by more than a 1.5-fold change, starting with those genes that were the most up- or down-regulated in the presence of the drugs.

1. Changes due to the presence of titanocene dichloride

There were a total of 503 probesets that were selected mathematically as down-regulated (fold change higher than -1.5) and 151 probesets selected as up-regulated (fold change higher than +1.5). We focused initially on 11 probesets which presented a fold change higher than -3, and 6 probesets which presented a fold change higher than +3 when titanocene dichloride was present. Table 9 shows, when available, the Gene Symbol, GenBank ID, Entrez Gene ID, UniGene ID, and a brief gene description along with the observed fold change for 8 of these 11 down-regulated probesets and 4 of the 6 up-regulated probesets. The identification of the probesets

excluded of this discussion is weak and they code for hypothetical proteins. When no reference is cited specifically, the information of these probesets and the corresponding gene and gene products was obtained from one or more of these mentioned sources.

Table 10. Probesets which showed a fold-change higher than -3 or +3 when comparing treatment with titanocene dichloride with no treatment (HT29 cells)

GenBankID Entrez Gene ID Gene Symbol UniGene ID	Gene Description	Observed Fold Change	Additional Comments
BC019341.1 10802 SEC24A Hs.211612	SEC24 related gene family, member A (*S. cerevisiae*)	-10.2	a
NM_016331.1 51193 ZNF639 Hs.22879	zinc finger protein 639	-6.6	a
AF279778.1 9910 RABGAP1L Hs.495391	This probeset may detect an alternate exon, an alternate termination site, or overlapping transcript of RAB GTPase activating protein 1-like	-5.9	a, b, c
NM_001001716.1 4793 NFKBIB Hs.9731	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	-3.8	a
NM_001722.2 661 POLR3D Hs.148342	polymerase (RNA) III (DNA directed) polypeptide D, 44kDa	-3.7	N/A
BC037787.1 162655 ZNF519 Hs.352635	zinc finger protein 519	-3.6	a

Table 10 (*continued*). Probesets which showed a fold-change higher than -3 or +3 when comparing treatment with titanocene dichloride with no treatment (HT29 cells)

GenBankID Entrez Gene ID Gene Symbol UniGene ID	Gene Description	Observed Fold Change	Additional Comments
NM_014665.1 9684 LRRC14 Hs.459391	leucine rich repeat containing 14	-3.1	a
NM_030911.1 81602 CDADC1 Hs.388220	cytidine and dCMP deaminase domain containing 1	-3.1	a
NM_001024594.1 388722 C1orf53 Hs.61329	chromosome 1 open reading frame 53	3.3	a
NM_006890.1 1087 CEACAM7 Hs.74466	carcinoembryonic antigen-related cell adhesion molecule 7	3.5	a
NM_022113 63971 KIF13A Hs.189915	kinesin family member 13A	3.6	a
NM_005484 10038 PARP2 Hs.409412	poly (ADP-ribose) polymerase family, member 2	3.6	N/A

a. More than one probeset detects this transcript
b. This probeset may detect multiple splice varients
c. Caution, check this probeset carefully

SEC24A is a protein-coding gene located in chromosome five. In yeast, SEC24 is part of a complex which coats vesicles that mediate protein transport from the

endoplasmic reticulum (ER) to the Golgi apparatus, and has been associated to membrane organization and biogenesis. SEC24A is one of several mammalian proteins that show structural and functional homology to yeast SEC24. It is known that protein transport between ER and Golgi complex is blocked in mitotic cells although the mechanism is unknown,[143] but saying that titanocene dichloride is a mitogen does not necessarily correlate with its *in vitro* killing effect. Other genes which could be related to this type of protein were also found to be down-regulated. SEC22L3 and SEC22L2 are vesicle trafficking proteins with a -2.0 and -1.6 fold-change. GOSR2, the golgi SNAP receptor complex member 2, which is a trafficking membrane protein, had a -1.9 fold-change.

ZNF639 is a protein-coding gene located in chromosome three. As a zinc-finger protein, it is involved in transcriptional control. Kruppel-like zinc finger proteins have been identified as being involved in neoplastic transformation,[144] although their functional roles in carcinogenesis remains unknown. Stating that titanocene dichloride anticancer effect is correlated with deregulation of this type of transcription factor is speculative.

RABGAP1L is a gene which codes for the RAB GTPase activating protein 1-like protein. GTPase Rab constitutes the largest family of potential membrane trafficking proteins that are conserved in all eukaryotic cells.[145] The human genome encodes almost 70 of these proteins.[146] Down-regulation of this probeset could be related to the down-regulation of SEC24A since G-proteins are required for membrane recruitment in budding pathways.[147]

NFKBIB is a protein-coding gene located in chromosome 19. It codes for the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor. This factor inactivates nuclear factor kappa B by trapping it in the cytoplasm. The phosphorylation of this factor by kinases marks them for destruction via the ubiquitination pathway, thereby allowing NF-kappa-B activation.[148] When activated, it can enter the nucleus and up-regulate gene expression. Titanocene dichloride presence is correlated with down-regulation of this factor, thereby permitting up-regulation of genes via the NF-kappa-B pathway. Other genes related to NFKBIB were deregulated in the presence of titanocene dichloride. NFRKB, a gene related to the kappa B binding protein which was down-regulated with a fold-change of -1.9, is involved in T-cell proliferation via overexpression of the interleukin 2 membrane receptor. We looked up this receptor in the data and did not find it to be deregulated, but there is no doubt that transcriptional control mechanisms were altered in the presence of the drug. The NF-kappa-B pathway is one of the main pathways responsible for control of cellular events (See Figure 24). It is involved in cellular responses to stimuli such as stress, cytokines and free radicals.[2] The presence of titanocene dichloride is correlated with deregulation of members of this signaling pathway, which plays an important role in apoptosis events and in response to inflammation. It is interesting to note that titanocene dichloride and other derivatives have been reported in the past to enhance Natural Killer cells function in Ehrlich ascites tumour-bearing mice.[178-179] Also, these compounds are able to modulate the cytokine imbalance characteristic of tumor-induced immunosuppression.[180] Within the same pathway, some caspases, a family of

cysteine proteases involved in apoptosis, participate in the nuclear factor kappa pathway, and one of the final targets of caspases is poly(ADP-ribose) polymerase, which was the highest up-regulated protein in the presence of titanocene dichloride as will be shown below. This signaling pathway must be studied further as to establish the mechanism of action of this drug in HT29 cells.

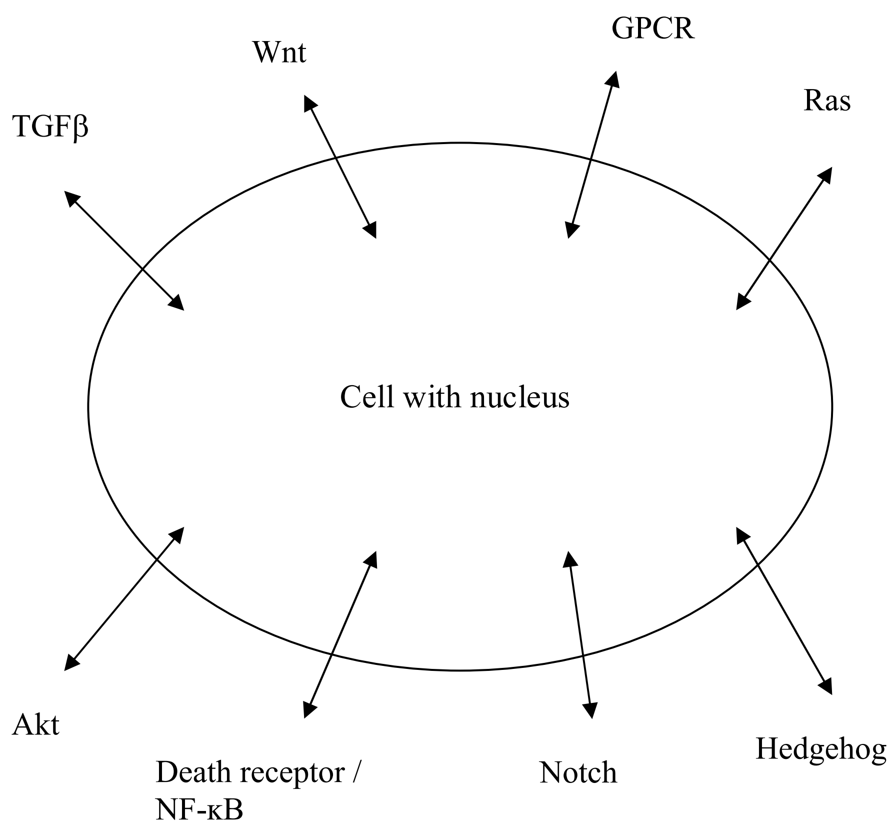


Figure 24. Main pathways in human cancer.[2]

POLR3D is a gene located in chromosome eight which codes for the DNA directed RNA polymerase III polypeptide D. The RNA polymerase III synthesizes

RNA components of the protein synthesis, pre-mRNA splicing, and tRNA processing apparatuses.[149] Yeast RNA polymerase III has been well defined and all of its subunits have been characterized, but much less is known about the human counterpart. It is interesting that up to this point of the analysis, three genes involved in protein biogenesis (SEC24A, RABGAP1L and POLR3D) are down-regulated in the presence of titanocene dichloride. Although it is generally unknown how specifically the cell cycle machinery converts an antiproliferative signal in a cellular response, we could speculate that titanocene dichloride is able to induce cell cycle growth arrest, in which the cell could try to repair DNA damage caused by the drug. Specifically, the machinery of transcription was deregulated as can be seen in the regulation of RNA polymerases. The polypeptide E of this complex was also down regulated with a -1.7 fold change. The transcription termination factor of RNA pol II was down-regulated with a -2.2 fold change, the small nuclear RNA activating complex required for polymerase II and III was also down-regulated with a -1.8 fold change, and the TATA box binding protein – associated factors of RNA pol I and pol II were down-regulated with a -2.6 and -1.5 fold change. Growth arrest and DNA-damage-inducible genes alpha and beta were up-regulated by factors of 1.6 and 1.9, respectively. These are part of a group of genes that respond to growth arrest conditions and treatment with DNA-damaging agents. The function of these genes or their protein products is involved in the regulation of growth and apoptosis. These genes mediate activation of the p38/JNK pathways which are important in apoptosis mediated by TGFβ and Ras signaling (see Figure 24). Although we could not find evidence of the activation of the

downstream members of this pathway, this is probably due to the limited time window which we are able to observe via the microarray.

ZNF519 is a gene located in chromosome 18 which codes for a polypeptide called zinc finger protein 519. It contains the Kruppel-associated box. It is predicted to be involved in critical functions such as cell proliferation and differentiation, apoptosis and neoplastic transformation. The prediction of its function as a zinc-finger protein is made on the sole basis of its sequence as coded by the DNA sequence. Anyhow, besides the above mentioned ZNF639 and ZNF519, other zinc fingers were found to be down-regulated, indicating that many transcriptional control mechanisms were altered in the presence of titanocene dichloride. ZNF311, ZNF42, ZNF655, ZNF198, ZFYVE27, KLF9, ZNF265, ZNF294, ZNF148, ZNF700, ZDHHC17, ZNF765, ZNF313, ZBTB41, ZNF746, ZNF289, ZNF638 and ZNF720 had fold changes which ranged from -2.2 to -1.5, and only one zinc finger protein, ZNF36, was up-regulated.

LRRC14 is a gene located in chromosome eight which codes for the leucine-rich repeat containing 14. LRR are sequence motifs consisting of a beta strand and an alpha helix present in many proteins that participate in protein-protein interactions and have different functions and cellular locations. Leucine-rich repeat motifs have been identified in a large number of functionally unrelated proteins, making it difficult to correlate their expression with the presence of our drug.

CDADC1 is a gene located in chromosome 13, which codes for the Homo sapiens cytidine and dCMP deaminase domain containing 1 mRNA. Deoxyctidylate deaminase catalyzes the deamination of dCMP to dUMP, providing the nucleotide

substrate for thymidylate synthase. This enzyme binds Zn^{2+}, which is required for catalytic activity. This type of enzyme is though to be involved in secondary antibody diversification, by initiation of immunoglobulin diversification processes; it has a mutagenic effect.

The highest positive fold change was observed for PARP2, which is a gene located in chromosome 14 and encodes the Homo sapiens poly (ADP-ribose) polymerase 2, a member of a family of enzymes that catalyze the poly(ADP-ribosyl)ation of proteins. It is one of the only PARP enzymes whose catalytic activity has been shown to be induced by DNA-strand breaks, providing strong support for key shared functions in the cellular response to DNA damage by alkylating agents or hydrogen peroxide oxidative stress.[150] In fact, ADP-ribose polymers target specific domains of topoisomerase I and reprogram the enzyme to remove itself from cleaved DNA and close the resulting gap. PARP2 act as a carrier of poly(ADP-ribose) to stalled topoisomerase I sites and induce efficient repair of DNA strand breaks, acting as positive regulators of genomic stability in eukaryotic cells.[151] PARP2 has also been found to interact with proteins involved in the base excision repair pathway, including DNA polymerase β and DNA ligase III.[152] Since PARP2 is overexpressed in HT29 cells following titanocene dichloride treatment, and metal-coordination to DNA is considered an alkylating-like disruption, we can conclude DNA damage is important in titanocene cytotoxic activity. As was discussed earlier, PARP is a target of caspases, from the death receptor signaling pathway (see Figure 20). This provides

further evidence that this pathway is very important for the mechanism of action of titanocene dichloride and should be explored in further detail in the near future.

KIF13A is a gene located in chromosome six which codes for the kinesin family member 13A protein. This is a candidate oncogene contributing to malignant progression of retinoblastoma.[153] It is a microtubule-dependent motor protein, which has roles in vesicle and organelle transport. In neurons, this family of proteins generates and drives the axonal anterograde transport (from the cell body to the synapse) and has been suspected to play important roles in neuronal pathfinding and migration.[154]

CEACAM7 is a gene located in chromosome 19 which codes for the Homo sapiens carcinoembryonic antigen-related cell adhesion molecule 7, member of a subfamily of genes expressed in normal and transformed colon cells. The expression of CEACAM7, also known as CGM2, is usually lower in colonic adenocarcinomas when compared to adjacent colonic mucosa.[155] In fact, the carcinoembryonic antigen itself is of great clinical value as a tumor marker and as a predictor of metastatic recurrence.[156] The down-regulation of this gene at the transcriptional level in colonic adenocarcinomas is similar to the down-regulation of the CEACAM1 gene, which indicates a potential tumor suppressor function of these genes. It has been shown for CEACAM7 that its down-regulation is an early even in colorectal tumorigenesis, with colorectal tumors not expressing the gene, while adenomas express it poorly and normal mucosa express it normally.[157] The presence of titanocene dichloride correlated with an up-regulation of this tumor suppressor gene enable us to infer that

the observed killing effect of HT29 colon adenocarcinoma cells is, in fact, an antineoplastic effect of our drug and not merely a toxic effect resulting in cell death.

C1orf53 is the open-reading frame 53 of chromosome one. Chromosome one, the largest human chromosome, encompasses about 4,200 genes (3,141 genes and 991 pseudogenes) and contains the most known genetic diseases of any human chromosome; over 350 diseases are associated with disruptions in the sequence of this chromosome.[158] Unfortunately, an open-reading frame only tells us that a protein could be encoded from the surrounding sequence.

Other genes were deregulated in the presence of titanocene dichloride and require our attention. The retinoblastoma binding protein 1 (RBBP1) is a cofactor involved in retinoblastoma-mediated suppression of cell proliferation. It has transcriptional repression activity by inhibiting E2F dependent gene expression when up-regulated, but this gene was down-regulated by a -2.2 fold change. The retinoblastoma binding protein 6 was also down-regulated by a -1.5 fold change. This is a splicing-associated protein that interacts with both p53 and Rb, and when up-regulated plays a role in the induction of apoptosis and regulation of the cell cycle. Unfortunately, no information was readily obtained from the data as to ascertain the effect of the down-regulation of these proteins. Anyhow, further study of this system should include both p53 and Rb related proteins since they are important for all types of cancers, even though p53 is mutated in HT29 cells.

Although titanium and platinum are two completely different metals in terms of acidity, size, polarizability and coordination behavior, it is usually required to

compare the effect of any metal-based anticancer drug with the behavior of platinum-based anticancer drugs. From the previously discussed results, we can conclude that titanocene dichloride, like platinum-based drugs, has a direct antineoplastic effect on colon adenocarcinoma HT29 cells, in which DNA damage is an important part of its mechanism of action. The specific titanium-DNA interactions possible have been studied using DNA and its models, such as polynucleotides and nucleotides, as was discussed in Chapter 2 of this thesis. From the present data it can be deduced that titanocene dichloride exerts a direct DNA-alkylating-like action. The mechanism by which the cell responds to this damage needs to be studied in further detail, but special attention should be given to apoptosis.

2. Changes due to the presence of the titanium-maltolato complex

There were a total of 205 probesets that were selected mathematically as down-regulated (fold change higher than -1.5) and 38 probesets selected as up-regulated (fold change higher than +1.5). We focused initially on 6 probesets which presented a fold change higher than -3, and 7 probesets which presented a fold change higher than +2 when the new titanium-maltolato complex was present. Table 10 shows, when available, the Gene Symbol, GenBank ID, Entrez Gene ID, UniGene ID, and a brief gene description along with the observed fold change for 3 of the 6 down-regulated probesets and 4 of the 7 up-regulated probesets. The identification of the probesets excluded of this discussion is weak and they code for hypothetical proteins.

Table 11. Probesets which showed a fold-change higher than -3 or +2 when comparing treatment with the new titanium-maltolato complex with no treatment (HT29 cells)

GenBankID Entrez Gene ID Gene Symbol UniGene ID	Gene Description	Observed Fold Change	Additional Comments
NM_001037631.1 1493 CTLA4 Hs.247824	cytotoxic T-lymphocyte-associated protein 4	-9.2	a, b
NM_178120.4 1745 DLX1 Hs.407015	distal-less homeo box 1	-3.6	a, b
NM_015915.3 51062 SPG3A Hs.241503	spastic paraplegia 3A (autosomal dominant)	-3.2	b
NM_005119.2 9967 THRAP3 Hs.160211	thyroid hormone receptor associated protein 3	2.0	a
NM_000945.3 5534 PPP3R1 Hs.280604	protein phosphatase 3 (formerly 2B), regulatory subunit B, 19kDa, alpha isoform (calcineurin B, type I)	2.0	a
NM_015049.1 66008 ALS2CR3 Hs.152774	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 3	2.0	a
NM_022045.2 27085 MTBP Hs.553528	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse) binding protein, 104kDa	2.1	a

a. More than one probeset detects this transcript
b. This probeset may detect multiple splice varients

The most down-regulated probeset corresponds to CTLA4, cytotoxic T-lymphocyte-associated protein 4, with a -9.2 fold-change when comparing treatment with the titanium-maltolato complex with no treatment of HT29 cells. This gene is present in chromosome two and is a member of the immunoglobulin superfamily and encodes a protein which transmits an inhibitory signal to T cells. Blockade of this antigen have been associated with cancer regression in patients with metastatic melanoma.[159-161] This regression is mediated by autoimmune manifestations in the patients. Complete responses have been obtained for some patients. Partial responses have been observed in tumor metastasis in the lungs, lymph nodes, liver and brain.

The most well-studied T-cell receptors for costimulatory molecules are CD28 and the CTLA-4 (also called CD152). These molecules have antagonistic functions. CD28 engagement enhances T-cell activation, proliferation and interleukin-2 production. CTLA-4 antagonizes T-cell activation by interfering with IL-2 secretion and with expression of the IL-2 receptor. Thus activation of T-cells requires at least two signals. The interaction of the T-cell receptor and the specific antigen in a major histocompatibility complex represents the first signal. But subsequent engagement of additional receptors on the surface of the T cell can enhance or inhibit the response. No evidence was found in the data regarding CD28 deregulation. Anyhow, this does not poses a major concern for the analysis since there is a clear correlation between CTLA-4 down-regulation and the presence of the new titanium-maltolato complex, allowing us to speculate that treatment with this complex could result in an autoimmune manifestation *in vivo*. T-cell activation is related to activation of NF-κB,

an important transcription factor in the death receptor / NF-κB signaling pathway (Figure 24). This poses interesting as members of this signaling pathway were deregulated in the presence of titanocene dichloride.

DLX1, the distal-less homeo box 1, is a gene located in chromosome 2 which encodes a member of a homeobox transcription factor gene family. The encoded protein is localized to the nucleus where it may function as a transcriptional regulator of signals from multiple TGF-{beta} superfamily members in a wide variety of cellular contexts.[162] For this reason, it is difficult to correlate the presence of the new titanium complex with down regulation of this gene. Anyhow, the p38/JNK signaling pathway, which was implicated in DNA-damage inducible genes in the presence of titanocene dichloride, is activated from TGFβ, allowing us to infer there could be similarities in the mechanism of action of both titanium drugs.

SPG3A, spastic paraplegia 3A (autosomal dominant), is a gene located in chromosome 14 which codes for atlastin 1, a member of GTPases required for ER and Golgi morphogenesis.[163] When this protein is mutated, a neurodegenerative disorder is observed because abnormal morphogenesis of the ER and Golgi results in improper membrane distribution or polarity. No information was obtained as to the effect of deregulation of this gene.

The thyroid hormone receptor associated protein 3 is the gene product of THRAP3, the first up-regulated gene that will be discussed. This gene presented a 2.0 fold change when comparing titanium-maltolato treatment with untreated HT29 cells. It functions as a co-activator of transcription with the thyroid hormone receptor, a

nuclear hormone receptor that traduces the signals of small lipophilic hormonal ligands.[164-165] The TRAP complex is a member of the Mediator complexes which interact with RNA polymerase II helping to recruit it to ligand-activated promoters.[166] Although we could not find in the data direct evidence of RNA polymerase II up-regulation, we did find up-regulation by a 1.6 factor of TAF15, TATA box binding protein associated factor 15 of RNA pol II. Unfortunately, the precise role of each type of coactivator in the Mediator complex and their interactions during transcription have not been clearly defined. Anyhow, this data allows us to state that deregulation of transcriptional mechanisms physically related to the nucleus area of the cells is correlated to the presence of the new titanium-maltolato complex.

PPP3R1 is a gene located in chromosome 2 which codes for the protein phosphatase 3, specifically the alpha isoform of regulatory subunit B. This enzyme, also called Calcineurin, can play a critical role in transcriptional regulation and growth control in T lymphocytes by a mechanism believed to involve dephosphorylation of the nuclear factor NF-AT which is essential for transcription of the interleukin-2 gene.[167] Although we could not find evidence in the data regarding deregulation of these downstream members, this is probably due to the limited time window we are able to evaluate with the microarray experiment. Anyhow, we encounter another member of the nuclear factor pathway which is deregulated in the presence of the new titanium-maltolato complex. Up-regulation of this gene represents further evidence that an autoimmune response could be a consequence of titanium-maltolato treatment

in vivo. T-cell activation should be explored in the near future as to ascertain the direct effect of the new complex in tumor models.

LS2CR3 is a gene which codes for the trafficking protein kinesin binding 2 (TRAK2). Unfortunately, their function as adaptors in the transport of organelles to synapses[168] does not provide us with a clear picture of their role in the cytotoxic effect of the titanium-maltolato complex.

The most up-regulated gene in the presence of the new complex was the gene MTBP, which codes for the MDM2 binding protein. This gene presented a -2.9 fold change when compared with untreated HT29 cells. MDM2 when overexpressed confers some tumorigenic potential to cells since it binds to p53, preventing it to exert its functions in cell cycle arrest and apoptosis, among others. MDM2 and p53 are part of an autoregulatory feedback loop, so we decided to look up for evidence of p53 and MDM2 deregulation in our data. The proteins p53 and MDM2 were neither up-regulated nor down-regulated in the presence of the titanium-maltol complex. Although this is consistent with an increased presence of MTBP, since it functions in maintaining p53/MDM2 homeostasis in cells, HT29 express a mutant inactive p53, so lack of deregulation of p53 in our data is somewhat irrelevant. But overexpression of MTBP has been shown to induce growth arrest in a p53-independent manner.[169] We can conclude that the new titanium-maltolato complex is correlated with growth-arrest or apoptosis. This could be a direct effect of the interaction with DNA or an indirect effect involving damage to other important molecules.

There is no doubt that transcriptional control mechanisms are being altered both in the presence of titanocene dichloride and the new titanium-maltolato complex, when examined alone, with nuclear factor and transforming growth factor signaling as major pathways in the mechanism of action of these drugs. The following section will provide an analysis of the most deregulated genes in the presence of either drug. This will allow us to infer about the commonalities of the possible mechanisms of action of the cytotoxic effect of the titanium drugs in HT29 cells.

3. Changes due to the presence of either drug

There were a total of 1692 probesets that were selected mathematically as down-regulated (fold change higher than -1.2) and 513 probesets selected as up-regulated (fold change higher than +1.2). We focused initially on 2 probesets which presented a similar negative fold change in the presence of both drugs, and 4 probesets which presented a similar positive fold change in the presence of both drugs. The number of change and the degree of change observed in the presence of either drug suggests without a doubt that there is a significant effect to the cells at this early stage. No attempt will be made as to analyze all 2205 deregulated genes. Table 11 shows, when available, the Gene Symbol, GenBank ID, Entrez Gene ID, UniGene ID, and a brief gene description along with the observed fold change for the six above-mentioned probesets. When no reference is cited specifically, the information on the probesets, the gene and the gene products was obtained from one or more of the mentioned sources.

Table 12. Probesets which showed a similar positive or negative fold-change when comparing treatment with either titanocene dichloride or titanium-maltolato complex with no treatment (HT29 cells)

GenBankID Entrez Gene ID Gene Symbol UniGene ID	Gene Description	Observed Fold Change[1]	Additional Comments
NM_138962 124540 MSI2 Hs.185084	musashi homolog 2 (Drosophila)	-7.6, -5.3	a, c
NM_033142.1 94027 CGB7 Hs.348402	chorionic gonadotropin, beta polypeptide 7	-6.0, -6.9	d
NM_004480.3 2530 FUT8 Hs.118722	fucosyltransferase 8 (alpha (1,6) fucosyltransferase)	3.1, 4.3	a, b
NM_001005335.1 3191 HNRPL Hs.446623	heterogeneous nuclear ribonucleoprotein L	3.2, 4.2	a, b
XM_941370.1 90133 KRT8L2 Hs.101651	keratin 8-like 2	3.6, 4.2	b
NM_000422.1 3872 KRT17 Hs.2785	keratin 17	4.7, 7.9	a

1. First value corresponds to titanocene dichloride and second value corresponds to titanium-maltolato complex
a. More than one probeset detects this transcript
b. This probeset may detect multiple splice varients
c. Weak identification
d. This probeset may detect multiple genes

MSI2 is a gene located in chromosome 17 which encodes for a putative protein that contains two conserved tandem RNA recognition motifs. Similar proteins in other species function as RNA-binding proteins and play central roles in posttranscriptional gene regulation. This is the product of a fusion gene obtained recurrently in the disease progression of chronic myeloid leukemia. The fact that the presence of titanium drugs is correlated to the down-regulation of this gene is interesting because this gene is believed capable of blocking cellular differentiation. Although a specific mechanism of action is not being described for the effect of the MSI2 gene on such blocking, cellular differentiation involves genetic transformation of cells. The correlation of titanium drugs with the presence of such blocking allows us to infer that titanium drugs are able of disrupting cellular events.

CGB7 is a gene located in chromosome 19 which is a member of the glycoprotein hormone beta chain family and encodes the beta 7 subunit of chorionic gonadotropin (CG). Glycoprotein hormones are heterodimers consisting of a common alpha subunit and a unique beta subunit which confers biological specificity. CG is produced by the trophoblastic cells of the placenta and stimulates the ovaries to synthesize the steroids that are essential for the maintenance of pregnancy. Different serum levels of this protein have been associated with the presence or absence of both benign and malignant tumors.[171] Since seven non-allelic genes code for the CGbeta, it would be irresponsible to point this gene as a measurement of titanium activity.

FUT8 is a gene located in chromosome 14 which codes for the alpha (1,6) fucosyltransferase. The expression of this gene may contribute to the malignancy of

cancer cells and to their invasive and metastatic capabilities. Among new mechanisms by which cells acquire resistance to anticancer drugs, the relationship of glycans in this mechanism is not well understood. Epirubicin (EPI)--and mitoxantrone (MIT)--resistant cell lines (HLE-EPI and HLE-MIT) from the human hepatocellular carcinoma cell line have been used and FUT8 overexpression was correlated with acquired tolerance to the drugs,[172] but an specific mechanism by which this tolerance is conferred have not been elucidated.

HNRPL is a gene located in chromosome 19 which codes for the heterogeneous nuclear ribonucleoprotein L. HNRPL is among the proteins that are associated with heterogeneous nuclear RNAs complexes, and which play a role in mRNA biogenesis, transport and function, mainly in splicing events.[173] Splicing regulators can function as activators or repressors, as is the case of HNRPL, which has been described as a global regulator in the level of mRNA splicing. Presence of titanium drugs is correlated with up-regulation of this gene, allowing us to confirm the abundant transcriptional deregulation activity following titanium-based drug treatment.

KRT8L2 is a gene predicted by computational analysis to encode a protein like keratin 8, a protein member of the intermediate filaments, the major components of the cytoskeleton and nuclear envelope in most types of eukaryotic cells.[174] No analysis will be made as to correlate titanium presence with a hypothetical protein.

Last gene in this group corresponds to down-regulated KRT17, which codes for the keratin 17, a member of the keratin family which for the cytoskeleton of the

cells. An overexpression of this gene could be related to a reorganization of the keratin filaments in the cell.[175] Reorganization of the cytoskeleton of a cell could only imply that some level of stratification, from what usually would be a single-layer of cells, is occurring. This phenomenon is observed when cells loose their contact-dependent growth control mechanisms, as in the case of transformed cells. The level of expression of this keratin in titanium-treated cells when compared with untreated cells could suggest a mitogenic effect of what is known to be an antiproliferative drug as previously determined and reported for these complexes. An alternate explanation of this effect is the ability of titanium drugs to alter cytoskeleton organization in cells, regardless of cell division or proliferation issues. This last phenomenon could confirm the ability of titanium complexes to disrupt important cellular events in HT29 colon adenocarcinoma cells.

In summary, the microarray data supports a direct DNA-damaging action from titanium-bases drugs, which is more evident for titanocene dichloride than to the titanium-maltolato complex. Since the new maltolato complex is apparently inert in character, it should not be surprising that fewer changes are associated with its presence when compared with treatment with titanocene dichloride. Anyhow, both drugs are able to induce a wide variety of cellular events. The specific mechanisms of action by which the observed cytotoxicity is exerted need to be confirmed by further study of this system. Special interest should be given to apoptosis for titanocene dichloride and to the NF-κB signaling pathway, as well as T-cell activation, for the titanium-maltolato complex. It is interesting to note that inhibition of NF-κB has been

reported to sensitize cancer cells to the effects of platinum drugs.[177] One important mechanism for resistance to platinum drugs is the avoidance of cells entering the apoptotic pathway, and NF-κB is important in the final response of cells to these drugs. On the other hand, the new titanium-maltolato complex was correlated with up-regulation of calcineurin, essential for transcription of the interleukin-2 gene, and titanocene dichloride was correlated with down-regulation of the NF-κB inhibitor, allowing activation of the NF-κB. This opposing information raises the question about if the mechanisms of resistance to platinum drugs are the same for titanium drugs. The mechanisms of resistance to titanocene dichloride have been correlated to the resistance to gallium complexes, but only because they are able to enter the cells via the transferrin receptor.[7] Our studies clearly show that transferrin is not necessary for the activity of titanium compounds in HT29 cells. Also, there is lack of information regarding the ability of titanium drugs to enter the cells via the copper transporter protein Ctr1, involved in resistance to cisplatin due to reduced uptake. Although data is limited and more information is necessary to sustain the following observation, since different metals behave differently, it is unlikely that tumors will develop cross-resistance to all metal compounds. This leaves us with the possibility of combination therapy with different metal drugs to overcome resistance to one of the drugs.

Chapter V. Conclusions

In this study, we have correlated the aqueous stability and the binding properties to human apo-transferrin to the cytotoxicity on the HT29 cell line of a selected group of titanium(IV) complexes. While all titanocene complexes donate their Ti(IV) ion to human apo-transferrin, the presence of this protein in the cell medium does not enhance their cytotoxic activities in HT29 cells. The titanium-maltolato complex, by contrast, showed a level of cytotoxic activity that exceeds those of titanocene dichloride and $(Cp\text{-}COOEt)_2TiCl_2$ and $(Cp)(Cp\text{-}COOEt)TiCl_2$ but still is unable to donate Ti(IV) to apo-transferrin. All complexes which were inactive, in addition to titanocene dichloride and the maltolato complexes, were evaluated in a transferrin-enriched environment, but this did not result in increased activity in any of the complexes studied. Of all the complexes studied, the titanium-maltolato complex is the most stable in water, being hydrolytically stable at and above physiological pH; it is, in addition the most cytotoxic.

$[Ti_4(maltolato)_8(\mu\text{-}O)_4]$ belongs to a prototype of coordination compounds with antitumor activity such as budotitane, cis-diethoxybis(1-phenylbutane-1,3-dionate)titanium(IV).[17,59] Interestingly, while budotitane hydrolyzes extensively being one of the major concerns with regard to which species is biologically active, titanium-maltolato complex overcomes such complications. It has been proven that the asymmetry of the β-diketone is responsible for this compound's antitumor

activity.[120,17] Likewise, in [Ti_4(maltolato)$_8$(μ-O)$_4$] the maltolato ligand possesses such asymmetry. Of particular interest is that while for budotitane complex several stereoisomers exist, we have been able to isolate only one stereoisomer.

The antitumor activity of [Ti_4(maltolato)$_8$(μ-O)$_4$] raises a question about the role of transferrin. While titanocene dichloride and all the derivatives were able to donate the titanium ion to the protein in a timely fashion, this did not result in an improved cytotoxic behavior. In this regard, transferrin may be important in protecting the titanium center from hydrolysis in a hostile environment such as plasma, but that could also be achieved by selecting ligands that could result in hydrolytically stable, yet active, complexes.

There are reports which demonstrate titanium is mostly found on nucleic-acid rich regions on cells, such as the nucleus, allowing us to infer the main site of interaction is DNA. We presented DNA-interactions of the new titanium-maltolato complex in order to discard that the improved cytotoxic behavior shown responds to a different mechanism of action than titanocene-like complexes. Although our studies does not discard other biomolecules as possible sites for interaction of titanium-based drugs, the attack to nucleic acids such as DNA apparently are the cause of the antiproliferative activity shown by titanium complexes in cancer cells. However, the mechanism of action of titanocene dichloride and the titanium-maltolato complex differs in terms of the possible interactions.

With regard to the cytotoxicity of ferrocene complexes we have found that increasing the lipophylic character of the substituents increased their antiproliferative

effects. This could be the results of better cell membrane permeability. In addition, a lower oxidation potential for these complexes would make them better producers of reactive oxygen species and this was not correlated with increased activity.

Lastly, the presence of both titanocene dichloride and the new titanium-maltolato complex is correlated with cellular changes at the expression level. We can conclude that titanocene dichloride, like platinum-based drugs, has a direct antineoplastic effect on colon adenocarcinoma HT29 cells, in which DNA damage is an important part of its mechanism of action. From the present data, we can conclude that titanocene dichloride, like platinum-based drugs, has a direct antineoplastic effect on colon adenocarcinoma HT29 cells, in which a direct DNA-alkylating-like action is an important part of its mechanism of action. For the titanium-maltolato complex the most notable changes are correlated to cell cycle arrest and apoptosis. Since the new maltolato complex is apparently inert in character, it should not be surprising that fewer changes are associated with its presence when compared with treatment with titanocene dichloride. Anyhow, both drugs are able to induce a wide variety of cellular events in HT29 colon adenocarcinoma cells. The mechanism underlying these events and how the cell responds to the damage caused by titanium drugs needs to be studied in further detail, but special attention should be given to growth arrest and apoptosis, as well as immune responses.

Chapter VI. Future Work

Since science research itself functions as some sort of auto-regulated feedback loop, this thesis would not be complete if it does not recommends further study of the work presented here.

First of all, a more comprehensive variety of cell lines should be obtained. Adenoma-like cell lines should be used as they encompass the majority of cancers diagnosed, but they must represent different organs – kidney, ovaries, prostate, lung, bladder and liver. Also, if further study of this system raises the question about the activity of titanium drugs in cisplatin-resistant cells, cell lines representative of the three major resistance mechanisms should be acquired and studied.

Further work must be done as to define the exact nature of the new titanium-maltolato complex. We have presented evidence in which total intercalation to DNA bases can be discarded as responsible for the observed cytotoxic effect, but partial intercalation, either combined or not with covalent bonding, can not be ruled out as a possible mode of interaction with DNA.

Regarding expression profiling, this system should be studied more comprehensively. Titanocene dichloride, as parent drug, and the new titanium-maltolato complex can be used with different time intervals. This will allow us to see a movie instead of a snapshot of what is actually happening at the cellular level when these drugs are used. Five to six time intervals in a period of 24 hours could be used

with both drugs and all results validated with other techniques such as real-time polymerase chain reaction. If the same methodology is to be used, DNA as well as protein extracts should be obtained along with the RNA. This would allow confirmation of an overexpressed protein, for example.

Regardless of the choice made for continuing study of this system, special attention should be given to the new titanium-maltolato complex. As a pseudo-inert titanium complex, aqueous chemistry studies as well as interaction with biomolecules could be performed with a wide variety of spectroscopic and other techniques. Information of the type of interaction, along with a more complete profile of activity in different cancer cell lines, should provide a solid ground basis for continuing development of inorganic titanium complexes as an alternative to the unstable metallocene counterpart.

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Appendixes

A. Verification of Reader 340 ATTC

The Reader 340 ATTC from SLT Lab Instruments is a fully automatic, microprocessor controlled reader, designed to measure the light absorbance (optical density) of samples in microplates. Major features that can be highlighted are that it has a temperature control unit to heat the microplate before and during reading, it can make agglutination measurements (20 different absorbance readings per well), it reads the eight wells of a column simultaneously, and can shake the microplate before the readings. Also, the instrument can be used alone or interfaced to a computer. If it is to be used alone, the equipment has a membrane pad, which in conjunction with the digital display, allows normal running of the instrument and programming steps. When interfaced to a computer, it is controlled with the winSeLecT software package.

The most important part of the functionality of the Reader 340 ATTC is its optical system. Briefly, a halogen lamp emits a light beam, which passes through the diaphragm plate to the heat absorbing filter, and then to the optical lens, to be focused. It is passed through the selected filter to obtain the correct wavelength. The beam falls onto a glass fiber optic unit, which distributes the available light into nine beams, one for reference and eight measurement beams.

The reference beam is passed through a hole in the mounting block to the reference diode, which measures and controls the amount of light passing through the

filter. The reference diode is used to keep the level of light passing through the filter constant for each measurement and for each filter. The light level is kept constant by altering the voltage supplied from the power supply board to the lamp.

The intensity of the light beams, after passing through the samples, is measured by eight diodes, which are located directly above the sample wells. The light is then amplified and converted into a digital value by the analog/digital board. This digital value is used to calculate an optical density.

The instrument can measure the microplate with single or dual wavelength measurements, with blanking (three different blanking modes) and shaking (four different shaking modes). The dual wavelength eliminates any influence caused by the plate itself, nonspecific absorbance of samples and finger prints, scratches, dust, etc. It should be noted that the best results are obtained by using an optically clear (visible range) flat bottom microplate. The best repeatability is obtained at λ_{max}, the accuracy is decreased at small volumes, and formation of meniscus causes inaccuracies (shaking reduces this last problem). The instrument's measuring range is 0 – 2 absorbance units. The instrument provides readings with three (0.000) decimal places.

A.1. Instrument Filters

The instrument filters were verified to be working correctly (producing the right wavelength) using a set of solutions of a known absorbance spectrum and known concentration. The dye MTT formazan was selected because it was the future analyte

in the real experiments. Solutions used were of the highest precision possible. The balance used was an OHAUS Analytical Plus S/N: M18820.

A.2. Instrument repeatability

After verifying the filters, the instrument reproducibility within a same measurement (the same plate) was verified with all possible filters in a low-absorbing situation and a higher absorbance situation. The low-absorbance substance was an empty 96-well plate, with and without lid. Although the data is not shown, the findings were as follows:

In the lowest absorbance range (highest lambda and no lid in plate) the maximum standard deviation [absolute (relative)] for a row or a column was 0.002 (5.9%) absorbance units but the standard deviation for the whole 96-well plate was 0.001 (3.3%). In a higher absorbance range (lowest lambda and lid with plate) the maximum standard deviation for a row or a column was 0.005 (2.4%) but the standard deviation for the whole 96-well plate was 0.004 (1.9%).

A.3. Limit of linearity of instrument

For an analysis of the instruments limits of linearity, quantification and detection, eight set of five solutions each were prepared, whose absorbance values range from 0.016 to 1.885 absorbance units. Although no standard statistical protocol was used to obtain LOD, LOQ or LOL ranges, a linear relationship was found for all

sets of solutions. The correlation factors were found to decrease as absorbance values increased. Table A1 summarizes the findings.

Table A1. Correlation factor of the absorbance-concentration linear relationship for sets of alcoholic solutions of MTT formazan

Absorbance range	Correlation factor	Absorbance range	Correlation factor
0.016 to 0.029	0.9561	0.452 to 0.681	0.9992
0.042 to 0.069	0.9949	0.977 to 1.440	0.9991
0.084 to 0.138	0.9947	1.386 to 1.816	0.9878
0.191 to 0.298	0.9987	1.819 to 1.885	0.8879

A.4. Solvent optimization for MTT formazan produced in vitro

After starting experiments with cells (HT29), I started to develop calibration curves to relate cell number and absorbance readings. I started working with 0.1N HCl in isopropanol as a solubilizing agent for the MTT formazan (the purple precipitate which forms upon metabolic conversion in mitochondrially active cells) because it is what is described as the solubilizing solution sold by Sigma. Wells in which there were suppose to be cells as well as wells in which no cells were seeded gave similar absorbance values. An analysis was made to compare different solvents for the formazan. The results are described below.

The 0.1N HCl solution in isopropanol dissolves MTT formazan completely but the color disappears within a short time. DMSO, pure isopropanol and pure ethanol

provide good correlation factors without color fading. Formazan solutions in DMSO have a λ_{max} farther from the instrument filter's wavelength than alcoholic solutions (data not shown). 10% Triton in isopropanol provided the highest correlation factor of all. Below is a graph for this last solvent.

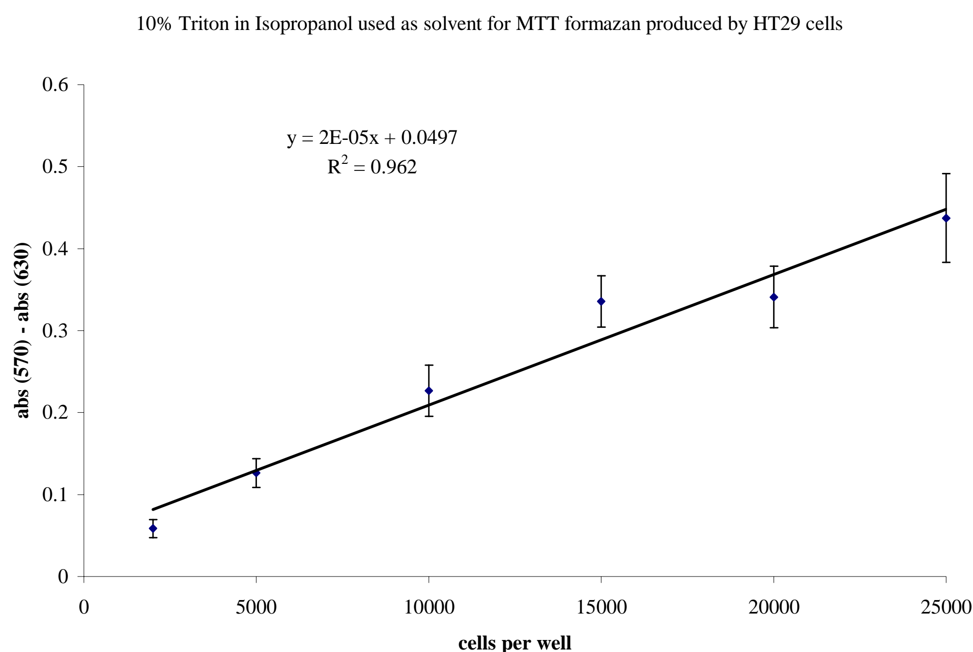

Figure A1. Linear relationship for a set of MTT formazan solutions using 10% Triton in Isopropanol as solvent. MTT formazan was produced *in vitro*.

A.5. Optimization of plate washing procedure

A problem was found then with the plate washing procedure and an analysis was made as to which PBS temperature produced the best results. Three exact plates seeded with HT29 cells were allowed to recover overnight and MTT was added to all wells. After MTT formazan was produced, plates were washed with PBS at three different temperatures: 37°C, 22°C and 4°C. It was found that cell loss is higher when

warm PBS is used to wash the plates, followed by PBS at room temperature. Cell loss is minimized when cold PBS is used to wash the cells after the MTT-containing media is removed.

A.6. Calibration curve with MTT formazan produced in vitro by HT29 cells

Then, after cell seeding was mastered and all problems related to solvents or washing procedures were overcome, a calibration curve for the HT29 cells was developed in a wide range of both cell count and absorbance. The calibration curve produced is shown below.

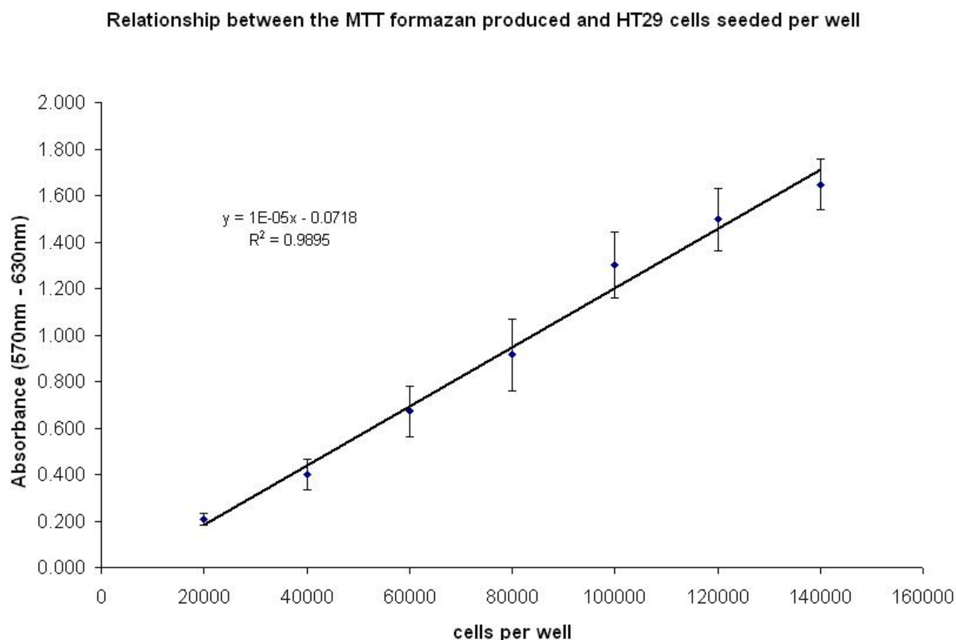

Figure A2. Linear relationship between the MTT formazan produced *in vitro* and HT29 cells seeded per well in 96-well plate.

A.7. Experiments started with complexes' concentrations in μM range

Trials were started with Ti(IV) complexes being dosed to plates' wells in different concentrations. Experiments were performed in quadruplicate plates from the beginning. If a set of concentrations was able to provide an IC50 value, then all repetitions were obtained in the same set of conditions and a standard deviation was calculated for each value. For example, titanocene dichloride and functionalized cyclopentadienyl complexes preliminary evaluation is described below.

For titanocene dichloride, a preliminary IC50 value of 1.6mM was obtained in HT29 cells. Since the IC50 value was obtained with one end of the data set, a new experiment using this value as a reference was determined to be necessary.

I dosed again a plate (quadruplicate) with Cp_2TiCl_2 on a less wide concentration range (log M = -2.7 to -3.9). Although the solubility of titanocene dichloride is a limiting factor for the experiment, the concentration range attainable was a little bit higher than previous experiments. An IC50 value was determined for this complex at a 72 hours drug exposure. The value 1.53mM was comparable to the approximate value of 1.6mM determined previously. This value of 1.53mM (and all values reported in this book and the articles already published) was determined with a fitted curve using the SigmaPlot software. An example of such curve along with the data points which are used to generate it is seen in Figure A3.

An additional experiment was performed with titanocene dichloride at 72hours in a less wide concentration range but centered in this already determined IC50 value.

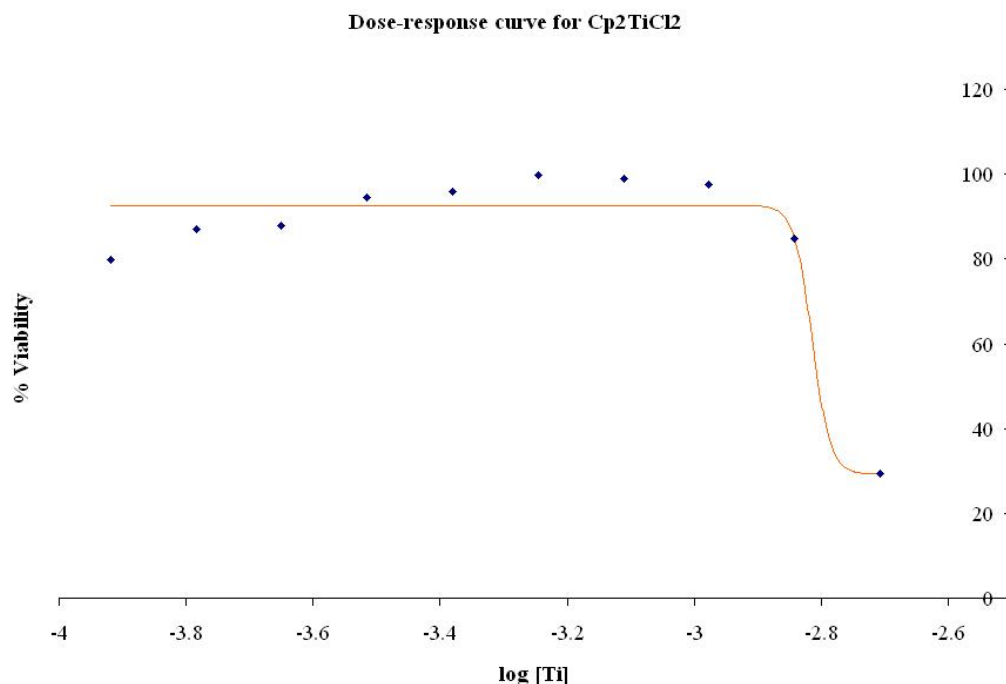

Figure A3. Graphic relationship of the data set between titanocene dichloride concentration used and percent cell viability of HT29 cells as obtained with MTT assay. Solid curve represents fitted equation obtained with SigmaPlot software.

The complex $(C_5H_4COOCH_3)_2TiCl_2$, obtained from LiMing Gao, was initially tested but an IC50 was not obtained. All concentrations tested resulted in 100% cell viability upon 72hours of drug exposure. Highest concentration tested was 130μM. The complex $(C_5H_4COOCH_2CH_3)_2TiCl_2$, obtained from LiMing Gao, was tested but an IC50 was not obtained. All concentrations tested resulted in 100% cell viability upon 72hours of drug exposure. Highest concentration tested is 180μM

The complexes $(C_5H_4COOCH_3)CpTiCl_2$ and $(C_5H_4COOCH_2CH_3)CpTiCl_2$, obtained from LiMing Gao, were tested but an IC50 was not obtained. All

concentrations tested resulted in 100% cell viability upon 72hours of drug exposure. Highest concentration tested was 303 and 336μM, respectively.

The titanium-maltolato complex and the three titanium-aminoacid complexes were also tested at a 72 hours (3 days) schedule in a low concentration range and the same behavior was obtained. All concentrations resulted in 100% cell viability. These experiments were used as a starting point to determine the concentration range needed in order to obtain an IC_{50} value.

If more or detailed information is needed on the calibration verification of the equipment or the different preliminary experiments performed with and without cells, feel free to contact Dr. Enrique Meléndez, Principal Investigator of the research group, to make an appointment and read the laboratory notebooks for this project.

B. Cytotoxic Activity of Titanium Complexes on MCF-7 cells

The cytotoxicity of titanocene dichloride and the titanium-maltolato complex on the MCF7 breast adenocarcinoma cell line was measured using a slightly modified MTT assay as described in the Chapter 3 of this book. The concentration range studied for titanocene dichloride and the maltolato complex was 13-1245 and 5-472μM, respectively. The drug exposure time was 72 hours. The IC_{50} values obtained were $5.7(5)x10^{-4}$ M for titanocene dichloride and $1.6(1)x10^{-4}$ M for the maltolato complex. Figure B1 depicts the curves from the MTT assays, which clearly show the lowest activity of titanocene dichloride compared to the titanium-maltolato complex.

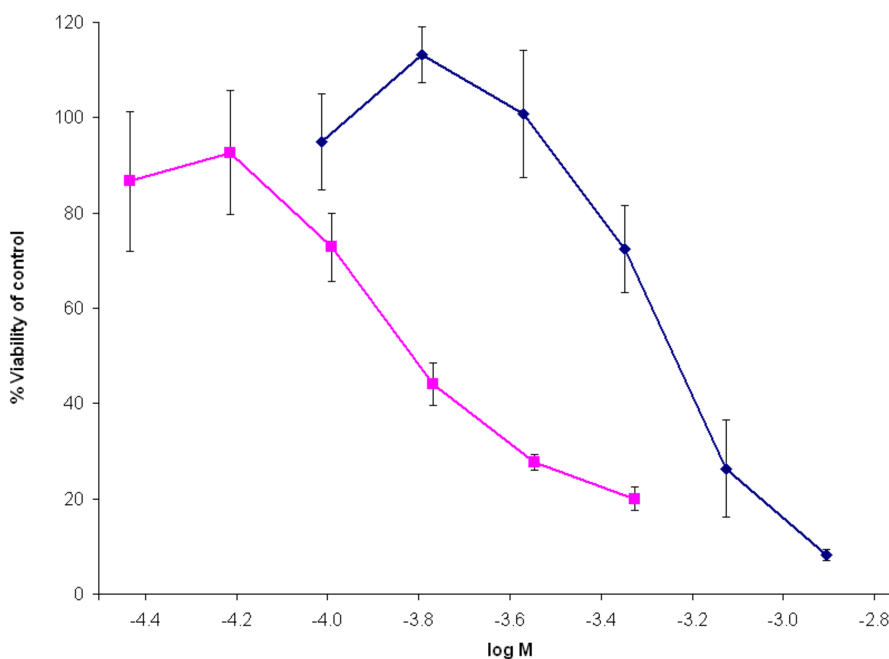

Figure B1. Titanocene dichloride (diamonds) and Titanium-maltolato complex (squares) cytotoxic activity against MCF7 breast cancer cells as measured by MTT colorimetric assay.

It is interesting that the IC_{50} values obtained for titanocene dichloride and the new titanium-maltolato complex are similar in both HT29 and MCF-7 cell lines. These two cell lines have different p53 status. HT29 cells express mutant p53, while MCF7 have normal p53 function but does not readily undergo p53-dependent apoptosis. Although p53 status is not a universal predictor of treatment response, it is a common defect in human tumors, especially those with multidrug resistance. In terms of DNA mismatch repair (MMR) proteins, both MCF-7 and HT29 cells are MMR proficient. Anyhow, the low degrees of resistance acquired due to loss of MMR function are nevertheless of substantial clinical significance. Regarding multidrug resistance proteins (MRPs), MCF-7 cells express negligible MRPs, while HT29 cells express

MRP1 and 3 glutathione conjugate efflux pumps, but no MRP2 and P-170 glycoprotein. Anyhow, IC_{50} values obtained for both drugs are higher than those for cisplatin, which shows values in the medium micromolar range for both cell lines (see table 5, page 62).

C. Cytotoxic data of niobocene dichloride and cyclodextrin inclusion complexes on HT29 cells

Experiments were performed as to evaluate the cytotoxic activity of niobocene dichloride cyclodextrin inclusion complexes on HT29 cells, but an unfortunate miscalculation of the initial cell density lead us to use a lower cell density than needed for obtaining accurate reproducible results as determined for this cell line in our experimental conditions. Preliminary results, which should not be taken as determinant, are shown in table C1.

Table C1. Preliminary cytotoxic data of niobocene-cyclodextrin inclusion complexes on HT29 cells.

Complex	IC_{50} (M)
Cp_2NbCl_2	$3.5x10^{-4}$
β-CD inclusion complex	$2.2x10^{-4}$
γ-CD inclusion complex	$5.5x10^{-4}$

Since there is no notion as to the precision of these measurements, it would be somewhat irresponsible to jump to any conclusions from this set of data.

Anyhow, it is worth mentioning that this is an area of research which has not been fully explored. As a research group, we will repeat these experiments as to establish clear relationships between the structure and binding of these and other inclusions and their cytotoxic activity on this and other cell lines.

Similar experiments have been performed with ferrocene derivatives[A26] and molybdenocene dichloride[C1] with mixed results. Unmodified cyclodextrin inclusion complexes of ferrocene derivatives has not lead to an increased cytotoxicity on Ehrlich ascites tumor cells, while it neither improves the activity nor decrease the toxicity of molybdenocene dichloride on HeLa cells. The use of modified cyclodextrins has resulted in an improved cytotoxic behavior both of ferrocene derivatives and molybdenocene dichloride.