Antiproliferative Activity of Some Polar Substituted Titanocene Dichlorides

by

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DEDICATION

To the beautiful bride-to-be, Kathy

ABSTRACT

This work describes the cytotoxic activity of polar substituted titanocene dichlorides against small cell lung cancers.

Two polar substituted metallocene dichloride compounds $Cp(C_5H_4CO_2Me)TiCl_2$ and $(C_5H_4CO_2Me)_2TiCl_2$ as well as Cp_2TiCl_2 were synthesized and tested for cytotoxicity against small cell lung cancer cell lines. The *in vitro* activity of Cp_2TiCl_2 was shown to be 100 fold less effective than the clinical standard, cis-platin. Activity of the mono substituted titanocene dichloride was increased compared to Cp_2TiCl_2 but was found to be 40 fold less potent than cis-platin. The disubstituted titanocene dichloride caused the maximum increase in cytotoxicity and was found to be only 2-3 times less potent than cis-platin.

This study provides justification for the further development of these compounds for the treatment of cancer.

ACKNOWLEDGEMENTS

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LIST OF SYMBOLS AND ABBREVIATIONS

DNA - Deoxyribonucleic acid

VT-NMR - Variable Temperature Nuclear Magnetic Resonance

Cp - Cyclopentadienyl

MTT - a yellow tetrazolium salt

IC_{50} - Inhibitory Concentration causing 50% of the cells to die

ID_{50} - Conentration effecting 50% inhibition of cellular proliferation *in vitro*

T/C - Ratio of tumor weights of treated and untreated (control) tumors. Values less than 50% are considered significant

ILS - Increase in Life Span

ED_{90} - Dose causing complete tumor regression in 90% of the animals treated

T.I. - Therapeutic Index, defined as the relation of a lethal dose (LD_{50}) to a therapeutic dose (ED_{90})

LD_{10}, LD_{50}, LD_{100} - Dose killing 10, 50, or 100%, respectively, of the animals treated

^1H NMR - Proton Nuclear Magnetic Resonance

TMS - Tetramethylsilane

DMSO - Dimethyl sulfoxide

THF - Tetrahydrofuran

MHz - Megahertz

IR - Infrared

UV - Ultraviolet spectrum

$CDCl_3$ - chloroform

CH_2Cl_2 - methylenechloride

Tdc - Titanocene Dichloride

JB1 - (η^5-Methoxycarbonylcyclopentadienyl)(η^5-cyclopentadienyl) dichlorotitanium

JB2 - Bis(η^5-Methoxycarbonylcyclopentadienyl)dichlorotitanium

°C - Degrees Celsius

δ - Chemical shift in ppm

η^n - Hapticity

mL - Millilitre

μL - Microlitre

mmole - milimole

mol - mol

ppm - parts per million

X - Halogen

M - Metal center of a metallocene

w - weak absorbance (IR)

s - strong absorbance (IR)

GLOSSARY

Metastasis - the spread of cancer from one part of the body to another. Cells in the metastatic (secondary) tumor are like those in the original (primary) tumor.

Immunotherapy therapy - treatment to stimulate or restore the ability of the immune system to fight infection and disease.

Drug-Resistance - when tumor cells become resistant to chemotherapy. Some tumour cells will be chemo-sensitive and are killed by anticancer drugs; the cells that remain are likely to be more resistant. Thus by selection it is the most resistant cells survive and divide, they may be resistant to a particular drug, a class of drugs, or all drugs.

In Vitro - in an artificial environment. For example many cancer research experiments are in vitro (in the test tube), using cell cultures (cells grown in the lab); either from established cell lines or from material collected at biopsy/surgery.

In Vivo - within the living body.

Leukemia – a progressive, malignant disease of the blood and blood-forming organs, characterized by over-proliferation and development of leukocytes (a type of white blood cell). There are many different forms of leukemia.

Nephrotoxicity - some anti cancer drugs may have the side effect of damaging the kidneys, for example ifosfamide and cisplatin are known to be nephrotoxic. There are two categories; glomerular and tubular toxicity relating to the two main areas of the nephron. In studies of ifosfamide the degree of nephrotoxicity is thought to be related to the cumulative dose, but there is a good deal of variability between patients.

Tumor - an abnormal mass of tissue that is not inflammatory, arises from cells of pre-existent tissues, and serves no useful purpose.

Oncogene - a gene that when altered in certain ways helps transform normal cells to cancerous cells.

Apoptosis - programmed cell death.

CHAPTER ONE - INTRODUCTION

1.1 Scope of Research

The accidental discovery by Rosenburg[1] that cis-diamminedichloroplatinum(II) (cis-platin) (Figure 1) is a potent cytotoxic agent was a significant advancement in the treatment of cancer. Cis-platin is commonly used for the treatment of testicular, ovarian, breast, lung, head and neck carcinomas. A large majority of testicular cancers are curable due to the availability of cis-platin in the clinic.[2,3] However, a therapeutic plateau has been reached for the use of this drug against cancer and the development of cis-platin resistance in the clinic is a major problem. Therefore the need to develop non-platinum anticancer agents for the treatment of cancers resistant to cis-platin i.e. non-small cell lung cancer, and in particular, cancers of the colon, is clear.[4] Kopf-Maier *et al* found that titanocene dichloride (Figure 1) is indeed effective against these carcinomas *in vivo*.[5] In addition, this compound was also found to have a less pronounced and different pattern of organ toxicity compared to cis-platin. As such, titanocene dichloride is currently undergoing early phase clinical trials.

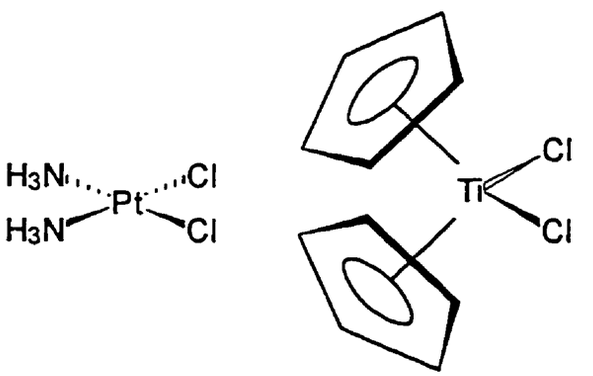


Figure 1 - Structure of Cis-Platin and Titanocene Dichloride

In the next sections, the anti-cancer properties of the metallocenes will be listed following a brief discussion of the biological events leading to cancerous growth and the techniques used to treat this disease.

1.2 Cancer-Defining the Disease

1.2.1 Carcinogenesis

A cancer arises from a process known as multistep carcinogenesis.[6] The initiation step begins when a normal cell undergoes a genetic change stimulated by one of the following: a virus, a chemical carcinogen, radiation or heredity. This process is followed by promotion, which is clonal expansion of the cancer cells forming a preneoplastic lesion. The next stage, termed conversion, involves another genetic change where a malignant lesion is formed. At this point, there occurs progression, which is stimulated by another genetic change and finally invasion and/or metastasis. According to Pitot, a genetic change begins with at least one or some combination of the following events: the activation of proto-oncogene(s), the inactivation of tumor suppressor gene(s) or antimetastasis gene(s), and/or apoptosis-regulatory genes.[7] As such, it is the subtle genetic diversity of cancerous cells which make them difficult to distinguish from normal cells and which is the major problem in treating cancer today.

1.3 Cancer-Treating the Disease

1.3.1 Cancer Therapies

The three most commonly used methods of cancer treatment are: surgery, radiation and chemotherapy. Some cancer therapies currently being used include surgery and/or intense doses of ionizing radiation. These are termed local treatments and are applied to eradicate or control the growth of the primary tumor. The latter therapy is effective because the cancer cells do not recover from the radiation whereas many normal cells will. In general, smaller tumours are more likely to be cured with local measures such as surgery or radiation. Other forms of treatment include chemoprevention[8] , which involves the use of drugs and/or diet to prevent or disrupt the various stages of carcinogenesis. Another area of intense research is immunotherapy, a form of biological therapy, which can involve virus-induced stimulation of the immune system such that the host will reject a tumor.[9]

One of the most common ways to combat cancer is chemotherapy. This therapy is based on the fact that cancer cells generally proliferate faster than normal cells. As such, the mechanism of action of many anticancer agents involves the disruption of DNA synthesis by interacting with critical enzymes, substrate-enzyme systems, or both.[10] The anticancer drug then causes the tumor to stop growing, having cytocidal character or die exhibiting a cytotoxic character. However, vivacious regenerating tissues such as bone marrow,

gastrointestinal epithelium and hair follicles are also effected by the nonspecific cytotoxicity.[10] Therefore, the need for meticulous pre-clinical evaluation of new anticancer drugs exists.

1.3.2 Anticancer Drugs and Toxicity

There are many anticancer drugs known, and most are categorized with respect to their proposed cellular mode of action and fall into one of the following groups: alkylating agents, antitumor antibiotics, plant alkaloids, antimetabolites, and hormonal agents. One of the most effective cytostatic agents used in the clinic is cis-platin. It was the first inorganic anticancer molecule introduced into the clinic, and has a remarkable potency against many forms of cancer. The mechanism of action of cis-platin is quite complex (see Chem. Rev. 87, 1987, pgs 1153-1181) and only a generalized explanation is given below.

The major cellular interaction of cis-platin involves the inhibition of DNA replication by coordinating to the N7 nitrogens of two adjacent guanine residues; this is called intrastrand crosslinking (Figure 2). Only the cis- form is active because it is believed that chelation to the DNA is necessary for cytotoxicity. Inside the cell the chloride ligands are substituted with water molecules forming a very reactive species which is believed to bind to the DNA. Thus one could picture the first aquo ligand being displaced by a guanine residue, rotation occuring about the guanine-Pt bond and then the second aquo being displaced by an adjacent guanine nucleobase. Conversely, trans-platin chelation to the DNA (i.e. intrastrand crosslinking) is not possible for steric reasons, and no cytotoxic activity is apparent. Furthermore, due to this apparent chelation, the

DNA substrate is altered and replication is no longer possible. In a similar sense, cis-platin is believed to induce interstand crosslinking of the DNA but to a much smaller extent.

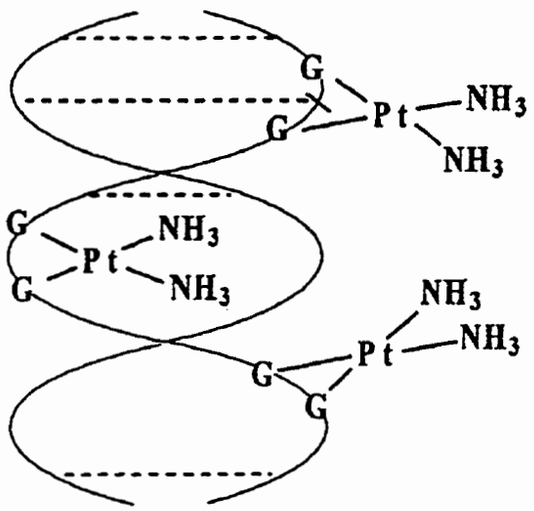


Figure 2 - Intrastrand Cross-link of DNA by Cis-Platin

The major drawback of using this compound and other cytotoxic substances is their inherent organ toxicity. The major dose-limiting toxicities of cis-platin are nephrotoxicity, bone marrow toxicity and neurotoxicity. Efforts to ameliorate this problem have led researchers to develop second generation platinum compounds that include carboplatin, spiroplatin and iproplatin (Figure 3). The spectrum of activity of these compounds resembles that of cis-platin, but with a markedly reduced nephrotoxicity. The primary objective of the third generation cis-platin analogues was to reduce the phenomenon of acquired cis-platin resistance. Some third generation cis-platin analogues include compounds where the chloride ligands have been substituted by steroid molecules. Future

development of second and third generation cis-platin analogues may provide the first orally administered platinum anti-cancer drugs.

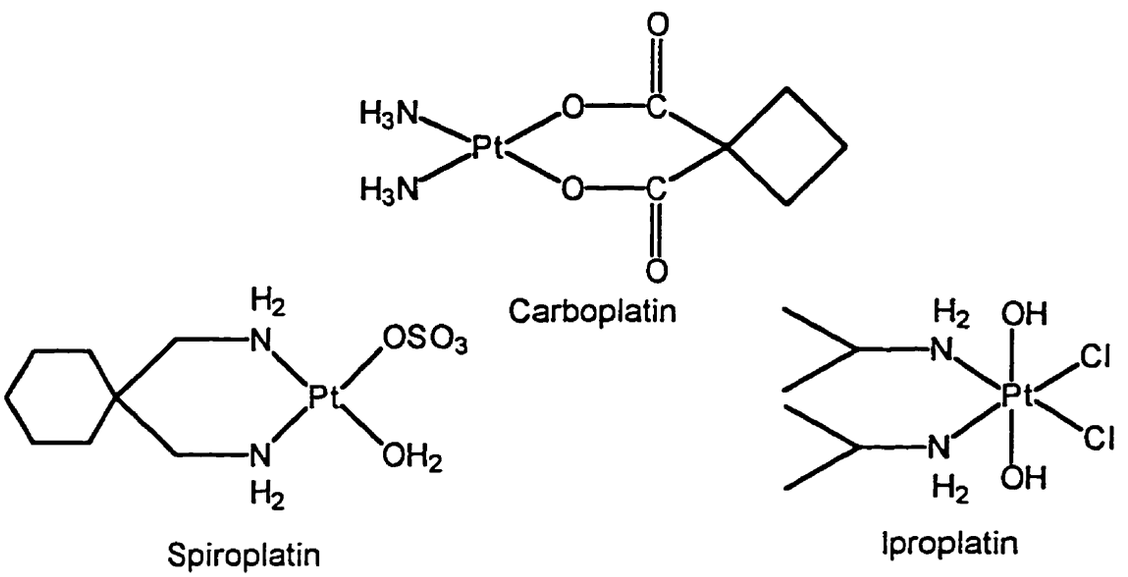


Figure 3 - Second generation cis-platin analogues

Although these compounds have efficacy against various forms of cancer, most anticancer drugs have little to no activity against colorectal cancer.[4,11] Thus fundamentally different cytotoxic agents must be developed to treat these and other cancers where no treatment is available.

1.4 Titanium Antitumor Compounds

1.4.1 Octahedral Titanium Compounds

Another class of cytostatic agents was discovered in the 1980's, prompted by the need for **non-platinum** antitumor drugs to treat a broader spectrum of tumors and a reduced toxic effect on the body. The first inorganic non-platinum

antitumor compound introduced into early clinical trials was a bis(β-diketonato) metal complex, cis-diethoxybis(1-phenylbutane-1,3-dionato)titanium(IV) (budotitane) shown in Figure 4.[12]

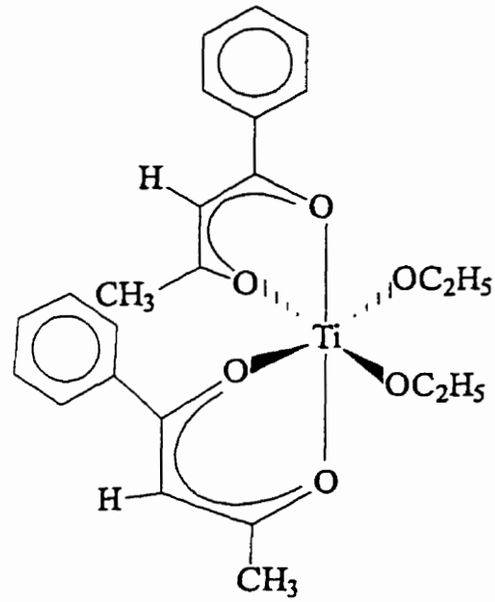
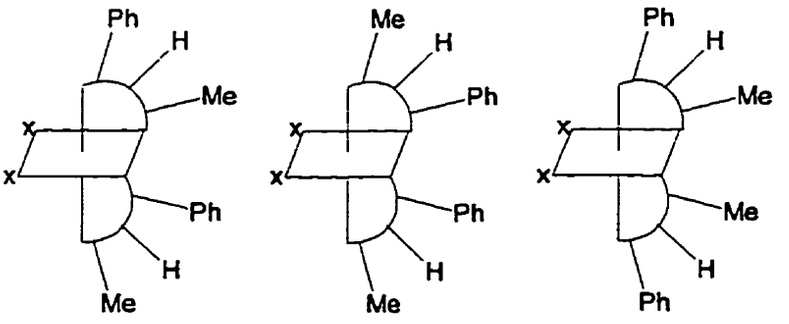


Figure 4 – Structure of Budotitane

This molecule was shown to be the most potent drug from a large arsenal of derivatives and showed the best activity against many types of cancers.[12-15] Although this compound was not as effective as cis-platin, its organ toxicity was significantly less, thus making it an excellent candidate for clinical use.

One obvious similarity between budotitane and cis-platin is the cis-conformation of the anionic ligands necessary for anticancer activity. However, the cis-conformers of this titanium drug are chiral. There exists three enantiomeric pairs of the cis-conformers and two for the trans (Figure 5).[11]



cis-cis-cis cis-cis-trans cis-trans-cis

Figure 5 – Enantiomeric Forms of Budotitane

The room temperature behavior of budotitane is that of a fluxional geometry between the three enantiomeric pairs arising from a "twist mechanism" and chirality was only observed (VT-NMR) at lower temperature.[11] This attribute may or may not be important to the antitumor activity of budotitane.

Another similarity between cis-platin and budotitane is that water hydrolyzes the cis-ligands (chloride of cis-platin and alkoxy of budotitane, refer to Figure 1 and 4 resp.) of the two compounds (refer to Figure 4) over time. This was more of a problem with budotitane due to the oxophilicity of titanium compared to platinum. Due to the sensitivity and solubility of budotitane to water, a formulation was made which minimized hydrolytic decomposition.[11]

1.4.2 Pseudotetrahedral Metallocene Dihalides

A metallocene is an organometallic compound containing two σ-bonded uninegative anionic ligands (X) and two η^5-bonded anionic, aromatic, cyclopentadienyl (Cp) rings attached to a central metal atom M in a pseudo

tetrahedral geometry (Figure 1). The central metal atom M is widely variable (M=Ti, Zr, Hf, Nb, V, etc...) and most are oxidatively and thermally stable. Metallocenes with titanium and vanadium in the +4 oxidation state will be discussed in this work as they showed the best antitumor activity.

1.4.3 Aqueous Chemistry of Titanocene Dichloride

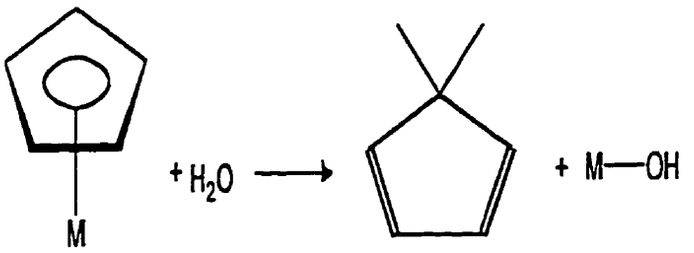
Similar to budotitane, titanocene dichloride is also susceptible to water hydrolysis. The displacement of the chloride ligands with water is illustrated in the following equations.

$$Cp_2TiCl_2 + 2H_2O$$
$$\rightleftharpoons$$
$$[Cp_2TiCl(OH_2)]^+ + Cl^- + H_2O$$
$$\rightleftharpoons$$
$$[Cp_2Ti(OH_2)_2]^{2+} + 2Cl^-$$
$$\rightleftharpoons$$
$$Cp_2TiOH(OH_2)^+ + H^+ + 2Cl^-$$
$$\rightleftharpoons$$
$$1/2[Cp_2(H_2O)Ti{-}O{-}Ti(OH_2)Cp_2]^{2+} + H^+ + 2Cl^- + 1/2H_2O$$

Scheme 1 – Aquation of Titanocene Dichloride

The first chloride displacement is very fast and the second chloride hydrolysis occurs with a half-life of around 25–45 minutes. For comparison purposes the half-lives for chloride hydrolysis in cis-platin are 154 and 890 minutes respectively.[16] This seems to agree with the fact that titanium is more oxophilic than platinum.

Another aspect of the aqueous chemistry of titanocene dichloride is protonolysis of the Cp ligands, as shown in the following diagram.



Scheme 2 - Protonolysis of Aromatic Cp Ligand

According to Toney and Marks, cyclopentadiene does not appear for days in unbuffered, low-pH solutions having the chloride concentration present in the human plasma (103 mM).[16] However, increasing the pH to 5.5 greatly increases Cp protonolysis. The initial rate of Cp protonolysis occurs with a rate constant of 6.8×10^{-3} $hour^{-1}$ at pH=7.5 while the second is lost over a period of days. The hydrolysis products of titanocene dichloride make clinical application difficult and must be corrected with galenic formulations.

1.4.4 Substituted Metallocene Dichlorides

It is possible to substitute one or more hydrogen atoms on the cyclopentadienyl ring of a metallocene with functional group (R). Since the Cp ring is aromatic, a multitude of substituted metallocene products can be directly accessible. This is true with ferrocene, as alkyl, formyl, acyl, and sulfonyl substitution products can be synthesized directly.[17] Some acetyl ferrocenes are shown in Figure 6.

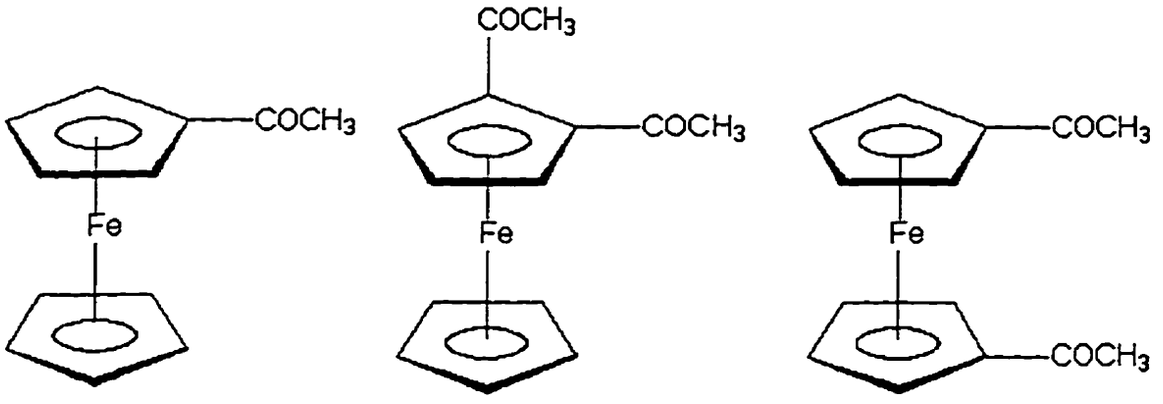
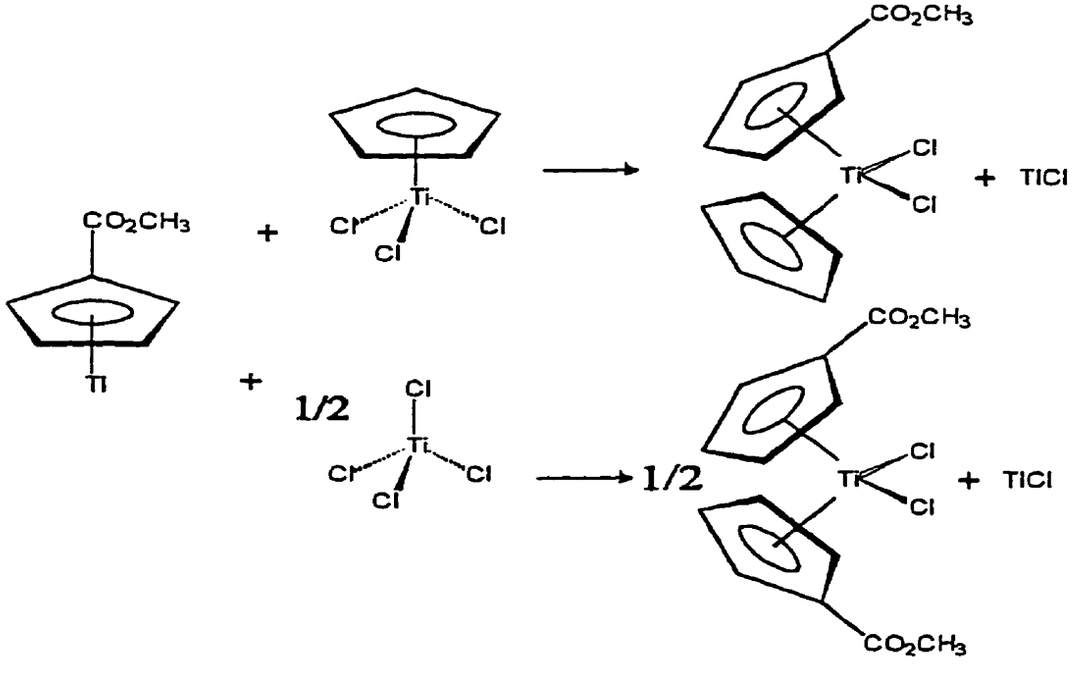


Figure 6 - Some Polar Substituted Ferrocenes

Other methods of preparation of the substituted metallocenes include the separate synthesis of the substituted cyclopentadienyl salt and then reaction with the metal chloride, either $CpTiCl_3$ or $TiCl_4$ depicted below.



Scheme 3 – Synthetic Routes to the Substituted Metallocene Dichlorides

Substituted cyclopentadienide anions with an electron withdrawing carbomethoxy substituent are generally more air stable than the unsubstituted dienide anion.[18] This may be due to a delocalization of electron density onto the substituent group forming resonance stabilized enolate anions shown in figure 6.

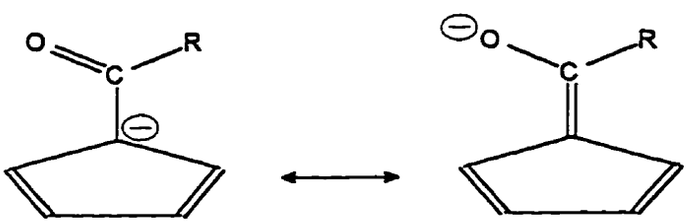


Figure 7 - Resonance Stabilization of Carbomethoxy Group, R=OMe

Bearing this fact in mind, and owing to the oxophilic nature of titanium (IV), very few substituted titanocene dichlorides with oxygen containing, polar functionalities, are known.

However, a large spectrum of non-polar substituted titanocene dichlorides is known, and the following substituted titanocene dichloride compounds have shown antitumor activity *in vivo*.[5]

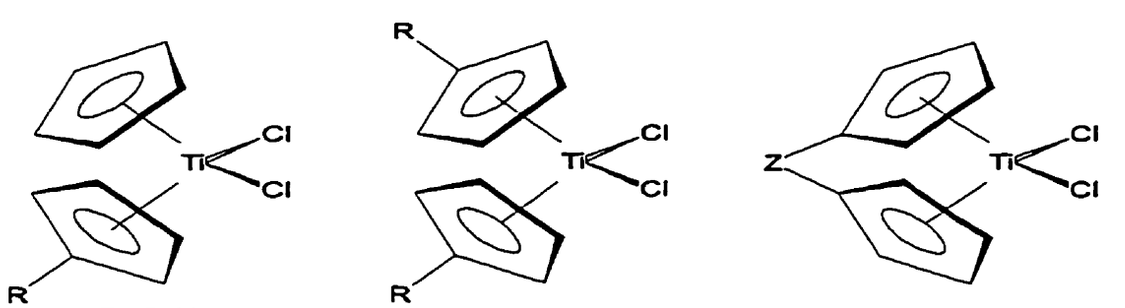


Figure 8 - Substituted Metallocene Dichlorides with Antitumor Properties

1.5 *In vitro* Testing of Titanocene and Vanadocene Dichloride

1.5.1 Chemosensitivity Assays and MTT

In vitro assays involve the culturing of tumor cells in a petri dish and then selectively applying protocols where the cell density and length of experiment are relevant to the drug being tested. The MTT assay, designed by Mossman[19] and adapted by Cole[20] and then Campling[21] , is a simple colorimetric test used for the preclinical screening of anticancer drugs against human cancer cell lines. This assay is practical because of its simplicity, and some researchers have shown correlations between *in vitro* drug responsiveness and clinical response with a variety of cancer lines and lung cancer. Furthermore, this assay has been used for the chemosensitivity screening of new anticancer drugs.[22] The data obtained from the MTT assay are absorbance values (lower values indicate cytotoxicity), and these are plotted versus drug concentration. The drug activity is summarized as a Dose Response Curve from which the Inhibitory Concentration 50 (IC_{50}) is calculated (see Dose Response Curve 1, p.53). The IC_{50} value is the drug concentration inducing 50% cell death. Another term used to described the dose activity relationship is the Inhibitory Dose 50 (ID_{50}), where a 50% reduction in cell growth is apparent.

1.5.2 Ehrlich Ascites Tumor

The anti-proliferative properties of titanocene and vanadocene dichloride have been measured using animal Ehrlich ascites, human KB and human HeLa tumor cells. These compounds were also tested against human embryonic fibroblasts (non-cancerous). The results are expressed as an ID_{50}. These values and one for the efficacy of cis-platin are shown in Table 1.[5]

CELL LINE	COMPOUND	ID_{50} (mol/L)
Ehrlich ascites tumor cells	Titanocene Dichloride	4×10^{-4}
Ehrlich ascites tumor cells	Vanadocene Dichloride	3×10^{-6}
Ehrlich ascites tumor cells	Cis-platin	8×10^{-7}
Human KB tumor cells	Titanocene Dichloride	3×10^{-4}
Human KB tumor cells	Vanadocene Dichloride	3×10^{-6}
Human HeLa tumor cells	Titanocene Dichloride	4×10^{-4}
Human HeLa tumor cells	Vanadocene Dichloride	4×10^{-6}
Human embryonic fibroblasts	Titanocene Dichloride	5×10^{-4}
Human embryonic fibroblasts	Vanadocene Dichloride	3×10^{-6}

Table 1 - *In vitro* Activities of Titanocene Dichloride, Vanadocene Dichloride and Cis-Platin

The experimental conditions used for each experiment can be found elsewhere.[5,23] These results did not show specificity towards animal or human tumor cells or towards normal, non-transformed cells. Moreover, the potency of cis-platin is 500 times greater than titanocene dichloride and about 10 times greater than vanadocene dichloride against Ehrlich ascites tumor cells. This result is surprising considering the pattern of *in vivo* activity (see below). According to Kopf *et al*, either metabolic activation or extensive hydrolytic

degradation of titanocene dichloride at low concentration levels in the aqueous culture medium may be responsible factors.[5] The *in vitro* activity of titanocene dichloride is much less pronounced than that of cis-platin, whereas the *in vivo* activity is more comparable. Thus a brief look at the *in vivo* activity will follow.

1.6 *In vivo* Testing of Titanocene and Vanadocene Dichloride

1.6.1 *In vivo* Experiments and Statistical Information

In vivo testing can be done several ways. The most common is by growing both fluid and solid tumor cells in the peritoneal cavity of a mouse and then injecting the drug directly to this site. Another method involves transplanting the human tumor into nude mice and applying the drug via i.v. or intra muscular techniques. The *in vivo* results obtained from treatment of **solid** tumors can be expressed as a ratio of treated responses vs control animals (T/C). However, the tumor growth **inhibition** is generally used and is calculated by 100%-(T/C), where T/C = the mean weight of the tumors from a treated groupx100/mean weight of the tumors from a control group. For fluid tumors, e.g. sarcoma 180 and leukemias L1210 and P388, the activity is expressed as a percent Increase in Life Span (ILS) calculated by the median survival time of the treated animalsx100/median survival time of the control animals. The fluid Ehrlich Ascite Tumor results with the metallocenes are expressed as cure rates calulated by the number of treated animals alive at the end of the experimentx100/the number of animals treated. Finally, when more than 90% of

the animals alive at the end of the experiment, a Therapeutic Index (T.I.) was calculated, where T.I. = LD_{10}/ED_{90}.

1.6.2 Fluid Ehrlich Ascites Tumor

The optimum dose range of titanocene dichloride was found to be 40-60 mg/kg.[23,24] Since more than 90% of the mice lived, the therapeutic index (T.I.) was calculated to be 3.3. Furthermore, Kopf *et al* showed that substitution of the two chlorides of titanocene dichloride with two bromides increased the T.I. of the metallocene to 4.5.[25] Similarly, substituting the two chloride ligands of titanocene dichloride with two carboxylates groups increased the T.I. to 5.5, the best T.I. for all the metallocenes tested.[26]

However, Kopf *et al* showed that modifying the cyclopentadienyl ligands, as shown in Table 2, significantly decreased the activity. The cure rates as well as the LD_{50} and LD_{100} values are shown in Table 2.[5] The maximum cure rate, listed at 80% for all the substituted cyclopentadienyl compounds, was exhibited by the trimethylsilyl derivative. In addition, the toxic dose range (LD_{50} and LD_{100}) of this compound was approximately equal to titanocene dichloride. Similarly, the activity is mostly lost with the disubstituted and bridged species, but the LD_{50} doses were significantly greater when compared to the more active titanocene dichloride. Thus it appeared that the degree of modification of the cyclopentadienyl ring was proportional to a loss in anticancer activity. This seems logical if the mechanism of action of the metallocenes is similar to cis-

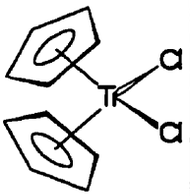
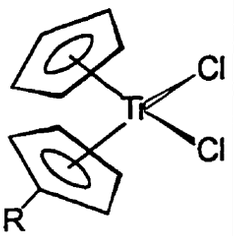
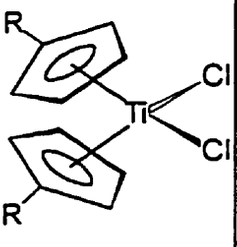
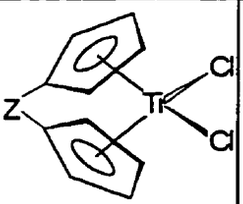
	Compound	Optimum Dose Range (mg/kg)	Optimum Cure Rate (%)	LD_{50} (mg/kg)	LD_{100} (mg/kg)
		40-60	100	100	140
	$R=C_2H_5$	50-70	60	100	120
	$R=Si(CH_3)_3$	40-70	80	100	120
	$R=N(CH_3)_2$	40-60	30	70	90
	$R=Si(CH_3)_3$	280-300	20	360	460
	$R=Si(CH_3)_2n-C_4H_9$	320-360	20	420	500
	$R=Ge(CH_3)_3$	240-320	28	400	460
	$R=N(CH_3)_3$	80-160	10	200	260
	$Z=CH_2$	220-260	13	360	440
	$Z=CHCH_3$	200-260	10	320	380
	$Z=SiHCH_3$	160-220	30	300	400
	$Z=Si(C_2H_5)$	140-200	15	220	300
	$Z=Ge(CH_3)_2$	220-260	13	280	360

Table 2 - *In vivo* Activity of Metallocene Dichlorides Against Ehrlich Ascites Tumor

platin, i.e. chelation to the DNA would be sterically hindered. However, the mechanism of action of the two molecules are not likely the same because of the soft Lewis acid character of platinum compared to the hard Lewis acid character of titanium. In any case, it seemed apparent that improving the potency of titanocene dichloride by substituting a proton on the Cp ring with a non-polar, electron donating group was not possible.

1.6.3 Leukemias L1210 and P388

Lymphoid leukemias (L1210 and P388) are standard fluid cell lines from the National Cancer Institute and are used for the screening of new anticancer agents. These tumor lines are sensitive to most anticancer agents but resistant to many alkylating agents such as vincristine, and organometallic metal complexes of gold, copper, and germanium.[5]

The activities of titanocene and vanadocene dichloride were markedly less pronounced against these leukemias in comparison to Ehrlich Ascites Tumor.[23] After a single (optimum) dose of titanocene dichloride, an ILS of 26% was observed with L1210 and 30% with P388. Similarly, vanadocene dichloride induced an optimum ILS of 29% for L1210 and 24% for P388.

1.6.4 Small Cell Lung Cancers

The tumour growth inhibition of titanocene dichloride were also significant against lung cancers L182 and L261.[27] The growth suppression also persisted beyond the end of the treatment period. In comparison, cis-platin and

cyclophosphamide, an anti-cancer drug used for the treatment of lung cancer, caused a similar effect.[28]

1.6.5 Human Colorectal Carcinomas

Human adenocarcinomas derived from the colon are generally insensitive to common cytostatic agents and only a few drugs are of clinical importance for the treatment of this disease.[5] For example, fluorouracil, the best anti-cancer drug known against colorectal cancer, has a response rate of only 21% and cis-platin exhibited a response rate of only 1%.[29] New cytostatic agents under development for the treatment of this form of cancer include the metallocenes, and early preclinical screening of titanocene dichloride showed excellent tumor inhibition.[5] In particular, the CX1 tumor, which is a standard human tumor from the National Cancer Institute, was suppressed by 50-70%. Furthermore, the growth inhibitions of the tumors persisted for several weeks after the treatment period. In comparison, cis-platin and fluorouracil did not induce similar growth suppressions as titanocene dichloride. Only at LD_{10} doses did these compounds slow down tumor proliferation. This result was quite exciting because this tumour line is multidrug resistant.

1.6.6 Organ Distribution and Toxicity

The most important feature of titanocene dichloride, aside from its broad spectrum of cytotoxicity, is reduced organ toxicity compared to cis-platin at

optimal drug doses. The toxicity of titanocene dichloride was determined by administering ED_{90} doses (40mg/kg) to normal mice.[30] For comparison purposes, the ED_{90} value of cis-platin (8mg/kg) was used.[28,31] ED_{90} doses of cis-platin causes severe nephrotoxicity. Equivalent doses of titanium do initially accumulate in the kidneys, but do not damage the renal function. The content of titanium in the kidneys decreases after several hours and the concentration of titanium in the liver increases. The serum content of some typical liver enzymes increased, reflecting damage to liver cells. However, this effect was reversible after 4 days. With higher doses of titanocene dichloride, single cell necroses of liver cells was apparent but disappeared after 16-32 days.[5]

1.7 Aims of Research

The major goal of this research was to improve the cytotoxic behaviour of titanocene dichloride *in vitro*. This may possibly be addressed by improving the solubility of Cp_2TiCl_2 in an aqueous medium. Replacing a proton on one or both Cp ligands with a polar functionality should provide products that are more amenable to chemotherapeutic testing. However, the literature contains very few examples of polar substituted titanocene dichlorides. Most examples are of late transition metallocenes of Fe, Co, Mn, Ni, Ru, Os and Re.[17] The two compounds to be assessed are $Cp(C_5H_4CO_2Me)TiCl_2$ and $(C_5H_4CO_2Me)_2TiCl_2$. These molecules are synthesized by first generating the substituted cyclopentadienide salt, which is reacted with $CpTiCl_3$ and $TiCl_4$ respectively.

Upon successful synthesis and purification, these compounds are tested against the small cell lung cancers NCI-H209, NCI-H69 and JN-M. The cytostatic activity is measured using a standard MTT-assay and compared to the parent compound, titanocene dichloride, under identical conditions. Further comparisons are made with a galenic formulation of titanocene dichloride, MKT4, (Medac GmbH), and with cis-platin.

CHAPTER TWO - EXPERIMENTAL

2.1 Physical and Analytical Methods

2.1.1 ^{1}H Nuclear Magnetic Resonance Spectroscopy

^{1}H NMR samples were prepared using ~5 mg solid and ~0.5 mL dry, deoxygenated deuterated solvent filtered (celite) into 5 mm o.d. NMR tubes. The FIDs were collected on a Bruker AM-200 with a dual $^{1}H/^{13}C$ probe at 200.132 MHz. Each spectrum was internally referenced to the residual protons of the deuterated solvents with respect to TMS. Thus chemical shifts reported as postive numbers are downfield from TMS.

2.1.2 IR Spectroscopy

Solution samples were prepared using dry, deoxygenated solvent and syringed into a sodium chloride cell. Solid samples were prepared using dry KBr and pressed into a pellet. The scans were recorded on a Bruker IFS 25 FT IR.

2.1.3 UV Spectroscopy

Absorbance readings for the MTT-assays were made using a Dynatech MR 600 microtitre plate spectrometer at 570nm.

2.1.4 Air Sensitive Manipulations

All air and moisture sensitive reagents were handled under a nitrogen atmosphere using standard Schlenk Line techniques. The nitrogen was deoxygenated and dried by passing it over a BASF catalyst and then over type 4Å molecular sieves. Air sensitive solids were manipulated and stored in a glove box.

2.2 Chemical Supplies

2.2.1 NMR Solvents

All solvents were obtained from Aldrich, MSD Isotopes and C/D/N Isotopes. $CDCl_3$ was dried using excess calcium hydride and filtered under a nitrogen atmosphere. DMSO-d_6 was dried by refluxing in the prescence of calcium hydride for 2 days and then fractionally distilled. Acetone-d_6 was dried using type 4Å molecular sieves.

2.2.2 Drying of Solvents

THF, benzene and hexanes were dried by refluxing over sodium ribbon. Benzophenone was added to form the blue benzophenone ketyl which is sensitive to oxygen and water. CH_2Cl_2 was dried by refluxing over CaH_2.

2.2.3 Other Chemicals Used

$TiCl_4$ (Aldrich), dicyclopentadiene (Aldrich), and N,N-dimethylhydroxyamine hydrochloride (Aldrich) were used without further purification. $CpTiCl_3$ (Aldrich) was sublimed at 40°C and collected as bright yellow crystals before use. Triethylamine (Fisher Scientific) was distilled and then dried with CaH_2 under nitrogen before use. Dimethyl Carbonate (Aldrich) was distilled under nitrogen before use. Distilled water was deoxygenated by bubbling nitrogen through the solution for 0.5 hours. Methane sulfonylchloride (Aldrich) was vacuum distilled under nitrogen before use.

2.2.4 HITES Medium

The medium was prepared from RPMI 1640 supplemented with hydrocortisone (10 nM, Sigma), insulin (10 mg/mL, Sigma), transferrin (10 μg/mL, Sigma), estradiol (10 nM, Sigma), and sodium selenite (30 nM). The medium was prepared and kept under sterile conditions.

2.3 Synthesis of Organic Reagents

2.3.1 Cracking of Dicyclopentadiene

This synthesis was following according to Buchannon *et al.* A 100 mL round bottom flask was attached to a condenser connected to a hot water source

(~50°C). To the round bottom flask was syringed 50.0 mL (49.3 g, 0.373 moles) of dicyclopentadiene. The condenser was connected to a distillation head and then to three collection flasks cooled to -78°C with dry ice in isopropanol. The dicyclopentadiene was heated and the fraction which boiled at 48°C was collected into a 50 mL Schlenk equipped with a side-arm. Approximately 40 mL of cyclopentadiene was collected at -78°C.

2.3.2 Synthesis of N,N-dimethyl-O-(methylsulfonyl)hydroxylamine

This synthesis was modified from the Boche *et al.*[32] N,N-Dimethylhydroxyamine hydrochloride (0.500 g, 5.13 mmole) was added (in glove box) to a 3-neck 500 mL round bottom flask connected to a dropping funnel. Methanesulfonylchloride (0.587 g, 0.397 mL, 5.13 mmole) was added to 10 mL of deoxygenated CH_2Cl_2 in the dropping funnel. To the round bottom flask was added 10 mL deoxygenated CH_2Cl_2. A yellow suspension resulted. The suspension was cooled to -20°C and triethylamine (2.14 mL, 15.4 mmoles) was syringed into the stirring suspension. A white precipitate was immediately formed. The suspension was allowed to warm to -15°C and the methanesulfonylchloride solution was added over 1.5 hours. After complete addition of methanesulfonylchloride 10 mL cold H_2O was added to the reaction flask. The white precipitate dissolved and the organic layer was collected using a separatory funnel. Another 5 mL H_2O was added to the organic layer and the aqueous layer was discarded. The solvent was removed under reduced pressure and a slightly yellow/colorless oil resulted.

^{1}H NMR ($CDCl_3$): δ 2.84 (6H, s, N-Me_2) δ 2.76 (3H, s, O-Me).

2.3.3 Attempted Synthesis of N,N-Dimethyl-1,3-cyclopentadieneamine

A 500 mL 3-neck round bottom flask containing the methylsulfonamide oil (146.87 g) was weighed. THF (10 mL) was added to the round bottom flask and then the solution was syringed into a 250 mL round bottom flask. The remaining solvent in the 500 mL flask was removed under reduced pressure and the flask was reweighed (147.34 g). Thus 0.47 g (3.4 mmoles) of the methylsulfonamide was added to a stirring solution of Cp^-Li^+ (0.243 g, 3.38 mmole) at -20°C. A red/brown solution resulted. After 1 hour the solvent was removed at -20°C under reduced pressure. A brown solid remained and 20 mL pentane (-40°C) was added to dissolve the diene. The solid did not dissolve and the pentane was removed under reduced pressure. According to the literature, a yellow oil is characteristic of the N,N-dimethyl-1,3-cyclopentadienylamine. It was obvious that this was not apparent and further characterization was not attempted.

2.4 Synthesis of Cyclopentadienyl Compounds

2.4.1 Synthesis of (Cyclopentadienyl)sodium

A 250 mL round bottom flask was dried and placed under nitrogen using standard techniques. THF (250 mL) was deoxygenated using three freeze-thaw-degass cycles and was syringed into the round bottom flask. The round bottom

flask was cooled to -78°C with dry ice in isopropanol. To this solution was added 35.0 mL (28.2 g, 0.427 moles) of freshly cracked cyclopentadiene. Next, ~24 g of 44% sodium dispersion was syringed into the stirring solution. A pinkish/orange suspension was stirred for 0.5 hours at room temperature. The solvent was removed under reduced pressure resulting in a white solid (31.52 g, 83% yield) that was stored in the glove box.

^{1}H NMR (DMSO-d_6): δ 5.36 ppm (s, 5H, H(1-5)).

Lit. (DMSO-d_6): δ 5.35

2.4.2 Synthesis of (Carbomethoxycyclopentadienyl)sodium

In the glove box, 10.00 g (0.114 moles) of Cp^-Na^+ was placed in a 3 neck 500 mL round bottom flask equipped with a pressure equalizing side arm and a condenser. To this solution was added 100 mL of deoxygenated THF. Next, 27.7 mL (30.8g, 0.341 moles) of freshly distilled dimethylcarbonate was syringed into the stirring solution. After 10 minutes at room temperature, the reaction mixture was refluxed for 2 hours. When the mixture cooled to room temperature, the solvent was removed under reduced pressure. A beige, air sensitive solid remained (7.82 g, 47.0%) and was stored in the glove box.

^{1}H NMR (DMSO-d_6): δ 6.07 (t, 2H, H(2,5)), δ 5.48 (t, 2H, H(3,4)), δ 3.44 ppm (s, 3H, CH_3)).

Lit: (D_2O, ext. TMS): δ 6.56 (M, 2H, H(2,5)), δ 6.06 (m, 2H, H(3,4)), δ 3.75 ppm (s, 3H, CH_3).[33]

2.4.3 Synthesis of (Carbomethoxycyclopentadienyl)thallium

Cp-$CO_2Me^-Na^+$ (4.78 g, 0.0325 moles) was dissolved in 90 mL THF. The solution was cooled to -20°C and 1.25 mL of degassed distilled water was added dropwise through an addition funnel. The mixture was stirred for 5 hours. The color changed from clear to light brown. Next, Tl_2SO_4 (7.89 g, 0.0162 moles) and 17.56 g of KOH were dissolved in 125 mL H_2O and added to the reaction mixture. A white precipitate formed and the reaction was stirred overnight. The mixture was filtered and the solid washed first with 20 mL H_2O then 20 mL ether and finally with 7 mL absolute ethanol. The white solid was re-washed (poor ^1H NMR purity in DMSO-d_6) with 20 mL H_2O then 20 mL ether and finally 10 mL absolute ethanol. A gray solid (6.32 g, 59.5% yield) remained on the frit.

^1H NMR (DMSO-d_6): δ 6.29 (t, 2H, H(2,5)), δ 5.70 (t, 2H, H(3,4)), δ 3.53 ppm (s, 3H, CH_3).

Lit. (DMSO-d_6): δ 6.39 (t, 2H, H(2,5)), δ 5.78 (t, 2H, H(3,4)), δ 3.58 ppm (s, 3H, CH_3).[34]

2.5 Synthesis of Substituted Metallocene Dichlorides

2.5.1 Synthesis of (η^5-Methoxycarbonylcyclopentadienyl)(η^5-cyclopentadienyl)dichlorotitanium (JB1)

$Tl^+CpCO_2Me^-$ (2.00 g, 6.09 mmol) was dissolved in 50 mL degassed THF in a 200 mL 2-neck round bottom flask attached to an addition funnel. $CpTiCl_3$ (1.33 g, 6.09 mmol) was placed in the addition funnel and dissolved in 20 mL of degassed THF. The $CpTiCl_3$ solution was added over 30 minutes. A red solution and a white precipitate resulted immediately and the reaction was allowed to stir overnight. The mixture was filtered and the solvent was removed under reduced pressure. The remaining dark red solid was filtered and washed with hexanes (~30 mL) until the filtrate was colorless. Next, the solid (0.429 g, 1.40 mmol, 23.0% yield) was dissolved in hot CH_2Cl_2 (~10 mL) and then placed in the freezer overnight. A deep red solid (61 mg) was filtered at 0°C and finally the solvent was removed under reduced pressure at room temperature.

^{1}H NMR ($CDCl_3$): δ 7.08 (t, 2H, H(2,5)), δ 6.62 (s, 5H, Cp), 6.54 (t, 2H, H(3,4)), δ 3.88 ppm (s, 3H, CH_3).

^{1}H NMR Lit. ($CDCl_3$): d 7.14 (t, 2H, H(2,5)), d 6.65 (s, 5H, Cp), d 6.65 (t, 2H, H(3,4)), d 3.91 ppm (s, 3H, CH_3).[34]

IR (KBr): 1721s, 1156s cm^{-1}

IR Lit. (KBr): 1722s, 1153s cm^{-1}[134]

2.5.2 Synthesis of Bis(η^5-methoxycarbonylcyclopentadienyl) dichlorotitanium (JB2)

Into a 3-neck 100 mL round bottom flask equipped with a pressure equalizing side arm was added $Tl^+Cp\text{-}CO_2Me^-$ (0.2982 g, .9101 mmol) Deoxygenated benzene (10 mL) was syringed into the flask. Next, $TiCl_4$ (0.0864 g, 0.0501 mL, 0.455 mmol) was quickly syringed into the stirring benzene solution (note: CAUTION* $TiCl_4$ is extremely moisture sensitive!!!). After 24 hours of stirring the solvent was removed under reduced pressure. A brown/red solid remained and was transfered to a Soxhlet thimble in the glove box. Next, 80 mL deoxygenated hexanes was syringed into the apparatus through a septum on the condenser. Then the septum was replaced with a ground glass pressure equalizing adapter. The extraction was continued for 4 hours, until the filtrate went from yellow to colorless. The round bottom flask was replaced with another containing 80 mL deoxygenated CH_2Cl_2. The extraction was continued for 3 hours, until the filtrate went from red to colorless. The solvent was removed under reduced pressure and a bright red solid (45.1 mg, 0.124 mmol, 27.2% yield) remained.

^{1}H NMR ($CDCl_3$): δ 7.15 (t, 2H, H(2,5)), δ 6.54 (t, 2H, H(3,4)), δ 3.88 ppm (s, 3H, CH_3).

Lit. ($CDCl_3$): δ 7.20 (t, 2H, H(2,5)), δ 6.58 (t, 2H, H(3,4)), δ 3.91 ppm (s, 3H, CH_3).[34]

IR (KBr): 1720s, 1160s cm^{-1}

Lit. (KBr): 1720s, 1158 cm^{-1} [34]

2.6 Attempted Ester Hydrolysis of (η^5-Methoxycarbonylcyclopentadienyl) (η^5-cyclopentadienyl)dichlorotitanium

2.6.1 With Concentrated HCl

$Cp(C_5H_4CO_2Me)TiCl_2$ (30 mg, mmole) was added to a 250 mL Schlenk. Concentrated HCl (15 mL) was added to the Schlenk and the solution was allowed to stir of 1 hour. A red solution remained. The HCl was removed under reduced pressure and a red solid remained.

^{1}H NMR ($CDCl_3$): δ 7.08 (t, 2H, H(2,5)), δ 6.62 (s, 5H, Cp), δ 6.54 (t, 2H, H(3,4)), δ 3.88 ppm (s, 3H, CH_3).

These signals corresponded to the starting material, signifying that no reaction had occurred.

2.6.2 With 60% v/v HCl/H_2O

$Cp(C_5H_4CO_2Me)TiCl_2$ (30 mg, mmole) was added to a 50 mL Schlenk. Concentrated HCl (2.0 mL) was added to 4.0 mL H_2O and this solution was

added to the Schlenk and stirred for 4 hours. A red suspension resulted and the solvent was removed under reduced pressure and a red solid remained.

^{1}H NMR ($CDCl_3$): δ 7.08 (t, 2H, H(2,5)), δ 6.62 (s, 5H, Cp), δ 6.54 (t, 2H, H(3,4)), δ 3.88 ppm (s, 3H, CH_3).

These signals corresponded to the starting material, signifying that no reaction had occurred.

2.6.3 With Concentrated H_2SO_4

$Cp(C_5H_4CO_2Me)TiCl_2$ (13 mg, mmole) was added to a 100 mL Schlenk. Concentrated H_2SO_4 (7.5 mL) was syringed into the Schlenk and the mixture was allowed to stir for 2 days. The solution was placed under reduced pressure but this failed to remove the liquid as the boiling point was too high. Precipitation of the desired product was attempted with CH_2Cl_2 but a two phase system resulted. An uncharacterizable acid phase remained.

2.6.4 With Triethylamine

$Cp(C_5H_4CO_2Me)TiCl_2$ (20 mg, 0.0652 mmole) was dissolved in 20 mL MeOH. Triethylamine (0.10 mL, mmole) was added dropwise to the stirring solution. The mixture was refluxed for 5 hours at 100°C. The solvent was removed under reduced pressure.

^{1}H NMR ($CDCl_3$) δ 3.07 (q, 2H, CH_2) δ 1.38 ppm (t, 3H, CH_3).

These signals are likely due to coordinated triethylamine to the titanium metal, signifying the desired products were not produced.

2.6.5 With CF_3COOH

$Cp(C_5H_4CO_2Me)TiCl_2$ (10 mg, 0.0326 mmole) was dissolved in 10 mL CH_2Cl_2. Trifluoroacetic acid (0.50 mL, 0.74 g, 6.5 mmole) was added to 10 mL CH_2Cl_2 and 5 mL of this solution was syringed into the stirring solution at room temperature. The mixture was allowed to stir overnight and an orange/yellow solution resulted. The solvent was removed under reduced pressure.

^{1}H NMR ($CDCl_3$): δ 7.10-6.51 (mult.), δ 3.92-3.66 ppm (mult.).

These signals did not correspond to the desired metallocene product.

2.7 General Procedure for MTT Assay

2.7.1 Preparation of SCLC Cells

SCLC cells were obtained from Iva Kosatka in a 100 mL test tube. It was centrifuged for 4 minutes and the supernatant was discarded. The pellet was shaken and the cells were resuspended in 5.0 mL HITES. The cells were disaggregated using a 21 gauge needle by drawing the solution up and down three times. Two quadrants in the hemacytometer were counted and the cells

were diluted to make a final cell density of 2.5×10^5 cells/well. Thus, if 170 cells were counted and 30 mL of cells required, 4.4 mL would have to be added to 25.6 mL HITES. The cells were dispensed into the 96 well microtitre plate using a 100 µL multichannel pipette according to the following schematic. 100 µL of HITES was added to the wells with no cells. The plates were incubated (37°C in a humidified atmosphere with 5% CO_2).

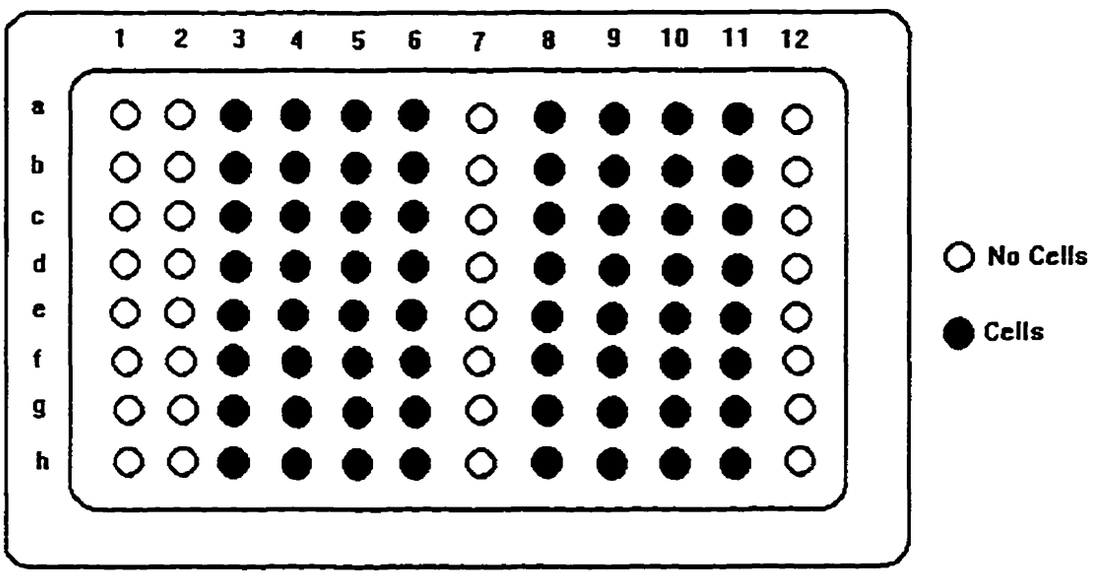


Figure 9 - 96 Well Microtitre Plate Depicting the Position of Cells

2.7.2 Drug Dilution

The drug (8 mgs) was weighed and placed into a dry glass vial. Acetone (2.0 mL) was added into the vial and a deep red solution resulted. From this solution was taken 0.20 mL and added to 1.80 mL of HITES medium resulting in a concentration of 400 µg/mL. A bright yellow solution resulted. Three ten fold dilutions were made resulting in concentrations of 40 µg/mL, 4 µg/mL and 0.4 µg/mL. Next, 0.64 mL of the 400 µg/mL solution was added to 1.46 mL HITES to

make a concentration of 130 μg/mL. Two more ten fold dilutions were made from this solution resulting in final concentrations of 13 μg/mL and 1.3 μg/mL.

2.7.3 Chemosensitivity Studies

Four 100 μL aliquots of each drug concentration were added to the cells approximately 16 hours after incubation in the following arrays: 400 μg/mL (3a-3d, 8a-8d), 130 μg/mL (3e-3h, 8e-8h), 40 μg/mL (4a-4d, 9a-9d), 13 μg/mL (4e-4h, 9e-9h), 4.0 μg/mL (5a-5d, 10a-10d), 0.13 μg/mL (5e-5h, 10e-10h) and 0.40 μg/mL (6a-6d, 11a-11d). Also, 100 μL of the 400 μg/mL solution was added to 2a and 7a (no cells) to serve as a control for the absorbance readings. The final concentrations were 200, 64, 20, 6.4, 2.0, 0.64 and 0.20 μg/mL. After addition of the drugs the plates were incubated for 4 days.

2.7.4 Development of the Plates

The cells were inspected for viability and amount. Then 100 μL of medium was carefully removed from each well using a multichannel pipette. After this procedure the plate was inspected again to insure the amount of cells remained constant. Next, MTT (25 μL) was added to each well using a multichannel pipette and the cells were incubated. After 6 hours, 100 μL of 1 N HCl:isopropanol (1:24) was syringed into each well using a multichannel pipette and mixed vigorously using a 300 μL multichannel pipette to dissolve the formazan crystals. After complete mixing the plate was placed in the hot room

(37°C for 1/2 hour). Finally, the absorbance readings were made on a Dynatech MR 600 microtitre plate spectrometer and the data printed. The plate was discarded and the data were transcribed into MS Excel.

2.8 MTT Data of Acetone Control

The following tables contain the absorbance data obtained from the acetone controls using the MTT-assay. The absorbance is measured at 570 nm, the λ_{max} of the dissolved formazan crystals.

2.8.1 NCI-H209

This experiment was performed exactly as described in Section 2.7 with the exception that no drug was in the glass vial.

Compound Tested=Acetone
Cell Line=H209

Conc.	#1	2	3	4	5	6	7	8	average	S.D.
200	0.894	0.900	0.911	0.852	0.895	0.784	0.792	0.698	0.8408	0.0759
64	1.013	0.949	0.981	1.047	0.928	0.996	1.036	1.038	0.9985	0.0435
20	0.897	0.941	0.963	0.936	0.875	0.927	0.894	0.941	0.9218	0.0299
6.4	1.011	1.040	0.963	1.138	1.006	1.060	1.047	1.072	1.0421	0.0521
2.0	1.038	1.026	0.959	0.911	1.002	1.024	1.013	1.020	0.9991	0.0429
0.64	0.975	0.971	1.011	1.055	1.006	1.060	1.047	1.072	1.0246	0.0393
0.20	0.967	0.993	0.916	0.927	1.060	1.045	0.973	1.121	1.0003	0.0702
0	1.004	1.077	1.268	1.351	1.397	1.408	1.506	1.499	1.3138	0.1862

2.8.2 NCI-H69

Compound Tested=Acetone
Cell Line=H69

Conc.	Trial #1	#2	#3	#4	#5	#6	#7	#8	average	S.D.
200	1.105	1.293	1.228	1.277	1.035	1.204	1.19	1.217	1.193625	0.085933
64	1.102	1.228	1.184	1.221	1.081	1.099	1.124	1.187	1.15325	0.058441
20	0.938	1.207	1.153	0.88	1.138	1.289	1.269	1.261	1.141875	0.154236
6.4	1.033	1.228	1.162	1.246	1.156	1.197	1.153	1.11	1.160625	0.067701
2	1.138	1.25	1.231	1.235	0.893	1.254	1.217	1.21	1.1785	0.12094
0.64	1.105	1.207	1.265	1.156	1.074	1.177	1.089	1.084	1.144625	0.068542
0.2	0.881	1.207	1.257	1.281	1.011	1.181	1.184	1.19	1.149	0.134828
0	1.239	1.099	1.328	1.306	1.228	1.273	1.214	1.217	1.238	0.07009

2.8.3 NCI-JNM

Compound Tested=Acetone
Cell Line=JN-M

Conc.	#5	#6	#7	#8	averag	S.D.
200	0.477	0.58	0.562	0.556	0.544	0.046
64	0.584	0.572	0.532	0.451	0.535	0.06
20	0.595	0.651	0.606	0.633	0.621	0.025
6.4	0.624	0.65	0.589	0.494	0.589	0.068
2	0.536	0.638	0.603	0.685	0.616	0.063
0.64	0.557	0.608	0.537	0.526	0.557	0.036
0.2	0.581	0.635	0.611	0.605	0.608	0.022
0	0.659	0.735	0.736	0.643	0.693	0.049

2.9 MTT Data of Titanium Containing Compounds

The following tables contain the absorbance data obtained from the MTT-assays of the various titanocene dichloride compounds. The absorbance is measured at 570 nm, the λ_{max} of the dissolved formazan crystals.

2.9.1 Drug=Tdc Cell Line=NCI-H209

Compound Tested=Tdc
Cell Line=H209

Conc.	#1	2	3	4	5	6	7	8	average	S.D.
100	0.775	0.875	0.936	0.992	0.455	0.691	0.612	0.623	0.7449	0.1832
32	0.943	1.007	1.056	1.018	0.640	0.695	0.857	0.959	0.8969	0.1542
10	0.617	0.800	0.773	0.883	0.483	0.727	0.720	0.957	0.7450	0.1482
3.2	0.813	0.921	0.977	0.975	0.861	0.963	0.910	0.943	0.9204	0.0582
1.0	0.472	0.686	0.715	0.788	0.459	0.623	0.821	0.758	0.6653	0.1376
0.32	0.784	0.876	0.804	0.724	0.765	0.896	0.674	1.058	0.8226	0.1199
0.10	0.408	0.535	0.662	0.703	0.401	0.513	0.637	0.663	0.5653	0.1186
0	0.706	0.722	0.788	0.893	0.710	0.818	0.827	0.776	0.7800	0.0657

Compound Tested=Tdc
Cell Line=H209

Conc.	#1	2	3	4	5	6	7	8	average	S.D.
100	0.775	0.875	0.936	0.992	0.455	0.691	0.612	0.623	0.744875	0.183178
32	0.943	1.007	1.056	1.018	0.640	0.695	0.857	0.959	0.896875	0.154196
10	0.617	0.800	0.773	0.883	0.483	0.727	0.720	0.957	0.745	0.148215
3.2	0.813	0.921	0.977	0.975	0.861	0.963	0.910	0.943	0.920375	0.058165
1.0	0.472	0.686	0.715	0.788	0.459	0.623	0.821	0.758	0.66525	0.137583
0.32	0.784	0.876	0.804	0.724	0.765	0.896	0.674	1.058	0.822625	0.119887
0.10	0.408	0.535	0.662	0.703	0.401	0.513	0.637	0.663	0.56525	0.118556
0	0.706	0.722	0.788	0.893	0.710	0.818	0.827	0.776	0.78	0.065729

Compound Tested=Tdc
Cell Line=H209

Conc.	#1	2	3	4	5	6	7	8	average	S.D.
200	0.666	0.690	0.624	0.690	0.745	0.770	0.698	0.723	0.7008	0.0456
64	0.813	0.904	0.826	0.608	1.016	0.718	1.085	0.907	0.8529	0.1651
20	0.980	1.051	0.978	1.010	1.027	0.947	1.120	0.870	0.9979	0.0741
6.4	0.870	0.921	0.887	0.783	0.848	0.653	0.906	0.760	0.8285	0.0908
2.0	0.738	0.789	0.799	0.833	0.698	0.496	0.930	0.766	0.7547	0.1357
0.64	0.947	0.904	0.873	0.750	0.830	0.918	0.859	0.728	0.8511	0.0782
0.20	0.768	0.863	0.909	0.860	0.896	0.890	0.873	0.801	0.8656	0.0466
0	0.978	1.117	1.053	0.934	0.986	1.030	1.053	0.930	1.0101	0.0647

2.9.2 Drug=MKT 4 Cell Line=NCI-H209

Compound Tested=MKT 4
Cell Line=H209

Conc.	#1	2	3	4	5	6	7	8	average	S.D.
100	0.237	0.189	0.200	0.209	0.195	0.237	0.227	0.249	0.2179	0.0225
32	1.184	1.215	1.076	1.111	1.039	1.025	1.066	1.013	1.0911	0.0741
10	1.002	0.329*	1.219	1.184	0.943	1.264	1.114	1.184	1.1300	0.1178
3.2	1.315	1.310	1.272	1.108	1.204	1.184	1.198	1.046	1.2046	0.0947
1.0	0.957	1.229	1.170	1.233	0.977	1.073	1.158	1.146	1.1179	0.1059
0.32	1.229	1.276	1.377	1.086	1.108	1.215	1.289	1.034	1.2018	0.1165
0.10	1.000	1.103	1.000	1.068	1.066	1.020	1.092	1.022	1.0464	0.0409
0	1.069	1.097	1.256	1.079	1.105	0.996	1.168	0.891	1.0826	0.1085

***omitted from average value**

2.9.3 Drug=MKT 4 Cell Line=NCI-H69

Compound Tested=MKT 4
Cell Line=H69

Conc.	Trial #1	#2	#3	#4	#5	#6	#7	#8	average	S.D.
200	0.155	0.144	0.144	0.147	0.144	0.136	0.118	0.144	0.1415	0.010823
64	0.393	0.667	0.661	0.66	0.405	0.66	0.668	0.688	0.60025	0.124586
20	0.839	0.988	1.029	1.085	1.124	1.092	1.092	1.151	1.05	0.099453
6.4	1.12	1.029	0.983	1.162	1.088	1.11	1.062	1.162	1.0895	0.062819
2	1.018	0.953	1.068	1.085	1.039	1.092	1.029	1.117	1.050125	0.05182
0.64	1.051	0.985	1.021	1.127	0.99	1.051	1.051	1.062	1.04225	0.045137
0.2	1.095	1.021	0.988	1.062	0.974	0.969	0.978	0.953	1.005	0.05012
0	1.259	1.042	1.085	1.184	1.162	1.085	1.107	1.241	1.145625	0.078624

2.9.4 Drug=MKT 4 Cell Line=NCI-JNM

Compound Tested=MKT 4
Cell Line=JN-M

Conc.	Trial #1	#2	#3	#4	#5	#6	#7	#8	average	S.D.
200	0.154	0.15	0.154	0.127	0.157	0.165	0.17	0.166	0.155375	0.013416
64	0.244	0.229	0.246	0.244	0.281	0.263	0.249	0.29	0.25575	0.020686
20	0.434	0.473	0.437	0.466	0.458	0.439	0.421	0.446	0.44675	0.017597
6.4	0.585	0.528	0.557	0.565	0.509	0.489	0.589	0.542	0.5455	0.035472
2	0.529	0.609	0.568	0.595	0.523	0.549	0.537	0.598	0.5635	0.03384
0.64	0.624	0.664	0.615	0.626	0.554	0.545	0.615	0.603	0.60575	0.039067
0.2	0.574	0.57	0.615	0.637	0.544	0.426	0.592	0.538	0.562	0.064296
0	0.742	0.758	0.773	0.733	0.695	0.75	0.752	0.75	0.744125	0.022987

2.9.5 Drug=JB1 Cell Line=NCI-H209

Compoud Tested=JB1
Cell Line=H209

Conc.	#1	2	3	4	Average	St.Dev.
200	0.091	0.082	0.081	0.074	0.0820	0.0070
41.5	0.151	0.184	0.188	0.230	0.1883	0.0324
20	0.578	0.597	0.572	0.591	0.5845	0.0115
4.15	0.601	0.587	0.624	0.644	0.6140	0.0252
2.0	0.652	0.666	0.645	0.754	0.6793	0.0506
0.415	0.645	0.639	0.610	0.641	0.6338	0.0160
0.20	0.449	0.621	0.608	0.698	0.5940	0.1045
0.02	0.681	0.642	0.658	0.627	0.6520	0.0231

Compound Tested=JB1
Cell Line=H209

Conc.	#1	2	3	4	5	6	7	8	average	S.D.
200	0.071	0.074	0.083	0.116	0.114	0.077	0.093	0.103	0.0914	0.0179
64	0.390	0.357	0.393	0.389	0.299	0.275	0.299	0.422	0.3530	0.0547
20	0.522	0.587	0.529	0.485	0.470	0.553	0.605	0.478	0.5286	0.0504
6.4	0.550	0.590	0.657	0.534	0.555	0.526	0.728	0.699	0.6049	0.0790
2.0	0.614	0.603	0.579	0.495	0.659	0.683	0.655	0.577	0.6081	0.0598
0.64	0.603	0.639	0.704	0.739	0.680	0.632	0.685	0.779	0.6826	0.0582
0.20	0.650	0.597	0.604	0.500	0.685	0.616	0.712	0.566	0.6163	0.0672
0	0.683	0.782	0.860	0.829	0.696	0.754	0.770	0.797	0.7714	0.0606

2.9.6 Drug=JB2 Cell Line=NCI-H209

Compound Tested=JB2
Cell Line=H209

Conc.	#1	2	3	4	5	6	7	8	average	S.D.
200	0.084	0.089	0.123	0.125	0.140	0.127	0.142	0.062	0.1115	0.0293
64	0.113	0.184	0.230	0.321	0.139	0.268	0.267	0.389	0.2389	0.0925
20	0.316	0.322	0.340	0.352	0.485	0.414	0.425	0.411	0.3831	0.0597
6.4	0.321	0.358	0.437	0.618	0.432	0.476	0.516	0.639	0.4746	0.1132
2.0	0.337	0.295	0.300	0.332	0.553	0.456	0.549	0.471	0.4116	0.1084
0.64	0.330	0.456	0.509	0.697	0.654	0.732	0.754	0.841	0.6216	0.1731
0.20	0.261*	0.234*	0.241*	0.261*	0.554	0.586	0.607	0.598	0.5863	0.0232
0	0.478*	0.637	0.818	1.053	1.149	1.127	1.213	1.181	1.0254	0.2159

***omitted from average value**

Chapter Three

Results and Discussion

3.1 General

The results obtained in this work are divided in two parts:

i) the synthesis and purification of the organometallic reagents and

ii) the biological testing of the titanocene dichloride compounds.

3.2 Synthesis of Ligands

3.2.1 Synthesis of Sodium Carbomethoxycyclopentadienide

The synthesis of this compound was done according to literature methods in 49% yield.[35,36] The ^{1}H NMR (DMSO-d_6) of the product showed two triplets: δ 6.07 (t, 2H, H(2,5)) and δ 5.48(t, 2H, H(3,4)) and one singlet: δ 3.44(s, 3H, CH_3). The literature ^{1}H NMR spectrum was done in D_2O/TMS and exhibited two multiplets at δ 6.56 (m, 2H, H(2,5)) and 6.06 (M, 2H, H(3,4)) and one singlet at 3.75 ppm (s, 3H, CH_3). It is likely that the respective solvents would cause different electronic effects on the cyclopentadienyl salt, producing different chemical shift values. However, the ^{1}H NMR of $Tl^+C_5H_4CO_2Me^-$ (synthesized from the compound described here) was done in the same solvent as that described in the literature and was practically identical. Thus it is likely that the ^{1}H NMR of $Na^+C_5H_4CO_2Me^-$ in DMSO-d_6 was correct. The only difficulty encountered was accurate integration of the two triplets. This arises because the near planar Cp protons have long relaxation times. The correct integration

was achieved by increasing the Relaxation Delay (RD) setting on the spectrometer to 5 seconds.

The desired polar substituted Cp ring has been synthesized, but this molecule was shown to be a poor reagent in the reaction with titanium chlorides. Rausch *et al* reported that the reaction of $Na^+C_5H_4CO_2Me^-$, with $TiCl_4$, in benzene produced an intractable product. Similarly, they showed that an analogous reaction with $CpTiCl_3$, produced $Cp(C_5H_4CO_2Me)TiCl_2$, but only in 22% yield. This seems a little strange since it is well known that the metathesis reactions of Na^+Cp^-, with $FeCl_2$, or CpTiCl3 proceed with an excellent yield to produce the repective metallocene. This synthetic dilemma was remedied by the substitution of sodium with thallium, for reasons that are as yet unclear but will be discussed in the next section.

3.2.2 Synthesis of Carbomethoxycyclopentadienylthallium

The synthesis of the thallium salt was modified from the procedure of Rausch *et al.*[34] Instead of thallium(I) chloride, which is sparingly soluble in water, thallium(I) sulfate (very soluble in water) was used. This modification allowed the use of a stirring bar instead of a blender. The purification step described in this synthesis was also modified from the literature where a double washing with water, ether and absolute ethanol produced the purified product.

The ^1H NMR (DMSO-d_6) showed two triplets at δ 6.29 (t, 2H, $H^{2,5}$) and 5.70 (t, 2H, $H^{3,4}$) and a singlet at δ 3.53 (s, 3H, CH_3). The literature values were 6.39, 5.78 and 3.58 ppm respectively (DMSO-d_6). The chemical shift values of

the two spectra differ only by ~0.1 ppm and this effect probably arises because of different experimental conditions (concentration) used to obtain the literature values.

The two triplet resonances correspond to an AABB spin system. The lower field triplet corresponding to the protons labeled $H^{2,5}$ (6.29ppm) are proposed[34] to be the pair close to the ester functionality due to the electron withdrawing effect of the carbonyl group. Thus the higher field triplet at 5.70 ppm corresponds to the remaining Cp protons (H(3,4)). Finally, the singlet at 3.53 ppm unambiguously corresponds to the methyl resonance of the ester group.

Comparing ^1H NMR spectra (both in DMSO-d_6) of $Tl^+C_5H_4CO_2Me^-$ and $Na^+C_5H_4CO_2Me^-$, it is apparent that exchanging sodium for thallium produced a downfield shift in all the protons. The triplet resonance of the thallium salt at 6.29 ppm was shifted downfield by 0.22 ppm compared to the sodium salt at 6.07 ppm. Similarly, the triplet resonance of the thallium salt at 5.70 ppm was shifted downfield by 0.22 ppm compared to the sodium salt at 5.48 ppm. Finally, the methyl resonance of the thallium salt at 3.53 was shifted downfield by only 0.09 ppm. The latter observation is most likely due to the number of bonds between the methyl protons and the Cp ring. However, the large 0.22 ppm shift may possibly be explained by the fact that thallium(I) has a more positive effective nuclear charge (Z_{eff}) and thus a stronger electron affinity than sodium(I). This seems logical because a shift to lower frequency of the Cp protons was

observed in the ^1H NMR. As a result, a tighter solvent separated ion pair may exist with the thallium salt compared to the sodium analogue. Due to this electronic effect, the thallium ion pair may possibly reduce the likelihood of formation of the resonance stabilized enolate ion, which may react with the highly oxophilic titanium(IV) center. Therefore, it seems plausible that $Tl^+C_5H_4CO_2Me^-$ reacts in better yields with titanium halides.

3.3 Synthesis of Polar Substituted Titanocene Dichlorides

3.3.1 Synthesis of (η^5-Methoxycarbonylcyclopentadienyl)(η^5-cyclopentadienyl)dichlorotitanium

The synthesis of the metallocene was achieved in good yield by modifying a procedure by Rausch *et al.*[34] Instead of using a Soxhlet extractor to remove any unreacted $CpTiCl_3$, the red solid was washed with hexanes. This proved useful as the Soxhlet extraction in hexanes removed much of the desired compound and a poor yield was obtained.[36] A clean ^1H NMR spectrum was obtained by one recrystallization in CH_2Cl_2 reflecting an acceptable degree of purity for the known compound.

The ^1H NMR spectrum showed two triplets at 7.08 and 6.54 ppm, both integrating to two protons, and a singlet at 6.62 ppm integrating to five protons. The literature chemical shift values were 7.14, 6.65, and 6.65 ppm respectively. Once more, it has been speculated that the downfield triplet (7.08 ppm) corresponds to the aromatic H(2,5) protons closest to the electron withdrawing

carbonyl functionality. Thus, the higher field aromatic triplet resonance at 6.62 ppm was assigned to H(3,4). The methyl ester singlet at 3.88 ppm correlated closely with the literature value of 3.91 ppm.

Comparing the aromatic triplet resonances of $Tl^+C_5H_4CO_2Me^-$ and $C_5H_4CO_2MeCpTiCl_2$, a dowfield shift was observed. This can explained by η^5-coordination to the electropositive titanium(IV) metal center.

The IR spectrum of the metallocene exhibited a strong C=O stretching frequency at 1721 cm^{-1} and a C-O stretching frequency at 1156 cm^{-1} (KBr). The respective literature values were 1722 and 1153 cm^{-1}.[36] Further characterization of this known metallocene was not necessary because of the unambiguous ^1H NMR and IR spectra.

3.3.2 Synthesis of Bis(η^5-methoxycarbonylcyclopentadienyl) dichlorotitanium

The synthesis of this compound was done according to literature methods.[34] The yield was extremely poor, 15%, which may in part, be due to the extreme sensitivity of $TiCl_4$ to water (i.e. syringing the $TiCl_4$ liquid using standard Schlenk techniques does not prevent extreme fuming from the syringe). This poor yield may also be attributed to large amounts of reaction byproducts, such as $(C_5H_4CO_2Me)TiCl_3$, $(C_5H_4CO_2Me)CpTiCl_2$, and more likely via a reaction between the carbonyl oxygen of $Tl^+C_5H_4CO_2Me^-$ with the titanium center. The latter reaction route would produce unwanted inorganic/organometallic mixtures

of varying complexity, i.e. one or more ligands would react with the titanium center.

Most of the unwanted compounds were removed by Soxhlet extraction with hexanes. The desired disubstituted species was then extracted with CH_2Cl_2 and then further purified by washing with hexanes because stopcock grease was still apparent in the ^1H NMR spectrum. The purified compound showed two ^1H NMR triplet resonances at 7.15 and 6.54 ppm (Lit.: 7.20 and 6.58 ppm) which were assigned (by arguments mentioned above) to H(2,5) and H(3,4) and one 1H NMR singlet resonance at 3.88ppm (Lit.: 3.91) corresponding to the methyl group. The IR spectrum showed a characteristic C=O stretching frequency at 1720 cm^{-1} and a C-O stretching vibration at 1158 cm^{-1} (KBr). The literature values (KBr) were 1720 cm^{-1} and 1158 cm^{-1} respectively. The ^1H NMR and IR values corresponded nicely with the literature, demonstrating proof of the successful synthesis of the disubstituted titanocene dichloride.

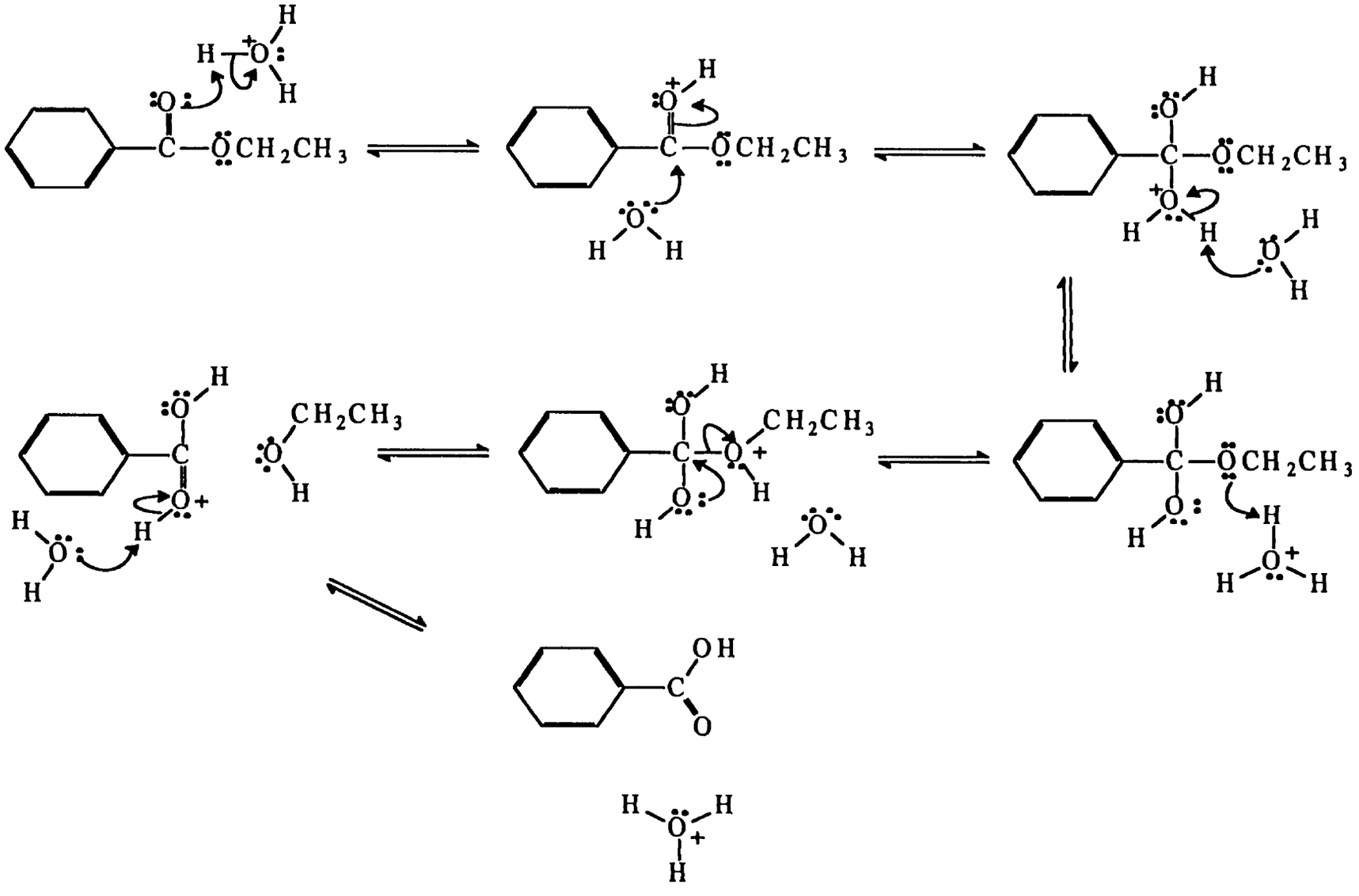
3.4 Attempts Made to Hydrolyze the Ester

The main goal of this work was to produce compounds more amenable to cancer testing. It was believed that this goal could be achieved by improving the water solubility of the metallocenes by placing polar substituents on the Cp ring. The above compounds, while being more polar than titanocene dichloride were not soluble in water. Thus attempts were made to hydrolyze the ester bond to produce the carboxylic acid derivative, an even more polar substituted titanocene dichloride.

It is well known[37] that ester hydrolysis can proceed either by a base catalyzed or an acid catalyzed mechanism. Due to the Lewis acidity character of the titanium metal center, acid catalyzed reactions were preferred; only triethylamine (a weak base) was used as a base. Additionally, attempts were not made using stronger bases to hydrolyze the ester because the titanocene is susceptible to extensive Cp-loss at pH~7 and higher.[16] The mechanism depicting the acid-catalyzed ester hydrolysis of ethyl benzoate is shown in Mechanism 1.[37] This mechanism should be analogous to the acid-catalyzed aromatic ester hydrolysis of the $C_5H_4CO_2Me$ ligand, the only difference being an ethyl group instead of a methyl and a benzene ring instead of a Cp ring. A convenient probe for this reaction is the appearance of an alcoholic resonance in the ^1H NMR. The successful hydrolysis would be apparent by a shifting of the methyl ester resonance to a methanolic resonance and the appearance of an acidic resonance at higher chemical shift (~10 ppm).

Concentrated HCl failed to produce the desired result. Indeed, the ^1H NMR of the reaction products did not differ from that of the starting material. Another experiment was done where a couple of drops of concentrated HCl were added to 4 mL of H_2O. This solution was syringed into a Schlenk containing the metallocene, but again, only the starting material was present in the reaction products. Additional experiments with concentrated H_2SO_4, CF_3CO_2H and $N(Et)_3$ did not hydrolyze the ester (^1H NMR) but did react with the titanium metal center to produce decomposition products. This result likely arises from the strong Lewis acid character of the titanium center, and further attempts to hydrolyze the

ester via base hydrolysis, i.e. NEt_3, or via acid hydrolysis using an oxygen containing acid should be avoided. It is certain though that the ether linkage is quite robust and more energetic conditions are necessary to cleave this bond.



Mechanism 1 - Acid-Catalyzed Hydrolysis of Ethyl Benzoate

3.5 *In Vitro* Cytotoxicity Studies of the Synthesized Titanocene Dichlorides

3.5.1 General – Drug Dilutions and Assay Protocols

The *in vitro* testing was done in an aqueous medium, which posed an immediate problem, as the titanocenes did not dissolve in water. However, all of the compounds were very soluble in acetone and it was chosen to solubilize the compounds for testing. Control experiments were done with acetone to measure the effect of acetone on cell viability.

Dissolving the metallocene in acetone and then adding a small volume of this solution to a pre-measured volume of aqueous medium produced the desired drug dilution. However, the inverse of this procedure, adding the aqueous media to the acetone solution, produced a white precipitate that was discarded in every case. This white precipitate may be oligomeric titanium species, titanium oxides, or compounds resulting from hydrolysis of either the chloride ligand(s) or protonolysis of the Cp ligand(s). In any case, it is not likely that the biologically active species is present in this precipitate.

The homogeneous drug solution was then added to the cancer cells and incubated for 4 days. This duration was chosen because of previously published results from Kopf-Maier *et al.*[38] In this publication, the authors showed that the cytotoxicity of a 90 minute exposure of titanocene dichloride (i.e. the cells were washed after 90 minutes, removing the drug from the medium) increased to a maximum after 3 days. This result may imply that the uptake of drug into the cells occurs within 90 minutes but cytotoxicity does not occur until 3 days post treatment. The metallocene activity was measured by the presence of formazan,

which absorbs in the uv spectrum at 570 nm. Thus number of viable cells is directly proportional to the amount of formazan. The results were displayed on a Dose Response Curve, where the drug concentration is plotted versus the % control absorbance. The IC_{50} values were measured from these plots where a 50% reduction in % control absorbance was observed (provided that at least one drug concentration produced 100% cell death).

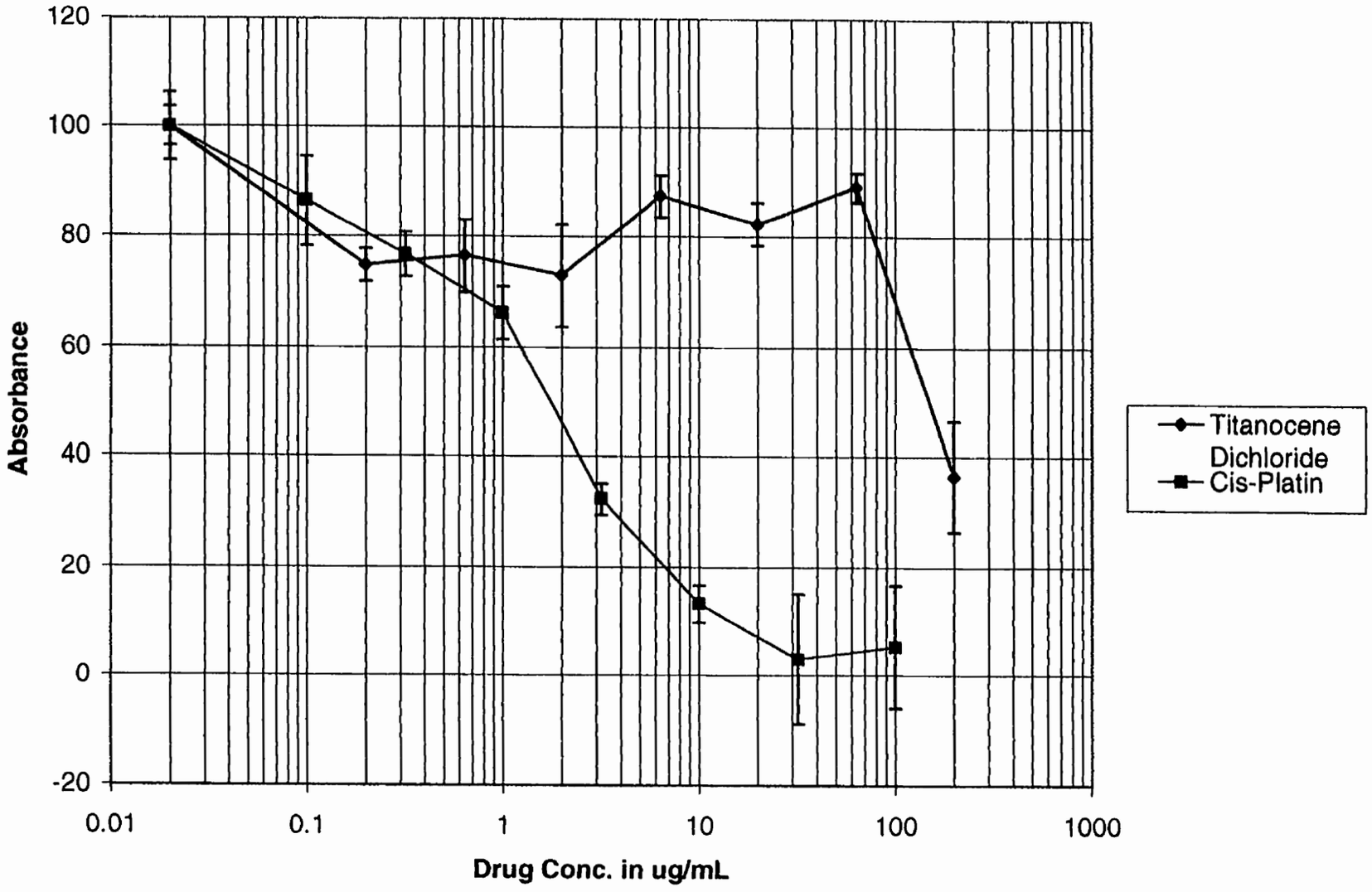
The two standards used in this study were titanocene dichloride and MKT 4. The former compound was used under identical conditions as the substituted titanocene dichlorides. The latter compound was used as an industry standard, and is a galenic formulation of titanocene dichloride in lactose solution. According to the literature available on this formulation, it seems that the lactose is necessary to facilitate the manipulation and application of titanocene dichloride in the clinic, i.e. an organic solvent is not necessary. The two ways of applying titanocene dichloride into a biological system exhibited two different activities *in vitro*. For simplicity, the words "titanocene dichloride" will refer to the *in vitro* test where acetone was used to dissolve the drug in the aqueous medium, and MKT 4 will refer to the galenic formulation of titanocene dichloride in lactose where only HITES medium was used.

3.5.2 Activity of Titanocene Dichloride *In vitro*

The first test was done to measure the activity of titanocene dichloride under identical conditions used for the testing of the polar substituted compounds. The result is compared to cis-platin and shown in Dose Response

Curve 1. Complete cytotoxic acitivity (~0% of control absorbency) was not apparent at a concentration of 200 μg/mL. In comparison, cis-platin showed complete cytotoxic activity at a concentration of 32 μg/mL. The IC_{50} value of titanocene dichloride was estimated as 150 μg/mL, because the highest concentration did not induce complete cytoxicity. This value is much greater than the IC_{50} value of cis-platin which was shown to be 2 μg/mL. Thus a 100-fold decrease in the potency of titanocene dichloride compared to the clinical standard cis-platin is apparent with this cell line. A similar result was also reported by Kopf-Maier *et al* when comparing the *in vitro* activity of titanocene dichloride.[5] This result is not consistant with the *in vivo* studies done with titanocene dichloride dissolved in DMSO. It has been speculated by Kopf-Maier *et al* that titanocene dichloride is metabolically activated *in vivo*, producing a drug potency only 10-fold less than cis-platin *in vivo*.[5] Another explanation was significant decomposition of titanocene dichloride at very low concentration *in vitro*. This decomposition may possibly arise from extensive Cp-loss producing mono-Cp and/or bridged titanium compounds that may be cytostatically inactive.

Other anti-cancer agents such as doxorubicin[39] , carboplatin, and spiroplatin also have different effective doses compared to cis-platin. For example doxorubicin is more potent than cis-platin whereas carboplatin is similarly potent to cis-platin, depending on the cell line. Furthermore, titanocene dichloride appears to be less active (~10x) *in vitro* compared to cis-platin. However, the *in vivo* studies with titanocene dichloride showed a similar biological effect only when a 10x increase in the dose of titanocene, compared



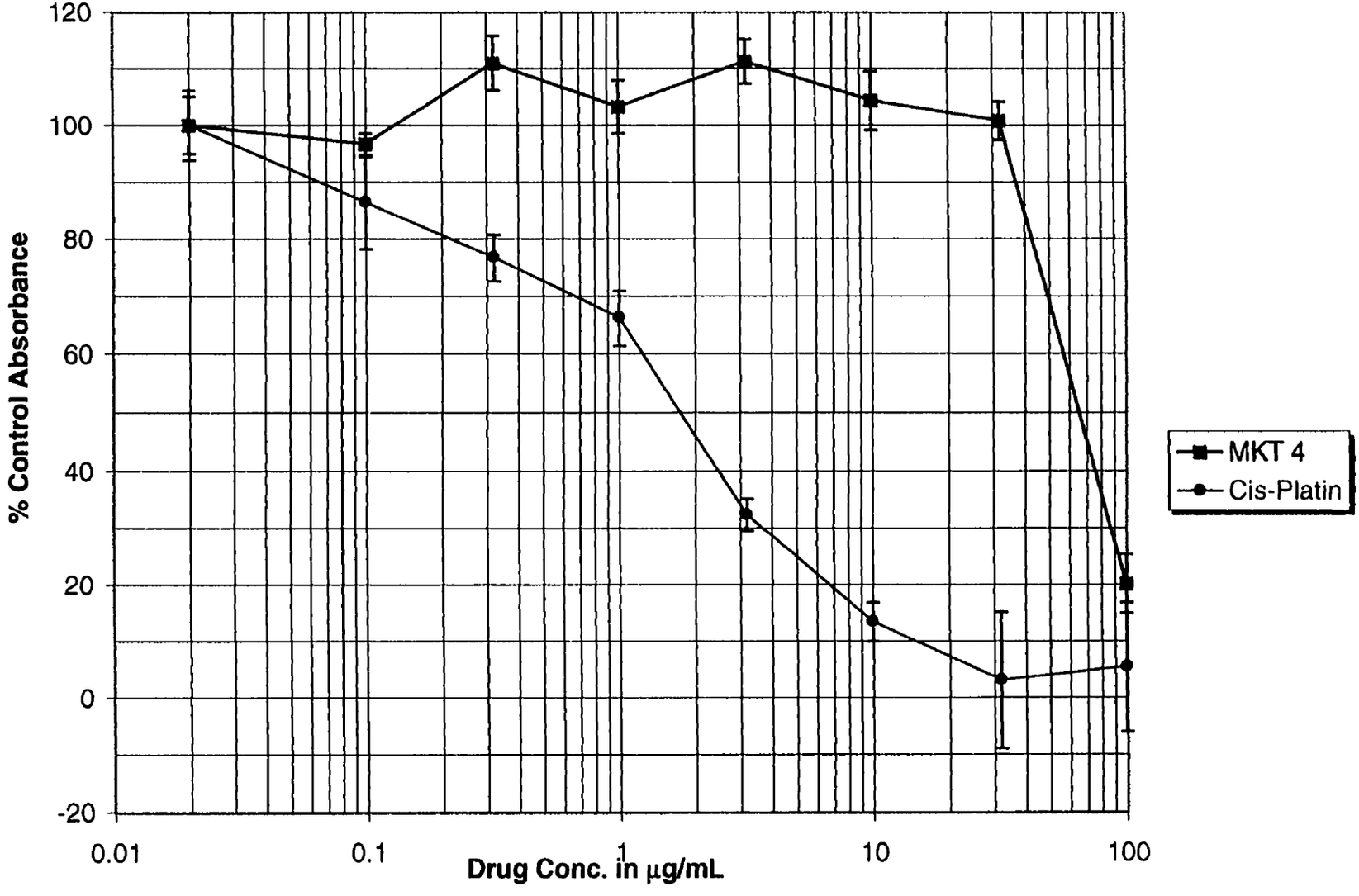
Dose Response Curve 1 - In Vitro Activity of Titanocene Dichloride and Cis-Platin Against SCLC NCI-H209

to cis-platin was given. This increased dose was tolerated *in vivo* due to the lower toxicity of titanocene dichloride compared to cis-platin.

The *in vivo* activities of zirconocene and hafnocene dichlorides were much less pronounced than titanocene dichloride but showed a similar activity *in vitro*.[38] However, comparing the *in vitro* zirconocene and hafnocene dichloride studies with the *in vitro* vanadocene dichlordie studies, a much less pronounced activity was apparent. This result was also consistent with the *in vivo* studies with the same metals. An explanation of this result was also ascribed to the increased lability of the Cp ligand in the trend V<Ti<Zr<Hf. This explanation makes sense except for the fact that the *in vivo* activity of titanocene dichloride was marginally better than vanadocene dichloride. This is likely due to different interactions with the respective cellular targets.

Next, the activity of MKT 4 was measured. After dissolving this compound in aqueous medium a bright yellow solution resulted. This colour was identical to the previously tested titanocene dichloride after addition to the aqueous medium. Again it is speculated that hydrolysis product(s) described in scheme 1 are being generated. This result is compared to cis-platin and is illustrated in the Dose Response Curve 2.

MKT 4 showed greater activity at lower concentrations than titanocene dichloride, and induced complete cytotoxic activity at a concentration of 100μg/mL. The IC_{50} value of MKT 4 was calculated as 65μg/mL and is 32 times less potent than cis-platin. This result shows that NCI-H209 is much more sensitive to cis-platin than to MKT 4.



Dose Response Curve 2 - In Vitro Activities of MKT 4 and Cis-Platin against SCLC NCI-H69

This result is interesting, as only a 10-fold decrease in potency of MKT 4 has been observed for ovarian cell lines, *in vitro*[39] and *in vivo*.[40] Furthermore, MKT 4 was shown to be effective *in vivo* against other Lewis Lung Carcinomas with doses only 10 times that of cis-platin.[28] In addition, it has been shown that titanocene is much less toxic to the kidneys than cis-platin and the 10-fold increase in dose is tolerated *in vivo*. Thus protecting the Cp rings from protonolysis has improved the *in vitro* activity of titanocene dichloride.

The increased sensitivity of MKT 4 compared to titanocene dichloride may possibly be explained by an increased stability of the Cp ring towards protonolysis in an aqueous medium. This protection may be ascribed to the formation of lactose micelles with an exterior hydrophilic surface and an interior hydrophobic pocket. The non-polar Cp rings would force the titanocene unit to the interior of the micelle, thus protecting the titanium metal center from nucleophilic water molecules.

Also, it was with this "Cp-protection/stabilization" theory in mind that prompted the testing of the bridged cyclopentadienyl metallocenes for anti-cancer activity. The Cp rings would be more stable by the well known chelate effect. Unfortunately this theory proved to be incorrect, as the *in vivo* studies showed that the bridged metallocenes showed a decrease in potency compared to titanocene dichloride.

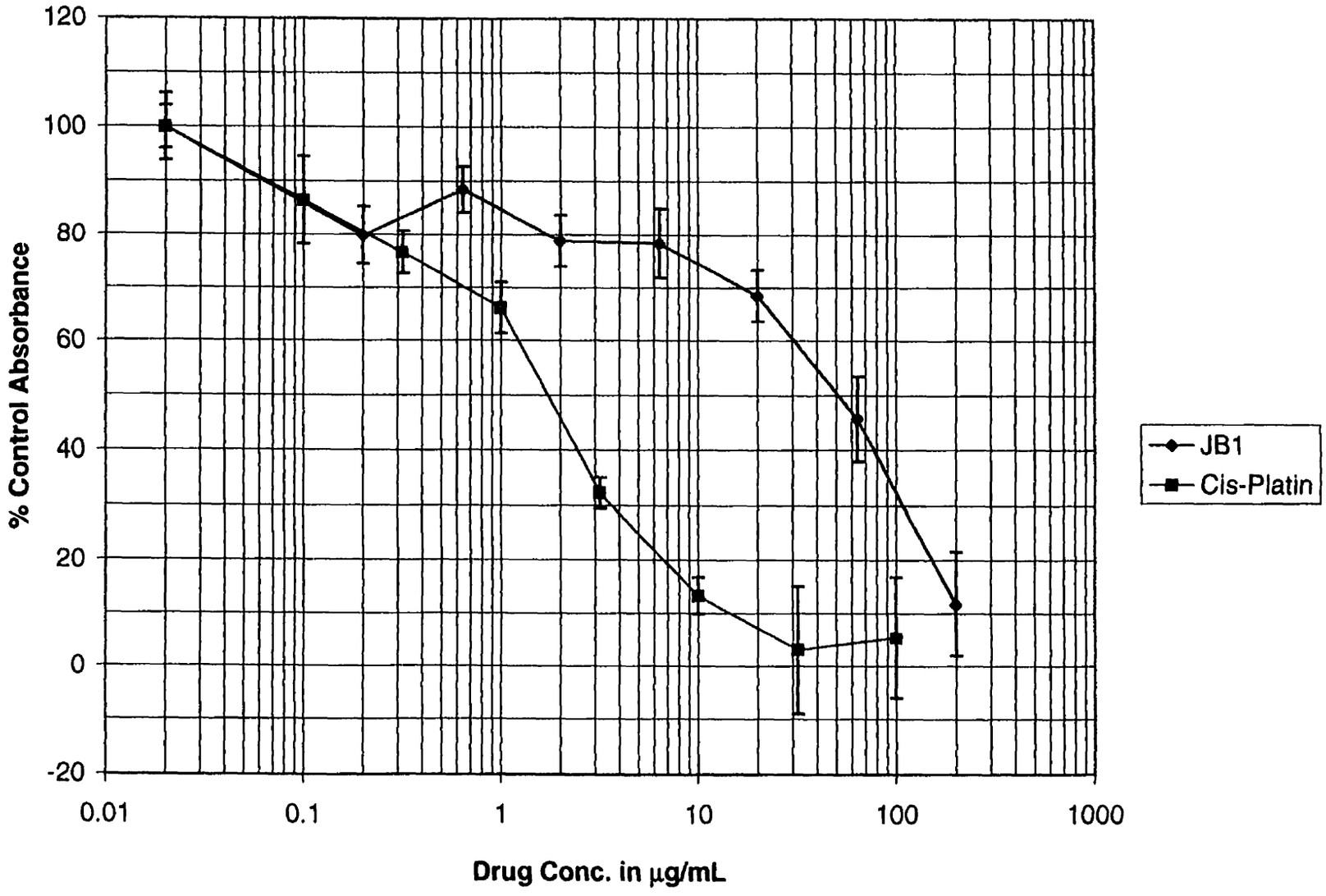
However, it was shown by Toney *et al* that very rapid hydrolysis of the first chloride ligand of titanocene dichoride was apparent and the second chloride hydrolysis occurs more slowly, exhibiting a half-life of 25-45 minutes.[16] It

seems likely that the observed yellow color may be attributed to a mono aqua or a mono hydroxy titanium species, the latter occuring by the loss of a proton from the water ligand. Thus some important aqueous chemical events of MKT 4 may be initial loss of one chloride ligand and one proton followed by incorporation of the intact titanocene unit into a lactose micelle. Furthermore, the micelle protects the Cp rings from protonolysis until the titanocene unit enters the cell via a passive or an active pathway.

3.5.3 *In vitro* Activity of (η^5-Methoxycarbonylcyclopentadienyl)(η^5-cyclopentadienyl)dichlorotitanium (JB1)

The next step was to test the two polar substituted titanocene dichlorides against NCI-H209. The monosubstituted compound was dissolved in acetone and diluted to the desired concentrations with HITES medium. A yellow color was apparent when the red acetone solution was added to the HITES medium. As mentioned above, this was probably due to rapid hydrolysis of a chloride ligand followed by loss of a proton from the water ligand. The cytotoxic activity of JB1 is illustrated in Dose Response Curve 3.

Compared to MKT 4, the monosubstituted compound showed a slight increase in potency. Cytotoxic activity is first observed at a drug concentration of 20 μg/mL, but 100% morbidity of the cells only occurred at the highest drug concentration. The IC_{50} value of the mono substituted compound was calculated to be 50 μg/mL, a moderate improvement compared to MKT 4 (65 μg/mL) and significantly better than titanocene dichloride (150 μg/mL) in acetone.



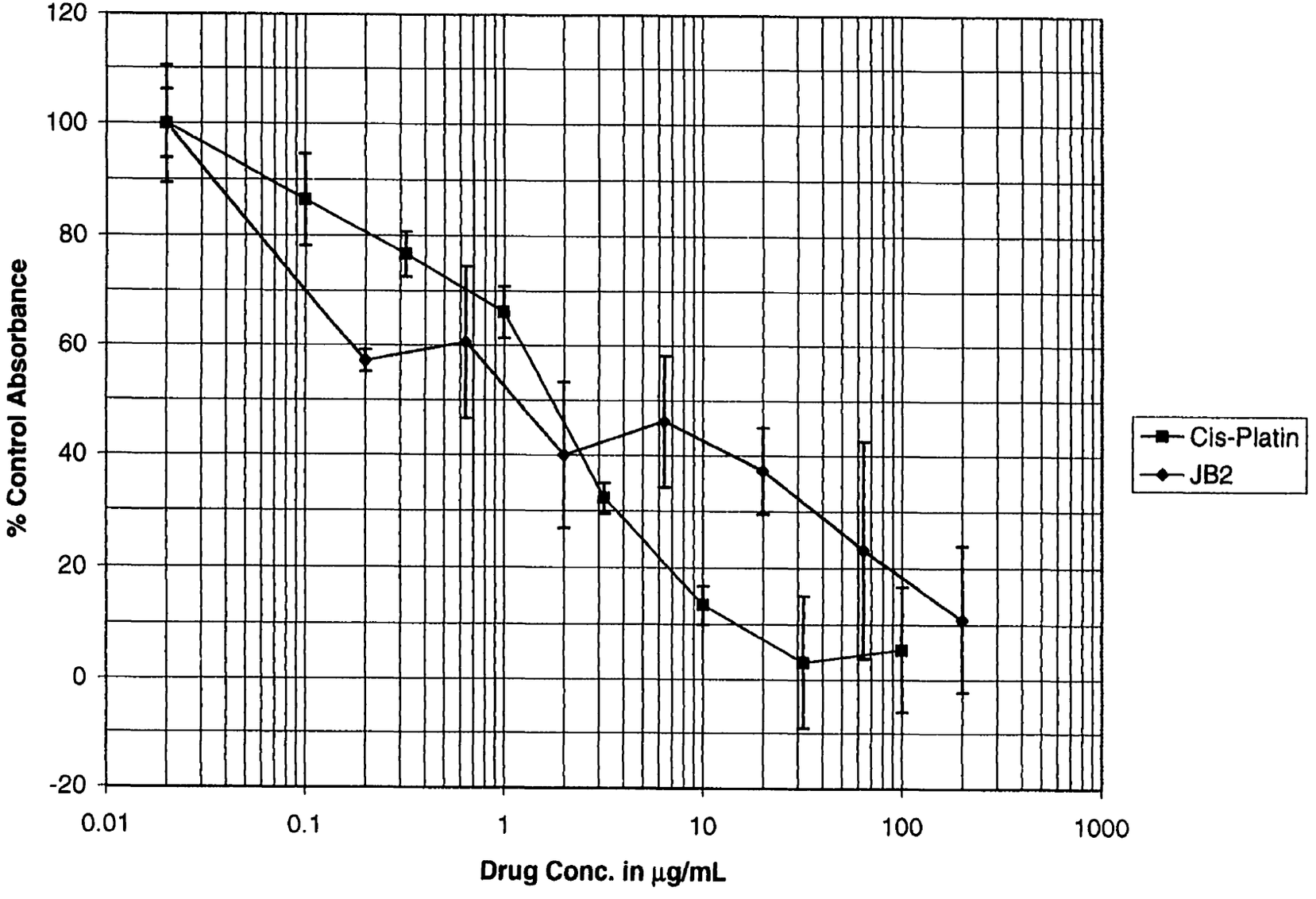
Dose Response Curve 3 - In Vitro Activity of JB1 and Cis-Platin Against NCI-H209

It is apparent then that a trend may possibly be developing where a polar, electron withdrawing substituent covalently bonded to the Cp ring increases the *in vitro* cytoxicity of titanocene dichloride. This trend was continued with the disubstituted compound.

3.5.4 *In vitro* Activity of Bis(η^5-methoxycarbonylcyclopentadienyl) dichlorotitanium (JB2)

The disubstituted compound, (JB2), differs from titanocene dichloride as both Cp rings attached to the titanium metal center are $C_5H_4CO_2Me^-$ ligands. This compound was tested against NCI-H209 and the result is shown in Dose Response Curve 4. The disubstituted compound produced an almost linear dose response curve. The IC_{50} value was calculated to be 1 μg/mL, which is slightly better than cis-platin (2 μg/mL).

One explanation of the increased potency may be that the presence of an electron-withdrawing substituent has fortified bonding interactions with the cellular targets (possibly DNA). The carbomethoxy group may induce a net shift of electron density away from the titanium center, causing it to be more Lewis acidic. This may then produce a more reactive titanium species which forms more stable adducts with the cellular targets. In addition, it was shown that substituting a Cp ring with an electron donating functionality caused a decrease in the activity of the titanocenes (see table 2, section 1.6.2). The reduced activity may possibly be explained by weaker cellular interactions with the less



Dose Response Curve 4 - In Vitro Activity of JB2 and Cis-Platin against SCLC NCI-H209

Lewis acidic titanium metal. Extending this electronic hypothesis, the polar functionality may also form hydrogen bonds with other nucleic acids and increase the cytotoxic effects of these drugs. These intermolecular forces may alter the 3-dimensional structure of the DNA effecting the inhibition of replication enzymes. This effect may force the cell to start apoptosis, thus effecting the ultimate anti-proliferation of the cell.

Another explantion of the increase antiproliferative properties of these polar substituted titanocenes may be described by increasing the stability of the substituted Cp ring towards protonolysis. This could be attributed to the fact that the carbomethoxy substituent has influenced the acid/base chemistry at the titanium metal center. Recall that the electron withdrawing substituent may cause the metal center to be more electron deficient. This would result in a lowering of the pKa of the protons on a coordinated water molecule. This would result in rapid loss of a proton into the buffered medium resulting in a titanium hydroxide species. Furthermore, it was shown by Toney *et al.*[16] that increasing the pH does induce Cp-loss, possibly via nucleophilic substitution at the titanium center of a Cp ligand with a hydroxide ion. The titanium metal center of this titanocene hydroxide species may then be sufficiently electron rich to prevent nucleophilic substitution of another hydroxide molecule for a Cp ligand at higher pH.

Hydrolysis of just one Cp ligand may indeed destroy the anti-cancer activity of the metallocene. It was apparent that only a mild increase in activity was apparent with the monosubstituted compound, but that a dramatic increase

in cytotoxic behaviour was apparent with the disubstituted compound. This observation may point to the fact that both Cp ligands must be present for cytotoxic activity of the metallocene. Thus the carbomethoxy group may indirectly maintain the integrity of the metallocene and increase the amount of active titanium molecules that reach the cellular targets.

3.6 Solubility in Water and Toxicity

It is known that many biologically important molecules have some degree of water solubility, which allows for efficient mobility in the body through various circulatory systems. Thus one may argue that an improved water solubility of the metallocenes will facilitate their clinical application into the body via absorption through tissues. Furthermore, this property may cause a decrease in the toxicity of the metallocenes, as excretion of the drug would be more facile. For example, some local toxicity of titanocene dichloride was apparent in the area the drug was injected. This may be attributed to the precipitation of titanocene dichloride causing local toxicity or by a local decrease in pH causing severe tissue damage.

However, increasing the water solubility (polarity) of the drug decreases the ability to permeate into the cell. This event relies more on hydrophobic, (intermolecular) forces to enable passive diffusion through the lipid bilayer. Other interactions involve active pathways where drug recognition occurs. Unfortunately, no active pathways have been identified for the specificity of titanocene dichloride for the treatment of cancer. The concept of an efficient

anti-cancer drug is best described by the notion that a drug must be sufficiently polar to be soluble in water but must also be sufficiently non-polar to diffuse through the cell membrane by passive diffusion. It seems apparent that a common ground must be established for a drug to be biologically active.

The fact that titanocene dichloride was active *in vivo* against various forms of malignant cancers initiated the search for better, stronger more efficient metallocenes. Some studies done in the early 1980's explored the anti-cancer properties of ionic titanocenes.[41] Four molecules showed activity *in vivo* and are shown in Table 3.

It is apparent that these compounds have excellent water solubility but as a result of replacing the Cl^- ligands with bidentate, chelating ligands, a reduced potency and a narrower therapeutic index was observed compared to titanocene dichloride. This decreased activity may be a result of the strong bonding interactions of the chelating ligands with the titanium center. This may affect the ability of the titanium center to interact with the cellular targets. Similarly, it was shown that the very strongly bonded titanocene chalcogeno molecules, Cp_2TiS_5 and $Cp_2Ti(SR)_2$, did not exhibit any anti-tumor activity *in vivo*.[5] However, this was most likely due to the insoluble nature of these molecules in an aqueous medium.

In another study, Kopf-Maier *et al* tested four metallocenes where the chloride ligands were substituted for a polar functional group. These molecules are shown in Table 4.[26] The water solubility of these compounds was significantly improved compared to titanocene dichloride, and three of the four

were completely soluble in water. Additionally, it was shown that the toxicological properties of these hydrophilic metallocenes were significantly improved. The least toxic metallocene was the trichloroacetic acid derivative, which exhibited a therapeutic index of 5.5, an improvement from 3.3 exhibited by titanocene dichloride. This result was impressive but disturbing because it was the only compound of the four that was not soluble in water. Although this minor discrepancy exists, it is apparent that substitution of the chloride ligands can produce molecules that are soluble in water and improve the biological properties of the metallocenes. Unfortunately, the reasons for this improvement are unclear at this time.

Another aspect of toxicity apparent in the literature was the observation that most metallocenes that are not active are also non-toxic. This was apparent with the substituted cyclopentadienyl titanocene compounds. Conversely, a more toxic effect was apparent with the more potent niobocene dichloride. The optimum dose range of which was 20-25 mg/kg. In addition, the therapeutic index of niobocene dichloride was 3.5, nearly identical to titanocene dichloride. It becomes apparent that the biological activity of the metallocenes is directly correlated to its inherent toxicity. However, as mentioned before, a 10-fold increase in the dose of titanocene dichloride was necessary to produce a similar tumor inhibition and less toxic effects compared to cis-platin. Thus one may deduce that 90% of the metallocene is being degraded or metabolized before it reaches the cancerous cells. Blocking this pathway would allow for smaller

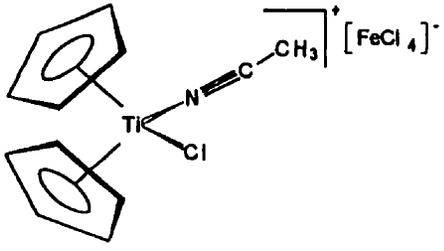
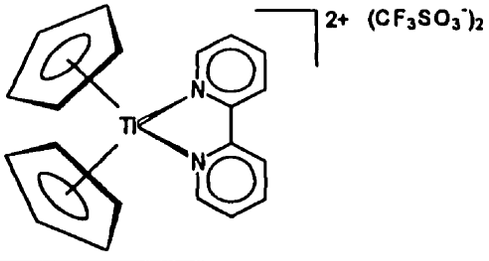
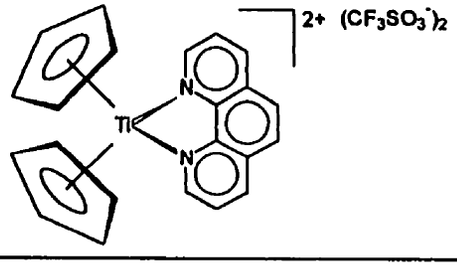
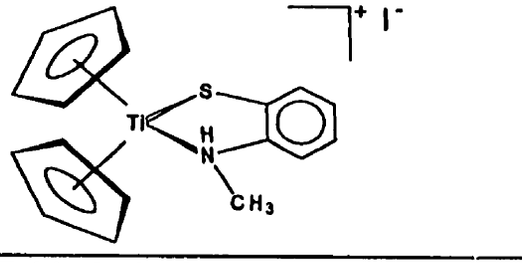
Compound	Optimum dose range (mg/kg)	Optimum cure rate (%)	T.I.	LD_{50} (mg/kg)	LD_{100} (mg/kg)
	80-140	75	-	180	220
	200-220	100	1.6	260	300
	140-220	67	-	240	340
	200-320	100	1.7	380	500

Table 3 - Ionic Metallocene Compounds and Their Anti-Cancer Properties

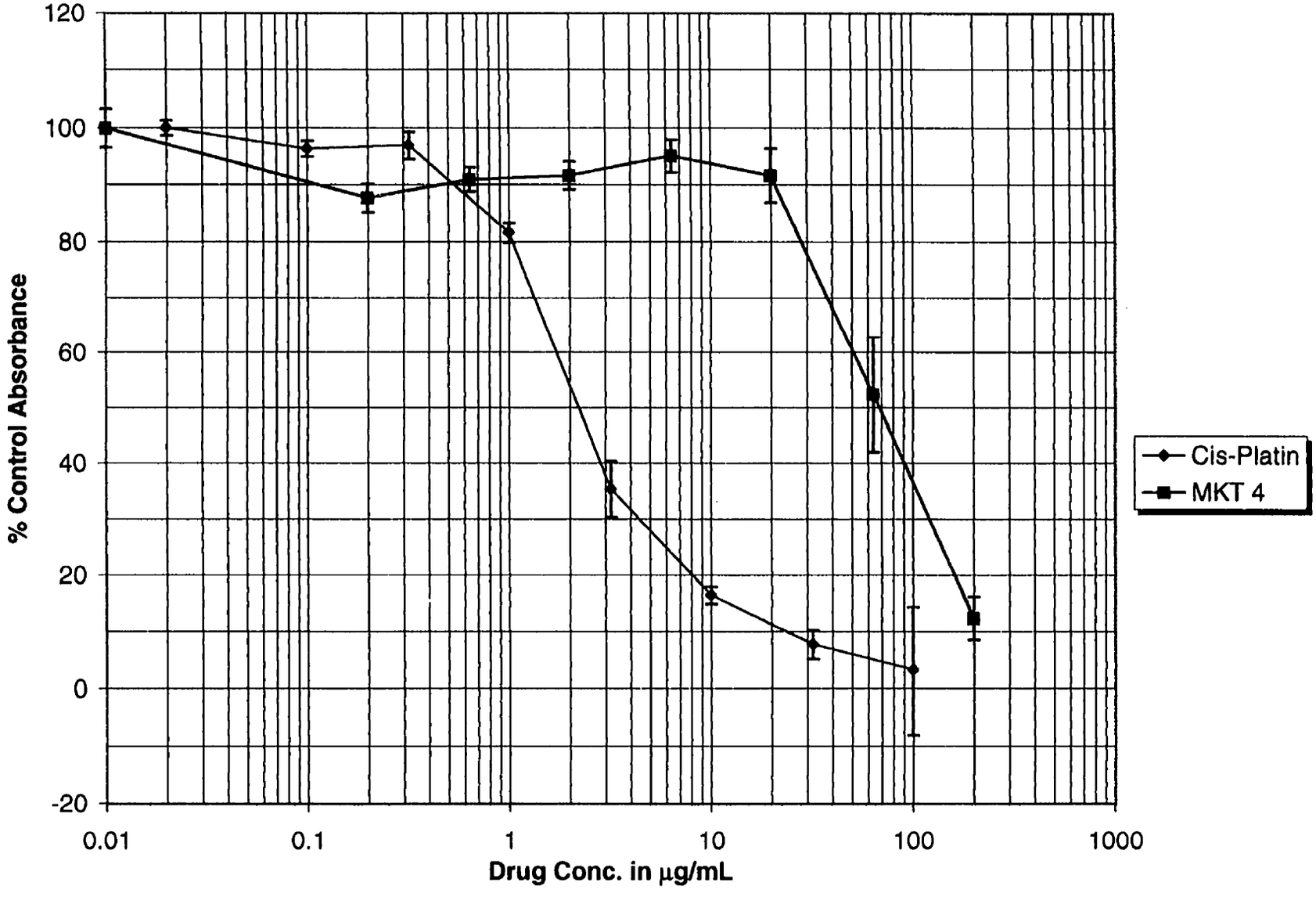
chemotherapeutic doses, an important attribute for future development of this drug.

3.7 Activity of MKT 4 Against Cis-Platin Resistant SCLC Cell Lines

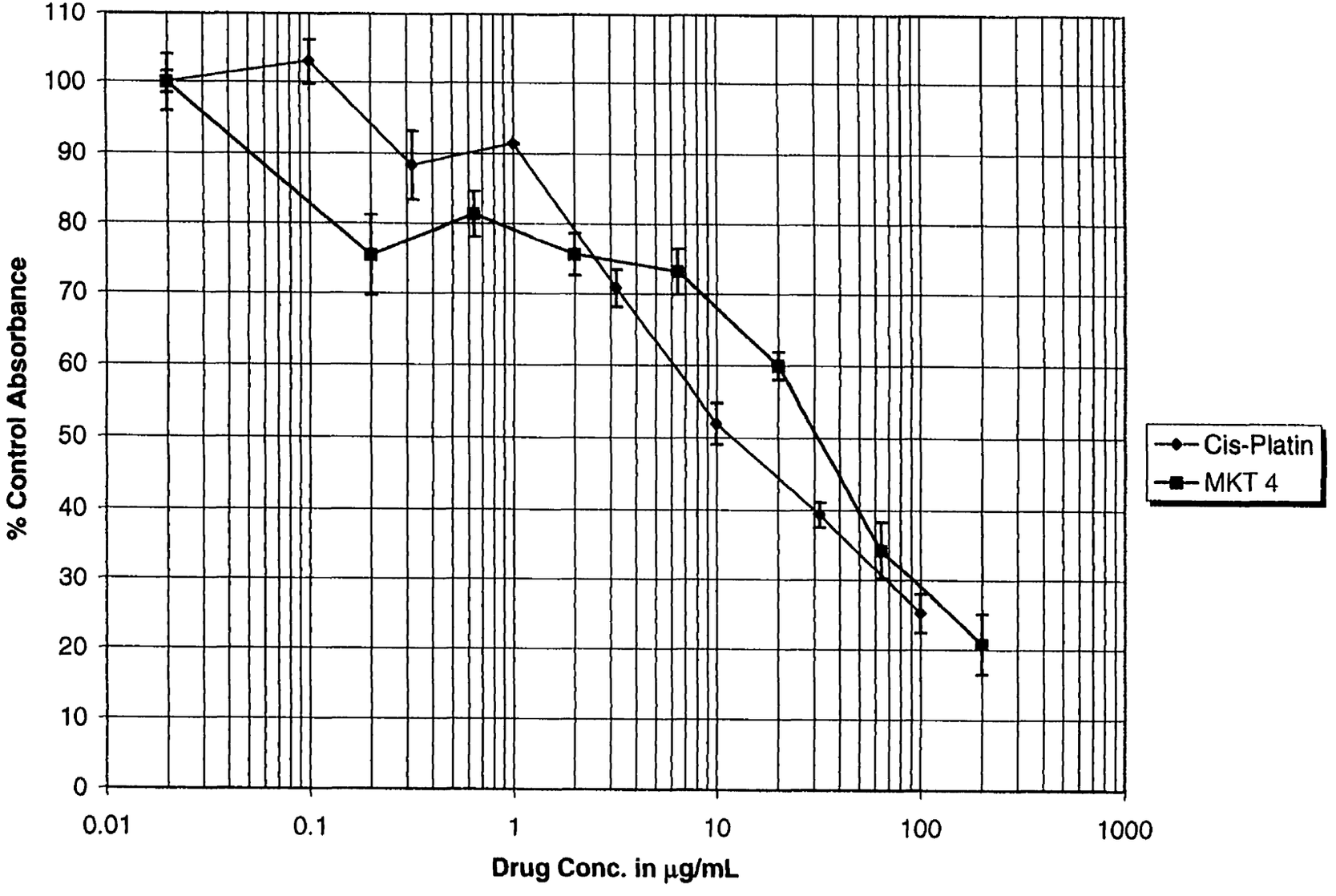
In an effort to determine whether titanocene dichloride exhibits cross resistance with cis-platin, MKT 4 was tested against two SCLC cell lines that are resistant to cis-platin. These two cell lines, NCI-H69 and JN-M, were chosen because they are increasingly more resistant towards cis-platin compared to NCI-H209. The activity of MKT 4 and cis-platin against JN-M and NCI-H69 are illustrated in the Dose Response Curves 5 and 6, respectively. The IC_{50} values for MKT 4 against NCI-H69 was 65 μg/mL. In comparison, the IC_{50} value of cis-platin against the same cell line was 2 μg/mL.

This result depicts a 30-fold increase in the dose requirement for MKT 4 compared to cis-platin to induce an identical pattern of cytotoxicity seen with SCLC. When MKT 4 was tested against JN-M, the metallocene exhibited an activity very similar to cis-platin, where the IC_{50} value of MKT 4 was 30 μg/mL and that of cis-platin was 12 μg/mL. This result provides evidence that titanocene dichloride is not cis-platin cross-resistant against SCLC. That is, the cell lines that are resistant towards cis-platin are not equally resistant to titanocene dichloride. This is evidence for different cellular targets of titanocene dichloride in comparison to cis-platin.

In comparison, other cis-platin resistant ovarian cancers were not cross-resistant to MKT 4 *in vitro*.[39] In fact, MKT 4 was active in eight of seventeen



Dose Response Curve 5 - In Vitro Activity of MKT 4 and Cis-Platin against SCLC NCI-H69



Dose Response Curve 6 - In Vitro Activity of MKT 4 and Cis-Platin against SCLC JN-M

cis-platin resistant tumors. The estimated IC_{50} values for MKT 4 against cis-platin tumors were 19 μg/mL and 11 μg/mL. The respective cis-platin IC_{50} values were estimated at 3.6 μg/mL and 15 μg/mL. The dosages required to induce cancer regression are very close to the LD_{10} of cis-platin (8 mg/kg) thus making these cancers difficult to combat. Futhermore, since titanocene dichloride is only slightly toxic in comparison to cis-platin, it is an excellent candidate for the effective chemotherapeutic treatment against these and perhaps other resistant tumors.

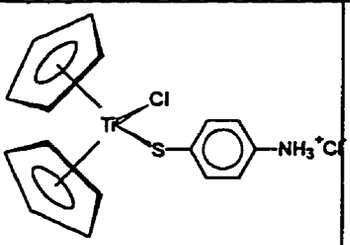
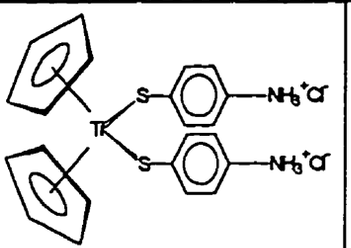
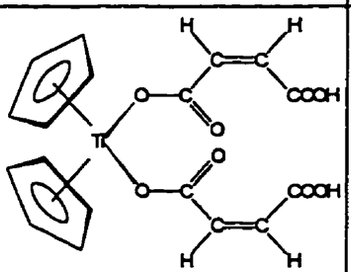
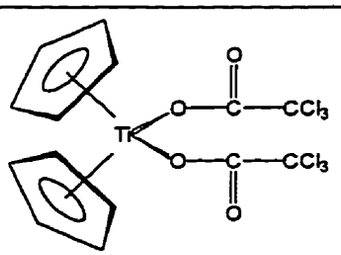
Compound	Optimum dose range (mg/kg)	Optimum cure rate (%)	T.I.	LD_{50} (mg/kg)	LD_{100} (mg/kg)
	60-140	100	4.6	180	200
	40-120	100	4.1	165	200
	60-140	100	3.7	175	240
	100-360	100	5.5	440	500

Table 4 - Anti-Cancer Properties of Four Polar Substituted Titanocenes Modified by Substituting Both Chlorides with Polar Functional Groups

Chapter Four - Conclusions

The anti-cancer activity of two polar substituted titanocene dichloride compounds were shown to be better than a galenic formulation of titanocene dichloride (MKT 4). The potency increase may possibly be ascribed to a carbomethoxy group covalently linked to one or both cyclopentadienyl rings. This electron withdrawing functional group may possibly be strengthening the cellular interactions with the titanocene unit. The disubstituted molecule (JB2) exhibited greater cytotoxicity than cis-platin against the SCLC NCI-H209. Lack of cis-platin cross resistant was also found with MKT 4 against NCI-H69 and JN-M.

These results are contradictory to those published by Kopf and Kopf-Maier where they said that modification of the Cp ring was detrimental to cytotoxic activity. It was found that modifying the Cp ring with a carbomethoxy substituent actually improves the potency of the metallocene. These results seem to point to that fact that electronic effects at the titanium metal center may influence the anti-cancer properties of the metallocenes. Further development of these compounds as anti-cancer agents should bear this fact in mind.

Chapter Five - Future Study

5.1 Synthesis of Polar Substituted Cyclopentadienyl Ligands

Polar substituted metallocene dichloride compounds which are analogous to the ones used in this study are not apparent in the literature. However, many polar substituted cyclopentadienyl salts are known. For example, the lithium, sodium and thallium salts of Cp-$(CO_2Me)_n$ (n=2-5) are known.[18] Similarly, sodium salt of the amide compound, Cp-NHOCOMe, is known. However, these compounds do not react with $CpTiCl_3$ or $TiCl_4$ to generate the respective titanocene dichloride. This observation may stem from the fact that resonance stabilized enolate and imine complexes react rapidly with the electron deficient titanium center.

Advances in this area were made by Rausch *et al* when they synthesized the mono and di substituted carbomethoxytitanocene dichlorides by mixing the carbomethoxycyclopentadienyl sodium salt together with $CpTiCl_3$ and $TiCl_4$ respectively.[34] However, only moderate yields were attainable by this reaction scheme. Exchanging sodium for thallium made an improvement to this synthesis, but the reasons for this are unclear. A speculative explanation of this phenomenon is that a more electron withdrawing cation produces a tighter solvent separated ion pair, thus reducing the need for resonance stabilization. Finally, reaction with $CpTiCl_3$ or $TiCl_4$ may be cleaner and more efficient, resulting in very active anti-tumor titanocene dichloride complexes.

5.2 *In vivo* Studies of Polar Substituted Titanocene Dichlorides

These molecules exhibit an excellent pattern of *in vitro* activity against SCLC. It was shown that substituting the Cp ring with polar, electron withdrawing carbomethoxy groups an increase in potency was produced. The disubstituted compound (JB2) exhibited an IC_{50} value better than cis-platin. The next logical step in the development of these compounds as chemotherapeutic drugs would be to examine their *in vivo* properties.

It was shown by Kopf-Maier *et al.* that substituting polar groups for the chloride ligands produced compounds that were water soluble and less toxic.[26] This decreased toxicity would be beneficial to the comfort of the patient being treated and would allow for higher more effective doses to kill all of the cancer cells. This less toxic effect may be apparent with the polar substituted titanocene dichlorides. In addition, it was already shown the potency of the titanium drug was improved and may possibly be attributed to the electron withdrawing character of the ester. However, this may increase the reactivity of the metallocene and increase its role in interfering with other biological processes that lead to toxicity. This effect cannot be calculated, it must be tested.

It would also be extremely helpful for the future development of the titanocene compounds to isolate the intrinsically active species. This would be beneficial for the rational drug design of future complexes that may be much more active, and potentially specific to cancerous cells.

Another aspect of this research that should be developed is the use of titanium molecules for multidrug therapies for cancer patients. This approach has been used first for treating cancer patients and for the treatment of AIDS patients with excellent results. Furthermore, cis-platin cross resistance is not apparent with MKT 4 and this may be due to the nature of the different metal centers. This research could then be extended to search for mechanisms of cis-platin resistance and the discovery of new approaches to avoid such problems. In conclusion, there is much potential for the use of these compounds in cancer chemotherapy and combination chemotherapy.

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