UNIVERSIDADE D
COIMBRA

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New Pt and Pd-based Anticancer Agents Towards Colon Cancer

Dissertação no âmbito do Mestrado em Bioquímica orientada pela professora Doutora Maria Paula Matos Marques e coorientada pela Doutora Ana Lúcia Marques Batista de Carvalho e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Outubro de 2021

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Em memória do meu pai,

De quem a saudade não cabe no peito

.

Aknowledgments/ Agradecimentos

A realização deste trabalho só podia ter sido possível com o apoio que tive de várias pessoas que estiveram sempre por perto e atentas, mostrando-se sempre prestáveis e, que de certa forma, me ajudaram muito a concretizar os meus objetivos na tese.

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Abreviatures

cDDP	*cis*-diaminodichloro platinum(II) **(**cisplatin)
Pd_2Spm	Spermine di-Paladium(II)
CRC	Colorrectal Cancer
CC	Colon Cancer
FDA	Food and Drug Administration
5-FU	5-Flourouraril
OXA	(trans-R,R-cyclohexane-(1,2-diamine)oxalatoplatinum(II)), (Oxaliplatina)
TS	Thymidylate Synthase
CPT-11	Irinotecan
GI	Gastrointestinal
TP	Thymidine Phosphorylase
MDR	Multidrug Resistance
ATP	Adenosine Triphosphate
SARs	Structure-Activity Relationships
FTIR	Fourier-Transform Infrared Spectroscopy
SNR	Signal-Noise Ratio
PBS	Phosphate-Buffered Saline
ENEM	Eagle's Minimal Essential Medium
DMSO	Dimethyl Sulfoxide
FBS	Fetal Bovine Serum
SRB	Sulforhodamine B
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
EDTA	Ethylenediaminetetraacetic Acid
IC_{50}	Half Maximal Inhibitory Concentration
PCA	Principal Component Analysis
PC	Principal Component
Pen/strep	Penicillin-Streptomycin
QFM-UC	Unidade de I&D Química-Física Molecular
Spm	Spermine
Tyr	Tyrosine
vs	versus
PA	Poliamine
Phe	Phenylalanine

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Resumo

Apesar das abordagens inovadoras que têm sido implementadas em quimioterapia, ainda existe uma falha no que diz respeito à redução dos efeitos tóxicos causados por este tipo de fármacos, bem como a falta de eficácia da maioria. Isso explica a motivação que a comunidade científica tem em desenvolver, cada vez mais, novos tipos de fármacos com um espectro de ação maior, para que seja possível obter um resultado citotóxico melhor e com menos efeitos colaterais negativos muito vezes presente nas outras terapias existentes.

Para a realização deste trabalho, foi observada a ação quimioterapêutica de compostos que têm metal na sua constituição e a sua análise foi feita através da exposição induzida numa linha celular de adenocarcinoma de cólon (Caco-2). Estes modelos de linhas celulares de cancro são importantes para as etapas pré-clínicas dos estudos, uma vez que podem ajudar a compreender os mecanismos e as funções que são influenciadas, quando um composto anti-tumoral é adicionado.

A cisplatina e o Pd_2Spm foram os dois compostos usados e observados por métodos biológicos, através dos ensaios de MTT e SRB, onde a administração de ambos os fármacos induziu uma resposta não proliferativa, terminando com a apoptose celular que é essencial para ajudar a diferenciar as células que ficaram viáveis das não viáveis ou as células ainda com ou sem conteúdo proteico, após o tratamento.

Foi a partir da descoberta do efeito citotóxico e potencialmente quimioterapêutico da cisplatina na década de 1960, que surgiu o interesse em desenvolver novos complexos análogos do Pd(II), com potencial para gerar um mecanismo de ação semelhante ao da cDDP, mas com menos mecanismos de resistência responsáveis pelo negativo impacto nefrótico.

Além de métodos biológicos, técnicas de espectroscopia vibracional, como o FTIR e o Raman, foram realizadas para se puder obter uma caracterização molecular do que é afetado com a adição do fármaco e, com base nas contribuições geradas pelo Pd_2Spm nas duas concentrações estudadas (4 e 8 μM) foi verificado um efeito farmacológico sobre o DNA, em proteínas e também em lipídios, onde um efeito citotóxico ficou evidenciado como era previsto já de alguns estudos anteriores, uma vez sintetizado com base no seu composto ‘lead’, a cisplatina.

Os resultados da análise de componentes principais (PCA) mostraram-se também significativos no que toca às alterações presentes nas características moleculares em ambas as concentrações. Esses resultados foram importantes para confirmar a resposta intracelular ao Pd_2Spm, o que acaba por encorajar a realização de novos estudos a este tipo de análogos de Pd(II) para terapias contra o cancro.

Palavras-Chave: Cancro do Cólon; cisplatina; Paládio; antiproliferativo; contribuições; ADN; Caco-2

Abstract

Despite the innovate approaches on cancers, there is still a lacune when it comes to the lower toxicity effects caused by these types of drugs and so the lack of effectiveness. This explains why the scientific community aims so much to develop each more, new types of drugs with an increased spectrum of action, so it can lead to a better cytotoxic effect with less negative side effects very much seen in the others therapies already used.

For this work, the chemotherapeutic action of metal-based compounds was studied by analyzing their inducted exposure on a cell line from the colon adenocarcinoma (Caco-2). Cancer model systems are important for pre-clinical stages of studies once they can help understand the mechanisms and functions influenced, when an antitumoral compound is added.

Cisplatin and Pd_2Spm were the two complexes used and observed using biological methods, MTT and SRB assays, where the administration of both was observed to induce a non-proliferative response, ending with the cell apoptosis which helps differentiating the viable from nonviable cells or the cells with protein content and with not, after the treatment.

It was the discovery of the cisplatin's cytotoxic and its potential chemotherapeutic effect back to 1960s, that brought out the interest to develop these new Pd(II) analogue chelates, that can potentiate a similar mechanism of action but with less resistance mechanism that could turn into nephrotic effects.

Besides biological methods, vibrational spectroscopy techniques, such as FTIR and Raman were performed to give us a characterization of what is affected once the therapy was induced and based on the concentrations of Pd_2Spm used (4 and 8 μM) it was verified a pharmacological effect on DNA, on proteins and also in lipids which evidenced the cytotoxic effect predicted on previous literatures, based also on its 'lead' compound, cisplatin.

Results from the principal component analysis (PCA) also showed significative results about the alterations on the molecular features with both concentrations. Those results were important to confirm the intracellular response to Pd_2Spm which helps to encourage further studies to this type of Pd(II) chelates for cancer therapies.

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Keywords: Colon Cancer; cisplatin; Palladium; antiproliferative; contributions; Caco-2

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1. Introduction

1.1. Cancer: A Worldwide Problem

Cancer is one of the leading causes of death worldwide and according to the WHO (World Health Organization), in 2018 there were 18.1 million cases (Figure 1), lung and breast cancers being the highest incidence ones (11.6%), followed by colorectal (10.2%) and prostate cancers (7.1%).

The main factors involved on the cancer appearance are generic which can easy lead to the development of the disease. Genetic predisposition can result from inherited mutations in genes that are directly involved in cell growth and proliferation [1].

Despite the continuous acknowledgment about the disease, the advances in technologies and the development in areas such as biology cellular and molecular or genetics there is no great treatment for tumors, leading to a frustrating search of the effective one.

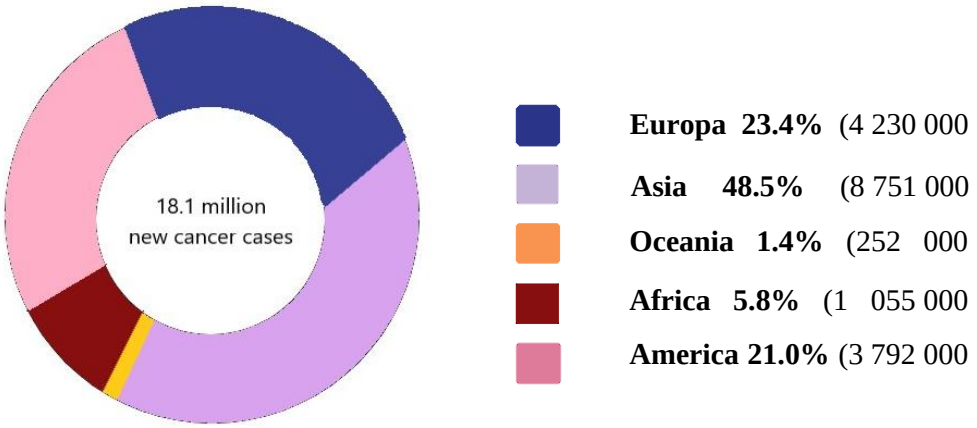

Figure 1: Global cancer incidence trough the different continents. (Adapted from Globocan website).

Cancer is defined as a multifactorial disease with a fast and aggressive behavior in which an initial neoplastic transformation quickly transforms into an uncontrolled proliferation of abnormal cells, spreading and infiltrating to various parts of the body, and finally destroying healthy body tissues [2]. In fact, there are very possible causes to trigger the proliferation of neoplastic cells and they can be present daily.

The proliferation process is mainly originated by damage or mutation of proto-oncogenes that code for proteins which are involved in the differentiation pathways or by tumor suppressor genes essential to lead to cell growth and/or stimulate apoptosis.

Those alterations will eventually drive cells to develop an tumor, once favored by mutations in the tumor susceptibility genes, accompanied by DNA damage [3].

As the world develops, lifestyle, diet or environment changes can have a negative impact on the population. Besides those examples, the lack of activity, exposure to radiation (essentially UV), tobacco smoke, virous or infections can also have a negative impact on the human organism. A healthy lifestyle, a balanced diet and avoiding exposure to toxic agents can be ideal ways to prevent the appearance of tumors. Some genetic changes can also be in the source of some cancers, especially when an historical of the disease is observed among a family generation.

Surgery and radiotherapy are the most effective and currently, the first recommended treatments for local and non-metastatic cancers but turn out inefficient when in an advanced stage of the cancer. Surgery is the process of removing cancer by doing operation and it is generally used only when cancer is localized. Radiation therapy uses high doses of radiation to shrink or kill cancer cells [4].

According to National Cancer Institute, there are other conventional techniques used in the treatment of cancer including immunotherapy with monoclonal antibodies, **i**mmune system modulators or vaccines; radiotherapy, target therapy (with monoclonal antibodies, small molecule drugs, angiogenesis inhibitors, who work as designed proteins that attach to specific targets found on cancer cells) and gene therapy (where to the cells are added one or more genes, that working as cytokine genes, produce pro-inflammatory immune stimulating molecules or highly antigenic protein gene [5].

Cancer drugs for treatments, such as chemotherapy, hormone and biological therapies, are prescribed when the diagnosis shows a metastatic cancer, since they are able to reach every organ in the body via the bloodstream [3,6]. Chemotherapeutic drugs are based on toxic compounds that aims to inhibit the fast proliferation of the cancer cells but, unfortunately, they also have some side effects and low specificity, inhibiting also the rapid growth needed for the maintenance of hair follicles, bone marrow and gastrointestinal tract cells.

The first anticancer drugs were approved in the 40s and 50s, by the Food and Drug Administration (FDA) for the treatment of hematological cancers and solid tumors and since that, the search and develop for new and more efficient chemotherapeutic drugs have evolved. The main progresses in the cancer treatment were in the combination of drugs as well as the practice of adjuvant chemotherapies with approval anticancer drugs, such as cisplatin and paclitaxel. However, is due to the toxicity still present on the treatments and the indiscriminate destruction of cells, leading to the undesirable side effects, that the scientific community aims to study and look for new

and better ways to approach cancer with high efficacy. This opens the door to the finding and testing the potential of target therapy.

The idea of targeting the delivery of chemotherapy agents specifically directed to cancer cells and also minimizing the death of normal cells, so consequently, avoiding undesirable side effects, seemed a great new perspective to explore [3].

It is a fact that scientific advances are being made during the last decades, but cancer still remains a challenging disease with many constraints, but fortunately, nowadays, there are already some highly treatable cancers, such as the breast, skin (nonmelanomas), Hodgkin lymphoma, testicular, thyroid, prostate and cervical and most of the childhood malignancies (both solid and hematolymphoid).

1.2. Colon Cancer: Etiology, Risk Factors and Therapeutics

Colon cancer (CC) and rectal cancer (RC) are synonymously called colorectal cancer (CRC). Colorectal cancer (CRC) is the third most incident cancer around the world and consequently, one of the most observed and studied following lung and breast cancer. Data from Globocan in 2020, concluded that there were 1 148 515 of new cases of colon cancer worldwide, in which 576 858 resulted in death, being the fifth most deadly cancer. Rectum cancer had also 732 210 new cases in the same year, ending with 339 022 deaths, being in the 10th place on the List of the cancer cases.

Data from the Laboratory of Demography at the University of Evora in 2015, showed that there was an incidence of CRC of 10.3% for women and 9.5% for men. In Portugal, it is estimated about 10 270 cases of CRC per year, mainly in the population over 70 years, featuring 17.6% of all cancers. Lifestyle habits such as, alcohol intake also increases the probable risk factor for colorectal polyps, as well as, dietary factors. Red meat consumption or fat intake were proven to be indeed, responsible to the aggravation of the prognostic [7]. Also, based on observational epidemiological evidence, a reduced risk is associated with regular physical activity with an estimative between 25 and 30%, when comparing the most active to least active participants in these studies [8]. Besides lifestyle and diet, genetic and familial history also play an important role in colon cancer appearance. The family history of colorectal cancer (CRC) has proven to be also a high-risk factor, mostly observed in younger patients, in a way that their historical, once verified, increases the risk of the disease (data already observed by some studies [7].

According to various medicine-related websites, patients with CRC can experience some symptoms that will depend on the size and location of the tumor, in the large intestine. It can be observed a sudden weight loss or narrow without an

explanation, ribbon-like stools, rectal bleeding or anemia caused by iron deficiency and then, among the time and as cancer grows, symptoms can develop to constipation, diarrheas, more rectal bleeding and others more serious effects to a point where cancer is already installed and highly spread, affecting the entire body. Endoscopy is a method that includes sigmoidoscopy and colonoscopy, examinations that allow to localize the tumor and take part of the large intestine for histological examination or further studies. Endoscopy is indeed a less invasive method which analyses large intestine, from the rectum to the sigmoid colon and usually large intestine, from the rectum to the sigmoid colonoscopy, which is a more invasive procedure, aiming to analyze all colorectal system, meaning large intestine, rectum and may include ileum (end of small intestine).

CRC incidence can be different in both genders due to numerous reasons (Figure 2). The fact that male individuals are more affected than females, mostly on rectal tumors, can be correlated with the different biological, molecular and clinical characteristics on both. Those particularities will differ on how the organism of each sex works, once formed from different anatomical locations.

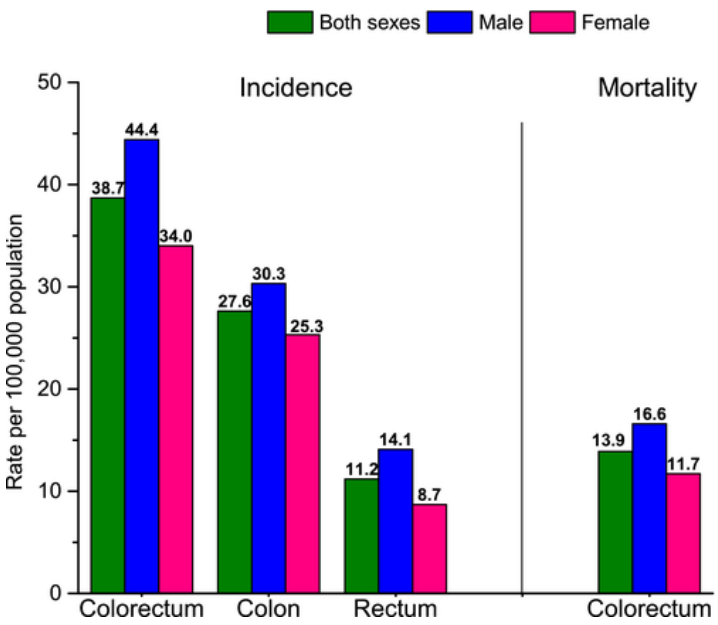

Figure 2: Colorectal Cancer Incidence (2012 to 2016) and Mortality (2013 to 2017) based on gender in United States. Rates are age adjusted to the 2000 US standard population. Incidence: North American Association of Central Cancer Registries (NAACCR), 2019; Mortality: National Center for Health Statistics (NCHS), 2019. From [9].

Age is a natural factor that is related with a higher incidence, mainly from 50 years old, also has the age recommended to do a CRC screening, which explains the lower bands in the following years, specially between 51 and 55 (Figure 3).

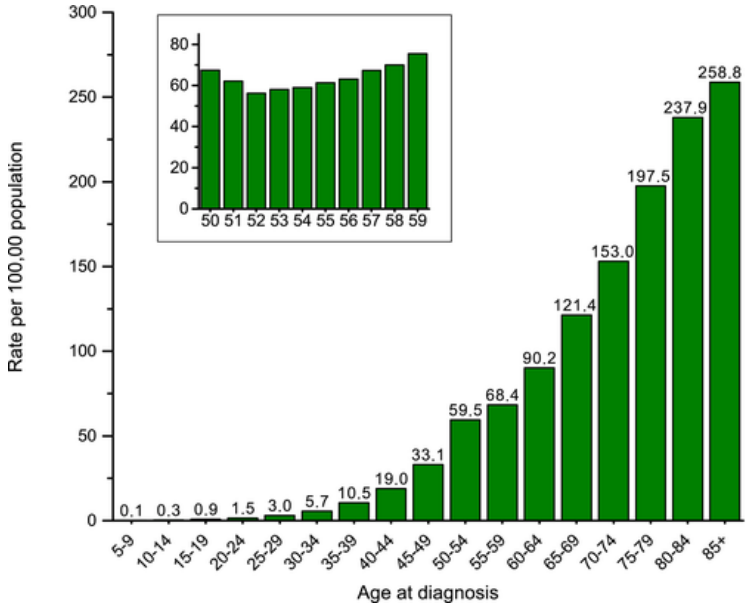

Figure 3: Incidence of CRC related with the age of diagnosis. in United States, between 2012 and 2016. Rates are age adjusted to the 2000 US standard population. Source: Main figure, NAACCR, 2019; Inset: Surveillance, Epidemiology, and End Results Program (SEER), 2019. From [9].

Disparity can also be found between races or ethnics (Figure 4) and the differences come from the low access to medical cares, lack of clinical resources such as, diagnosis and the correspondent as well as your follow-up. These circumstances are specially from countries with a poor developed socioeconomical situation. Also, regions with a higher tendency for an exposure to risk factors adds the tendency to a major incidence to the cancer.

NHB (Non-Hispanic Blacks) are the group with the highest incidence of CRC, essentially due the lack of clinical resources to follow-up these individuals and perform a regular diagnostic screening, coupled to a poor education regarding the risk factors. Individuals from ANs (Alaska Natives) also experience a high incidence of CRC which may be assigned to their diet, very rich in animal fat and poor in fruits/vegetables.

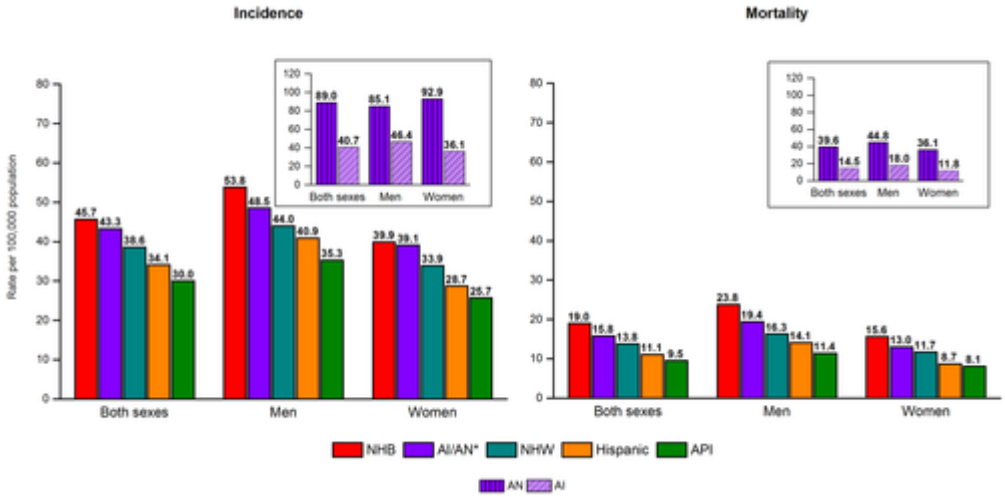

Figure 4: Colorectal Cancer Incidence (2012 to 2016) and Mortality (2013 to 2017). Rates by Race/Ethnicity and Sex, United States. Rates are age adjusted to the 2000 US standard population. *Statistics are based on data from Purchased/Referred Care Delivery [1]. (NHB (Non-Hispanic Blacks); AI (American Indian); AN (Alaska Native); NHW (Non- Hispanic Whites); Hispanic; API (Asia Americans/Pacifics Islanders)). From North American Association of Central Cancer Registries, 2019; Mortality: National Center for Health Statistics, 2019. From [9]

Fortunately, knowledge on CRC has increased, as well as the available approaches to fight it. A major education to the risk factors and diagnostic screening tests also enhances the patients´ prognosis, . All these parameters explain why CRC incidence on both sexes has become lower over the years (Figure 5).

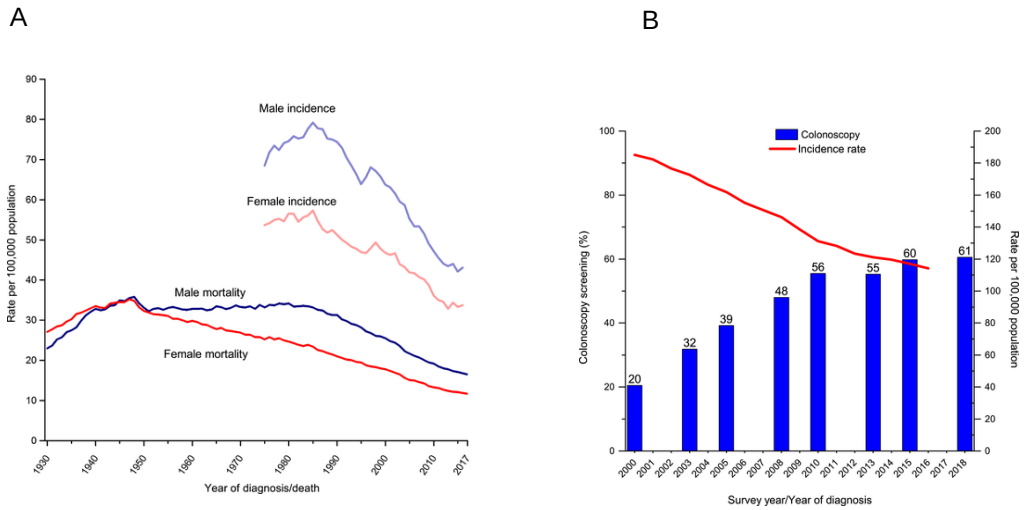

Figure 5: (A) Trends in Colorectal Cancer Incidence starting from 1975 to 2013) and Mortality between 1930 and 2014. Rates by Sex, United States. Incidence rates exclude the appendix and are age adjusted to the 2000 US standard population. Source: Incidence: SEER Program, 2019; Mortality: NCHS, 2019; (B) Trends in Colonoscopy Prevalence (2000 to 2018) and Colorectal Cancer Incidence Rates (2000 to 2016), Adults ≥50 Years, United States. Prevalence was based on colonoscopy within the past 10 years of the survey date. Incidence rates exclude the appendix and are age adjusted to the 2000 US standard population and adjusted for reporting delays. Source: Colonoscopy prevalence: National Health Interview Survey, 2019; Incidence: NAACCR, 2019. From [9].

1.2.1. Chemotherapeutic Drugs Against CRC

Based on the stage and behavior of the cancer, chemotherapy is usually included into the treatment regimen, in combination with surgery or radiation or even both. The type of tumor will determine the approach executed as well as, the drug dose required will depend on its nature and the condition of the patient, which can be comprise as a single drug or be used in combination with others via intraperitoneal, intravenous and oral administration [7].

Following surgery, patients can experience a complete adjuvant chemotherapy. [6] Currently, four chemotherapeutic drugs can be used as a first-line therapy in advanced CRC with metastasis, being Oxaliplatin, Fluoropyrimidines (5-Fluorouracil and Capecitabine) and Leucovorin, each one with potential to work against tumoral proliferation [7].

5-fluorouracil (5-FU) is a based neoadjuvant chemoradiation, which has become the preferred approach to the treatment of patients with stage II/III rectal cancer, due to its capacity to inhibit thymidylate synthase by impairing DNA synthesis in the S phase, preventing proliferation and repair process to take place[6]. Thanks to its metabolites and being an uracil analogue, 5-FU can be incorporated into RNA and DNA and interfere with the normal protein production necessary for cell growth, by an competitive inhibition of Thymidine synthase (TS) [10]. However, despite its many advantages, clinical applications have been greatly limited once drug resistance was observed [10].

Combined treatments for advanced CRC using 5-FU started to become one possible way to overcome the treatment tolerance, such as with leucovorin (5FU/FA). Leucovorin (FA) is a 5-formyl derivative of folic acid that have gain interest from the scientific community, to be use as a palliative treatment of colon cancer, once some studies observed its potential to prolong survival in patients, when used in combination with 5-fluorouracil. This potential comes from the fact FA potentiates the cytotoxic effects of 5-FU, once it is responsible for the inhibition of the fluoronucleotide biosynthesis by competing with the natural substrate of thymidylate synthase (TS) [1112]. Once capable to enhance the therapeutic effect of 5-FU when combined, the drug was approved by Food and Drugs Administration (FAD) in 2002. However, once 5-FU has its unique drug resistance, which is closely related to the expression of thymidylate synthase (TS), patients with high TS expression (primary target of 5-FU) display a worse overall survival (OS) than patients with lower TS expression in tumor tissue. In fact, nearly half of mCRC patients are resistant to 5-FU-based chemotherapies, so as

expected, the same tolerance can occur with5FU/FA treatment as well [11]. Later, other types of combinations were also performed with 5FU/FA, being oxaliplatin and irinotecan (FOLFOX and FOLFIRI, respectively), two of them.

Oxaliplatin (trans-R,R-cyclohexane-(1,2-diamine)oxalatoplatinum(II)), is an alkylating agent also approved for the treatment of CRC that can form platinated intra-strand cross-links between two adjacent guanine bases or two adjacent guanine–adenine bases. The principle adducts formed are 1;2-GP intra-strand and 1;2-GA inter-strand, which are responsible for the DNA lesions capable to damage DNA, conceding the anti-cancerigeneous activity of OXA. Also, the difference between oxaliplatin and others platinum-based chemotherapeutic drugs is that, oxaliplatin possesses a 1,2-diaminocyclohexane ligand (DACH) (Figure 6), which can lead to a more difficult process to repair the DNA, hereby improving its tumor cell killing potential, where replication and repair processes of DNA are inhibited as well as RNA synthesis. OXA can be used in treatment with Leucovorin and 5-FU, being the combination of these drugs referred to as FOLFOX and currently, it displays one of the first-line chemotherapies combination used in CRC. In fact, the viability of the treatment was already verified in some studies in the beginning of the 2000s, in which patients treated [11]. The improvement in efficacy coupled with a favorable toxicity profile, turned this combination one of the most approached on CRC [13]. However, drug resistance and tolerance to the drugs can also be observed in this group of drugs. As a heterogeneous disease, FOLFOX resistance can come from various heterogeneous subgroups that will affect each drug separately, creating individual resistance mechanism but concluding in the treatment failure. This can explain why combining cytotoxic effects of various drugs can in fact increase the effectiveness of the combination, but at the same time have more than one resistance factor observed [14].

Irinotecan (CPT-11), as well as OXA, was found to have activity against advanced colorectal cancer. This chemotherapeutical drug was approved by the FDA for CRC treatment in 1996 when it was verified by several studies the potential to do a selectively inhibition of topoisomerase I (Topo I). Topo I, is involved in the cleavage and reannealing of single-strand DNA during replication and transcription, so by inhibiting that, it can lead to an accumulation of damage to the DNA and consequently, an eventual uncontrolled proliferation of cancerigeneous cells [1415]. This concludes that, the higher the concentration of Topo I, the more sensitive the cells become to irinotecan. Also, Irinotecan or its active metabolite SN-38, can form a topoisomerase-inhibitor-DNA complex, which can affect the DNA function.

The addition of CPT-11 to the standard regime of 5FU/FA treatment created a new combination of drugs named FOLFIRI and around 2000, it became a second-line treatment of patients with mCRC, once it was verified to increase the effectiveness of treatment, where a higher survival benefit was observed unlike to patients who received 5FU/leucovorin alone [16].

In various studies, advantages of the addition to LV5FU2 of irinotecan (FOLFIRI) and oxaliplatin (FOLFOX) were concluded, when those regimes showed better results than performing LV5FU2 alone [14]. In fact, both treatments have been verified to cause similar results but with different toxicity profiles, being the FOLFIRI therapy the most toxic, causing side effects such as, gastrointestinal effects and alopecia [17]. Despite the patients with multiple comorbidities do experience a higher likelihood when receiving FOLFIRI over FOLFOX treatments, the lack of survival benefit still exists as well as some toxic effects, once the presence of more than one drug. In fact, FOLFOX is more likely to cause neuropathy and FOLFIRI is more likely to cause GI toxicities [14].

Lastly, Capecitabine was another chemotherapy drug approved for CRC treatment, by FDA in 2005. As an alternative to intravenous 5-FU, being prescribe as an oral drug. The advantages of its approach rely on its mechanism of action that starts with its metabolization process. Capecitabine goes through several steps in the organism, being firstly converted to 5'-deoxy-5-fluorocytidine (5'-OFCR) and 5'-deoxy-5-fluorouridine (5'-DFUR). 5'-DFUR eventually suffers hydrolyzation by TP (Thymidine phosphorylase) and converts into 5-FU, which will determine its cytotoxic effect. As expected, the resistance mechanisms involved in 5-FU resistance will be shared by Capecitabine and explains why TP plays an important role in the resistance of this drug. In contrast to 5-FU mechanism, patients with higher expression levels of TP will have better responses to capecitabine and consequently, loss of its functions will cause resistance mechanisms [15]. The cytotoxic action of capecitabine showed up to be equivalent to 5-FU in terms of response rate and various trials, concluded that its use was indeed associated with an improved response rate [16]. However, toxic effects of Capecitabine still compromises tests and ends up to their discontinuation [16].

Resistance to anticancer drugs is an obstacle facing the development of new antitumor drugs, when enhanced tolerance started to be observed with the performance of some therapies. During the progression of cancer and specially Colon cancer, several parameters can lead to a decreased efficacy of the treatment and consequently, to the aggravation of the tumor once cancer cells gain resistance to multiple chemotherapeutic drugs with different structures and mechanisms [18].

Cancer cells often develop some multi-resistance (MDR) to chemotherapeutic drugs which leads to a low disponibility whereas tissues are affected and a decreased action from them, resisting pro-apoptotic signaling, which will lead to an invulnerability to conventional treatment [8]. Drug efflux can be observed in these types of situations and might be due to the over-expression of ABC transporters, such as P-gp, which when co-expressed in tumors, are responsible for increasing the efflux of the drugs and consequently, to cause a decrease of the drug concentration on the target tissues/organs [19].

This happens because the glycoprotein (P-gp) is encoded by the MDR-1 gene, that is a member of the large ATP-binding cassette protein superfamily. After binding to P-gp, liposoluble drugs are constantly pumped outside of the cell because of a process powered by ATP hydrolysis, inducing a continuous decline of the intracellular drug levels. This will lead that to a decreased drug toxicity on cancer cells and its efficacy as well, to a point where drug resistance in cancer cells will be generated [20]. This low bioavailability encourages the efforts to perform combination therapy using more than one medication at the same time, in a way it becomes possible to overcome MDR.

1.3. PLatinum Compounds: A new approach for CRC?

The important role of Pt-based complexes, when it comes to approach tumors, began with the discovery of cisplatin {cis- [PtCl2(NH3)2]} in the 1960s, which led to a subsequent clinical generated interest in the use of metal compounds in cancer treatments [21]. The interest of cisplatin (cDDP), commercialized as Platinol, came from the fact being an inorganic complex with a Pt(II) metal center ion covalently linked to two chloride ions (known as leaving groups) and two ammonium groups in a *cis* position. As a neutral molecule with a square planar structure (figure 6), cDDP also has a moderate solubility in water. Currently, this compound can be administered to the patients through intravenous route (the most common) and once it is neutral by nature, it can easily diffuse into cells by passive diffusion. The interest to cisplatin enhanced when it was verified that the cytotoxicity mediated by the drugs was directly proportional to the amount of cisplatin bound to DNA. In fact, the bound between both was observed to be the one responsible for the damage on DNA, which explains apoptosis processes and other type of deregulations such as, necrosis, which end up bringing a new perspective of use to this chemotherapeutic drug [17].

Currently, cisplatin is responsible for the cure of over 90% of testicular cancer cases and its cytotoxic effect plays a vital role in the treatment of cancers such as ovarian, head and neck cancer, bladder cancer, cervical cancer, as well as melanoma and lymphomas [18].

Besides the great efficacy demonstrated by cisplatin, its severe deleterious side effects (e.g., nephrotoxicity, myelosuppression) and acquired resistance have prompted the search for second- and third-generation Pt(II) agents, aiming to an improved therapeutic effectiveness and safety broader spectrum of activity, to avoid serious collateral effects coupled to a higher cell growth-inhibiting activity [22]. Thus, from the thousands of mononuclear analogues that were synthesized as potential anticancer agents, only two entered clinical trials and were approved for clinical use – carboplatin and oxaliplatin [23] (Figure 6).

Carboplatin (cis-diammine(cyclobutane-1,1-dicarboxylate-O,O')platinum(II)) (commercialized as Paraplatin) was developed to face the toxic and severe side effects from cisplatin, by changing the kinetics of activation. This translates to a slower hydrolysis of the leaving groups (which are different from the cisplatin (Figure 6)). This led to a lower toxicity without affecting the efficacy of the drug, and to a broader chemotherapeutic effect.

Oxaliplatin (commercialized as Eloxatin) was designed to decrease resistance mechanisms, by changing the platinum-based structure (Figure 6), The presence of the ring linked to two NH_2 groups leads to steric hindrance relative to parallel reactions during the pharmacokinetic phase (*e.g.* with glutathione) thus enhancing drug bioavailability at the target tissue/organ. . With a decreased resistance, the drug dosage can also be lower (*i.e.* decreased side effects) and still effective. Oxaliplatin is effective towards several types of cancer, including those resistant to cisplatin.

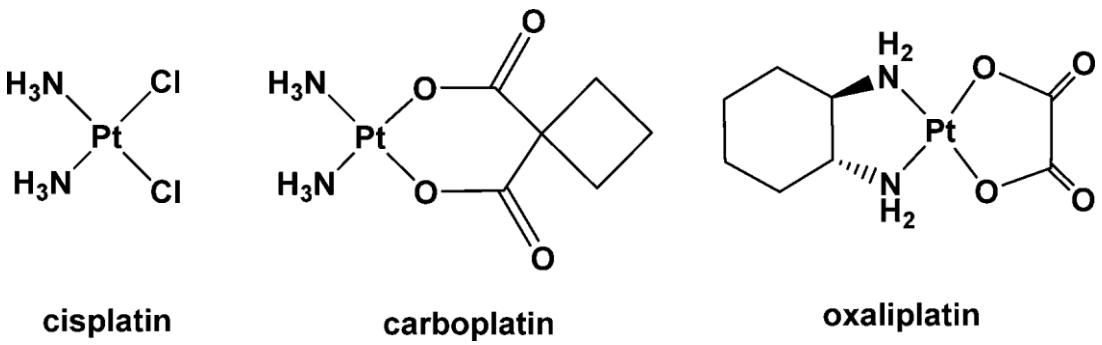

Figure 6: Structure of the three Pt(II)-based drugs approved for clinical use in cancer treatment. From [6].

1.3.1.Platinum and Palladium Complexes with Polyamine Ligands

Apart from Pt(II)-based agents, Pd(II) compounds have been shown to display anticancer properties, through DNA damage, inducing cellular apoptosis [2021]. However, besides these mononuclear metal-complexes, polynuclear chelates have also been the object of intense research since the 90s. The presence of more than one metal center in these complexes allows them to establish non-conventional interactions with DNA, not accessible to monomeric complexes such as cisplatin, that render them particularly effective as anticancer agents. Thanks to their high biocompatibility and presence of several nitrogen donor atoms, linear polyamines (*e.g.* biogenic polyamines such as spermine) emerged as possible ligands for this new multinuclear metal chelates. Their aliphatic carbon chain confers flexibility and a certain degree of lipophilicity to the metal complexes, which can favor their transport across cell membranes in their way to the pharmacological target [22].

Examples of cisplatin-like polynuclear complex with a polyamine as linker are Pt_2Spm and Pd_2Spm (Spm=spermine, $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$)) (Figure 7 (A)). One other advantage of these polynuclear complexes as antineoplastic drugs is that one of metal centers may bind to DNA while the other is free to interact with other biomolecules (*e.g.,* a protein). Also, while cisplatin preferentially yields short-range intrastrand adducts with DNA (mainly *via* adenine and guanine), these new generation polynuclear complexes allow multiple interactions with DNA through long-range interstrand adducts (Figure 8). These severe and less reparable perturbations of the native DNA conformation lead to an enhancement of the antineoplastic effect of these Pt/Pd-based compounds in comparison with the currently used mononuclear drugs [22].

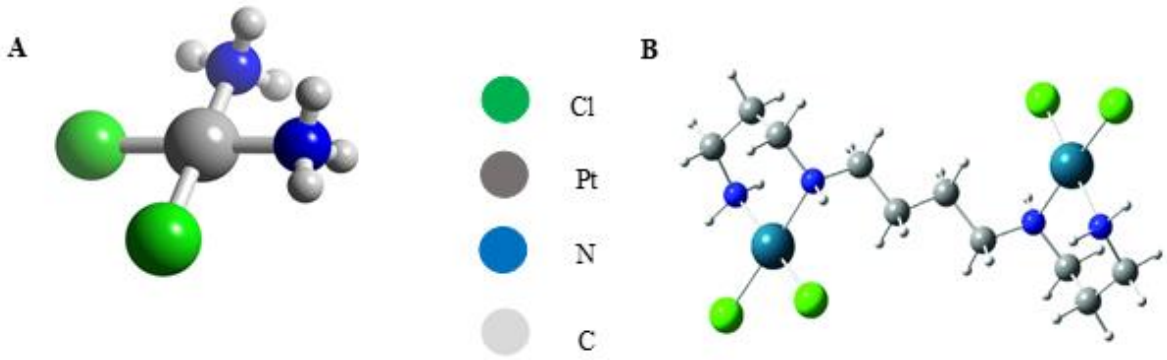

Figure 7: Structure of Pd_2Spm (A) and cisplatin (B) where the main differences are the presence of 2 Pt ions, the presence of cisplatin-like portions and the amino points, where the different carbonated

strands can determine lipophilicity and flexibility, as well as the length of the strand gives the distance to the metal center. Adapted from [22].

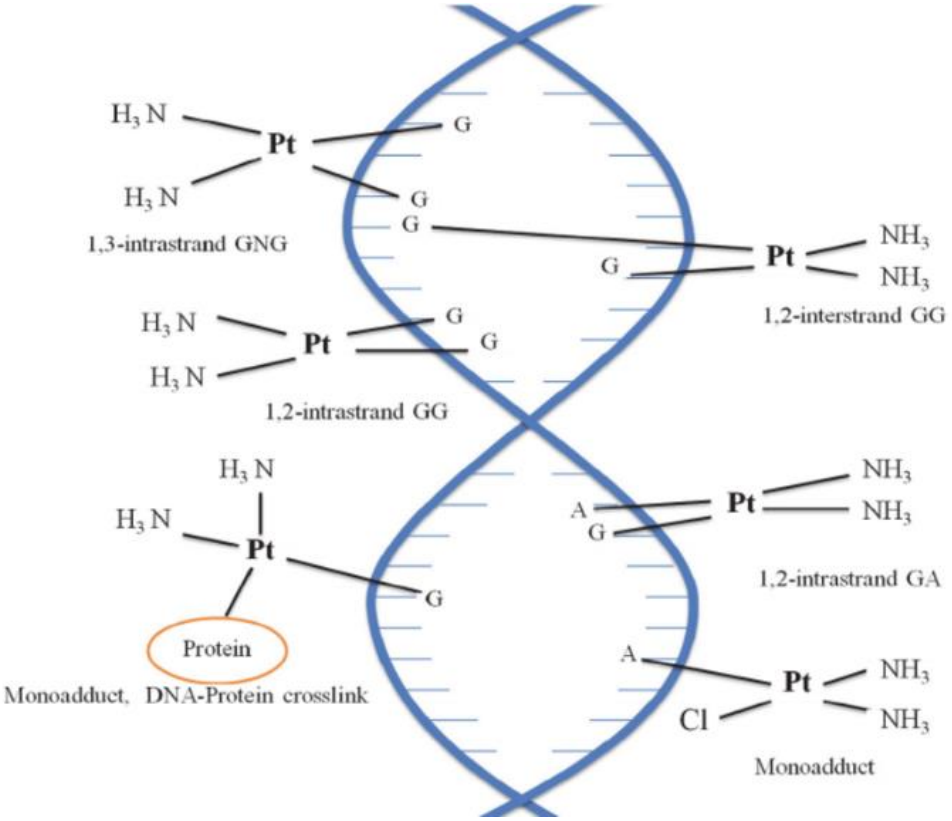

Figure 8: Schematic representation of the possible intra- and interstrand crosslinks between cisplatin and DNA. From [24].

Focusing on the cytotoxicity mechanisms from metal-based compounds in order to enhance antitumor capacity, while decreasing toxic side effects and acquired resistance, many unconventional Pt(II) and Pt(IV) compounds have been tested, mainly based on pre-established Structure-Activity Relationships (SARs), as well as complexes with other metals such as ruthenium (Ru(II) and Ru(III)) titanium (Ti(IV)) and gold (Au(I) and Au(III Many researches have been developed at the I&D Unity of "Quimica-Física Molecular" from Universidade de Coimbra with synthesized Pt(II) and Pd(II)-polyamine complexes with promising anticancer properties , such as Pt_2SpmCl_4 and Pd_2SpmCl_4,. To study the chemotherapeutic against human metastatic breast cancer, those developed complexes: Pd_2SpmCl_2 (or Pd_2Spm) were in fact, found to display an important cytotoxicity role towards a cDDP-resistant cell line [25]. Also, techniques such as, Raman microspectroscopy and synchrotron-radiation infrared microspectroscopy (SR-IRMS) were later applied to probe the effects of their interaction with the cellular components.

Effects of NSpd, Pd-NSpd or PtNSpd treatment on three human breast cancer cell lines were also evaluated in a previous study [26]. Administration with those complexes was observed to result in the decreased cell viability and in the increased growth inhibition in a dose- and time-dependent manner, proving once more the impact of those polyamines-based complexes on cancer cell lines.

More specific studies to elucidate the effect of Pt-spermine and Pd-spermine on the expression of genes in the polyamine pathway in an ovarian carcinoma cell line, have been performed. The results thus obtained highlighted the important role of these dinuclear metal- complexes in cancer therapy, since a different mechanism of action was revealed as compared to cDDP. This supports the synergetic interaction when these two agents are co-administered as an advantage to damage DNA as a fundamental cytotoxic effect against tumors and consequently, chemotherapy/adjuvant strategy [27].

Therefore, drug cytotoxicity at a molecular level is based on strict structure–activity relationships (SARs). These can be accurately determined by combined structural and biological methods such as the ones described in this work: (i) *in vitro* assays to evaluate anticancer activity (in human cancer cell lines) and (ii) vibrational spectroscopy of the cellular samples, with and without drug, to determine bioavailability and metabolic impact. This will assure a tailored design of new and more efficient cisplatin-like anticancer agents [28].

1.4. Vibrational Spectroscopy Techniques

The basic principle of spectroscopy is the interaction an incident radiation with the sample, which induces transitions within the molecules. Vibrational spectroscopy techniques such as infrared (IR) and Raman are based on sensitive phenomena, molecular vibrations being detected upon interaction with optical radiation, due to absorption (IR) or scattering (Raman) events (Fig. 9). Both IR and Raman are used in order to perform chemical analysis of the compounds of interest, with high sensitivity and unique fingerprint characteristics. With the use of these techniques, it became possible to evaluate the chemical composition of numerous types of samples, from simple molecules to complex heterogeneous biological matrices.

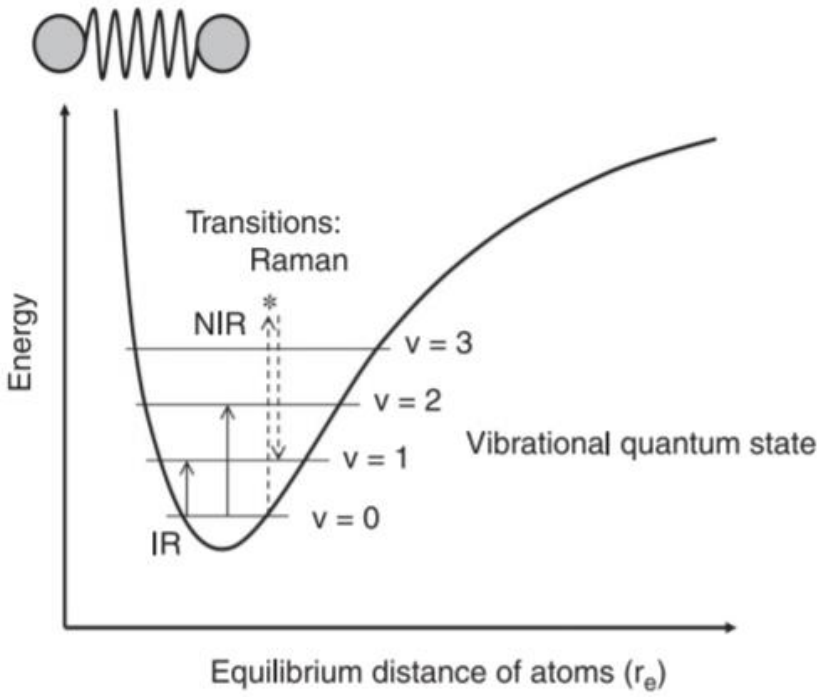

Figure 9: Schematic drawing of a vibrational potential function and the energy level transitions measured by IR, (NIR) and Raman spectroscopy, * denotes a virtual excited state. From [27].

1.4.1. Infrared Spectroscopy

1.4.1.1 FTIR Microspectroscopy

Each atom or molecule may exist in different energy levels, associated to a *quantum number* and every time a radiation interacts with a molecule a quantum of energy (photon) will be emitted or absorbed, according to an energy gap such as E_1-E_0; E_2-E_1, etc. [26] (Figure 9). Plus, the energy of the quantum is associated with the frequency by the following equation:

$$\Delta E = hv$$

Hence, the frequency resulting from the emission/absorption of a radiation (transition between energy levels (e.g. E_0 and E_1)), is given by the following equation:

$$U = \frac{(E1 - E0)}{h}$$

In infrared spectroscopy (IR), those variations can involve changes in bond length (stretching), bond angle (bending) or dihedral angle (torsion). These can include symmetrical or asymmetrical stretching vibrations or types of bending vibration where symmetry is not affected. For these vibrations to give rise to the absorption of IR radiation, a change in dipole moment must occur ($\Delta\mu \neq 0$) and, consequently, the larger this change, the most intense the IR band will be. In organic molecules, there are well-defined frequencies at which certain bond types or functional groups are expected to absorb [29]. As an example, for a carbonyl group (Figure 10) the difference in electronegativity between the carbon and the oxygen is permanently polarized (Figure 10), which means that when the bond C=O undergoes stretching its associated dipole moment will change and cause an intense IR absorption band [26].

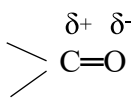

Figure 10: The dipole moment in a carbonyl group

Therefore, unsymmetrical vibrations are usually more intense than symmetrical ones will be weaker. Also, the bending or stretching modes involving atoms that are widely separated in the periodic table, will naturally lead to an intense band [30].

Throughout the years, it became possible to understand and define absorption bands and correlate them to each functional group, according to the specific vibrations of the molecules, coming from the bond strength and the type of atom. Therefore, an unequivocal identification of the compounds is attained by IR spectroscopy with a high sensitivity [23].

Initially, one of the limitations of the infrared technique was the presence of narrows slits at the entrance and exit, which could limit the wavenumber range of the radiation detected in the detector and, as very quick measurement was essential. This led to search new and more efficient techniques, but sensitive as well. Due to that, Fourier-Transform Infrared (FTIR) spectrometers were included in the laboratories as an alternative, where the presence of two beams became essential to help produce an interference when a radiation signal is given. The basic components of an IR spectrometer are shown schematically in the scheme:

Figure 11: Basic components of an FTIR spectrometer. Adapted from [31].

In Fourier transform infrared (FTIR) spectroscopy, the transmitted light is collected and focused onto an IR detector, where it is converted into an electric light and a single- beam spectrum is produced by a fast Fourier transform. FTIR spectrometers present several significant advantages in comparison with dispersive instruments, one of the most important being the higher SNR that can be achieved coupled to a higher scan speed. [26]. The radiation emerges from a source of IR light and passes through the interferometer directed to the sample before reaching the detector. Then, the signal is amplified, after high frequencies contributions have been eliminated by a filter and data is converted into an electrical signal so it can be collected to a computer to create a spectrum. The most common interferometer used is a

Michelson interferometer, as shown in Figure 11. were the two perpendicularly plane mirrors are bisected from a reflecting film, the beamsplitter, which its material must be chosen according to the region for further examination. Technically, half of the incident radiation will be reflected to one of the mirrors back and the other half will be transmitted to the other mirror. When the two beams are reflected from the mirrors, they will recombine, once back to the beamsplitter and interfere with each other, being only 50% of the reflected beam from the fixed mirror directed to the beamsplitter and the remaining 50% reflected to the source. The beamsplitter works as a transmitted beam and it is the one detected in the IR interferometer and responsible to forward the radiation to the detector.

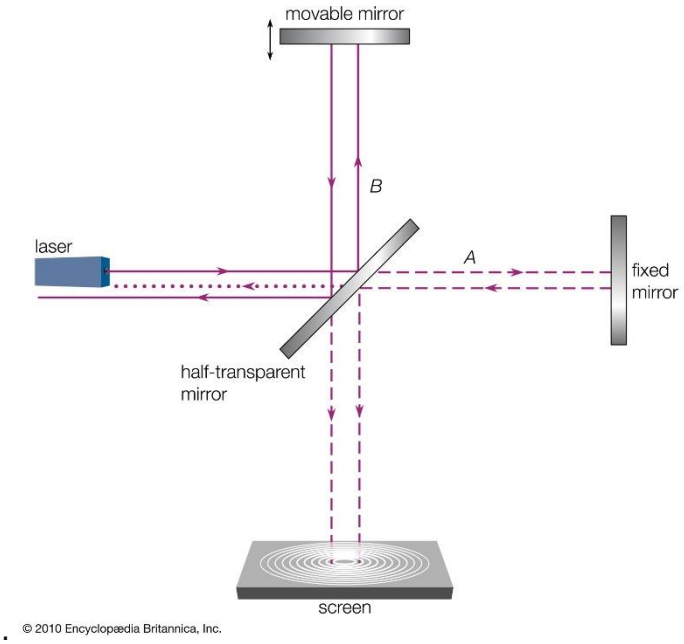

Figure 12: Schematic representation of a Michelson interferometer. From [32].

An essential step to obtain an IR spectrum is to produce an interferogram with and without sample in the beam, to form a ratio of the former and then, a double-beam dispersive spectrum. The source will depend on the region that the performer wants to detect (near-infrared region, far-infrared region or medium-infrared region) as well as the detector, also chosen taking into account the desired sensitivity. . For IR spectroscopy, it is necessary to switch the glass lens once glass can absorb IR light. This way, optical elements such as, gold or aluminum-coated or even transparent windows are used and cannot interfere with the incident IR light [30].

Infrared spectra can be acquired in three distinct ranges: far-IR (up to *ca.* 400 cm^{-1}), mid-IR (400-4000 cm^{-1}, comprising the fingerprint region, Figure 12) and near-IR (above 4000 cm^{-1}).

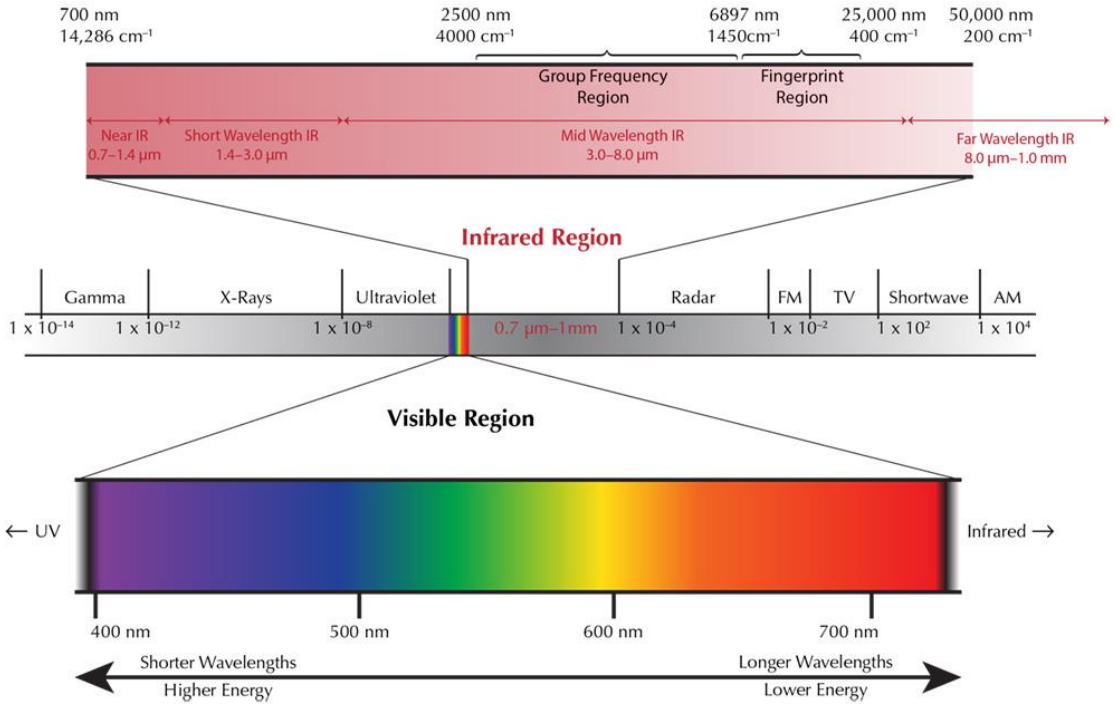

Figure 13: The electromagnetic spectrum, and the region of molecular vibrations, in the mid-infrared. From [33].

The great advantage of IR spectroscopy is its ability to yield a high signal-to-noise ratio (SNR), which increases with the number of scans, which may be summed to increase the signal while it decreases the noise.

When infrared light irradiates the sample absorption can occur at different frequencies, according to the different functional groups present, and a spectrum results from all the frequencies detected at the same time within a broad energy window. Those frequencies are specific for each atom, the spectrum being a unique fingerprint of that particular sample. It is thus possible to know, very accurately, which components are present in the sample and therefore determine its structure [29].

1.4.2. Raman Spectroscopy

Raman spectroscopy has been used as an analytical technique for more than 70 years, having been particularly applied to the characterization of a diverse number of samples upon the development of the laser, in the late 1960´s [25]. Raman spectroscopy is a vibrational technique complementary to IR, but it is based on a dispersion of the incident radiation (photons) instead of an absorption.

The process involves shining a monochromatic radiation onto the sample which, upon interaction with the molecule, generates signals due to inelastic dispersion of light (the Raman effect) [3435]. The energy change within the sample can be dispersed with either a higher or a lower energy, coming initially from the ground stage (Stokes) or from the first virtual stage (anti-Stokes) (Figure 13). Once the lower energy level has a higher population, Stokes transitions give rise to stronger Raman bands. This dispersion is only Raman active (*i.e.* gives rise to a Raman signal) if involves a change in the polarization of the molecular vibration ($\Delta\alpha \neq 0$), which means that the electron cloud suffers a deformation [28,31].

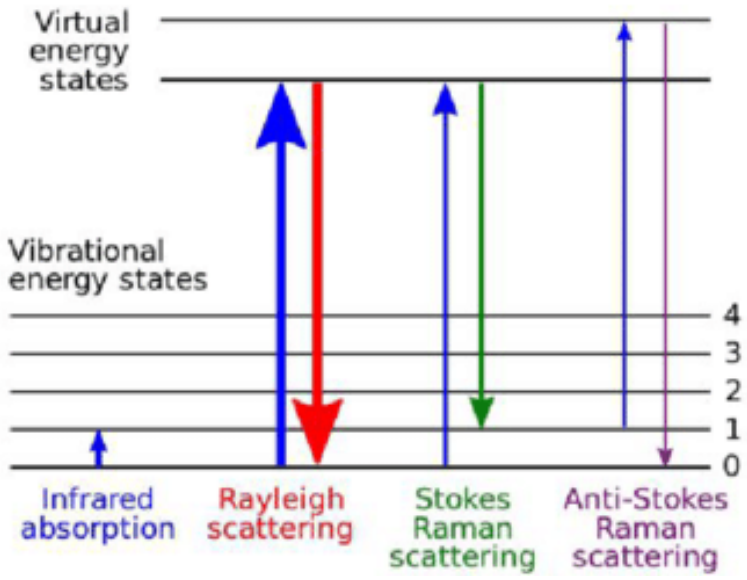

Figure 14: Schematic representation of the infrared absorption and Raman dispersion phenomena. (The Rayleigh band corresponds to an elastic scattering, thus yielding a very intense band). From [36].

Raman spectroscopy is a very specific technique but has a low sensitivity. Thus, phenomena that can compete with it may easily hide the characteristic Raman dispersion bands, such as emission of fluorescence which can be intrinsic to the sample.

What distinguishes IR and Raman techniques is that while in the former the light is absorbed by the sample, in Raman there is no absorption episode and the incident monochromatic light is scattered inelastically by the sample, resulting in anti-Stokes and Stokes lines (Figure 13) [37]. Raman spectroscopy is an important tool used in the characterization of many compounds. As we have already seen, because the physical phenomena underlying the Raman effect (change in polarizability) is different from that associated to infrared (change in the dipole moment), there are some vibrations that are active in one technique but not in the other – complementary methods.

This means that between the two, the analyst should be able to get a complete picture of the vibrational modes of a molecule [38].

The activity of a potential drug does not rely solely on its chemical properties, but it is also dependent on its conformational preferences, which determine its activity and mode of action – namely its bioavailability, metabolic impact and interaction with the target. Vibrational spectroscopy is an especially useful technique for probing these properties since it yields unique and accurate chemical and structural information on both the drug molecule and its environment (*e.g.,* a cell or tissue).

2. Hypothesis and Aims

The antiproliferative and cytotoxic activities of a dinuclear Pd(II) complex (Pd_2Spm) was evaluated towards a human colon cancer cell line (Caco-2).

In vitro tests are essential for assessing the compound´s efficacy as a potential anticancer agent (as compared to cisplatin, taken as a reference drug). Animal cell lines are important models to evaluate the growth-inhibiting activity as well as the toxicity of a specific compound.

In addition, the agent´s impact on cellular metabolism was determined, with high specificity and sensitivity, by vibrational spectroscopy methods (both Raman and FTIR) of drug-treated and untreated cells.

This multidisciplinary approach allowed us to better comprehend the potential anticancer activity and mode of action of the new compound, at the molecular level.

3. Experimental

Table 1: List of reagents, material, equipment and software used along this work.

Reagents	
Acetic and acid glacial (99.7%)	Sigma-Aldrich - Portugal
Acetone (≥ 95%)	Sigma-Aldrich – Portugal
Cloridric acid (37%)	Sigma-Aldrich – Portugal
cis-dichlorodiamine platinum(II) (cisplatin) (99.9%)	Sigma-Aldrich – Portugal
Dimethyl sulfoxide (DMSO) (≥ 99%)	Sigma-Aldrich – Portugal
Minimum Essential Medium (EMEM) w/ Earle's Balanced Salts w/ 2.0mM L-Glutamine w/o Sodium Bicarbonate	Sigma-Aldrich – Portugal
Ethanol (99.8%)	Sigma-Aldrich - Portugal
Ethylenediaminetetraacetic acid (EDTA) (≥ 98.5%)	Sigma-Aldrich – Portugal
Fetal Bovine Serum (FBS) (EU Approved(South America))	Gibco-Life Technologies, Porto, Portugal
Formalin solution, neutral-buffered, 10%	Sigma-Aldrich – Portugal
Methanol (≥ 99.8%)	Sigma-Aldrich – Portugal
Non-Essential Aminoacids Solution (NEAA) (100x) without L-glutamine, liquid, sterile-filtered, Bioreagent, suitable for cell culture	Sigma-Aldrich – Portugal
Sodium Bicarbonate (≥ 99.7%)	Sigma-Aldrich – Portugal
Pd_2spm	Sigma-Aldrich – Portugal
Sulforhodamine B (SRB) (75%)	Sigma-Aldrich - Portugal
Tris (hydroxymethyl)aminomethane (Tris)	Absolve, Odivelas, Portugal
Trypan Blue (0.4% *(w/v)* solution)	Sigma-Aldrich – Portugal
Trypsin (10x solution, 25g porcine trypsin *per* liter in 0.9% sodium chloride)	Sigma-Aldrich – Portugal
Gelatin	Sigma-Aldrich - Portugal
Material	
12 wells plates	OrangeScientic, Frilabo, Portugal
24 wells plates	OrangeScientic, Frilabo, Portugal
96 wells plates	OrangeScientic, Frilabo, Portugal
T 75 cm^2 culture flasks	OrangeScientic, Frilabo, Portugal
T 25 cm^2 culture flasks	OrangeScientic, Frilabo, Portugal
5 mL syringes	BD Falcon, Enzifarma, Portugal
15 and 50mL conic tubes	OrangeScientific, Frilabo, Portugal
1.5 mL micro-tubes	OrangeScientific, Frilabo, Portugal

MgF_2 (Raman grade, 2x20mm) windows	Crystran, United Kingdom
CaF_2 (UV-grade, 1x13mm) windows	Crystran, United Kingdom
Equipment	
Analytical balance (Toledo AB54)	Metler, Rotoquímica, Portugal
pH-meter (BASIC 20 +)	Crison, Rotoquímica, Portugal
Shaker “Vortex” (MS2 Minishaker)	IKA® Works, Frilabo, Portugal
Water purification apparatus Mili-q (Gen Pure)	TKA, Frilabo, Portugal
Parafilm PM-992	
Centrifuge with cooling (MPW-350R)	MPW, Frilabo, Portugal
Incubator MCO-19AIC (UV)	Sanyo, Frilabo, Portugal
Laminar flow hood (BW 100) (flow rate: 1050 m^3/h)	BioWizard, Frilabo, Portugal
Microplate reader µQuant MQX200	BioTek, Portugal
Microscope CRX41 coupled to a DP20 camera	Olympus®, Portugal
Nitrogen cooled Bruker Hyperion 3000 FT-IR microscope	
Bruker Optics Vertex 70	Bruker, Portugal
WITec confocal Raman microscope system alpha300 R	
Ultra-High-Throughput-Spectrometer UHTS 300 VIS-NIR	
Software	
GraphPad Prism 8.4.3 Software	
MatLab 20121	
OPUS 7.2	
OriginPro 9.1	
Gen5 1.11 Ink	

3.1. Experimental methods

3.1.1. Synthesis of metal-based compounds with polyamine ligands –Pd_2Spm

Synthesis of the Pd_2Spm was carried out based on already published procedures at QFM-UC [39,40]. Briefly 2 mmol of K_2PdCl_4 were dissolved in a minimal amount of water and an aqueous solution containing 1 mmol of spermine was added dropwise under continuous stirring. The reaction occurred for 24h, after which the resulting yellow powder was filtered off and washed with acetone. Recrystallization of this product from water afforded needle-shaped crystals (yellow).

Preparation of solutions

Table 2: *Solutions used along the experimental work.*

Solution	Components	pH	Storage
Cell culture			
Phosphate Buffered Saline (PBS) 10 x	2.0 g KH_2PO_4 (15 Mm) 6.1 g Na_2HPO_4 (43mM) 2.0 g KCl (27 mM) 87.7 g NaCl (1.5 M) 1000.0 mL ultrapure water	7.4	Room temperature
PBS 1x	100.0 mL PBS 10x 900.0 mL ultrapure water	7.4	Room temperature
Minimum Essential Medium (EMEM)	9.53g/L (EMEM) 2.2g sodium bicarbonate 1L water miliQ	6.9 - 7.1	4ºC
Trypsin-EDTA 1x	90.0 ML pbs 1x 100mL Trypsin 10x 20.0 mg EDTA	7.4	4ºC
Tested agents			
cisplatin 1mM	3.0 mg cisplatin 10.0 mL PBS 1x		-20ºC
Pd_2Spm 500 uM	2.8 mg Pd_2Spm 10.0 mL PBS 1x		-20ºC
MTT colorimetric assay			
MTT	8.5 mg MTT 17 mL PBS		-20ºC
SRB colorimetric assay			
Methanol 1% (v/v) in Acetic acid	495.0 mL Methanol 5.0 mL Acetic acid		-20ºC
1% Acetic acid	990.0 mL denoised water 10.0 mL Acetic acid		Room temperature
SRB 0.5%	2.5 mg Sulforhodamine B 500.0 mL 1% Acetic acid solution		Room temperature
Tris 10 mM	1.2 g Tris base 1000.0 mL ultrapure water	10	Room temperature

Spectroscopic experiments			
Formalin 4%	400 mL 60.0 mL NaCl 0.9%		4ºC
Coating solution	Gelatin solution 4% (100 mL water miliQ + 2g of gelatin BSA 2% (100 mL ultrapure water + 2g of BSA)		4ºC

3.1.2. *In vitro* assays

3.1.2.1. Cell culture

In this study, for the cytotoxic assays, a human colon adenocarcinoma cell line (Caco-2) isolated from human epithelial colorectal adenocarcinoma cells was used.

The Caco-2 cell line was grown as a monolayer in EMEM medium with Earle's Balanced Salts, with 2.0 mM L-glutamine and sodium bicarbonate, supplemented with 10% fetal bovine serum (FBS) (Gibco BRL / Life Technologies Ltd), 1% NEAA, 1% sodium pyruvate (in one of the media), 10% serum and kept in a humidified atmosphere of 5% CO_2 in an incubator at 37 ˚C.

3.1.2.2. General Maintenance

During the cellular maintenance, it is necessary to examine the appearance of the cells throughout the experiment. Before the beginning of the experiment, the growth medium of healthy adherent cells should be clear, considering that a contaminated culture will appear turbid

Once cells are ready to manage, for propagation in culture flasks Caco-2 cells are seeded in a concentration of 105 cells/cm^2. Medium should be changed every 3 days to maintain 80 % confluence, which is typically after 4–5 days with a splitting of 1:4 before further cultivation. For trypsinization of Caco-2 cells it is first required rinsing with PBS (volume at least the double of Trypsin added). Next, 2mL trypsin/EDTA solution are added with further incubation at 37ºC for 2 min least, so enzyme activation can occur. In this step, cells should detach from the flask and immerse into the medium. If most of the cells remain adhered to the flask, the flask should be further incubated for several minutes. However, the incubation with trypsin should be as short as possible as this process will affect cell viability. As soon as the cells are detached, trypsinization is stopped by adding complete medium containing fetal calf serum. Then cells are

transferred to a test tube to let cell aggregates and debris sediment after centrifugation (1100rpm, 5minutes). Lastly, the supernatant is transferred to a new test tube, and an aliquot is taken to count the cells after checking cell viability. The content of dead cells should not exceed 5%.

3.2. Evaluation of antitumoral activity

3.2.1. Determination of antiproliferative activity

The antiproliferative activity tests were performed at the QFM-UC laboratories.

3.2.1.2. Determination of the cellular viability

The Trypan blue colorimetric assay was used to assess the number of morphologically intact cells (cells with undamaged cell membranes), by color discrimination. For this purpose, after the cell suspension is prepared, 0.5mL is taken to an eppendorf, an essential step to then provide 20 µL of it into each of two different micro-tubes, with 20 µL of Trypan blue. Cells are then counted by observation under the optical microscope using a Neubauer-counting chamber. As a result, the number of cells with intact membranes and, therefore, dye excluding was expressed in terms of mean ± standard deviation.

In sum, living cells will stay colorless while the death ones will be stained blue.

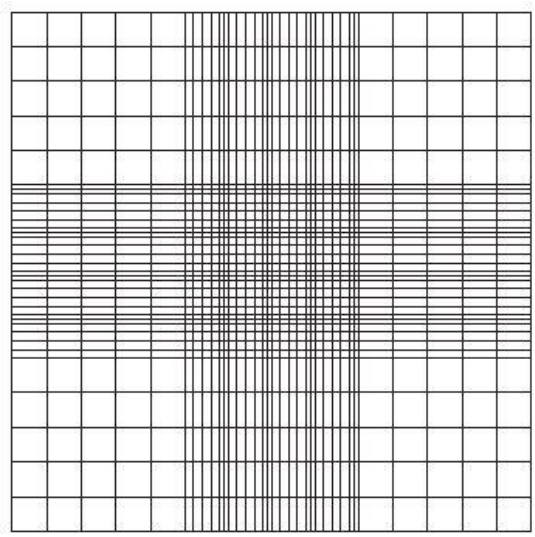

Figure 15: Scheme of the counting grid of a hemocytometer.

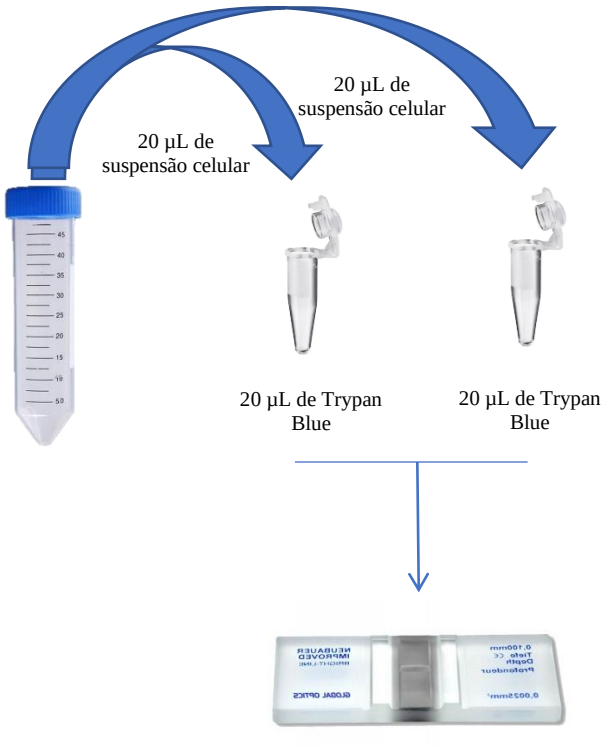

Figure 16: Schematic design of the Trypan blue method.

MTT (Thiazolyl blue tetrazilium bromide) assay

MTT is an important colorimetric method to verify the cellular viability of the samples. This assay is based on the reduction of a yellow tetrazolium salt (MTT) to purple formazan crystals by metabolically active cells, once it is caused by the activity of mitochondrial dehydrogenases (Figure 16). Formazan crystals that once precipitated, are observed with an intense coloration and the stronger the coloration, the higher is the number of viable cells. These MTT parameters helps to comprehend the autofluorescence of mitochondria, which seem to be specifically related to metabolic changes and only present in viable cells.

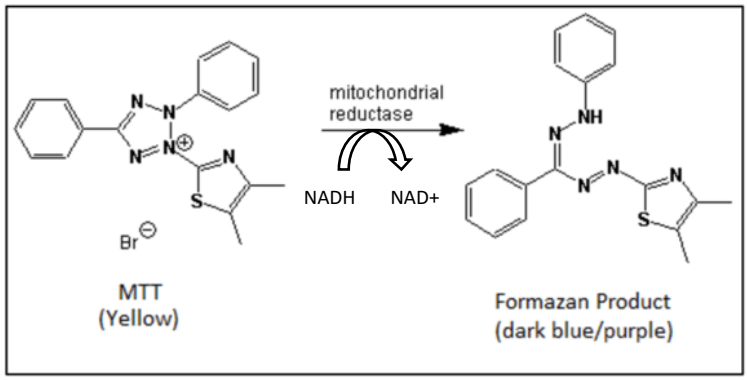

Figure 16: Representation of the MTT reduction reaction. Adapted from [41].

To determine cellular viability, the medium of each well is removed and washed with 500 µL of PBS, then 250 µL of MTT is added to all of them and then plates are incubated at 37ºC for 2 hours (light protected). The viable cells contain NAD(P)H-dependent oxidoreductase enzymes which are responsible to reduce the MTT to formazan. After the MTT solution is discarded, the insoluble formazan crystals are dissolved using a highly polar organic reagent that has exceptional solvent properties for organic and inorganic chemicals, (250 µL DMSO) resulting in a colored solution that is quantified below by measuring absorbance at 570 nM, in a multi-well plate reader. Cellular viability is determined according to the following formula:

$$\% \text{ cellular viability} = \frac{A_{sample}}{A_{control} - A_{blank}} \times 100$$

The assay starts with cells being plated in triplicate in 24-well microplates by addition of the cell culture prepared and complete medium (EMEM) previously mentioned. Cisplatin is added after 24h on the microplates in different amounts, in order to obtain final concentrations of 32, 16, 8, 4 and 2 µM. No cisplatin solution was added either on control wells (cells that have not been exposed to the cDDP) or blank wells (empty wells). (Figure 17) The viability was assayed with MTT after 24, 48 and 72h of incubation with cDDP, in which 250µL of the reagent acts during 2h on the cells. (Figure 17). The blue MTT formazan crystals are then solubilized in 250 µL of DMSO and absorbance is measured at 570 nm and the outcome cell viability is expressed in percentage and compared with the control.

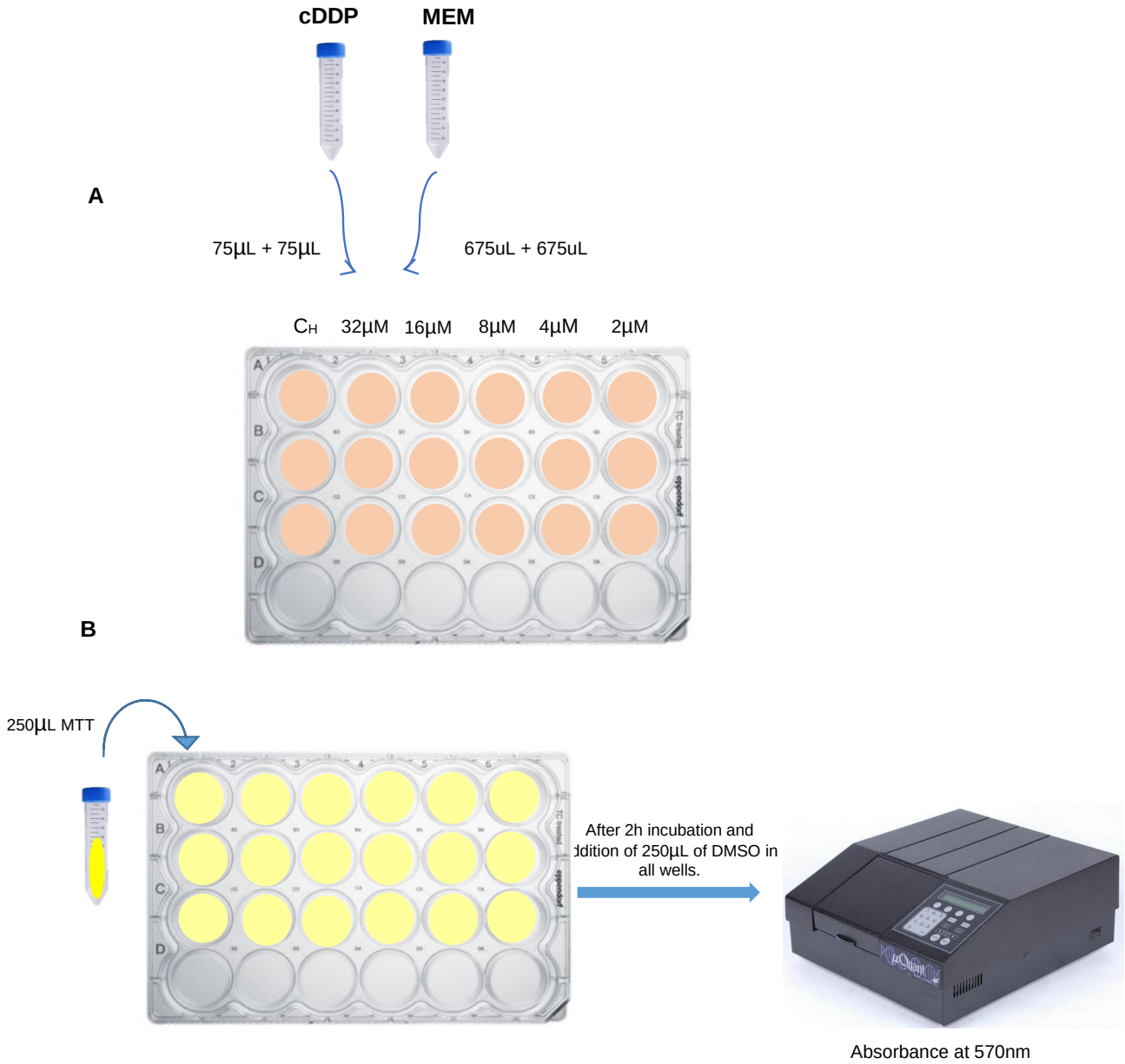

Figure 17: (A) Schematic representation of decreased concentrations of cDDP among the wells, after CH (24 h;48 h;72 h) and 32 µM, 16 µM, 8 µM, 4 µM and 2 µM have already 750 µL of MEM. The passages between decreasing concentrations are always in cells of the same row. After the last concentration, the same amount of volume is discarded, so that all wells have the same volume. (B) Addition of 250 µL of MTT in every well and respective measurement at 570nm.

Sulforhodamine B colorimetric assay

The Sulforhodamine B (SRB) is a fluorescent dye used to quantify the cellular proteins presents on cultured cells, based on binding of the dye to basic amino acids of cellular proteins. This essential method provides an estimate of total protein mass, which is related to cell number.

To add SRB on each well, previous wash must be done. Every well is washed with 500 µL of PBS following by 500 µL of Mili-Q water (aspiration step at the same time) and then, after incubation at 37ºC for 2 hours, each well is fixed with acetic acid/methanol and frozen overnight (Figure 18). After thawing in a stoven, 250 µL of SRB solution is added, followed by incubation at 37ºC for 1 hour, after which cells are then carefully washed with 1% acetic acid and left to dry overnight. This assay is finished with the addition of 1 mL of 10mM-Tris (pH = 10) to dissolve the protein-bound SRB so the absorbance can be read at 540 nm. The percentage of proliferation or cellular growth is calculated by:

$$\% \text{ cell grow} = \frac{A_{sample} - A_{0\ day}}{A_{control} - A_{0\ day}} \times 100$$

A_{sample} represents the absorption of the solution exposed to the tested compound and $A_{0\ day}$ refers to the absorption of the control at 0h (no compound added).

SRB works the same way as MTT assay until the step of cell culture, with the same decreasing concentrations among the cells. However, the 24-well plate adds a C_0 triplicate to observe cells at time 0, being the plate all full and the following ones (48 h and 72 h), with the same organization as the 24-well plates of MTT

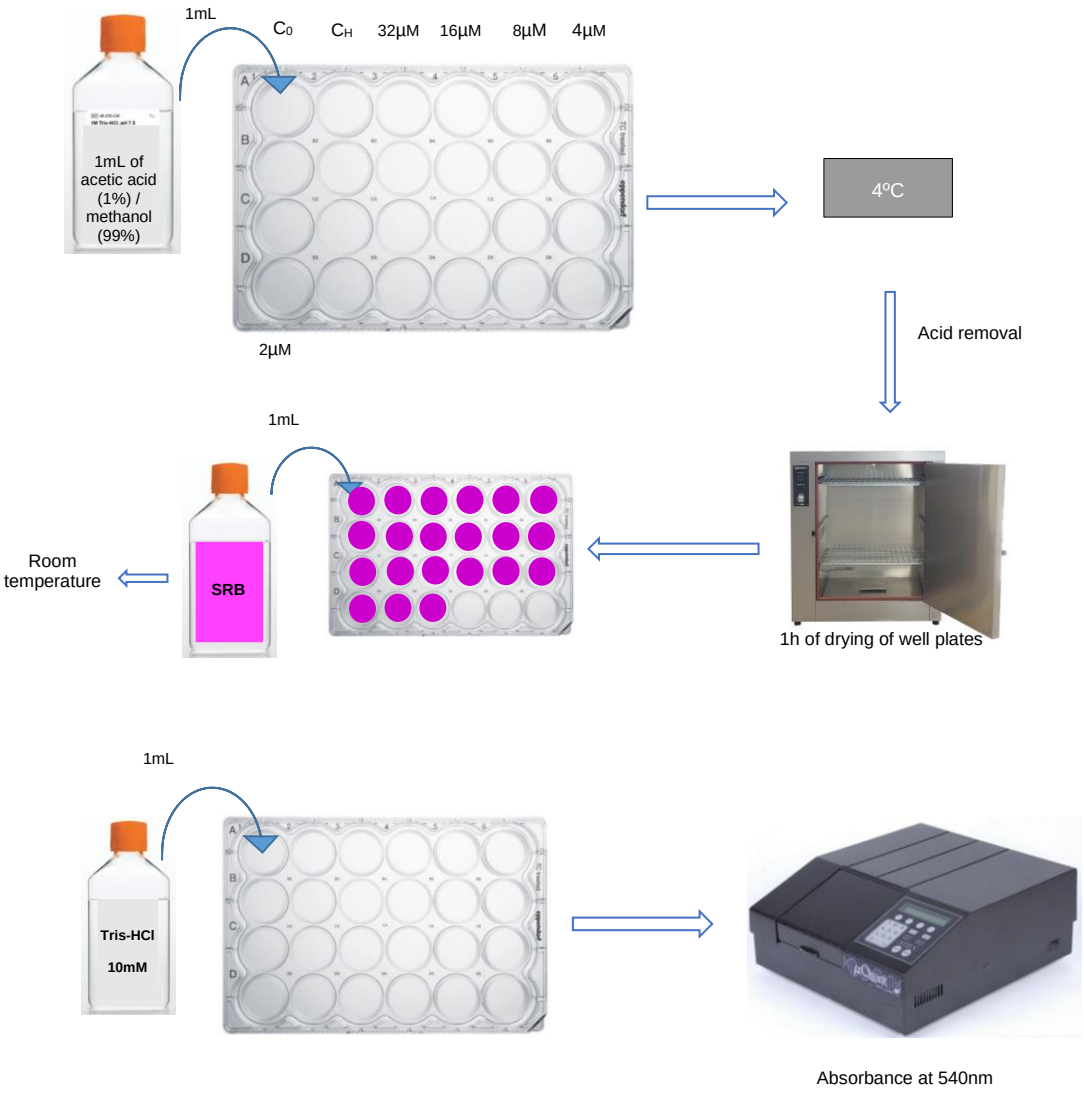

Figure 18: Schematic representation of SRB assay after addition of culture cell and respective 24h incubation, followed by the washing procedures.

3.3. Vibrational Spectroscopy

3.3.1. Sample preparation for spectroscopic analysis

Prior to plating the cell culture for spectroscopic analysis, a coating solution that works for at least 1 hour in the incubator is added to the disks. This step is necessary so the cell substrate can adhere to the disks and make the subsequent process more viable and safer, without major material losses.

After the addition of trypsin, the cells are collected and undergo a centrifugation (1100 rpm, 5 minutes) where the pellet is then resuspended with medium, to a density of $3x10^4$ cells/cm^2 This culture medium is plated on 12-plates with MgF_2 disks and with CaF_2 on 24 well-plates for persuade their data analysis on Raman and FTIR techniques, respectively. Both methods contain triplicate disks for the control and for the two chosen concentrations, 4 µM and 8 µM, previously autoclaved and washed with 70% ethanol and. This step ends with the plates in the incubator until cell adhesion to the disk is observed. For this study, the metal-based compound was added 24h after addition of the cells in medium and then, 2 days after the process was stopped (48h of drug exposure).

To prepare cells for later analysis on spectroscopical techniques, firstly the medium is removed, the cells are washed with PBS and then fixed with formalin (4%) for 10 minutes and then washed with milli-Q water. The last washing step is done enough times until there is no formalin residue, so the plates are maintained in the fridge (temperature of 4ºC to avoid fungal contamination), where cells are ready to be read.

3.3.2. Raman spectroscopy

The Raman spectra of Caco-2 fixed cells on MgF_2 disks, both untreated and treated with Pd_2Spm, were obtained at room temperature with Micro-Raman Witec alpha 300 spectrometer. Samples with untreated cells were used as a control.

Raman acquisition was performed at QFM-UC in a WITec confocal Raman microscope system alpha300 R coupled to an Ultra-High-Throughput-Spectrometer UHTS 300 VIS-NIR, using the diode laser 785 nm line. The power of the laser was kept

at 20 mW and the measurements were achieved using 5 accumulations of 7s per spectra with a 100x Zeiss objective.

Besides control samples, treated samples with 4 and 8 µM (48 h exposure) were analyzed. An average of 50 spectra were collected per sample (in different cells), with 2 accumulations and 120s of exposure.

3.3.2.1. FTIR microspectroscopy

In the ATR-FTIR technique, the analysis of Caco-2 cells required previously fixation to CaF_2 disks v untreated and treated with Pd_2Spm, following with the acquisition of spectra at room temperature. Untreated cells were used as a control.

To obtain an absorption spectrum in the mid-IR range 400-4000 cm^{-1} FTIR acquisition was performed at QFM-UC, in a nitrogen cooled Bruker Hyperion 3000 FT-IR microscope coupled to a Bruker Optics Vertex 70 spectrometer, in transmission mode. Each acquisition was performed with 8 cm^{-1} resolution and 64 scans using a 15x objective.

Samples containing drug at 4 and 8 µM concentrations were analyzed, as well as the untreated samples (without drug) and an average of 150 to 200 spectra were collected *per* sample. The spectrum of each sample was obtained with a resolution of 4 cm^{-1} with an average of 128 accumulations. All samples were performed in three replicates.

5. Results and Discussion

In this study, the growth inhibitory activity of the chemotherapeutic drugs Pd_2Spm and cisplatin against Caco-2 cell line was investigated. Results showed that both agents can have a significant growth inhibitory effects, in a dose-dependent manner.

Since various resistance episodes of cancer cell lines when using cisplatin as the treatment of choice have been reported to happen, many other compounds based on its structure and mechanism of action have been developed. Pd(II)-based complexes are the new potential drugs when it comes to approach cancer, once several advantages have been observed with its use, beginning with the major spectrum of action and the better specificity to the pharmalogical target, DNA, which leads to the inhibition of the proliferative and survival mechanisms of the tumor cells, causing its apoptosis [42]. Caco-2 cell line was used to study the hypothetic cytotoxic effect that can happen when cisplatin and one analogue complex is introduced (Pd_2Spm) [40,43].

The cytotoxic activity of both metal-based compounds was evaluated by addition of the drug in a medium with concentrations from 2 to 32 µM on a cultured human colon adenocarcinoma cell line (Caco-2) for 24h, 48h and 72h and the results are presented on Figure 19. MTT assay was the method of choice to observe the biological changes altered from the induced exposure of cDDP and Pd_2Spm. The cytotoxic effect is evaluated by the rate of formation of formazan that will correspond to the function of essential cellular processes like respiration. This happens because the reduction of the reagent MTT is mainly by the mitochondrial metabolism, which is responsible to convert the MTT into formazan crystals, that will be measured spectrophotometrically. A purple pigmentation is verified among the wells and it is expected a lighter color on nonviable cells, once a lower mitochondrial activity might be presented (3.2.1.2 on Experimental section).

Effect of cDDP at 24h was higher than the one observed with treatment, although this last one showed a decreased behavior among the concentrations used (16 and 32 µM with the biggest differences), while in the cisplatin exposure no big differences were verified (Figure 19) at that time. At 48h, cDDP showed a bigger cytotoxic effect on Caco-2 than Pd_2Spm, however both treatments were more efficient with 16 and 32 µM. In fact, 32 µM was the dose of cisplatin that induced the death of 50% of the tumoral cells.

It was at 72h that the major antitumoral effect was observed for Pd_2Spm, where a population < 50% have not survived with concentrations of 8, 16 and 32µM, which shows a negative impact caused by Pd(II) complex, confirming the promising use of the candidate and its antitumoral potential. Besides the lower cytotoxic effect, results were similar in cDDP treatment. (Figure 19).

The lower rates of survival with Pd_2Spm, comparing to cDDP, may suggest that the Pd(II) analogue chelate has indeed a better anti-tumoral performance than cDDP, as already observed by several studies Complexes like Pd(II) that shares a polyamine as a ligand, such as spermine, are synthetized and tested based on their structure-activity relationships (SARs) to potentiate a better cytotoxic effect. Pd_2Spm has a spermine as ligand that allows the long-distance interactions between the amine and DNA, mostly inter and intrastrand crosslinks in the helix and those interactions will be responsible to conduct the cells to their death and justify the higher antitumoral effect when compared to cisplatin [44].

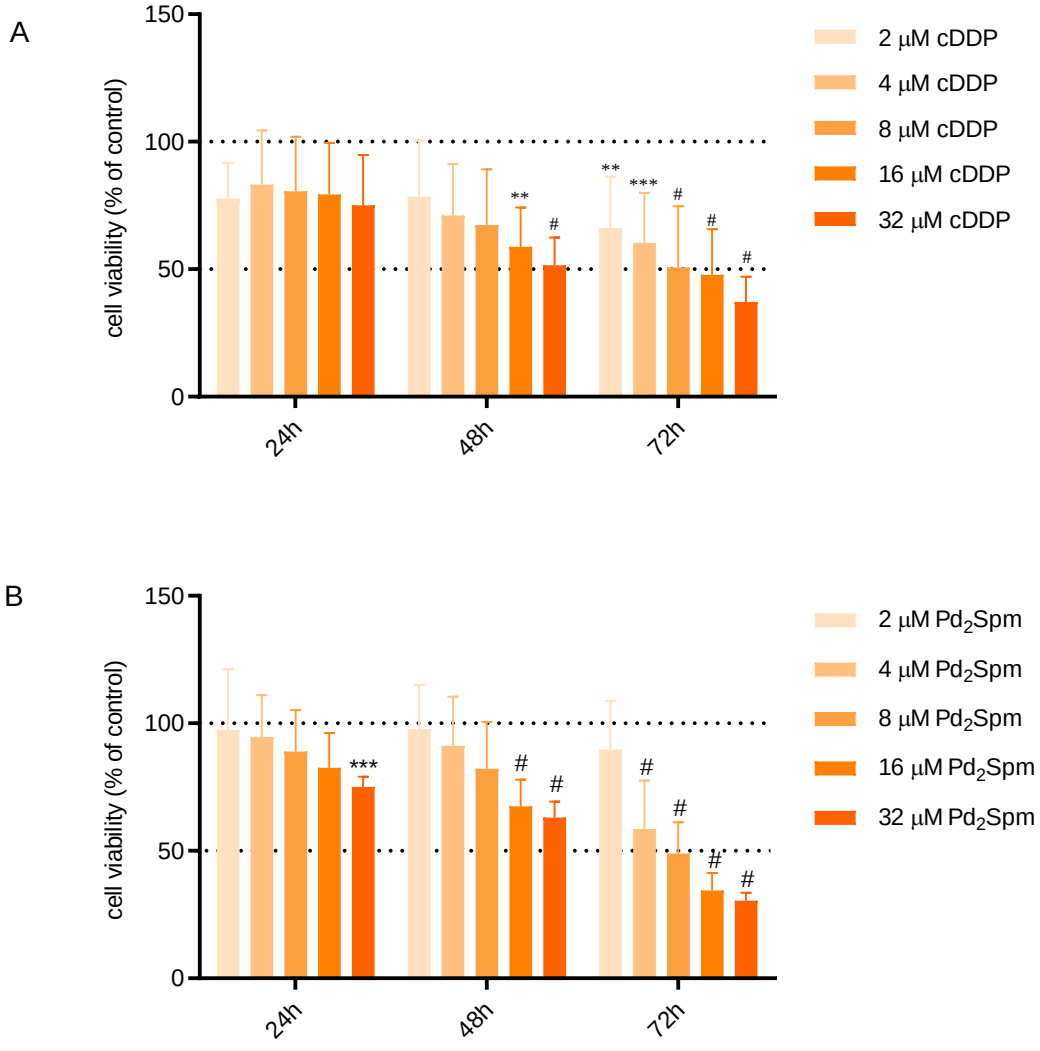

Figure 19: Antiproliferative effect of cDDP (A) and Pd_2Spm (B) on Caco-2 cell line with concentrations ranging from 2 to 32µM. The MTT assay was performed as described in the section of Material and Methods (3.2.1.3) for 24h, 48h and 72h and the results are expressed in % of cellular proliferation ± standard deviation, obtained in triplicate. Data was treated statistically on the GraphPad

Prism 8.4.3 program with the one-way ANOVA method. The one-way ANOVA statistical analysis was used, and the Dunnett's post-test was carried out to verify the significance of the obtained results (*p<0.05, **p<0.01, ***p<0.001, #p<0.0001 vs the control for the same time-points).

For each inhibition assay, a dose-response curve was generated which gave the information related to IC_{50}. Figure 20 shows the percentage of inhibition of the cell growth with the concentrations studied in this work during 24h, 48h and 72h for the Caco-2 cancer cell line. The new synthetized Pd(II) based-complex showed dose and time-dependency, as an antitumor and anti-proliferative behavior was observed among the concentrations and hours, respectively. Comparing the two drugs used, both had the greatest effect at 72h, however Pd_2Spm was more cytotoxic once it led to a decrease of a higher number of cells capable to proliferate and survive. Moreover, the difference between the effect of the drug at 72h comparing with the other two timings (24 and 48h) is bigger with Pd_2Spm treatment, which suggests the greatest cytotoxic effect carried by the drug, when an exposure of 72h is applied onto the cancer cell line used.

Caco-2 proved to be more sensitive to Pd_2Spm therapy compared to cDDP, data that also can be sustained by the IC_{50} values obtained at 72h (8.68 µM vs 10.20 µM, respectively). The IC_{50} values were obtained by linear regression using GraphPad Prism 8. This data is in accordance with the literature [45], where values ranged from 1µM to 7 µM, for Colon adenocarcinoma cells (HT-29), a study also based on the cytotoxic effect of some palladium complexes on various tumor cells. Despite this information, it is important to acknowledge that each cell line has its own response even when comparing the same drug, but those values still are important, once they give us a promising approach and can turn Pd_2Spm a high potential cancer treatment, being an alternative to overcome cisplatin's resistance.

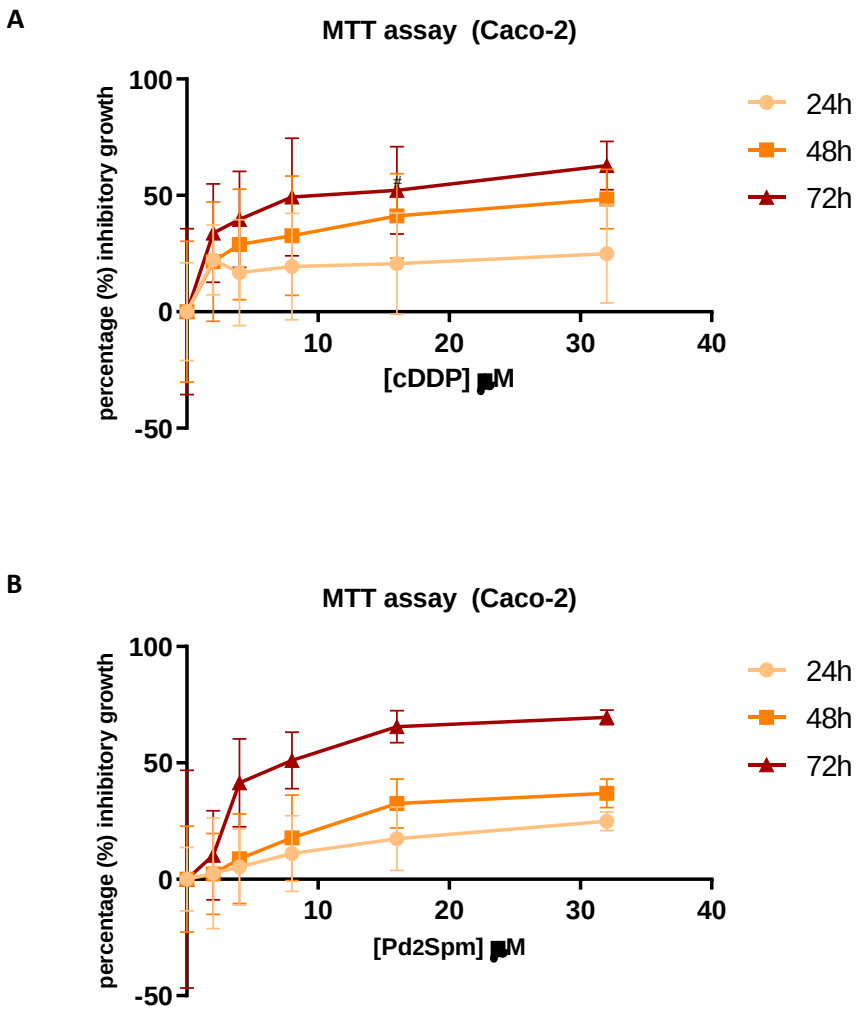

Figure 20: Quantitative MTT assay results in the presence of increasing concentrations of (A) cDDP and (B) Pd_2Spm and (according to 3.2.1.2 Experimental section). The results are expressed as a percentage of the control ± SEM. The one-way ANOVA statistical analysis was used, and the Dunnett's post-test was carried out to verify the significance of the obtained results (*p<0.05, **p<0.01, ***p<0.001, #p<0.0001 vs the control for the same time-points).

Besides the effectiveness of the MTT assay on evaluating the cytotoxic effect of a compound, this method does not provide any more information regarding the cell death mechanism by discrimination on which cells are and are not viable. The SRB assay is an important method once the dye only binds to the amino acids of the proteins, which only happens if the integrity of the membrane is compromised. Thus, the amount of binding will be directly proportional to the cell mass, because it will depend on the intact membrane of adherent cells. Viable cells have intact membranes and exclude the dyes. Presuming that the dying ones were washed before, this assay can give additional data about the cytotoxic effect of the drugs, based on the cellular density observed.

Results presented on Figure 21 showed lower cellular density at 72h to the both metal-based compounds, which comparing to the MTT data it could mean that besides the viability of cells is compromised, their membrane can remain intact and maybe a mechanism of resistance it is present.

The SRB assay is characterized to be a better linear method, suitable to study chemosensitivity of subconfluent monolayers and multilayer cell clusters.[46] Plus, a higher number of cells will translate in a higher binding to the reagent.

When observing the results at 24 h, no high values were observed among the concentrations used for cDDP treatment, which goes in accordance with the MTT results. At this time, the drug still can not induce sufficient cytotoxic effect that inhibits a large number of cells to proliferate or to cause a disruption of the membrane. This can be presumed by the low cellular density verified on the figure 21, based on the SRB dye binding onto the amino acids of the proteins. On the other side, in Pd_2Spm treatment, besides the little effect observed on the cellular viability (Figure 19), we still can observe satisfactory results in relation to the cellular density at 24 h. Those can confirm the presence of an intact membrane once no significant differences for cellular density between the control and all the concentrations used, was observed.

At 48 h time point, besides the decrease of the cellular viability seen on MTT (Figure 19) for both metal-based compounds (especially with 16 and 32 μM), the increased values observed on the SRB assay (Figure 21), may confirm the cytotoxic effect of the drugs, once it is expected a less intact membrane which translated to the entrance of the dye onto to the cells and its binding to the amino acids, leading to the higher results on the graph.

As seen in the MTT assay, at 72h it was confirmed the highest antitumoral effect on both cDDP and Pd_2Spm treatments. However, based on the outcome observed in Figure 21, at 72 h, the cellular density remained almost the same with the cDDP treatment for concentrations of 2 and 4 μM, but for 8 and 16 μM it was verified a significative decrease and a much more for 32 μM. This can be explained by several reasons, being a resistance mechanism an hypothesis or a lack of correlation between the two assays. This has already been discussed to be a disadvantage of the use of both on this type of studies, mainly because the effects of mitochondrial dysfunction occur early, whereas effects that compromise cell membranes occurs later in the cell death process. [47]

Based on the antiproliferative studies above, our data could confirm the importance of both assays when it comes to evaluate the effect of chemotherapeutical drugs and the consequents molecular changes by its induced exposure, showing that Pd_2Spm has indeed, a better effect on tumoral cells when it comes to decrease its

survival functions to proliferate. This could suggest that the metal-based compound has a higher effect than cisplatin and can be a potential antitumoral therapy for cancer cell lines, but further studies should be performed to understand better the effects and alterations shown at 72h, where a higher effect was verified.

Results from 48 h from both assays showed to be the best correlated, once the drop of the cellular viability observed by the MTT data was also accompanied by the disruption of the membrane integrity, a result translated in the SRB results with the increase of cellular density observed.

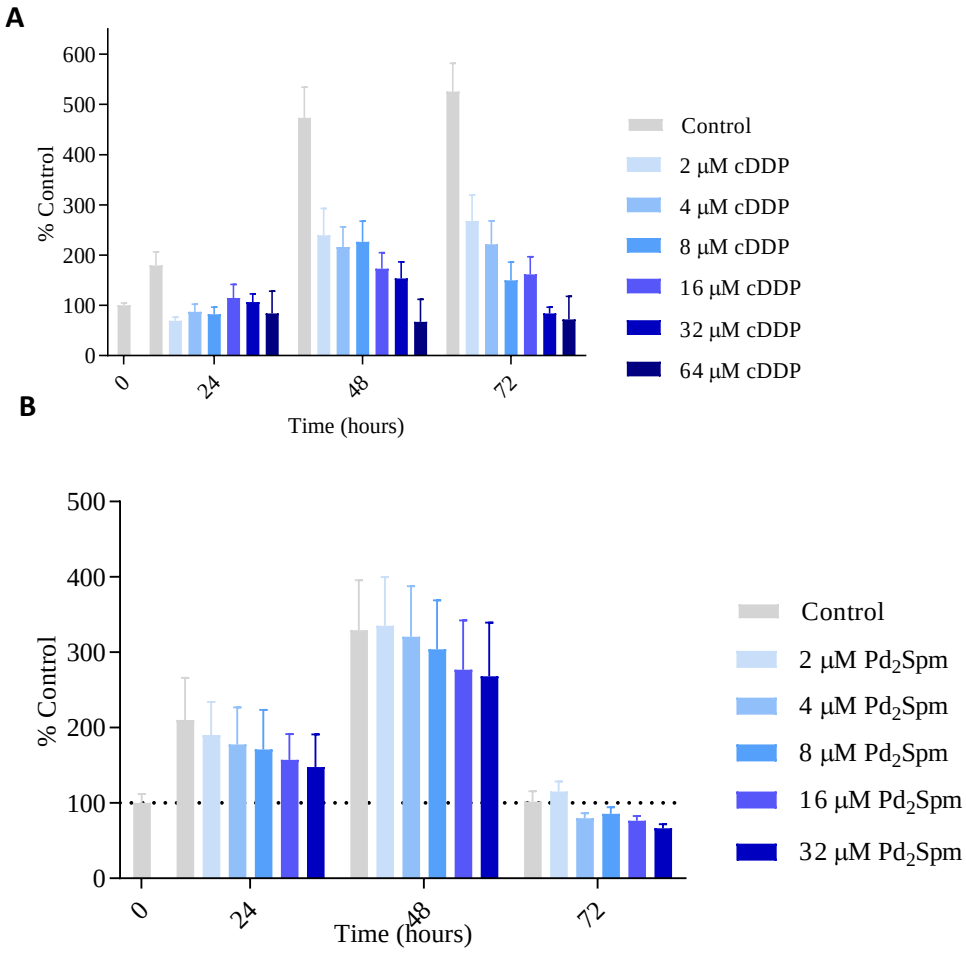

Figure 21: The protein content by amino acids binding with the SRB dye, after exposure to cDDP (A) and Pd_2Spm (B) for 24h, 48h and 72h on Caco-2 cell line, using 2,4,8,16 and 32 µM as the concentrations of test by performing the SRB assay already described in the Experimental section. The one-way ANOVA statistical analysis was used, and the Dunnett's post-test was carried out to verify the significance of the obtained results (*p<0.05, **p<0.01, ***p<0.001, #p<0.0001 vs the control for the same time-points).

a. Metabolic impact on cells

In order to obtain the spectral signatures of the cells, with and without the tested compounds, average FTIR and Raman spectra were acquired for control cells (without drug). This allows us to characterize each drug-induced variation observed in the spectra. The cisplatin-like dinuclear compound Pd_2Spm is able to interact with DNA, as a pharmacological target, leading to a series of changes in the conformation of this biopolymer. The effective mechanism of action of Pt- and Pd(II) based-complexes was shown to be effective by causing DNA disruption (mainly due to the presence of more than one metal center), through covalent binding to nucleophilic sites such as the N-7 atom of purine bases. Thus, interstrand cross- links are formed, possibly at more than one position, the 1,2- and 1,3-crosslink intrastrand adducts being the most frequent. These interactions will be responsible to drive cells to cell cycle arrest and inevitable apoptosis, once basic cell functions like DNA transcription and replication are compromised. [51]

Structural changes of the cisplatin-like agents (mono- *versus* polynuclear or variations in the bridging ligands), may be responsible for different DNA binding properties, namely the relative amounts of interstrand cross-links. This may explain the higher efficiency of the dinuclear metal complexes as compared to mononuclear ones (*e.g.* cisplatin). [39]

Both FTIR and Raman data evidenced that Caco-2 cells are sensitive to Pd_2Spm, once it was found to trigger several alterations in the bands assigned to the different cellular constituents. This interaction underlies the cytotoxic effect of the drug, by inducing changes in the biomolecular structure and consequently in cellular function.

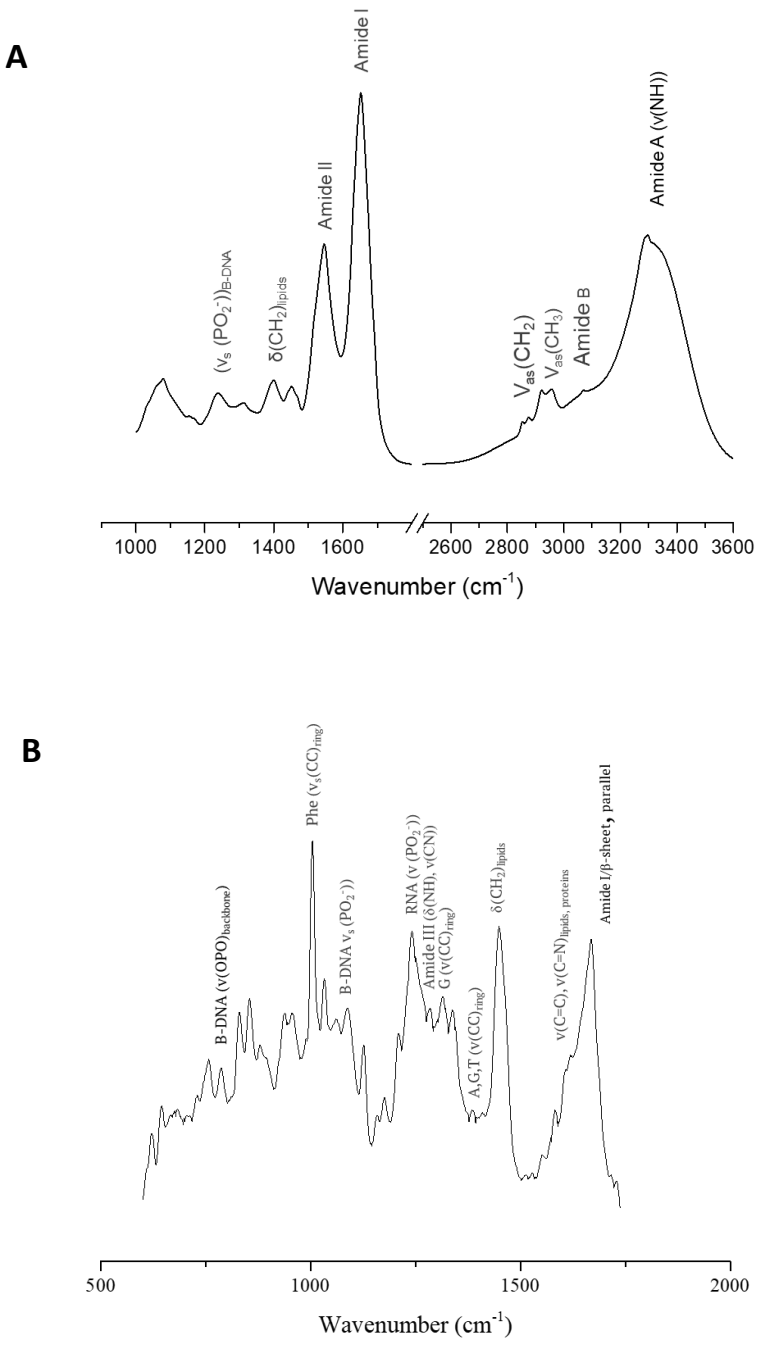

Figure 22: Average FTIR (A) and Raman (B) spectra of human of human colon cancer cells (Caco-2) (formalin-fixed).

Table 3: Raman and infrared bands for human colon cancer cells (Caco-2). Features from specific drug-prompted DNA and protein conformational rearrangements are in red. The signal mainly detected by infrared are shaded in grey.

Band (cm^{-1})	Assigment[0]			
	Nucleic acids	Proteins	Lipids	Carbohydrates
782-795	C,T,U ($v(CC)_{ring}$)			
812	RNA ($v(OPO)$ backbone)	Pro, Tyr ($v(CC)$)		
935-940		V(CC) (α-helix)	Glycolipids ($v(COC)$)	$v(COC)_{glycosidic}$
1092	B-DNA (v_s (PO_2^-))			
1095	Z-DNA (v_s (PO_2^-))		v(CC), v(CN)	V(CC), v(CC)
1099	A-DNA (v_s (PO_2^-))			$\delta(CH_2)$
1175	C,G,T ($v(CC)_{ring}$)	Tyr, Phe ($\delta(CH)$)		
1300	RNA/A,C ($v(CC)_{ring}$)			
1440-1450			$\delta(CH_2)$	
1550-1560		amide II ((δ(CN-H)/v(CN))		
1660	DNA (δ NH))	amide I (v(C=0))/α-helix	v(C=C)	
1612-1690		amideI/β-sheet, antiparallel		
1640-1650		amide I/random coil		
2850-2875		$V_s(CH_2)$	$v_s(CH)$, $_s(CH_2)$/CH)	Vs(CH), s(CH2)/(CH)
2900-2935		$V_{as}(CH_2)$	$V_{as}(CH_2)$	$V_{as}(CH_2)$
3300-3350			V(OH)	V(OH)

A- Adenine; C- cytosine; dG – deoxyguanine; G- guanine; Phe – phenylalanine; Pro – proline v – stretching; s – symmetric.

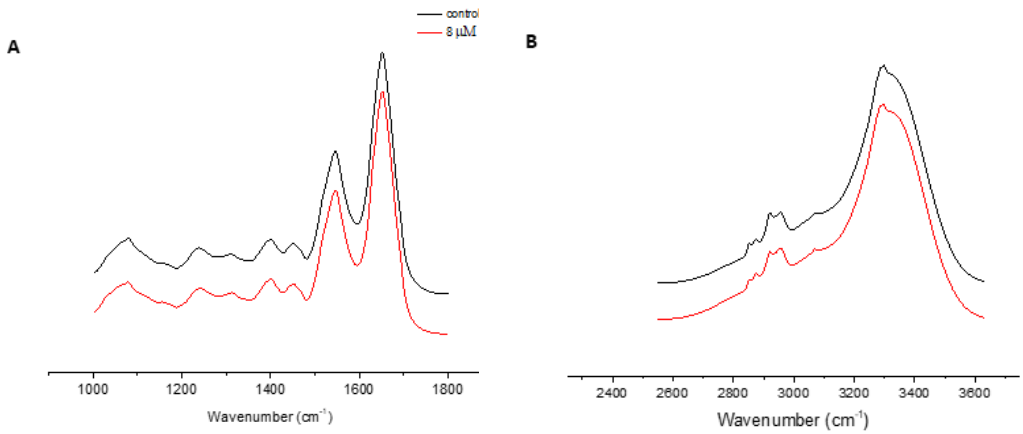

Figure 23: (A) Average FTIR spectrum of both untreated and Pd2Spm-treated (8 µM) human colon cancer cells (Caco-2), in the 1000 to 1800 cm-1 (B) and 2400 to 3800 cm-1 spectral regions.

Comparing both FTIR spectra of human colon cancer cells (Caco-2) (Figure 23 (A)), a slightly lower intensity was observed for the band assigned to B-DNA/deoxyribose (ν(CO), at 1078 cm^{-1}), evidencing a small influence of the drug on this specific mode.

In turn, the infrared high wavenumber region (Figure 23 (B)) showed no significant differences between untreated and treated cells, at a naked-eye observation. This confirms the importance of applying dediocated multivariate statistical analysis methodologies such as the principal component analysis (PCA), with a view to obtain an accurate and detailed information on the cytotoxic effect of Pd_2Spm, allowing to discriminate (when possible) between control and drug-exposed cells as well as the main variations elicited by the drug on the cellular constituents.

Figures 24 and 25 comprise the score and loading plots for the FTIR and Raman data, respectively, measured for control and Pd_2Spm-incubated Caco-2 cells. These plots evidenced a good discrimination between control and treated cells (at both 4 and 8 μM), mostly according to PC-3 (high wavenumber region) and to PC-2 (low wavenumber region).

The effect of the drug according to its concentration can be discussed once the two groups (for each concentration) appear separately in some the PC plots. This distinction will allow a further analysis according to the loading plots, which gives information on the effect of the compound on specific spectral bands (biomarkers) assigned to particular cellular components (*e.g.* proteins, lipids, DNA), namely at 1087 cm^{-1} - ν(CC), ν(CN) from polypeptide and glycogen chains, and $\nu_s(PO_2^-)$ from phospholipids.

The observed spectral changes showed that there is a strong interaction between Pd_2Spm and proteins, lipid and carbohydrate constituents of the cell. Variations on the proteins' structures might be caused by drug binding to N and S atoms within their amino acid residues, which was reflected in the characteristic Amide A band from β-sheet and random coil conformations observed at *ca.* 1650 cm^{-1}. Besides, an signal due to antiparallel β-sheet was detected at 1612 and 1691 cm^{-1}. An effect of the drug could also be observed in the Amide II mode (δ(CN-H)/ν(CN)) at 1544 cm^{-1}.

Through the analysis of the PC-3 loading, the significant drug impact on proteins, lipids and carbohydrates was reflected in both the symmetrical and ant symmetrical CH_2 stretching bands, at 2885 cm^{-1} and 2908 cm^{-1}, respectively. A major impact could also be observed on the ν(OH) vibrational mode due to lipids and carbohydrates (at 3307 cm^{-1} and 3340 cm^{-1}, respectively), as well as on the amide A signal at 3291 cm^{-1}.

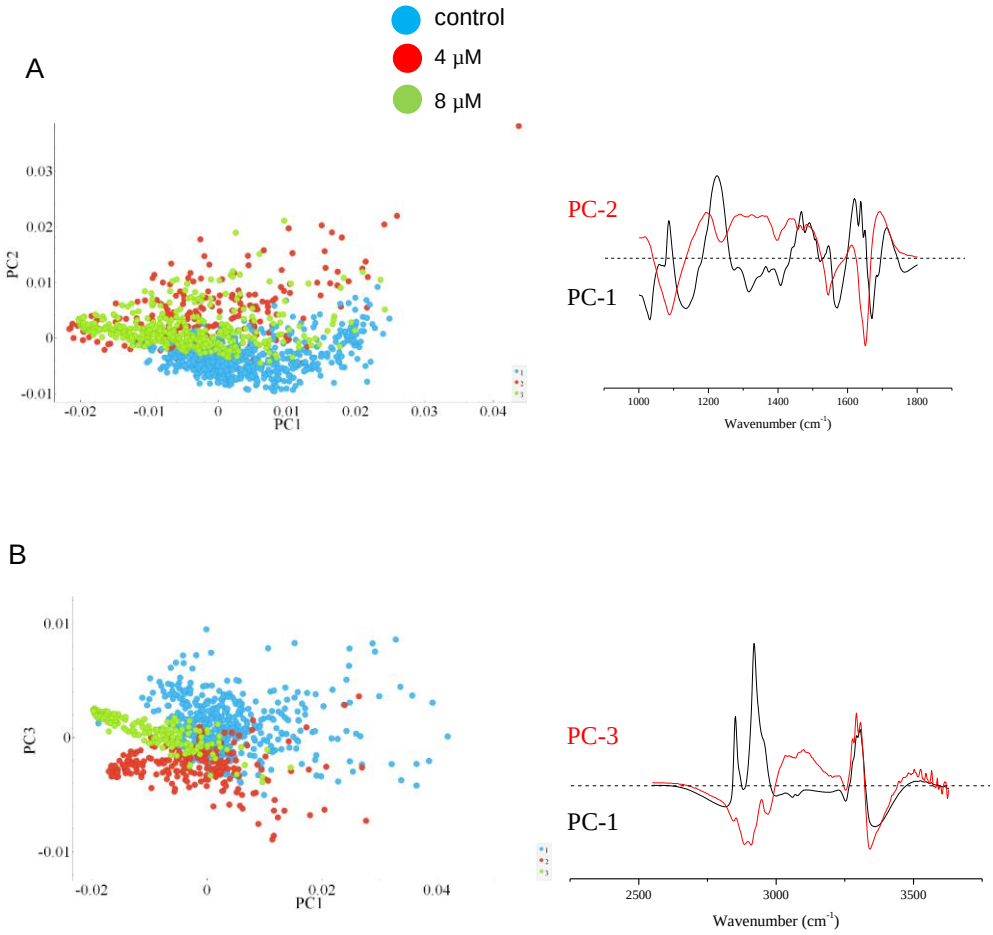

Figure 24: PCA score and loading plots of FTIR data of both untreated and Pd2Spm-treated (4 and 8 µM) human colon cancer cells (Caco-2), in the 1000 to 1800 cm-1 (A) and 2500 to 4000 cm-1 (B) spectral regions.

For clarity the loadings are offset, the dashed horizontal lines indicating zero loading.

Both infrared and Raman data reflected a clear dose-dependent response of Caco-2 cells to Pd_2Spm, with a major effect on intracellular metabolism, specifically on DNA (the preferential target), lipids and proteins. Vibrational modes of the DNA deoxyribose-phosphate backbone that were observed with higher sensitivity by Raman spectra, showed to be suitable biomarkers of drug activity for this type of metal-based agents, once they reflect base stacking and base-pairing within DNA´s double helix.

Differences were found between the two concentrations used (4 and 8 µM), discrimination between the control and Pd_2Spm treated samples having been achieved only for the highest concentration (through FTIR results). The cellular biochemical profile evidenced alterations on the drug´s main pharmacological target (DNA) – bands at 1097 cm^{-1} (ν_s (PO_2^-)) and 1655 cm^{-1} (δ NH_2). This corroborates the conformational

rearrangements due to drug binding (mainly to guanine and adenine), upon formation of stable interstrand long-range adducts [51].

The effect of Pd_2Spm on cellular lipids, in turn, can be explained by several factors: an enhanced lipid peroxidation; a reduction in the total lipid cellular content, once degradation processes can occur at the membrane (which translates to a decreased cell viability [42]); or change in the unsaturation degree of membrane phospholipids (associated to membrane fluidity and ultimately to metastasis). Furthermore, the influence of the drug on proteins could also be observed, *via* changes in the signals at 938 cm^{-1}, from $\nu(CC)_{\alpha\text{-helix}}$ and $\nu(COC)_{\text{glycolipids, carbohydrates}}$; at 1300 cm^{-1}, due to(ν(CC) from Pro and Tyr, and to RNA´s backbone; and at 1450 cm^{-1}, assigned to $\delta(CH_2)$ from lipids and polypeptide chains. In particular, variations in the bands due to biomolecule´s backbones may evidence drug-induced dynamical changes, fluctuations and/or distortions [51], such as those detected at 832 cm^{-1} ($\nu(OPO)_{\text{DNA backbone}}$). Also, intense bands from ring breathing modes can be explained by an increased exposure of aromatic amino acid side chains to the external medium due to protein drug-prompted unfolding [42].

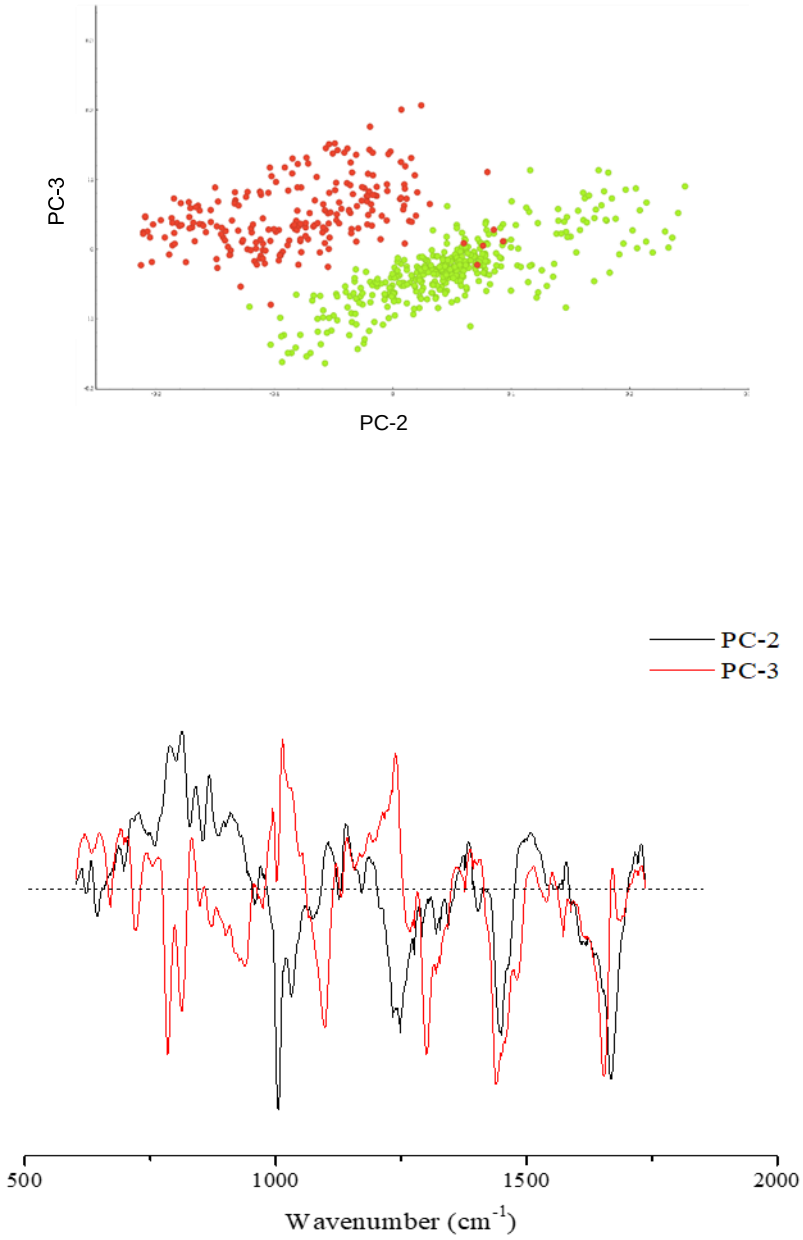

Figure 25: PCA score and loading plots of Raman data (600 to 1800 cm-1) of both untreated and Pd2Spm-treated (4 and 8 µM) human colon cancer cells (Caco-2).

For clarity the loadings are offset, the dashed horizontal lines indicating zero loading.

In summary, the overall effect of Pd_2Spm on the vibrational features from the main cellular components in Caco-2 cells is consistent with data reported in the literature for an epithelial human breast cancer cell line MDA-MB-231, where it was also observed by vibrational spectroscopy techniques (FTIR and Raman) that cells

were sensitive to the metal-based tested compounds, including Pd_2Spm and cisplatin, with main variations observed in the DNA spectral profile[51]. Changes in the bands specific for cytoplasmic proteins and lipids were also detected, consistent with conformational alterations such as membrane disruption and lipid accumulation, which is in accordance with the results obtained in the present work.

This type of experiments can help to understand, at a molecular level, the chemotherapeutic effect and the cellular response to the drug, by monitoring changes in the cellular biochemical profile that underlie functional impairment and apoptotic cell death.

6. Conclusion

The present work aimed to investigate the effect of cisplatin and the dinuclear Pd(II) spermine complex (Pd_2Spm)) towards a human cancer cell line from colon adenocarcinoma – antiproliferative and cytotoxic activities, and metabolic impact. This effect was evaluated at a biological level through *in vitro* methods – MTT and SRB assays – while further biochemical impact was determined by vibrational spectroscopy (Raman and infrared) of fixed cells to obtain the vibrational signature of drug-treated and untreated samples.

In accordance with the biological assays, 16 and 32 μM were found to be the best Pd_2Spm concentrations for inducing significant growth-inhibitory and cytotoxic effects on Caco-2 cells.

A major biochemical effect of Pd_2Spm was found to be on DNA, which is in accordance with the known mechanism of action of mononuclear platinum agents (such as cisplatin), as well as of polynuclear Pt(II) and Pd(II) complexes (recently investigated at QFM-UC), DNA being considered the main pharmacological target of these metallodrugs. In addition, an impact on lipids and proteins was also unveiled upon drug exposure, which reveals distinct types of targets for the Pd-complex within the cellular medium. PCA analysis of the spectral data led to a discrimination between drug-treated and untreated cells, although no differentiation between both concentrations used was possible.

The combined use of *in vi*tro methods and spectroscopic techniques is an innovative approach in this type of drug development studies, aiming to link biological changes to spectral signatures of cellular biochemistry upon exposure. This may contribute to an improved understanding of the mode of action of these metal-based potential anticancer agents, at a molecular level, with high accuracy and sensitivity.

However, further investigations are needed in order to achieve a more detailed analysis of the antitumor potential of Pd(II) complexes in human cancer cell lines, namely by testing distinct types of cancers (to assess specificity of the drug effect) and different complexes (structurally modified in either the ligand or the metal centers)

7. References

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