Universidade de Évora - Escola de Ciências e Tecnologia

Mestrado em Bioquímica

Dissertação

Study of the cytotoxic effect of modified titanium compounds in a cellular model of resistance to cisplatin

Cristina Isabel Branca Mendes

Orientador(es) | Rosario Serrano Vargas
Fernando Capela e Silva
Isabel Martinez Argudo

Évora 2025

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A dissertação foi objeto de apreciação e discussão pública pelo seguinte júri nomeado pelo Diretor da Escola de Ciências e Tecnologia:

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Abstract

Cancer is still a major cause of death in developed countries, and the search for better therapies to fight this disease has been increasing. This study focuses on evaluating the effectiveness of two newly developed titanium-based compounds, Myr-Ti and Ole-Ti, against ovarian cancer cells that do not respond to standard cisplatin treatment.

We employed a range of methods, including western blot analysis, MTT assays, flow cytometry, and DNA binding tests, to determine which compound more effectively targets cancer cells. The results revealed that both compounds showed significant cytotoxic effects, with Ole-Ti demonstrating superior efficacy in reducing cell viability, particularly in cisplatin-resistant cell lines.

These promising findings suggest that Myr-Ti and Ole-Ti could serve as potential alternatives for patients who do not respond to traditional therapies. Further research is needed to explore the mechanisms of action and therapeutic potential of these compounds in the ongoing battle against cancer.

Key words: Titanium compounds, ovarian cancer cells, cisplatin, cytotoxicity

Resumo

Título: *Estudo do efeito citotóxico de compostos de titânio modificados num modelo celular de resistência à cisplatina*

O cancro continua a ser uma das principais causas de morte em países desenvolvidos, e a procura por terapias mais eficazes para combater essa doença continua a aumentar. Este estudo foca-se em avaliar a eficácia de dois novos compostos à base de titânio, Myr-Ti e Ole-Ti, contra células de cancro do ovário que não respondem ao tratamento padrão com cisplatina.

Foram utilizados métodos como ensaios MTT e citometria de fluxo que revelaram que ambos os compostos apresentaram efeitos citotóxicos significativos, com Ole-Ti a demonstrar uma maior eficácia na redução da viabilidade celular, particularmente em linhas celulares resistentes à cisplatina.

Estes resultados sugerem que Myr-Ti e Ole-Ti podem ser potenciais alternativas para pacientes que não respondem às terapias tradicionais, sendo necessárias pesquisas adicionais para explorar os mecanismos de ação e o potencial terapêutico desses compostos no tratamento do cancro.

Palavras-chave: Compostos de titânio, células do cancro do ovário, cisplatina, citotoxicidade.

List of Acronyms

BSA: Bovine Serum Albumin

CDK1/CDK2: Cyclin-dependent kinases 1 and 2

DMSO: Dymethil sulfoxide

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

Myr-TiY - [TiCpY2{(OOC)2py-O-Myr}]

Ole-Ti - [TiCp2(OOC)2py-O-Ole]

PBS: Phosphate-Buffered Saline

PI: Propidium Iodide

TiCp2Cl2: Titanocene dichlorid

7-AAD: 7-Aminoactinomycin D

1. Introduction

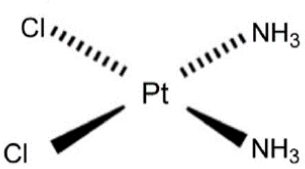
Cancer is characterized by the presence of abnormal cells that proliferate uncontrollably due to mutations, which can occur either spontaneously or as a result of external factors. It is one of the leading causes of death in developed countries, second only to cardiovascular diseases. Beyond demographic factors and heredity, the incidence of cancer is significant, with an estimated 19 to 20 million people diagnosed each year [1–4]. Among the possible treatments for cancer, we have radiation therapy, surgery for solid tumors, systemic therapies, immunotherapy, and chemotherapy, with the latter being one of the most widely used treatments for a wide variety of cancers [5].

1.1 Cisplatin in cancer treatment and its mechanisms of action

Cisplatin ((SP-4-2)-diamminedichloridoplatinum(II)), which was discovered by mere chance during an experiment using platinum electrodes to analyze the effect of electric fields on bacterial growth, is the most widely used compound in the treatment of a vast variety of solid cancers. Since the discovery of its cytotoxic effects in 1960, cisplatin began to gain special attention, becoming the first platinum compound to be approved for cancer treatment in 1978 [4,6–8].

Cisplatin has a square-planar structure with platinum bonded to two ammonia ligands and two chloride ligands (Fig. 1).

A



B

M_w	301.1g/mol
ρ	3.74 g/cm^3
T_m	270 °C
S_w	2,53 g/L at 25 °C
log K_{ow}	2,19

Figure 1. *Chemical structure (A) and physicochemical properties of Cisplatin (B). Adapted from Dasari et al. (2014) [8]*

Its mechanism of action primarily involves binding to cellular DNA and activating various signal transduction pathways (Fig. 2).

When injected into the bloodstream, which is its usual method of administration, cisplatin remains in its neutral and unchanged state due to the relatively high concentration of chloride in the blood. Once it crosses the cell membrane, where the chloride concentration is significantly lower, hydrolysis of the chloride molecules occurs, leading to the formation of cationic compounds. These cationic forms then react with the nitrogenous bases of the DNA [7,9,10].

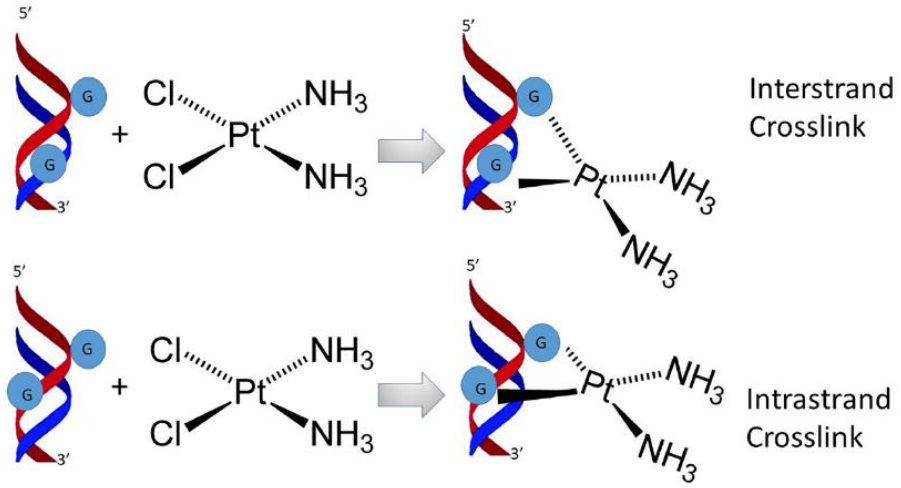


Figure 2. *Interaction of Cisplatin wit DNA chains (Image reproduced by Romani et al. (2022)* [11]

The cationic compounds resulting from the hydrolysis of chloride molecules become reactive and preferentially bind to the nitrogenous bases adenine and guanine through covalent bonds. This affinity for adjacent adenine and guanine bases leads to the formation of intra- and inter-strand adducts. These adducts cause distortions in the DNA helix and may also lead to strand breaks due to this distortion, triggering DNA repair checkpoints. Activation of these checkpoints results in a temporary halt in the S phase and a more prolonged inhibition of the G2/M transition to allow for DNA repair before the cell cycle progresses. These cell cycle alterations are linked to the activation of the p53 protein, a tumor suppressor that can either halt the cell cycle or induce apoptosis depending on the extent of DNA damage, as well as the MAPK signaling pathways, which regulate cell proliferation, differentiation, survival, and apoptosis [6,10].

Another common cytotoxic mechanism of cisplatin is the induction of oxidative stress in the cell through the generation of radicals such as hydroxyl and superoxide. These radicals lead to lipid peroxidation, depletion of sulfhydryl groups, alterations in signaling pathways, and DNA damage. Since mitochondria are the primary target of reactive oxygen species, there is a loss of mitochondrial membrane integrity, which can result in its rupture. The rupture of the mitochondrial membrane releases cytochrome C and procaspase-9, allowing procaspase-9 to join with the apoptotic protease activating factor (Apaf-1) and ATP, forming the apoptosome. Activation of caspase-9 occurs through the apoptosome, initiating the caspase cascade, where caspase-9 interacts with and activates caspases 3, 6, and 7. This activation leads to the cleavage of key cellular substrates, resulting in programmed cell death [6,11].

1.2 Ovarian cancer

Among the cancers of the female reproductive system, ovarian cancer is one of the most common. Although it is not the most prevalent, ranking seventh among the most diagnosed cancers and being less common compared to cervical cancer and endometrial cancer, ovarian cancer has a higher mortality rate than the others [12,13]. Additionally, early diagnosis is very difficult due to the commonality of the initial symptoms, resulting in more than 70% of cases not being diagnosed until the disease is in stage III or IV [14,15].

Depending on the tissue, ovarian cancer can be characterized into three types: epithelial, germ cell, and sex cord-stromal cells [16,17]. Epithelial cancer is the most prevalent among these types, and it is usually diagnosed at a later stage. It is also the most recurrent in developing resistance to chemotherapy treatments [16,18,19]. In addition, it is further divided into four subtypes: serous carcinomas, mucinous carcinomas, endometrioid carcinomas, and clear cell carcinomas [16,20].

1.2.1 Cancer and its mechanisms of resistance to cisplatin

The main problem with cisplatin treatment for ovarian cancer is its high incidence of resistance. It is quite common for patients to experience relapses due to this resistance [10,21]. Cisplatin resistance is caused by numerous factors, such as lowering intracellular drug accumulation, detoxification by glutathione (GSH), and DNA repair. However, cisplatin presents five possible scenarios for the development of resistance:

before cisplatin's entry into tumor cells, during the influx and efflux of cisplatin through the cell membrane, in the presence of cisplatin in the cytoplasm, during the interaction of cisplatin with nuclear DNA, and after post-targeting interactions [21–25] .

Cisplatin interacts with plasma proteins, particularly human serum albumin, by binding to histidine and methionine residues. These interactions not only affect albumin's ability to transport essential ions, like zinc, leading to zinc imbalances in patients undergoing treatment, but also contribute to the metabolic inactivation of the drug. Furthermore, a significant portion of intravenously administered cisplatin rapidly binds to proteins containing sulfhydryl groups, such as glutathione, further reducing the amount of active drug available to act on tumor cells. Although neutral cisplatin species are more likely to diffuse passively into cells, studies suggest that active transport mechanisms also exist. The formation of stable bonds between cisplatin and methionine residues can hinder its uptake by cells. Furthermore, cisplatin can be converted into carbonate complexes, which are anionic species that may be transported by organic anion transporters, leading to its accumulation in tissues. This accumulation could help explain some of the side effects, such as nephrotoxicity, observed in patients [21,26].

Additionally, the interactions between cisplatin and membrane proteins, lipids, and other biomolecules affect both the influx and efflux of the drug. Cisplatin enters cells primarily through passive diffusion and specific transporters, such as organic cation transporters, which facilitate its uptake. However, downregulation of these transporters can reduce cisplatin absorption, while efflux systems, including copper-transporting ATPases (ATP7A and ATP7B) and multidrug resistance protein 1 (P-glycoprotein), can expel cisplatin from the cells before it reaches its intracellular target [21,27,28] .

Cisplatin resistance in the cytoplasm is closely linked to glutathione (GSH), which, due to its high affinity for cisplatin, competitively inhibits the drug's binding to

DNA, leading to drug resistance. Studies have shown that elevated levels of GSH in cisplatin-resistant cell lines contribute to the inactivation of cisplatin and reduce the production of reactive oxygen species (ROS), which are essential for the drug's efficacy and sensitivity [21,27,28].

The interaction of cisplatin with nuclear DNA is another key factor in resistance (Fig. 3). Since DNA is the primary target of cisplatin, the binding of cisplatin to cysteine thiol groups in metallothionein results in the loss of the drug's activity. Metallothionein, a low molecular weight protein, is important for the homeostasis and detoxification of metal ions and has been associated with tumor cell resistance to cisplatin, particularly in cell lines like A2780CIS, where metallothionein expression is significantly higher [21,27,28].

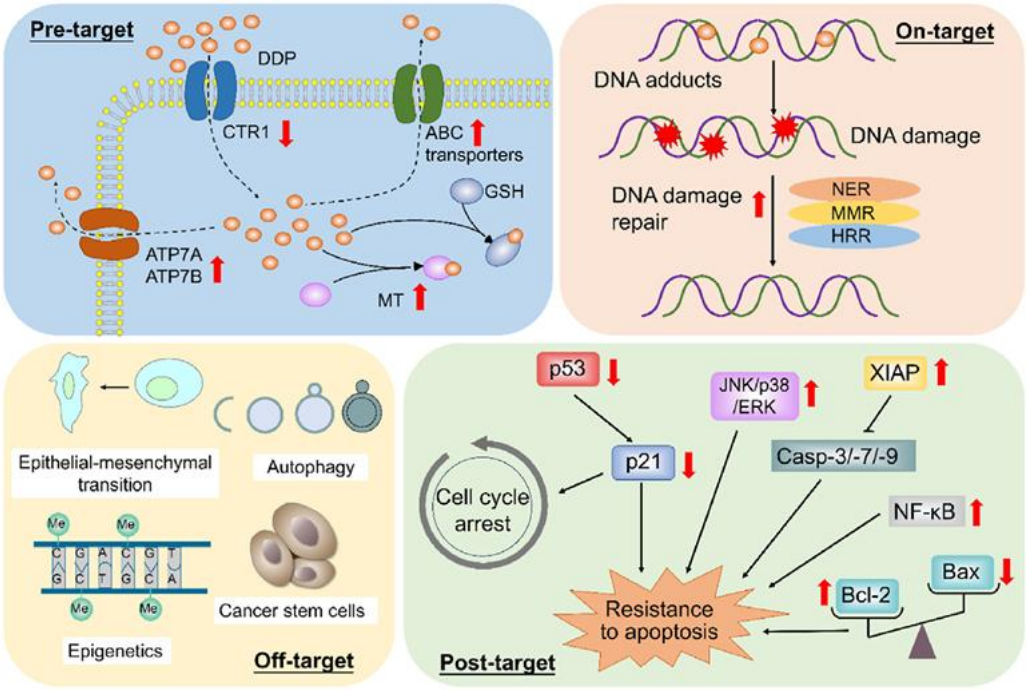


Figure 3. *Proposed mechanisms mediating cisplatin resistance in ovarian cancer cell. CTR 1, copper influx transporter copper transport protein 1; ABC transporters, ATP-binding cassette transporters; ATP7A/B, ATPase copper-transporting alpha/beta; GSH, glutathione; MT, metallothionein; NER, nucleotide excision repair; MMR, mismatch repair; HRR, Homologous recombination repair. (Image reproduced by Song et al.(2022) [12])*

Moreover, cisplatin resistance can also occur post-target, where non-coding RNAs (ncRNAs) play a crucial role. These RNAs, which include microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs), are emerging as important research targets in ovarian cancer resistance. miRNAs, by post-transcriptionally silencing

target genes, can contribute to cisplatin resistance. Studies have shown that the downregulation of certain miRNAs, such as miR-29 and miR-216b, is linked to resistance in ovarian cancer cells. On the other hand, long non-coding RNAs which are over 200 nucleotides in length, can promote cisplatin resistance in various cell line [21,27,28]. The circular RNAs, due to their stable closed-loop structure, are also becoming a significant target of study for their potential to regulate chemotherapy sensitivity by forming competing endogenous RNA networks with miRNAs. Understanding the interactions between all ncRNAs and their relationship with DNA may offer new insights for the development of more effective therapies [21,27,28].

These interconnected factors, from cisplatin's binding to proteins in the blood, interactions with cellular transporters, DNA repair mechanisms, and ncRNA-mediated regulation, limit the drug's efficacy and further contribute to the nephrotoxicity observed in patients [21,27,28].

1.3 Titanocene based anticancer compounds

In order to create safer and more effective alternatives to platinum-based treatments, which are well-known for their high nephrotoxicity and myelotoxicity, titanium compounds have garnered significant interest in the scientific community over the past few years. These compounds stand out due to their lower associated toxicity and considerable efficacy in tumors resistant to cisplatin, making them a promising option to overcome the limitations of existing treatments [29,30].

The medicinal properties of organometallic complexes of transition metals, such as titanium complexes, were not explored until 1979, when the first metallocene with antitumor activity, dichlorotitanocene (Cp_2TiCl_2), was published [31]. This compound stood out among the tested metallocenes, demonstrating significant antitumor activity, particularly against colon, lung, and breast cancers, with no apparent signs of nephrotoxicity or myelotoxicity, marking an important advancement over platinum complexes. These characteristics led to the testing of dichlorotitanocene in clinical trials, solidifying the potential of Ti(IV) complexes as an innovative alternative for chemotherapy, with mechanisms of action distinct from those of platinum complexes [31]. Despite these initial advances being promising, significant difficulties arose in the transition of these compounds to clinical use. Dichlorotitanocene (Cp_2TiCl_2), although

promising in preclinical studies, exhibited a high hydrolysis rate, resulting in reduced efficacy *in vivo* and limiting its clinical application. Additionally, the compound was associated with nephrotoxicity as a dose-limiting effect in clinical studies. Two phase II clinical trials were conducted in patients with advanced renal cell carcinoma and metastatic breast cancer; however, the results were not sufficiently promising to warrant further studies, leading to the discontinuation of the clinical development of Cp_2TiCl_2 [31,32].

Another relevant compound in this area is budotitane, which stood out for its efficacy against tumors such as sarcoma 180 and MAC 15A colon carcinoma, demonstrating superior activity compared to 5-fluorouracil (5-FU) and cisplatin in these tumor models. However, budotitane faced similar challenges to dichlorotitanocene, such as its high hydrolysis rate, as well as stability and solubility issues in aqueous solution, which also hindered its clinical use. A phase I clinical trial initiated in 1986 aimed at determining the maximum tolerated dose showed that, like dichlorotitanocene, budotitane did not progress to more advanced stages of clinical trials due to these pharmacokinetic challenges [31,33].

Considering the promising anticancer capabilities, research in this area has focused on finding new titanium compounds that address the solubility, hydrolysis, and cytotoxicity issues presented by previously developed compounds. An example of this new generation of compounds is PhenolaTi (Fig. 4), which is a promising titanium (Ti(IV)) complex with anticancer properties, as it exhibits high stability and cytotoxicity [34].

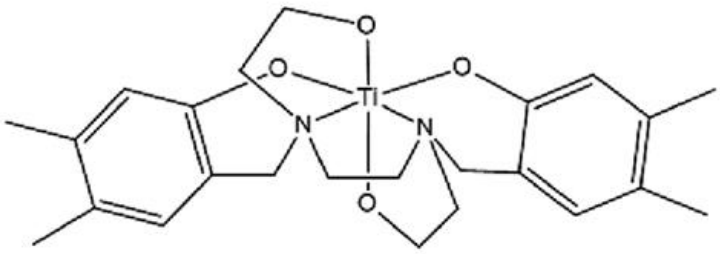


Figure 4. *Chemical structure of PhenolaTi (Reproduced by Shpilt et al. (2023) [34])*

Its cellular mechanism is associated with endoplasmic reticulum (ER) stress; however, it has been demonstrated that the presence of reactive oxygen species (ROS) does not affect the cytotoxicity of PhenolaTi, suggesting that their formation is a side

effect rather than the main mechanism of action. Although there are already some consistent hypotheses, the detailed molecular mode of action of PhenolaTi still requires further investigation [34].

This work focuses on the cytotoxic evaluation of the compounds [$TiCpY_2${$(OOC)_2$py-O-Myr}] and [$TiCp_2(OOC)_2$py-O-Ole]. These two compounds were designed by the research group to address the issues presented by titanium compounds thus far. The compound [$TiCpY_2${$(OOC)_2$py-O-Myr}] features a tridentate ligand that enhances its stability and includes a fragment of the aliphatic chain of myristic acid, which is an endogenous ligand of albumin, thereby increasing its affinity for this protein [30]. The compound [$TiCp_2(OOC)_2$py-O-Ole], which also contains the same tridentate ligand, incorporates a fragment of oleic acid, known for its good lipophilicity and higher affinity for albumin [35,36].

2. Problem and Objectives

Pharmacological cancer treatment is based on chemotherapeutic compounds, such as cisplatin and its derivatives, which often cause serious side effects in patients. As a consequence, the dosage that becomes possible to administer is often insufficient to eliminate all tumor cells, leading to the formation of tumors resistant to this type of drug. It is estimated that 90% of cases of unsuccessful chemotherapy treatment in metastatic patients are due to this innate or acquired resistance of tumor cells [37–40]. However, despite all the side effects, cisplatin and its derivatives still represent 40% of all chemotherapeutic treatments [38]. Other kind of metallic compounds, such as compounds derived from titanium, have been shown to be promising, due to its effectiveness against tumors that are resistant to cisplatin. Notwithstanding some of the existing titanium compounds presents problems related to stability and solubility [41,42]. Furthermore, one of the biggest concerns with tumor drugs is their low specificity for target cells, making their use in clinical practice difficult [43]. With these problems in mind, the host research group synthesized a family of titanium complexes containing a titanocene fragment, a tridentate ligand to improve its stability in water and a medium to long aliphatic chain, designed to facilitate its non-covalent bond with albumin.

This way, the aim of this study was to characterize the two best novel synthetized titanium-derived compounds, focusing on their cytotoxic potential and in vitro mechanism of action.

3. Methodology

3.1 Synthesis of titanocene compounds

The titanium compounds [TiCp2(OOC)2py-O-Myr] (Myr-Ti) and [TiCp2(OOC)2py-O Ole] (Ole-Ti) were synthetized by the Dr[a]. María José Ruiz García from the Inorganic Chemistry Area of the University of Castilla La Mancha. Both compounds are titanocene complexes formed by a ligand tridentate dicarboxylate, differing in the hydrocarbon chain from the corresponding fatty acid. Both compounds were dissolved in ethanol for biological assays (Fig. 5).

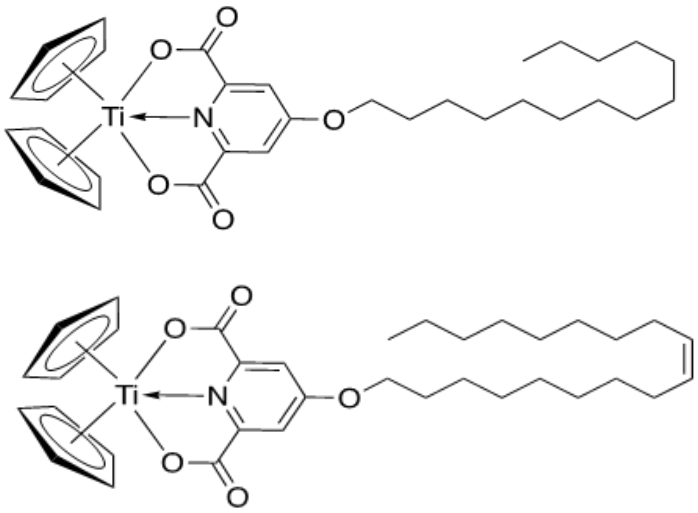


Figure 5. *Chemical structure of myristic titanocene (above) and oleic titanocene (below). Images provided by Dr. María José Ruiz García*

3.2 Cell lines and maintenance of cell cultures

The cell line A2780 and its respective cisplatin-resistant cell line A2780cis from human ovarian tumor used for this work were provided by commercial house European Collection of Authenticated Cell Cultures (ECACC).

Cell culture was performed in Roswell Park Memorial Institute (RPMI) 1640 Medium (Bio Whittaker, Lonza), supplemented with 10% FBS (Fetal Bovine Serum) (Gibco) and 1% penicillin/streptomycin (Bio Whittaker, Lonza). Furthermore, A2780cis cell line was maintained in the presence of cisplatin (Sigma-Aldrich) at 1 μM in the culture medium.

The cell maintenance of the cultures was reproduced through standard protocols, performing the cell passes with a frequency determined by the cell confluence, keeping them always in an exponential growth phase, and cells were lifted by enzymatic treatment with trypsin-EDTA(Gibco). The cultures were incubated in a humidified atmosphere with 5% CO2 at 37 ºC.

3.3 Cell viability assays

To calculate the cell viability of A2780 and A2780cis in response to titanium compounds, cells were cultured in a 96-well plate at a confluence of $3x10^4$cells/well ($3x10^5$ cells/mL) and treated with increasing concentrations of each compound (1.25, 2.5, 3.75, 5, 10, 15, and 20 µM), with untreated batches as control. Three replicates were performed for each condition in each experiment. The plate was incubated at 37 ºC for 72h after the treatment and then a solution of MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at 0.5 mg/mL was added to each well and incubated for 45 minutes at 37ºC at 5% CO2. For absorbance measurement by spectrophotometry, dimethyl sulfoxide (DMSO) (Thermo Fisher Scientific) was used to dissolve the formazan crystals. Absorbance was measured at 555 nm and 690 nm using a Synergy H.T reader (BioTek). The cell viability of each group was calculated as ($OD_{\text{Target sample}} - OD_{\text{blank}}$)/ ($OD_{control} - OD_{white}$) × 100%. At least three independent experiments were carried out or each cell line and drug. The MTT assay is a colorimetric technique that gauges the reduction of the MTT dye catalyzed by mitochondrial enzymes within viable cells, leading to the formation of a formazan product. This resultant formazan product can be quantitatively assessed through spectrophotometry, with the absorbance readings directly correlating with the number of viable cells.

The IC50 of each of the drugs was calculated for each of the cell lines using a nonlinear regression analysis applied to the dose-response curve obtained for each of the compounds.

3.4 Analysis of the Cell cycle by flow cytometry

For the analysis of the influence of Myr-Ti and Ole-Ti on the cell cycle of both cell lines, the cells were cultured in a 12-well plate, at a confluence $3x10^5$ cells/well ($3x10^5$ cells/mL) and treated with concentrations of 5,10,15 µM of each drug. After 48h the cells were trypsinized and centrifuged and their pellet was resuspended in ethanol to permeabilize the cell membrane. After 30 minutes of incubation, the pellet was washed with a PBS (Phosphate-Buffered Saline)/3% BSA (Bovine Serum Albumin) and centrifuged for 5 min at 300xg. Cell cycle analysis was measured in linear mode by flow cytometry Accuri C6 BD (Becton Dickinson) after an incubation of 15 minutes in a propidium iodide solution (PI/RNAse, Immunostep). A total of 10,000 events were acquired per sample. Debris and cell aggregates were excluded using FSC-H vs FSC-A and PI-W vs PI-A gating strategies to ensure analysis of single cells.

The cell cycle analysis by flow cytometry is based on the quantification of DNA content within the cells using the fluorescent DNA-binding dye, propidium iodide (PI). Propidium iodide binds to the DNA present in the cell nucleus, and the fluorescence intensity emitted is directly proportional to the quantity of DNA, allowing the quantification of cells in the different phases of the cell cycle.

3.5 Analysis of cell apoptosis by flow cytometry

Cells were cultured in a 12-well plate, at a confluence of $3x10^5$ cells/well ($3x10^5$ cells/mL) and treated with concentrations of 5,7.5,10 and 15µM of each drug. After 48h the cells were trypsinized and centrifuged and their pellet was resuspended in Annexin-binding buffer after a wash with Phosphate Buffered Saline (PBS) (Bio Whittaker, Lonza). Subsequently 5 µL of 7AAD (7-Aminoactinomycin D) and 4 µL of Annexin V were added and incubated for 15 min at room temperature following instructions from the manufacturer. After incubation 100 µL of binding buffer was added and the analysis was performed by flow cytometry Accuri C6 BD (Becton Dickinson).

A total of 10,000 events were recorded per sample, and debris and doublets were excluded using FSC-H vs FSC-A gating strategies to ensure single-cell analysis. Data were acquired in linear scale and quadrant gating was used to distinguish between viable, early apoptotic, and late apoptotic or necrotic cell populations.

Flow cytometry, in this context, serves to detect and characterize apoptotic cells based on alterations in cell morphology, membrane integrity, and DNA content. Two fluorescent markers, namely Annexin V and 7-AAD, were employed for this analysis. 7-AAD binds to the DNA within the cell nucleus, while Annexin V attaches to phosphatidylserine exposed on the outer membrane during apoptosis. The content of Annexin V in conjunction with the level of DNA, were utilized to categorize cells into various stages of apoptosis. Specifically, a viable cell is characterized by Annexin V-negative and 7-AAD-negative staining, while a cell in the early apoptotic phase is Annexin V-positive and 7-AAD-negative. On the other hand, a late apoptotic or necrotic cell is Annexin V-positive and 7-AAD-positive.

3.6 Immunodetection of proteins by SDS-PAGE

A2780 and A2780cis cell lines were seeded in p100 plates until they reach 80% confluence. Then cells were treated with 10µM of the corresponding drug. After 48h cells were trypsinized and centrifuged for 5 min at 300 x g. The pellet was lysed using RIPA buffer (1 M Tris-HCl pH 7.2; 0.5 M NaCl; 10% Triton X-100; 10% sodium deoxycholate; 10% SDS and EDTA 100 mM) along with a cocktail of protease inhibitors (1 mM EDTA, 2 mM EGTA, 1mM DTT, 1 mM PMSF, 10 ug/ml leupeptin, 10 ug/ml aprotinin, 1 ug/ml pepstatin) and phosphatases (5 mM NaF, 0.05 M Na3VO4) and subsequently centrifuged at 4ºC for 10 minutes at 13.000 rpm, to recover the supernatant.

The quantification of the proteins obtained was carried out by the bicinchoninic acid (BCA) method (Thermofisher) according to the manufacturer´s instructions.

Protein extracts were prepared with loading buffer (65.8 mM Tris-HCl pH 6.8; 2.1% SDS; 26.3% glycerol; 0.01% bromophenol blue and β-mercaptoethanol) and incubated 10 minutes at 95ºC. Proteins were separated by electrophoresis on an SDS polyacrylamide gel (30%), using Precision Plus ProteinTM (BIO-RAD) as molecular weight markers. Proteins were blotted (BIO-RAD miniTrans-Blot Cell) to a nitrocellulose membrane

(BIORAD). Membranes were blocked with 5% skimmed milk solution in PBS/0.05% Tween-20 for 1 hour shaking at room temperature. Membranes were incubated overnight at 4°C with the primary antibody specific for each protein to be tested (Table 2). After this incubation membranes were washed 3 times for 5 minutes with PBS/0.05% Tween 20 and subsequently incubated for 1 hour at room temperature with the corresponding secondaries antibodies (Table 2). After washing Detection was carried out with the SuperSignal West Pico ECL solution (Thermo Fisher Scientific Inc.) and proteins visualized using the GenSys program.

Table 1. *Antibodies that were assessed in the present study*

Primary antibody	Dilution	Molecular weight (Kda)	Company	Secondary Antibody	Dilution	Company
Cdk1/Cdk2	1:1000	33	Santa Criz Biotechnology	Mouse	1:2000	Abcam
Cyclin B1	1:1000	60	Santa Cruz Biotechnology	Mouse	1:2000	Abcam
Cyclin E	1:1000	53	Santa Cruz Biotechnology	Mouse	1:2000	Abcam
Actin	1:2000	43	Sigma	Mouse	1:5000	Abcam

3.7 DNA Binding Analysis

Plasmid DNA extraction (pBR322) was carried out from a bacteria culture using the QIAprep Spin Miniprep Kit (QIAGEN). Mixtures of the plasmid (200 nanograms) were prepared with increasing concentrations of the titanium derivative compounds of interest (0, 5, 10, 20, 30 and 50μM). As a control, the same plasmid was used, without the addition of any drug. Reactions were incubated for 20 hours at 37°C shaking in the dark (100 rpm). Finally, an electrophoretic separation was carried out in a gel of 0.8% (w/v) agarose for 90 minutes at 70 V, using Hyperladder TM 1kb tracer (Bioline). After the separation was complete, the gel was incubated in a RedSafe solution (iNtRON Biotechnology) diluted 1:10.000 in 1X TAE buffer. The bands were visualized using an ultraviolet (UV) transilluminator (GBOX-CHEMI, Fisher Scientifics Inc.) at 260 nm.

3.8 Statistical Analysis

All experiments were performed at least three times independently. Data are presented as the mean ± SEM.

Statistical significance between treated cells and control or between Ole-Ti and Myr-Ti treated cells was calculated using Student's t-test (Graph Pad Prism 9.5, Graph Pad Software, Inc, San Diego). P values less than 0.05 were considered to be statistically significant.

4. Results and Discussion

4.1 Effect of Myr-Ti and Ole-Ti on cell viability

To elucidate the impact of Myr-Ti and Ole-Ti on cell viability and to determine the corresponding half maximal inhibitory concentration values (IC_{50}), an MTT assay was performed.

The IC_{50} value, denoting the concentration of a compound necessary to inhibit 50% of a specific biological activity, was computed through sigmoidal regression based on cell viability. The Y-coordinate was established at 50%, reflecting the threshold at which 50% of the cells remain viable after treatment with the compound, allowing for the determination of the concentration required to achieve this level of inhibition.

Figure 6 show cell viability data for A2780 and A2780cis cell lines, sensitive and cisplatin-resistant, 72h hours after treatment, along with the respective IC_{50} (Table 3) values for Myr-Ti and Ole-Ti.

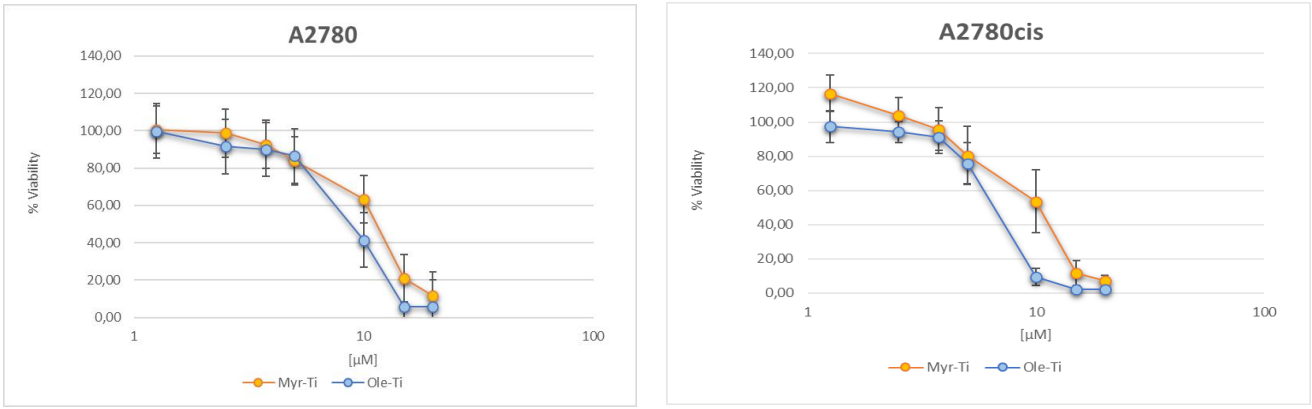


***Figure 6**. Cytotoxicity evaluation of Ole-Ti and Myr-Ti, 72 hours after treatment, for A2780 and A2780cis cell line*

***Table 2**. IC_{50} values obtained for A2780 cell line (A) and A2780cis cell line (B)*

A

	IC_{50} Value	***p-Value***
Myr-Ti	10,0 ± 1,9 µM	0,49
Ole-Ti	8,4 ± 0,6 µM	

B

	IC_{50} Value	***p-Value***
Myr-Ti	8,9 ± 2,2 µM	0,29
Ole-Ti	6,1 ± 0,6 µM	

In the parental line, A2780 cell line, it is observed that both compounds do not seem to affect cell viability up to a concentration of 5 µM. A noticeable decrease becomes meaningful at the 10µM concentration, with a 35% decrease in cell viability for Myr-Ti treatment and a 60% decrease in cell viability for Ole-Ti treatment. Both compounds show a decrease in cell viability greater than 90% starting from a concentration of 15 µM.

For A2780Cis cell line, cisplatin-resistant cell line, similar to the parental line, the concentration of 5 µM does not seem to have significant relevance in the decrease of cell viability, becoming evident only at the concentration of 10 µM where the decrease in cell viability assumes values from 45% for Myr-Ti treatment and 90% for Ole-Ti treatment. At a concentration of 15 µM, the compound Myr-Ti shows a decrease in viability greater than 90%, and for the same concentration, the decrease presented by the compound Ole-Ti is 100%. It is important to note that both cell types exhibit greater cytotoxicity when exposed to the Ole-Ti compound compared to Myr-Ti, indicating that both compounds exert significant cytotoxic effects on both cell types, with Ole-Ti having a more pronounced impact, with Ole-Ti having a more pronounced cytotoxic effect. Specifically, the IC_{50} values are observed to be 10.0 µM for the Myr-Ti compound and 8.4 µM for the Ole-Ti compound in cisplatin sensitive cells. For cisplatin-resistant cells, the IC_{50} values are determined as 8.9 µM for Myr-Ti and 6.1 µM for Ole-Ti.

Although the difference in IC50 values for both compounds did not reach statistical significance, as only four assays were conducted, limiting statistical accuracy, its trend of being lower for Ole-Ti suggests a potentially greater efficacy of Ole-Ti in

inhibiting cell viability. This is particularly relevant when considering its effectiveness in cisplatin-resistant cells, where the IC_{50} values are lower.

Additionally, the results presented here are consistent with the findings presented by Serrano et al. (2021) [30], which reported a significant antiproliferative effect of TiCp2{(OOC)2py-O-myr on human neuroblastoma cell lines, with antiproliferative concentrations observed between 10-15 µM. While Serrano et al. did not assess Ole-Ti, both studies reported low IC50 values for the Myr-Ti compound, reinforcing its potential as an antitumor agent. This alignment, together with the trends observed for Ole-Ti in the present study, further highlights the therapeutic potential of these titanium-based compounds.

4.2 Analysis of the Effect of Titanium-Derived Compounds, Myr-Ti and Ole-Ti, on the Cell Cycle

In order to investigate the impact of the compounds on the cell cycle and gain insights into their potential effects on proliferative status in tumor cells, cell cycle analysis was carried out by flow cytometry.

Consequently, the results from this analysis allow us to quantify the effect of titanium compounds on the cell cycle phases as shown in Figures 7 and 8.

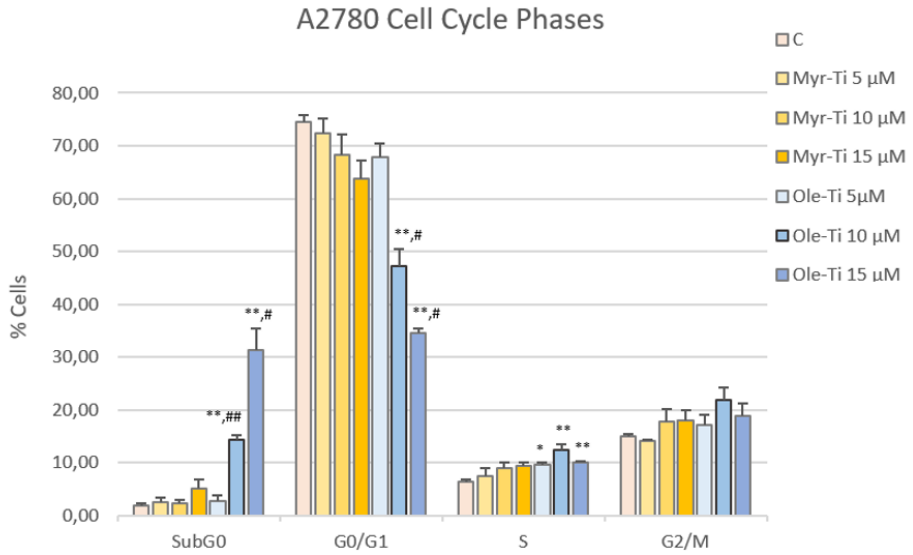


Figure 7. *Analysis of cell cycle phases after 48h of treatment with Myr-Ti and Ole-Ti in A2780 cell line. (*p<0,05; ** p<0,01 vs control cells; # p<0,05; ## p<0,01 vs Myr-Ti treated cells)*

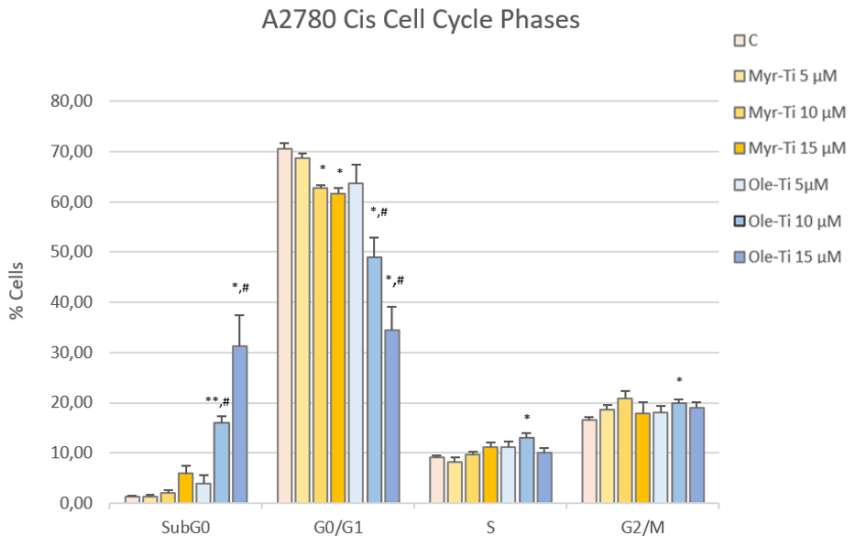


***Figure 8**. Phases of cell cycle after 24h of treatment with Ole-Ti and Myr-Ti for A278cis cell line. (*p<0,05; ** p<0,01 vs control cells; # p<0,05; ## p<0,01 vs Myr-Ti treated cells)*

When examining the Sub-G0 phase, in the A2780 cell line, there does not appear to be an increase in cells in the SubG0 phase at concentrations of 5 and 10 µM for the Myr-Ti compound, with only a slight increase noticeable at a concentration of 15 µM. In contrast, for the Ole-Ti compound, the results are significant starting from a concentration of 10 µM, with an increase of about 15% compared to the control values, becoming highly expressive at a concentration of 15 µM with an increase of about 30%. In the A2780Cis cell line, the pattern observed is very similar as that presented by the parental cell line, with similar rates of decline in both compounds. These cells in Sub-G0 phase exhibit decreased DNA content, often attributed to genetic material fragmentation, a primary factor culminating in cellular apoptosis.

In the G0/G1 phase for the A2780 cell line, there is a decrease that is not very significant but noticeable and proportional to the increase in concentration compared to the control, for the Myr-Ti compound. For the Ole-Ti compound, there is a decrease of about 20% at a concentration of 10 µM and about 35% at a concentration of 15 µM. For the A2780Cis cell line, the same pattern can be observed, with the Myr-Ti compound showing a slight decrease in the G0/G1 phase, while Ole-Ti also exhibits reductions very similar to those observed in the A2780 cell line compared to the control. This indicates that both Myr-Ti and Ole-Ti can impact the cell cycle, but Ole-Ti appears to have a more pronounced effect on reducing the population of cells in the G0/G1 phase, especially at higher concentrations.

This decrease appears to correspond to the increase in cells in the Sub-G0 phase since many of these cells have entered the phase of cellular quiescence, and as we can observe, this is an effect that is highly significant in response to the Ole-Ti compound and, not so notable for Myr-Ti compound, in both cell lines.

S Phase plays pivotal roles in ensuring the duplication and precisely regulated division of cells, thereby safeguarding genomic integrity, and facilitating tissue growth, repair, and renewal. During this phase, in both cell lines, there is no significant cell increase in the cells treated with Myr-Ti. However, a slight increase is observed in the populations of cells treated with Ole-Ti at all concentrations in the parental cell line and at a concentration of 10 µM in the cisplatin-resistant cell line. Furthermore, when analyzing the G2/M phase in the parental line, we see that the increase observed in the S phase for the cells treated with Ole-Ti does not continue into the G2/M phase. This suggests that the cells appeared to attempt continued duplication but became arrested in the S phase. Unable to progress to the G2/M phase, they likely underwent apoptosis, leading to an increase in the number of cells in the SubG0 phase. In the cisplatin-resistant cell line, the population of cells treated with 10 µM Ole-Ti, which showed an increase in the S phase, also exhibited a rise in the G2/M phase. This pattern indicates that, despite the blockage in these phases, the cells could not proceed to the G1 phase, possibly reflecting an adaptive response by the resistant population at this concentration, ultimately resulting in apoptosis.

Comparing with the literature, in the study by Gomez-Lopez et al. (2024) [44], where the compound $TiCpY_2\{(OOC)_2py\text{-}O\text{-}Myr\}]$ (Myr-TiY) was studied in the same cancer cell lines, A2780 and A2780Cis, the occurrence of a sub-G0 phase was observed, along with significant cell cycle arrest during the G0/G1 phase and a notable inhibition of cell proliferation during the S phase. The study by Abdel-Ghany et al. (2020) [45], which investigated the effect of titanium dioxide nanoparticles (TiO_2 NPs) on breast cancer cells, demonstrated an increase in the sub-G0 phase, accompanied by an increase in the G1 phase. This suggests a cell cycle interruption during these phases, with a large number of cells being prevented from progressing to the replication phase. Both studies clearly show that these compounds interfere with the cell cycle. However, based on these observations, we can suggest that Myr-Ti and Ole-Ti operate through a mechanism that does not fundamentally disrupt the cell cycle but instead leads to complete cell death, likely due to the induction of cellular apoptosis, as indicated by the increase in the

population of cells in the Sub-G0 phase. The Ole-Ti compound stands out with more promising results at concentrations of 10 and 15 µM, showing a more pronounced pre-apoptotic cell profile compared to Myr-Ti. This, in turn, supports the promising results of oleic acid presented by Jiang et al. (2017) [46], which demonstrate cell cycle arrest in the G0/G1 phases in a concentration-independent manner, along with a significant increase in the apoptotic population after treatment with oleic acid in tongue squamous cell carcinomas.

To corroborate the findings regarding the impact of both compounds on the cell cycle, an analysis of various proteins that play crucial roles in cell cycle regulation was undertaken. This analysis included proteins CDK1/CDK2 (Cyclin-dependent kinases 1 and 2), Cyclin E, and Cyclin B1. The investigation of these proteins was carried out through Western blotting immunoassays, as depicted in Figure 9.

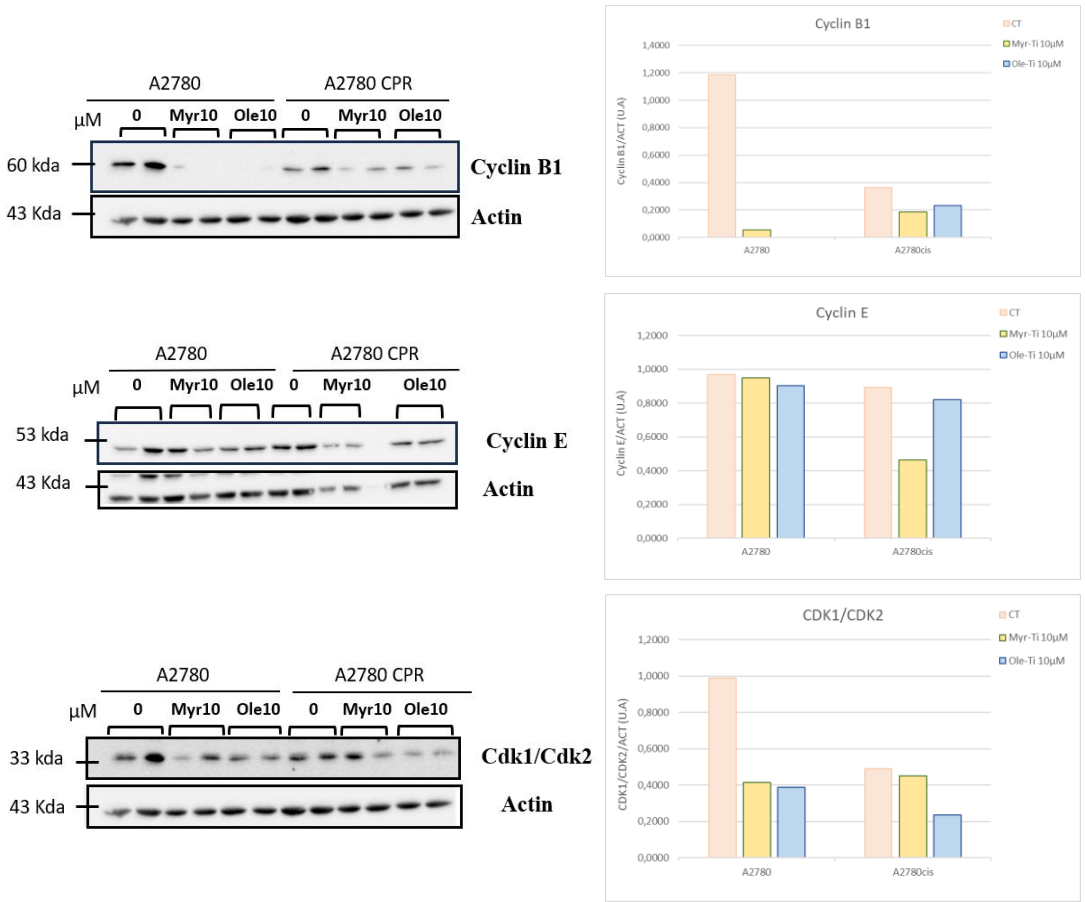


Figure 9. *Western blotting analysis of Cyclin B1, Cyclin E, and CDK1/CDK2 in A2780 anA2780Cis cell lines treated with Myr-Ti and Ole-Ti*

Through the analysis of Figure 9, a decreasing trend in the expression of Cyclin B1, as well as in the expression of CDK1/CDK2, by Myr-Ti and Ole-Ti can be observed in both cell lines. For Cyclin B1, the compound Ole-Ti at a concentration of 10 µM is able to completely inhibit the expression of Cyclin B1 in A2780 cells. For Cyclin E, only a clear decrease in protein level was seen in Myr-Ti 10 µM A2780cis-treated cells compared to control cells. CDK1/CDK2 show a decrease in protein quantity relative to the control for both compounds, with no apparent differences between Myr-Ti and Ole-Ti at 10 µM.

For the A2780cis cell line, a decrease in CDK1/CDK2 activation is also notable. Myr-Ti shows a very modest decrease, and Ole-Ti shows the smallest reduction in protein quantity, with a decrease of approximately 0.2 U.A. However, for Cyclin E and Cyclin B1 proteins, the analysis does not seem to demonstrate a conclusive result due to the irregularity presented in the data. Although the graph presented by the A2780cis cell line for Cyclin E and Cyclin B1 does not allow for a better interpretation of the results, we can observe from the graphs for these proteins in the A2780 cell line and the results for CDK1/CDK2 in both cell lines that the decrease in the activation of CDK1/CDK2, Cyclin E, and Cyclin B1 proteins suggests an inhibition of the Cyclin B1/CDK1 and Cyclin E/CDK2 complexes by the compounds Myr-Ti and Ole-Ti.

Cyclin B1 and Cyclin E are key regulators of the cell cycle, promoting progression through different phases in association with their respective cyclin-dependent kinases, CDK1 and CDK2. Cyclin B1 forms a complex with CDK1, activating the M phase of the cell cycle, while Cyclin E forms a complex with CDK2, promoting the transition from G0/G1 to the S phase. Therefore, inhibition of these proteins would lead to a disruption in the normal progression of the cell cycle. When cyclin B1 decreases, cells cannot proceed to mitosis and are retained in the G2/M phase. Similarly, when cyclin E decreases, cells are unable to advance to replication, remaining in the G0/G1 phase.

In this study, we can deduce that the accumulation of cells in the G2/M phase coincides with the observed decrease in Cyclin B1. The reduction in Cyclin B1 levels prevents the formation of the Cyclin B1/CDK1 complex, thereby blocking the entry of cells into the mitotic phase. This effect is particularly evident in the A2780 cell line, where a significant decline in Cyclin B1 levels is associated with an increased number of cells in the S phase, suggesting an arrest in DNA replication.

In contrast, in the A2780Cis cell line, the decrease in Cyclin B1 levels is less pronounced, and the surviving cells appear to be adapting differently. Rather than accumulating in the S phase as seen in A2780 cells, these cells continue to progress through the cycle, albeit with potential alterations in checkpoint regulation. This difference suggests that while Cyclin B1 downregulation contributes to cell cycle arrest in A2780 cells, A2780Cis cells might rely on alternative mechanisms to bypass this arrest and sustain proliferation. The effect of Cyclin B1 dysregulation is highlighted in a study by Liu et al. (2019) [35], where the dysregulation of Cyclin B1, induced by Aloperine, led to cell cycle arrest in the G2/M phase in hepatocellular carcinoma cells.

However, the data regarding Cyclin E are less clear, and there does not appear to be a significant retention of cells in the G0/G1 phase, suggesting that DNA replication may not be inhibited. Based on the literature, we might have expected a more conclusive decrease in Cyclin E, given the modest cell cycle arrest during the S phase in both cell lines. Previous studies conducted by Chappuis et al. (2025) [47] suggest that inhibition of Cyclin E expression promotes cell cycle arrest and prevents tumor cell proliferation in breast cancer, with elevated Cyclin E levels being a negative prognostic marker for this type of cancer.

4.3 Analysis of the Effect of Titanium-Derived Compounds, Myr-Ti and Ole-Ti, on the apoptotic Cell death

To validate the outcomes obtained in the cell cycle analysis for SubG0 phase, an apoptosis assay utilizing flow cytometry was conducted. This was carried out to corroborate the findings observed in the Sub G0 phase and to affirm the compounds' effectiveness in triggering programmed cell death.

In Figure 10, we can observe the results of quantification of early apoptotic cells (Annexin V positive/7AAD negative) in both cell lines, shedding light on the compounds' impact on inducing apoptosis in these cells.

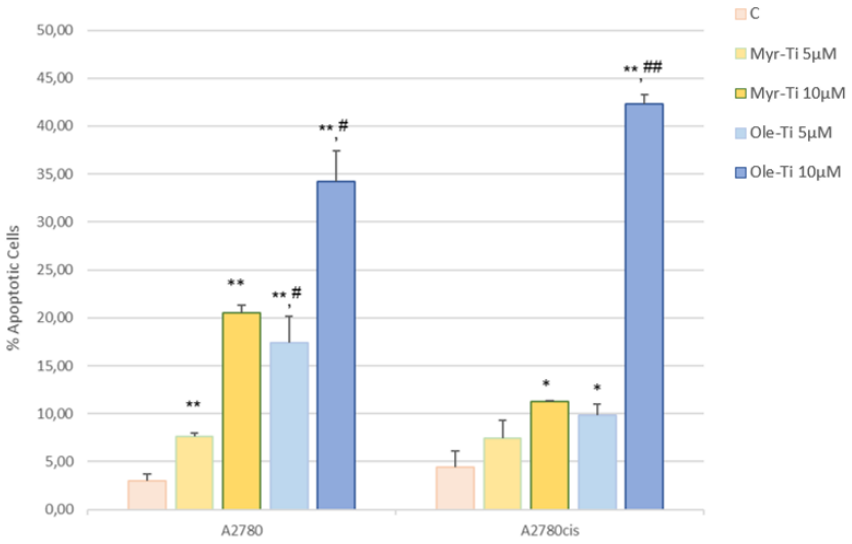


Figure 10. *Analysis of early apoptotic cells using Annexin V and AAD, after 48h treatment with Myr-Ti and Ole-Ti in A2780 and A2780cis cell line (*p<0,05; ** p<0,01 vs control cells; # p<0,05; ## p<0,01 vs Myr-Ti treated cells)*

Through the analysis of the graph, we can observe that the concentration of 5 µM of Myr-Ti does not seem to be effective for the cisplatin-resistant cell line, showing significance only for the parental cell line. In contrast, the Ole-Ti compound shows significance at a concentration of 5 µM for both cell lines. For both the parental and cisplatin-resistant cell lines, the Ole-Ti compound at a concentration of 10 µM achieves the highest values of cell apoptosis, being over 30% in the A2780 cell line and over 40% in the A2780Cis cell line. For the Myr-Ti compound at 10 µM, the results are relatively lower, with cell apoptosis values slightly above 20% for the A2780 cell line and values above 10% for the A2780Cis cell line. A substantial apoptotic effect exerted by the Ole-Ti compound compared to the Myr-Ti compound in both cell lines, with Ole-Ti at 15 µM registering the highest levels of cellular apoptosis, is observed. However, we can still observe that the results seem to indicate a higher sensitivity of the parental cell line to the Myr-Ti compound and a greater response to the 10 µM concentration of Ole-Ti when compared to the cisplatin-resistant cell line. These results reveal a proportional increase in apoptosis with the increase in the compound concentrations, showing the compounds' potential to induce programmed cell death. They highlight the superior cytotoxic effect of Ole-Ti, especially at higher concentrations, as an effective agent in promoting apoptosis within the experimental context. This allows us to infer that the results obtained for apoptosis align with the observations made regarding the cytotoxic effects of the

compounds and their influence on the cell cycle. The induction of the apoptosis results observed in this study for the Myr-Ti and Ole-Ti compounds align with those reported by Gomez-Lopez et al. (2024) [44] where a significant induction of apoptosis was also noted in both A2780 and A2780Cis cell lines when treated with the Myr-TiY and TiY compounds. Specifically, Myr-Ti demonstrated slightly higher levels of apoptosis compared to Myr-TiY in both cell lines, with a more pronounced effect observed at higher concentrations. It is important to note that the Ole-Ti compound was not studied in these prior works, and therefore, a direct comparison of its effects with those of Myr-TiY and TiY is not possible. However, our findings reveal that Ole-Ti exhibits even more robust apoptotic activity than Myr-TiY and TiY, inducing over 30% apoptosis in the A2780 cell line and over 40% in the A2780Cis line at a concentration of 10 µM.

Notably, the study by Serrano et al. (2021) [30], provides insights into how structural modifications in titanium compounds, particularly the incorporation of myristic acid, can enhance their biological activity. In that study, the presence of a myristic chain in the compound [TiCp2{(OOC)2py-O-myr}], significantly increased its cytotoxicity and ability to induce apoptosis, especially in cancer cell lines such as neuroblastoma, while also promoting strong interactions with proteins like human serum albumin (HSA).

Furthermore, while our study demonstrated that Myr-Ti was more effective in inducing apoptosis in the parental A2780 cell line, the substantial apoptotic effect of Ole-Ti, particularly in the cisplatin-resistant A2780Cis cells, underscores its potential as a superior cytotoxic agent. This aligns with the findings from Serrano et al. (2021)[30]study, where compounds with specific structural modifications, like aliphatic chains, exhibited selective cytotoxicity and higher efficiency in cancer cells. The proportional increase in apoptosis with rising concentrations of both Myr-Ti and Ole-Ti in our study further reinforces the cytotoxic potential of these titanium-based compounds.

4.4 DNA Binding Analysis

To ascertain whether DNA is a molecular target for our titanium derivates, a DNA binding assay was conducted to investigate the interactions between these compounds and DNA. In the event of compound-DNA interactions, they could potentially induce cleavage in the supercoiled form of plasmid DNA (SC), leading to an increase of the relaxed circular (OC) or linear (L) forms. All the three forms share an identical base pair

length but exhibit distinct conformations, thereby yielding differing migration patterns in an agarose gel. The supercoiled form exhibits higher mobility and advances further owing to its compactness, whereas the linear and relaxed forms display comparatively reduced migration. Figure 11 illustrates the outcomes following electrophoretic separation of plasmid DNA treated with Myr-Ti and Ole-Ti.

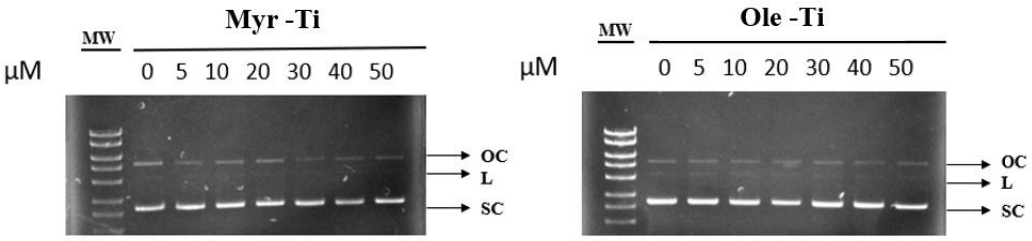


Figure 11. *Agarose gel electrophoresis of pBR322 DNA in the presence of Myr-Ti and Ole-Ti*

Previous studies have shown that cisplatin compounds induce alterations at the DNA level, as demonstrated in the study by Keck et al. (1992) [48]. In this study, the interference of Cisplatin, cis-[Pt(NH3)2(N8-Etd)Cl]$^{2+}$, and cis-[Pt(NH3)2(N3-Etd)Cl]$^{2+}$ with DNA was observed.

In contrast, the results presented in this study indicate that the compounds Myr-Ti and Ole-Ti do not induce detectable conformational changes in plasmid DNA at any of the concentrations tested. These findings are consistent with those reported by Gomez-Lopez et al. (2024) [44], who observed similar results with the titanium compound MyrTiY. These findings suggest that DNA may not be the primary target of Myr-Ti and Ole-Ti, and their biological activity might occur through alternative mechanisms, such as interactions with proteins, cell membranes, or other cellular pathways. This is supported by the observed decrease in Cyclin B1 levels, which indicates that there is no retention in the S phase of the cell cycle; instead, a post-S arrest occurs, preventing the cells from dividing. Thus, the reduction in Cyclin B1 provides a rationale for the lack of DNA damage, further emphasizing the need to consider alternative pathways in understanding the effects of Myr-Ti and Ole-Ti.

5.Conclusions

Ovarian cancer remains one of the most lethal gynecological malignancies due to its late-stage diagnosis and the high incidence of chemoresistance, particularly against platinum-based therapies like cisplatin. Despite its effectiveness, cisplatin treatment is often limited by severe toxicity and the eventual development of resistance, leaving a critical need for more effective and less toxic therapeutic alternatives. Given these challenges, the search for novel agents that can both enhance therapeutic efficacy and overcome resistance mechanisms is imperative.

Titanium-based compounds have emerged as promising candidates in the field of cancer therapy, owing to their distinct anticancer properties and potential for reduced toxicity compared to conventional platinum-based drugs. Early studies on these compounds have shown significant anticancer activity, encouraging further exploration into modifications that could improve their pharmacokinetics, bioavailability, and selectivity toward cancer cells. In this context, two novel titanium-derived compounds, Myr-Ti and Ole-Ti, were synthesized and investigated for their cytotoxic potential in ovarian cancer cell lines, including those resistant to cisplatin.

The primary objective of this study was to assess the cytotoxic effects of Myr-Ti and Ole-Ti, focusing on their ability to induce cell death and disrupt the cell cycle in both cisplatin-sensitive (A2780) and cisplatin-resistant (A2780Cis) ovarian cancer cells. Viability assays revealed significant reductions in cell survival at concentrations as low as 10 µM, with both compounds exhibiting strong cytotoxic effects, particularly Ole-Ti, which caused total cell death at 15 µM in cisplatin-resistant cells. These findings were further supported by apoptosis assays and IC50 values, where Ole-Ti demonstrated superior pro-apoptotic activity compared to Myr-Ti.

Further mechanistic studies revealed that Ole-Ti induced significant pre-apoptotic activity, particularly through an accumulation of cells in the Sub-G0 phase, suggesting a pronounced disruption in cell cycle progression. Notably, cell cycle analysis indicated an arrest at the G2/M phase in Ole-Ti-treated cells, implying that while cells attempt to replicate DNA, they are driven into apoptosis before completing the cell cycle. Immunoblotting confirmed a marked reduction in key regulatory proteins, such as Cyclin B1 and CDK1/CDK2, especially in response to Ole-Ti.

Interestingly, DNA binding studies showed that neither Myr-Ti nor Ole-Ti induced detectable conformational changes in plasmid DNA, even at higher concentrations. These findings suggest that their antitumor activity may not be mediated through direct DNA interaction but through alternative mechanisms such as protein interactions, membrane disruption, or interference with other cellular pathways.

Although the number of replicates was limited, the data highlight the potential of these compounds, particularly Ole-Ti, as promising candidates for ovarian cancer treatment, including in cisplatin-resistant cases. Moreover, the lipophilicity of Ole-Ti may facilitate its entry into tumor cells, allowing for more effective targeting [35]. Additionally, its higher affinity for albumin could contribute to prolonged circulation time in the bloodstream, enhancing therapeutic efficacy [36]. Reinforcing these aspects, responses at the membrane level through specific receptors may also play a significant role in its action [49], making Ole-Ti a strong candidate for potential new treatments. Future in vivo studies will be necessary to evaluate the toxicity, pharmacokinetics, and safety profiles of these compounds. Nonetheless, this study opens new therapeutic avenues and brings hope for the development of more effective treatments for ovarian cancer.

6.References

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