LEBANESE AMERICAN UNIVERSITY

Novel platinum II and platinum IV complexes exhibit potent cytotoxicity and selectivity towards several cancer cell lines

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A thesis
Submitted in partial fulfillment of the requirements
For the degree of Master of Science in Molecular Biology

School of Arts and Sciences
April 2020

ACKNOWLEDGEMENT

I was honored to have Dr. Daher as my advisor and grateful for his overwhelming support, appreciation, advices and unlimited kindness. He taught me how a successful biologist should act when faced to unexpected obstacles, and how a researcher should always be ready to start all over again, “that’s why it’s called re-search”. All the work and experiments presented in this thesis were designed and supervised by him.

I would also like to thank Dr. Robin Taleb and Dr. Rony Khnayzer who were always ready to help at any moment during my Master’s journey. They taught me a lot about research and specially the chemistry-related part which wouldn’t have been easy without their help. I am very thankful for our numerous instructive meetings with Dr. Daher that always benefited me and helped me to reach my goal.

I am also very thankful for the support of my committee members Dr. Roy Khalaf and Dr. Christian Khalil, and for their great interest in my project. Additionally, I would like to thank Dr. Wassim Shebaby for always being available whenever I needed help in my experiments.

I cannot but express my love and gratitude to my family and specially my mother who will always be my number one supporter. Finally, I was extremely lucky to work in a lab full of caring and supporting colleagues: Najwa Mansour, Stephanie Mehanna, Petra krtenova, and Maria Elias who were always ready to sacrifice their time in order to teach me new techniques and to help me in my experiments whenever needed. Thank you for being my second family.

Last but not least, I would like to thank my very dear friend and lab partner Christelle Fayad, with whom I spent the best moments during this Master’s journey, helping and supporting me all the way. Thank you for everything we did together and I would have never accomplished all this without your help.

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ABSTRACT

The discovery of cisplatin and its antineoplastic potential directed research toward the usage of platinum-based complexes as chemotherapeutical drugs. The aim of the present study is to test the anticancer activity of two novel platinum complexes Pt(II) and Pt(IV) on lung cancer cells (A549), breast cancer cells (MDA-MB-231), melanoma cells (A375), and mesenchymal stem cells (MSC), and evaluate the mechanism of action involved. Cell viability was assessed 72 hours post-treatment using MTS assay. The cellular uptake of both compounds was measured by ICP-MS. Their effect on DNA fragmentation was then tested using Comet assay. The type of cell death was assessed by flow cytometry. Evaluation of apoptotic proteins' expression was performed using western blots. Results showed that both complexes significantly reduced cancer cell viability and exhibited 7 to 20-fold higher cytotoxicity compared to cisplatin. Pt(IV) showed remarkable selectivity towards different cancer cells (17-22 fold) compared with mesenchymal stem cells. While both complexes were actively transported into the cells, Pt(II) showed a faster and higher uptake compared to its platinum IV analogue. Pt(IV) but not Pt(II) treatment caused significant DNA fragmentation in A549 cells. Western blot analysis demonstrated an upregulation of Bax/Bcl-2 ratio and cytochrome c, downregulation of procaspases 3 and 9 and cleavage of Parp for both Pt(II) and Pt(IV), which indicates that apoptosis was induced through the intrinsic pathway. No effect on procaspase 8 was observed, eliminating the involvement of the extrinsic apoptotic pathway. Flow cytometry analysis confirmed the apoptotic cell death caused by both platinum complexes. In conclusion, Pt(II) and Pt(IV) may be considered as promising anti-cancer drugs with Pt(IV) being

more selective to cancer cells, which might provide a robust alternative for currently approved anticancer platinum medications.

Keywords: Platinum, Cancer, Pt(II), Pt(IV), Cisplatin, Cytotoxicity, Selectivity, DNA Fragmentation, Intrinsic, Apoptosis.

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LIST OF ABBREVIATIONS

UV: ultraviolet

NSCLC: non-small cell lung cancer

SCLC: small cell lung caner

BRCA1: breast cancer 1 tumor suppressor gene

BRCA2: breast cancer 2 tumor suppressor gene

PTEN: Phosphatase and tensin homolog

BMI: Body mass index

TNF: tumor necrosis factor

TNFR1: tumor necrosis factor receptor 1

TNF-α: tumor necrosis factor alpha

DISC: death-inducing signaling complex

AIF: Apoptosis Inducing Factor

CAD: Caspase-activated DNase

ICAD: inhibitor of Caspase-activated DNase

Bax: Bcl2-Associated X Protein

Bad: Bcl2 Associated Agonist of Cell Death

Bak: Bcl2 homologous antagonist killer

Bcl-2: B-cell lymphoma 2

BH3: Bcl-2 Homology 3

Bcl-x: B-cell lymphoma x

DIABLO: Direct Inhibitor of Apoptosis-Binding protein with LOw pI

SET: Nucleosome assembly protein

DNA: Deoxyribonucleic acid

PARP: Poly (ADP-ribose) polymerase

NUMA: Nuclear mitotic apparatus protein

FDA: Food and Drug Administration

ATP: adenosine triphosphate

ATPase: adenosine triphosphatase

Ctr1: High affinity copper uptake protein 1

MAPK: mitogen-activated protein kinase

Akt: Protein Kinase B

DCA: dichloroacetate

H_2O_2: hydrogen peroxide

pH: potential hydrogen

MTS: (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)

PMS: phenazine methosulfate

MSC: mesenchymal stem cells

ICP-MS: Inductively coupled plasma mass spectrometry

IC_{50}: Inhibitory concentration 50

DMEM: Dulbecco's Modified Eagle's Medium

PBS: Phosphate Buffered Saline

Pen/Strep: Penicillin-streptomycin

FBS: Fetal bovine serum

SDS: Sodium dodecyl sulphate

BSA: Bovine serum albumin

APS: Ammonium persulfate

TBS: Tris-buffered saline

TBE: Electrophoresis buffer

TBS-T: Tris-buffered saline with Tween 20

HRP: Horseradish peroxidase

ACUC: Animal Care and Use Committee

PI: Propidium iodide

PS: Phosphatidyl serine

Rpm: Rounds per minute

LMA: Low melting agarose

PVDF: Polyvinylidene fluoride

SEM: Standard error of the mean

TMI: Tail moment index

CASP: Comet assay software package

KDa: Kilodalton

BID: BH3 interacting-domain death agonist

CO_2: Carbon dioxide

$KMnO_4$: Potassium permanganate

NaCl: Sodium chloride

HCl: Hydrochloric acid

NaOH: Sodium hydroxide

ROS: Reactive oxygen species

FITC: Fluorescin Isothiocyanate

EtOH: Ethanol

HNO_3: Nitric acid

Chapter One

Literature Review

1.1 Cancer

1.1.1 Overview

Cancer occurs when normal cells of the body start replicating and dividing in an abnormal and uncontrollable manner leading to their infiltration and destruction of the normal tissues of the body. These cancer cells might acquire the ability to spread all over the body in a phenomenon called metastasis (Mayo Clinic, 2018) (figure 1). Because of this ability, cancerous tumors are considered malignant which are, unlike benign tumors, capable of invading nearby tissues by breaking the basal membrane and traveling through blood or lymph to distant parts of the body (Dana-Farber Cancer Institute, 2019). Cancer tumors are mainly classified based on the type of tissue of origin. Based on this criterion, cancer can be subdivided into 6 major categories including solid and non-solid tumors (carcinoma, sarcoma, myeloma, leukemia, lymphoma and mixed types) (National Cancer Institute, 2015).

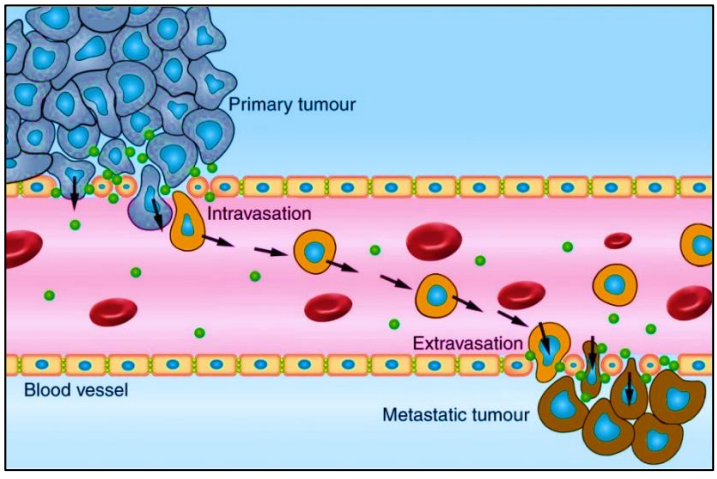


Figure 1. Metastasis of cancerous tumors. Cancer cells acquire the ability to metastasize and travel to distant parts of the body (Peng et al., 2019).

1.1.2 Differences between normal and cancer cells

The main difference between cancer and normal cells is proliferation. Normal cells divide and proliferate in a controlled manner and are destroyed by the body whenever they become old or damaged. However, in cancer cells, this is not the case. In fact, cancer cells continue to grow uncontrollably and escape apoptosis through different ways (National Cancer Institute, 2015). (figure 2). Additionally, cancer cells can detach from one another by losing surface proteins in order to migrate and metastasize to different body parts which does not occur in normal body cells. Other differences include abnormal cell signaling in cancer cells, lack of differentiation of these cells and presence of several mutations in different genes such as tumor suppressors genes and proto-oncogenes in cancer cells (Cancer Research, 2014).

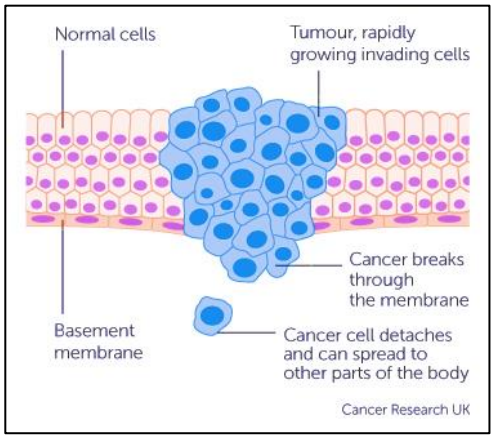


Figure 2. Difference in morphology and behavior between cancer and normal cells. Unlike normal cells, cancer cells can rapidly grow breaking through the membrane and spreading to other parts of the body (Cancer Research, 2014).

1.1.3 Causes of cancer

Cancer does not result from a single factor. In fact, the combination of many different genetic and environmental factors might lead to this disease (figure 3). Genetic alterations are often found in cancerous tumors including mutations in oncogenes leading to an uncontrollable cell growth and proliferation, mutations in tumor suppression genes which favors the development of the tumor and mutations mismatch-repair genes increasing the mutational rate because of unrepaired damaged DNA. Additionally, several risk factors might increase the probability of developing cancer including smoking,

obesity, alcohol, family history, pollution, UV radiation etc. (Cancer Research Institute, 2019; Lewandowska, Rudzki, Rudzki, Lewandowski, & Laskowska, 2019).

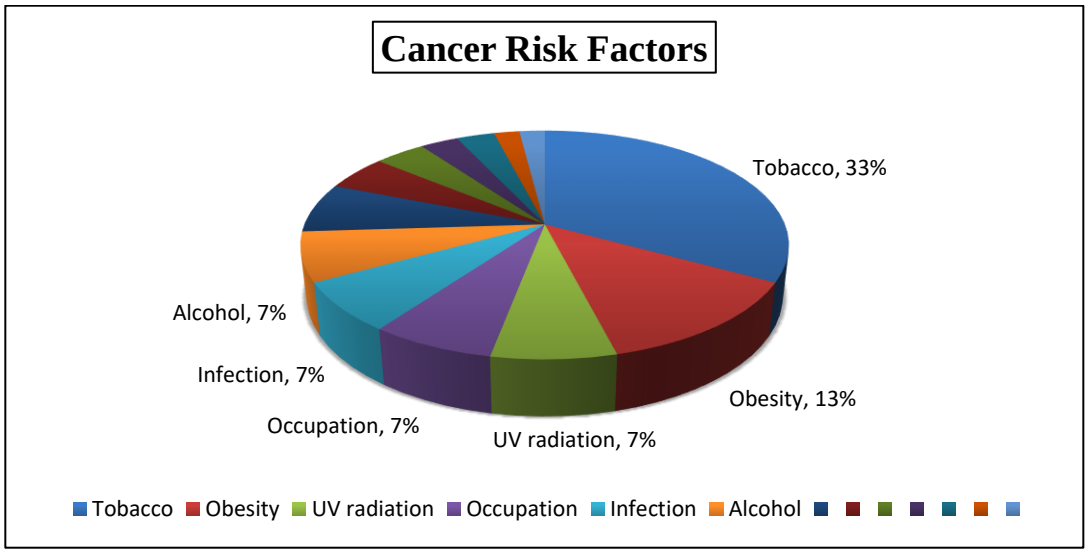


Figure 3. Risk factors for cancer. The main risk factors increasing the risk of cancer diagnosis include tobacco smoking, obesity and UV radiation (Cancer Research, n.d.).

1.2 Lung cancer

1.2.1 Overview

According to the World Health Organization, cancer is the second most frequent death-causing disease, with over 9 million deaths recorded in 2018. In fact, around 17% of death cases are attributed to cancer (World Health Organization, 2018). This number is still increasing with 1,762,450 new cancer cases of which 606,880 deaths recorded in the U.S in 2019 (Siegel, Miller, & Jemal, 2019). Different cancer types are responsible for this high mortality, among which are skin, prostate and breast cancers (figure 4). However, causing more than 25% of cancer-related death cases in the world, and with a very low 5-year survival rate (4-17%), lung cancer was classified as the most frequent deadly cancer type in the world (American Cancer Society, 2020; Hirsch et al., 2017).

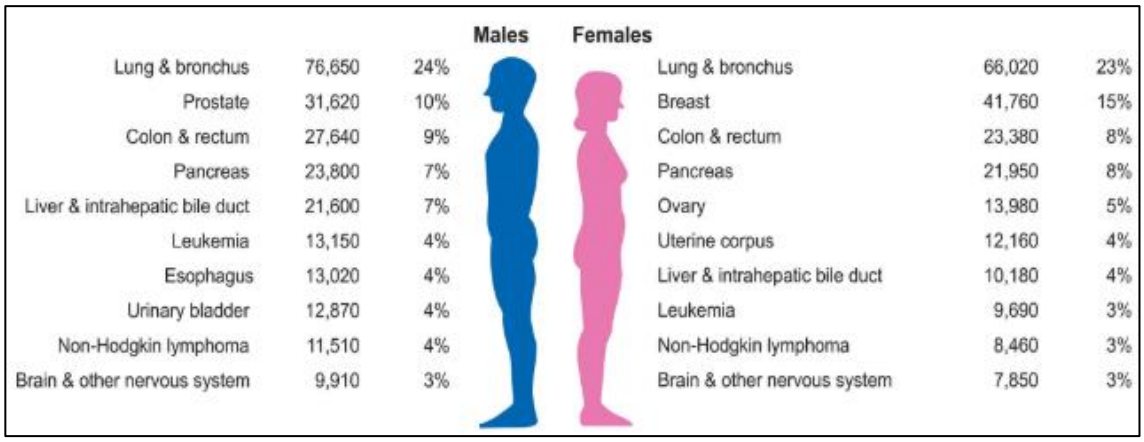


Figure 4. Ten most abundant types of cancer in both sexes. Number of cancer cases per year due to different cancer types. As it is clearly shown, lung cancer is the most frequent death-causing type of cancer in both sexes. Modified from (Siegel et al., 2019).

1.2.2 Risk factors

Although everyone is differently vulnerable to develop lung cancer based on his genetic background or lifestyle, different risk factors might increase the probability of acquiring this deadly disease. The major cause of lung cancer and responsible of more than 80% of death cases is tobacco smoking (American Cancer Society, 2020). This factor strongly affects the individuals in a direct or indirect way (secondhand smoking) and, in combination with other additional factors, increases the probability of acquiring lung cancer. More than 7000 chemicals are found in tobacco smoke and a high number of these chemicals is known to cause cancer (American Lung Association, 2019) . In fact, continuous exposure to the different carcinogens present in cigarettes leads to an increased damage in the cells lining the lungs, increasing the rate of mutations in these cells leading to lung cancer (Mayo Clinic, 2019) . However, many other factors could similarly contribute to this disease including pollution via exposure to several chemicals. Radon gas, in elevated levels, is an important risk factor as well as secondhand smoke which is leading to the death of more than 41000 people in world yearly (American Lung Association, 2019) . Additionally, genetic changes can be considered as an important risk leading to lung cancer. Mutations in different oncogenes and tumor suppressor genes might increase this risk in addition to family history, diet and radiation (Cancer Research, 2019).

1.2.3 Classes and stages

Lung cancer is classified into two major categories: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) (figure 5). This classification is based on different criteria including prognosis, appearance under microscope and treatment. NSCLC is the most common class of lung cancer and is present in approximately 85% of the cases. This class includes additional subtypes: adenocarcinoma which is considered to be the most frequent subtype of lung cancer in the United States, squamous cell carcinoma and large cell carcinoma with a percentage of 30% and 10% respectively (Gridelli et al., 2015). The second class of lung cancer (SCLC) is less common than the previously mentioned one but is most frequently found in the case of heavy smokers (Mayo Clinic, 2019).

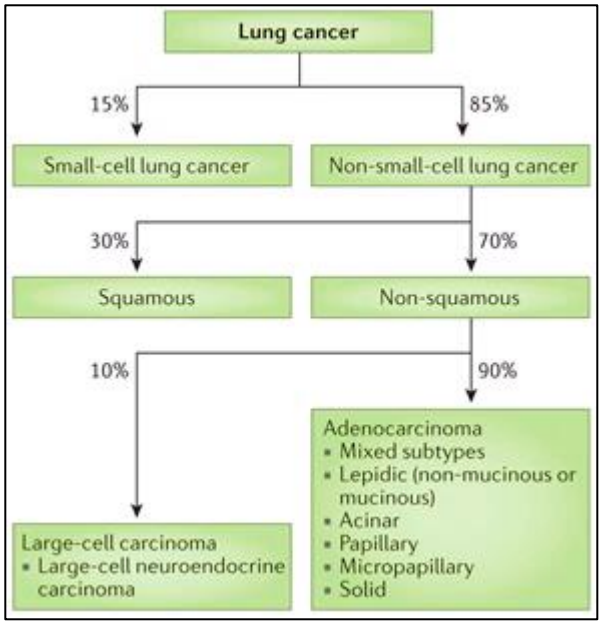


Figure 5. Classification of lung cancer. Lung cancer is mainly classified into small and non-small cell lung cancer. The latter is further classified into two additional subgroups: squamous and non-squamous (Gridelli et al., 2015).

Concerning the stages of the disease's progression, a different classification is attributed to the 2 different classes of lung cancer because of the difference in the progression of cancer in each class. In fact, while NSCLC exhibits four different stages of progression, only two stages exist in SCLC (Chheang & Brown, 2013) . In NSCLC, stage I occurs when cancer is localized in the lungs without spreading outside the affected area. When cancer starts to spread to nearby lymph nodes, stage II takes place. In the more

advanced stage III, the disease progresses and spreads to more distal lymph nodes in the middle of the chest and this stage can be further divided into two subtypes: stage IIIA in which the spreading of the disease occurs exclusively to lymph nodes located on the same side of the primary tumor's location, and stage IIIB in which the affected lymph nodes are located on the opposite side of the chest. The most advanced stage IV is characterized by the metastasis of lung cancer to distal organs of the body after reaching both lungs and plural membranes (Lemjabbar-Alaoui, Hassan, Yang, & Buchanan, 2015) . In SCLC, only 2 stages take place: limited and extensive stage. In the limited stage, cancer is only found in one hemithorax and no significant metastasis is detected. However, in the extensive stage, cancer spreads to the opposite lung and distant organs and nodes (Kalemkerian, 2012). Staging is a very important factor related to survival. Patients diagnosed with lung cancer at earlier progression stages will have higher survival rate than those diagnosed with more advanced stages (Cancer Research, 2015)

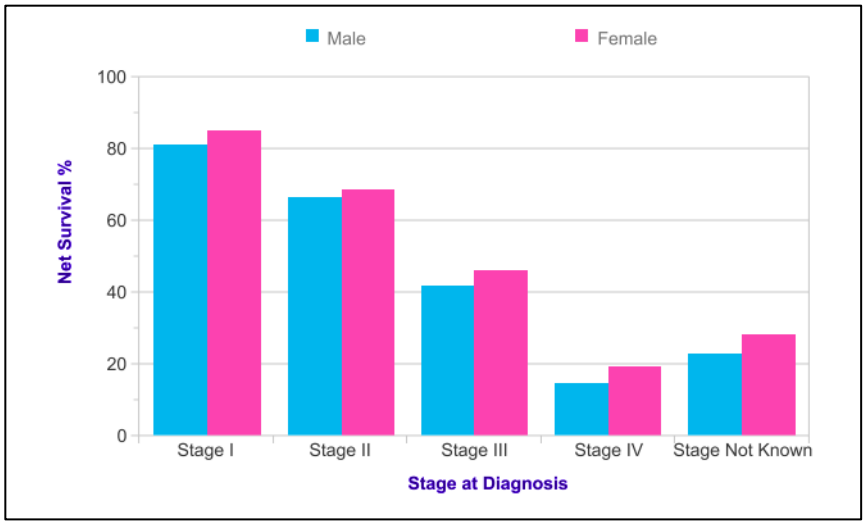


Figure 6. One-Year Net Survival (%) by Stage. Around 80% of patients diagnosed at stage I lung cancer from both sexes had one-year net survival compared to less than 20% of patients diagnosed at the more advanced stage IV (Cancer Research, 2015).

1.2.4 Treatment

The first option used to treat lung cancer is surgery, which consists of removing the entire area in which the tumor is localized (CancerCare, 2019) (figure 7). The risk of this treatment depends on the exact size and location of the tumors, as well as on the health of the individual. Surgery is usually applied in the case of stage I, II and IIIa of NSCLC (Zappa & Mousa, 2016).

Chemotherapy, and particularly combination chemotherapy, is another effective treatment used to treat more advanced lung cancer cases (Chan & Coward, 2013) . It is usually applied after surgery in order to increase the probability of curing. This treatment consists of injecting drugs in order to kill cancer cells via different ways including microtubule breakdown, preventing the assembly of microtubules, chelating agents interfering with DNA etc. Combination chemotherapy was shown to be more effective in cancer treatment compared to the effect of individual chemotherapeutic agents (Fossella et al., 2003; Schiller et al., 2002). However, one of the main problems of this treatment method is the fact that these drugs affect normal dividing cells leading to several side effects such as hair loss that is usually seen in patients undergoing chemotherapy (American Cancer Society, 2019) .

Radiotherapy is also used as a primary treatment by applying X-rays on cancer cells in order to kill them. Approximately, half of the malignancy cases are treated using this method (Buckley, Lynam-Lennon, O'Neill, & O'Sullivan, 2020). Radiation is an important treatment that could also be applied post-surgery in order to remove all remaining cancerous cells escaping surgery that could develop into new tumors after a short period of time (Amini, Yeh, Gaspar, Kavanagh, & Karam, 2014; Zappa & Mousa, 2016). Targeted therapy using FDA approved drugs against specific genes on which cancer cells depend on is another treatment option. In this case, cancer cells are killed in a more selective way than the one used in chemotherapy or radiation, reducing the side effects of this treatment method (Ke, X. & Shen, 2017) .

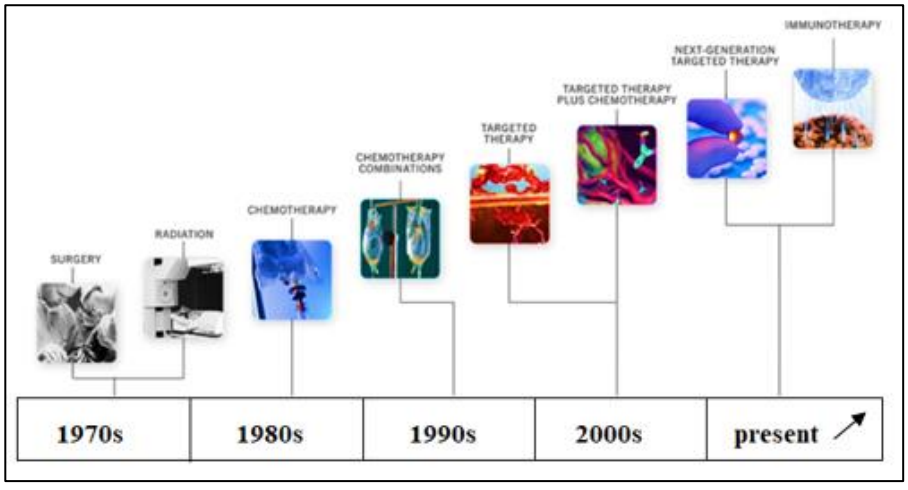


Figure 7. Lung cancer treatments. Different treatment options for lung cancer existed over the years, the earliest being surgery in the 1970s. Modified from (Chaudhary, 2016).

1.3 Breast cancer

1.3.1 Epidemiology and risk factors

According to American cancer society, breast cancer is considered to be one of the most commonly diagnosed cancer in women, causing more than 42,000 expected death cases among women in 2020 (American Cancer Society, 2020). Several risk factors can increase the probability of developing breast cancer including age, genetic background, personal and familial history, etc. (Shah, Rosso, & Nathanson, 2014). Age is considered one of the most important risk factors of breast cancer. In fact, as women get older, the probability of getting diagnosed with this disease increases from 0.44% to 3.5 % beyond the age of 60 (BreastCancer.ORG, 2018). This risk factor plays an essential role in diagnosis and it could be incorporated in the optimization of the diagnosis model in order to be improved (Feng et al., 2019). Other uncontrollable factors include family history and genetic predisposition. Having a family history in which breast cancer is present can increase the risk of being diagnosed with this disease; a woman having a mother previously diagnosed with breast cancer is 1.69 times more likely to develop this disease compared to a woman with no affected relatives (Colditz, Kaphingst, Hankinson, & Rosner, 2012). Important genetic factors can also increase the probability of developing this cancer including mutations in several genes like BRCA1 and BRCA2, mutations in different tumor suppressor genes including P53 and PTEN, the latter being the most frequently lost tumor suppressors in cancer (McCready, Littlewood, & Jenkinson, 2005) . A high exposure to estrogen over a long time period is also one the important risk factors (Shah et al., 2014).

Controllable risk factors include obesity. Obese and over-weight women with increased BMIs are at higher risks of developing breast cancer compared to women with normal weight (Lee, Kruper, Dieli-Conwright, & Mortimer, 2019). In fact, in postmenauposal women, the lack of estrogen production by ovaries is compensated by another estrogen source: adipose tissues. Increasing fat levels lead to increased estrogen hormones which, as previously mentioned, constitute an important risk factor (Picon-Ruiz, Morata-Tarifa, Valle-Goffin, Friedman, & Slingerland, 2017). Obesity is strongly related to another important risk factor: physical activity. The lack of exercising increases the risk of developing breast cancer, while this probability was found significantly

decreased in physically active women (Siewierska et al., 2018). Additionally, other lifestyle factors can increase the risk of breast cancer including excessive alcohol consumption and tobacco smoking (Shah et al., 2014).

1.3.2 Screening

Several screening methods have been used by patients and clinicians in order to achieve early detection of breast cancer, which leads to better treatment, therapy or possible cure (McCready et al., 2005). Self-examination is the simplest and most basic screening methods, which should be performed monthly for better and earlier detection of this cancer (Dewi, Kesuma Massar, Karlijn, Ruiter, & Leonardi, 2019) . Starting the age of 40, women are recommended to perform this screening method clinically each year for safety approaches, which was found effective in reducing breast-cancer-related mortality (Moss et al., 2015).

One of the most important and frequently used screening methods is mammography, which consists of applying low energy X rays to women breast in order to detect neoplastic tissues (Seely & Alhassan, 2018). Compared to other basic screening methods, mammography was shown to reduce mortality rate in a high efficacy (Hanley, Hannigan, & O'Brien, 2017). However, research shows controversial results regarding this mortality rate, and controversies remain concerning the recommended age, effectiveness and utility of this screening method (Swain, Jeudy, & Pearlman, 2016). Magnetic resonance imaging and ultrasound are two additional screening methods. The former presents multiple advantages over mammography including higher specificity and sensitivity (Warner et al., 2008), and is used in cases of high-risk patients or in case of failure in the mammography's detection because of the breast augmentation. (Kriege et al., 2004).

1.3.3 Treatment

Previously mentioned treatment methods for lung cancer also applies in the case of breast cancer. This includes surgery which could be either lumpectomy (removing only the tumor with margin cells) or mastectomy (removing the whole breast tissue), in addition to chemotherapy and radiotherapy that can also serve as treatment methods in several

cases (American Cancer Society, 2019). Another additional therapy would be hormonal therapy that only applies in the case of hormone-receptor-positive breast cancers. This therapy consists decreasing the effects of estrogen in the body by decreasing its levels or blocking its effects on cancer cells (Awan & Esfahani, 2018).

1.4 Skin cancer

1.4.1 Occurrence

Melanoma occurs as a result of several genetic mutations in cells responsible of pigment production (melanocytes), found in several organs in the body including eyes and skin (Domingues, Lopes, Soares, & Pópulo, 2018). The number of patients diagnosed with melanoma is rapidly increasing worldwide, which could cause several problems socially and economically (Rastrelli, Tropea, Rossi, & Alaibac, 2014). This malignant disease is considered today one of the most common malignancies affecting women at a very young age (Todd & Driscoll, 2017). The frequency of this cancer type has increased by more than 250% (in the U.S) in less than 30 years (Rastrelli et al., 2014), including a 47% increase worlwide in the past decade (2010-2020) (Skin Cancer Foundation, 2020). Additionally, a 2% increase is expected in the number of skin cancer cases in 2020 (Skin Cancer Foundation, 2020).

1.4.2 Risk factors

Several biological and environmental risk factors can lead to melanoma. The differences in the frequency and occurrence of skin cancer between the two genders can be explained by multiple biological factors including the nature of hormones and the dissimilarity in the levels of these hormones between males and females, in addition to the difference in oxidative stress between genders (Roh, Eliades, Gupta, Grant-Kels, & Tsao, 2015). Other biological factors shaping the risk of melanoma are related the number of melanocytic nevi and family history (Rastrelli et al., 2014). Moreover, as the individual gets older, the probability of skin cancer occurrence will also increase along with a decrease in the effectiveness of diagnosis (Friedman & Wolchok, 2017). Environmental risk factors include the usage of indoor tanning beds specially at a very young age, increasing the probability of melanoma by 59%. Long Exposures to ultraviolet radiation

from sunlight due to their genotoxic effect can also lead to skin cancer specially during childhood years (Roh et al., 2015).

1.4.3 Prevention and treatment

Melanoma can be mainly prevented by protecting the skin from UV radiations. This includes minimizing long exposure to the sun, wearing protective clothing and sunscreen, and avoiding tanning beds (Mayo Clinic, 2019). Treatment includes the classical methods used in cancer treatment including chemotherapy, combination chemotherapy, immunotherapy, and targeted therapy (Domingues et al., 2018). Biochemotherapy, the combination of both chemotherapy and immunotherapy, is also used in case of melanoma, and recorded increased clinical responses. However, the overall survival wasn't improved because of the high toxicity resulting from this treatment method (wilson, m. & Schuchter, 2015).

1.5 Cells lines

A549 (non-small cell lung cancer) cell line was initially isolated from a Caucasian male suffering from pulmonary adenocarcinoma. This cell line was initially used in several domains of cancer research; However, scientists started using it in respiratory research because of their importance and belongness to the alveolar type 2 pneumonocytes that can produce surfactants(Giard et al., 1973). MDA-MB-231 cell line was originally extracted from a female patient diagnosed with metastatic breast cancer. These triple negative breast cancer cells are widely used by scientists in cancer/medical research because of their high aggressivity and invasiveness (Welsh, 2013). A375 (human melanoma cell line) was originally isolated from a 54-year-old female who was suffering from skin cancer by explant culture of her solid tumor. This cell line was widely used in research in studying several pathways of metastasis and tumor growth (Imanis Life Sciences, n.d.).

1.6 Apoptosis

1.6.1 Definition and characteristics

Apoptosis, also known as programmed cell death, is a process that occurs in cells as a result of intercellular or extracellular microenvironmental perturbations (DNA damage, replication stress etc.) in order to maintain homeostasis (Galluzzi et al., 2018). It can be used as a defense mechanism to protect cells from various infections, viruses and toxic agents (Hsu, 2019). Apoptotic cells are characterized by their round shape, reduced size, chromatin condensation and a dense darkly stained cytoplasm (figure 8) (Karch & Molkentin, 2015). Following a process known as budding, apoptotic cells will transform into apoptotic bodies with significant deviations in shape compared to normal cells. These bodies will then be engulfed and phagocytized by different immune cells, mainly macrophages in order to be totally degraded by phagolysosomes (Gordon & Plüddemann, 2018; Xu, Lai, & Hua, 2019). Although different immune cells play a certain role in this process, apoptosis does not elicit an inflammatory response for several reasons, but most importantly because of the lack of production or release of cytokines and chemokines (Wallach & Kovalenko, 2014).

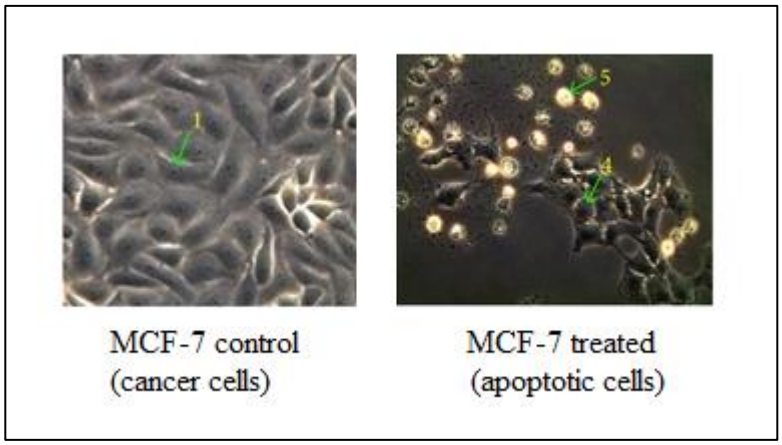


Figure 8. Multiple differences between normal and apoptotic cells. Normal and apoptotic cells differ in several characteristics including shape and morphology; apoptotic cells having a rounder shape and a more reduced size compared to normal ones. Modified from (Syed Abdul Rahman, Syarifah Nur, Abdul Wahab, & Abd Malek, 2013).

1.6.2 Difference between apoptosis and necrosis

Another mechanism of cell death is necrosis. Unlike apoptosis, necrosis is not a naturally occurring event taking place daily as cells age and mature. Rather, it is an unregulated process caused by different external factors leading to an abnormal premature cell death (Adigun, Basit, & Murray, 2019) . Moreover, several mechanistic and

morphological differences exist between these two events (table 1), but one of the most important difference is related to energy dependence. Whereas apoptosis is an active energy-dependent event, necrosis does not depend on energy and is an uncontrollable passive process (Karch & Molkentin, 2015). Another crucial difference between these two cellular events is the induction of inflammation. Necrosis eventually leads to an inflammatory response after the release of different cytoplasmic molecules resulting from the disruption of the cytoplasmic membrane. However, this is not the case in apoptosis because the rapid engulfment of the cells prevents the recruitment of immune cells, preventing any inflammation (Wallach & Kovalenko, 2014).

Table 1. Differences between apoptosis and necrosis. In addition to the presence/absence of inflammation, necrotic and apoptotic cells differ in several other aspects including membrane integrity and cell behavior. Modified from (Elmore, 2007).

Apoptosis	Necrosis
Single cells or small clusters of cells	Often contiguous cells
Cell shrinkage and convolution	Cell swelling
Pyknosis and Karyorrhexis	Karyolysis, pyknosis, and karyorrhexis
Intact cell membrane	Disrupted cell membrane
Cytoplasm retained in apoptotic bodies	Cytoplasm released
No inflammation	Inflammation usually present

1.6.3 Mechanisms of apoptosis

Apoptosis is not a simple passive process that depends on a simple pathway leading to cell death. Instead, many different cascades and events are involved in this process (Galluzzi et al., 2018). Two main independent apoptotic pathways can take place: the intrinsic and extrinsic pathways (Singh, Letai, & Sarosiek, 2019) (figure 9).

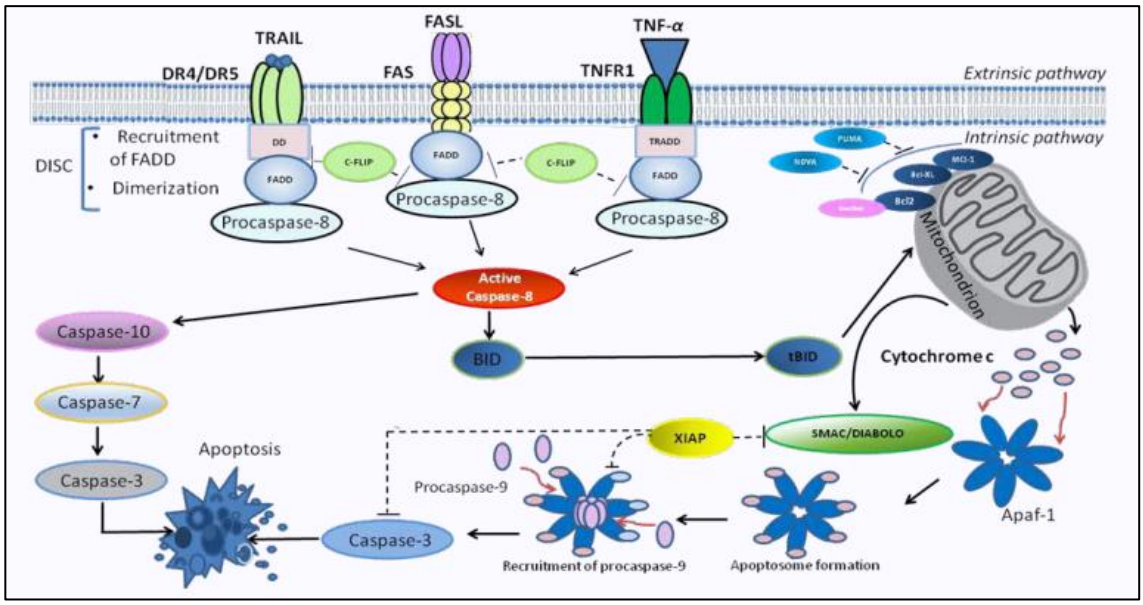


Figure 9. Different apoptotic pathways. The two main apoptotic pathways are the intrinsic and the extrinsic pathways, and multiple protein families and caspases play some crucial roles during this process (Belkacemi, 2018)**.**

1.6.3.1 Extrinsic pathway

The extrinsic pathway, also known as death receptor pathway, involves an interaction between a ligand and its appropriate receptor in order to transduce the death signals into the cells leading to their death (Barreiro-Iglesias, Sobrido-Cameán, & Shifman, 2017) . Most of these receptors belong to a family of genes called tumor necrosis factor TNF. For example, a well characterized receptor that is part of this pathway is the TNFR1 which binds its corresponding ligand TNF-α. The main event taking place during this series of events is the activation of procaspase 8 by the DISC complex; the latter is formed after the binding of the ligand to its receptor leading the binding of another adaptor protein and the recruitment of several proteins (Singh et al., 2019). The activation of caspase 8 leads to the activation of another protein belonging to the same family: caspase 3 which is part of the execution pathway that causes the formation of the apoptotic bodies leading to cell death (Jakubowska et al., 2016).

1.6.3.2 Intrinsic pathway

Unlike the extrinsic pathway that relies on the binding of a ligand to its death receptor, this model of interaction is not observed in the intrinsic pathway. Instead, this

latter can be activated by withdrawal of several growth factors, environmental insults and developmental cues (Ke, F. F. S. et al., 2018). These stimuli can act in two different ways: either negatively by reversing the suppression of apoptosis, or positively directly affecting a main organelle involved in this pathway: the mitochondria (Elmore, 2007). This will lead to the liberation of pro-apoptotic proteins like cytochrome c and DIABLO into the cytosol, after affecting the permeability of the mitochondria by forming several pores in its membrane. These proteins from a complex called apoptosome that is responsible for the activation of caspase 9, followed by the activation of the execution pathway (Singh et al., 2019). An additional role is played by another group of pro-apoptotic proteins including AIF, endonuclease G and CAD. These proteins lead to the fragmentation of the DNA after reaching the nuclei of the cells, and chromatin condensation also takes place in two stages: satge I and II with the latter being the more severe one (Benítez-Guzmán, Arriaga-Pizano, Morán, & Gutiérrez-Pabello, 2018; Joza et al., 2001).

An important family of proteins: the Bcl-2 family has an important effect on all the previous events because it plays an initial role in controlling the mitochondrial membrane permeability, thus regulating the release of the pro-apoptotic protein cytochrome c (Kale, Osterlund, & Andrews, 2018). The members of this family, controlled by the tumor suppressor protein p53 (Kim et al., 2017), can be either anti-apoptotic or pro-apoptotic (Warren, Wong-Brown, & Bowden, 2019)

1.6.3.3 Bcl- 2 Family

One important property of cancer cells is their ability to avoid apoptosis, which constitutes an advantage over normal cells in terms of survival. Thus, current cancer treatments are targeting specific protein families that play an important role in cell survival and cancer progression (Radha & Raghavan, 2017). One of these families is the Bcl-2 family (figure 10) previously mentioned in the intrinsic pathway. This family is composed of two different subfamilies of proteins: proapoptotic proteins promoting apoptosis i.e. Bax and Bak (Barillé-Nion, Bah, Véquaud, & Juin, 2012) and anti-apoptotic proteins promoting the survival of the cells i.e. Bcl- 2 and Bcl- x. Anti-apoptotic proteins bind to proapototic proteins preventing them from causing apoptosis, because of the lack of release of cytochrome c from outer mitochondrial membrane, which is usually caused by

proapoptotic proteins like Bax (Singh et al., 2019). Anti-cancer treatments lead to the activation of proapoptotic proteins having BH3 domain, which prevent the binding of anti-apoptotic proteins to proapoptotic proteins. The latter can consequently permeabilize the outer mitochondrial membrane leading to apoptosis (Delbridge, Grabow, Strasser, & Vaux, 2016).

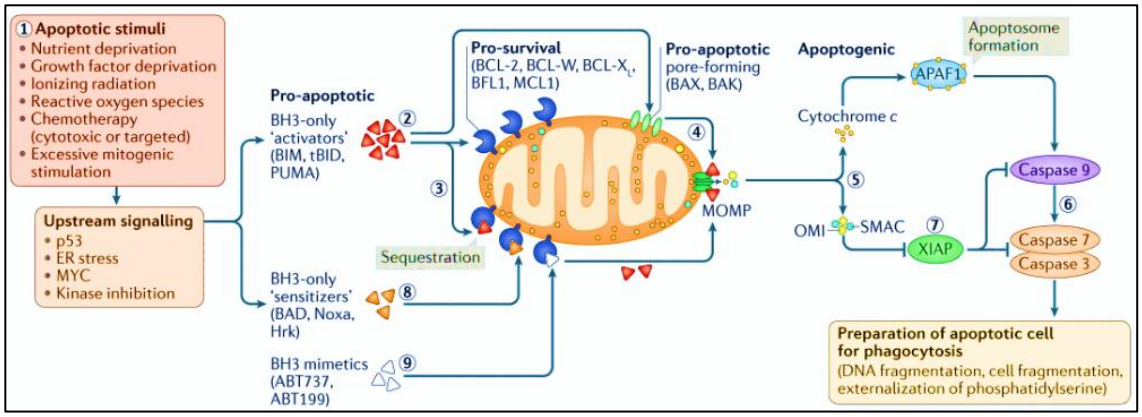


Figure 10. Bcl-2 family of proteins involved in the intrinsic pathway of apoptosis. Different pro-apoptotic and anti-apoptotic proteins belong to the Bcl-2 family, playing an important role in the intrinsic apoptotic pathway, along with several caspases and downstream proteins (Singh et al., 2019).

1.6.3.4 Perforin/granzyme pathway

Granzymes A and B secreted by cytotoxic T cells into the transmembrane pores created by perforins have distinct roles in this apoptotic pathway. While granzyme B (directly and indirectly) leads to the activation of procaspase 10 and caspase 3 respectively, granzyme A activation releases the inhibition of an important tumor suppressor gene NM23-H1 by cleaving the SET complex, leading to DNA fragmentation and chromatin breakdown causing apoptosis (Fan, Beresford, Oh, Zhang, & Lieberman, 2003; Voskoboinik, Whisstock, & Trapani, 2015).

1.6.3.5 Execution pathway

All the three previously discussed pathways converge at one point: the execution pathway in which caspase 3, or any similar execution caspase, play a central role. These caspases are responsible for the apoptotic events including DNA fragmentation, degradation of nuclear proteins etc. by cleaving various proteins like PARP and NuMA

(Jan & Chaudhry, 2019). The activation of the previously mentioned caspases leads to the morphological and molecular damage within cells including chromatin condensation and nuclear fragmentation (Goldar, Khaniani, Derakhshan, & Baradaran, 2015). A last step before phagocytosis is the externalization of phosphotidylserine on apoptotic cells triggering their engulfment by immune cells. Since this uptake is fast and takes place without the release of any cytokines, no inflammation results (figure 11). Research recently showed that this step is not unique to apoptosis only, but it can also take place in necroptotic cells (Shlomovitz, Speir, & Gerlic, 2019).

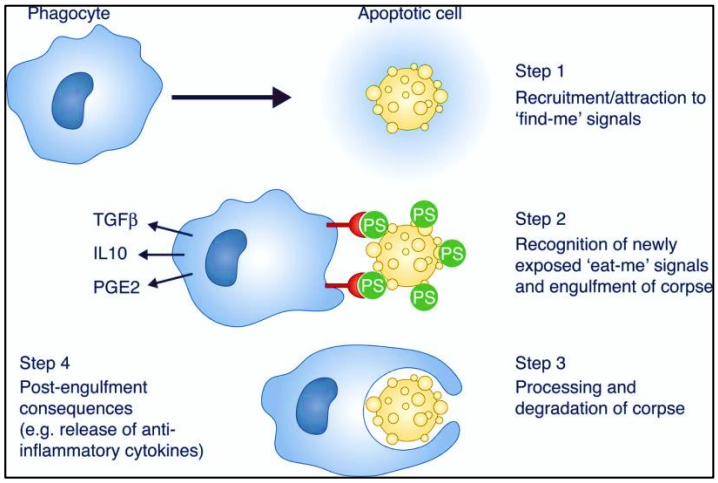


Figure 11. Recognition of apoptotic cells by phagocytes due to the externalization of the phosphatidylserine. Once apoptosis takes place, phosphatidylserine which is restricted to the inner leaflet of the membrane starts flipping to the outer leaflet, enabling phagocytes to recognize apoptotic cells, leading to their engulfment (Ravichandran, 2010).

1.7 Platinum based anti-cancer drugs

1.7.1 Discovery

In the past years, different traditional cancer treatments were used including surgery, radiotherapy and chemotherapy. However, due to resistance and several side effects resulting from these treatment methods, research lied towards developing new treatments with higher efficacy and selectivity (Yang et al., 2020). In the 1960s, Rosenberg was testing the relation between electric field and cellular division. In his experiments, bacterial division was inhibited (Hoeschele, 2014) . It turned out later that this inhibition was not caused by the electric field itself, but by the fact that the used electrodes were platinum based, and their electrolysis products, including a complex that

turned out to be cisplatin, were inducing this effect (Muggia, Bonetti, Howell, Rozencweig, & Hoeschele, 2015). Since then, this metal has become the interest of many scientists because it could be used in the synthesis of several anticancer drugs (figure 12) (Sutton, McDevitt, Yglesias, Cunningham, & DeRose, 2019) among which the previously mentioned cisplatin, that was the first platinum compound showing high efficacy in treating several tumor types including ovarian and testicular cancers, thus receiving the FDA approval in 1978 (Hazlitt, A., Min, & Zuo, 2018; Makovec, 2019).

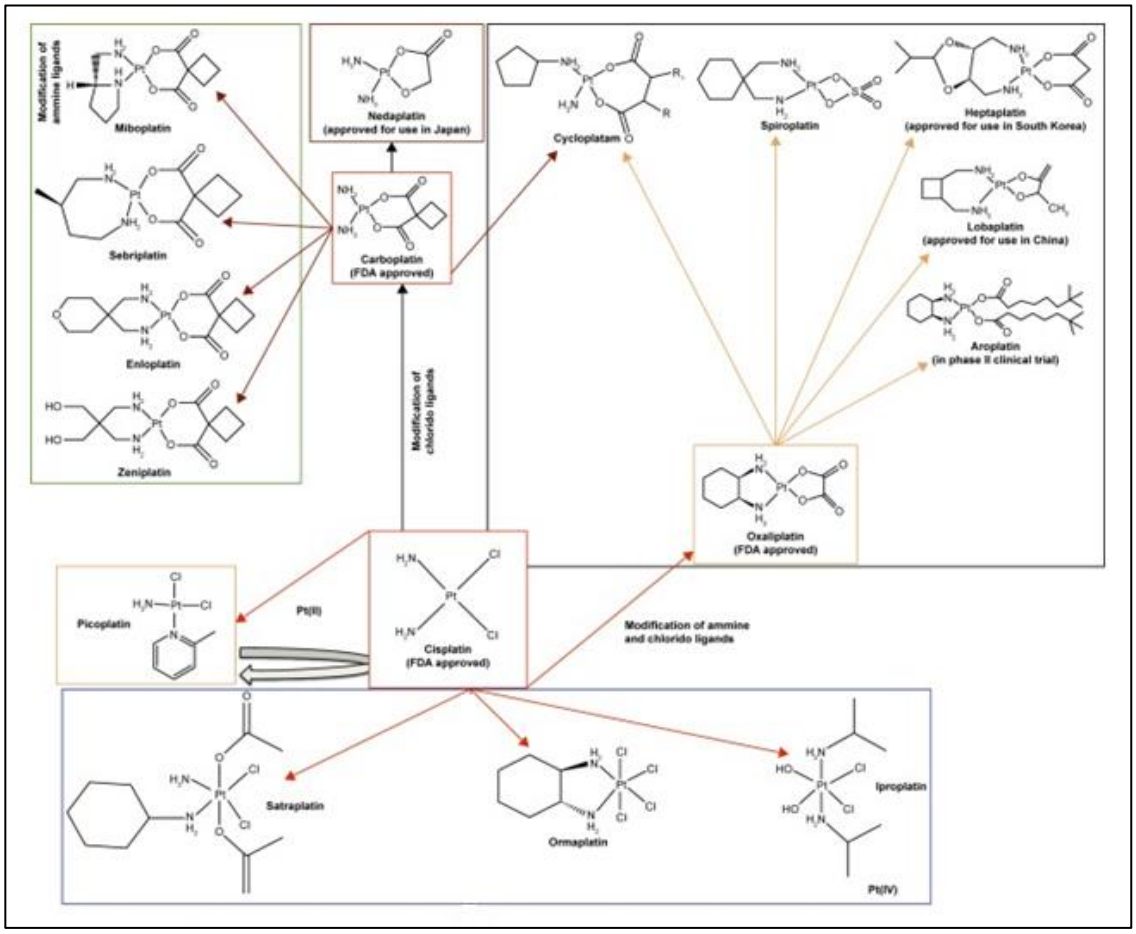


Figure 12. Development of platinum drugs for cancer therapy. Since the accidental discovery of the antineoplastic effect of cisplatin, several platinum complexes were later synthesized and used as anticancer drugs after receiving FDA approval (Ndagi, Mhlongo, & Soliman, 2017).

1.7.2 Platinum (II) drugs

1.7.2.1 Structure

A platinum (II) compound has a planar geometry and its general formula is ML2X2, where M represents the platinum ion present at the center, L representing the stable ligand and X the leaving group (Monroe, Hruska, Ruggles, Williams, & Smith, 2018) . As previously stated, *cis*-diamminedichloroplatinum (II), commonly known as cisplatin, was the first FDA-approved platinum-based anticancer drug (1978) (Apps, Choi, & Wheate, 2015). In case of cisplatin, the two inert ligands are amine groups while the unstable ones are chlorides. The bonds between the ionic center and the two chlorides are weak, and these two leaving groups, because of the low concentration of chloride inside the cell, fall off allowing the platinum ion to bind DNA inside the nucleus (Corinti et al., 2017; Goodsell, 2006). Seen the success of cisplatin, multiple novel platinum II drugs were developed after 1978 in order to treat different cancer types with lower side effects compared to cisplatin (figure 13) (Makovec, 2019). In fact, by slightly changing the structure of this compound, different analogues were obtained and many of them showed significant anticancer activity on several cancer types like carboplatin and oxaliplatin, with additional advantages over cisplatin (Yu, Xiao, Yang, & Cao, 2015).

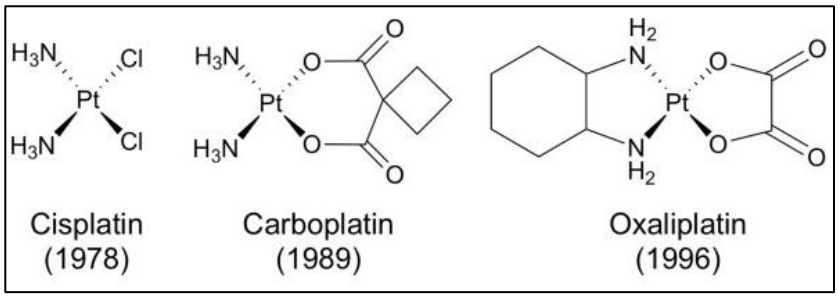


Figure 13. Different cisplatin analogues used as drugs for cancer treatment. Several platinum-based complexes were synthesized and tested for their anti-cancer activity including carboplatin and oxaliplatin that showed high efficacy against several cancer types (Sutton et al., 2019).

1.7.2.2 Mechanisms of action

The purpose of using different platinum II compounds was to inhibit cancer progression and development. To do so, these drugs must penetrate into cells causing their death via several mechanisms (Dasari & Bernard Tchounwou, 2014). In order to be transported inside the cells, some platinum-based complexes, including cisplatin, use membrane transporters (efflux ATPases or solute carriers like Ctr1) that mediate their

entry into mammalian cells (Martinho, Santos, Florindo, & Silva, 2019). Other uptake pathways include active, passive and facilitated transports (Dasari & Bernard Tchounwou, 2014).

After replacing its 2 chlorides groups with water molecules, cisplatin becomes electrophilic and get attacked by the N7 position of purines, damaging the DNA of the cells by forming several cross links leading to cellular division arrest and apoptosis (figure 14) (Nejdl et al., 2014).

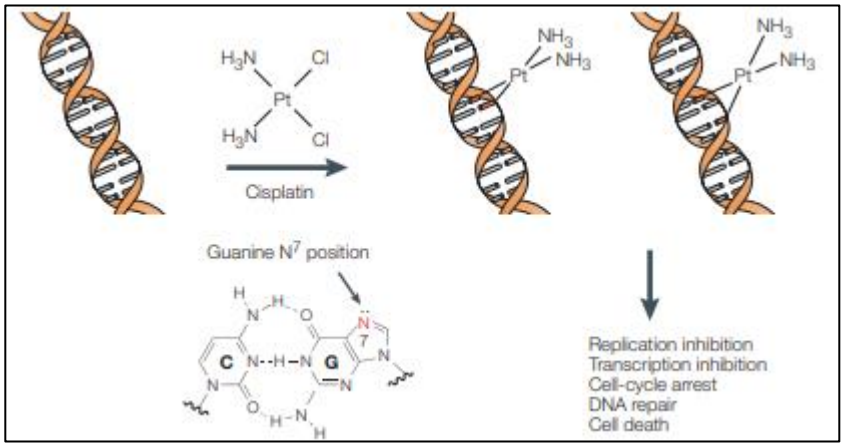


Figure 14. Formation and effects of cisplatin adducts. Once inside the cell, the dissociation of the two chloride groups of cisplatin because of the low chloride concentration enables the complex to form several cross links with DNA (Wang, Dong & Lippard, 2005)**.**

This final apoptotic state is the result of several different mechanisms through which platinum compounds kill cancer cells. For instance, cisplatin can induce oxidative stress, leading to the malfunctioning of the mitochondria by reducing its membrane potential and reducing its proteins (Soni, Kaminski, Gangaraju, & Adebiyi, 2018). This mitochondrial damage can also result from the dysregulation of the calcium homeostasis in these cells following the entry of cisplatin, leading to decreased ATP levels and inhibition of the mitochondrial function (Saad, Najjar, & Alashari, 2004). Platinum II drugs can also overexpress reactive oxygen species, which also trigger apoptosis via its two intrinsic and extrinsic pathways, affecting many protein families implicated in these mechanisms like cytochrome c, BCl_2 family and several caspases (Kleih et al., 2019). Other several molecular pathways also play a role in transmitting the effects of platinum II complexes to the cells including Protein kinase C pathway, MAPK pathway etc. and

many different proteins are affected like p53 and AKt (Dasari & Bernard Tchounwou, 2014).

While some platinum II compounds, such oxaliplatin and nedaplatin, mimic cisplatin's mechanism of action (Johnstone, 2014; Shimada et al., 2013), others react differently inside the cells. These complexes include trans-platinum (II) with heteroaromatic ligands, iminoether ligands, asymmetric aliphatic amine ligands, monofunctional platinum (II) complexes and noncovalently binding platinum (II) complexes (Johnstone, Suntharalingam, & Lippard, 2016). In fact, because of the π-conjugated heterocyclic bidentate ligands, this latter group of complexes cannot covalently bind DNA. Instead, the chemotherapeutic potential of these compounds, like other mononuclear platinum (II) metalointercalators, resides in their intercalation between the base pairs of DNA using dipole−dipole interactions and π−π stacking, distorting its topology without forming any covalent bonds (Jennette, Lippard, Vassiliades, & Bauer, 1974; Long & Barton, 1990; Wu, Koch, Abratt, & Klump, 2005).

The final effect of all the previous mechanisms and pathways is cell death, which could be either induced by apoptosis or necrosis, or autophagy in some cases (Buss & Jaehde, 2017; Wang et al., 2018). Because of the observed significant effects these compounds had on cancer cells, many platinum II compounds were continuously being used as anticancer drugs including cisplatin, carboplatin and oxaliplatin showing high efficacy in several types of cancer and receiving FDA approval in 1978, 1989 and 1991 respectively (Wang & Lippard, 2005)**.**

1.7.2.3 Side effects and disadvantages

Despite the high chemotherapeutic potential that platinum II complexes exhibited throughout the years, multiple severe side effects resulted from their treatment, limiting their usage for safety reasons (Oun, Moussa, & Wheate, 2018). For instance, although cisplatin treatment offered high efficacy against lung, testicular and ovarian cancer, this drug affected some patients' hearing, damaged their nervous system, increased blood pressure in other patients and elevated their risk of nephrotoxicity. Other patients reported less severe cases of vomiting, nausea and diarrhea (Pouryasin et al., 2014). Beside these important side effects, a major problem limited the effectiveness of cisplatin when cells

started developing resistance to this drug (De Luca et al., 2019). This resistance was acquired through several mechanisms including internalization of CTR1 (less uptake of cisplatin), upregulation of efflux pumps (ejecting the drug out of the cells), and the overexpression of thiol-rich molecules to which platinum has a very high affinity (Wang & Lippard, 2005). To overcome these barriers, scientists synthesized different analogues of cisplatin, including carboplatin which showed high cytotoxicity against several cancer types (including lung and ovarian cancer), with less nephrotoxicity, vomiting and nausea, along with a higher half-life (Ho, Woodward, & Coward, 2016). However, patients receiving this treatment showed a high number of thrombocytopenia, anemia and neutropenia cases (Cheng et al., 2016). Following carboplatin, novel platinum II drugs were developed (oxaliplatin, satraplatin, picoplatin, etc.) in order to increase the efficacy of the treatment while minimizing the previous side effects. However, these chemotherapeutic agents shared several drawbacks including over reactivity, instability and low selectivity which shifted research towards higher efficacy, lower undesired cytotoxicity platinum IV complexes (Li, X., Liu, & Tian, 2018).

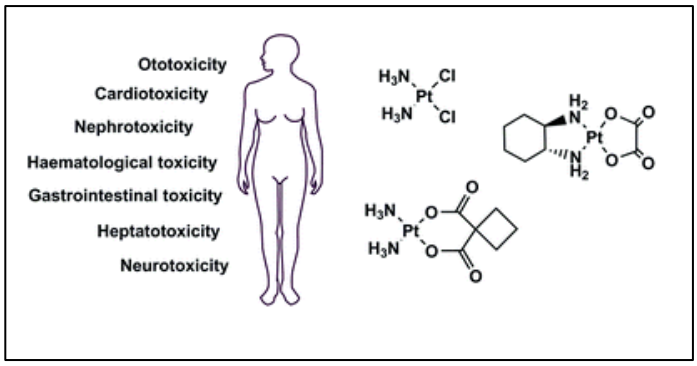


Figure 15. The side effects resulting from the usage of cisplatin and its platinum analogues. Several side effects accompanied the treatment of patients with platinum II drugs including Neurotoxicity and nephrotoxicity (Oun et al., 2018)**.**

1.7.3 Platinum (IV) drugs

1.7.3.1 Structure

Compared to the square-planarity characterizing platinum II complexes, Platinum IV compounds possess an octahedral geometry (Wexselblatt & Gibson, 2012). Their general formula is ML2X2R2, M representing the platinum ion at the center, to which 6 ligands are attached: two leaving groups (represented by the letter X), two inert ligands (represented by the letter L), and two additional axial ligands (represented by the letter R)

(Johnstone et al., 2016). The latter characterize the platinum IV complexes (because of their absence in the platinum II analogues), play a very important role in the structure-activity relationship and provide these pro-dugs several additional advantages over platinum II complexes (figure 15).

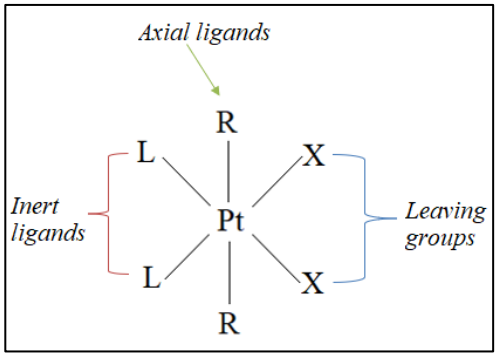


Figure 16. General formula and composition of platinum IV complexes. Because of the two additional axial ligands, a platinum IV complex has a general formula of ML2X2R2, where L represents the inert ligand, X the leaving group and R the axial ligand. Modified from (Wilson & Lippard, 2014).

1.7.3.2 Mechanisms of action

The mechanism of action of platinum IV complexes is similar to that of their platinum II analogues. Once inside the cell, because of the reducing environment, platinum IV complexes lose their two axial ligands, and turn into platinum II complexes with only four ligands attached to the central platinum ion (figure 17) (Ong, Lim, Le, & Ang, 2019). Once this reduction occurs, the platinum complexes exhibit the same mechanisms of action previously listed for platinum II (DNA cross links, DNA non-covalent intercalation, etc.) (Johnstone et al., 2016).

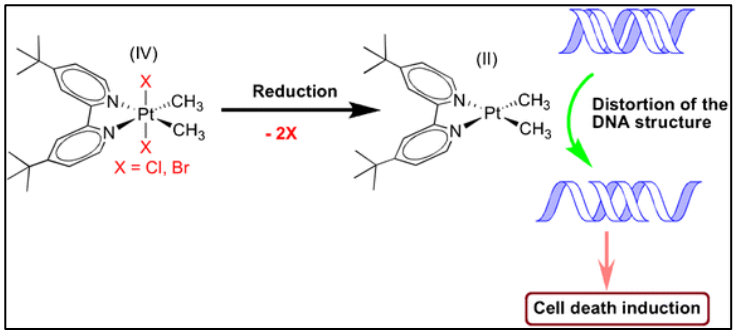


Figure 17. The reduction of octahedral Pt (IV) into the square planar Pt(II). Once inside the cell, the platinum IV complex is reduced to its platinum II analogue because of the reducing environment, and this platinum II complex induces cell death through several mechanisms (Pouryasin et al., 2014).

This mechanism of action is performed by several platinum IV complexes like iproplatin, ormaplatin and straplatin (figure 18), which undergo reduction to platinum II complexes once inside the cell (Kenny, Chuah, Crawford, & Marmion, 2017). All the three drugs underwent several clinical trials in different cancer types but were rejected by the FDA because of elevated neurotoxicity, low survival rates and no overall improved effectiveness compared to cisplatin (Johnstone et al., 2016).

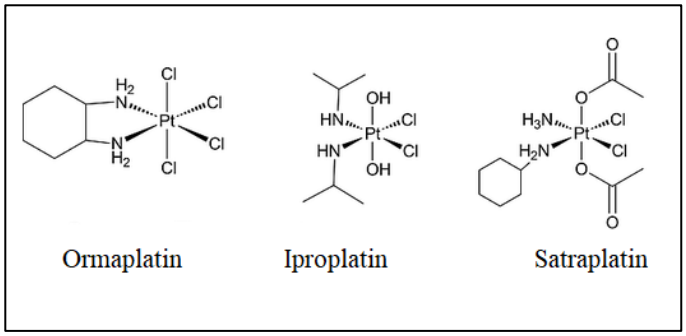


Figure 18. Chemical structures of platinum (IV) complexes. The platinum IV complexes iproplatin, ormaplatin and straplatin were synthesized and tested for their anticancer activity. However, because of several drawbacks, FDA didn't approve any of these complexes (Kenny et al., 2017)**.**

Other platinum IV complexes follow additional mechanisms like in the case of mitaplatin. The structure of the latter is composed of a cisplatin complex, in addition to two dichloroacetate ligands (Johnstone et al., 2013). Upon reduction of mitaplatin inside the cell, the two dichloroacetate ligands are lost and cisplatin follows the previously mentioned traditional mechanism of action (DNA cross links) (Dhar & Lippard, 2009). However, these two DCA ligands also play a role in cytotoxicity by inhibiting pyruvate dehydrogenase kinase. This affects aerobic glycolysis and induces apoptosis through the release of cytochrome c from the mitochondria. Since cancer cells rely on aerobic glycolysis more than normal cells do, this drug offers a big advantage in terms of selectivity, killing cancer cells in a higher frequency (Xue et al., 2012)

Other platinum IV drugs have totally different mechanisms of action, including the oxidation of guanine catalyzed by the platinum II centers following the reduction of the prodrugs (Choi et al., 2006) . Another discovered mechanism reveals the role of H_2O_2 in cell death, where the coupling of this molecule with the platinum IV complex was shown to be responsible of the cleavage of the doubly stranded DNA causing the

cytotoxicity effects of this prodrug, exhibiting a non-cisplatin-like mechanism of action (Johnstone & Lippard, 2014). Additional mechanisms display the idea of photoactivation, offering enhanced selectivity where irradiation applied only on the tumorigenic area activates the toxic compounds leading to cancerous cell death (Smith & Sadler, 2013).

1.7.3.3 Advantages compared to platinum II complexes

The structural difference between platinum II and platinum IV complexes give the latter additional advantageous properties. Having an octahedral configuration, platinum IV compounds are fine-tuned easier, increasing their inertness and uptake by the cells (Reithofer et al., 2008). Second, the two additional ligands provide extra stability, thus less reactivity with the non-target molecules outside the cells, reducing the resulting side effects (Kenny et al., 2017). Finally, because of the previous property, these drugs can be administered orally, leading to an easier and less costly therapy. Another major advantage is selectivity. In fact, the environment of normal cells is different in many aspects from that of cancer cells in which pH is lower, oxygen levels are decreased, and glutathione is upregulated (Li, An, Lin, Tian, & Yang, 2019). Because platinum IV complexes get reduced to platinum II in order to be toxic, axial ligands can be fine-tuned so that the reduction takes place at a lower pH, killing only cancerous cells (Ma et al., 2018)**.** Despite these additional advantages, none of the previously mentioned platinum IV complexes succeeded in receiving the FDA approval because of several limitations including neurotoxicity in case of ormaplatin, myelosupression caused by iproplatin and neutropenia with thrombocytopenia in case of satraplatin treatment (Johnstone et al., 2016).

1.8 Aim of the study

Since studying novel chemotherapeutic drugs is essential to find more effective/safer treatments, the purpose of our current study is to measure the antineoplastic potential of eighteen novel platinum II and platinum IV complexes against non-small cell lung cancer (A549), triple negative breast cancer cells (MDA-MB-231) and melanoma cells (A375), and to select two of these complexes (Pt(II) and Pt(IV)) that showed remarkable cytotoxicity in order to assess their mechanism of action.

Chapter Two
Materials and Methods

2.1 Platinum complexes and cell lines

The eighteen novel platinum complexes were synthesized in the lab of Professor Janice Aldrich-Wright (Western Sydney University) for a collaborative research project aiming to develop novel anticancer agents. The complex number, chemical formula and molecular weight of each complex are mentioned in figure 19 (appendix). The cytotoxicity of all these complexes was measured on three different cell lines: human alveolar adenocarcinoma A549, triple negative human breast adenocarcinoma MDA-MB-231 and human melanoma A375 cell lines purchased from ATCC (www.atcc.org). The platinum (II) complex, complex 3 (designated as Pt(II)) and its platinum (IV) analogue, complex 4 (designated as Pt(IV)) were then selected in the present study for comprehensive investigation due to their sub-micromolar IC_{50} values and the difference in oxidation state and geometry.

2.2 Cell survival assay

Cell survival assays were performed on human alveolar adenocarcinoma A549, triple negative human breast adenocarcinoma MDA-MB-231, human melanoma A375 cell lines and mesenchymal stem cells (MSC). Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS and 1% penicillin/Streptomycin was used to maintain the cells. Incubation took place in a humidified chamber at 37 °C and 5% CO_2. Cells were then plated in 96 well cell culture flat bottom plates at a concentration of 10^4 cells per well. Pt(II) was added to achieve a 50 µM concentration in the first well, then 3-fold serial dilution was applied by pipetting 50 µL of media from each well to the other, resulting in 8 different final concentrations of the drug (0.023 µM, 0.069 µM, 0.206 µM, 0.617 µM, 1.85 µM, 5.56 µM, 16.67 µM and 50 µM). The same procedure was applied for Pt(IV). In order to investigate the cytotoxic effect of our complexes on the three cell lines, cells mitochondrial damage was assessed using the MTS assay (CellTiter 96® Aqueous non-

radioactive cell proliferation kit, Promega) 72 post-treatment. The supernatant was removed, and cells were incubated with 100 µl of MTS-PMS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)- 2-(4-sulfophenyl)-2H-tetrazolium, inner salt-phenazine methosulfate] solution at 37 °C for 2 hours. Absorbance was measured at 492 nm using a Labsystem Multiskan MS plate reader. Three independent experiments were performed, and each sample was run in triplicate

2.3 Isolation of mesenchymal stem cells (MSCs) from rat bone marrow

Rats (12-weeks-old) were provided by the animal facility at the Lebanese American University and were maintained under optimal laboratory conditions receiving food and water ad libitum. All experiments were approved by the University's Animal Care and Use Committee (ACUC) and complied with the Guide for the Care and Use of Laboratory Animals. MSCs were isolated from rat bone marrow according to the following procedure. Briefly, rats were sacrificed by CO_2 asphyxiation and both hind legs were aseptically removed. Femoral and tibial bones were then isolated and washed with EtOH and 1% PBS. The bone marrows were flushed out using a needle filled with DMEM containing 10% FBS and 1.5% pen-strep. The cells collected were then cultured in a 75 cm^2 flask and incubated at 37 °C with 5% CO_2. After 48 h incubation, cells were washed with 1% PBS to remove non-adherent cells and the medium was changed frequently until cells reached high confluency. MSCs were identified by their spindle-shaped morphology as observed under inverted microscope (Nikon Eclipse TE300)

2.4 Quantification of cellular uptake by ICP-MS

Human alveolar adenocarcinoma cells (A549) were seeded in 6-well plates at a final concentration of 10^5 cells/mL and could adhere overnight at 37 °C in the presence of 5% CO_2. Cells were then treated with Pt (II) or Pt (IV) at a final concentration of 1.29 and 2.34 µM, respectively. After, 1, 3, 6, 12 or 24 hours, the medium was removed, and cells were washed thrice with PBS and harvested by scraping in the presence of PBS. The resulting suspension was centrifuged at 800 g for 10 min and the supernatant was removed. Following air drying, the pellet was resuspended in 20 µL milliQ water and transferred to a glass vial containing 500 µL of 65% HNO_3. Samples were completely

digested at 120 ℃ and were finally suspended in 2% HNO_3. Cellular uptake of Pt (II) and Pt (IV) was quantified by ICP-MS based on external standards.

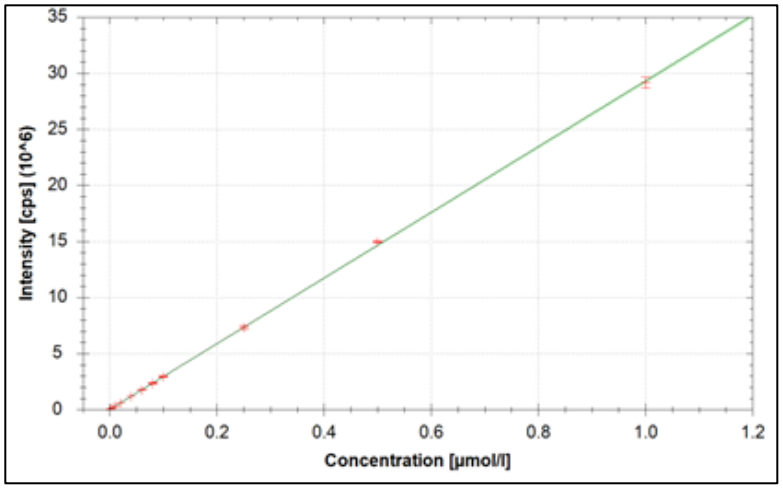


Figure 20: Calibration curve generated by plotting the peak areas (measured by the ICP-MS) against known concentrations. This curve was used to quantify the cellular uptake of both Pt(II) and Pt(IV); $y= 2.9\times10^7x + 1.9\times10^4$ and $R^2= 0.9999$

Table 2: Conditions and parameters selected on the ICP-MS machine

Parameter	Value
Plasma RF power	1550 W
Nebulizer gas flow rate	1.0 $L.min^{-1}$
Auxiliary gas flow rate	0.8 $L.min^{-1}$
Collision gas flow rate (He)	4.7 $L.min^{-1}$
KED voltage	3 V
Extraction lens	-250 V
Isotope monitored	^{194}Pt
Dwell times	10 ms

2.5 Cell death analysis

Annexin V-fluorescin Isothiocyanate (Annexin V-FITC) and PI (Annexin V-FITC apoptosis staining/detection kit (Abcam, Cambridge, USA) were used to determine the type of cell death taking place 72 h post-treatment with Pt (II) and Pt (IV). A549 cells were incubated with these two drugs (0.43 µM and 0.78 µM, respectively) in 6 well plates at 37º C and 5% CO2. After 72 hours, the 2 ml media were removed from each well and kept in labeled conical tubes on ice. Cells were then trypsinized with 200 µl of trypsin, and the detached cells were then added to their corresponding media previously kept in

the labeled conical tubes. Cells were centrifuged for 5 minutes at 1500 rpm at 4º C. After removal of the supernatant, pellets were dissolved in 200 µl of PBS. 10 µl of this solution was then added to 10 µl of trypan blue in order to count the cells under the inverted microscope (Nikon Eclipse TE300). Calculations were made in order to obtain a final concentration of 500 cells per microliter. The calculated initial volumes were then pipetted and put in new labeled Eppendorf tubes, followed by another centrifugation for 5 minutes at 1500 rpm at 4º C. After removal of the supernatant, pellets were dissolved in 200 µl of binding buffer, 2 µl of Annexin V-FITC (2.5 mg/ml) and 2 µl of PI (5 mg/ml), pipetted into a 96 well plate, and kept for 5 minutes in the dark. Cells were then read using the Guava® easyCyte 8HT Benchtop Flow Cytometer. Annexin V/PI data was measured on FL1-H versus FL2-H scatter plot.

2.6 Western blot

A549 human lung cancer cells were plated in 6 well plates and treated with two different concentrations of Pt (II) (0.43 µM and 0.8 µM) and Pt (IV) (0.78 µM and 1.5 µM) for 72 hours. Supernatants were then transferred into labeled conical tubes and centrifuged at 2000 rpm for 5 minutes at 4 °C, and only pellets were kept in each tube. Simultaneously, 200 µl of Ripa buffer were added in each well of the 6 well plate and cells were scraped on ice, added over their corresponding pellet and put on the shaker for 10 minutes at 4 °C. Centrifugation took place at 13000 rpm for 15 minutes at 4 °C, and supernatants containing the proteins were transferred to new Eppendorf tubes. 2 µl of each protein lysate were mixed with 3 µl of water and 200 µl of diluted Bradford reagent. The mixture was pipetted in a 96 well plate and absorbance was read at 595 nm using a Labsystem Multiskan MS plate reader. Based on the results and on a generated standard curve, specific volumes of laemmli buffer, Ripa buffer and proteins were used in order to insure equal loading.

The mixture was then heated at 100 °C for 5 minutes, and equal volumes (15 or 20 µl) were loaded to 10% SDSPAGE. The voltage was set at 90 V for 30 minutes, then at 120 V for 90 minutes. Meanwhile, PVDF membranes and filter pads were cut, soaked in methanol followed by water, then left in transfer buffer until later use. Once the run is over, the gels were transferred to PVDF membrane (Pall Corporation, Ann Arbor, USA)

and blocked with blocking buffer (1× TBS, 0.1% Tween-20, 5% skim milk) for 1 hour. The membranes were then probed with primary antibodies against several apoptotic and antiapoptotic proteins at 4 ºC overnight. Membranes were then washed thrice for 5 minutes with TBST in order to remove the primary antibodies, treated with secondary antibodies for 90 minutes, and washed thrice for 10 minutes with TBST afterwards. Detection of proteins was performed using the chemiluminescence ECL kit (Bio-Rad, U.S.A).

500 µl of peroxide solution were mixed with 500 µl of luminol/enhancer solution and pipetted/spread all over the membrane. This membrane was then left in dark for five minutes before imaging. Blot images were finally obtained with the ChemiDoc MP imaging system (Bio-Rad, U.S.A).

2.7 Comet assay

A549 human lung cancer cells were plated in 6 well plates at a density of 1×10^5 cells/ ml and treated with 0.43 µM of Pt(II) and 0.78 µM of Pt(IV) for 72 hours. The comet assay was performed using Trevigen comet assay silver staining kit. Low melting agarose (LMA) was melted in a boiling water bath for 5 min, then placed at 37 °C until ready for use. The prepared lysis solution was stored at 4 °C for 20 min before use. Cells were mixed with LMA (at 37 °C) at a ratio of 1:10 (v/v) and 50 µL of the mixture were immediately spread all over the comet slides. Incubation took place at 4 °C in dark for 30 min to insure agarose solidification.

Slides were then immersed in the lysis solution at 4 °C for 60 min, then in an alkaline solution for 40 min at room temperature in dark. Slides were washed and immersed in 1 X TBE buffer (pH 8.3) for 5 min, then transferred to horizontal electrophoresis apparatus. Slides immersed in Electrophoresis buffer (TBE, pH 8.3) were subjected to electrophoresis at 1 V cm−1 for 10 min. Then, excess TBE was tapped off and slides were dipped in 70% ethanol for 5 min, air-dried and desiccated overnight at room temperature for silver staining.

The silver staining method involved covering slides with 100 ml of fixation solution for 20 min at room temperature. Slides were then rinsed with deionized water for 30 min and sample areas were covered with 100 ml of staining solution for 5–20 min at room

temperature until the comets acquired the desired intensity. 100 ml of 5% acetic acid was added to the samples for 15 min in order to stop the reaction, followed by a deionized water wash. Slides were air-dried and stored in dark until ready for analysis.

2.8 Statistical analysis

Data and results are reported as Mean ± SEM from three independent trials. Results were analyzed by one-way ANOVA. The statistical difference between groups was analyzed via independent t-test and was considered significant when $p<0.05$ (Tukey's multiple comparisons test).

Chapter Three

Results

3.1. The cytotoxic effects of novel platinum complexes on cancer cells survival

The effect of eighteen novel platinum complexes, along with cisplatin, was examined on A549, A375 and MDA-MB-231 cancer cell lines for 72 hours. Cells were treated with eight different concentrations of each drug and the IC_{50} values were determined using a non-linear regression curve (GraphPad Prism 6). All platinum-based complexes exhibited a dose dependent activity with IC_{50}s presented in table 3. The IC_{50}s ranged from a minimum of 0.19 µM (complex 16; A375) to a maximum of >50 µM (complex 2; A375 and complexes 8, 9 and 10; A549, A375 and MDA-MB-231). Overall, the highest activity (in the sub-micromolar range) was recorded with complex 3 followed by complex 4. The latter two complexes were then named as Pt(II) and Pt(IV) respectively. All cell lines showed resistance to treatment with complexes 8, 9 and 10.

Table 3: Cytotoxic effect of novel platinum complexes and cisplatin on the survival of A549, A375 and MDA-MB-231 cancer cells.

	IC_{50} (µM)			
Drug	**MDA-MB-231**	**A375**	**A549**	**Oxidation State**
Complex 1	5.67 ± 2.25	8.56 ± 1.32	6.97 ± 3.72	Platinum (II)
Complex 2	31.4 ± 6.6	> 50	18.13 ± 0.86	Platinum (IV)
Complex 3	0.32 ± 0.01	0.36 ± 0.03	0.43 ± 0.01	Platinum (II)
Complex 4	0.60 ± 0.11	0.68 ± 0.19	0.78 ± 0.1	Platinum (IV)
Complex 5	0.89 ± 0.05	0.99 ± 0.04	1.18 ± 0.05	Platinum (II)
Complex 6	1.15 ± 0.01	1.23 ± 0.19	0.90 ± 0.03	Platinum (IV)
Complex 7	2.92 ± 0.31	6.63 ± 3.69	2.0 ± 0.48	Platinum (II)
Complex 8	> 50	> 50	> 50	Platinum (IV)
Complex 9	> 50	> 50	> 50	Platinum (II)
Complex 10	> 50	> 50	> 50	Platinum (II)
Complex 11	1.42 ± 0.34	1.67 ± 0.14	1.48 ± 0.48	Platinum (II)
Complex 12	5.25 ± 0.17	9.99 ± 0.91	7.45 ± 0.93	Platinum (II)
Complex 13	1.12 ± 0.1	1.52 ± 0.16	1.43 ± 0.55	Platinum (IV)
Complex 14	1.15 ± 0.21	1.52 ± 0.22	1.49 ± 0.36	Platinum (IV)
Complex 15	1.50 ± 0.17	1.65 ± 0.04	1.37 ± 0.09	Platinum (IV)
Complex 16	2.67 ± 0.19	0.19 ± 0.02	3.21 ± 0.01	Platinum (IV)

Complex 17	1.27 ± 0.17	1.44 ± 0.05	1.16 ± 0.04	Platinum (IV)
Complex 18	11.33 ± 3.53	0.78 ± 0.02	4.89 ± 0.66	Platinum (IV)
Cisplatin	4.43 ± 0.02	6.32 ± 0.13	8.54 ± 1.18	Platinum (II)

3.2. The cytotoxic effects of Pt(II), Pt(IV) and cisplatin on cell survival

3.2.1. A549 cancer cells

The effect of Pt(II), Pt(IV) and cisplatin was examined on A549 cancer cells for 72 hours (figure 21). Cells were treated with multiple concentrations of each drug. Results showed a dose-dependent activity and IC_{50} values of 0.43 µM, 0.78 µM and 8.54 µM for Pt(II), Pt(IV) and cisplatin respectively.

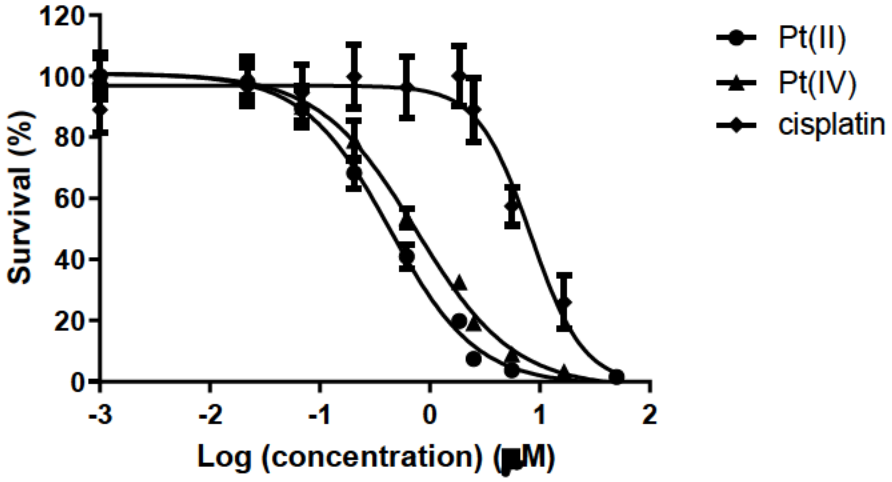


Figure 21: Cytotoxic effect of Pt(II), Pt(IV) and cisplatin on the survival of A549
A549 cells were treated with several concentrations of Pt(II), Pt(IV) and cisplatin over 72 hours. Data points denote mean ± SEM. n = 3 from three independent experiments where samples were run in triplicate.

3.2.2. A375 cancer cells

The effect of Pt(II), Pt(IV) and cisplatin was examined on A375 cancer cells for 72 hours (figure 22). Cells were treated with multiple concentrations of each drug. Results showed a dose-dependent activity and IC_{50} values of 0.36 µM, 0.68 µM and 6.32 µM for Pt(II), Pt(IV) and cisplatin respectively.

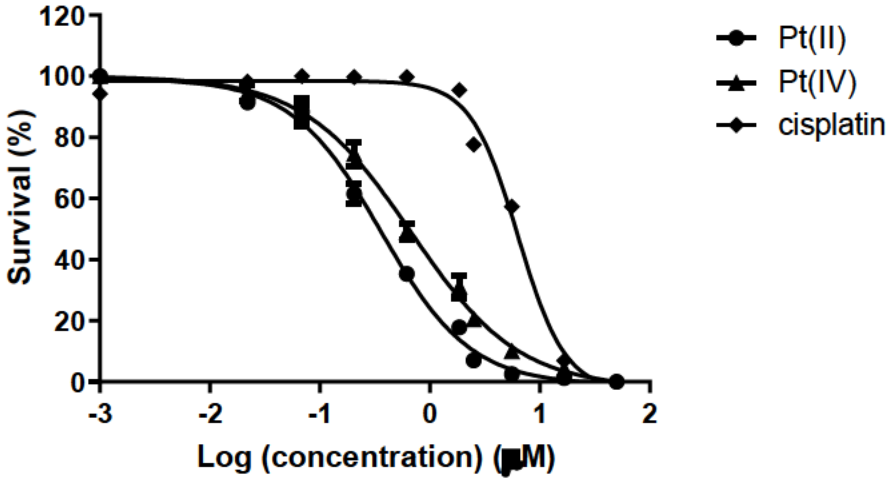


Figure 22: Cytotoxic effect of Pt(II), Pt(IV) and cisplatin on the cell survival of A375 A375 cells were treated with several concentrations of Pt(II), Pt(IV) and cisplatin over 72 hours. Data points denote mean ± SEM. n = 3 from three independent experiments where samples were run in triplicate.

3.2.3. MDA-MB-231 cancer cells

The effect of Pt(II), Pt(IV) and cisplatin was examined on MDA-MB-231 cancer cells for 72 hours (figure 23). Cells were treated with multiple concentrations of each drug. Results showed a dose-dependent activity and IC_{50} values of 0.32 μM, 0.6 μM and 4.43 μM for Pt(II), Pt(IV) and cisplatin respectively.

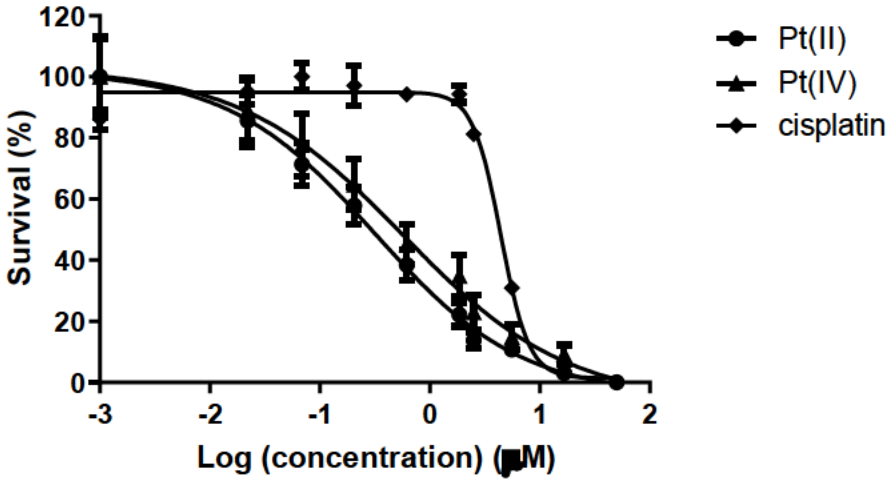


Figure 23: Cytotoxic effect of Pt(II), Pt(IV) and cisplatin on the cell survival of MDA-MB-231. MDA-MB-231 cells were treated with several concentrations of Pt(II),

Pt(IV) and cisplatin over 72 hours. Data points denote mean ± SEM. n = 3 from three independent experiments where samples were run in triplicate.

3.2.4. Mesenchymal stem cells

The effect of Pt(II), Pt(IV) and cisplatin was examined on mesenchymal stem cells for 72 hours (figure 24). Cells were treated with multiple concentrations of each drug. Results showed a dose-dependent activity and IC_{50} values of 0.69 µM, 13.15 µM and 2.00 µM for Pt(II), Pt(IV) and cisplatin respectively.

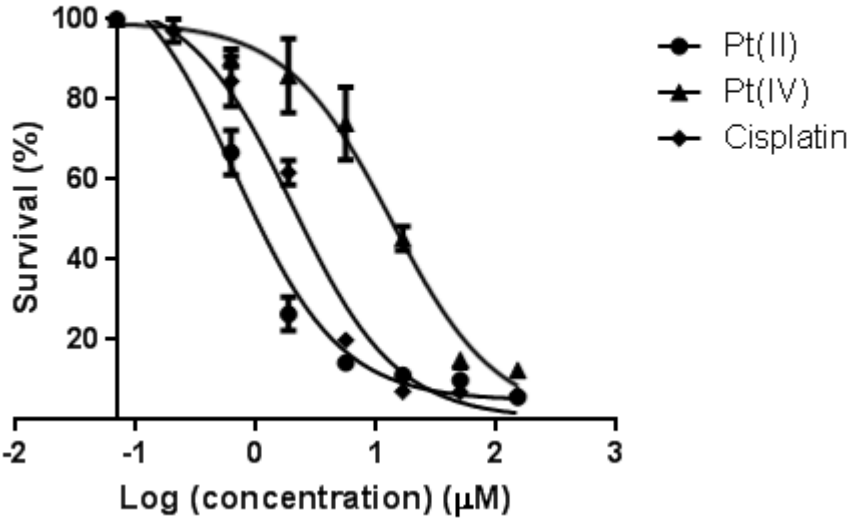


Figure 24: Cytotoxic effect of Pt(II), Pt(IV) and cisplatin on the survival of mesenchymal stem cells. Mesenchymal stem cells extracted from a rat's bone marrow were treated with several concentrations of Pt(II), Pt(IV) and cisplatin over 72 hours. Data points denote mean ± SEM. n = 2 from two independent experiments where samples were run in triplicate.

Table 4: Cytotoxic effect of Pt(II), Pt(IV) and cisplatin on A549, MDA-MB-231, A375 and MSC survival

	IC_{50} (µM)			
Drug	**MDA-MB-231**	**A375**	**A549**	**MSC**
Pt(II)	0.32 ± 0.01	0.36 ± 0.03	0.43 ± 0.01	0.69 ± 0.12
Pt(IV)	0.60 ± 0.11	0.68 ± 0.19	0.78 ± 0.1	13.15 ± 4.51
Cisplatin	4.43 ± 0.02	6.32 ± 0.13	8.54 ± 1.18	2.00 ± 0.21

3.3 Cellular uptake of Pt(II) and Pt(IV) in A549 cancer cells

Human alveolar adenocarcinoma cells (A549) were treated with Pt(II) and Pt(IV) at a final concentration of 1.29 and 2.34 µM, respectively. After 1, 3, 6, 12 or 24 h, cellular uptake of both complexes was quantified by ICP-MS based on external standards (table 5; figure 25). Both Pt(II) and Pt(IV) were actively uptaken into the cells, shown by their high intracellular/extracellular ratio, with Pt(II) showing a faster (higher mean cellular concentration after 1 h of treatment) and a higher uptake (higher mean cellular concentration 24 hours post-treatment) compared to Pt(IV).

Table 5: Measurements of the mean cellular concentration and the ratio of intracellular/extracellular concentrations of Pt(II) and Pt(IV) in A549 cancer cells. n=3

Time (in hours)		**1**	**3**	**6**	**12**	**24**
Mean cellular concentration (µM)	**Pt(II)**	5.29±1.76	9.12±0.29	20.83±1.23	44.26±4.26	114.71±8.82
	Pt(IV)	1.04±0.15	1.04±0.15	4.56±0.15	29.41±5.88	53.73±9.52
Ratio (intracellular /extracellular)	**Pt(II)**	4.1±1.37	7.07±0.23	16.15±0.95	41.12±7.06	88.92±6.84
	Pt(IV)	1.34±0.45	1.34±0.45	1.95±0.06	12.57±2.51	19.19±1.89

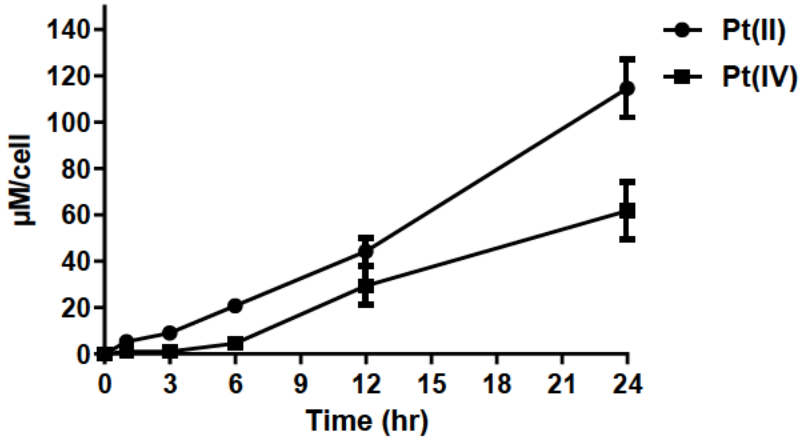


Figure 25: LC-MS/MS analysis for the uptake of Pt(II) and Pt(IV) by A549 cells. Data points denote mean ± SEM and are expressed in µM per cell. n = 3 from three independent experiments where samples were run in triplicate.

3.4 Effect of Pt(II) and Pt(IV) on the expression of apoptotic proteins in A549 cells

3.4.1 Procaspase 3

A549 cells were treated with two different concentrations of Pt(II) (0.43 µM and 0.86 µM) and two different concentrations of Pt(IV) (0.78 µM and 1.56 µM) for 72 hours. Proteins were then extracted from treated and control cells, and the levels of procaspase 3 were measured by Western blot. Bands showed a significant decrease in the expression of procaspase 3 in treated cells compared to control. *ImageLab* was used for quantification, and the values obtained for procaspase 3 under each condition were normalized against actin and plotted as a Bar graph (figure 26).

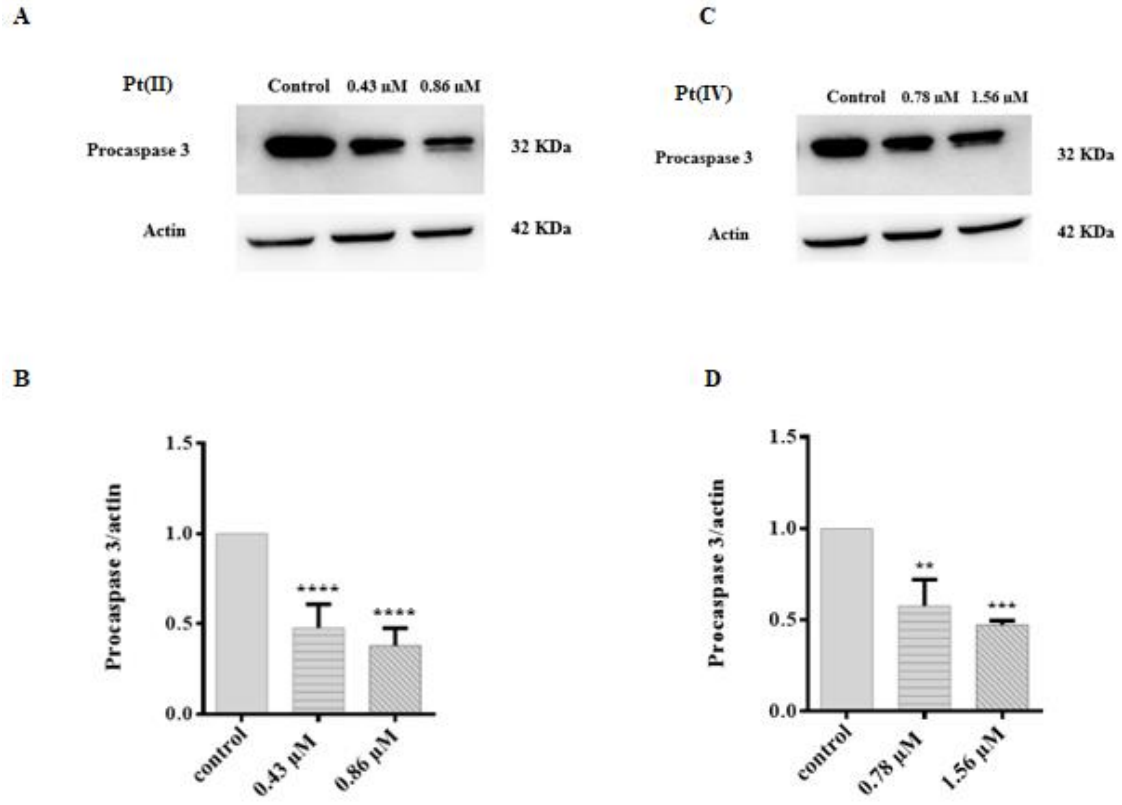


Figure 26: Level of Procaspase 3/actin in A549 cells treated with Pt(II) and Pt(IV). A549 cells were treated with two different concentrations of Pt(II) (0.43 µM and 0.86 µM) and two different concentrations of Pt(IV) (0.78 µM and 1.56 µM) **(A)/(C)** Western blot results presented as bands visualized by the ChemiDoc imaging system. **(B)/(D)** Bands quantificed by *ImageLab*, normalized against actin and plotted as a Bar graph. Bars denote mean ± SEM. n = 3 from three independent experiments.

3.4.2 B-cell lymphoma 2 (Bcl-2)

A549 cells were treated with two different concentrations of Pt(II) (0.43 µM and 0.86 µM) and two different concentrations of Pt(IV) (0.78 µM and 1.56 µM) for 72 hours. Proteins were then extracted from treated and control cells, and the levels of Bcl-2 were measured by Western blot. Bands showed a significant decrease in the expression of Bcl-2 in treated cells compared to control. *ImageLab* was used for quantification, and the values obtained for Bcl-2 under each condition were normalized against actin and plotted as a Bar graph (figure 27).

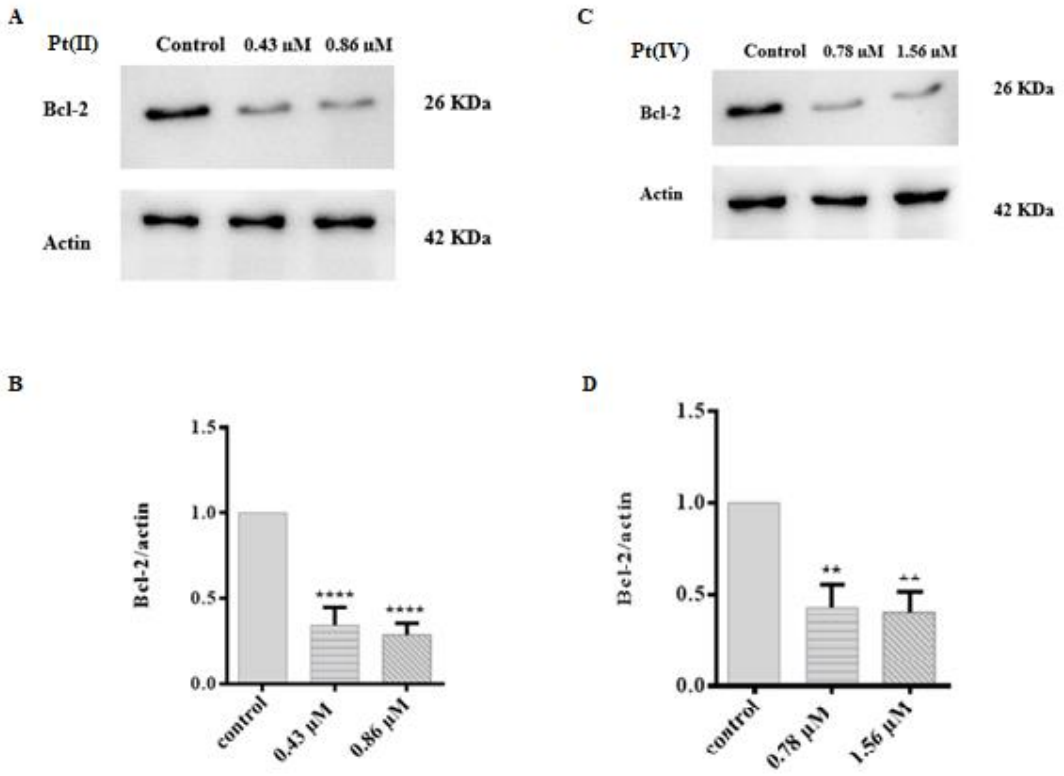


Figure 27: Level of Bcl-2/actin in A549 cells treated with Pt(II) and Pt(IV). A549 cells were treated with two different concentrations of Pt(II) (0.43 µM and 0.86 µM) and two different concentrations of Pt(IV) (0.78 µM and 1.56 µM) **(A)/(C)** Western blot results presented as bands visualized by the ChemiDoc imaging system. **(B)/(D)** Bands quantificed by *ImageLab*, normalized against actin and plotted as a Bar graph. Bars denote mean ± SEM. n = 3 from three independent experiments.

3.4.3 Bcl2-Associated X Protein (Bax)

A549 cells were treated with two different concentrations of Pt(II) (0.43 µM and 0.86 µM) and two different concentrations of Pt(IV) (0.78 µM and 1.56 µM) for 72 hours. Proteins were then extracted from treated and control cells, and the levels of Bax were measured by Western blot. Bands showed no significant difference in the expression of Bax in treated cells compared to control. *ImageLab* was used for quantification, and the values obtained for Bax under each condition were normalized against actin and plotted as a Bar graph (figure 28).

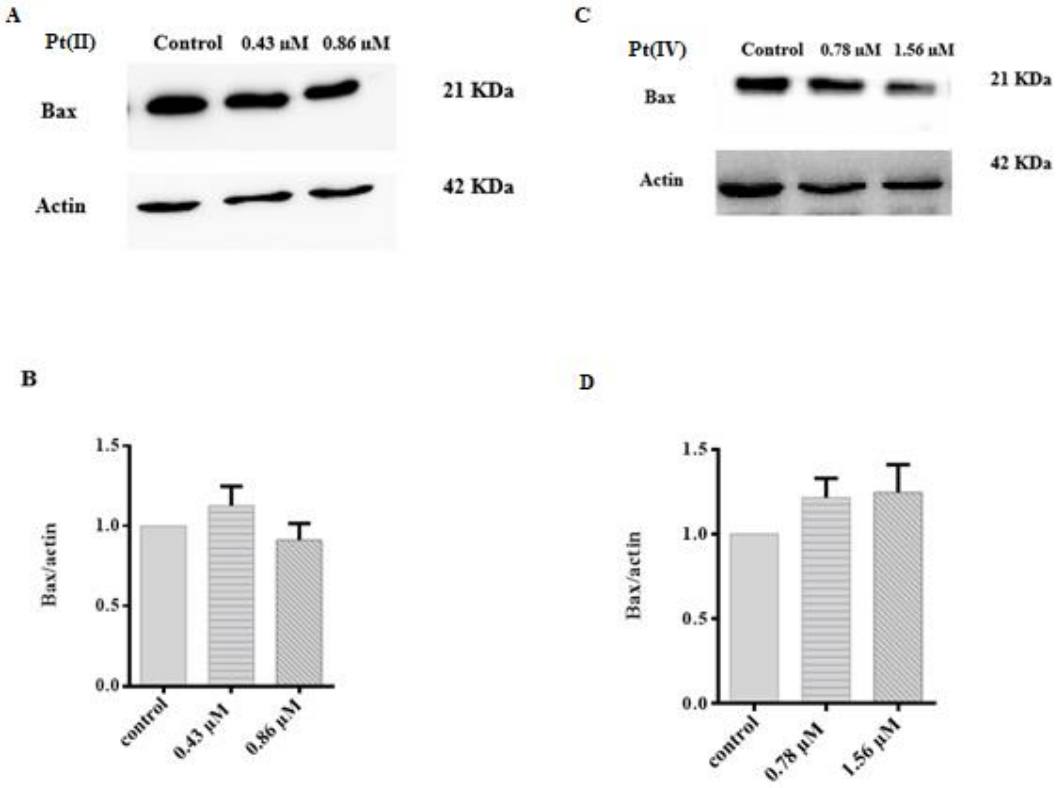


Figure 28: Level of Bax/actin in A549 cells treated with Pt(II) and Pt(IV). A549 cells were treated with two different concentrations of Pt(II) (0.43 µM and 0.86 µM) and two different concentrations of Pt(IV) (0.78 µM and 1.56 µM) **(A)/(C)** Western blot results presented as bands visualized by the ChemiDoc imaging system. **(B)/(D)** Bands quantificed by *ImageLab*, normalized against actin and plotted as a Bar graph. Bars denote mean ± SEM. n = 3 from three independent experiments.

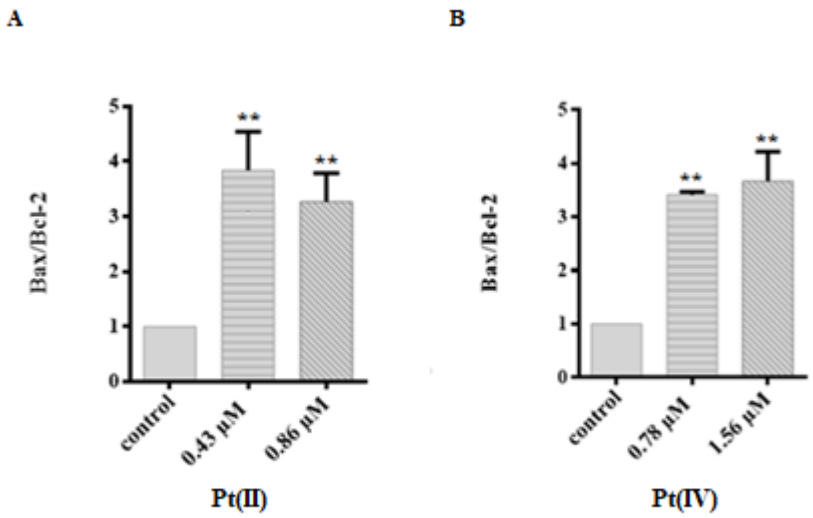


Figure 29: Bax/Bcl-2 in A549 cells treated with Pt(II) and Pt(IV). The ratio of Bax/Bcl-2 was calculated for both cells treated with **(A)** Pt(II) and **(B)** Pt(IV). This ratio was shown to be upregulated in treated cells compared to the control.

3.4.4 Poly (ADP-ribose) polymerase (Parp)

A549 cells were treated with two different concentrations of Pt(II) (0.43 µM and 0.86 µM) and two different concentrations of Pt(IV) (0.78 µM and 1.56 µM) for 72 hours. Proteins were then extracted from treated and control cells, and the levels of Parp were measured by Western blot. Bands showed a significant decrease in the 116 KDa band in treated cells compared to control (figure 30), along with a significant increase in the cleaved 89 KDa band (figure 31). *ImageLab* was used for quantification, and the values obtained for Parp under each condition were normalized against actin and plotted as a Bar graph (figures 30 and 31).

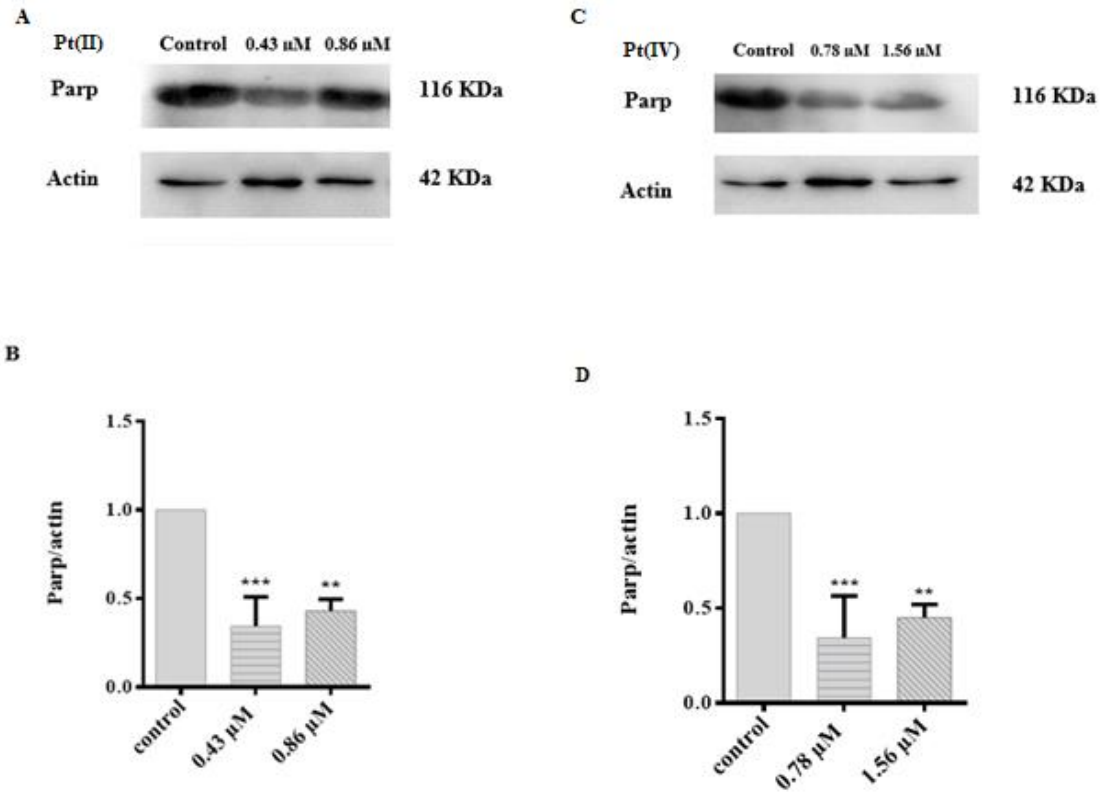


Figure 30: Level of Parp/actin in A549 cells treated with Pt(II) and Pt(IV). A549 cells were treated with two different concentrations of Pt(II) (0.43 µM and 0.86 µM) and two different concentrations of Pt(IV) (0.78 µM and 1.56 µM) **(A)/(C)** Western blot results presented as bands visualized by the ChemiDoc imaging system. **(B)/(D)** Bands quantificed by *ImageLab*, normalized against actin and plotted as a Bar graph. Bars denote mean ± SEM. n = 3 from three independent experiments.

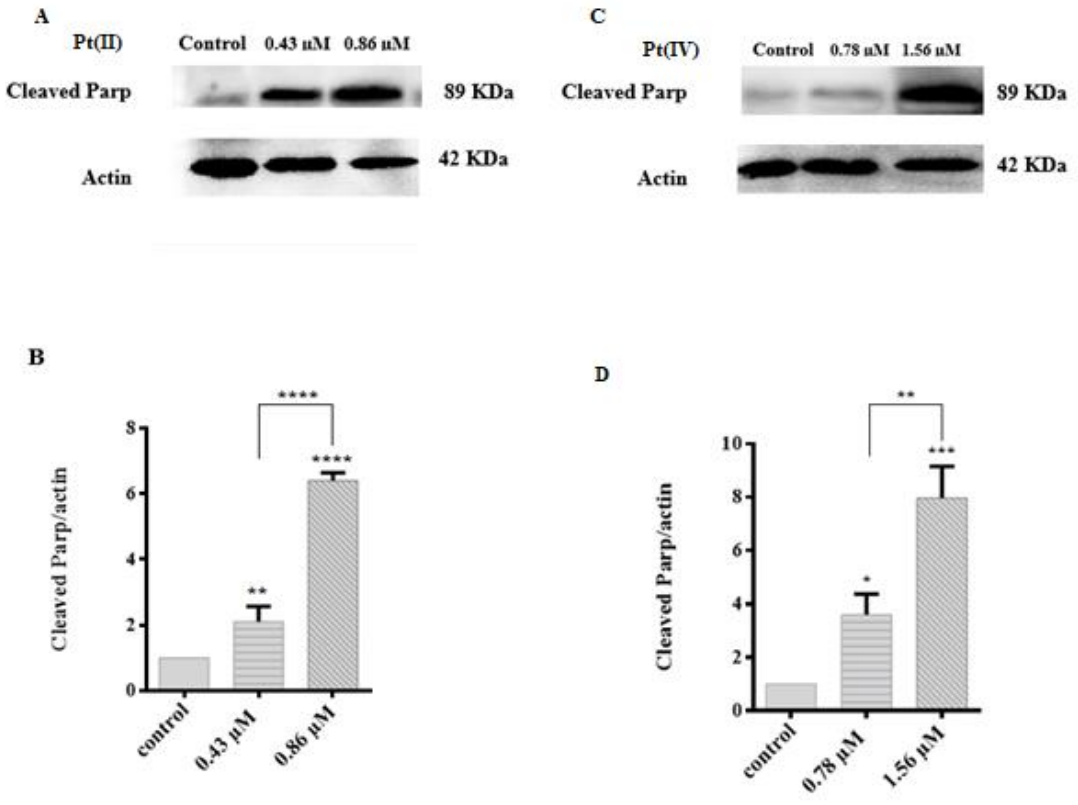


Figure 31: Level of cleaved Parp/actin in A549 cells treated with Pt(II) and Pt(IV). A549 cells were treated with two different concentrations of Pt(II) (0.43 µM and 0.86 µM) and two different concentrations of Pt(IV) (0.78 µM and 1.56 µM) **(A)/(C)** Western blot results presented as bands visualized by the ChemiDoc imaging system. **(B)/(D)** Bands quantificed by *ImageLab*, normalized against actin and plotted as a Bar graph. Bars denote mean ± SEM. n = 3 from three independent experiments.

3.4.5 Procaspase 9

A549 cells were treated with two different concentrations of Pt(II) (0.43 µM and 0.86 µM) and two different concentrations of Pt(IV) (0.78 µM and 1.56 µM) for 72 hours. Proteins were then extracted from treated and control cells, and the levels of procaspase 9 were measured by Western blot. Bands showed a significant decrease in the expression of procaspase 9 in treated cells compared to control. *ImageLab* was used for quantification, and the values obtained for procaspase 9 under each condition were normalized against actin and plotted as a Bar graph (figure 32).

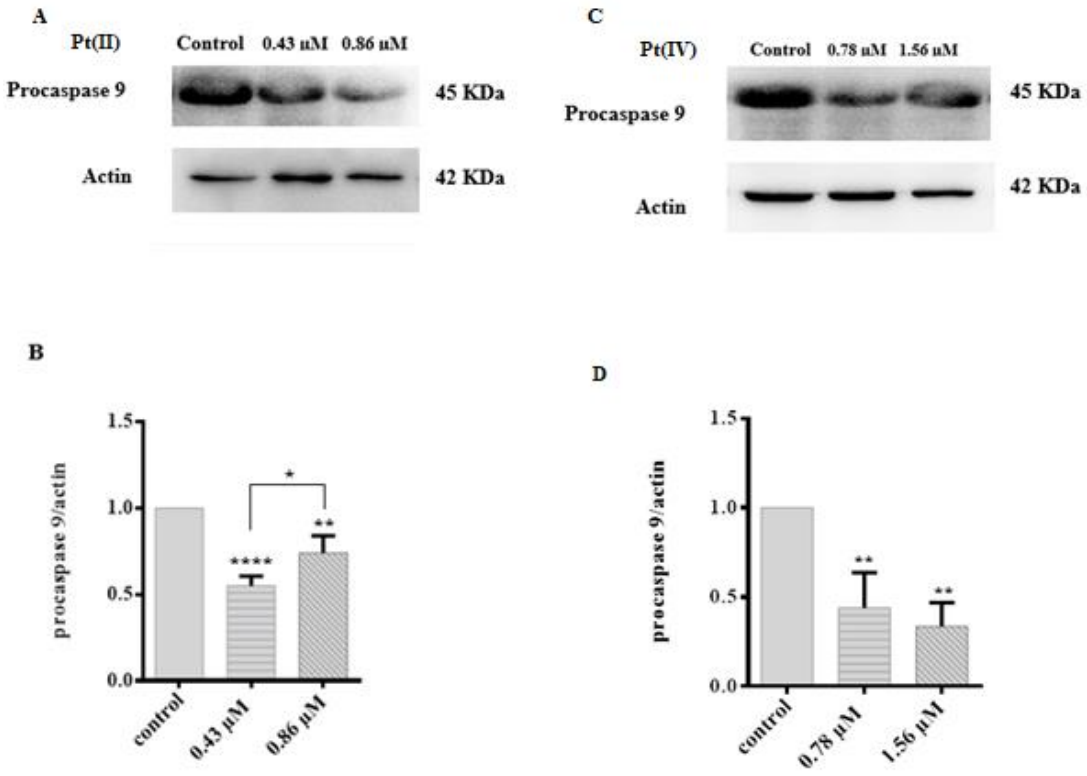


Figure 32: Level of procaspase 9/actin in A549 cells treated with Pt(II) and Pt(IV). A549 cells were treated with two different concentrations of Pt(II) (0.43 µM and 0.86 µM) and two different concentrations of Pt(IV) (0.78 µM and 1.56 µM) **(A)/(C)** Western blot results presented as bands visualized by the ChemiDoc imaging system. **(B)/(D)** Bands quantificed by *ImageLab*, normalized against actin and plotted as a Bar graph. Bars denote mean ± SEM. n = 3 from three independent experiments.

3.4.6 Cytochrome c

A549 cells were treated with two different concentrations of Pt(II) (0.43 µM and 0.86 µM) and two different concentrations of Pt(IV) (0.78 µM and 1.56 µM) for 72 hours. Proteins were then extracted from treated and control cells, and the levels of cytochrome c were measured by Western blot. Bands showed a significant increase in the expression of cytochrome c in treated cells compared to control. *ImageLab* was used for quantification, and the values obtained for cytochrome c under each condition were normalized against actin and plotted as a Bar graph (figure 33).

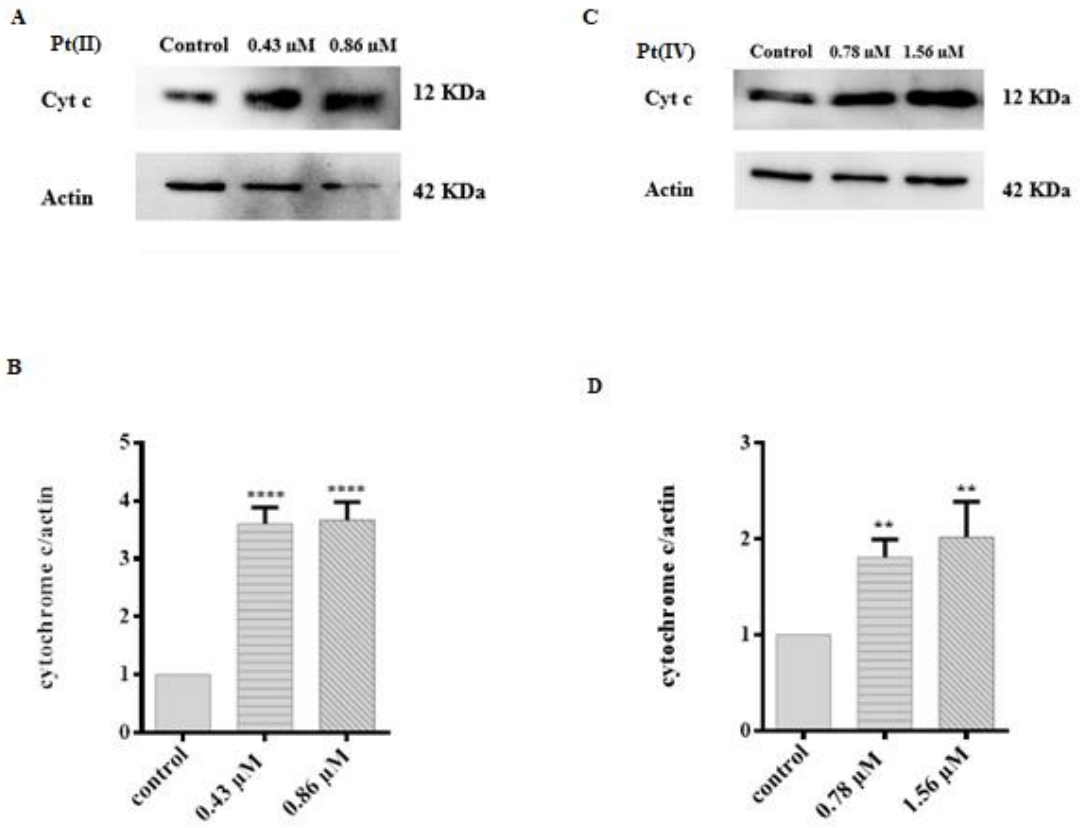


Figure 33: Level of cytochrome c/actin in A549 cells treated with Pt(II) and Pt(IV). A549 cells were treated with two different concentrations of Pt(II) (0.43 µM and 0.86 µM) and two different concentrations of Pt(IV) (0.78 µM and 1.56 µM) **(A)/(C)** Western blot results presented as bands visualized by the ChemiDoc imaging system. **(B)/(D)** Bands quantificed by *ImageLab*, normalized against actin and plotted as a Bar graph. Bars denote mean ± SEM. n = 3 from three independent experiments.

3.4.7 Procaspase 8

A549 cells were treated with two different concentrations of Pt(II) (0.43 µM and 0.86 µM) and two different concentrations of Pt(IV) (0.78 µM and 1.56 µM) for 72 hours. Proteins were then extracted from treated and control cells, and the levels of procaspase 8 were measured by Western blot. Bands showed no significant difference in the expression of procaspase 8 in treated cells compared to control. *ImageLab* was used for quantification, and the values obtained for procaspase 8 under each condition were normalized against actin and plotted as a Bar graph (figure 34).

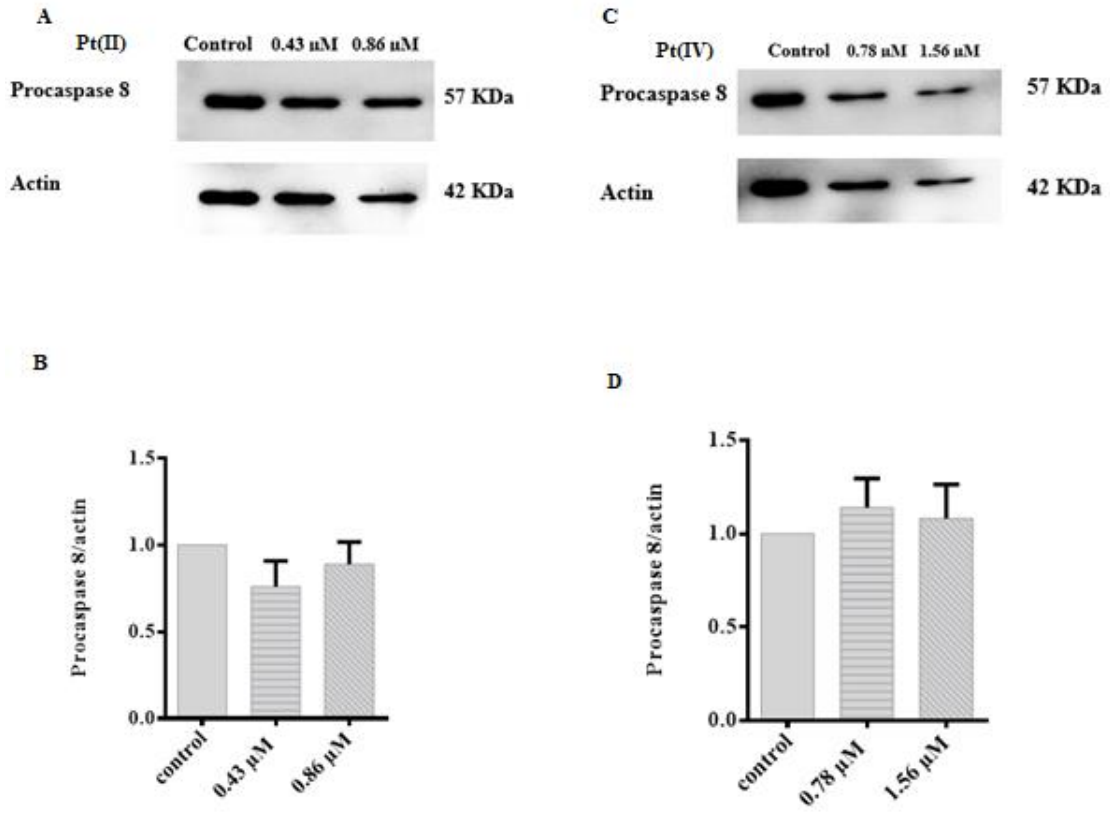


Figure 34: Level of procaspase 8/actin in A549 cells treated with Pt(II) and Pt(IV). A549 cells were treated with two different concentrations of Pt(II) (0.43 µM and 0.86 µM) and two different concentrations of Pt(IV) (0.78 µM and 1.56 µM) **(A)/(C)** Western blot results presented as bands visualized by the ChemiDoc imaging system. **(B)/(D)** Bands quantificed by *ImageLab*, normalized against actin and plotted as a Bar graph. Bars denote mean ± SEM. n = 3 from three independent experiments.

3.5 Effect of Pt(II) and Pt(IV) on DNA in A549 cells

The DNA damaging potential of Pt(II) and Pt(IV) was assessed using alkaline comet assay. The DNA damage in cells exposed to both complexes was measured using multiple parameters including head length, tail length, comet length, head DNA content, tail DNA content and tail moment (table 6). To reflect DNA damage, the tail moment index (TMI) was calculated by multiplying the tail DNA content by the tail length divided by 1000. Data shown in figure 35 revealed a significant increase in TMI in both cells treated with $KMnO_4$ (positive control) and Pt(IV) compared with negative control and Pt(II) groups. The latter group did not show any significant change in TMI compared with the negative control group.

Table 6: Effect of Pt(II) and Pt(IV) treatment on DNA in A549 cells. DNA damage in A549 cells exposed to Pt(II), Pt(IV) and $KMnO_4$ was measured by the comet assay and CASP (Comet assay Software package) was used to measure the parameters present in the table below. n=2

	Negative control	Positive control	Pt(II) (0.43 µM)	Pt(IV) (0.78 µM)
Head length	30.6±3.9	49.0±7.0	38.3±8.2	33.3±9.9
Tail length	3.1±0.1	39.1±5.7	3.4±0.2	10.4±1.7
Comet length	33.7±3.9	89.7±11.5	46.4±8.2	34.0±7.3
Head DNA content	99.1±1.5	25.9±5.9	89.9±4.0	71.6±3.5
Tail DNA content	0.62±0.27	77.0±5.9	2.51±0.03	24.5±4.1
Tail moment	0.04±0.06	29.6±6.1	0.30±0.10	2.68±0.69
Overall Tail Moment	0.19±0.08	18.0±3.5	1.59±0.86	3.47±1.02

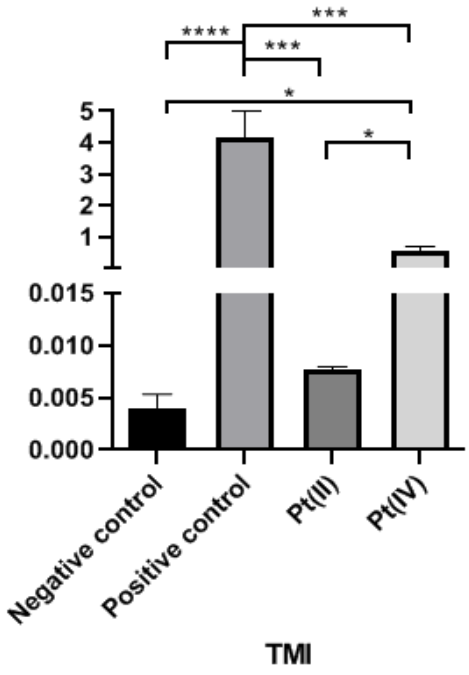


Figure 35: Tail moment index as calculated from the comet assay data (n=2)
TMI= $[Tail\ DNA\ content\ x\ Tail\ length] \div 1000$

3.6 Analysis of cell death post- Pt(II) and Pt(IV) treatment of A549 cells

Annexin V/PI staining was used in order to determine the type of cell death following the treatment of A549 cell line with 0.43 µM of Pt(II), 0.78 µM of Pt(IV) and 8.5 µM of cisplatin (positive control). Flow cytometry result demonstrated a 3-4 fold increase in the percentage of annexin V/ propidium iodide double positive cells post-treatment with Pt(II) and Pt(IV) respectively compared to the control cells (figure 36). These data confirm apoptotic cell death.

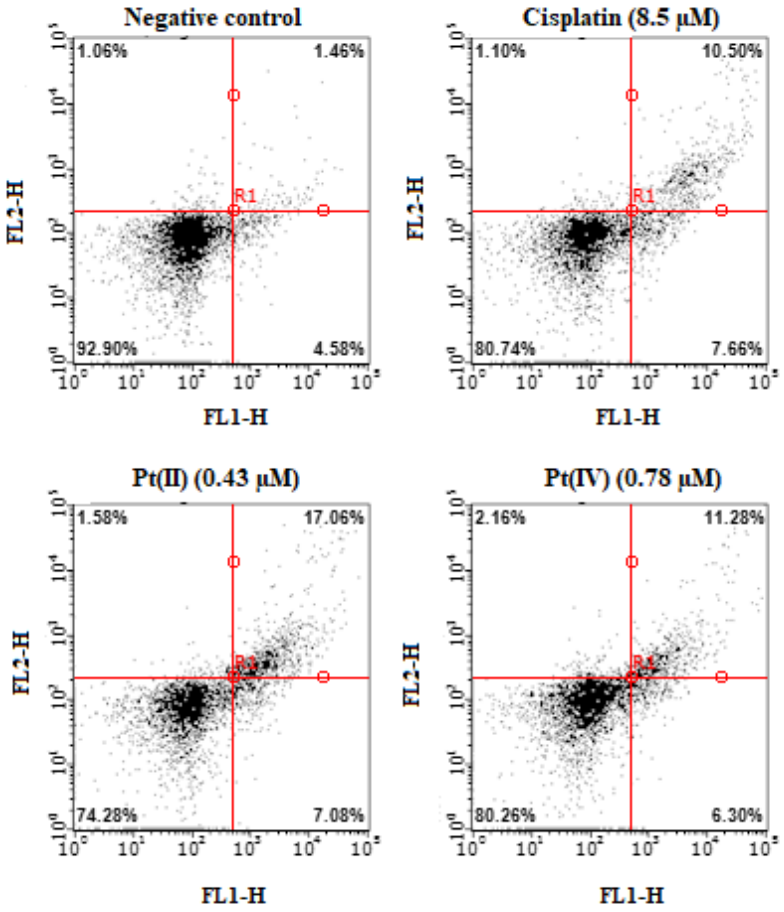


Figure 36: Effect of Pt(II) and Pt(IV) on the death of A549 cells. A549 cells were subjected to treatment with Pt(II), Pt(IV) and cisplatin for 72 hours. Guava easyCyte 8HT Benchtop Flow Cytometer was used to generate the images above.

Chapter Four

Discussion

The discovery of cisplatin and its antineoplastic potential directed research toward the usage of platinum-based complexes as chemotherapeutical drugs (Qin et al., 2020). Several platinum complexes (including but not limited to oxaliplatin, carboplatin and nedaplatin) were then synthesized and analyzed by scientists in order to test their biological activity on cancer cells (Shimada et al., 2013). Some of these drugs were found to have high cytotoxicity against several cancer cell lines and were then used to treat multiple cancer types in patients, after receiving their FDA approval (Johnstone et al., 2016). Several drawbacks, however, resulted from the usage of these FDA-approved drugs, reducing their safety and usage capacity in patients (Ma et al., 2018). This paved the way to extensive research towards discovering novel platinum complexes that would show high chemotherapeutic potential, along with less side effects compared to the previously synthesized platinum drugs (Muhammad & Guo, 2014).

In our present study, novel platinum-based complexes were investigated for their biological activity. Assessment of their cytotoxicity using MTS assay showed that most of the complexes were effective against the three tested cancer cell lines: lung cancer cells (A549), triple-negative breast cancer cells (MDA-MB-231) and melanoma cells (A375). Except for complexes 2, 8, 9 and 10, all remaining 14 complexes had small IC_{50} values that are < 12 µM. Among these complexes, two (complexes 3 and 4) demonstrated a very high cytotoxicity on the three previously mentioned cell lines. Consequently, these two analogues were later designated as Pt(II) and Pt(IV) respectively, and used in the elucidation of the mechanism of action.

Pt(II) is a platinum II complex with a square-planar geometry. It has a central platinum ion surrounded by two bidentate organic ligands. Its platinum IV analogue, Pt(IV), has an octahedral geometry with two additional monodentate hydroxyl (-OH) ligands binding to the central atom and occupying the axial position. As previously mentioned, the anti-cancer activity of Pt(II) and Pt(IV) was first evaluated using MTS assay on A549, MDA-MB-231 and A375 cell lines. Results demonstrated that both Pt(II) and Pt(IV) exhibited a dose dependent growth inhibition against all three cell lines with IC_{50s} in the sub-

micromolar range. Cisplatin displayed IC_{50} values of 4.43 ± 0.02 µM, 6.32 ± 0.13 µM and 8.54 ± 1.18 µM against MDA-MB-231, A375 and A549 cells respectively which are 7 to 20-fold higher compared to Pt(II) and Pt(IV). Consequently, Pt(II) and Pt(IV) complexes might provide a robust alternative for cisplatin and further experimentation should be conducted to reveal the mechanisms of action involved.

Compared to normal cells, cancerous cells possess a more reducing environment because of the lower pH, decreased oxygen levels and upregulated glutathione (Li et al., 2019). This offers an advantage to platinum IV prodrugs compared to their platinum II analogues. Because platinum IV complexes get reduced inside the cells in order to be active, and since cancer cells have a more reducing environment compared to normal cells, these complexes can be selective with a significantly higher cytotoxicity against cancer cells (Ma et al., 2018). This characteristic was tested by performing the MTS assay on mesenchymal stem cells (MSC) extracted from rats' bone marrows. Data showed that the platinum II complexes, Pt(II) and cisplatin, showed no selectivity in cytotoxicity against either cancer or normal cells, with similar IC_{50} values on all tested cell lines (A549, MA-MB-231, A375 and MSC). Pt(IV), however, recorded an IC_{50} value of 13.37 µM, which is 17 to 22-fold higher than its IC_{50} values on the three tested cancer cell lines. This selectivity gives an additional advantage to our platinum IV complex, increasing its efficacy in treating cancer cells.

Cellular uptake of both complexes was then assessed using inductively coupled plasma mass spectrometry (ICP-MS). In order to be transported inside the cells, platinum-based complexes use membrane transporters including efflux ATPases or solute carriers like Ctr1 that mediates the entry of cisplatin into mammalian cells. Other uptake pathways include active, passive and facilitated transports (Dasari & Tchounwou, 2014). In case of passive transport, previous studies on platinum-based complexes suggested a proportional relationship between cellular uptake and lipophilicity of the compound: as the lipophilicity of a platinum-based complex increased, its cellular uptake was consequently found increased (Ghezzi, Aceto, Cassino, Gabano, & Osella, 2004). Our results showed that both complexes are actively transported into the cells as shown by the high ratio of intracellular to extracellular concentration post-treatment. This energy-dependent transport was previously reported by several other platinum complexes (Göschl et al., 2016; Ma et al.,

2018; Wang et al., 2018). Accumulation of our complexes started as early as 1 h post-treatment, when Pt(II) showed a faster cellular uptake compared to Pt(IV) (mean cellular concentrations of 5.29 µM and 1.04 µM for Pt(II) and Pt(IV) respectively). This concentration increased significantly with time, reaching 114.71±8.82 µM 24 hours post-treatment with Pt(II), that showed a higher cellular uptake compared to Pt(IV) whose cellular concentration was 53.73±9.52 µM after 30 hours treatment. This difference in the cellular uptake can explain the slight difference observed in their cytotoxicity. In fact, compared to Pt(IV), Pt(II) recorded a slightly lower IC_{50} value on A549 cells (0.43 µM vs. 0.78 µM for Pt(II) and Pt(IV) respectively) , which could be explained by its higher cellular uptake.

Some platinum II compounds, such as oxaliplatin and nedaplatin, mimic cisplatin's mechanism of action (Johnstone, 2014; Shimada et al., 2013). After replacing two of their unstable ligands with water, they become electrophilic and get attacked by the N7 position of purines, damaging DNA via formation of several cross links (Makovec, 2019). Other platinum II compounds, including trans-platinum (II) with heteroaromatic ligands, iminoether ligands, asymmetric aliphatic amine ligands, monofunctional platinum (II) complexes and noncovalently binding platinum (II) complexes, react differently inside the cells (Johnstone et al., 2016). Since Pt(II) has a similar structure to the latter group of platinum complexes, it is expected to bind non-covalently to DNA. Platinum IV compounds follow a similar mechanism of action, after being reduced to platinum II inside the cells (Pouryasin et al., 2014**).** However, they have more electrophilic binding sites on axial positions and consequently, different variations of ligands could be used generating multiple modes of action for these platinum complexes (Johnstone et al., 2016).

In order to determine if Pt(II) and Pt(IV) cause DNA damage, the comet assay experiment was conducted on A549 cells. Potassium permanganate ($KMnO_4$) treated cells were used as positive control, as the DNA damaging potential of this oxidizing agent was reported since decades (Akman, Doroshow, & Dizdaroglu, 1990). The tail moment index (reflecting DNA damage) was calculated by multiplying the tail DNA content by the tail length divided by 1000 (Khalil, Al Hageh, Korfali, & Khnayzer, 2018). Results showed no significant DNA damage in cells treated with Pt(II) as compared to the negative control. This can be explained by the chemical structure of our Pt(II) compound. Due to its π-

conjugated heterocyclic bidentate ligands, this complex cannot covalently bind DNA. Instead, it was shown that the chemotherapeutic potential of this compound, like other mononuclear platinum (II) metalointercalators, resides in its intercalation between the base pairs of DNA using dipole−dipole interactions and π−π stacking, distorting its topology without forming any covalent bonds (Jennette et al., 1974; Long & Barton, 1990; Wu et al., 2005). Pt(IV), however, caused significant DNA damage. This effect is attributed to the presence of the two additional labile axial ligands that, once dissociated, will enable Pt(IV) to intercalate and form several cross links damaging DNA (Hall, Dolwan, & Hambley, 2006). That's why, although Pt(IV) had a significantly lower cellular uptake compared with Pt(II), its DNA damaging potential was sufficient enough to compensate for the higher uptake observed with its platinum (II) analogue; both complexes having sub-micromolar IC_{50} values.

Cisplatin, along with other platinum complexes, is known to cause apoptotic cell death (Su, Wang, Wang, & Wang, 2020). To understand the mechanism by which the two complexes are causing cell death, western blots analysis was carried out against several proapoptotic and antiapoptotic proteins. Cleavage of procaspase 3 into caspase 3 is considered to be one of the main events in apoptosis, since caspase 3 is an effector caspase that can cleave hundreds of substrates inside the cell, leading to cellular death (Wang, Fangyang et al., 2015). Western blot analysis showed that, similar to previously studied novel platinum complexes (Czarnomysy et al., 2018), treatment of A549 cells with 0.43 µM and 0.86 µM of Pt(II) caused a significant decrease in the levels of procaspase 3 compared to the control cells. Similar results were obtained in A549 cells treated with 0.78 µM and 1.56 µM of Pt(IV). This indicates that the apoptotic pathway could be implicated in the mechanism of action of these two complexes.

Downstream of caspase 3, poly (ADP-ribose) polymerase (PARP) is known to be a very important regulator of apoptosis since its cleavage leads to an increase in DNA fragmentation inside the nucleus leading to cell death (Caron et al., 2019). When caspase 3 gets cleaved, it will subsequently cleave PARP constituting one of the most important hallmarks of apoptosis (Chaitanya, Alexander, & Babu, 2010). Western blots analysis showed a significant decrease in the 116 kDa PARP along with a significant increase in

the 89 kDa cleaved PARP in A549 cells treated with either Pt(II) or Pt(IV) compared to control cells. These results reinforce the idea that the mechanism of cell death of both complexes depends on the apoptotic pathway.

Caspase 3 is known to be activated mainly via two different apoptotic pathways: the intrinsic and the extrinsic. The former pathway is previously shown to be mainly targeted by cisplatin and other similar platinum complexes (Dasari & Tchounwou, 2014) and includes the components of an important protein family: the Bcl-2 family. This family is composed of many anti-apoptotic or pro-survival proteins including Bcl-2 and other proapoptotic proteins including Bax (Youle & Strasser, 2008). Bax is an important proapoptotic protein because its activation leads to the permeabilization of the outer mitochondrial membrane, causing the release of cytochrome c (Danial, 2007). Bcl-2, on the contrary, promotes cell survival by binding to Bax and inhibiting its proapoptotic activity. Following the release of cytochrome c from the mitochondria, an important protein (caspase 9), known to play a crucial role in apoptosis and belonging to the family of cysteine-dependent aspartate, is subsequently activated. This caspase 9 protein has an important role in cleaving caspase 3 which is the central effector caspase implemented in apoptosis (Würstle, Laussmann, & Rehm, 2012). The present data showed that upon treatment with either Pt(II) or Pt(IV), the levels of Bcl-2 in A549 cells were significantly downregulated, whereas the levels of the proapoptotic proteins Bax were relatively similar compared to control cells. This result was different than that of cisplatin whose previous studies revealed an upregulation in the expression of Bax proteins in A549 cells (Matsumoto, Nakajima, Seike, Gemma, & Tanaka, 2016). The ratio of Bax/Bcl-2 was then measured and found to be significantly upregulated in the treated cells, suggesting an intrinsic apoptotic cell death. Additionally, compared to control cells, the levels of cytochrome c were found to be significantly upregulated in treated A549 cells, whereas the levels of procaspase 9 were significantly downregulated confirming that these platinum complexes affect the intrinsic apoptotic pathway, leading to the observed cell death.

Since caspase 3 can also be activated extrinsically, it was important to measure the expression of proteins belonging to this extrinsic apoptotic pathway. The main event

taking place during this series of events is the activation of procaspase 8 by the DISC complex (Singh et al., 2019). The activation of caspase 8 can directly activate caspase 3 causing apoptosis or can lead to the activation and cleavage of BID, a BH3 domain-containing proapoptotic Bcl2 family member that facilitates the release of cytochrome c from the mitochondria activating the intrinsic apoptotic pathway (Huang et al., 2016). To test if our two complexes also target the extrinsic pathway, the levels of procaspase 8 was measured in A549 cells. Compared to control cells, the levels of procaspase 8 were not altered in A549 cells treated with either Pt(II) or Pt(IV). Altogether, these results suggest that Pt(II) and Pt(IV) induce cell death particularly through the intrinsic apoptotic pathway, similar to several previously studied platinum complexes (Wang, Feng-Yang et al., 2018).

Finally, to confirm the type of cell death induced by our platinum complexes, flow cytometry was conducted using A549 cells. Cells treated with 8.5 μM of cisplatin were used as a positive control, as cisplatin is known to induce apoptosis (Dasari & Tchounwou, 2014). Exposure of the lipid phosphatidylserine (PS) to the outer side of the cell membrane is one of the most important hallmarks of apoptotic cell death (Shlomovitz et al., 2019). Since annexin V is a protein known for its binding capacity to PS, it may be utilized in order to test the presence of apoptosis (Lizarbe, Barrasa, Olmo, Gavilanes, & Turnay, 2013). Propidium iodide (PI) was also used because this fluorescent agent can stain necrotic cells (Crowley, Marfell, Scott, & Waterhouse, 2016). Flow cytometry analysis confirmed apoptosis in A549 cells treated with either Pt(II) or Pt(IV) as shown by the 3-4 fold increase in the percentage of annexin V/ propidium iodide double positive cells compared to control cells.

Chapter Five

Conclusion

In conclusion, our study demonstrated that the two novel platinum analogues, Pt(II) and Pt(IV), exhibited a high cytotoxic activity against the three cancer cell lines (A549, A375 and MDA-MB-231), with Pt(IV) demonstrating additional selectivity towards cancer cells compared to mesenchymal stem cells. Both complexes were actively transported in to the cells, and Pt(II) showed a faster and higher uptake compared to Pt(IV). DNA damage was found in cells treated with Pt(IV) only. The inhibition of cellular proliferation, after treatment with either platinum complexes, was associated with the intrinsic apoptotic pathway. In vivo studies will follow to test the efficacy and safety of our complexes, and to confirm their chemotherapeutic effect on model organisms using DMBA/TPA skin carcinogenesis model and DMBA breast carcinogenesis model. Additionally, other platinum complexes that showed high cytotoxicity on the three tested cell lines will be used in order to elucidate their mechanisms of action. The importance of our study lies towards identifying two novel platinum complexes, considered as potent chemotherapeutic drugs, providing a robust alternative for cisplatin because of their higher cytotoxicity.

References

Adigun, R., Basit, H., & Murray, J. (2019). Necrosis, cell (liquefactive, coagulative, caseous, fat, fibrinoid, and gangrenous). *StatPearls* (). Treasure Island (FL): StatPearls Publishing. Retrieved from http://www.ncbi.nlm.nih.gov/books/NBK430935/

Akman, S. A., Doroshow, J. H., & Dizdaroglu, M. (1990). Base modifications in plasmid DNA caused by potassium permanganate. *Archives of Biochemistry and Biophysics, 282*(1), 202-205. doi:10.1016/0003-9861(90)90105-8

American Cancer Society. (2019). How chemotherapy drugs work. Retrieved from https://www.cancer.org/treatment/treatments-and-side-effects/treatment-types/chemotherapy/how-chemotherapy-drugs-work.html

American Cancer Society. (2020). Lung cancer statistics | how common is lung cancer. Retrieved from https://www.cancer.org/cancer/lung-cancer/about/key-statistics.html

American Lung Association. (2019). What's in a cigarette? Retrieved from https://www.lung.org/stop-smoking/smoking-facts/whats-in-a-cigarette.html

Amini, A., Yeh, N., Gaspar, L. E., Kavanagh, B., & Karam, S. D. (2014). Stereotactic body radiation therapy (SBRT) for lung cancer patients previously treated with conventional radiotherapy: A review. *Radiation Oncology (London, England), 9*, 210. doi:10.1186/1748-717X-9-210

Apps, M., Choi, E. H. Y., & Wheate, N. J. (2015). The state-of-play and future of platinum drugs. Retrieved from https://erc.bioscientifica.com/view/journals/erc/22/4/R219.xml#bib16

Awan, A., & Esfahani, K. (2018). Endocrine therapy for breast cancer in the primary care setting. *Current Oncology, 25*(4), 285-291. doi:10.3747/co.25.4139

Barillé-Nion, S., Bah, N., Véquaud, E., & Juin, P. (2012). Regulation of cancer cell survival by BCL2 family members upon prolonged mitotic arrest: Opportunities for anticancer therapy. *Anticancer Research, 32*(10), 4225-4233.

Barreiro-Iglesias, A., Sobrido-Cameán, D. & Shifman, M. I. (2017). Retrograde activation of the extrinsic apoptotic pathway in spinal-projecting neurons after a complete spinal cord injury in lampreys. Retrieved from https://www.hindawi.com/journals/bmri/2017/5953674/

Belkacemi, L. (2018). Exploiting the extrinsic and the intrinsic apoptotic pathways for cancer therapeutics. Retrieved from http://www.remedypublications.com/open-access/exploiting-the-extrinsic-and-the-intrinsic-apoptotic-pathways-for-cancer-therapeutics-704.pdf

Benítez-Guzmán, A., Arriaga-Pizano, L., Morán, J., & Gutiérrez-Pabello, J. A. (2018). Endonuclease G takes part in AIF-mediated caspase-independent apoptosis in mycobacterium bovis-infected bovine macrophages. *Veterinary Research, 49* doi:10.1186/s13567-018-0567-1

BreastCancer.ORG. (2018). Risk of developing breast cancer. Retrieved from https://www.breastcancer.org/symptoms/understand_bc/risk/understanding

Buckley, A. M., Lynam-Lennon, N., O'Neill, H., & O'Sullivan, J. (2020). Targeting hallmarks of cancer to enhance radiosensitivity in gastrointestinal cancers. *Nature Reviews Gastroenterology & Hepatology*, , 1-16. doi:10.1038/s41575-019-0247-2

Buss, I., & Jaehde, U. (2017). Platinum complexes. Retrieved from https://link.springer.com/referenceworkentry/10.1007%2F978-3-642-16483-5_4616

Cancer Research Institute. (2019). What are the causes of cancer? Retrieved from https://www.cancerresearch.org/blog/april-2019/what-causes-cancer-risk-factors

Cancer Research, U. K. (2014). How cancer starts. Retrieved from https://www.cancerresearchuk.org/about-cancer/what-is-cancer/how-cancer-starts

Cancer Research, U. K. (2015). Lung cancer survival statistics. Retrieved from https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/lung-cancer/survival

Cancer Research, U. K. (2019). Can radon gas cause cancer? Retrieved from https://www.cancerresearchuk.org/about-cancer/causes-of-cancer/air-pollution-radon-gas-and-cancer/can-radon-gas-cause-cancer

Cancer Research, U. K. (n.d.). Statistics on preventable cancers. Retrieved from https://www.cancerresearchuk.org/health-professional/cancer-statistics/risk/preventable-cancers

CancerCare. (2019). Lung cancer | treatment update. Retrieved from https://www.cancercare.org/publications/18-treatment_update_lung_cancer

Caron, M., Sharma, A. K., O'Sullivan, J., Myler, L. R., Ferreira, M. T., Rodrigue, A., . . . Masson, J. (2019). Poly(ADP-ribose) polymerase-1 antagonizes DNA resection at double-strand breaks. *Nature Communications, 10*(1), 1-16. doi:10.1038/s41467-019-10741-9

Chaitanya, G. V., Alexander, J. S., & Babu, P. P. (2010). PARP-1 cleavage fragments: Signatures of cell-death proteases in neurodegeneration. *Cell Communication and Signaling : CCS, 8*, 31. doi:10.1186/1478-811X-8-31

Chan, B. A., & Coward, J. I. G. (2013). Chemotherapy advances in small-cell lung cancer. *Journal of Thoracic Disease, 5*(5), S565-S578. Retrieved from http://jtd.amegroups.com/article/view/1515

Chaudhary, P. (2016). What you need to know about lung cancer treatment in india. Retrieved from https://www.vaidam.com/knowledge-center/your-guide-to-cause-and-treatment-of-lung-cancer

Cheng, Y., Wu, R., Cheng, M., Du, J., Hu, X., Yu, L., . . . Wan, F. (2016). Carboplatin-induced hematotoxicity among patients with non-small cell lung cancer: Analysis

on clinical adverse events and drug-gene interactions. *Oncotarget, 8*(19), 32228-32236. doi:10.18632/oncotarget.12951

Chheang, S., & Brown, K. (2013). Lung cancer staging: Clinical and radiologic perspectives. *Seminars in Interventional Radiology, 30*(2), 99-113. doi:10.1055/s-0033-1342950

Choi, S., Vastag, L., Leung, C., Beard, A. M., Knowles, D. E., & Larrabee, J. A. (2006). Kinetics and mechanism of the oxidation of guanosine derivatives by pt(IV) complexes. *Inorganic Chemistry, 45*(25), 10108-10114. doi:10.1021/ic061243g

Colditz, G. A., Kaphingst, K. A., Hankinson, S. E., & Rosner, B. (2012). Family history and risk of breast cancer: Nurses' health study. *Breast Cancer Research and Treatment, 133*(3), 1097-1104. doi:10.1007/s10549-012-1985-9

Corinti, D., Coletti, C., Re, N., Piccirillo, S., Giampà, M., Elisa Crestoni, M., & Fornarini, S. (2017). Hydrolysis of cis- and transplatin: Structure and reactivity of the aqua complexes in a solvent free environment. *RSC Advances, 7*(26), 15877-15884. doi:10.1039/C7RA01182B

Crowley, L. C., Marfell, B. J., Scott, A. P., & Waterhouse, N. J. (2016). Quantitation of apoptosis and necrosis by annexin V binding, propidium iodide uptake, and flow cytometry. *Cold Spring Harbor Protocols, 2016*(11) doi:10.1101/pdb.prot087288

Czarnomysy, R., Surażyński, A., Muszynska, A., Gornowicz, A., Bielawska, A., & Bielawski, K. (2018). A novel series of pyrazole-platinum(II) complexes as potential anti-cancer agents that induce cell cycle arrest and apoptosis in breast cancer cells. *Journal of Enzyme Inhibition and Medicinal Chemistry, 33*(1), 1006-1023. doi:10.1080/14756366.2018.1471687

Dana-Farber Cancer Institute. (2019,). Is a tumor cancer? Retrieved from https://blog.dana-farber.org/insight/2018/05/difference-cancer-tumor/

Danial, N. N. (2007). BCL-2 family proteins: Critical checkpoints of apoptotic cell death. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research, 13*(24), 7254-7263. doi:10.1158/1078-0432.CCR-07-1598

Dasari, S., & Bernard Tchounwou, P. (2014a). Cisplatin in cancer therapy: Molecular mechanisms of action. *European Journal of Pharmacology, 740*, 364-378. doi:10.1016/j.ejphar.2014.07.025

Dasari, S., & Bernard Tchounwou, P. (2014b). Cisplatin in cancer therapy: Molecular mechanisms of action. *European Journal of Pharmacology, 740*, 364-378. doi:10.1016/j.ejphar.2014.07.025

Dasari, S., & Tchounwou, P. B. (2014). Cisplatin in cancer therapy: Molecular mechanisms of action. *European Journal of Pharmacology, 740*, 364-378. doi:10.1016/j.ejphar.2014.07.025

De Luca, A., Parker, L. J., Ang, W. H., Rodolfo, C., Gabbarini, V., Hancock, N. C., . . . Dyson, P. J. (2019). A structure-based mechanism of cisplatin resistance mediated by glutathione transferase P1-1. *Proceedings of the National Academy of Sciences, 116*(28), 13943-13951.

Delbridge, A. R. D., Grabow, S., Strasser, A., & Vaux, D. L. (2016). Thirty years of BCL-2: Translating cell death discoveries into novel cancer therapies. *Nature Reviews. Cancer, 16*(2), 99-109. doi:10.1038/nrc.2015.17

Dewi, T., Kesuma Massar, Karlijn, Ruiter, R. A. C., & Leonardi, T. (2019). Determinants of breast self-examination practice among women in surabaya, indonesia: An application of the health belief model. Retrieved from https://bmcpublichealth.biomedcentral.com/articles/10.1186/s12889-019-7951-2

Dhar, S., & Lippard, S. (2009). Mitaplatin, a potent fusion of cisplatin and the orphan drug dichloroacetate. *Proceedings of the National Academy of Sciences of the United States of America, 106*(52), 22199-22204. doi:10.1073/pnas.0912276106

Domingues, B., Lopes, J. M., Soares, P., & Pópulo, H. (2018). Melanoma treatment in review. *ImmunoTargets and Therapy, 7*, 35-49. doi:10.2147/ITT.S134842

Elmore, S. (2007). *Apoptosis: A review of programmed cell death*. United States: SAGE Publications. doi:10.1080/01926230701320337

Fan, Z., Beresford, P. J., Oh, D. Y., Zhang, D., & Lieberman, J. (2003). Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. *Cell, 112*(5), 659-672. doi:10.1016/S0092-8674(03)00150-8

Feng, X., Li, J., Li, H., Chen, H., Li, F., Liu, Q., . . . Zhou, F. (2019). Age is important for the early-stage detection of breast cancer on both transcriptomic and methylomic biomarkers. *Frontiers in Genetics, 10* doi:10.3389/fgene.2019.00212

Fossella, F., Pereira, J. R., von Pawel, J., Pluzanska, A., Gorbounova, V., Kaukel, E., . . . Belani, C. P. (2003). Randomized, multinational, phase III study of docetaxel plus platinum combinations versus vinorelbine plus cisplatin for advanced non-small-cell lung cancer: The TAX 326 study group. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology, 21*(16), 3016-3024. doi:10.1200/JCO.2003.12.046

Friedman, C. F., & Wolchok, J. D. (2017). Checkpoint inhibition and melanoma: Considerations in treating the older adult. *Journal of Geriatric Oncology, 8*(4), 237-241. doi:10.1016/j.jgo.2017.04.003

Galluzzi, L., Vitale, I., Aaronson, S. A., Abrams, J. M., Adam, D., Agostinis, P., . . . Kroemer, G. (2018). Molecular mechanisms of cell death: Recommendations of the nomenclature committee on cell death 2018. *Cell Death & Differentiation, 25*(3), 486-541. doi:10.1038/s41418-017-0012-4

Giard, D. J., Aaronson, S. A., Todaro, G. J., Arnstein, P., Kersey, J. H., Dosik, H., & Parks, W. P. (1973). In vitro cultivation of human tumors: Establishment of cell

lines derived from a series of solid tumors. *JNCI: Journal of the National Cancer Institute, 51*(5), 1417-1423. doi:10.1093/jnci/51.5.1417

Goldar, S., Khaniani, M. S., Derakhshan, S. M., & Baradaran, B. (2015). Molecular mechanisms of apoptosis and roles in cancer development and treatment. *Asian Pacific Journal of Cancer Prevention, 16*(6), 2129-2144. doi:10.7314/APJCP.2015.16.6.2129

Goodsell, D. S. (2006). The molecular perspective: Cisplatin. *Stem Cells, 24*(3), 514-515. doi:10.1634/stemcells.2006-CSC2

Gordon, S., & Plüddemann, A. (2018). Macrophage clearance of apoptotic cells: A critical assessment. Retrieved from https://www.frontiersin.org/articles/10.3389/fimmu.2018.00127/full

Göschl, S., Varbanov, H. P., Theiner, S., Jakupec, M. A., Galanski, M., & Keppler, B. K. (2016). The role of the equatorial ligands for the redox behavior, mode of cellular accumulation and cytotoxicity of platinum(IV) prodrugs. *Journal of Inorganic Biochemistry, 160*, 264-274. doi:10.1016/j.jinorgbio.2016.03.005

Gridelli, C., Rossi, A., Carbone, D. P., Guarize, J., Karachaliou, N., Mok, T., . . . Rosell, R. (2015). Non-small-cell lung cancer. *Nature Reviews Disease Primers, 1*, 15009. doi:10.1038/nrdp.2015.9

Hall, M., Dolwan, R. & Hambley, T. (2006). Platinum(IV) anticancer complexes. Retrieved from https://www.researchgate.net/publication/8502185_PlatinumIV_anticancer_complexes

Hanley, J. A., Hannigan, A., & O'Brien, K. M. (2017). Mortality reductions due to mammography screening: Contemporary population-based data. *PLoS ONE, 12*(12) doi:10.1371/journal.pone.0188947

Hazlitt, R., A., Min, J., & Zuo, J. (2018). Progress in the development of preventative drugs for cisplatin-induced hearing loss. *Journal of Medicinal Chemistry, 61*(13), 5512-5524. doi:10.1021/acs.jmedchem.7b01653

Hirsch, F. R., Scagliotti, G. V., Mulshine, J. L., Kwon, R., Curran, W. J., Wu, Y., & Paz-Ares, L. (2017). Lung cancer: Current therapies and new targeted treatments. *The Lancet, 389*(10066), 299-311. doi:10.1016/S0140-6736(16)30958-8

Ho, G. Y., Woodward, N., & Coward, J. I. G. (2016). Cisplatin versus carboplatin: Comparative review of therapeutic management in solid malignancies. *Critical Reviews in Oncology/Hematology, 102*, 37-46. doi:10.1016/j.critrevonc.2016.03.014

Hoeschele, J. D. (2014). Biography of professor barnett rosenberg: A tribute to his life and his achievements. *Anticancer Research, 34*(1), 417-421. Retrieved from http://ar.iiarjournals.org/content/34/1/417.2

Hsu, H. (2019). Apoptosis as a primary defense mechanism in response to viral infection in invasive fire ant solenopsis invicta. *Virology, 531*, 255-259. doi:10.1016/j.virol.2019.03.015

Huang, K., Zhang, J., O'Neill, K. L., Gurumurthy, C. B., Quadros, R. M., & Luo, X. (2016). Cleavage by caspase 8 and mitochondrial membrane association activate bid during TRAIL-induced apoptosis. *Journal of Biological Chemistry*, , jbc.M115.711051. doi:10.1074/jbc.M115.711051

Imanis Life Sciences. (n.d.). A375 human melanoma reporter gene cell lines - imanis life sciences. Retrieved from https://imanislife.com/collections/cell-lines/a375-cells/

Jakubowska, K., Guzińska-Ustymowicz, K., Famulski, W., Cepowicz, D., Jagodzińska, D., & Pryczynicz, A. (2016). Reduced expression of caspase-8 and cleaved caspase-3 in pancreatic ductal adenocarcinoma cells. *Oncology Letters, 11*(3), 1879-1884. doi:10.3892/ol.2016.4125

Jan, R., & Chaudhry, G. (2019). Understanding apoptosis and apoptotic pathways targeted cancer therapeutics. *Advanced Pharmaceutical Bulletin, 9*(2), 205-218. doi:10.15171/apb.2019.024

Jennette, K. W., Lippard, S. J., Vassiliades, G. A., & Bauer, W. R. (1974). Metallointercalation reagents. 2-hydroxyethanethiolato(2,2′,2″-terpyridine)-platinum(II) monocation binds strongly to DNA by intercalation. *Proceedings of the National Academy of Sciences, 71*(10), 3839-3843.

Johnstone, T. C. (2014). The crystal structure of oxaliplatin: A case of overlooked pseudo symmetry. *Polyhedron, 67* doi:10.1016/j.poly.2013.10.003

Johnstone, T. C., Kulak, N., Pridgen, E. M., Farokhzad, O. C., Langer, R., & Lippard, S. J. (2013). Nanoparticle encapsulation of mitaplatin and the effect thereof on in vivo properties. *ACS Nano, 7*(7), 5675-5683. doi:10.1021/nn401905g

Johnstone, T. C., & Lippard, S. J. (2014). Reinterpretation of the vibrational spectroscopy of the medicinal bioinorganic synthon c,c,t-[pt(NH3)2Cl2(OH)2]. *Journal of Biological Inorganic Chemistry: JBIC: A Publication of the Society of Biological Inorganic Chemistry, 19*(4-5), 667-674. doi:10.1007/s00775-014-1109-6

Johnstone, T. C., Suntharalingam, K., & Lippard, S. J. (2016). The next generation of platinum drugs: Targeted pt(II) agents, nanoparticle delivery, and pt(IV) prodrugs. *Chemical Reviews, 116*(5), 3436-3486. doi:10.1021/acs.chemrev.5b00597

Joza, N., Susin, S. A., Daugas, E., Stanford, W. L., Cho, S. K., Li, C. Y. J., . . . Penninger, J. M. (2001). Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. *Nature, 410*(6828), 549-554. doi:10.1038/35069004

Kale, J., Osterlund, E. J., & Andrews, D. W. (2018). BCL-2 family proteins: Changing partners in the dance towards death. *Cell Death and Differentiation, 25*(1), 65-80. doi:10.1038/cdd.2017.186

Kalemkerian, G. P. (2012). Staging and imaging of small cell lung cancer. *Cancer Imaging : The Official Publication of the International Cancer Imaging Society, 11*(1), 253-258. doi:10.1102/1470-7330.2011.0036

Karch, J., & Molkentin, J. D. (2015). Regulated necrotic cell death: The passive aggressive side of bax and bak. *Circulation Research, 116*(11), 1800-1809. doi:10.1161/CIRCRESAHA.116.305421

Ke, F. F. S., Vanyai, H. K., Cowan, A. D., Delbridge, A. R. D., Whitehead, L., Grabow, S., . . . Strasser, A. (2018). Embryogenesis and adult life in the absence of intrinsic apoptosis effectors BAX, BAK, and BOK. *Cell, 173*(5), 1217-1230.e17. doi:10.1016/j.cell.2018.04.036

Ke, X., & Shen, L. (2017). Molecular targeted therapy of cancer: The progress and future prospect. *Frontiers in Laboratory Medicine, 1*(2), 69-75. doi:10.1016/j.flm.2017.06.001

Kenny, R. G., Chuah, S. W., Crawford, A., & Marmion, C. J. (2017). Platinum(IV) prodrugs – A step closer to ehrlich's vision? *European Journal of Inorganic Chemistry, 2017*(12), 1596-1612. doi:10.1002/ejic.201601278

Khalil, C., Al Hageh, C., Korfali, S., & Khnayzer, R. S. (2018). Municipal leachates health risks: Chemical and cytotoxicity assessment from regulated and unregulated municipal dumpsites in lebanon. *Chemosphere, 208*, 1-13. doi:10.1016/j.chemosphere.2018.05.151

Kim, E. M., Jung, C., Kim, J., Hwang, S., Park, J. K., & Um, H. (2017). The p53/p21 complex regulates cancer cell invasion and apoptosis by targeting bcl-2 family proteins. *Cancer Research, 77*(11), 3092-3100. doi:10.1158/0008-5472.CAN-16-2098

Kleih, M., Böpple, K., Dong, M., Gaißler, A., Heine, S., Olayioye, M. A., . . . Essmann, F. (2019). Direct impact of cisplatin on mitochondria induces ROS production that

dictates cell fate of ovarian cancer cells. *Cell Death & Disease, 10*(11), 1-12. doi:10.1038/s41419-019-2081-4

Kriege, M., Brekelmans, C. T. M., Boetes, C., Besnard, P. E., Zonderland, H. M., Obdeijn, I. M., . . . Klijn, J. G. M. (2004). Efficacy of MRI and mammography for breast-cancer screening in women with a familial or genetic predisposition. *The New England Journal of Medicine, 351*(5), 427-437. doi:10.1056/NEJMoa031759

Lee, K., Kruper, L., Dieli-Conwright, C. M., & Mortimer, J. E. (2019). The impact of obesity on breast cancer diagnosis and treatment. *Current Oncology Reports, 21*(5) doi:10.1007/s11912-019-0787-1

Lemjabbar-Alaoui, H., Hassan, O., Yang, Y., & Buchanan, P. (2015). Lung cancer: Biology and treatment options. *Biochimica Et Biophysica Acta, 1856*(2), 189-210. doi:10.1016/j.bbcan.2015.08.002

Lewandowska, A., Rudzki, M., Rudzki, S., Lewandowski, T., & Laskowska, B. (2019). Environmental risk factors for cancer. Retrieved from http://www.aaem.pl/Environmental-risk-factors-for-cancer-review-paper,94299,0,2.html

Li, X., Liu, Y. & Tian, H. (2018). Current developments in pt(IV) prodrugs conjugated with bioactive ligands. Retrieved from https://www.hindawi.com/journals/bca/2018/8276139/

Li, An, Lin, Tian & Yang. (2019). Smart nanomedicine agents for cancer, triggered by pH, glutathione, H2O2, or H2S.

Lizarbe, M. A., Barrasa, J. I., Olmo, N., Gavilanes, F., & Turnay, J. (2013). Annexin-phospholipid interactions. functional implications. *International Journal of Molecular Sciences, 14*(2), 2652-2683. doi:10.3390/ijms14022652

Long, E. C., & Barton, J. K. (1990). On demonstrating DNA intercalation. *Accounts of Chemical Research, 23*(9), 271-273. doi:10.1021/ar00177a001

Ma, J., Liu, H., Xi, Z., Hou, J., Li, Y., Niu, J., . . . Wang, P. G. (2018). Protected and de-protected platinum(IV) glycoconjugates with GLUT1 and OCT2-mediated selective cancer targeting: Demonstrated enhanced transporter-mediated cytotoxic properties in vitro and in vivo. *Frontiers in Chemistry, 6* doi:10.3389/fchem.2018.00386

Makovec, T. (2019). Cisplatin and beyond: Molecular mechanisms of action and drug resistance development in cancer chemotherapy. *Radiology and Oncology, 53*(2), 148-158. doi:10.2478/raon-2019-0018

Martinho, N., Santos, T. C. B., Florindo, H. F., & Silva, L. C. (2019). Cisplatin-membrane interactions and their influence on platinum complexes activity and toxicity. *Frontiers in Physiology, 9* doi:10.3389/fphys.2018.01898

Matsumoto, M., Nakajima, W., Seike, M., Gemma, A., & Tanaka, N. (2016). Cisplatin-induced apoptosis in non-small-cell lung cancer cells is dependent on bax- and bak-induction pathway and synergistically activated by BH3-mimetic ABT-263 in p53 wild-type and mutant cells. *Biochemical and Biophysical Research Communications, 473*(2), 490-496. doi:10.1016/j.bbrc.2016.03.053

Mayo Clinic. (2018). Cancer - symptoms and causes. Retrieved from https://www.mayoclinic.org/diseases-conditions/cancer/symptoms-causes/syc-20370588

Mayo Clinic. (2019). Lung cancer - symptoms and causes. Retrieved from https://www.mayoclinic.org/diseases-conditions/lung-cancer/symptoms-causes/syc-20374620

McCready, T., Littlewood, D., & Jenkinson, J. (2005). Breast self-examination and breast awareness: A literature review. *Journal of Clinical Nursing, 14*(5), 570-578. doi:10.1111/j.1365-2702.2004.01108.x

Monroe, J. D., Hruska, H. L., Ruggles, H. K., Williams, K. M., & Smith, M. E. (2018). Anti-cancer characteristics and ototoxicity of platinum(II) amine complexes with only one leaving ligand. *PLoS ONE, 13*(3) doi:10.1371/journal.pone.0192505

Moss, S. M., Wale, C., Smith, R., Evans, A., Cuckle, H., & Duffy, S. W. (2015). Effect of mammographic screening from age 40 years on breast cancer mortality in the UK age trial at 17 years' follow-up: A randomised controlled trial. *The Lancet Oncology, 16*(9), 1123-1132. doi:10.1016/S1470-2045(15)00128-X

Muggia, F. M., Bonetti, A., Howell, S. B., Rozencweig, M., & Hoeschele, J. D. (2015). Platinum antitumor complexes: 50 years since barnett rosenberg's discovery. Retrieved from https://ascopubs.org/doi/10.1200/JCO.2015.60.7481

Muhammad, N., & Guo, Z. (2014). Metal-based anticancer chemotherapeutic agents. *Current Opinion in Chemical Biology, 19*, 144-153. doi:10.1016/j.cbpa.2014.02.003

National Cancer Institute. (2015). Metastatic cancer. Retrieved from https://www.cancer.gov/types/metastatic-cancer

Ndagi, U., Mhlongo, N., & Soliman, M. E. (2017). Metal complexes in cancer therapy – an update from drug design perspective. *Drug Design, Development and Therapy, 11*, 599-616. doi:10.2147/DDDT.S119488

Nejdl, Kudr, Blazkova, Chudobova, Skalickova, Ruttkay-Nedecky, . . . Kizek. (2014). *Mechanisms of uptake and interaction of platinum based drugs in eukaryotic cells* Retrieved from https://link.springer.com/chapter/10.1007/978-3-662-44559-4_25

Ong, J. X., Lim, C. S. Q., Le, H. V., & Ang, W. H. (2019). A ratiometric fluorescent probe for cisplatin: Investigating the intracellular reduction of platinum(IV) prodrug complexes. *Angewandte Chemie International Edition, 58*(1), 164-167. doi:10.1002/anie.201810361

Oun, R., Moussa, Y. E., & Wheate, N. J. (2018). The side effects of platinum-based chemotherapy drugs: A review for chemists. *Dalton Transactions, 47*(19), 6645-6653. doi:10.1039/C8DT00838H

Peng, F., Setyawati, M. I., Tee, J. K., Ding, X., Wang, J., Nga, M. E., . . . Leong, D. T. (2019). Nanoparticles promote in vivo breast cancer cell intravasation and

extravasation by inducing endothelial leakiness. *Nature Nanotechnology, 14*(3), 279-286. doi:10.1038/s41565-018-0356-z

Picon-Ruiz, M., Morata-Tarifa, C., Valle-Goffin, J. J., Friedman, E. R., & Slingerland, J. M. (2017). Obesity and adverse breast cancer risk and outcome: Mechanistic insights and strategies for intervention. *Ca, 67*(5), 378-397. doi:10.3322/caac.21405

Pouryasin, Z., Yousefi, R., Nabavizadeh, S., Rashidi, M., Hamidizadeh, P., Alavianmehr, M., & Moosavi-Movahedi, A. (2014). Anticancer and DNA binding activities of platinum (IV) complexes; importance of leaving group departure rate. *Applied Biochemistry and Biotechnology, 172*(5), 2604-2617. doi:10.1007/s12010-013-0700-6

Qin, Z., Ren, G., Yuan, J., Chen, H., Lu, Y., Li, N., . . . Zhao, D. (2020). Systemic evaluation on the pharmacokinetics of platinum-based anticancer drugs from animal to cell level: Based on total platinum and intact drugs. *Frontiers in Pharmacology, 10* doi:10.3389/fphar.2019.01485

Radha, G., & Raghavan, S. C. (2017). BCL2: A promising cancer therapeutic target. *Biochimica Et Biophysica Acta (BBA) - Reviews on Cancer, 1868*(1), 309-314. doi:10.1016/j.bbcan.2017.06.004

Rastrelli, M., Tropea, S., Rossi, C. R., & Alaibac, M. (2014). Melanoma: Epidemiology, risk factors, pathogenesis, diagnosis and classification. *In Vivo, 28*(6), 1005-1011. Retrieved from http://iv.iiarjournals.org/content/28/6/1005

Ravichandran, K. S. (2010). Find-me and eat-me signals in apoptotic cell clearance: Progress and conundrums. Paper presented at the doi:10.1084/jem.20101157

Reithofer, M. R., Schwarzinger, A., Valiahdi, S. M., Galanski, M., Jakupec, M. A., & Keppler, B. K. (2008). Novel bis(carboxylato)dichlorido(ethane-1,2-diamine)platinum(IV) complexes with exceptionally high cytotoxicity. *Journal of Inorganic Biochemistry, 102*(12), 2072-2077. doi:10.1016/j.jinorgbio.2008.07.006

Roh, M. R., Eliades, P., Gupta, S., Grant-Kels, J. M., & Tsao, H. (2015). Cutaneous melanoma in women. *International Journal of Women's Dermatology, 1*(1), 21-25. doi:10.1016/j.ijwd.2015.01.001

Saad, S. Y., Najjar, T. A. O., & Alashari, M. (2004). Role of non-selective adenosine receptor blockade and phosphodiesterase inhibition in cisplatin-induced nephrogonadal toxicity in rats. *Clinical and Experimental Pharmacology & Physiology, 31*(12), 862-867. doi:10.1111/j.1440-1681.2004.04127.x

Schiller, J. H., Harrington, D., Belani, C. P., Langer, C., Sandler, A., Krook, J., . . . Johnson, D. H. (2002). Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. *The New England Journal of Medicine, 346*(2), 92-98. doi:10.1056/NEJMoa011954

Seely, J. M., & Alhassan, T. (2018). Screening for breast cancer in 2018—what should we be doing today? *Current Oncology, 25*(Suppl 1), S115-S124. doi:10.3747/co.25.3770

Shah, R., Rosso, K., & Nathanson, S. D. (2014). Pathogenesis, prevention, diagnosis and treatment of breast cancer. *World Journal of Clinical Oncology, 5*(3), 283-298. doi:10.5306/wjco.v5.i3.283

Shimada, M., Itamochi, H., & Kigawa, J. (2013). Nedaplatin: A cisplatin derivative in cancer chemotherapy. *Cancer Management and Research, 5*, 67-76. doi:10.2147/CMAR.S35785

Shlomovitz, I., Speir, M., & Gerlic, M. (2019). Flipping the dogma – phosphatidylserine in non-apoptotic cell death. Retrieved from https://biosignaling.biomedcentral.com/articles/10.1186/s12964-019-0437-0

Siegel, R. L., Miller, K. D., & Jemal, A. (2019). Cancer statistics, 2019. *CA: A Cancer Journal for Clinicians, 69*(1), 7-34. doi:10.3322/caac.21551

Siewierska, K., Malicka, I., Kobierzicky, C., Paslawska, U., Cegielski, M., Grzegrzolka, J., . . . Wozniewski, M. (2018). The impact of exercise training on breast cancer. *In Vivo, 32*(2), 249-254. doi:10.21873/invivo.11231

Singh, R., Letai, A., & Sarosiek, K. (2019). Regulation of apoptosis in health and disease: The balancing act of BCL-2 family proteins. *Nature Reviews Molecular Cell Biology, 20*(3), 175-193. doi:10.1038/s41580-018-0089-8

Skin Cancer Foundation. (2020,). Skin cancer facts & statistics. Retrieved from https://www.skincancer.org/skin-cancer-information/skin-cancer-facts/

Smith, N. A., & Sadler, P. J. (2013). Photoactivatable metal complexes: From theory to applications in biotechnology and medicine. *Philosophical Transactions. Series A, Mathematical, Physical, and Engineering Sciences, 371*(1995), 20120519. doi:10.1098/rsta.2012.0519

Soni, H., Kaminski, D., Gangaraju, R., & Adebiyi, A. (2018). Cisplatin-induced oxidative stress stimulates renal fas ligand shedding. *Renal Failure, 40*(1), 314-322. doi:10.1080/0886022X.2018.1456938

Su, X., Wang, B., Wang, Y., & Wang, B. (2020). Inhibition of TRIM32 induced by miR-519d increases the sensitivity of colorectal cancer cells to cisplatin. *OncoTargets and Therapy, 13*, 277-289. doi:10.2147/OTT.S235940

Sutton, E. C., McDevitt, C. E., Yglesias, M. V., Cunningham, R. M., & DeRose, V. J. (2019). Tracking the cellular targets of platinum anticancer drugs: Current tools and emergent methods. *Inorganica Chimica Acta, 498*, 118984. doi:10.1016/j.ica.2019.118984

Swain, M., Jeudy, M., & Pearlman, M. D. (2016). Controversies in screening mammography. *Clinical Obstetrics and Gynecology, 59*(2), 351-361. doi:10.1097/GRF.0000000000000205

Syed Abdul Rahman, Syarifah Nur, Abdul Wahab, N. & Abd Malek, S. N. (2013). In vitro morphological assessment of apoptosis induced by antiproliferative constituents from the rhizomes of curcuma zedoaria. Retrieved from https://www.hindawi.com/journals/ecam/2013/257108/

Todd, S. P., & Driscoll, M. S. (2017). Prognosis for women diagnosed with melanoma during, before, or after pregnancy: Weighing the evidence. *International Journal of Women's Dermatology*, *3*(1), 26-29. doi:10.1016/j.ijwd.2016.12.004

Voskoboinik, I., Whisstock, J. C., & Trapani, J. A. (2015). Perforin and granzymes: Function, dysfunction and human pathology . Retrieved from https://www.nature.com/articles/nri3839.pdf?origin=ppub

Wallach, D., & Kovalenko, A. (2014). Keeping inflammation at bay. *eLife*, *3*, e02583. doi:10.7554/eLife.02583

Wang, D., & Lippard, S. J. (2005). Cellular processing of platinum anticancer drugs. *Nature Reviews Drug Discovery, 4*(4), 307-320. doi:10.1038/nrd1691

Wang, F., Liu, Y., Wang, L., Yang, J., Zhao, Y., Wang, N., . . . Wu, C. (2015). Targeting procaspase-3 with WF-208, a novel PAC-1 derivative, causes selective cancer cell apoptosis. *Journal of Cellular and Molecular Medicine, 19*(8), 1916-1928. doi:10.1111/jcmm.12566

Wang, F., Tang, X., Wang, X., Huang, K., Feng, H., Chen, Z., . . . Liang, H. (2018). Mitochondria-targeted platinum(II) complexes induce apoptosis-dependent autophagic cell death mediated by ER-stress in A549 cancer cells. *European Journal of Medicinal Chemistry, 155*, 639-650. doi:10.1016/j.ejmech.2018.06.018

Wang, Tang, X., Wang, X., Huang, K., Feng, H., Chen, Z., . . . Liang, H. (2018). Mitochondria-targeted platinum(II) complexes induce apoptosis-dependent autophagic cell death mediated by ER-stress in A549 cancer cells. *European Journal of Medicinal Chemistry, 155*, 639-650. doi:10.1016/j.ejmech.2018.06.018

Warner, E., Messersmith, H., Causer, P., Eisen, A., Shumak, R., & Plewes, D. (2008). Systematic review: Using magnetic resonance imaging to screen women at high risk for breast cancer. *Annals of Internal Medicine, 148*(9), 671-679.

Warren, C. F. A., Wong-Brown, M. W., & Bowden, N. A. (2019). BCL-2 family isoforms in apoptosis and cancer. *Cell Death & Disease, 10*(3), 1-12. doi:10.1038/s41419-019-1407-6

Welsh, J. (2013). Chapter 40 - animal models for studying prevention and treatment of breast cancer. In P. M. Conn (Ed.), *Animal models for the study of human disease* (pp. 997-1018). Boston: Academic Press. Retrieved from http://www.sciencedirect.com/science/article/pii/B9780124158948000403

Wexselblatt, E., & Gibson, D. (2012). What do we know about the reduction of pt(IV) pro-drugs? *Journal of Inorganic Biochemistry, 117*, 220-229. doi:10.1016/j.jinorgbio.2012.06.013

wilson, m., & Schuchter, L. (2015). Chemotherapy for melanoma. Retrieved from https://link.springer.com/chapter/10.1007%2F978-3-319-22539-5_8

Wilson, & Lippard. (2014). Synthetic methods for the preparation of platinum anticancer complexes. *Chemical Reviews, 114*(8), 4470-4495. doi:10.1021/cr4004314

World Health Organization. (2018). Cancer. Retrieved from https://www.who.int/news-room/fact-sheets/detail/cancer

Wu, Y., Koch, K. R., Abratt, V. R., & Klump, H. H. (2005). Intercalation into the DNA double helix and in vivo biological activity of water-soluble planar [pt(diimine)(N,N-dihydroxyethyl-N'-benzoylthioureato)]+Cl- complexes: A study of their thermal stability, their CD spectra and their gel mobility. *Archives of Biochemistry and Biophysics, 440*(1), 28-37. doi:10.1016/j.abb.2005.05.022

Würstle, M. L., Laussmann, M. A., & Rehm, M. (2012). The central role of initiator caspase-9 in apoptosis signal transduction and the regulation of its activation and

activity on the apoptosome. *Experimental Cell Research, 318*(11), 1213-1220. doi:10.1016/j.yexcr.2012.02.013

Xu, X., Lai, Y., & Hua, Z. (2019). Apoptosis and apoptotic body: Disease message and therapeutic target potentials. *Bioscience Reports, 39*(1) doi:10.1042/BSR20180992

Xue, X., You, S., Zhang, Q., Wu, Y., Zou, G., Wang, P. C., . . . Liang, X. (2012). Mitaplatin increases sensitivity of tumor cells to cisplatin by inducing mitochondrial dysfunction. *Molecular Pharmaceutics, 9*(3), 634-644. doi:10.1021/mp200571k

Yang, L., Shi, P., Zhao, G., Xu, J., Peng, W., Zhang, J., . . . Cui, H. (2020). Targeting cancer stem cell pathways for cancer therapy. *Signal Transduction and Targeted Therapy, 5*(1), 1-35. doi:10.1038/s41392-020-0110-5

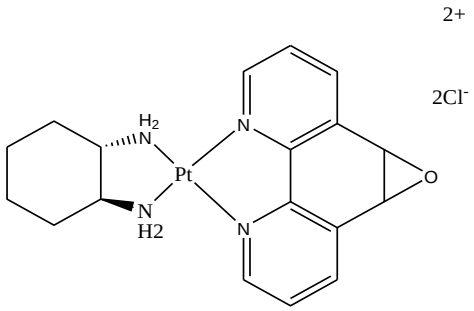
Youle, R. J., & Strasser, A. (2008). The BCL-2 protein family: Opposing activities that mediate cell death. *Nature Reviews. Molecular Cell Biology, 9*(1), 47-59. doi:10.1038/nrm2308

Yu, J., Xiao, J., Yang, Y., & Cao, B. (2015). Oxaliplatin-based doublets versus cisplatin or carboplatin-based doublets in the first-line treatment of advanced nonsmall cell lung cancer. *Medicine, 94*(27) doi:10.1097/MD.0000000000001072

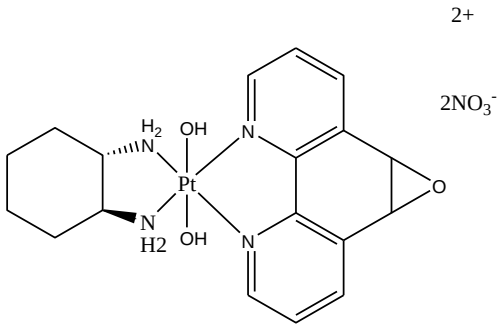
Zappa, C., & Mousa, S. A. (2016). Non-small cell lung cancer: Current treatment and future advances. *Translational Lung Cancer Research, 5*(3), 288-300. doi:10.21037/tlcr.2016.06.07

Appendix

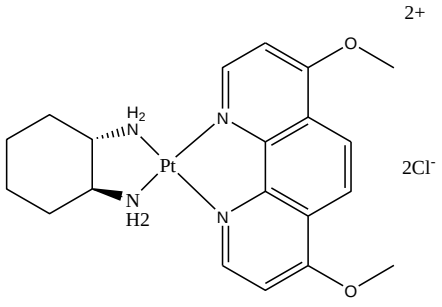
The following eighteen novel platinum-based complexes were synthesized in the lab of Professor Janice Aldrich-Wright (Western Sydney University) for a collaborative research project aiming to develop novel anticancer agents. The complex number, chemical formula and molecular weight of each complex are mentioned in the figure below.



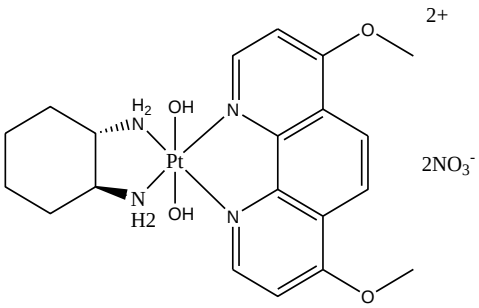
Complex 1
56OSS(II)
Molecular formula: $C_{18}H_{22}Cl_2N_4OPt$
Molecular weight: 576.38



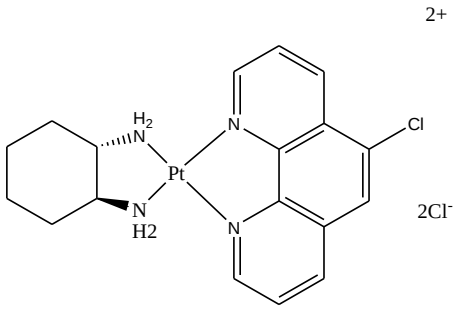
Complex 2
56OSS(IV)
Molecular formula: $C_{18}H_{24}N_6O_9Pt$
Molecular weight: 663.50



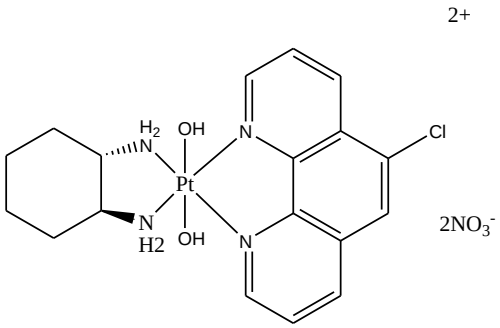
Complex 3 (Pt(II))
47OMESS(II)
Molecular formula: $C_{20}H_{26}Cl_2N_4O_2Pt$
Molecular weight: 620.44



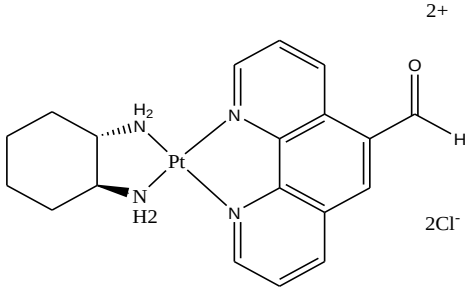
Complex 4 (Pt(IV))
47OMESS(IV)
Molecular formula: $C_{20}H_{28}N_6O_{10}Pt$
Molecular weight: 707.55



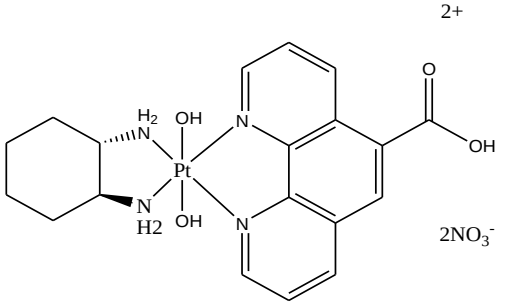
Complex 5
5CLSS(II)
Molecular formula: $C_{18}H_{21}Cl_3N_4Pt$
Molecular weight: 594.83



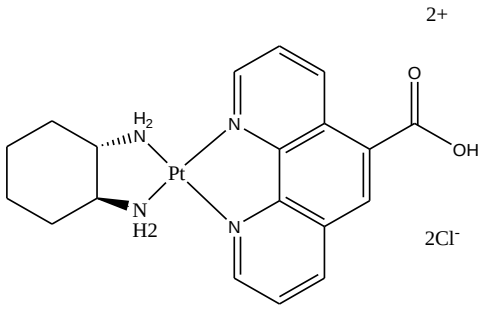
Complex 6
5CLSS(IV)
Molecular formula: $C_{18}H_{23}ClN_6O_8Pt$
Molecular weight: 681.95



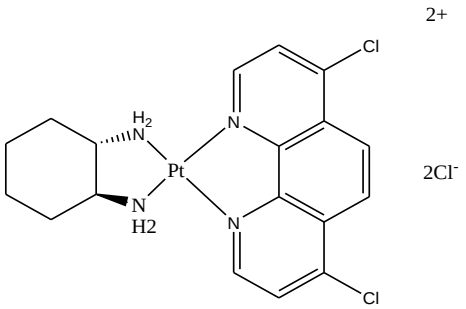
Complex 7
5CHOSS(II)
Molecular formula: $C_{19}H_{22}Cl_2N_4OPt$
Molecular weight: 588.40



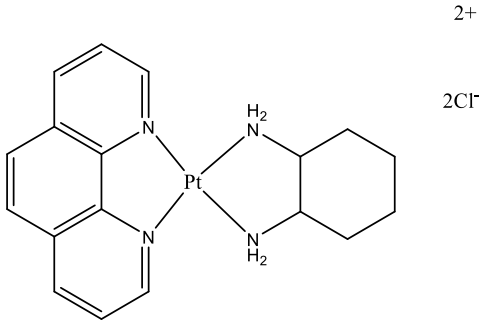
Complex 8
5COOHSS(IV)
Molecular formula: $C_{19}H_{24}N_6O_{10}Pt$
Molecular weight: 691.51



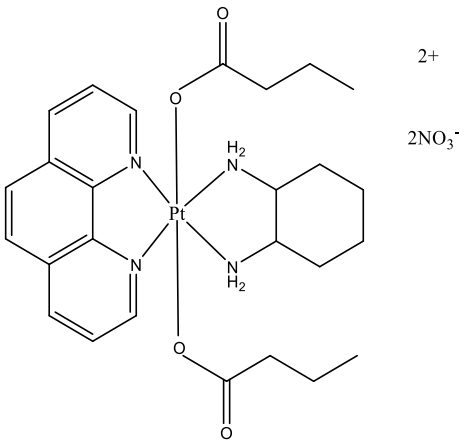
Complex 9
5COOHSS(II)
Molecular formula: $C_{19}H_{22}Cl_2N_4O_2Pt$
Molecular weight: 604.39



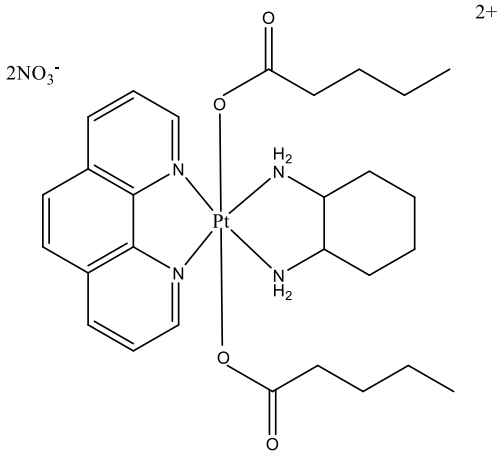
Complex 10
47CLSS(II)
Molecular formula: $C_{18}H_{20}Cl_4N_4Pt$
Molecular weight: 629.28



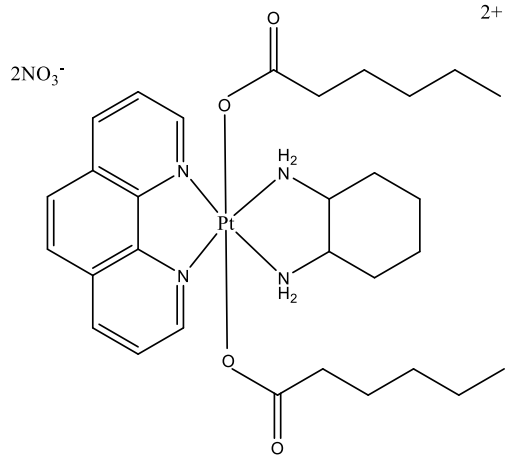
Complex 11/12
PHENSS/PHENRR
Molecular formula: $C_{18}H_{22}Cl_2N_4Pt$
Molecular weight: 560.39



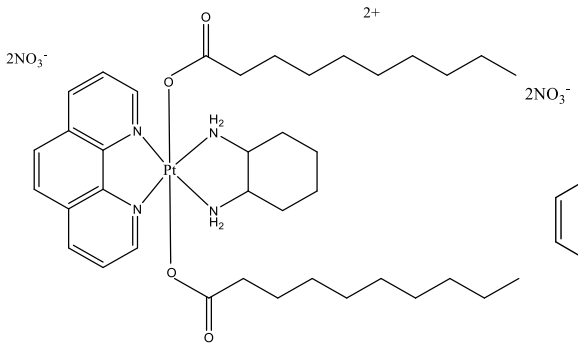
Complex 13
P-BUT
Molecular formula: $C_{26}H_{36}N_6O_{10}Pt$
Molecular weight: 787.69



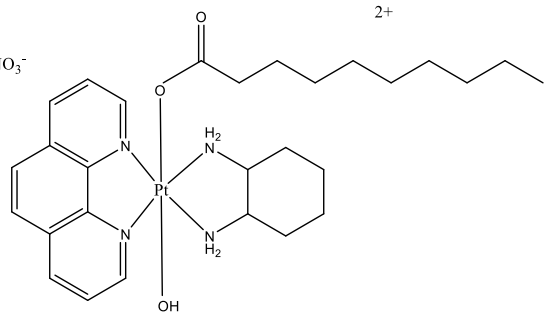
Complex 14
P-PENT
Molecular formula: $C_{26}H_{40}N_6O_{10}Pt$
Molecular weight: 815.75



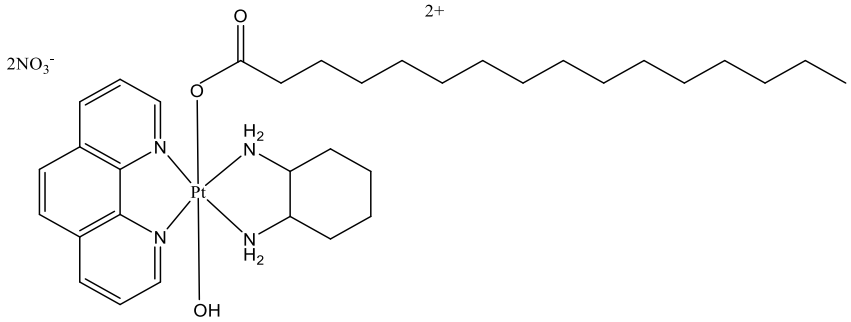
Complex 15
P-HEX
Molecular formula: $C_{30}H_{44}N_6O_{10}Pt$
Molecular weight: 843.80



Complex 16
P-Dec
Molecular formula: $C_{38}H_{60}N_6O_{10}Pt$
Molecular weight: 956.02



Complex 17
P-Dec (mono)
Molecular formula: $C_{28}H_{42}N_6O_9Pt$
Molecular weight: 801.76



Complex 18
P-HexaDec
Molecular formula: $C_{34}H_{54}N_6O_9Pt$
Molecular weight: 885.92

Figure 19: Structures, molecular formulas and molecular weights of the 18 tested platinum complexes. Eighteen novel platinum complexes were synthesized and tested for their cytotoxicity against several cancer cells lines.

Chemicals and reagents

Cell culture

DMEM, Dulbecco's Modified Eagle's Medium, with 4.5 g/L glucose / L-glutamine / sodium bicarbonate / sodium pyruvate, Dulbecco's Phosphate Buffered Saline, 1X, with MgCl2 and CaCl2 and Trypsin-EDTA, with phenol red, 1X were purchased from Sigma Aldrich, Missouri, USA. Penicillin-streptomycin solution for cell culture, 10K/10K stock, 10,000 U/mL Pen/Strep was purchased from Gibco, Germany and fetal bovine serum, heat inactivated, sterile-filtered, suitable for cell culture were purchased from Lonza, Germany.

Cell survival assay

CellTiter 96® Aqueous non-radioactive cell proliferation kit/ MTS-PMS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt-phenazine methosulfate] was purchased from Promega, USA

Western blot

The following chemicals were used in the preparation of the buffers used in the western blot experiment:

Triton X-100, sodium dodecyl sulphate (SDS), Glycine, Methanol, Polyoxyethylkene (20) sorbitan monolaurate (Tween 20), Bovine serum albumin (BSA) were purchased from Sigma Aldrich, Missouri, USA.

Ammonium persulfate (APS) and Tris Base were purchased from Fisher Scientific, USA.

Laemmli buffer and TEMED while Acrylamide/Bis solution was purchased from Bio-Rad, China.

NaCl was purchased from HiMedia, India.

The following buffers and solution were prepared:

- RIPA buffer: 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40 (NP-40) or 0.1% Triton X-100 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1 mM sodium orthovanadate.
- Resolving gel (10%) (for 2 gels): 8 ml type II water, 6,6 ml 30 % acrylamide, 5ml buffer pH=8.8, 100 µL 20 % SDS, 200 µL 10 % APS,10 µL TEMED.

- Stacking gel (10%) (for 2 gels): 4.2 ml type 2 water, 1.275 ml 30 % acrylamide, 1.875 ml buffer pH=6.8, 37.5 µL 10 % SDS, 75 µL 10 % APS, 7.5 µL TEMED
- Loading buffer (2x Laemmli buffer): 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl
- Buffer for separating gel (500 mL): 90.75 g Tris base, 500 mL distilled water, pH=6.8
- Buffer for stacking gel (200 mL): 12 g Tris base, 200 mL distilled water, pH=8.8
- Running buffer 5X (1L): 15g Tris Base, 72g glycine, 25 mL 20% SDS, pH=8.3
- Transfer buffer 5X (500 mL): 7.6g Tris Base, 37.5g glycine, pH=8.5
- Transfer buffer 1X (100 mL): 20 mL Transfer buffer 5X, 60 mL distilled water, 20 mL methanol
- TBS 10X: 24.2 g Tris Base, 80 g Glycine, pH=7.6
- Tris-buffered saline with Tween 20 (TBST) buffer: 40 mL TBS 10X, 360 mL distilled water, 0.1% Tween-20
- Blocking buffer: 5% bovine serum albumin (BSA) in TBST

The following antibodies were used: Anti-beta actin antibody: rabbit polyclonal, suitable for WB, species reactivity mouse, rat, human, Anti-caspase 3: rabbit monoclonal, Anti-bcl2: rabbit polyclonal, Anti-cytochrome c: rabbit monoclonal, Anti-caspase 9: rabbit monoclonal, Anti-caspase 8: rabbit monoclonal, Anti-bax: rabbit polyclonal and Anti-cleaved PARP-1: rabbit monoclonal were purchased from Abcam, Cambridge, USA. Goat Anti-Mouse secondary antibody, HRP Conjugate, and Goat anti-Rabbit secondary antibody, HRP Conjugate were purchased from Bio-Rad, Hercules, CA, USA.

Flow cytometry

Annexin V-FITC apoptosis staining/detection kit was purchased from Abcam, Cambridge, USA.

Comet assay

Comet assay Kit was purchased from Trevigen, Maryland, U.S.A.

The following chemicals were used in the preparation of the solutions used in the comet assay experiment: NaOH pellets and Methanol were purchased from Sigma Aldrich, Missouri, USA and acetic acid was purchased from BDH, England.

The following solutions were used:

- *Lysis Solution (For up to 10 slides):* 40 ml Lysis Solution (cat# 4250-050-01)
- Alkaline Unwinding Solution, pH>13 (200 mM NaOH, 1 mM EDTA): Per 50 ml of Alkaline Solution: 0.4 g NaOH Pellets, 250 µl 200 mM EDTA (cat # 4250-050-04), 49.75 ml dH_2O
- Alkaline Electrophoresis Solution, pH >13 (200 mM NaOH, 1 mM EDTA): 8 g NaOH pellets, 2 ml 500 mM EDTA, pH=8, add to 1-liter dH_2O (after NaOH is dissolved)
- Fixation solution (per sample): 10 µl 10X Fixation Additive (cat# 4254-200-05), 30 µl dH_2O, 50 µl methanol, 10 µl glacial acetic acid
- Staining solution (Per sample): 35 µl dH_2O, 5 µl 20X Staining Reagent #1 (cat# 4254-200-01), 5 µl 20X Staining Reagent #2 (cat# 4254-200-02) 5 µl 20X Staining Reagent #3 (cat# 4254-200-03). 50 µl 2X Staining Reagent #4.
- *Stop solution:* 5% acetic acid solution (100 µl per sample area is required)