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Synthesis and characterization of novel tumor-inhibiting platinum(IV) complexes

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Abstract

Cisplatin was introduced as chemotherapeutic substance after being approved by the FDA in the late 1970s. Despite its curative potential, cisplatin's major drawbacks remain its severe side-effects and either acquired or intrinsic resistance of several cancer types. Thus, further development of novel platinum-based anticancer drugs was carried out. Up to date, two additional platinum(II) agents gained worldwide market approval as chemotherapeutics, namely carboplatin and oxaliplatin. During the last decades, not only development of platinum-based agents in the oxidation state +II, but also that of platinum(IV) compounds gained more attention, since the latter show several advantages over their precursors: They allow a better pharmacological fine-tuning due to six instead of four coordination sites, and they open up the possibility of oral administration due to their kinetical inertness. Additionally, systemic toxicity might be reduced since platinum(IV) compounds are activated by reduction inside the tumor tissue.

The content of this PhD work was divided in three different projects: In the first project, reduction of four ^{13}C- and ^{15}N labelled platinum(IV) complexes, synthesized by oxidation and functionalization of either cisplatin or carboplatin, in cancer cell lysates was observed by means of 2D NMR spectroscopy.

Since several ferrocenyl containing compounds show cytotoxic activity, the aim of the second project was to synthesize and characterize the first anticancer platinum(IV) compounds bearing one or two ferrocene moieties in the axial position. By loss of axial ligands upon reduction inside the cell, the antiproliferative potential of both platinum and ferrocenyl compounds could be combined; for all of the synthesized complexes, IC_{50} values in the low micromolar range in SW480 and CH1 human cancer cell lines could be detected.

In the last project, platinum(IV) complexes were coupled onto biodegradable polyphosphazenes in order to design tumor-targeting agents by taking advantage of the EPR effect. Preliminary *in vitro* investigations showed promising antiproliferative activity as well as high cellular accumulation of the polymer-platinum(IV) conjugate in three different human cancer cell lines.

Zusammenfassung

Cisplatin wurde, nachdem es in den späten 1970er Jahren von der FDA zugelassen wurde, als Chemotherapeutikum eingeführt. Trotz dessen heilender Wirkung bleiben ernsthafte Nebenwirkungen und entweder erworbene oder intrinsische Resistenz die größten Nachteile von Cisplatin. Folglich wurde weiter geforscht, um neue platin-basierte krebshemmende Medikamente zu entwickeln. Bisher erhielten zwei weitere Platin(II) Wirkstoffe, nämlich Carboplatin und Oxalipaltin, die weltweite Zulassung als Chemotherapeutika. In den letzten Jahrzehnten gewann nicht nur die Entwicklung platinbasierter Wirkstoffe in der Oxidationsstufe +II, sondern auch Platin(IV) Verbindungen mehr Aufmerksamkeit, da diese einige Vorteile gegenüber ihren Vorgängern aufweisen: Sie ermöglichen eine bessere pharmakologische Abstimmung aufgrund von sechs anstelle von vier Koordinationsstellen, und sie ermöglichen aufgrund ihrer kinetischen Inertheit eine orale Verabreichung. Da Platin(IV) Komplexe durch Reduktion in der Zelle aktiviert werden, kann zusätzlich die systemische Toxizität reduziert werden.

Der Inhalt dieser Dissertation wurde in drei verschiedene Projekte unterteilt: Im ersten Projekt wurde die Reduktion von vier ^{13}C- und ^{15}N-markierten Platin(IV) Komplexen mittels 2D Spektroskopie in Krebszelllysaten untersucht.

Da einige ferrocenhältige Verbindungen zytotoxische Aktivität aufweisen, war die Synthese und Charakterisierung der ersten krebshemmenden Platin(IV)-Verbindungen, welche einen oder zwei Ferrocenreste beinhalten, das Ziel des zweiten Projekts. Durch die Abspaltung der axialen Liganden bei der Reduktion in der Zelle könnte die antiproliferative Wirkung der Platin- als auch von der Ferrocenyl-Verbindung kombiniert werden; für alle synthetisierten Komplexe konnten in den humanen Krebszelllinien SW480 und CH1 IC_{50} Werte im niedrigen mikromolaren Bereich gefunden werden.

Im letzten Projekt wurden Paltin(IV)-Komplexe an biologisch abbaubare Polyphosphazene gekoppelt, um tumorspezifische Wirkstoffe durch die Ausnutzung des EPR Effekts herzustellen. Erste *in vitro* Untersuchungen zeigten eine vielversprechende antiproliferative Aktivität sowie eine hohe zelluläre Akkumulation des Polymer-Platin(IV)-Konjugats in drei verschiedenen humanen Krebszelllinien.

Table of Contents

1. Introduction

1.1. Cancer

1.1.1. Defining cancer

The term "cancer" is used for comprising a family of more than 200 diseases involving the ability of uncontrolled cell proliferation. All of those diseases have common characteristics: Cancer starts when a cell undergoes malignant changes and turns into an abnormal cell which does no longer depend on external growth stimulation and hence grows unregulated into a mass of cells. A normal cell divides, replaces damaged or old cells and undergoes apoptosis controlled by a regulatory system. After approximately 50 mitoses, a healthy cell dies due to shortening of *telomeres* – the linear ends of chromosomes – after each DNA replication. Resistance to those apoptotic pathways is one of the main characteristics of cancer cells. By repression, subversion and destruction of healthy cells due to uncontrolled spread of cancer cells, the body's functions are detracted.[1]

Additionally, some types of cancer cells have the ability to metastasize, i.e. to detach from the *primary tumor*, spread into the body and generate a *secondary tumor*. The earlier cancer is diagnosed and treated, the higher the chances for cure of the affected person are.[2]

1.1.2. Metastatic spread

The *primary tumor* develops an own blood vessel system – a process called *angiogenesis* - in order to provide the required nutrients for the tumor tissue. The newly generated blood vessels don't only serve as nutrition system for the tumor, but also generate an escape route for tumor cells into the body's blood stream.[3] Some types of cancer cells have the ability to leave the primary tumor via the formed vessels and to form a secondary tumor in tissue located in a distant part of the body.[4] Angiogenesis is crucial for metastatic spread, since the tumor's vessels are highly permeable and immature with little basement membrane; there is evidence for direct correlation between vascular density and development of metastatic cells. Only 1 out of 1000-10000 tumor cells has the ability to metastasize, since various obstacles have to be overcome before this process can take place. Escape from the primary tumor has

to be successful, followed by survival in the blood stream, response to growth factors and finally entrance into the target organ and inducement of tumor angiogenesis.[5,6]

Metastatic spread is the most feared course of the disease, as its occurrence decreases patient survival time and response to conventional therapies.[7]

1.1.3. Types of cancer

Cancer is named by the organ in which the tumor occurs first. As there is a broad range of tumors, subdivision in the following categories was chosen1[,8,9]:

Cancer Type	Origin
Carcinoma	Epithelial cells that cover or line organs
Sarcoma	Connective and supportive tissue (i.e. bones, fat, muscles)
Leukemia	Blood or bone marrow; increase of immature white blood cells
Central nervous system cancer	Brain or the spinal cord
Lymphoma	Lymphatic tissue (B/T lymphocytes)
Myeloma	Bone marrow

The tumor types are classified by the different types of cells, according to their job in the human body.1 The majority of all cancers, rather 85%, derive from epithelial cells. In 2012, 8.2 million people died of cancer worldwide and 14.1 million new cases were diagnosed. The most frequent cases occurring in males are lung (17%), prostate (17%), colorectal (10%), and stomach cancer (10%). However, the most common cancer diagnosed in females is breast cancer (23%), followed by colorectal, cervix, and lung cancer, each of them accounting for 9%. Occurrence of cervix cancer could be reduced noticeably in countries with screening programs.[10,11]

1.1.4. Risk factors

The causes for mutation of cells are various and could not be completely investigated to date. Despite our imperfection of knowledge, several risk factors for cancer development could be identified:

- 5-10% of all diagnosed cancers are incident to ***genetic factors***.[12]

- Lifestyle plays a major role in carcinogenesis: ***Smoking*** was identified as the primary reason for lung cancer and increases the risk of development of additional thirteen types of cancer.[12] An unambiguous connection between ***diet*** and cancer development could be found in the past decades. Carcinogens could be found directly in food or they are produced during cooking, such as nitrates, nitrosamines, dioxins, and amino acids.[12] Increased consumption of red meat was found to be linked to 70% of diagnosed colorectal cancer cases. Another risk factor is ***obesity***, which is associated with increased mortality i.a. from colon, breast, kidney, prostate, and liver cancer.[12, 13] *In vivo* experiments have proven calorie restriction to be an effective strategy for suppression of carcinogenesis.[14]

- Several exogenous ***environmental factors*** play a major role in carcinogenesis: *Infection*-caused cancers are mainly caused by viruses, and represent 17.8% of neoplasms. It should be mentioned that the indicated percentage varies from 10% in high-income countries to 25% in low-income countries.[12] The main types of viruses which are known to be incident to cancer development are Hepatitis B and C viruses, HPV, HIV, and human T-cell lymphotrophic virus I. Furthermore, carcinogenesis may be caused by schistosoma, liver flukes, or *Helicobacter pylori*.[15] One further exogenous factor is ionizing or non-ionizing *radiation*, causing approximately 10% of all cancer cases. Occurrence of several cancer types, including skin cancer, leukemia, lung and breast carcinomas were brought in connection with radiation.[16] Furthermore, *environmental pollution*, including food additives (pesticides, nitrates etc.) and exposure to air pollutants such as polycyclic aromatic hydrocarbons (PAHs) increase cancer risk.[12,16]

Human biology is too complicated to understand and find out which of the numerous potential factors contributed actually to carcinogenesis. Besides genetic factors, lifestyle and exogenous factors, the patient's age and public health care play a major role whether a person

will develop cancer in a lifetime or not. In the US, cancer is the leading cause of death among men and women aged 40 to 79 years. As already mentioned, early diagnosis and screening programs are decisive for cancer prevention. [11,17]

1.1.5. Cancer treatment

During the past decades, several progresses could be achieved in development of cancer treatment. After diagnosis, a number of factors influence the choice of treatment. First, the organ in which the primary tumor occurred has to be determined. The stage of cancer has to be categorized according to the internationally recognized *TNM* (tumor, node, metastasis) *classification system*. In this way, tumor size, involvement of lymph nodes and assessment whether metastatic spread has occurred or not has to be identified.2 The patient's age and physical condition also plays a role for choosing a suitable treatment method:[18,19]

Local methods are the method of choice for neoplasms which can be localized in the body and are accessible for the medical practitioner.

Chemotherapy is a widely used treatment method for cancers which cannot be localized or removed completely by surgery. Administration of cytostatics may as well serve as adjuvant treatment after or before a surgery.

In contrast to cytostatics, ***tumor-targeting drugs*** have the ability to distinguish between healthy and cancer cells and thus attack cancer cells more specifically and reduce side-effects.

Immunotherapy is another type of treatment, helping the endogenous immune system by stimulation, to fight the tumor.

If the diagnosed tumor first occurred in hormone-sensitive tissue, a ***hormone-based anti-tumor therapy*** may be worthy of consideration for the affected person. In this case, treatment with synthetic hormones or antihormones is possible due to the high density of hormone receptors on the tissue surface.

If leukemia is diagnosed, treatment of several types demand destruction of the immune system by cytostatics or radiation and following ***stem cell transplantation***. Peripheral blood or bone marrow may be obtained by the patient himself – during the window of time when the disease is not active – or by a donor.

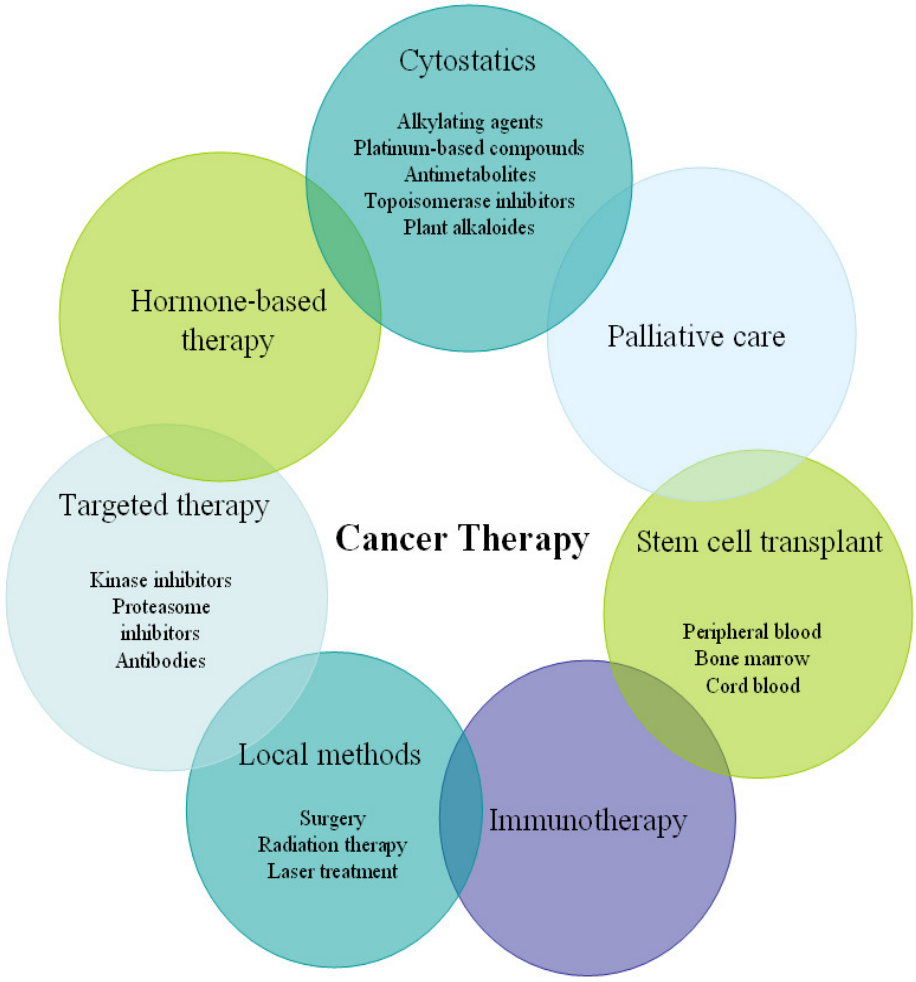


Figure 1-1. Depiction of different strategies of cancer treatment in nowadays clinical practice.

1.1.6. Tumor-specific characteristics

For development of more sophisticated anticancer drugs, first the biology of cancer has to be analyzed. Many advances have been made in cancer research during the past decades, resulting in a remarkable body of knowledge. In this section, a short overview of distinguishing features of cancer cells will be given:

Self-sufficiency in growth signals. Normal cells are not able to proliferate without exogenous growth stimulation. However, cancer cells are able to reduce their dependence on mitogenic growth factors by producing their own growth signals.[20]

Continuous angiogenesis. When the tumor reaches a size of 2-3 mm, angiogenesis is induced in order to ensure the supply with the needed nutrients. For healthy cells, development of the vasculature and blood system is regulated; tumor neovasculature shows

several irregularities compared to normal tissue: The blood vessels are leaky and irregular in shape, slow venous return has been observed, its endothelial cells show exceptionally high levels of proliferation and they are arranged unevenly.[21] Moreover, tumor vessels show poor lymph drainage and insufficiency of blood plasma components, thus enabling macromolecules and lipidic particles to enter and remain in the tumor tissue. This effect is called EPR (enhanced permeability and retention) effect, and is a key mechanism for design of tumor-targeting anticancer compounds.[22,23]

Tissue invasion and metastasis. Some cancer types have the ability to undergo this multistep process. It begins with escape of cancer cells into nearby blood and lymphatic vessels, continued by transit of the prevailing systems until they reach distant tissue – a process called *extravasation* – where new cancer cells and micrometastatic lesions are formed.[21]

Evading apoptosis. In healthy cells, sensors monitor occurrences inside the cell and activate the programmed cell death pathway if irregularities occur. Most types of cancer cells are able to evade the apoptotic pathway due to alterations of the apoptotic factors. The most common detected strategy for circumvention of apoptosis is mutation of the p53 tumor suppressor gene.[20,21]

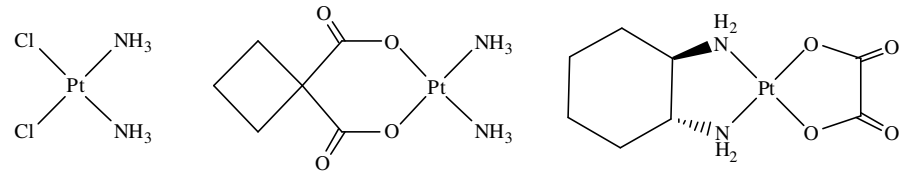
Insensitivity to anti-growth signals. Cancer cells possess the ability to evade apoptotic signals; disruptions of the retinoblastoma protein (pRb) pathway lead to insensitivity towards pRb and its relatives, p107 and p130.[20,21]

Unlimited proliferation potential. The number of consecutive growth-and-division cycles is limited in healthy cells before entering a crisis phase which leads to cell death. With each cycle, the telomeric DNA is shortened until its protective function is lost. Hence, after having passed several cell generations, normal cells undergo apoptosis. In contrast to healthy cells, the majority of cancer cells possess considerable levels of telomerase, a DNA polymerase adding telomeres to the ends of DNA. Thus, the replicative potential of most cancer cells is limitless.[20,21]

1.2. Metal-based Chemotherapy

1.2.1. Overview

In 1844, the Italian chemist Michele Peyrone was the first to synthesize cis-diamminedichloridoplatinum(II) (Figure 2-1).[24] At that time, he couldn't imagine the antiproliferative potential of his newly designed compound. More than one century later, Barnett Rosenberg was the one to rediscover accidentally cisplatin and its ability to suppress growth of *Escherichia coli* without killing them.[25] It was assumed that platinum compounds may have the ability to stop growth of fast growing tumor cells. Soon, a variety of different platinum(II) complexes were synthesized and tested as antitumor compounds; the *cis*-conFigured compounds showed cytotoxic activity, while the *trans*-conFigured did not.[26] In 1979, cisplatin gained worldwide market approval as chemotherapeutic; during the following decades, thousands of cisplatin analogues have been synthesized in order to reduce its severe side-effects and to overcome intrinsic or acquired resistance.[27,28] Ten years later, worldwide approval for carboplatin (Figure 2-1), a second-generation platinum compound, as anti-cancer agent against ovarian cancer was granted. Oxaliplatin (Figure 2-1) was introduced worldwide in clinical practice in 2002, being used for treatment of colorectal cancer.[29]



Cisplatin **Carboplatin** **Oxaliplatin**

Figure 2-1. Worldwide approved platinum-based chemotherapeutic agents.

Although cisplatin is involved in nearly 50% of chemotherapeutic treatments, its major problems remain intrinsic and acquired drug resistance and several severe side-effects.[30,31] Hence, intensive research is taking place in order to develop new, improved chemotherapeutics with reduced side-effects compared to cisplatin. Not only development of platinum(II) compounds is progressing, but also platinum(IV) compounds have been identified as active antineoplastic agents during Rosenberg's studies in the 1960s. In general, platinum(IV) compounds show several advantages over their precursors and open up the possibility of oral administration.[32]

During the last decades, the research area of complexes with antiproliferative potential has been extended to several other transition metal compounds. Up to date, various promising complexes have been designed: Ruthenium and gallium based complexes made it even up to clinical trials during the last years;[33,34] furthermore, osmium-, molybdenum-, copper- and rhodium-based complexes have been identified as agents with antiproliferative properties.[35] Although still an unproven chemotherapeutic system, ferrocene-containing agents have already displayed promising results *in vitro*.[36] In the following sections, some of the above mentioned topics and metals will be described in more detail. A timeline containing the major developments of platinum-based anticancer agents is given in Figure 2-2.

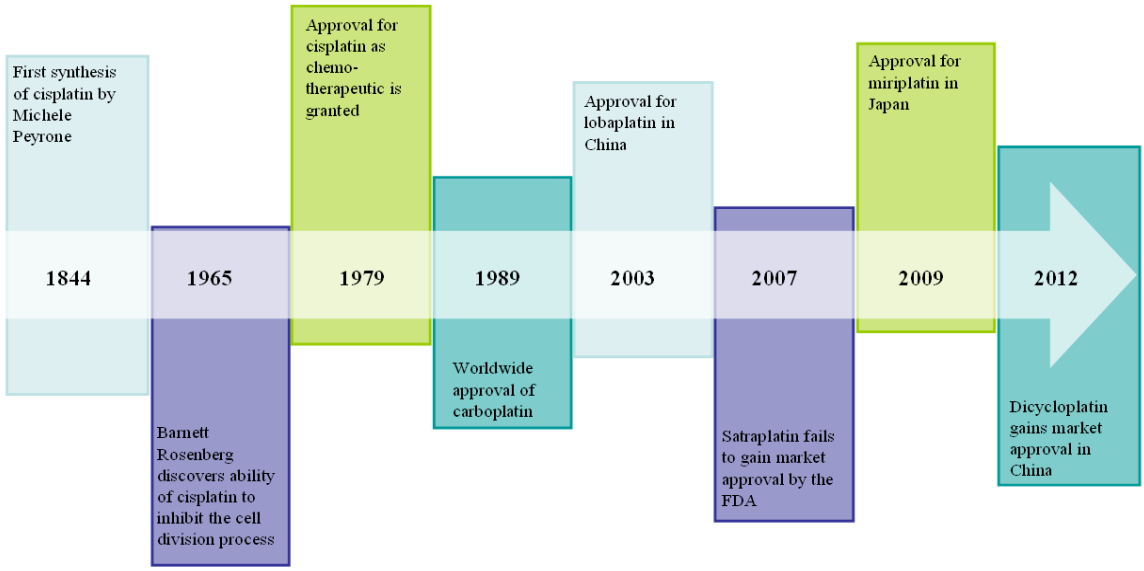


Figure 2-2. Timeline of the development of platinum-based anticancer therapeutics.

1.2.2. Cisplatin

Although cisplatin is one of the best studied complexes, its exact behavior and interactions within the intra- and extracellular environment in the human body remains unclear. In this chapter, mechanisms as well as new insights concerning the mode of action, resistance and side-effects of cisplatin will be discussed.

1.2.2.1. Mode of Action

1.2.2.1.1.. Administration and Cell Uptake

Administration and behavior in the blood stream. Cisplatin is administered intravenously. The desired behavior of the drug would be to circulate via the blood stream inside the body until it enters the cancer cell as the initial uncharged complex, where it can unfold the desired effect. In reality, it has been proven that cisplatin undergoes reaction with various constituents of the blood, including human serum albumin (HSA), L-cysteine, and hemoglobin.[37,38] Since the mentioned components possess thiol groups, they are most suitable for reactions with cisplatin due to the HSAB (hard and soft acids and bases) principle.[39]

Cisplatin binding to HSA may lead to side-effects, deactivation, or enhanced response to the drug. Additionally, it has been reported, that cisplatin binds not only to blood plasma components, but also to red blood cells.[40] Several investigations showed that 24 hours after infusion in patients, up to 93% of the drug was bound to plasma proteins.[37,41]

Reaching the cell. After transportation of the drug through the blood stream, a part of the initially administered amount reaches the cell membrane. Up to date, the exact entrance mechanisms of cisplatin are not completely defined. However, several accumulation pathways could be identified (depicted in Figure 2-3):

a. **Passive Diffusion.** It has been assumed for years, that passive diffusion plays the main role in cisplatin uptake. However, it could be confirmed that not only passive diffusion, but also active transport is decisive for cisplatin to reach the inner part of a cell.[29]

b. **Copper transporter-1 (CTR-1).** CTR-1, a plasma-protein transporter, plays a major role in cell uptake of cisplatin.[42] Lower levels of this transporter, which is implicated in copper homeostasis, lead to increased cisplatin resistance. CTR-1 seems to bind via its histidine- and methionine-rich region to cisplatin during the transport.[43] Not only influx of cisplatin is mediated by copper transporters, but also a part of the drug's efflux is carried out by copper efflux transporters, namely ATP7A and ATP7B.[44]

c. **Endocytosis.** Cisplatin uptake by endocytosis is also regarded as a possible mechanism.[45] During this process, the drug is attached to the cell membrane, followed

by invagination and membrane fusion. This route of cell entrance has been proven especially when cisplatin was encapsulated in nanoparticles.[46,47]

d. Organic cation transporter (OCT). Another active transport system involved in accumulation of cisplatin is the organic cation transporter (OCT). There are three OCTs (OCT1, OCT2, and OCT3) known so far, whereas only OCT1 and OCT2, containing 68-69% of sequence homology in humans, participate in cisplatin transport. Uptake of cationic species with M_r<400 Da is modulated via OCTs. According to *in vitro* studies, cisplatin accumulation is enhanced in OCT2 overexpressed cells compared to OCT1 overexpression.[43,48]

e. Sodium-dependent process. A process involving sodium and potassium ATPases has been identified as another potential cisplatin uptake mechanism.[29] By increasing the sodium- and potassium-level in the extracellular medium *in vitro*, cisplatin uptake was inhibited significantly.[49] Nevertheless, the exact mechanism of this process remains vague.

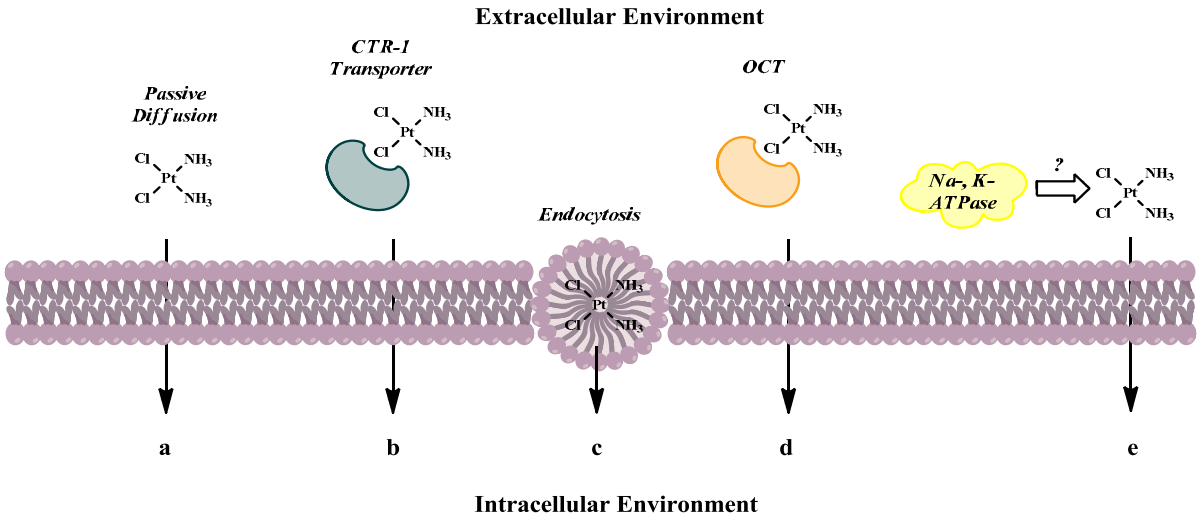


Figure 2-3. Depiction of different mechanisms for cisplatin to enter the cell: Entrance into the cell is possible via **(a)** passive diffusion, which was thought to be the main entrance pathway several years ago. Active transport is enabled either by **(b)** plasma-protein copper transporter CTR-1 or by **(d)** organic cationic transporters OCT1 and OCT2, whereas OCT2 seems to play the major role. The drug may also pass the cell membrane by **(c)** endocytosis or by a **(e)** sodium- and potassium-dependent pathway; the exact mechanism couldn't be identified to date.

1.2.2.1.2. Inside the cell

Aquation of cisplatin. Once inside the cell, the chloride ion concentration of ~100 mM in the blood stream is significantly lowered to 2-30 mM. Under these conditions, replacement of one or both chlorido ligands by water is facilitated.[50] The monoaquated, positively charged complex is believed to be the main species binding to DNA, since water is a better leaving group than chlorido ligands; aquation steps of cisplatin inside the cell are depicted in Figure 2-4.[51] It could be shown that the diaquated species plays a minor role in this process, since the second aquation step takes a longer period of time.[52]

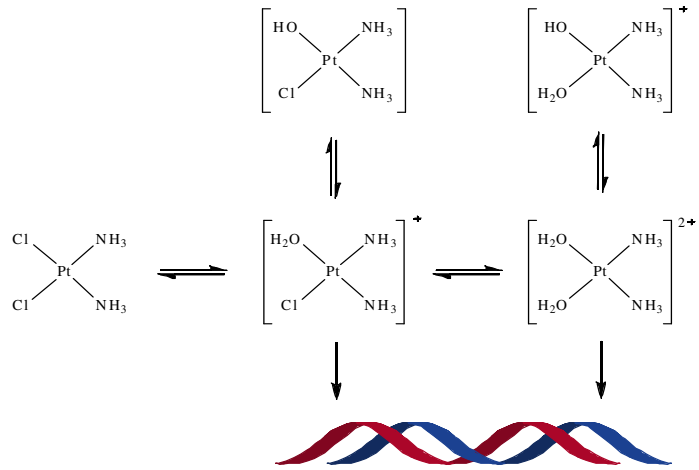
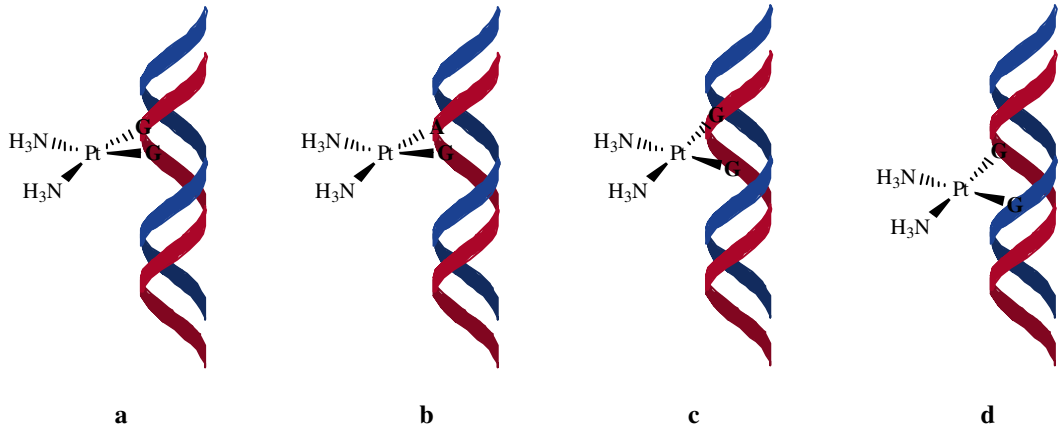


Figure 2-4. Possible aquation steps of cisplatin inside the cell. The monoaquated form plays the major role in DNA binding.

Binding to DNA. As already mentioned above, mainly the monoaqua species coordinates to its main cellular target DNA. In this activated form, cisplatin is able to bind to the N7 position of guanine or - in lower extent - to other purine bases.[43] 90% of the formed cisplatin DNA adducts are intrastrand cross-links with adjacent guanine and adenine bases (Figure 2-5).

Additional products are 1,3-intrastrand adducts and interstrand cross-links; monofunctional products could be identified as well in low extent.[53] By examination of human cells after treatment with cisplatin, it was found that only a small percentage of the drug reaches and binds to DNA after cell entrance.[54]



a **b** **c** **d**

Figure 2-5. Most frequently formed cisplatin-DNA adducts: **a.** 1,2-GpG intrastrand, **b.** 1,2-ApG intrastrand, **c.** 1,3-GpG intrastrand, and **d.** GpG- interstrand crosslink.

Binding to other intracellular targets. It is generally accepted that binding of cisplatin to DNA is the main trigger for cell death and thus also for its cytotoxic activity. Nevertheless, there is evidence for reaction of the drug with other cellular targets, since only a small fraction reaches genomic DNA. Cisplatin also interacts with mitochondrial DNA, phospholipids, and other intracellular agents containing thiol groups, such as glutathione, peptides, proteins, and RNA.[54, 55] Anticancer potential of cisplatin may be increased by binding to mitochondrial DNA, as in contrast to genomic DNA binding, mitochondria are unable to carry out nucleotide excision repair.[53,56]

Efflux. Cytotoxic activity of cisplatin is not only dependent on entrance into the cell, but staying inside the intracellular environment is also crucial for the drug's cytotoxic potential. Therefore, enhanced efflux mediated by several transporter proteins results in decreased cytotoxicity.[56]

Exporter proteins MRP1-7 have been associated with drug-detoxifying pathways. MRP2 seems to play the most important role amongst the MRP gene family concerning cisplatin efflux.[57 , 58] Apart from this gene family, two copper P-type adenosine triphosphate transporters, namely ATP7A and ATP7B, are involved in cisplatin efflux mechanisms. Cisplatin-resistant cells display enhanced levels of both transport protein MRP2 and copper-transport ATPase genes ATP7A and ATP7B.

Besides, other proteins involved in cisplatin efflux and resistance could be identified, such as ATP-binding cassette multidrug transporters, copper-transporting P-adenosine triphosphatases, or multidrug resistance-associated protein.[56]

1.2.2.1.3. Cisplatin resistance

One of the major drawbacks in platinum-based cancer therapy, besides undesired side-effects, is intrinsic or acquired resistance. If a certain type of cancer is already resistant to cisplatin before undergoing chemotherapy, it is called to be *intrinsically* resistant; however, *acquired* resistance occurs during treatment.[59] Typical intrinsically resistant cancer types are non-small cell lung (NSCL) and colorectal tumors, while small cell lung and ovarian cancers tend to develop cisplatin resistance upon treatment.[59,60] The key mechanisms of cisplatin resistance, which could be identified so far (depicted in Figures 2-6 and 2-7) and will now be described.

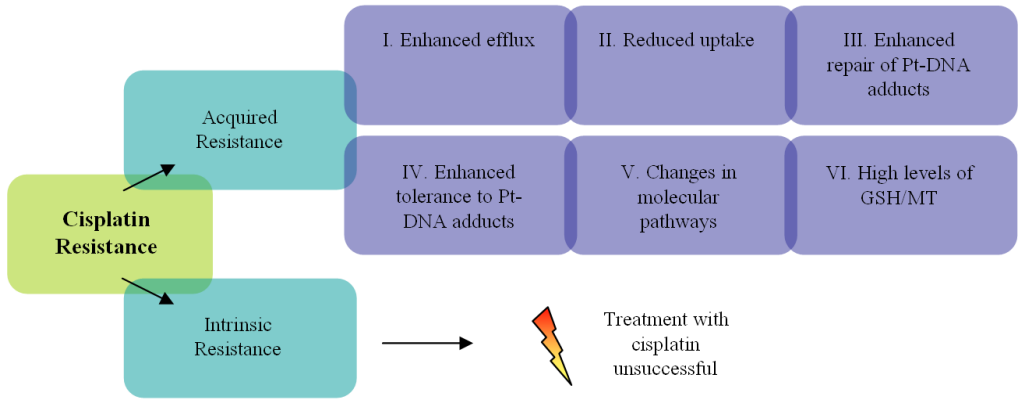


Figure 2-6. Pathways of cisplatin resistance and underlying mechanisms.

Reduced uptake and enhanced efflux. Reduced cisplatin accumulation is a known phenomenon of many resistant cell lines. Dependence on the content of cell membrane fluidity has been suggested to be involved in reduced drug uptake. Insufficient concentration of CTR-1 in tumor cells contribute to reduced uptake of cisplatin as well.[61] Enhanced efflux of cisplatin may occur due to increased export (described in chapter 1.2.2.1.2.), exocytosis, or secretion.[62]

Enhanced repair and tolerance of Pt-DNA adducts. Cisplatin-DNA adducts are repaired by the nucleotide excision repair (NER) system after recognition by proteins and enzymes.[63] The main protein associated with cisplatin DNA crosslink repair is the excision repair cross-complementation group 1 protein (ERCC1). Another key mechanism in cisplatin resistance is loss of the mismatch repair pathway (MMR); an increased tolerance of cisplatin and carboplatin DNA adducts occurs upon loss of the MMR system.[64]

Binding to glutathione and metallothionein. Glutathione (GSH) and metallothionein (MT) are cysteine-containing molecules, bearing a multitude of thiol groups which are likely to bind to cisplatin. Thus, enhanced levels of those two molecules as a result of intrinsic or acquired resistance of certain cancer types result in binding and detoxification of cisplatin.[64] The exact relevance of GSH and MT levels remains unknown.[60]

Changes in molecular pathways. Mediation via proteins, such as anti-apoptotic proteins bcl-2 and bcl-x_L, tumor suppressor protein p53, or members of the inhibitor of apoptosis family IAP, may prevent apoptosis upon binding of cisplatin to genomic DNA. Comparison of several cancer cell lines assumed that p53 mutated cell lines were more resistant to cisplatin than cell lines without p53 mutation. In addition, cancer patients with p53 wild-type tumors showed allover better response to cisplatin than patients suffering from tumors with p53 mutations.[64,65]

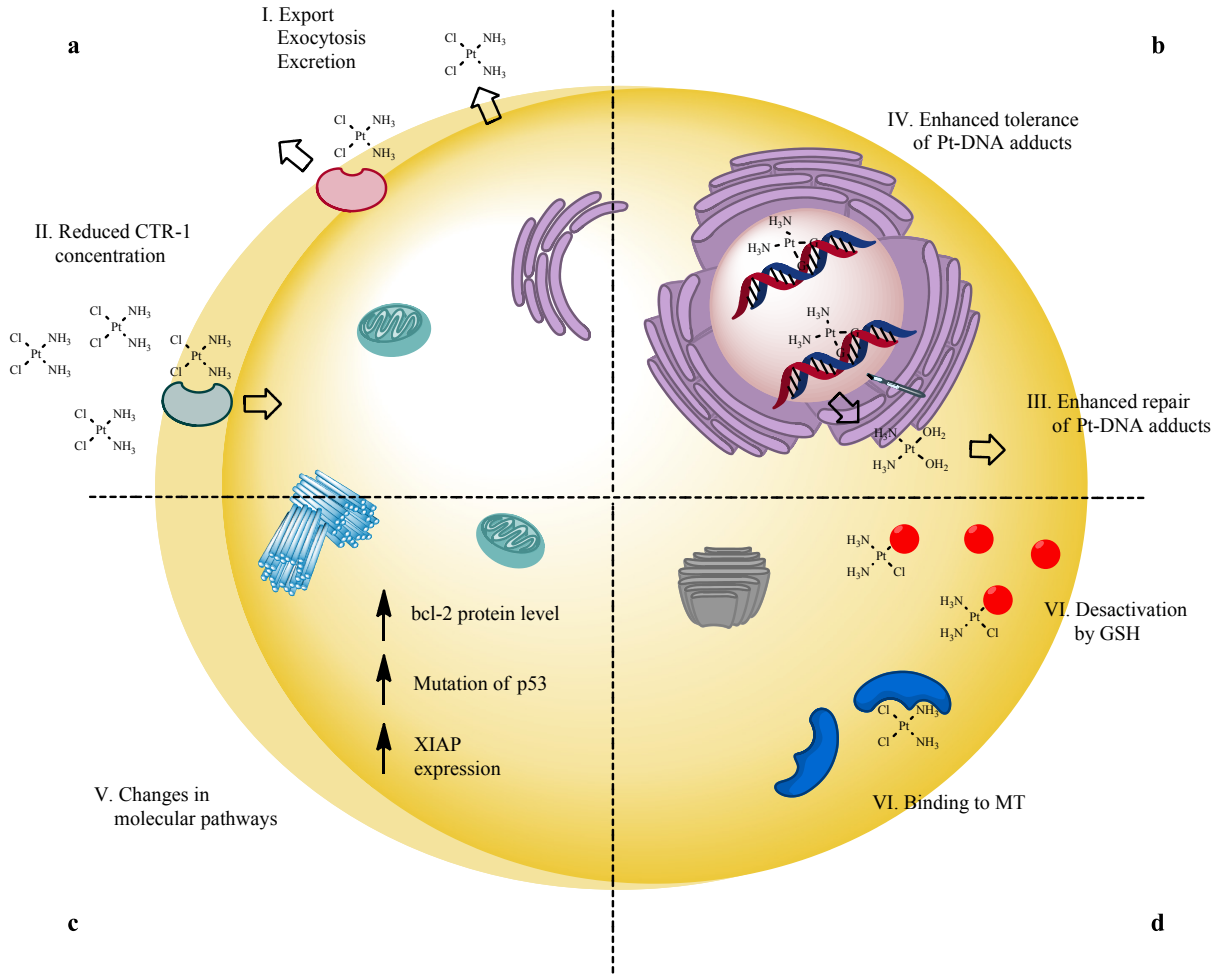


Figure 2-7. Several pathways leading to cisplatin resistance. **a.** Reduced uptake due to low CTR-1 concentration and an enhanced transport out of the cell leads to a lower cisplatin concentration inside the cell. **b.** Despite of cisplatin reaching its main target, cisplatin-DNA crosslinks are better tolerated or getting repaired. **c.** Impaired apoptosis during treatment with cisplatin occurs due to higher levels of several proteins which influence the molecular pathway. **d.** Cisplatin may react with glutathione or metallothionein and thus be desactivated.

Data concerning overexpression of bcl-2 and bcl-x_L proteins in tumor cells are controversial: In various cancer cell lines, cisplatin-induced apoptosis is hindered when enhanced expression of those two proteins occurs, whereas human ovarian cancer cell lines express an inverse correlation and show higher sensitivity to cisplatin at high levels of bcl-2.[65, 66] Preclinical studies showed that ovarian cancer cell lines developed cisplatin resistance at high levels of IAP, or more precisely, X-linked inhibitor of apoptosis XIAP. Nevertheless, the relevance of XIAP remains vague, since response to the protein varies between different cancer cell lines.[65]

1.2.2.2. Side-effects

For patients with testicular cancer, cisplatin in combination therapy displays a curative potential over 90%. Before its discovery, a 5% cure rate could be achieved by standard therapy with dactinomycin. Besides testicular cancer, cisplatin shows anti-cancer activity against several tumor types, including ovarian, non-small and small cell lung, neck, head, and bladder cancer.[27,67]

Despite its curative potential, cisplatin involves a number of non-desirable side-effects during treatment. Cisplatin is notoriously not only harmful for cancer, but also for healthy cells. Its dose-limiting side-effect is nephrotoxicity; patients treated with cisplatin also mainly suffer from neurotoxicity, nausea, vomiting, and ototoxicity. Approximately 39% lose high frequency hearing irreversibly as a consequence of treatment.[27,28,68]

In order to reduce toxicity and design novel platinum-based anti-cancer agents, further research is still necessary. Two additional platinum(II) compounds gained worldwide market approval so far, and will be discussed in the following sections.

1.2.3. Carboplatin

The second-generation platinum-based anticancer compound carboplatin has been designed to reduce side-effects of cisplatin, and gained market approval in 1989 for ovarian carcinoma.[29] The mechanism triggering cell death is identical for carboplatin and cisplatin: binding to DNA followed by bending and unwinding it. Nevertheless, several differences concerning side-effects and pharmacokinetics between the two drugs could be detected. *1,1*-Cyclobutanedicarboxylate is a more inert leaving group than chloride, resulting in a reduced toxicity profile compared to cisplatin. Although nephrotoxicity and toxicity to the gastrointestinal tract are lowered, its dose-limiting side-effects are myelosuppression and thrombocytopenia. Compared to the administered dose of cisplatin, a ratio of 4:1 of carboplatin is required to achieve comparable anti-tumor activity. Another difference between the two drugs is their metabolism and excretion: While 90% of carboplatin is excreted, only 25% of the administered amount of cisplatin is found in urine. [29,69]

1.2.4. Oxaliplatin

By now, oxaliplatin was the last platinum agent to obtain worldwide market approval as cancer therapeutic agent in 2002.[29] In contrast to cisplatin, oxaliplatin has two chlorido ligands replaced by a bidentate oxalato ligand in order to improve water-solubility of the complex; instead of the ammine ligands, *(trans-1R,2R-)*diaminocyclohexane serves as equatorial bidentate ligand.

Oxaliplatin is administered in combination with 5-fluorouracil in colon cancer treatment, whereas cisplatin does not show activity against this type of carcinoma.[70] One reason for the decreased curative potential of cisplatin in resistant cell lines might be the ability of mismatch repair proteins to distinguish between cisplatin and oxaliplatin DNA adducts.[71] Oxaliplatin binds to DNA, showing the same distribution of intrastrand and interstrand DNA adducts as cisplatin.[72] Although similar adducts are formed, the angle of bending and unwinding differs from cisplatin DNA adducts due to the bulky diaminocyclohexane ligand.[73]

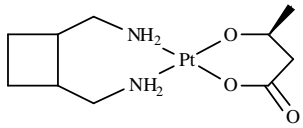
Oxaliplatin is considered being a better tolerated cytostatic than cisplatin, since neurotoxicity occurring upon administration of oxaliplatin is reversed faster in comparison to cisplatin.[70]

1.2.5. Regionally approved platinum compounds

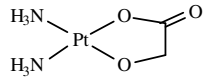
Several platinum-based compounds are currently in clinical use, but have not gained worldwide market approval so far. A short description including their chemical structures (Figure 2-8) is listed below.

Miriplatin is a platinum(II) agent bearing two myristate groups as equatorial ligands to improve lipophilicity.[74] The drug gained market approval for treatment of hepatocellular carcinoma in Japan in 2009.[75]

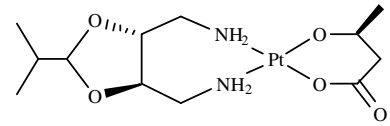
Since 1995, ***nedaplatin*** is in use as chemotherapeutic in Japan. It exhibits strong antitumor activity in treatment of head and neck, cervical, and lung cancer.[76,77]



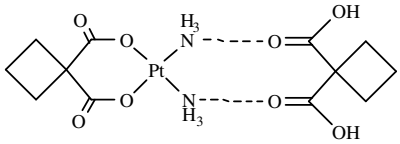
Lobaplatin



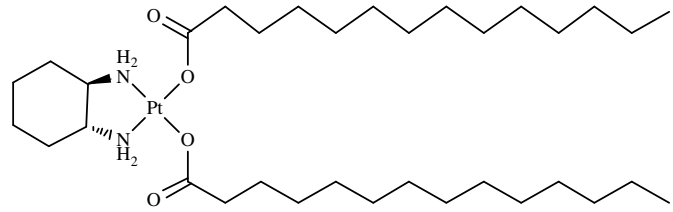
Nedaplatin



Heptaplatin



Dicycloplatin



Miriplatin

Figure 2-8. Chemical structures of regionally approved platinum drugs.

Lobaplatin is a third-generation platinum complex which is approved in China to treat inoperable metastatic breast cancer, chronic myelogenous leukemia, and small cell lung cancer.[27]

Heptaplatin is an approved anti-cancer drug in South Korea since 1999. The drug shows antitumor activity in several cisplatin-resistant cell lines and appears to have lower nephrotoxicity compared to cisplatin.[78,79]

The most recently regional approved platinum-based chemotherapeutic is ***dicycloplatin***, which was approved by the SFDA in China. [80,81]

1.2.6. Other metals as potential anti-cancer agents

As already mentioned, not only platinum-based compounds, but also complexes bearing several other metals are being considered as potential anticancer drugs.[33,35,82] In this section, complexes with ruthenium, osmium, and rhodium as metal center as well as ferrocene-bearing compounds and their role in development of chemotherapeutics will be discussed in more detail.

1.2.6.1. Ruthenium

There are various remarkable properties making ruthenium compounds suitable for clinical applications:

a. Ruthenium complexes have ***ligand exchange rates*** similar to those of platinum(II) complexes, which seem to be decisive for their antitumor activity. The exchange of their ligands takes place within one to two hours, comparable to the time in which many cell division processes take place.[83]

b. Ruthenium compounds are physiologically accessible in three ***oxidation states*** Ru(II), Ru(III), and Ru(IV), whereas Ru(III) compounds tend to be the biologically most inert one. One possible approach is administration of ruthenium compounds in the oxidation state +III, which is activated by reduction inside the cancer cell. The reducing environment in tumor cells allows reduction of Ru(III) to active Ru(II) complexes. If the active Ru(II) complex leaves the cancer cell, it might be oxidized to its initial oxidation state +III.[84]

c. Tumor cells have a faster cyclic activity than healthy cells and thus need more iron, which is transported via **binding to transferrin** inside the cell. According to the HSAB theory, complexes containing ruthenium as central atom interact primarily with soft ligands, such as molecules containing nitrogen and sulfur. Since transferrin contains domains with the desired atoms and due to its presence in blood plasma, it is easily accessible for reactions with intravenously administered ruthenium compounds. The number of transferrin receptors on the cancer cell surface is increased in order to ensure its iron supply by means of metal-bound transferrin. By mimicking iron to bind to transferrin, 2-12 fold increase of ruthenium in cancer cells in comparison to healthy cells could be found *in vivo*.[84,85]

Two Ru(III) complexes, known as NAMI-A and KP1019 (Figure 2-9), already completed clinical phase I studies. Remarkably, KP1019 displays cytotoxic activity against primary cisplatin-resistant colorectal tumors. The anti-tumor activity of both complexes seems not to be based on interactions with DNA.[85,86]

It has to be mentioned that neither the exact mode of action of ruthenium compounds, nor identification of DNA as main target for ruthenium anti-cancer agents could be identified so far.[83]

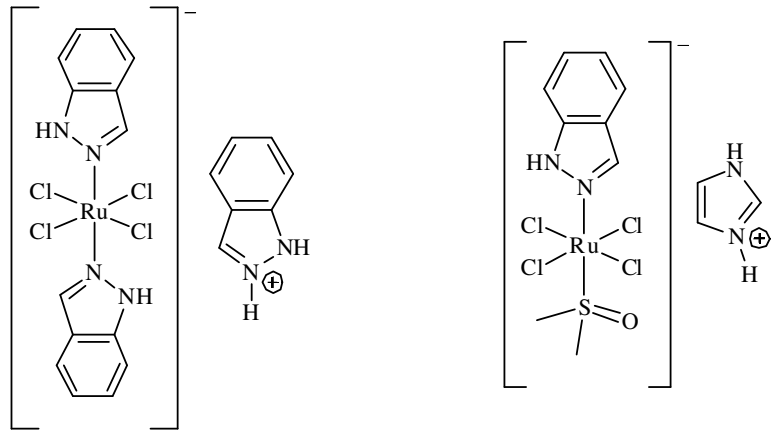


Figure 2-9. Ru(III) anticancer compounds in clinical trials: KP1019 and NAMI-A.

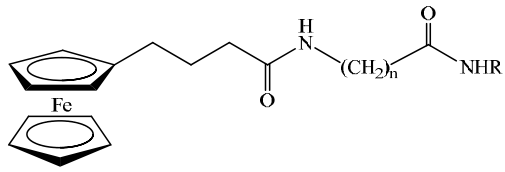
1.2.6.2. Osmium and rhodium

Osmium-based compounds gained only little attention concerning their investigation as chemotherapeutics, since Os(II) and Os(III) complexes seem to be substitution-inert. Despite this assumption, particular Os(II) complexes bearing arene ligands seem to display interesting IC_{50}-values *in vitro*. So-called piano-stool organometallic osmium arene complexes with pseudo-octahedral geometry seem to be promising agents and worth further investigations.[87,88] Synthesis of several osmium organometallic compounds with remarkably *in vitro* activity have been reported[89,90], whereas only in two *in vivo* studies osmium-arene complexes indicated tumor growth inhibition.[91]

Complexes containing **Rhodium** as central atom have also been considered as potential anti-cancer drugs. Dimeric rhodium(II) carboxylates as well as complexes including Rh(I) and Rh(III) have been subject of investigations to date. Toxic effects prevented the use of dimeric Rh(II) carboxylates, although there was evidence for their activity against Ehrlich ascites, L1210, and P388 tumor cells.[92] However, Rh(I) compounds, as acetylacetonate-1,5-cyclooctadienerhodium(I), display antitumor activity against Ehrlich ascites carcinoma comparable to cisplatin in mice and with decreased host toxicity.[93,94] Several Rh(III) derivatives as well as Rh(III) analogues of Ru(III) compounds have shown antineoplastic activity. Their exact mechanism of action is still not clear, but interactions with the DNA as well as inhibition of enzymes are possible mechanisms for rhodium complexes.[94]

1.2.6.3. Ferrocenyl derivatives

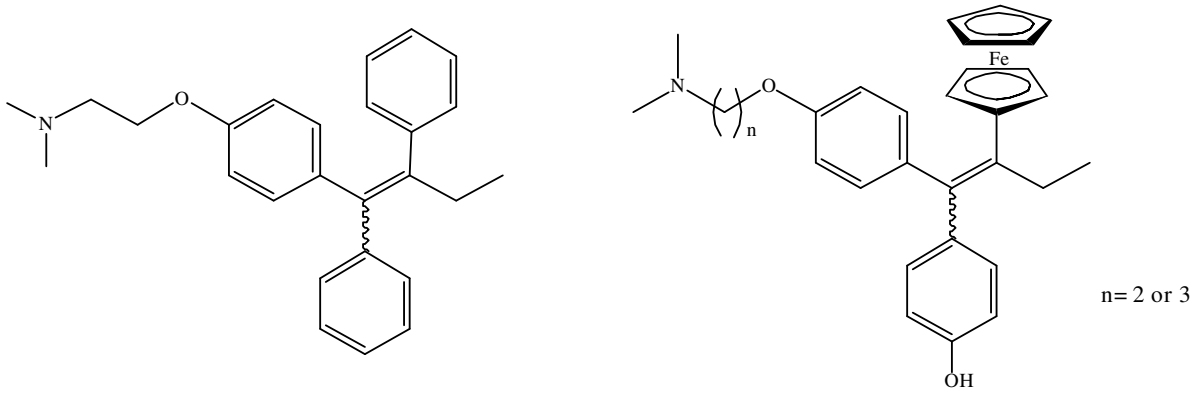
In 1978, Brynes et al. reported on the first ferrocene-bearing compound (Figure 2-10) with significant antitumor activity against lymphatic leukemia P-388.[95] During the past decades, numerous ferrocenyl including molecules have been developed and their antiproliferative and tumor-inhibiting properties examined. The most promising classes of ferrocenyl compounds which could be identified to date are salts, derivatives of selective estrogen modulators, conjugates with biologically active molecules, linker to DNA targeting moieties, androgens and anti-androgens, ferrocenyl polymers, and conjugates with other metals.[96,35] Some of the listed ferrocenyl compounds will be further discussed at this point.



1 n=3, R=H
2 n=3, R = $(CH_2)_2CONH_2$
3 n=2, R= $(CH_2)_3CONH(CH_2)_2CONH_2$

Figure 2-10. First ferrocene derivative found to have antiproliferative activity, synthesized by Brynes et al.

In the late 1990s, ferrocene was coupled for the first time to the antiestrogen tamoxifen, a drug that is widely used in therapy of hormone-dependent breast cancer.[97] Several tamoxifen-derived conjugates bearing ferrocene moieties, known under the generic term **ferrocifens**, have been synthesized and studied so far. They displayed remarkable results *in vitro*, and have shown even less toxicity than tamoxifen in *in vivo* studies with rats. Figure 2-11 shows the general structure of already investigated active hydroxyferrocifens; the hydroxyl group in this compound is essential, since 4-hydroxy-tamoxifen is the active metabolite of tamoxifen, with an enhanced affinity for the estrogen receptor. Competitive binding of tamoxifen to estrogen receptor ERα, which results in repression of estradiol-mediated DNA-transcription in the tumor cell, is responsible for its antitumor activity. [96,98,99,100]



Tamoxifen **Hydroxyferrocifen**

Figure 2-11. Chemical structures of the antiestrogens tamoxifen and hydroxyferrocene.

Another attempt to design ferrocenyl derivatives was coupling to **DNA-targeting compounds**, such as topoisomerase II inhibitors. According to in vitro studies, azalactone ferrocene and thiomorpholide ferrocene were found to be significantly active against topoisomerase IIβ activity.[101]

A promising attempt for development of potential anti-tumor agents containing ferrocene groups was **linking to transition metals**. For instance, complexes with palladium(II), platinum(II) or rhodium(I) as central atom and ferrocenyl-bearing ligands have been synthesized and biologically tested. Cytotoxic activity of those compounds was tested, and remarkable results could be found for some of them.[102,103,104] The first potential anti-cancer platinum(IV) and ferrocene bearing systems were synthesized in 2013, displaying promising IC_{50} values down to the low micromolar region.[32] However, initial design and synthesis of platinum(IV)-ferrocenyl conjugates was first published in 2008.[105]

1.3. Platinum(IV) compounds

1.3.1. Why platinum(IV)?

When B. Rosenberg discovered the antiproliferative activity of cisplatin, he contemporaneously found out that two platinum(IV) compounds, namely *cis*-tetrachloridodiammineplatinum(IV) and tetrachlorido(ethylenediammine)platinum(IV), were also able to exhibit antitumor activity.[106] Nevertheless, development of higher oxidation state platinum analogues didn't gain as much attention as platinum(II) compounds during the

following years. During the last decades, development of platinum(IV) compounds progressed, since they show numerous advantages over their platinum(II) precursors. [107]

In contrast to platinum(II) anticancer drugs, platinum(IV) compounds open up the possibility of oral administration, making chemotherapy a more convenient process. The higher oxidation state and d^6 configuration of the central ion results in kinetical inertness; thus, they are more stable and able to pass the gastrointestinal tract without degradation. Platinum(IV) compounds act as prodrugs, which are mainly getting reduced inside the cell. Since tumor tissue is a hypoxic environment, one possible approach for tumor targeting would be design of hypoxia-selective platinum(IV) agents by fine-tuning of their reduction potential.[108,109] The octahedral sphere of platinum(IV) compounds allows variation of six instead of only four coordination sites. Therefore, fine-tuning of the compound's lipophilicity, reduction potential, and pharmacological properties is enabled, and targeting moieties may be attached as leaving groups more easily.[107] Reduction of platinum(IV) compounds is accompanied by loss of two ligands in trans position; the exact square-planar sphere of the resulting platinum(II) complex depends on the reducing agent.[110]

Three platinum(IV) compounds (Figure 3-1), namely satraplatin, iproplatin, and tetraplatin, made it up to clinical trials so far. Satraplatin recently failed to be approved by the FDA, but is still being investigated in clinical trials.[111,112] Tetraplatin was abandoned in a clinical phase I trial due to its high neurotoxicity; however, iproplatin failed in a phase III clinical trial when being compared to treatment with carboplatin.[113]

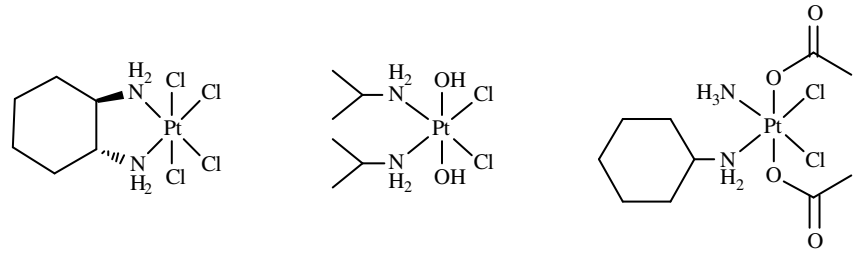


Figure 3-1. Platinum(IV) complexes which are or were undergoing clinical trials: Tetraplatin, Iproplatin, and Satraplatin.

Tetraplatin displays high toxicity, which might be explained by its high reduction potential; electronegative and bulky ligands promote a faster reduction of the platinum(IV) agent. Reduction studies carried out by Choi et al. showed that an increase of the reduction potential

depends on the axial ligands, following the order: OH < $OCOCH_3$ < Cl < $OCOCF_3$. Furthermore, small equatorial ligands might stabilize the coordination state of Pt(IV) agents and thus prevent its easy reduction.[114]

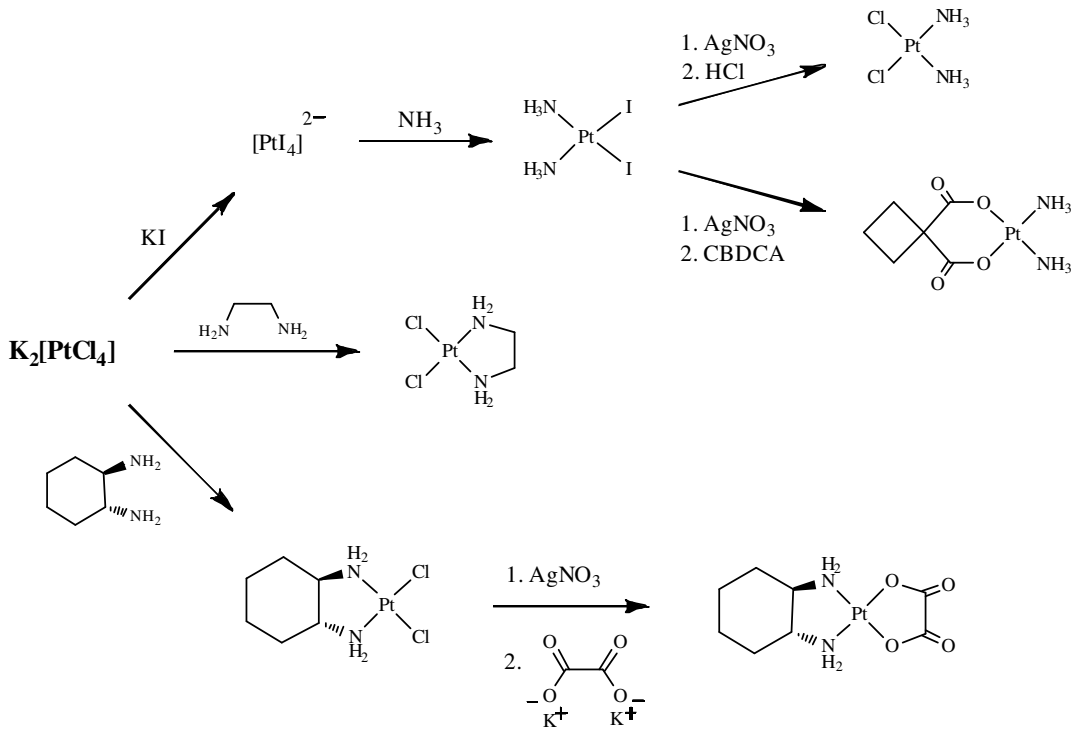
1.3.2. Synthetic strategies

1.3.2.1. Synthesis of platinum(II) precursors

Preparation of cisplatin described by Dhara is the most widely used method;[115] in the first step, the chlorido ligands of $K_2[PtCl_4]$ are replaced by iodido ligands upon addition of KI in order to enhance the trans-effect. Reaction with NH_3 yields *cis*-diamminediiodidoplatinum(II), and subsequent removal of the iodido ligands is performed with $AgNO_3$, thus forming AgI. Addition of HCl produces the final product cisplatin in high yields. Purification may be performed by storage of cisplatin in DMF at between 0 and 5°C, filtration of the adduct and subsequent removal of the solvent *in vacuo*.[116] If bidentate ligands serve as non-leaving ligands, as in case of oxaliplatin or dichlorido(ethane-*1,2*-diamine)platinum(II), replacement of chlorido ligands with iodido ligands is unnecessary, since substitution results in *cis*-configuration in anyway.[117]

Contrary to synthesis of cisplatin, *trans*-diamminedichloridoplatinum(II) is produced by reaction of $K_2[PtCl_4]$ with ammonia in excess, followed by addition of HCl (Scheme 3-1).[118]

To date, several thousand platinum complexes have been synthesized and tested concerning their cytotoxic properties. Thus, a variety of reaction pathways for the design of platinum(II) complexes already exists. In this section, particular attention was paid on the synthesis of platinum(II) compounds which served as precursors for later described platinum(IV) complexes.

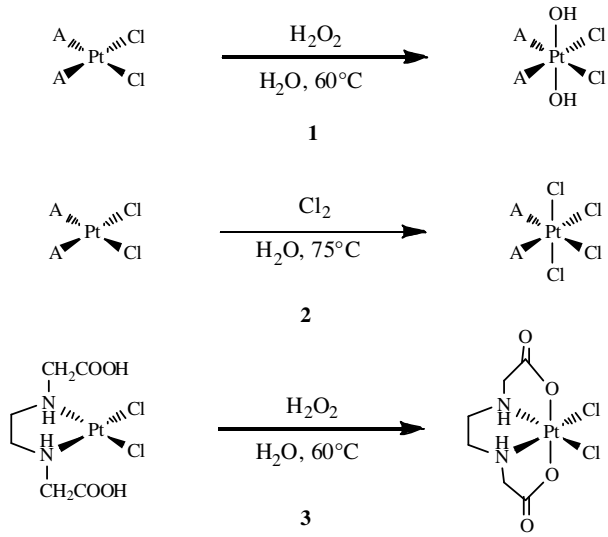


Scheme 3-1. Synthetic pathways leading to different platinum(II) precursors (CBDCA= cyclobutane-*1,1*-dicarboxylic acid).

1.3.2.2. Oxidation strategies

Several methods for oxidation of platinum(II) compounds have been reported during the last decades. In Scheme 3-2, an excerpt of methods for oxidation of platinum(II) compounds is depicted. Amongst various procedures for oxidation of platinum(II) to platinum(IV) compounds, reaction with hydrogen peroxide is the most widely used (reaction **1**).[118] Symmetric oxidation may also be achieved by treatment with chlorine (reaction **2**).[119] Oxidation with hydrogen peroxide is also being carried out for ring-closing reactions: In case of reaction **3**, the educt contains two uncoordinated carboxylic acid groups, allowing the equatorial ligand to bind in a tetradentate manner to the central ion upon oxidation.[120]

Symmetric oxidation leading to identical ligands in trans position is often limiting for drug design. Preparation of more versatile platinum(IV) drugs can be achieved by introduction of mixed axial ligands upon oxidation.

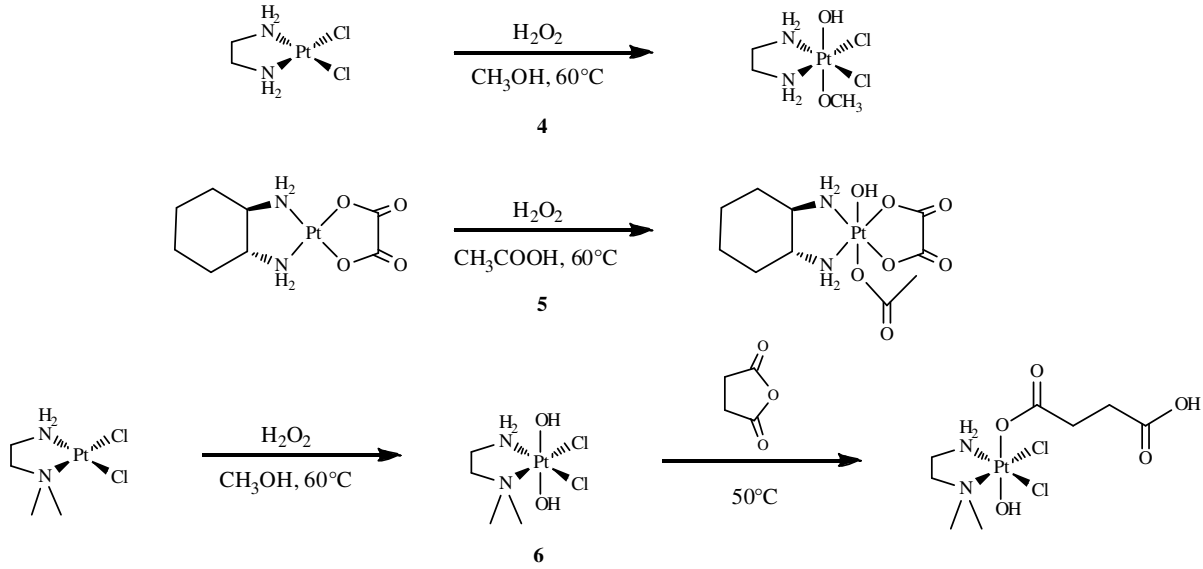


Scheme 3-2. Excerpt of published oxidation methods for platinum(II) compounds.

Oxidation with hydrogen peroxide in absolute methanol produces a monohydroxido platinum(IV) complex with one methoxido ligand in trans position (reaction **4**).[121]

For preparation of monocarboxylato platinum(IV) compounds, oxidation with hydrogen peroxide in carboxylic acid has been reported (reaction **5**); if the reaction is conducted in an excess of carboxylic acid, the main product is the monocarboxylato species with only one hydroxido ligand. The reaction has been carried out successfully with acetic acid, bromoacetic acid, hexanoic acid, and naphthyleneacetic acid.[122,123]

Another strategy for mono functionalization of platinum(IV) compounds is to design a precursor with specific equatorial ligands which sterically hinder carboxylation of one coordination site. In case of reaction **6**, the bidentate *N,N-dimethylethane-1,2-diamine* ligand prevents carboxylation of the hydroxido ligand close to the two methyl groups.[111]

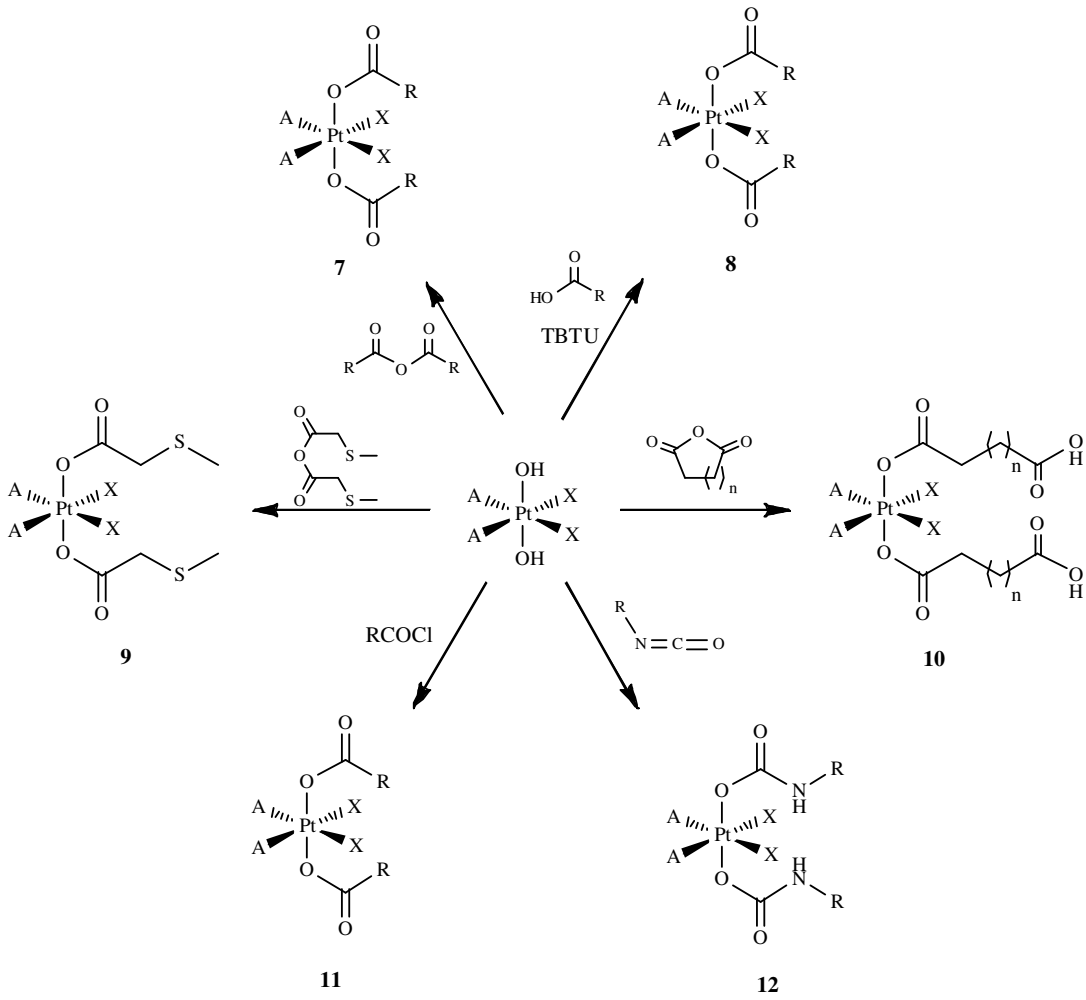


Scheme 3-3. Methods for unsymmetrical oxidation of platinum(II) compounds.

1.3.2.3. Derivatization of hydroxido ligands

For further derivatization of platinum(IV) compounds, ligand substitution is problematic due to their kinetic inertness. Complexes bearing one or more hydroxido moieties are convenient substrates for further reactions with electrophiles. Suitable reactants for carboxylation are e.g. anhydrides, acyl chlorides, or isocyanates (Scheme 3-4, reactions **7-12**).

Carboxylation with anhydrides is an established synthetic route for platinum(IV) compounds nowadays (reactions **7-10**). Introduction of thioethers (reaction **9**) which may be oxidized to sulfoxides or sulfones in additional steps is also possible via carboxylation with anhydrides.[124] In 2013, *Hambley et al.* presented another carboxylation method, in which a dihydroxido platinum(IV) complex is coupled to a carboxylic acid by DCC (*N*,*N*-dicyclohexylcarbodiimide) or TBTU (*O*-(benzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium tetrafluoroborate) (reaction **8**), respectively.[123,125]



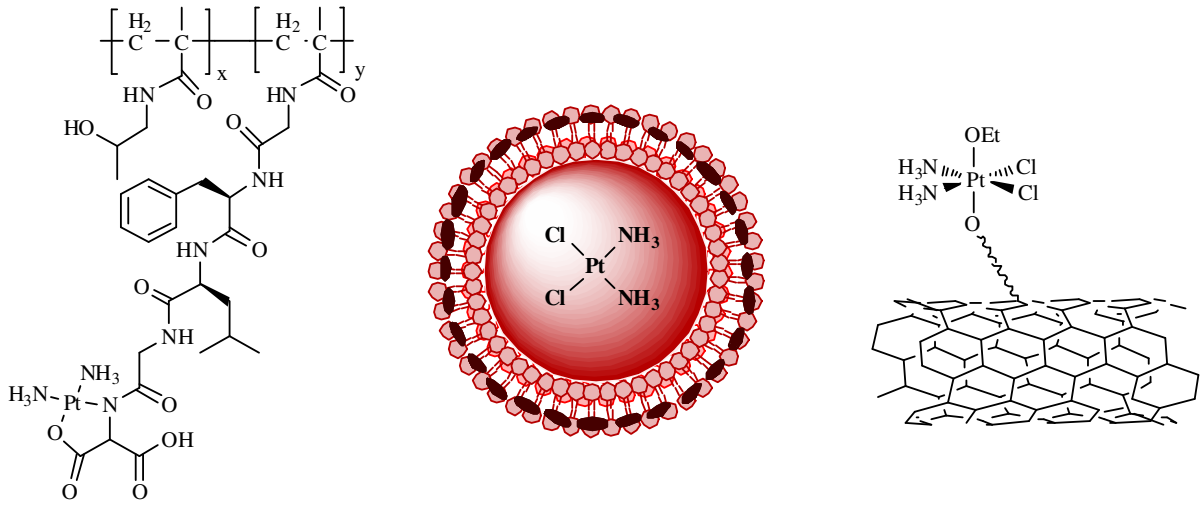
Scheme 3-4. Selection of common carboxylation strategies for platinum(IV) compounds.

Reaction with cyclic anhydrides, such as succinic or glutaric anhydride, yields compounds with free carboxylic groups (reaction **10**), which might be used for further derivatization steps.[118,121,126]

Acylation may also be carried out with acyl chlorides in the presence or absence of a base.[125,127] Another straightforward synthetic route resulting in compounds bearing carbamate functionalities is carboxylation with isocyanates.[124,128]

1.3.3. Towards targeted therapy

So far, several strategies have been adopted in order to develop platinum compounds with the ability to specifically attack tumor tissue.



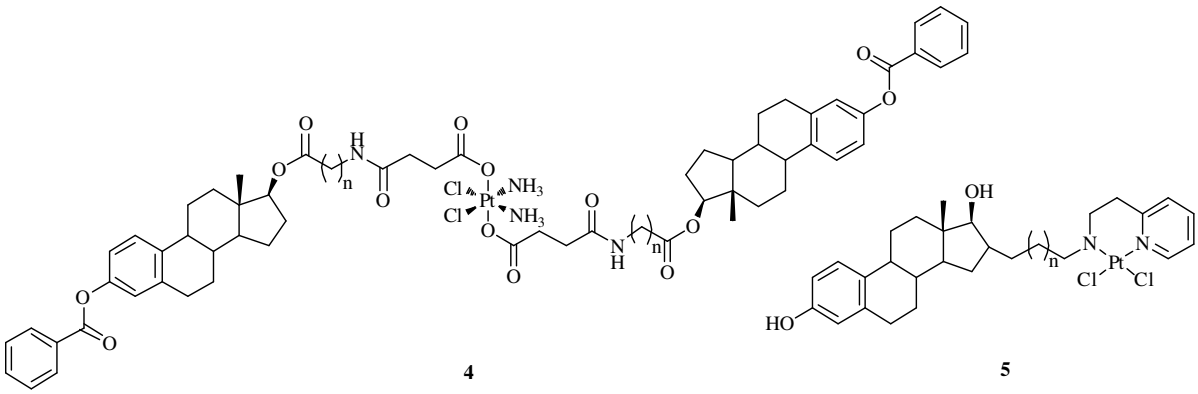
1 **2** **3**

Figure 3-2. Macromolecular molecules as platinum drug carrier: **1:** AP5280 is a polymer loaded with platinum drug and has already been investigated in clinical trials; **2:** lipoplatin consists of liposomal encapsulated cisplatin; **3:** a platinum(IV) compound attached to a SWNT (SWNT=single-walled carbon nanotube).

One approach is to take advantage of the EPR effect by attaching platinum compounds to macromolecular carriers. A *N*-(2-hydroxypropyl)methacrylamide polymer was linked to a diammine platinum(II) unit in a bidentate manner (AP5280, **1**) to serve as delivery system.[129] *In vivo* models showed decreased toxicity of the drug, displaying tumor growth inhibition comparable with that of carboplatin. Poly(organophosphazenes) and micelles have also already been loaded with anti-cancer platinum drugs to selectively accumulate in tumor tissue.[130] Lipoplatin (**2**), a liposomal formulation of cisplatin which has been designed to overcome systemic toxicity of cisplatin, has already been investigated in terms of its pharmacological properties in clinical phase I-III trials.[27,131] In 2007, *Feazell and coworkers* attached a cisplatin-derived platinum(IV) compound to a soluble single-walled carbon nanotube (SWNT); SWNTs are regarded as suitable carrier molecules, since they act as a longboat which can transport the drug through cell membranes via endocytosis.[132]

Tumor-targeting of novel platinum drugs may also be achieved by attachment of a bioactive moiety to the platinum precursor. Since overexpression of specific receptors in tumors occurs frequently, anti-cancer drugs might be linked to either the substrate for or the antibody against the prevailing receptor. Conjugation of estrogen to a platinum(IV) precursor (**4**) has already been reported in the literature. This molecule was designed to target [ER (+)] (receptor positive) breast cancer cells, where it may be reduced upon cell uptake to the active

platinum(II) species. A series of platinum(II) complexes coupled to estradiol have been synthesized by *Bérubé et al*. (**5**). *In vivo* investigations showed that some of these compounds were specific towards ovarian and hormone-dependent breast cancers in nude mouse xenograft models.[133,134]

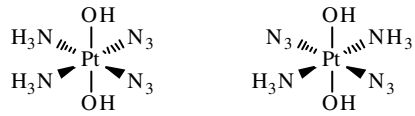


4 **5**

Figure 3-3. Chemical structure of a platinum(IV) drug with estrogen moieties in axial position (**4**), designed by *Lippard et al*.; Bérubé et al. synthesized platinum(II) compounds linked to estradiol (**5**).

Furthermore, *Dyson et al.* synthesized ethacraplatin, a conjunction of a platinum(IV) complex with two ethacrynic acid (a glutathione-S-transferase inhibitor, GST) molecules in axial position. Glutathione-S-transferase catalyzes binding of GSH to several anticancer drugs (such as cisplatin and oxaliplatin), resulting in an enhanced efflux of the drug.[127,135] Coupling of platinum compounds to peptides, carbohydrates or bisphosphonates is also object of investigations.[134]

Since platinum(IV) compounds act as prodrugs, selective activation upon reduction inside the cancer cell would be desirable. One possible strategy for specific and controlled activation is photoactivation in cancer tissue accessible to irradiation. Sadler et al. designed platinum(IV) complexes (**6** and **7**) that are nontoxic in the dark, but show high toxicity in human bladder cell lines 5673 upon radiation with UVA light.[135]



6 **7**

Figure 3-4. Two complexes enabling activation by UVA irradiation (synthesized by *Sadler et al.*).

1.4. Abbreviations

ATP	Adenosine triphosphate
CBDCA	Cyclobutanedicarboxylic acid
CTR	Copper transporter
DCC	*N,N*-Dicyclohexylcarbodiimide
DNA	Deoxyribonucleic acid
EPR	Enhanced permeability and retention
ERCC1	DNA excision repair protein
FDA	Food and drug administration
GSH	Glutathione
GST	Glutathione S-transferase
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSA	Human serum albumin
HSAB	Hard and soft acids and bases
IAP	Inhibitor of apoptosis
MMR	Mismatch repair
MRP	Multidrug resistance protein
MT	Metallothionein
NER	Nucleotide excision repair
OCT	Organic cation transporter
PAH	Polycyclic aromatic hydrocarbon
pRb	Retinoblastoma protein
SWNT	Single-walled carbon nanotube
TBTU	*O*-(Benzotriazol-*1*-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate

1.5. References

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2. Results

The content of this PhD work is based on two peer-reviewed publications and one manuscript which has been submitted on April 23rd, 2014. The results are presented chronologically and in the publisher's format or as manuscript.

1. "Platinum(IV) Complexes Featuring Axial (1,4–$^{13}C_2$)Succinato Ligands – Synthesis, Characterization, and Preliminary Investigations in Cancer Cell Lysates"

J. Banfić, M. S. Adib-Razavi, M. Galanski, and B. K. Keppler; Z. anorg. allg. Chem.**,** 639(8-9), 1613-1620, 2013.

2. "Platinum(IV) Complexes Featuring One or Two Axial Ferrocene Bearing Ligands – Synthesis, Characterization, and Cytotoxicity"

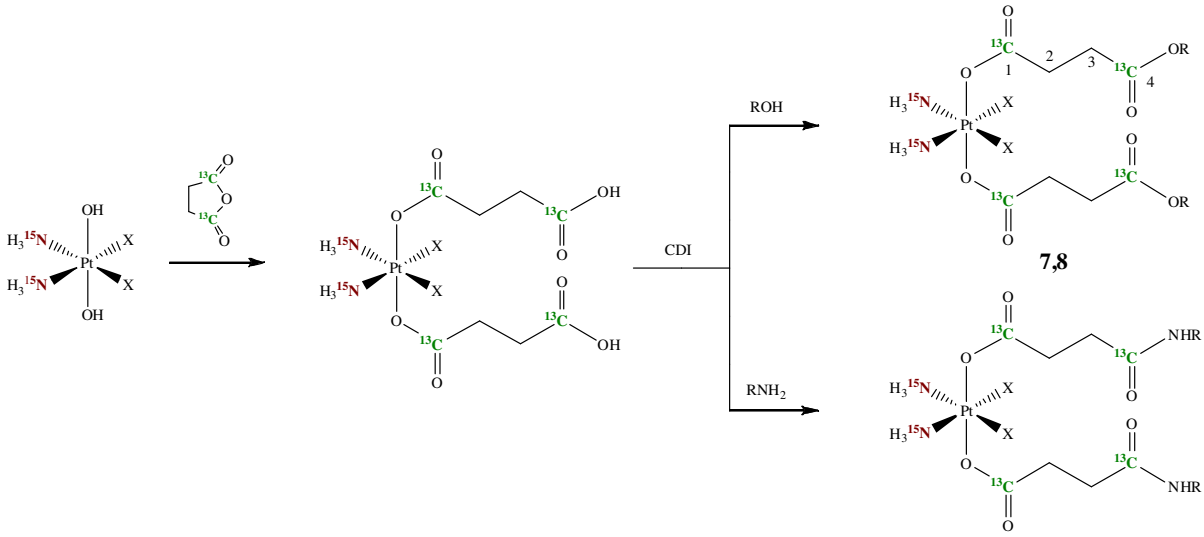
J. Banfić, A. A. Legin, M. A. Jakupec, M. Galanski, and B. K. Keppler; Eur. J. Inorg. Chem., 3, 484-492, 2014.

3. *"***Coupling of Cytotoxic Platinum(IV) Prodrugs to Biodegradable Polyphosphazenes: Synthesis, Antiproliferative Activity, and Cellular Accumulation"**

J. Banfić, H. Henke, K. Kryeziu, S. Theiner, W. Körner, P. Heffeter, M. Galanski, I. Teasdale, W. Berger, O. Brüggemann, B. K. Keppler; submitted to the Journal of Controlled Release on April 23rd, 2014.

2.1. Platinum(IV) Complexes Featuring Axial (1,4–$^{13}C_2$)Succinato Ligands – Synthesis, Characterization, and Preliminary Investigations in Cancer Cell Lysates

J. Banfić, M. S. Adib-Razavi, M. Galanski, and B. K. Keppler



Zeitung für anorganische und allgemeine Chemie, 639 (8-9), 1613-1620, 2013

J. Banfić synthesized and characterized all complexes, and evaluated NMR results. M. S. Adib-Razavi manufactured cancer cell lysates, M. Galanski carried out NMR measurements, evaluated and discussed results, and finalized the manuscript. B. K. Keppler supervised the work and discussed results.

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Platinum(IV) Complexes Featuring Axial (1,4–$^{13}C_2$)Succinato Ligands – Synthesis, Characterization, and Preliminary Investigations in Cancer Cell Lysates

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Keywords: Platinum(IV) complexes; NMR spectroscopy; Cancer cell lysates; Reduction; Isotopic labeling

Abstract. The chemistry of cytotoxic platinum(II) complexes is more or less restricted to ligand exchange reactions, derivatization of coordinated ligands is cumbersome, and subsequent purification in many cases impossible. Consequently, kinetically more inert platinum(IV) complexes found their way into the development of novel, promising anticancer drugs. Research has focused more and more during the last years on the use of platinum(IV) complexes featuring one or two axial succinato ligands in which one carboxylic acid moiety is available for further derivatization. In order to gain a deeper insight into the mechanism of action, isotopically labeled platinum(IV) complexes with axial (1,4–$^{13}C_2$)succinato ligands were synthesized and fully characterized by multinuclear (^{1}H, ^{13}C, ^{15}N, and ^{195}Pt) 1D- and 2D-NMR spectroscopy. Especially of note in this context is a long range ^{1}H,^{13}C shift correlation signal detected between the equatorial ammine protons and the axially coordinated carboxylato moiety. Furthermore, their behavior in extracts of SW480 cancer cells was investigated. Preliminary results demonstrate that cisplatin analogs are reduced significantly faster in comparison to carboplatin analogs.

Introduction

Since *Rosenberg* discovered the *anti*-proliferative properties of platinum complexes in the mid-1960s,[1] many efforts have been made in order to develop platinum-based chemotherapeutics. Around forty platinum complexes entered clinical trials;[2,3] however only three, namely cisplatin, carboplatin, and oxaliplatin (Figure 1), achieved worldwide marketing approval as cytostatics.[4]

Due to severe side-effects, intrinsic and acquired drug resistance, low selectivity for cancerous tissue, and unavailable inertness,[5–8] the development of novel platinum(IV) compounds gained more and more interest.[9,10] Some advantageous characteristics of platinum(IV) complexes in comparison to their platinum(II) analogs are: (i) kinetic inertness, providing a basis for oral absorption and decreasing in parallel the systemic toxicity, (ii) activation by reduction via loss of the axial ligands,[11] releasing the reactive platinum(II) complexes under reductive conditions as found in many solid tumors, and (iii) octahedral geometry, allowing the variation of six instead of four coordination sites, thereby enriching platinum chemistry and controlling more easily pharmacological properties (e.g., redox behavior and lipophilicity).

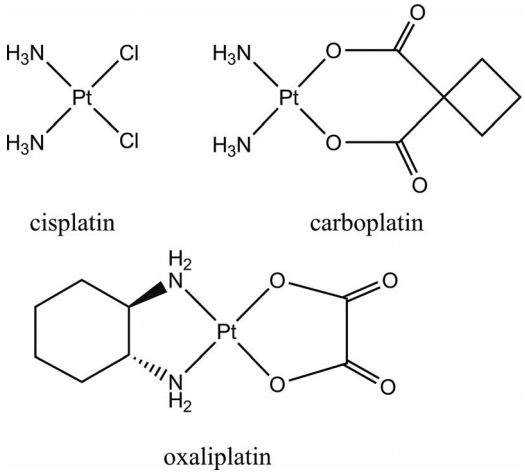

Figure 1. Platinum-based cytostatics which gained worldwide approval: cisplatin, carboplatin, and oxaliplatin.

Till now, three structurally significantly different platinum(IV) complexes entered clinical trials, tetraplatin (two axial chlorido ligands), iproplatin (two axial hydroxido ligands), and satraplatin (two axial carboxylato ligands) (Figure 2). A satraplatin-related complex, LA-12, in which cyclohexylamine

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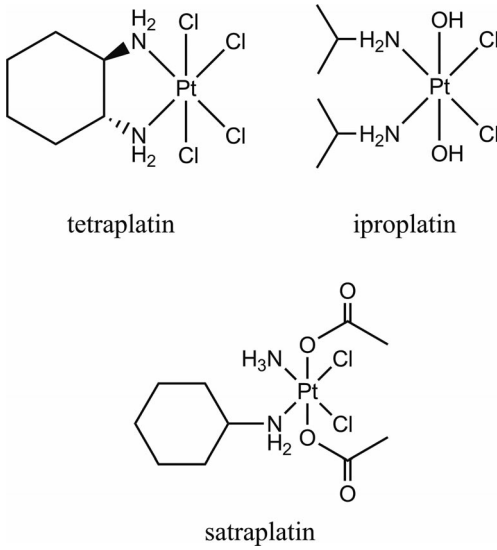

Figure 2. Chemical structures of tetraplatin (only one *trans* isomer shown, abandoned, too toxic), iproplatin (abandoned, not active enough), and satraplatin (still under clinical investigation).

was exchanged by adamantylamine, was under clinical investigation in phase I, but results have not been published yet.

Tetra- and iproplatin were abandoned, tetraplatin was too toxic, whereas iproplatin showed an activity not superior compared to carboplatin. This circumstance is often explained by their different redox potential; contrary to iproplatin, tetraplatin is reduced very fast in the blood. Satraplatin, although failing phase III clinical trials (SPARC study), is still under investigation in combination therapy. It has a redox potential which is situated between tetra- and iproplatin, which might be in an optimal window for cancer therapy.

The synthesis of satraplatin opened up the door not only to a new class of platinum compounds, it ultimately lead to a new synthetic strategy in platinum chemistry, the carboxylation of dihydroxidoplatinum(IV) complexes.[12–15] A further advance in the development of platinum(IV) complexes could be reached by carboxylation with cyclic anhydrides, resulting in the coordination of one carboxylate and leaving the second carboxylic group uncoordinated as platform for subsequent derivatization.

Series of novel platinum(IV) complexes were synthesized in which the terminal COOH group of the axial ligand was transferred into esters or amides, respectively.[16–26] With increasing lipophilicity, highly cytotoxic complexes featuring IC_{50} values down to the nanomolar range could be developed. Interestingly, within that class of complexes, amides were less cytotoxic than ester derivatives, although showing a comparable lipophilicity and redox potential. The terminal COOH group was also advantageously used for coupling biologically active molecules[27–30] or targeting moieties[31–37] to the platinum compounds.

It is generally accepted that the inert platinum(IV) complexes act as prodrugs,[38] which have to be activated via reduction. A direct interaction of platinum(IV) agents with the ultimate target, the DNA, via ligand exchange reaction is considered extremely slow and not likely in the organism[39]. Reduction to the respective platinum(II) complexes is normally accompanied by release of the axial ligands; however, *Gibson* et al. recently showed that this assumption has to be handled with care.[40] It was also assumed that the reduction (activation) of platinum(IV) complexes is mainly dependent on the redox potential, which is controlled by the nature of the axial ligands: (i) compounds with axial chlorido ligands are reduced easily, (ii) compounds with axial hydroxido ligands are reduced hardly, and (iii) compounds with axial carboxylato ligands are situated in between.[41] Very recently, *Gibson* et al. presented results, demonstrating that the rate of reduction and the reduction potential do not necessarily correlate[42].

One possibility to shed more light on the mechanism of action of platinum(IV) complexes trusts in the use of ^{15}N and/or ^{13}C labeled compounds being investigated in cancer cell lysates. Due to the isotopic labeling, two-dimensional NMR experiments can be performed at rather low concentrations (high micromolar range) in reasonable time (some minutes). The use of cancer cell extracts guarantees that a plethora of biomolecules is able to interact with the platinum complex. Consequently, it is not that artificial like the reaction of a complex with only one single constituent of the cell culture system. The behavior of platinum(IV) compounds with ^{13}C labeled axial acetato ligands has recently been investigated in cancer cell extracts by *Gibson* et al.,[43,44] showing that reduction rates varied in aqueous extracts of different cancer cell lines.

The aim of the present work was to synthesize and characterize isotopically labeled platinum(IV) compounds which were build up by the reaction of dihydroxidoplatinum(IV) derivatives with ^{13}C labeled succinic anhydride. To be on the safe side, the compounds were additionally provided with ^{15}N labeled ammine ligands in order to judge the oxidation state of the central platinum ion. One further aspect was to prepare the respective amides and esters. Last but not least, preliminary NMR spectroscopic investigations in cancer cell lysates should be performed in order to judge the usefulness of that system for further and in depth studies.

Results and Discussion

Synthesis and Characterization

^{15}N labeled cisplatin was synthesized in analogy to *Dhara*'s method;[45] K_2PtCl_4 was converted to K_2PtI_4 and subsequently brought to reaction with $^{15}NH_4Cl$ in the presence of NaOH (Figure 3).

The resulting bis{(^{15}N)ammine}diiodoplatinum(II) was suspended in H_2O and after addition of 1.95 equivalents of $AgNO_3$, AgI was precipitated. The aqua ligands were replaced by addition of HCl or 1,1-cyclobutanedicarboxylate to obtain ^{15}N labeled cisplatin (**1**) or carboplatin (**2**), respectively. Both complexes were oxidized by reaction with 30% hydrogen peroxide in water. Further carboxylation of **3** and **4** with (2,5–$^{13}C_2$)succinic anhydride in dry DMF yielded the desired carboxylatoplatinum(IV) complexes **5** and **6**.[16] The COOH groups were activated with 1,1′-carbonyldiimidazole (CDI);

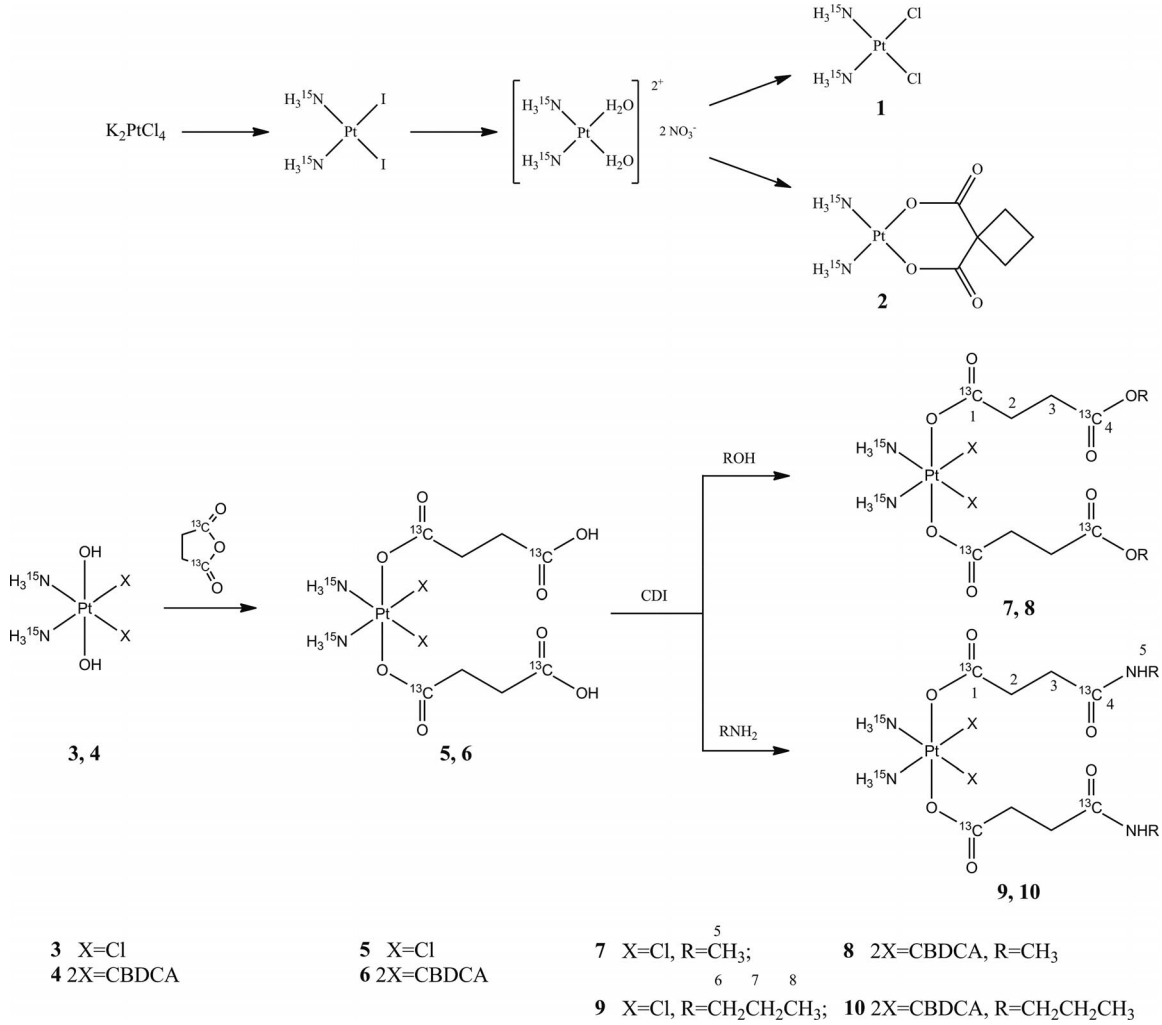

Figure 3. Synthesis and NMR numbering scheme of target complexes **7**–**10** (CBDCA = cyclobutanedicarboxylato ligand of carboplatin).

addition of sodium methoxide or propylamine resulted in the formation of esters **7**, and **8** or amides **9**, and **10**, respectively.

The latter substances **7**–**10** were characterized by elemental analysis, ESI mass spectrometry and multinuclear (^{1}H, ^{13}C, ^{15}N, and ^{195}Pt) 1D- and 2D-NMR spectroscopy.

NMR characterization of **7**–**10** is in principle straight forward, however, due to ^{13}C, and ^{15}N isotopic labeling, some interesting features can be observed in the NMR spectra which should be discussed in more detail exemplarily for one of the complexes, namely the cisplatin analog **7** featuring two axial methyl succinato ligands (see Figure 3 for NMR numbering scheme).

Oxidation state of the central platinum ion and kind of coordinated atoms can best be judged via acquiring a ^{195}Pt NMR spectrum (^{195}Pt chemical shifts span a wide range of several thousand ppm, e.g., K_2PtCl_4 and K_2PtCl_6 are separated by around 1620 ppm). The ^{195}Pt resonance of **7** was detected at δ = 2802 ppm (Figure 4, measured relative to K_2PtCl_4) being indicative for platinum in the oxidation state +IV and having a $Cl_2N_2O_2$ coordination sphere.

In unlabeled platinum complexes, the Pt resonance is broad due to the influence of coordinated ^{14}N (^{14}N is a quadrupolar nucleus leading to a fast relaxation of ^{195}Pt resulting in broad resonances), therefore normally not showing any coupling information. In contrast, in **7** platinum is attached to $^{15}NH_3$ in which the nitrogen isotope has a spin of ½. Consequently, a well resolved triplet (coupling of ^{195}Pt with two coordinated $^{15}NH_3$ nuclei) with a $^1J_{N,Pt}$ coupling constant of 252 Hz was observed in the proton decoupled ^{195}Pt NMR spectrum.

In the ^{13}C NMR spectrum, the nonlabeled atoms C-5, C-2, and C-3 (see Figure 3 for NMR numbering scheme) were hardly detected. Both methylene carbons (C-2, C-3, centered at δ = 29.7 and 30.6 ppm, Figure 4) coupled with the neighboring ^{13}C=O labeled carbon atom as well as with that being separated by one CH_2 moiety, resulting in a doublet of doublets with coupling constants of around $^1J_{C,C}$ = 57 Hz, and $^3J_{C,C}$ = 1.5 Hz. The signal centered at δ = 30.6 ppm could relatively easily be observed; whereas the second (centered at 29.7) was superimposed by one signal of the deuterated solvent (Figure 4, arrows). Methyl ester carbon C-5 (δ =51.1 ppm) split

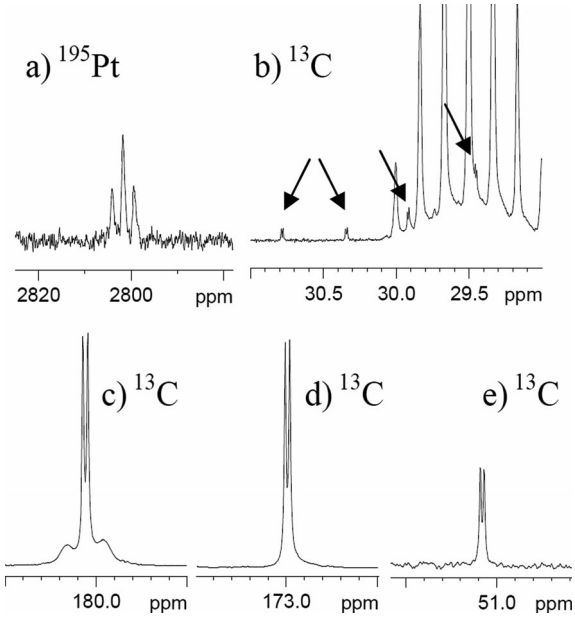

Figure 4. Sections of ^{195}Pt and ^{13}C NMR spectra of **7**; a) the triplet of the ^{195}Pt resonance is a consequence of the coupling with two coordinated $^{15}NH_3$ ligands, b) signals of C-2 and C-3 centered at δ = 29.7 and 30.6 ppm, respectively; resonances of C=O carbon atoms C-1 (c) and C-4 (d), and methyl carbon atom C-5 (e).

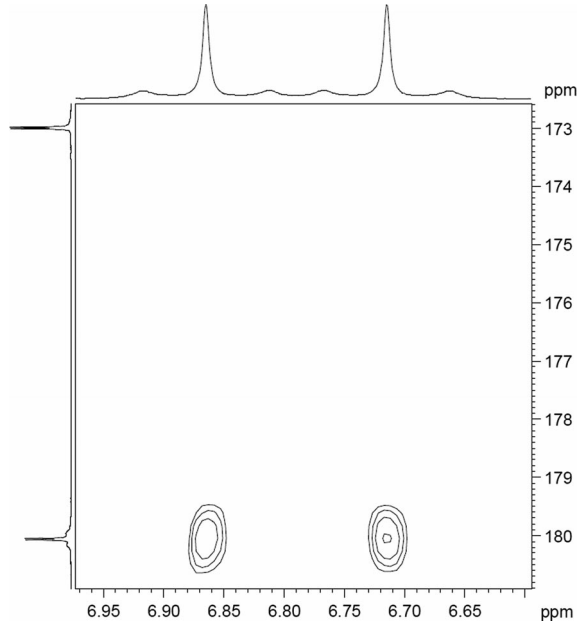

Figure 5. Section of the long-range ^{1}H,^{13}C HMBC NMR spectrum of **7** showing shift correlation signals between each resonance of the doublet of coordinated $^{15}NH_3$ (6.72, and 6.87 ppm) with the ^{13}C=O carbon atom at δ = 180.1 ppm. ^{195}Pt satellites are visible in the ^{1}H NMR spectrum, flanking each resonance of the doublet.

into a doublet, coupling with ^{13}C-4 by $^{2}J_{C,C}$ = 1.5 Hz, thereby proofing formation of the ester group.

Isotopically labeled quarternary atoms ^{13}C-1 (δ =180.1 ppm) and ^{13}C-4 (δ =173.0 ppm) could be distinguished via their long range couplings in the ^{1}H,^{13}C HMBC NMR spectrum; a shift correlation signal between methyl ester protons 5-H and C-4 was detected. Very remarkably, cross peaks between the ammine protons and ^{13}C-1 via three different hetero nuclei (N-Pt-O) could be observed (Figure 5). Additionally, atoms ^{13}C-1 and ^{13}C-4 could unambiguously be assigned via the broad $^{2}J_{Pt,C}$ satellites of around 25 Hz at ^{13}C-1. Besides, ^{13}C-1 and ^{13}C-4 coupled to each other with a $^{3}J_{C,C}$ coupling of 3.2 Hz (Figure 4).

In Figure 5, also the characteristic signal of ammine protons in the ^{1}H NMR spectrum at δ = 6.78 ppm is shown. It is shifted downfield by about 2.8 ppm in comparison to cisplatin; each signal (6.72, and 6.87 ppm) of the sharp doublet ($^{1}J_{N,H}$ = 75.2 Hz) is flanked by ^{195}Pt satellites ($^{2}J_{Pt,H}$ = 52.1 Hz). Further features in the ^{1}H NMR spectrum are the additional couplings of protons 2-H, 3-H, and 5-H with ^{13}C labeled nuclei, resulting in a doublet (5-H), and two quintets (2-H and 3-H) instead of a singlet and two triplets.

Recently, *Gibson* et al.[43] investigated the behavior of diamminedichloridoplatinum(IV) complexes, featuring ^{13}C labeled acetato ligands in the axial position, in cancer cell lysates. By doing that, they were able to evaluate the rate of reduction in dependence on the cell line chosen. Since more and more research groups work with platinum(IV) complexes with axially coordinated succinato ligands, it was the aim of the present work to transfer *Gibson*'s procedure to the novel class of compounds by introducing ^{13}C labeling, and to Figure out similarities and/or differences.

The feasibility of such studies is dependent on the following parameters: (i) the reducing capabilities of the cell line, (ii) the number of cells used for preparing the respective lysate, (iii) the concentration of the platinum complex, which should be high enough to measure appropriate NMR spectra in a relatively short time, but low enough to guarantee complete reduction by the components of the cell lysate, and (iv) to have NMR signals which can be integrated. The last point of course is most critical since it cannot be influenced very easily. For the investigations, it was decided to acquire the NMR spectrum within reasonable time (22 or 36 minutes) and at ambient temperature (26 °C), so that the measurement itself is fast enough compared to the reduction process.

In a first attempt, 251 μM of complex **5** were incubated with an extract from 80 million Jurkat (T-lymphocytic leukemia) cells. Within the first 100 hours, no reaction was detected. The same result was found for 482 μM of **5** when incubated with the lysate of 80 million SW480 (colon carcinoma) cells. Increasing the number of Jurkat cells to 180 million for preparation of the lysate (236 μM of complex **5**) did not result in a significant reduction of the platinum(IV) complex. Further and significant decrease of compound concentration was not possible from the NMR point of view; increase of the number of cells was not practicable. In a third attempt, 303 μM of **5** were incubated with an extract of 138 million SW480 cells. Under these conditions, reduction was completed in around 50 hours.

In this context it should be mentioned that accurate integration of shift correlation signals in the ^{1}H,^{13}C HMBC NMR spectrum was not possible due to an overlapping of product cross peaks with those of the platinum(IV) starting complex (Figure 6).

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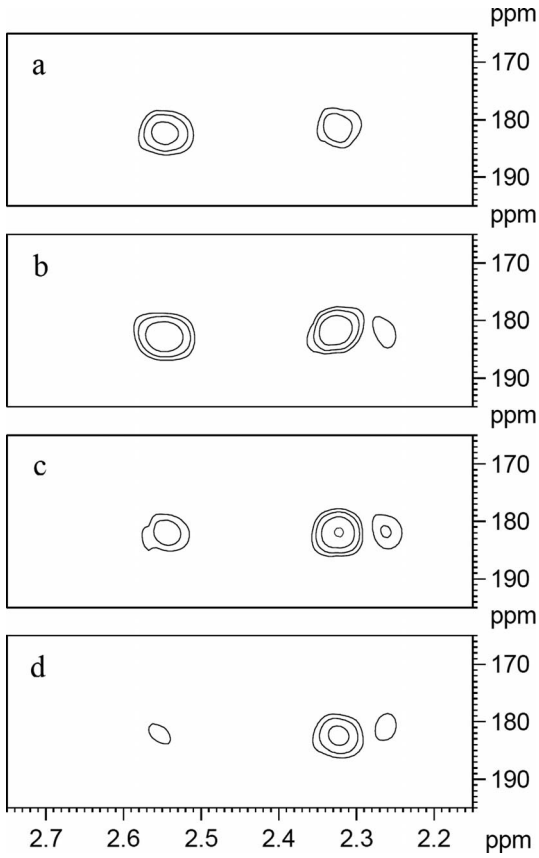

Figure 6. Incubation of 303 μM of **5** with an extract of 138 million SW480 cells, sections of the $^{1}H,^{13}C$ HMBC NMR spectra at the beginning (a), after 4.4 (b), 10.4 (c), and 27.5 (d) hours. Shift correlation signals derive from the long-range coupling of protons 2-H and 3-H with carbon atoms C-1 and C-4.

The only possibility of getting time dependent data, was to integrate just one signal of complex **5** (at 2.55/182.4 ppm) in relation to the second signal of **5** (2.33/181.4 ppm) together with signals coming up as result of the reduction process. A half time of around 13–14 h was found (Figure S1, Supporting Information)

In a next step, both methyl ester derivatives, **7** (cisplatin analog), and **8** (carboplatin analog), were investigated; 206 μM of **7** and 217 μM of **8** were incubated with lysates of 150 and 160 million SW480 cells, respectively (variation in the cell number was compensated by adapting the concentration of the complex). In that case, the cross peak of methyl ester protons with C-4 in the $^{1}H,^{13}C$ HMBC NMR spectra at around 3.6/176 ppm was set to 1 as a reference signal and shift correlation signals generated during reduction of the complexes were given relative to the former peak.

Cisplatin analog **7** was reduced faster in comparison to carboplatin analog **8** (Figures S2 and S3, Supporting Information). This is in accord with recently published reports, namely that tetrakis(carboxalato)platinum(IV) complexes show a significantly different rate of reduction although having comparable redox potentials to bis(carboxylato)dichlorido counterparts.[24,42] A ratio of 0.5 between the reference signal and product peaks was reached in the case of cisplatin analog **7** after around 12–13 hours (Figures S2, Supporting Information). In contrast, reduction of carboplatin analog **8** was remarkably slower reaching a ratio of 0.5 after more than 100 hours (Figures S3, Supporting Information). At this point, it should not remain unmentioned that hydrolysis of the methyl ester via esterases is in principle possible in cell lysates and that absolute kinetic values cannot be drawn from these investigations; however, when investigating two compounds in one and the same cell line lysate, comparison of the rate of reduction is still possible, as demonstrated here. Unfortunately, amide derivative **9** and **10** could not be investigated, due to a severe overlap of starting material and product signals. Therefore, one aim of the present work, namely to compare ester analogs **7** and **8** with amide derivatives **9** and **10** could not be performed.

In conclusion, ^{13}C labeled analogs as representatives of a novel class of cytotoxic platinum(IV) complexes were successfully synthesized and characterized. Preliminary investigations of their behavior in aqueous extracts of cancer cells were performed and it was manageable to follow the reduction under some assumptions for some derivatives. An accurate, time dependent evaluation resulting in half live times was however not possible because of overlapping signals in the NMR spectra. Consequently, subsequent and in depth investigations based on that setup will unfortunately not be conducted.

Experimental Section

2,5-^{13}C-labeled succinic anhydride was synthesized according to literature methods.[46] Syntheses, especially when platinum(II) complexes were involved, were carried out under light protection; glass coated magnetic stirring bars were used for each synthesis. K_2PtCl_4 was obtained from Johnson Matthey (Switzerland). All other chemicals were purchased from Aldrich, Fluka, Acros or Fisher and used without further purification. Methanol was dried by distillation over magnesium and stored under argon. For reactions performed in water, doubly distilled osmosis water was used.

Physical Measurements

Elemental analysis was carried out by the Microanalytical Laboratory of the University of Vienna with a 2400 CHN Elemental Analyzer manufactured by Perkin–Elmer. For each submitted substance, the percentage of the elements carbon, hydrogen and nitrogen was determined. Electrospray ionization mass spectra were recorded with a Bruker Esquire$_{3000}$ ion trap spectrometer with MeOH as solvent, the molecular mass was determined in the positive as well as in the negative mode. ^{1}H, ^{13}C, ^{15}N, and ^{195}Pt one- and two-dimensional NMR spectra were recorded with a Bruker Avance III 500 MHz instrument at 500.32 (^{1}H), 125.81 (^{13}C), 50.70 (^{15}N), and 107.55 (^{195}Pt) MHz. 2D NMR measurements were recorded using standard pulse programs. Chemical shifts were referenced relative to the solvent signal for ^{1}H and ^{13}C spectra; for ^{15}N and ^{195}Pt spectra, the external standards NH_4Cl and $K_2[PtCl_4]$ were used, respectively.

Synthesis of (2,5-$^{13}C_2$)-labeled succinic anhydride: (1,4–$^{13}C_2$)Succinic acid (1 g, 8.19 mmol) was placed in an argon atmosphere and suspended in acetic anhydride (3.16 mL, 28.67 mmol). The suspension was heated to 110 °C and stirred for 1.5 hours. The solution was cooled to room temperature and then placed into an ice bath. The white pre-

cipitate was filtered, washed with ice cold ether and dried in vacuo. The filtrate was reduced to dryness on a rotavapor and dried in vacuo for 16 hours. The two products were combined. $C_4H_4O_3$, white solid, 85 % yield. **^{1}H NMR** ([D$_6$]DMSO): δ = 2.91 (d, ^2J(C,H) = 3.0) ppm. **^{13}C NMR** ([D$_6$]DMSO): δ = 29.2 (dd, ^1J = 3.0, ^2J = 50.4, C-2), 173.5 (C-1) ppm.

Synthesis of (SP-4-2)-Bis((15N)ammine)dichloridoplatinum(II) (1): K_2PtCl_4 (1.5 g, 3.6 mmol) was dissolved in H_2O (30 mL) and filtered through a cotton plug. KI (3 g, 18 mmol) dissolved in H_2O (5 mL) was added and the solution was left stirring for 20 min at room temperature. Then, $^{15}NH_4Cl$ (5 mg, 9 mmol) in NaOH (2 M *solution*) was added and the solution was stirred for three more hours. The solution was cooled to 4 °C and the yellow precipitate ([PtI$_2$(^{15}NH$_3$)$_2$]) was filtered and washed with cold H_2O. $AgNO_3$ (500 mg, 3 mmol) was added to a suspension of ^{15}N-PtI$_2$(NH$_3$)$_2$ (720 mg, 1.48 mmol) in H_2O (12 mL) and stirred for 24 hours at room temperature. The solution was filtered through a glass filter with a paper filter disk (MN GF-3) to remove the obtained AgI. HCl (37 %, 306 µL, 3.7 mmol) was added to the filtrate, stirred for 3 hours and left in the fridge for 16 hours. The precipitate **1** was filtered off, washed with H_2O and dried in a vacuum desiccator over P_4O_{10}. $H_6Cl_2N_2Pt$, yellow solid 86 % yield. **^{1}H NMR** (H_2O/D_2O 9:1): δ = 3.97 (d+dd, $^1J_{N,H}$ = 73.0, $^2J_{Pt,H}$ = 63.5 Hz) ppm

Synthesis of (SP-4-2)-Bis((15N)ammine)(1,1-cyclobutanedicarboxylato)platinum(II) (2): $AgNO_3$ (245 mg, 1.4 mmol) was added to a suspension of ^{15}N-PtI$_2$(NH$_3$)$_2$ (357.6 mg, 0.74 mmol) in H_2O (8 mL) and stirred for 24 hours at room temperature. The solution was filtered through a glass filter with a paper filter disk (MN GF-3) to remove the obtained AgI. CBDCA (107 mg, 0.74 mmol) was dissolved in NaOH (2 M, 0.74 mL) to generate the disodium salt of CBDCA, which was added to the filtered solution, heated to 40 °C for 10 min. and stirred for 3 more hours at room temperature. Then, the solution was cooled to 4 °C and the white precipitate was filtered off. The filtrate was reduced almost to dryness with a rotavapor cooled to 4 °C and the white precipitate **2** was filtered off again. $C_6H_{12}N_2O_4Pt$, white solid, 64 % yield. **^{1}H NMR** (D_2O): δ = 1.80 (t, ^3J = 7.9 Hz, 2 H, CBDCA), 2.78 (m, ^3J = 7.9 Hz, 4 H, CBDCA) ppm.

Synthesis of (OC-6-33)-Bis((15N)ammine)dichloridodihydroxidoplatinum(IV) (3): ^{15}N-labeled cisplatin (383 mg, 1.27 mmol) was suspended in H_2O (8 mL) and H_2O_2 (30 %, 8 mL) was added. The solution was stirred for 30 min at room temperature, for 10 min at 50 °C and cooled to 4 °C for 16 hours. The precipitate (3) was filtered off, washed with a small amount of H_2O and dried in a vacuum desiccator over P_4O_{10}. $H_6Cl_2N_2Pt$, pale yellow solid 81 % yield.

Synthesis of (OC-6-33)-Bis((15N)ammine)(cyclobutane-1,1-dicarboxylato)dihydroxidoplatinum(IV) (4): ^{15}N-labeled carboplatin (409.7 mg, 1.09 mmol) was suspended in H_2O (6 mL), H_2O_2 (30 %, 6 mL) was added and the solution was stirred for 2 hours. The colorless solution turned into a white foam and was cooled to 4 °C. The pale yellow precipitate (4) was filtered off, washed with cold H_2O and dried in a vacuum desiccator over P_4O_{10}. The filtrate was cooled to 4 °C for 2 hours after reducing the volume. The pale yellow precipitate (4) was filtered off and dried over P_4O_{10}. The two precipitates were combined. $C_6H_{14}N_2O_6Pt$, 48 % yield. **^{1}H NMR** (D_2O): δ = 1.96 (t, ^3J = 8.1 Hz, 2 H, CBDCA), 2.59 (m, 4 H, CBDCA), 5.70 (d+dd, $^1J_{N,H}$ = 74.5, $^2J_{Pt,H}$ = 53 Hz) ppm.

Synthesis of (OC-6-33)-Bis((15N)ammine)bis[(3-(13C)carboxy(1-13C)propanato]dichloridoplatinum(IV) (5): 3 (353 mg, 1.052 mmol) and ^{13}C-labeled succinic anhydride (322.2 mg, 3.16 mmol) were suspended in DMF (5 mL) and stirred at 70 °C for 2 hours. DMF was evaporated in vacuo. The residue was suspended in acetone and filtered off. Et_2O was added to the yellow filtrate to precipitate **5**. The substance was filtered off and dried overnight in vacuo. $C_8H_{16}Cl_2N_2O_8Pt$, white solid, 81 % yield. **^{1}H NMR** ([D$_7$] DMF): δ = 2.48 (m, 4 H, 2-H/3-H), 2.56 (m, 4 H, 2-H/3-H), 6.79 (d+dd, $^1J_{N,H}$ = 75.2, $^2J_{Pt,H}$ = 51 Hz, 6 H, NH_3), 12.32 (br. s, 2 H, OH) ppm. **^{13}C NMR** ([D$_7$] DMF): δ = 29.8 (C-2/C-3), 30.6 (C-2/C-3), 173.9 (d, ^3J = 3.4 Hz, C-4), and 180.3 (d, ^3J = 3.6 Hz, 1-C) ppm. **^{15}N NMR** ([D$_7$] DMF): δ = –41.6 ppm. **^{195}Pt NMR** ([D$_7$] DMF): δ = 2803 (t, $^1J_{N,Pt}$ = 253) ppm.

Synthesis of (OC-6-33)-Bis((15N)ammine)bis[(3-(13C)carboxy(1-13C)propanato](cyclobutane-1,1-dicarboxylato)platinum(IV) (6): 4 (99 mg, 0.243 mmol) and ^{13}C-labeled succinic anhydride (75 mg, 0.730 mmol) were suspended in DMF (4 mL) and stirred at 50 °C for 2 hours. DMF was evaporated in vacuo. The residue **(6)** was suspended in acetone, filtered off and dried in vacuo. $C_{14}H_{22}N_2O_{12}Pt$, white solid, 69 % yield. **^{1}H NMR** ([D$_6$]DMSO): δ = 1.81 (m, 2 H, CBDCA), 2.37 (m, 6 H, 2-H/3-H/CBDCA), approx. 2.5 (underneath the DMSO-peak, 6 H expected, 2-H/3-H/CBDCA), 6.35 (m, $^1J_{N,H}$ = 74.4 Hz, 6 H, NH_3), and 12.07 (br. s, 2 H, OH) ppm. **^{13}C NMR** ([D$_6$]DMSO): δ = 16.2 (CBDCA), 30.0 (C-2/C-3), 30.9 (C-2/C-3), 31.7 (CBDCA), 56.0 (CBDCA), 174.2 (C-1), 179.1 (C-4), and 179.7 (CBDCA) ppm. **^{15}N NMR** ([D$_6$]DMSO): δ = –51.4 ppm.

Synthesis of (OC-6-33)-Bis((15N)ammine)dichloridobis{(4-methoxy)-4-oxo(1,4-13C$_2$)butanoato} platinum(IV) (7): CDI (153 mg, 0.94 mmol) was dissolved in DMF (5 mL) and added to a solution of ***5*** (250 mg, 0.46 mmol) in DMF (5 mL) under argon atmosphere and heated to 60 °C for 10 min. The solution was allowed to cool to room temperature and the CO_2 was removed by flushing with argon. A small piece of sodium, dissolved in methanol (in order to generate NaOMe) was added to the solution and stirred for 24 hours. DMF was removed in vacuo. The product was dissolved in MeOH and purified by column chromatography (EtOAc/MeOH 8:1). **7** was precipitated with EtOAc, filtered and dried in vacuo. $C_{10}H_{20}Cl_2N_2O_8Pt$, pale yellow solid, 22 % yield. **^{1}H NMR** ([D$_7$] DMF): δ = 2.50 (m, 4 H, 2-H), 2.57 (m, 4 H, 3-H), 3.64 (d, $^3J_{C,H}$ = 3.8 Hz, 6 H, 5-H), and 6.78 (d+dd, $^1J_{N,H}$ = 75.2, $J_{Pt,H}$ = 52.2 Hz, 6 H, NH_3) ppm. **^{13}C NMR** ([D$_7$] DMF): δ = 29.7 (dd, $^1J_{C,C}$ = 58.4, $^2J_{C,C}$ = 1.6 Hz, C-2/C-3), 30.6 (dd, $^1J_{C,C}$ = 55.4, $^2J_{C,C}$ = 1.6 Hz, C-2/C-3), 51.1 (d, $^2J_{C,C}$ = 2.7 Hz, C-5), 173.0 (d, $^3J_{C,C}$ = 3.2 Hz, C-4), 180.1 (dd, $^3J_{C,C}$ = 3.2, $^2J_{Pt,C}$ = 25 Hz, C-1) ppm. **^{15}N NMR** ([D$_7$] DMF): δ = –42.0 ppm. **^{195}Pt NMR** ([D$_7$] DMF): δ = 2802 (t, $^1J_{N,Pt}$ = 252) ppm. **Elemental Analysis:** $C_{10}H_{20}Cl_2N_2O_8Pt$ (M = 568,26); C 21.14 (20.71); H 3.54 (3.23); N 4.93 (4.78) %. **MS** (180 °C): *m/z* = 591 [M + Na$^+$], 567 (M-H$^+$), 603 (M+Cl$^-$).

Synthesis of (OC-6-33)-Bis((15N)ammine)(cyclobutane-1,1-dicarboxylato)bis{(4-methoxy)-4-oxo(1,4-13C$_2$)butanoato}platinum(IV) (8): CDI (2.05 equiv., 59 mg, 0.37 mmol) was dissolved in DMF (3 mL) and added to a solution of 6 (102 mg, 0.17 mmol) in DMF (3 mL) under argon atmosphere and heated to 60 °C for 10 min. The solution was allowed to cool to room temperature and the CO_2 was removed by flushing with argon. A small piece of sodium, dissolved in methanol (in order to generate NaOMe) was added to the solution and stirred for 24 hours. DMF was removed in vacuo. The product was dissolved in MeOH and purified by column chromatography (EtOAc/MeOH 3:1). 8 was precipitated with EtOAc, filtered and dried in vacuo. $C_{16}H_{26}N_2O_{12}Pt$, white solid, 37 % yield. **^{1}H NMR** ([D$_7$] DMF): δ = 2.07 (q, $^3J_{H,H}$ = 8.0 Hz, 2 H, CBDCA), 2.65 (m, 4 H, 2-H/3-H), 2.70 (m, 4 H, 2-H/3-H), 2.80 (t, 4 H, $^3J_{H,H}$ = 8.0 Hz, CBDCA), 3.79 (d, $^3J_{C,H}$ = 3.8 Hz, 6 H, 5-H), 6.88 (d+dd, $^1J_{N,H}$ = 74.6, $^2J_{Pt,H}$ = 53.6 Hz, 6 H, NH_3) ppm. **^{13}C NMR** ([D$_7$] DMF): δ = 16.1 (CBDCA), 29.9 (C-2/C-3), 30.0 (C-2/C-3), 32.0 (CBDCA), 51.2 (d, $^2J_{C,C}$ = 2.7 Hz, C-5), 56.5 (CBDCA), 173.0 (d, $^3J_{C,C}$ = 3.4 Hz, C-4), 176.9 (CBDCA), 179.4

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(d+d, $^3J_{C,C}$ = 3.3, $^2J_{Pt,C}$ = 25.4 Hz, C-1) ppm. **^{15}N NMR** ([D_7] DMF): δ = –53.9 ppm. **^{195}Pt NMR** ([D_7] DMF): δ = 3566 (t, $^2J_{Pt,N}$ = 294) ppm. **Elemental Analysis:** $C_{16}H_{26}N_2O_{12}Pt$ (M = 639.46); C 30.05 (29.64); H 4.10 (3.89); N 4.38 (4.51)%. **MS** (180 °C): *m/z* = 661 [M + Na^+], 638 (M-H^+), 674 (M+Cl^-).

Synthesis of (OC-6-33)-Bis((15N)ammine)dichloridobis{(4-propylamino)-4-oxo(1,4-$^{13}C_2$)butanoato}platinum(IV) (9): CDI (81 mg, 0.5 mmol) was dissolved in DMF (5 mL), added to a solution of **5** (132 mg, 0.24 mmol) in DMF (5 mL) under argon atmosphere and heated to 60 °C for 10 min. The solution was allowed to cool to room temperature and the CO_2 was removed by flushing with argon. N-Propylamine (50 μL, 0.6 mmol) in DMF (5 mL) was added to the solution and stirred for 24 hours. DMF was removed in vacuo.

The product was dissolved in MeOH and purified by column chromatography (EtOAc/MeOH 4:1). **9** was precipitated with EtOAc, filtered and dried in vacuo. $C_{14}H_{30}Cl_2N_4O_6Pt$, white solid, 26.1% yield. **^{1}H NMR ([D_7] DMF):** δ = 0.86 (t, ^3J = 7.4 Hz, 6 H, 8-H), 1.47 (m, 4 H, 7-H), 2.39 (m, 4 H, 2-H/3-H), 2.49 (m, 4 H, 2-H/3-H), 3.10 (m, 4 H, 6-H), 6.82 (d+dd, $^1J_{N,H}$ = 75.2, $^2J_{Pt,H}$ = 51.7 Hz, 6 H, NH_3), and 7.78 (m, 2 H, 5-H) ppm. **^{13}C NMR ([D_7] DMF):** δ = 11.1 (C-8), 22.7 (d, $^3J_{C,C}$ = 1.4 Hz, C-7), 31.8 (dd, $^1J_{C,C}$ = 55.4, $^2J_{C,C}$ = 1.4 Hz, C-2/C-3), 31.9 (dd, $^1J_{C,C}$ = 50.0, $^2J_{C,C}$ = 1.4 Hz, C-2/C-3), 40.8 (d, $^2J_{C,C}$ = 0.9 Hz C-6), 171.8 (d, $^3J_{C,C}$ = 3.2 Hz, C-4), 180.9 (d, $^3J_{C,C}$ = 3.2 Hz, C-1) ppm. **^{15}N NMR ([D_7] DMF):** δ = –42.5 ppm (NH_3), 93.7 (NH). **^{195}Pt NMR ([D_7] DMF):** 2800 (t, $^1J_{N,Pt}$ = 253 Hz) ppm. **Elemental Analysis**: $C_{14}H_{30}Cl_2N_4O_6Pt$ (M = 616.40); C 27.06 (27.01); H 3.73 (4.48); N 9.00 (9.08)%. **MS** (180 °C): *m/z* = 622 (M^+), 645 [M + Na^+], 621 (M-H^+), 657 (M+Cl^-).

Synthesis of (OC-6–33)-Bis((15N)ammine)(cyclobutane-1,1-dicarboxylato)bis{(4-propylamino)-4-oxo(1,4–$^{13}C_2$)butanoato}-platinum(IV) (10): CDI (49.9 mg, 0.307 mmol) was dissolved in DMF (4 mL) and added to a solution of 6 (96 mg, 0.15 mmol) in DMF (3 mL) under argon atmosphere and heated to 60 °C for 10 min. The solution was allowed to cool to room temperature and the CO_2 was removed by flushing with argon. N-Propylamine (51 μL, 0.625 mmol) in DMF (5 mL) was added to the solution and left stirring for 24 hours. DMF was removed in vacuo. The product was dissolved in MeOH and purified by column chromatography (EtOAc/MeOH 2:1). **10** was precipitated with EtOAc, filtered and dried in vacuo. $C_{20}H_{36}N_4O_{10}Pt$, pale yellow solid, 60% yield. **^{1}H NMR** ([D_7] DMF): δ = 0.87 (*t*, ^3J = 7.4 Hz, 6 H, 8-H), 1.45 (m, 4 H, 7-H), 1.89 (q, ^3J = 8.0 Hz, 2 H, CBDCA), 2.35 (m, 4 H, 2-H/3-H), 2.48 (m, 4 H, 2-H/3-H), 2.62 (*t*, ^3J = 8.0 Hz, 4 H, CBDCA), 3.09 (m, 4 H, 6-H), 6.73 (d+dd, $^1J_{N,H}$ = 74.4, $^2J_{Pt,H}$ = 53 Hz, 6 H, NH_3), 7.77 (m, 2 H, 5-H) ppm. **^{13}C NMR** ([D_7] DMF): δ = 11.0 (C-8), 15.9 (CBDCA), 22.7 (C-7, $^3J_{C,C}$ = 1.4 Hz), 31.2 (dd, $^1J_{C,C}$ = 55.6, $^2J_{C,C}$ = 1.7 Hz, C-2/C-3), 31.6 (dd, $^1J_{C,C}$ = 50.1, $^2J_{C,C}$ = 1.3 Hz, C-2/C-3), 31.8 (CBDCA), 40.8 (d, $^3J_{C,C}$ = 0.9 Hz, C-6), 56.3 (CBDCA), 171.5 (d, $^3J_{C,C}$ = 3.6 Hz, C-4), 176.8 (CBDCA), 180.1 (d, $^3J_{C,C}$ = 3.6 Hz, C-1) ppm. **^{15}N NMR** ([D_7] DMF): δ = –54.3 (NH_3), 93.6 (NH) ppm. **^{195}Pt NMR** ([D_7] DMF): δ = 3561 (t, $^1J_{Pt,N}$ = 294) ppm. Elemental Analysis: $C_{20}H_{36}N_4O_{10}Pt$ (M = 693.60); C 34.19 (33.74); H 5.16 (5.02); N 7.97 (8.14)%. **MS** (180 °C): *m/z* = 555 (M+Li^+-1 axial ligand), 571 (M+Na^+-1 axial ligand), 693 [M + H^+], 715 [M + Na^+], 691 (M-H^+).

Preparation of Cancer Cell Extracts

Jurkat (T-lymphocytic leukemia, human) and SW480 (colon carcinoma, human) cells were kindly provided by the cell bank of the Institute of Cancer Research, Department of Medicine I, Medical University of Vienna, Austria. Cell culture media and supplements were purchased from Sigma–Aldrich Austria. Cells were grown in 150-cm^2 culture flasks (Starlab), either in suspension in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 4 mM L-glutamine (Jurkat) or as adherent monolayers in Minimum Essential Medium supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 4 mM L-glutamine and 1% v/v non-essential amino acids from 100× ready-to-use stock (SW480), both without antibiotics at 37 °C under a humidified atmosphere containing 5% CO_2 and 95% air. Cells were harvested from several culture flasks (by the use of trypsin in the case of SW480 cells) and pooled as appropriate to obtain cell numbers ranging from 80–180 million Jurkat or 80–160 million SW480 cells. Cell suspensions were transferred to 15 mL tubes and centrifuged at 1200 g for 3 min, the supernatant was removed and cells were resuspended in 5 mL of the appropriate medium. Numbers of viable cells were inferred by counting dilutions of small aliquots (10 μL) of the cell suspension using trypan blue exclusion in a hemocytometer. Then, cells were washed three times by centrifugation at 1200 g for 3 min, removal of the supernatant and resuspending in 5 mL phosphate-buffered saline (PBS). Cell extracts were prepared as follows: after one more centrifugation step and removal of PBS, the cell pellet was suspended in 900 μL deionized water, transferred to 2 mL tubes, vortexed for 1 min, put on ice for 1 min, ultrasonicated for 3 × 15 sec with intervals of 1 min and centrifuged at 1200 g for 10 min at 4 °C. The supernatant was shock-frozen in liquid nitrogen and stored at –80 °C until shortly before the NMR spectroscopic experiments.

Incubation of Platinum Complexes with Cancer Cell Lysates and NMR Spectroscopic Investigation

Cell lysates of 80– 180 million cancer cells (from cell lines Jurkat or SW480) were freeze dried to a volume of 500 μL and filtered through cotton into a NMR tube in order to get rid of some insoluble material. D_2O (50 μL) was added to the extracts. Additionally, a solution of the respective complexes in H_2O (50 μL) was added and measured using a Bruker FT-NMR Avance III 500 MHz NMR spectrometer at 26 °C using standard pulse programs. Gradient-enhanced ^{1}H,^{13}C HMBC (Heteronuclear Multiple Bond Correlation) NMR spectra were acquired with an acquisition time of 0.1 s and a recycle delay of 1.5 s; typically, NMR spectra were recorded with 32 increments, and 24 or 40 transients.

Supporting Information (see footnote on the first page of this article): Figures showing the incubation of **5**, **7**, and **8** with an extract of SW480 cells.

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SUPPORTING INFORMATION

Title: Platinum(IV) Complexes Featuring Axial (1,4–$^{13}C_2$)Succinato Ligands – Synthesis, Characterization, and Preliminary Investigations in Cancer Cell Lysates

Author(s): J. Banfic, M. S. Adib-Razavi, M. Galanski,* B. K. Keppler*

Ref. No.: Z201300058

Supporting Information

Platinum(IV) complexes featuring axial (1,4-$^{13}C_2$)succinato ligands – synthesis, characterization, and preliminary investigations in cancer cell lysates

Jelena Banfic, Mahsa S. Adib-Razavi, Markus Galanski,* and Bernhard K. Keppler*

Contents

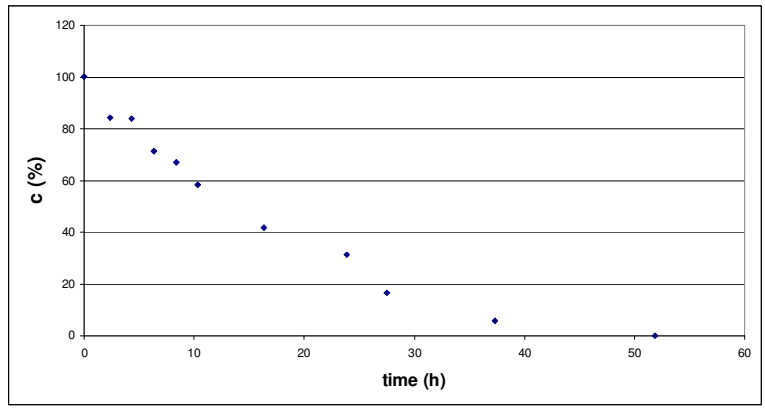


Figure S1. Incubation of 303 μM of **5** with an extract of 138 million SW480 cells, decrease of the starting complex measured via $^{1}H,^{13}C$ HMBC NMR spectroscopy.

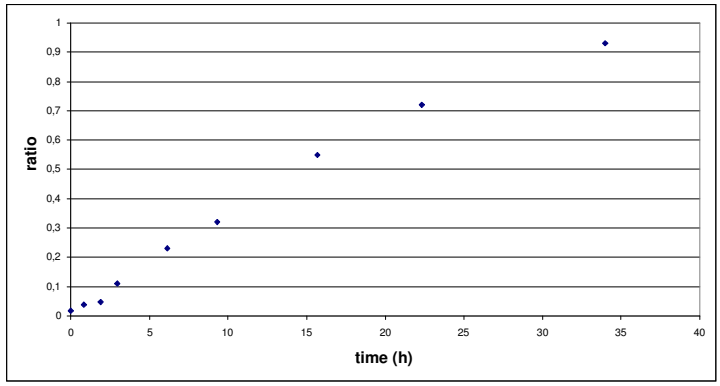


Figure S2. Incubation of 206 μM of the methyl ester derivative **7** (cisplatin analog) with an extract of 150 million SW480 cells, product cross peaks generated during reduction of the complex are given relative to the shift correlation signals of methyl ester protons with C-4 in the $^{1}H,^{13}C$ HMBC NMR spectra (the latter were set to 1).

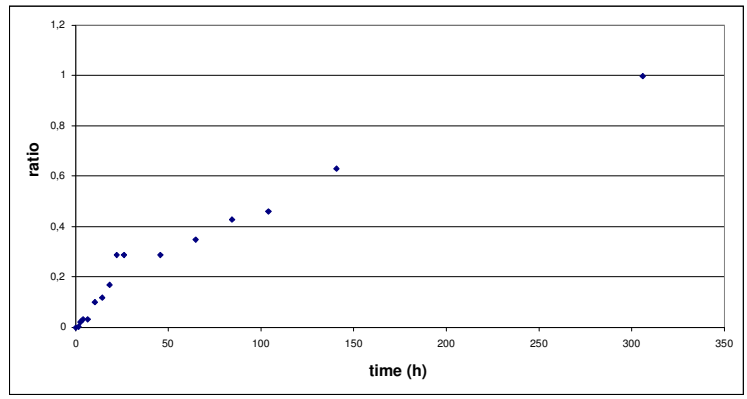
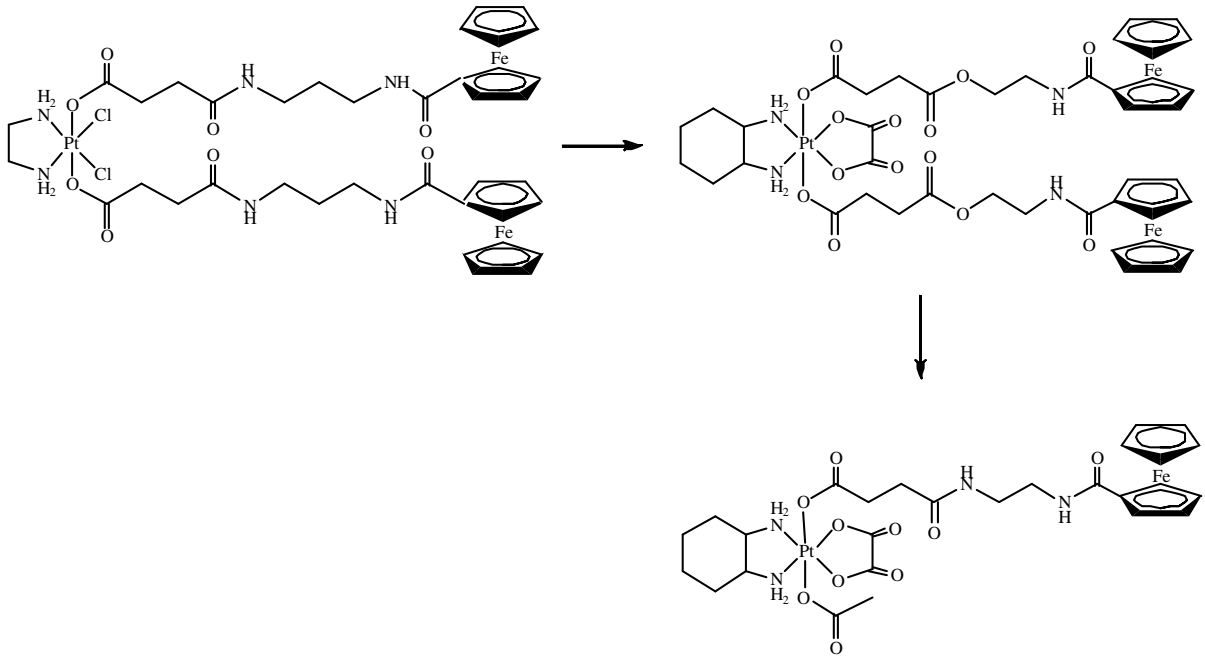


Figure S3. Incubation of 217 μM of the methyl ester derivative **8** (carboplatin analog) with an extract of 160 million SW480 cells, product cross peaks generated during reduction of the complex are given relative to the shift correlation signals of methyl ester protons with C-4 in the $^{1}H,^{13}C$ HMBC NMR spectra (the latter were set to 1).

2.2. Platinum(IV) Complexes Featuring One or Two Axial Ferrocene Bearing Ligands – Synthesis, Characterization, and Cytotoxicity

J. Banfić, A. A. Legin, M. A. Jakupec, M. Galanski, and B. K. Keppler



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J. Banfić synthesized and characterized all precursors and complexes, A. A. Legin carried out cell culture experiments, M. A. Jakupec supervised and discussed results of the cell culture experiments. M. Galanski discussed results, carried out NMR measurements, supervised the synthetic part of the work, and finalized the manuscript. B. K. Keppler supervised the work and discussed results.

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Platinum(IV) Complexes Featuring One or Two Axial Ferrocene Bearing Ligands – Synthesis, Characterization, and Cytotoxicity

Jelena Banfić,[a] Anton A. Legin,[a] Michael A. Jakupec,[a] Markus Galanski,*[a] and Bernhard K. Keppler*[a,b]

Keywords: Platinum / Ferrocene / Antitumor agents / Cytotoxicity / Metallocenes

Ferrocenyl compounds show interesting antiproliferative properties. Consequently, ferrocene bearing moieties were prepared and coupled for the first time to anticancer platinum(IV) complexes. The compounds, featuring either one or two axially coordinated ferrocene-containing ligands, were fully characterized by ESI-MS and multinuclear (^{1}H, ^{13}C, ^{15}N, and ^{195}Pt) one- and two-dimensional NMR spectroscopy. Their cytotoxicity was investigated in three human cancer cell lines deriving from ovarian carcinoma (CH1), colon carcinoma (SW480), and non-small-cell lung carcinoma (A549) by means of the colorimetric MTT assay. Promising IC_{50} values in the low micromolar range in CH1 and SW480 human cancer cells were found.

Introduction

In 1965, Barnett Rosenberg discovered accidentally the ability of cisplatin to inhibit cell proliferation.[1] After cisplatin had been granted approval in 1978 as a chemotherapeutic,[2] many efforts have been made to develop novel platinum compounds with anti-proliferative potential. Besides cisplatin, two so-called second- and third-generation platinum drugs, namely carboplatin and oxaliplatin, gained worldwide approval as antineoplastic drugs (Figure 1). Three other platinum-based compounds, nedaplatin, lobaplatin, and heptaplatin, gained regional approval in Japan, China, and South Korea, respectively.[3] Due to severe side-effects of cisplatin and its analogues and tumor resistance, and due to the fact that platinum(II) compounds are kinetically labile agents, the investigation of platinum(IV) compounds has recently received more and more interest. Platinum(IV) agents are kinetically inert, thus opening up the possibility of being administered orally and thereby enabling chemotherapy to become a more convenient treatment. Furthermore, six instead of only four coordination sites may be varied, facilitating a better fine-tuning of the pharmacological properties of the drug. By now, four platinum(IV) compounds have found their way into clinical trials, namely tetraplatin, iproplatin, satraplatin, and LA-12.[4]

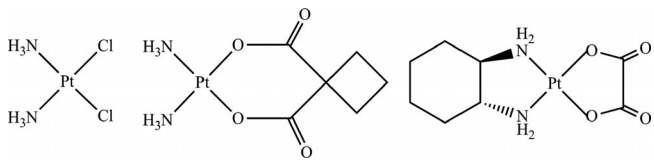

Figure 1. Worldwide approved platinum-based drugs: cisplatin (left), carboplatin (middle), and oxaliplatin (right).

Tetraplatin was abandoned due to its high toxicity in vivo, whereas iproplatin showed a lack of activity on account of its slow reduction rate.[5] Satraplatin failed to gain approval by the FDA but is still being investigated in combination therapy, while its derivative, LA-12 was in phase I clinical trials after having shown good in vivo results in mice.[4] Besides platinum-based metallodrugs, transition-metal-based complexes, such as ferrocene-bearing compounds, have shown strong antiproliferative effects in vitro as well as antitumor effects in vivo.[6,7] The most intensively investigated ferrocene-based compounds with anticancer potential are ferrocifens, prepared by replacing a phenyl group with ferrocene in tamoxifen, a selective estrogen receptor modulator (SERM) used in hormone-dependent (ER+) breast cancer treatment. In 1996, Jaouen et al. coupled ferrocene to 4-hydroxytamoxifen and modified the length of the carbon chain in order to optimize its pharmacological properties (Figure 2).[8] The hydroxyferrocifens show antiproliferative activity in hormone-dependent and in hormone-independent cells, whereas tamoxifen and hydroxytamoxifen are inactive in the latter.[9] Investigation of ferrocene-based anticancer agents ranged from design of ferrocenium salts (first investigated by Köpf-Maier et al. in

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1984)[10] over combination of ferrocenyl groups with transition-metal complexes and to coupling of ferrocene moieties to drug delivery systems, such as polymers and nanoparticles.[9] During the past two decades, the synthesis of several platinum(II) compounds bearing ferrocene moieties has been accomplished.[9,11–13] Some of these compounds have shown promising IC_{50} values (Figure 3): (i) Nieto et al. recently published the successful synthesis and first cell culture tests of a *cis*-configured ferrocene-platinum derivative, which showed in vitro activity in human breast, cervix, lung and colon cancer cells in the low micromolar range (partially exceeding the antiproliferative activity of cisplatin);[11] (ii) a *trans*-dichloridoplatinum(II) complex featuring two ferrocene-bound phosphane units was designed by Schulz et al., which yielded low IC_{50} values in the micromolar range in the ovarian cancer cell line A2780.[13] A platinum(IV)-ferrocenyl system was described recently.[14] However, with the aim of combining the advantages of anticancer platinum(IV) complexes with the therapeutic potential of ferrocene-based compounds, we report here the synthesis, characterization, and antiproliferative potential of the first anticancer platinum(IV) complexes bearing ferrocene moieties as axial ligands.

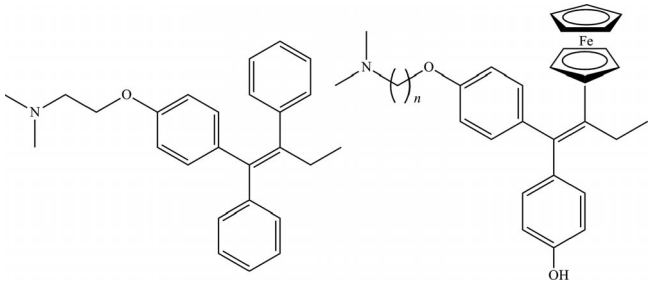
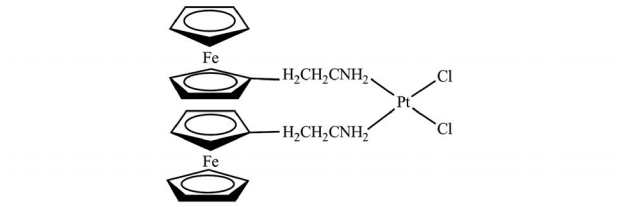


Figure 2. Molecular structures of SERM tamoxifen (left) and hydroxyferrocifens (right).



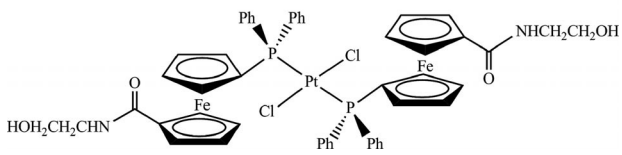


Figure 3. Cytotoxic platinum(II) complexes with ferrocene-bearing ligands.

Results and Discussion

Synthesis

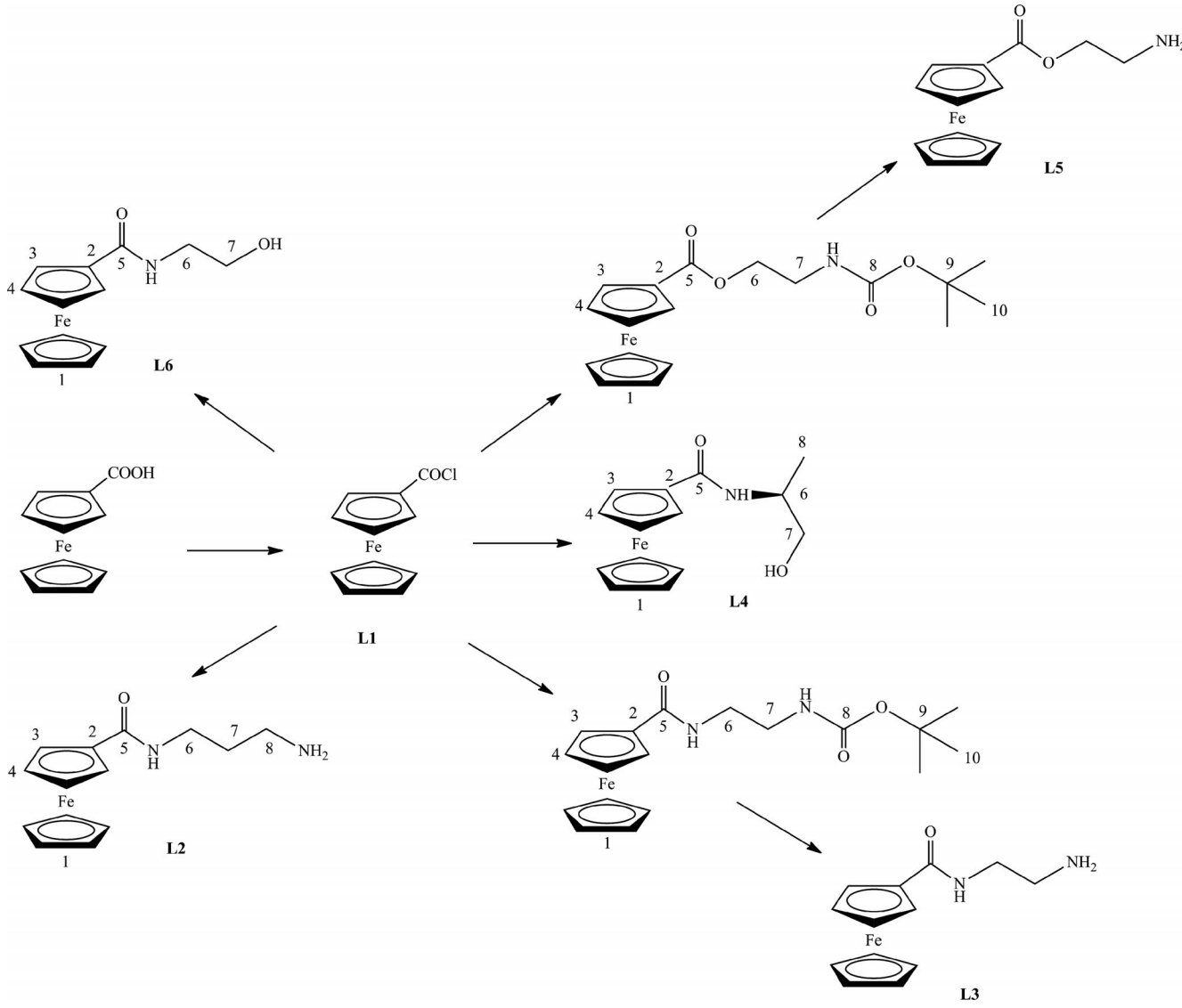
For generation of ferrocene precursors **L2**–**L6**, the synthesis started with preparation of ferrocenecarboxylic acid according to literature methods (Scheme 1).[15] The acid was converted into the acyl chloride, subsequently brought to reaction with the desired diamine, *N*-*boc*-ethylenediamine, *N*-*boc*-ethanolamine or amino alcohol, and characterized by ^{1}H NMR spectroscopy. Precursors **L2**, **L4**, and **L6** could directly be used without further purification for coupling with compounds **2**, **8**, and **10** (Schemes 2 and 3), respectively. Before amidation, the *N*-*boc* protecting groups were removed from precursors of **L3** and **L5** by reaction with conc. trifluoroacetic acid (TFA).

All investigated complexes were prepared from $K_2[PtCl_4]$ according to literature methods by conversion into two different platinum(II) precursors, dichlorido(ethane-1,2-diamine) platinum(II) and (*1R*,*2R*-diaminocyclohexane)(oxalato)platinum(II). Both complexes were oxidized subsequently in the presence of hydrogen peroxide to yield dihydroxidoplatinum(IV) complexes **1** and **7** (Schemes 2 and 3). Preparation of dicarboxylic acids **2** and **8** was performed with succinic anhydride. Activation of the uncoordinated carboxylic acid groups followed by conversion into amides was accomplished by 1,1′-carbonyldiimidazole (CDI). The formed platinum(IV) imidazolides were brought to reaction in situ with ferrocene-bearing amines **L2**, **L3**, and **L5**, which resulted in amides **4**, **3**, and **11**, respectively. Synthesis of esters **5** and **12** was carried out by using the peptide coupling reagent *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine. Since complexes **3**, **4**, **5**, **11**, and **12** show only poor solubility in water, analogous complexes with only one axial ferrocene fragment were prepared. For that purpose, (1*R*,2*R*-diaminocyclohexane)oxalatoplatinum(II) was oxidized with peracetic acid in acetic acid, which yielded acetato(1*R*,2*R*-diaminocyclohexane)hydroxido(oxalato)platinum(IV) (**9**). Thus, carboxylation with succinic anhydride of only one axial coordination site was facile, which resulted in an enhanced solubility in water of the substance when converted into esters and amides, respectively. Esterification and amidation were achieved by the same reaction pathways as described above. The final products **13**, **14**, and **15** were isolated in moderate yields of 20–41%.

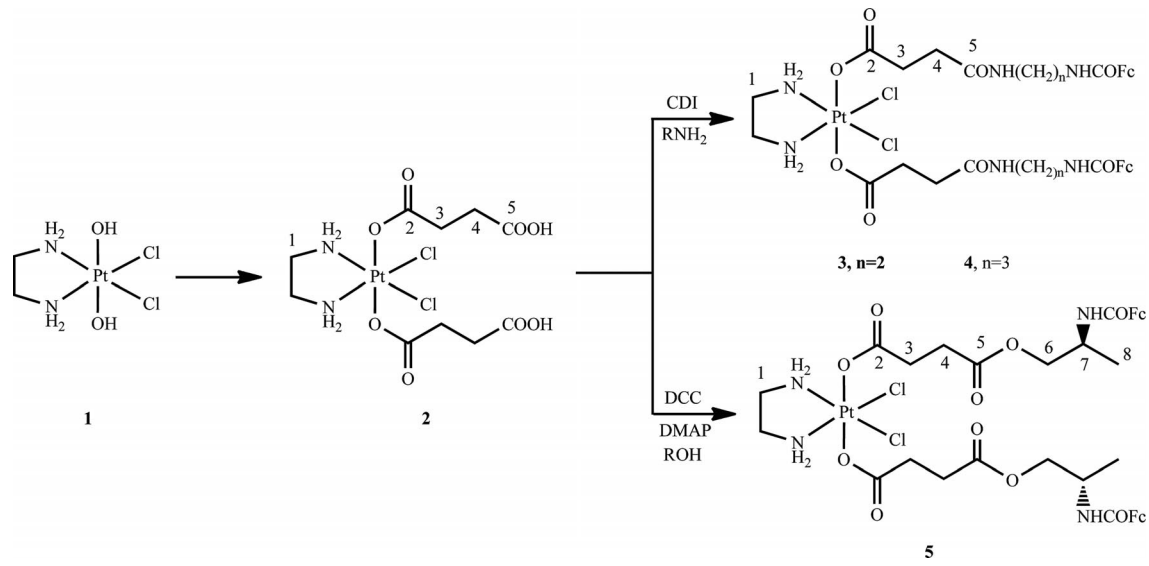
Spectroscopic Characterization

The final products **3**–**5** and **11**–**15** were fully characterized by elemental analysis, ESI-MS and multinuclear (^{1}H, ^{13}C, ^{15}N, ^{195}Pt) 1D and 2D NMR spectroscopy, whereas structures of ferrocene precursors **L2**–**L6** were verified by ^{1}H NMR spectroscopy. Carboxylation and further derivatization can best be judged by NMR spectroscopy.

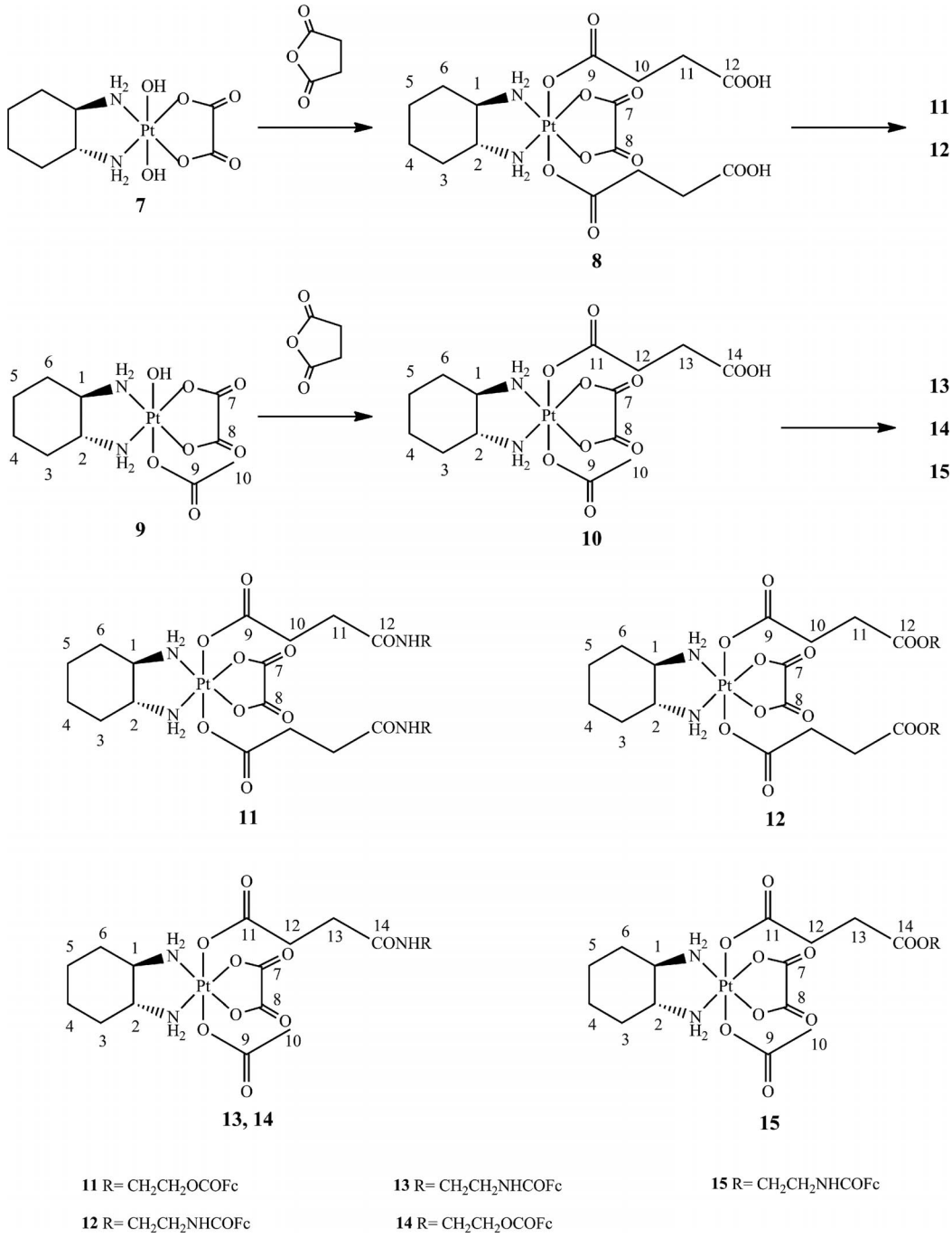
By analysis of the 2D NMR spectra, all protons of the final products could be assigned except for the two methylene groups in the axial position nearest to the platinum nucleus because of several overlaps. It was possible to distinguish between the three quaternary C=O carbon atoms because of the long-range shift correlation signals arising from the carbon atoms and protons of the cyclopentyl ring or protons belonging to the residue of the coupled ligand.



Scheme 1. Synthesis of ferrocene precursors **L2**–**L6** and NMR numbering scheme.



Scheme 2. Synthesis of complexes **3**–**5** and NMR numbering scheme.



Scheme 3. Synthesis of platinum(IV) complexes **11**–**15** and NMR numbering scheme.

As described in the literature,[16] novel Pt^{IV} complexes with the $Cl_2N_2O_2$ coordination sphere show ^{195}Pt resonances in the region around 2656 ppm, whereas resonances for oxaliplatin-type platinum(IV) complexes appear in the region of 3230 ppm. As anticipated, the ^{195}Pt resonances are comparable for oxaliplatin-based Pt^{IV} complexes **13**, **14**, and **15** with one acetato and one derivatized carboxylato ligand in the axial position to those for their biscarboxylated analogues. ESI-MS spectra were measured in the positive as well as in the negative mode. The highest peak observed in each spectrum in the positive mode is $[M + Na]^+$, which is also in accordance with the calculated values.

Cytotoxicity

The cytotoxicity of dichlorido(ethane-1,2-diamine)platinum(IV) complexes **3**–**5**, as well as of the oxaliplatin derivatives **11**–**15**, was determined in cisplatin-sensitive CH1 (ovarian carcinoma) and in intrinsically cisplatin-resistant SW480 (colon carcinoma) as well as in A549 (non-small-cell lung carcinoma) cancer cells by means of the MTT colorimetric assay (Table 1). In general, the antiproliferative potency of novel ferrocene-containing complexes is highest in the ovarian carcinoma cell line CH1 with promising IC_{50} values in the low micromolar range (0.84–2.3 μM). Remark-

ably, the equatorial ligand sphere and the number of ferrocene moieties of the platinum compounds seem to have no pronounced influence on these properties. In contrast, the cytotoxicity of all tested complexes in the lung carcinoma cell line A549 is only moderate with IC_{50} values well above 20 μM. Antiproliferative potency in SW480 colon carcinoma cells was observed in an intermediate range, and a clear-cut structure–activity relationship in this cell line could be drawn. Compounds **3**–**5** featuring more cisplatin-like equatorial ligands, i.e. ethane-1,2-diamine and dichlorido, were less cytotoxic (IC_{50} = 32–46 μM) than oxaliplatin-type complexes **11**–**15** (IC_{50} = 2.7–5.6 μM).

Table 1. Cytotoxicity of complexes **3**–**5** and **11**–**15** in CH1, A549, and SW480 cancer cells.

IC_{50} [μM][a]			
Compound	CH1	SW480	A549
3	1.5 ± 0.4	43 ± 5	69 ± 15
4	1.6 ± 0.4	32 ± 11	65 ± 8
5	2.3 ± 0.5	46 ± 9	84 ± 1
11	0.86 ± 0.20	4.4 ± 1.4	>50
12	1.2 ± 0.1	3.9 ± 1.3	38 ± 4
13	0.84 ± 0.13	2.7 ± 1.0	24 ± 6
14	1.6 ± 0.1	4.8 ± 1.3	48 ± 2
15	1.3 ± 0.0	5.6 ± 0.9	49 ± 12
cisplatin[b]	0.14 ± 0.03	3.3 ± 0.4	1.3 ± 0.4
oxaliplatin	0.18 ± 0.01	0.29 ± 0.05	0.98 ± 0.21
8[c]	55 ± 28	44 ± 9	–
L6	>280	>320	>320

[a] 50% inhibitory concentrations in the MTT assay (96 h exposure). Values are means (± standard deviations) obtained from at least three independent experiments. [b] Data taken from ref.[17] [c] Data taken from ref.[18].

The latter behavior was expected, since oxaliplatin is approved for clinical treatment of metastatic colorectal carcinoma and shows above-average in vitro activity in many intrinsically cisplatin-resistant colon carcinoma cell lines such as SW480. As could be shown by evaluating monoacetato complexes **13**, **14**, and **15**, the linking fragment between the carboxylatoplatinum(IV) moiety and the ferrocene part ($NH–CH_2–CH_2–NH$ vs. $NH–CH_2–CH_2–O$ vs. $O–CH_2–CH_2–NH$) has nearly no influence on cytotoxicity. Furthermore, no substantial differences in the antiproliferative behavior of bis- and mono(ferrocene) derivatives (**11** vs. **14** and **12** vs. **15**) could be observed.

There is also no difference depending on the length of the linking methylene moiety ($NH–CH_2–CH_2–NH$ vs. $NH–CH_2–CH_2–CH_2–NH$) when comparing the cytotoxicity of compounds **3** and **4**. Generally, all complexes are less active by factors of 6–16 in the cisplatin-sensitive cell line CH1 than cisplatin. Likewise, oxaliplatin derivatives **11**–**15** are by an order of magnitude less potent than oxaliplatin in SW480 cells. All novel complexes presented in this manuscript are hence less effective than cis- and oxaliplatin, but it should be kept in mind that cis- and oxaliplatin are platinum(II) complexes, whereas the central platinum ion in complexes **3**–**5** and **11**–**15** has the oxidation state +4. This means that they act as prodrugs and, consequently, have to be activated in the organism by reduction. Therefore, their suitability according to the prodrug concept cannot be assessed based on these in vitro investigations alone. Further experiments, especially in vivo, are warranted.

Exemplarily, one of the ferrocene compounds, namely **L6**, was tested for its antiproliferative potential. IC_{50} values were mostly not reached with maximum tested concentrations of 320 μM, attesting a very low cytotoxicity. The platinum(IV) precursor **8** shows also a low antiproliferative activity (Table 1), however, when **L6** and **8** were brought to reaction resulting in ester **12**, a significant increase in cytotoxicity (one order of magnitude as compared to **8**) was observed, warranting further studies.

Conclusions

Platinum(IV) complexes featuring axially coordinated ferrocene-bearing ligands were synthesized for the first time and fully characterized by multinuclear NMR spectroscopy. Taking into account that platinum(IV) complexes act as prodrugs, which have to be activated in the organism, they show promising IC_{50} values in the low micromolar range in CH1 and SW480 human cancer cells.

Experimental Section

$K_2[PtCl_4]$ was obtained from Johnson Matthey (Switzerland), ferrocenecarboxylic acid from Alfa Aesar. All other chemicals were purchased from Aldrich, Fluka, Acros, or Fisher and used without further purification. When platinum(II) complexes were involved, syntheses were carried out under light protection; glass-coated magnetic stirring bars were used for each synthesis. For reactions in water, doubly distilled osmosis water was used.

Starting compounds **L1**–**L6** and precursors,[19–22] (SP-4–2)-(*trans*-1R,2R-diaminocyclohexane)oxalatoplatinum(II) (**6**), (*OC*-6–33)-bis(3-carboxypropanoato)dichlorido(ethane-1,2-diamine)platinum(IV) (**2**), (*OC*-6–33)-bis(3-carboxypropanoato)(*trans-1R,2R*-diaminocyclohexane)oxalatoplatinum(IV) (**8**), **9**, and **10**,[23–26] were synthesized according to literature methods.

Physical Measurements: Elemental Analysis was carried out by the Microanalytical Laboratory of the University of Vienna with a 2400 CHN Elemental Analyzer manufactured by Perkin–Elmer. For each submitted substance, the percentage of the elements carbon, hydrogen, and nitrogen was determined. Electrospray ionization mass spectra were recorded with a Bruker Esquire3000 ion trap spectrometer with MeOH as solvent, the molecular mass was determined in the positive as well as in the negative mode. NMR spectra were recorded on a Bruker FT-NMR Avance III 500 MHz instrument at 500.32 (^{1}H), 125.81 (^{13}C), 50.70 (^{15}N), and 107.55 (^{195}Pt) MHz. 2D NMR measurements were recorded by using standard pulse programs. Chemical shifts were referenced relative to the solvent signal for ^{1}H and ^{13}C spectra; for ^{15}N and ^{195}Pt spectra, the external standards $^{15}NH_4Cl$ and $K_2[PtCl_4]$ were used, respectively.

Synthesis of Ferrocene Precursors

Ferrocenyl Chloride (L1): Ferrocenecarboxylic acid was dissolved in abs. CH_2Cl_2 (typically 700 mg in 5 mL of abs. CH_2Cl_2) under an argon atmosphere, and oxalyl chloride (2 equiv.) was added. The solution was stirred for 20 min at room temperature, and the solvent was removed under reduced pressure. The obtained dark red

substance was dissolved in CH_2Cl_2 and used without purification for subsequent reactions.

***N*-3-(Aminopropyl)ferrocenecarboxamide (L2):** Compound **L1** (3.04 mmol) was dissolved in abs. CH_2Cl_2 (5 mL) and added slowly over 2 h at –23 °C to a solution of 1,3-diaminopropane (2.5 mL, 30.4 mmol) in abs. CH_2Cl_2 (50 mL). The solution was filtered (in order to get rid of the dimer) and extracted three times with water; the organic phase was dried with $MgSO_4$. After removal of the solvent under reduced pressure, the product was suspended in diethyl ether and filtered through a G-4 glass filter. The product was dried in vacuo. Yield: 235 mg, 33%. ^{1}H NMR ($CDCl_3$): δ = 6.82 (br. s, 1 H, NH), 4.69 (t, ^3J = 1.9 Hz, 2 H, H3), 4.35 (t, ^3J = 1.9 Hz, 2 H, H4), 4.22 (s, 5 H, H1), 3.53 (m, 2 H, CH_2), 2.94 (m, 2 H, CH_2), 1.76 (m, 2 H, CH_2) ppm.

***N*-2-(Aminoethyl)ferrocenecarboxamide (L3):** Compound **L1** (3.48 mmol) was dissolved in abs. CH_2Cl_2 (6 mL) and then added via a Teflon cannula to a stirred solution of *N*-*boc*-ethylenediamine (826 µL, 5.22 mmol) and triethylamine (964.7 µL 6.96 mmol) in abs. CH_2Cl_2 under an argon atmosphere and left to stir for 1 h. The solution was extracted three times with water, the organic phase was dried with $MgSO_4$, and the solvent was removed under reduced pressure. Yield: 512 mg, 33% of the *N*-*boc*-protected product. ^{1}H NMR ($CDCl_3$): δ = 6.30 (br. s, 1 H, NH), 4.67 (t, ^3J = 1.8 Hz, 2 H, H3), 4.31 (t, ^3J = 1.9 Hz, 2 H, H4), 4.18 (s, 5 H, H1), 3.42 (m, 2 H, CH_2), 2.90 (m, 2 H, CH_2), 1.63 (s, 9 H, H10) ppm. The product was dissolved in trifluoroacetic acid (4 mL) and left to stir for 5 min. Subsequently, trifluoroacetic acid was removed under reduced pressure, and the crude product was extracted three times with CH_2Cl_2 and with a saturated $NaHCO_3$ solution. The organic phase was dried again over $MgSO_4$, and the solvent was removed under reduced pressure.

(*S*)-*N*-(1-Hydroxypropan-2-yl)ferrocenecarboxamide (L4): Compound **L1** (2.17 mmol) was dissolved in abs. CH_2Cl_2 (4 mL) and then added via a teflon cannula to a stirred solution of (*S*)-2-amino-1-propanol (606 µL, 4.34 mmol) and triethylamine (604.9 µL 4.34 mmol) in abs. CH_2Cl_2 under an argon atmosphere and left to stir for 1 h. The solution was extracted three times with water, and the organic phase dried with $MgSO_4$. The solvent was removed under reduced pressure. Yield: 171 mg, 28%. ^{1}H NMR ($CDCl_3$): δ = 5.83 (m, 1 H, NH), 4.72 (m, 1 H, H3), 4.69 (m, 1 H, H3), 4.39 (s, 2 H, H4), 4.25 (s, 5 H, H1), 3.79 (m, 1 H, H6/H7), 3.65 (m, 1 H, H6/H7), 2.96 (m, 1 H, H6/H7), 1.30 (d, ^3J = 6.9 Hz, 3 H, H8) ppm.

[(2-Aminoethoxy)carbonyl]ferrocene (L5): Compound **L1** (3.04 mmol) was dissolved in abs. CH_2Cl_2 (6 mL) and then added via a teflon cannula to a stirred solution of *N*-*boc*-ethylenediamine (705.4 µL, 4.56 mmol) and triethylamine (847 µL 6.04 mmol) in abs. CH_2Cl_2 under an argon atmosphere and left to stir for 1 h. The solution was extracted three times with water, and the organic phase dried over $MgSO_4$. The solvent was removed under reduced pressure. Yield: 384 mg, 32.5% of the *N*-*boc*-protected product. ^{1}H NMR ($CDCl_3$): δ = 4.85 (t, ^3J = 1.8 Hz, 2 H, H3), 4.45 (t, ^3J = 1.8 Hz, 2 H, H4), 4.32 (m, 2 H, CH_2), 4.24 (s, 5 H, H1), 3.52 (m, 2 H, CH_2), 1.48 (s, 9 H, H10) ppm. The product was dissolved in trifluoroacetic acid (4 mL) and left to stir for 5 min. Subsequently, trifluoroacetic acid was removed under reduced pressure, and the crude product was extracted three times with CH_2Cl_2 and with a saturated $NAHCO_3$ solution. The organic phase was dried again over $MgSO_4$, and the solvent removed under reduced pressure.

{[(2-Hydroxyethyl)amino]carbonyl}ferrocene (L6): Compound **L1** (3.04 mmol) was dissolved in abs. CH_2Cl_2 (6 mL) and then added via a teflon cannula to a stirred solution of ethanolamine (550.4 µL, 9.12 mmol) and triethylamine (847 µL 6.04 mmol) in abs. CH_2Cl_2 under an argon atmosphere and left to stir for 1 h. The solution was filtered and extracted three times with water. The organic phase was dried with $MgSO_4$, and the solvent was removed under reduced pressure. Yield: 380 mg, 50%. ^{1}H NMR ($CDCl_3$): δ = 6.15 (br. s, 1 H, NH), 4.71 (t, ^3J = 1.5 Hz, 2 H, H3), 4.40 (t, ^3J = 1.6 Hz, 2 H, H4), 4.25 (s, 5 H, H1), 3.84 (t, ^3J = 4.9 Hz, 2 H, CH_2), 3.59 (m, 2 H, CH_2) ppm.

Synthesis of precursors 9 and 10

(*OC*-6–44)-Acetato(*1R*,*2R*-diaminocyclohexane)hydroxido(oxalato)platinum(IV) (9): Compound **6** (3 g, 7.6 mmol) was suspended in acetic acid (60 mL), peracetic acid (3.3 mL, 2.5 equiv.) was added, and the solution stirred for 30 min. The solution turned clear and was left to stir for 24 h. Acetic acid was removed under reduced pressure; the residue was dissolved in acetic acid (20 mL) and evaporated again. The product was suspended in ethyl acetate, methanol was added until the appearance of the suspension did not change any more, and the suspension was filtered. Solvents were removed on a rotary evaporator, and the white solid was dried in vacuo. Yield: 2.43 g, 66%. ^{1}H NMR ([D_6]DMSO) = 8.59 (br. m, 1 H, NH_2), 8.18 (br. m, 1 H, NH_2), 7.80 (br. m, 1 H, NH_2), 7.10 (m, 1 H, NH_2), ≈2.55 (H1/H2, underneath the DMSO peak), 2.07 (m, 2 H, H3/4/5/6), 1.92 (s, 3 H, H10), 1.54–1.29 (br. m, 4 H, H3/4/5/6); 1.13 (m, 2 H, H3/4/5/6) ppm.

(*OC*-6–44)-Acetato(3-carboxypropanoato)(*1R*,*2R*-diaminocyclohexane)oxalatoplatinum(IV) (10): Compound **9** (1 g, 2.04 mmol) and succinic anhydride (306.2 mg, 3.06 mmol) were suspended in abs. DMF (15 mL) and stirred for 24 h at 50 °C. The solvent was removed under reduced pressure, and the crude product was suspended in acetone. Methanol was added stepwise until the amount of insoluble solid did not change any more. The solution was filtered, the solvents were removed under reduced pressure, the residue was dissolved in acetone again, and ethyl acetate was added until no further precipitation was observed. The product was filtered, washed with diethyl ether, and dried in vacuo. Yield: 945 mg, 79%. ^{1}H NMR ([D_6]DMSO): δ = 12.13 (br. m, 1 H, OH), 8.11–8.52 (br. m, 4 H, NH_2), 2.58 (m, H1/H2), 2.40 (m, 2 H, H12/H13), 2.12 (m, 2 H, H3/H4/H5/H6), 1.97 (s, 3 H, H10), 1.51 (m, 2 H, H3/H4/H5/H6), 1.41 (m, 2 H, H3/H4/H5/H6), 1.17 (m, 2 H, H3/H4/H5/H6) ppm.

General Procedure for the Synthesis of Complexes 3, 4, 11, 13, and 14: 1,1′-Carbonyldiimidazole and the prevailing complex (**2**, **8** or **10**) were dissolved in abs. DMF stirred for 10 min at 60 °C and simultaneously flushed with argon. The solution was cooled down to room temperature, the required amine was added (**L2**, **L3** or **L5**), and the solution was left to stir for 24 h. The solvent was removed under reduced pressure; the crude product was dissolved in acetone and precipitated with diethyl ether. The product was filtered and subsequently purified by column chromatography.

General Procedure for the Synthesis of Complexes 5, 12, and 15: To a stirred solution of 4-dimethylaminopyridine, carboxylic acid (**2**, **8** or **10**), and the alcohol (**L4** or **L6**) was added *N*,*N*′-dicyclohexylcarbodiimide over 100 min. under an argon atmosphere. The solution was left to stir for 24 h at room temperature. The volume was reduced to 3 mL, and the suspension was filtered. The remaining solution was evaporated, and the crude product was purified by column chromatography.

(*OC*-6–33)-Dichlorido(ethane-1,2-diamine)bis(4-{[2-ferrocenoylamino]ethyl}amino)-4-oxobutanoatoplatin(IV) (3): 1,1′-Carbonyldiimidazole (92 mg, 0.57 mmol), **2** (144 mg, 0.26 mmol) in abs. DMF (6 mL), **L3** (175 mg, 0.64 mmol) in abs. DMF (2 mL). The

crude product was purified by column chromatography (EtOAc/MeOH, 2:1). The obtained product was dried in vacuo. Yield: 69 mg, 26%, yellow powder. $C_{36}H_{46}Cl_2Fe_2N_6O_8Pt·4H_2O$: calcd. C 37.91, H 4.77, N 7.37; found C 37.69, H 4.25, N 7.11. ^{1}H NMR ([D$_6$]DMSO): δ = 8.45 (br. s, 2 H, NH$_2$), 7.94 (br. m, 2 H, NH), 7.83 (br. m, 2 H, NH–COCp), 4.76 (br. m, 4 H, H10), 4.34 (br. m, 4 H, H11), 4.16 (s, 10 H, H12), 3.22 (m, 4 H, H7), 3.18 (m, 4 H, H6), 2.67 (br. m, 4 H, H1), ≈2.5 (H3/H4, underneath the DMSO peak), 2.32 (t, ^3J = 7.3 Hz, 4 H, H3/H4) ppm. ^{13}C NMR ([D$_6$]-DMSO): δ = 181.7 (C2), 172.0 (C5), 169.6 (C8), 77.0 (C9), 70.4 (C11), 69.9 (C12), 68.6 (C10), 49.2 (C1), 39.3 (C6/7), 39.1 (C6/7), 32.2 (C3/C4), 31.8 (C3/C4) ppm. ^{15}N NMR ([D$_6$]DMSO): δ = 91.4 (NH), 85.8 (NH–COCp), –4.4 (NH$_2$) ppm. ^{195}Pt NMR ([D$_6$]-DMSO): δ = 2655 ppm. MS (180 °C): *m*/*z* = 1091 [M + Na$^+$].

(*OC*-6–33)-Dichlorido(ethane-1,2-diamine)bis(4-{[3-ferrocenoylamino]propyl}amino)-4-oxobutanoatoplatin(IV) (4): 1,1′-Carbonyldiimidazole (109 mg, 0.67 mmol), **2** (184 mg, 0.33 mmol) in abs. DMF (7 mL), **L2** (235 mg, 0.82 mmol) in abs. DMF (2 mL). The crude product was purified by column chromatography (CHCl$_3$/MeOH, 5:1). The obtained product was dried in vacuo. Yield: 73 mg, 20%, yellow powder. $C_{38}H_{50}Cl_2Fe_2N_6O_8Pt·3H_2O$: calcd. C 39.67, H 4.91, N 7.30; found C 39.55, H 4.57, N 7.20. ^{1}H NMR ([D$_6$]DMSO): δ = 8.45 (br. s, 2 H, NH$_2$), 7.85 (br. m, 2 H, NH), 7.75 (br. m, 2 H, NH–COCp), 4.77 (br. m, 4 H, H11), 4.34 (br. m, 4 H, H12), 4.16 (s, 10 H, H13), 3.18 (m, 4 H, H8), 3.12 (m, 4 H, H6), 2.66 (br. m, 4 H, H1), ≈2.5 (H3/H4, underneath the DMSO peak), 2.31 (t, ^3J = 7.1 Hz, 4 H, H3/H4), 1.61 (m, 4 H, H7) ppm. ^{13}C NMR ([D$_6$]DMSO): δ = 181.7 (C2), 171.7 (C5), 169.3 (C9), 77.3 (C10), 70.3 (C12), 69.8 (C13), 68.5 (C11), 49.1 (C1), 36.8 (C6/C8), 36.8 (C6/C8), 32.3 (C3/C4), 31.8 (C3/C4), 30.1 (C7) ppm. ^{15}N NMR ([D$_6$]DMSO): δ = 94.1 (NH), 87.9 (NHCOCp), –4.8 (NH$_2$) ppm. ^{195}Pt NMR ([D$_6$]DMSO): δ = 2656 ppm. MS (180 °C): *m*/*z* = 1119 [M + Na$^+$], 1095 [M – H$^+$].

(*OC*-6–33)-Dichlorido(ethane-1,2-diamine)bis(4-{*S*-2-[ferrocenoylamino]propoxy})-4-oxobutanoatoplatin(IV) (5): 4-Dimethylaminopyridine (4.9 mg, 0.04 mmol), *N*,*N*′-dicyclohexylcarbodiimide (84.2 mg, 0.41 mmol) in abs. DMF (5 mL), **2** (111.4 mg, 0.20 mmol) in abs. DMF (7 mL), **L4** (171.4 mg, 0.60 mmol). The crude product was purified by column chromatography (EtOAc/MeOH, 2:1). The obtained product was dried in vacuo. Yield: 55 mg, 26%, yellow powder. $C_{38}H_{48}Cl_2Fe_2N_4O_{10}Pt·3H_2O$: calcd. C 39.60, H 4.72, N 4.86; found C 39.67, H 4.31, N 4.82. ^{1}H NMR ([D$_6$]DMSO): δ = 8.46 (br. s, 2 H, NH$_2$), 8.43 (br. s, 2 H, NH$_2$), 7.58 (d, ^3J = 8.3 Hz, 2 H, NH), 4.84 (br. m, 2 H, H11), 4.80 (br. m, 2 H, H11), 4.35 (br. m, 4 H, H12), 4.21 (m, 2 H, H7), 4.17 (s, 10 H, H13), 4.11 (m, 2 H, H6), 3.95 (m, 2 H, H6), 2.65 (br. s, 4 H, H1), 2.56 (m, 2 H, H3/H4), 2.50 (H3/H4, underneath the DMSO peak), 1.15 (d, ^3J = 6.7 Hz, 6 H, H8) ppm. ^{13}C NMR ([D$_6$]DMSO): δ = 180.0 (C2), 172.7 (C5), 169.1 (C9), 76.8 (C10), 70.5 (C12), 70.4 (C12), 69.9 (C13), 68.9 (C11), 68.5 (C11), 67.0 (C6), 49.2 (C1), 43.9 (C7), 31.3 (C3/C4), 30.1 (C3/C4), 17.7 (C8) ppm. ^{15}N NMR ([D$_6$]-DMSO): δ = 96.3 (NH), –5.1 (NH$_2$) ppm. ^{195}Pt NMR ([D$_6$]-DMSO): δ = 2658 ppm. MS (180 °C): *m*/*z* = 1121 [M + Na$^+$].

(*OC*-6–33)-(trans-*1R*,*2R*-Diaminocyclohexane)bis{4-[2-(ferrocenoyloxy)ethylamino]-4-oxobutanoato}oxalatoplatin(IV) (11): 1,1′-Carbonyldiimidazole (85.6 mg, 0.53 mmol), **8** (151.8 mg, 0.24 mmol) in abs. DMF (6 mL), **L5** (234 mg, 0.6 mmol) in abs. DMF (2 mL). The crude product was purified by column chromatography (CHCl$_3$/MeOH, 8:1). The obtained product was dried in vacuo. Yield: 112 mg, 41%, yellow powder. $PtC_{42}H_{50}Fe_2N_4O_{10}$: calcd. C 44.18, H 4.41, N 4.91; C 43.86, H 4.11, N 4.74. ^{1}H NMR ([D$_6$]-DMSO): δ = 8.36 (br. m, 2 H, NH$_2$), 8.15 (br. m, 2 H, NH$_2$), 8.07 (t, ^3J = 5.5 Hz, 2 H, NH), 4.77 (m, 4 H, H17), 4.50 (m, 4 H, H18), 4.23 (s, 10 H, H19), 4.13 (t, ^3J = 5.8 Hz, 2 H, H14), 3.37 (m, 2 H, H13), 2.69 (m, 2 H, H1/H2), ≈2.5 (H10/H11 underneath the DMSO peak), 2.35 (m, 2 H, H10/H11), 2.10 (m, 2 H, H3/H6), 1.50 (m, 2 H, H4/H5), 1.40 (m, 2 H, H3/H6), 1.18 (m, 2 H, H4/H5) ppm. ^{13}C NMR ([D$_6$]DMSO): δ = 180.7 (C9), 172.0 (C12), 171.1 (C15), 163.8 (C7/C8), 71.8 (C18), 71.1 (C16), 70.3 (C17), 70.1 (C17/C19), 62.9 (C14), 61.2 (C1/C2), 38.3 (C13), 31.6 (C3/C6/C10/C11), 31.4 (C3/C6/C10/C11), 24.0 (C4/C5) ppm. ^{15}N NMR ([D$_6$]DMSO): δ = 88.8 (NH), –6.3 (NH$_2$) ppm. ^{195}Pt NMR ([D$_6$]DMSO): δ = 3233 ppm. MS (180 °C): *m*/*z* = 1164 [M + Na$^+$], 1040 [M – H$^+$].

(*OC*-6–33)-(trans-*1R*,*2R*-Diaminocyclohexane)bis[4-(2-ferrocenoylamino)ethoxy]-4-(oxobutanoato)oxalatoplatin(IV) (12): 4-Dimethylaminopyridine (7.3 mg, 0.06 mmol), *N*,*N*′-dicyclohexylcarbodiimide (136.2 mg, 0.66 mmol) in abs. DMF (5 mL), **8** (190.4 mg, 0.30 mmol) in abs. DMF (8 mL), **L6** (247 mg, 0.90 mmol). The crude product was purified by column chromatography (CHCl$_3$/MeOH, 8:1). The obtained product was dried in vacuo. Yield: 67 mg, 20%, yellow powder. $C_{42}H_{50}Fe_2N_4O_{10}Pt·H_2O$: calcd. C 43.50, H 4.52, N 4.83; found C 43.27, H 4.21, N 4.78. ^{1}H NMR ([D$_6$]DMSO): δ = 8.35 (br. m, 2 H, NH$_2$), 8.17 (br. m, 2 H, NH$_2$), 7.89 (t, ^3J = 5.5 Hz, 2 H, NH), 4.79 (m, 4 H, H17), 4.35 (s, 4 H, H18), 4.23 (s, 10 H, H19), 4.12 (t, ^3J = 5.7 Hz, 2 H, H13), 3.42 (m, 2 H, H14), 2.69 (m, 2 H, H1/H2), ≈2.5 (H10/H11, underneath the DMSO peak), 2.15 (m, 2 H, H10/H11), 2.10 (m, 2 H, H3/H6), 1.50 (m, 2 H, H4/H5), 1.38 (m, 2 H, H3/H6), 1.13 (m, 2 H, H4/H5) ppm. ^{13}C NMR ([D$_6$]DMSO): δ = 179.9 (C9), 172.8 (C12), 169.8 (C15), 163.9 (C7/C8), 76.8 (C16), 70.5 (C18), 69.9 (C19), 68.7 (C17), 63.3 (C13), 61.3 (C1/C2), 38.2 (C14), 31.4 (C3/C6), 30.9 (C10/C11), 30.0 (C10/C11), 24.0 (C4/C5) ppm. ^{15}N NMR ([D$_6$]-DMSO): δ = 82.9 (NH), –6.4 (NH$_2$) ppm. ^{195}Pt NMR ([D$_6$]-DMSO): δ = 3238 ppm. MS (180 °C): *m*/*z* = 1164 [M + Na$^+$].

(*OC*-6–44)-Acetato(trans-*1R*,*2R*-diamminocyclohexane)[4-(2-ferrocenoylamino)ethylamino]-4-(oxobutanoato)oxalatoplatin(IV) (13): 1,1′-Carbonyldiimidazole (63.4 mg, 0.40 mmol), **10** (216.8 mg, 0.36 mmol) in abs. DMF (9 mL), **L3** (250 mg, 0.92 mmol) in abs. DMF (2 mL). The crude product was purified by column chromatography (CHCl$_3$/MeOH, 8.5:1). The obtained product was dried in vacuo. Yield: 100 mg, 34%, yellow powder. $C_{27}H_{35}Fe_2N_3O_{11}Pt·2H_2O$: calcd. C 37.55, H 4.67, N 6.49; found C 37.34, H 4.27, N 6.30. ^{1}H NMR ([D$_6$]DMSO): δ = 8.41 (br. m, 1 H, NH$_2$), 8.31 (br. m, 1 H, NH$_2$), 8.26 (m, 1 H, NH$_2$), 8.20 (m, 1 H, NH$_2$), 7.93 (br. m, 2 H, NH), 7.80 (br. m, 2 H, NH), 4.75 (br. m, 4 H, H19), 4.35 (br. m, 4 H, H20), 4.15 (s, 10 H, H21), 3.22 (br. m, 2 H, H15), 3.18 (bm, 2 H, H16), 2.67 (m, 1 H, H1/H2), 2.55 (m, H1/H2), ≈2.5 (H12/H13, underneath the DMSO peak), 2.31 (m, 2 H, H12/H13), 2.11 (m, 2 H, H3/H6), 1.96 (s, 3 H, H10), 1.50 (m, 2 H, H4/H5), 1.41 (m,2 H, H3/H6), 1.17 (m, 2 H, H4/H5) ppm. ^{13}C NMR ([D$_6$]DMSO): δ = 180.6 (C11), 179.1 (C9), 172.0 (C14), 169.6 (C17), 163.9 (C7/C8), 163.8 (C7/C8), 77.0 (C18), 70.4 (C20), 69.9 (C21), 68.6 (C19), 61.5 (C1/C2), 61.2 (C1/C2), 39.2 (C15), 39.1 (C16), 31.6 (C12/C13), 31.5 (C12/C13), 31.3 (C3/C6), 24.0 (C4/C5), 23.9 (C4/C5), 23.4 (C10) ppm. ^{15}N NMR ([D$_6$]DMSO): δ = 91.3 (NH), 85.4 (NH), –6.3 (NH$_2$) ppm. ^{195}Pt NMR ([D$_6$]DMSO): δ = 3236 ppm. MS (180 °C): *m*/*z* = 850 [M + Na$^+$].

(*OC*-6–44)-Acetato(trans-*1R*,*2R*-diaminocyclohexane)[4-(2-ferrocenoyloxy)ethylamino]-4-(oxobutanoato)oxalatoplatin(IV) (14): 1,1′-Carbonyldiimidazole (60.6 mg, 0.37 mmol), **10** (201.1 mg, 0.34 mmol) in abs. DMF (9 mL), **L5** (200 mg, 0.51 mmol) in abs. DMF (2 mL). The crude product was purified by column chromatography (CHCl$_3$/MeOH, 7:1). The obtained product was dried in vacuo. Yield: 78 mg, 28%, yellow powder.

$C_{27}H_{36}Fe_2N_4O_{10}Pt{\cdot}H_2O$: calcd. C 38.31, H 4.41, N 5.07; found C 38.41, H 4.01, N 4.95. ^{1}H NMR ([D$_6$]DMSO): δ = 8.42 (br. m, 1 H, NH_2), 8.31 (br. m, 1 H, NH_2), 8.25 (br. m, 1 H, NH_2), 8.24 (br. s, 1 H, NH_2), 8.07 (t, ^3J = 5.6 Hz, 2 H, NH), 4.77 (t, ^3J = 1.9 Hz, 2 H, H19), 4.50 (t, ^3J = 1.9 Hz, 2 H, H20), 4.23 (s, 5 H, H21), 4.13 (t, ^3J = 5.8 Hz, 2 H, H16), 3.37 (m, 2 H, H15), 2.71 (br. s, 1 H, H1/H2), ≈2.55 (m, H1/H2, underneath the DMSO peak), ≈2.50 (H12/H13, underneath the DMSO peak), 2.35 (m, 2 H, H12/H13), 2.11 (m, H3/H6), 1.96 (s, 3 H, H10), 1.50 (m, 2 H, H4/H5), 1.41 (m, 2 H, H3/H6), 1.17 (m, 2 H, H4/H5) ppm. ^{13}C NMR ([D$_6$]DMSO): δ = 180.6 (C11), 179.0 (C9), 172.0 (C14), 171.1 (C17), 163.9 (C7/C8), 163.8 (C7/C8), 71.9 (C20), 71.1 (C18), 70.3 (C19), 70.1 (C21), 62.9 (C16), 61.4 (C1/C2), 61.2 (C1/C2), 38.3 (C15), 31.5 (C3/C6/C12/C13), 31.4 (C3/C6/C12/C13), 24.1 (C4/C5), 23.9 (C4/C5), 23.4 (C10) ppm. ^{15}N NMR ([D$_6$]DMSO): δ = 87.6 (NH), –6.6 (NH_2) ppm. ^{195}Pt NMR ([D$_6$]DMSO): δ = 3236 ppm. (180 °C): m/z = 851 [M + Na$^+$], 826 [M – H$^+$].

(*OC*-6–44)-Acetato(trans-*1R*,*2R*-diaminocyclohexane)[4-(2-ferrocenoylamino)ethoxy]-4-(oxobutanoato)oxalatoplatin(IV) (15): 4-Dimethylaminopyridine (6.6 mg, 0.05 mmol), *N*,*N*′-dicyclohexylcarbodiimide (61.3 mg, 0.30 mmol) in abs. DMF (5 mL), **10** (160 mg, 0.27 mmol) in abs. DMF (8 mL), **L6** (133 mg, 0.49 mmol). The crude product was purified by column chromatography ($CHCl_3$/MeOH, 8:1). The obtained product was dried in vacuo. Yield: 67 mg, 30%, yellow powder. $C_{27}H_{35}Fe_2N_3O_{11}Pt{\cdot}1.5H_2O$: calcd. C 37.91, H 4.48, N 4.91; found C 37.73, H 4.05, N 4.82. ^{1}H NMR ([D$_6$]DMSO): δ = 8.41 (br. m, 1 H, NH_2), 8.33 (br. m, 1 H, NH_2), 8.26 (br. m, 1 H, NH_2), 8.21 (br. s, 1 H, NH_2), 7.89 (t, ^3J = 5.7 Hz, 2 H, NH), 4.79 (br. m, 2 H, H19), 4.35 (br. m, 2 H, H20), 4.16 (s, 5 H, H21), 4.11 (m, 2 H, H15), 3.41 (m, 2 H, H16), 2.57 (br. s, 2 H, H1/H2), ≈2.5 (m, H12/H13, underneath the DMSO peak), 2.10 (br. s, 2 H, H3/H6), 1.50 (m, 2 H, H4/H5), 1.40 (m, 2 H, H3/H6), 1.15 (m, 2 H, H4/H5) ppm. ^{13}C NMR ([D$_6$]DMSO): δ = 179.8 (C11), 179.0 (C9), 172.8 (C14), 169.8 (C17), 163.9 (C7/C8), 163.8 (C7/C8), 76.8 (C18), 70.5 (C20), 69.9 (C21), 68.6 (C19), 63.3 (C15), 61.4 (C1/C2), 61.2 (C1/C2), 38.2 (C16), 31.4 (C3/C6), 31.3 (C3/C6), 31.0 (C3/C6/C12/C13), 30.0 (C12/C13), 24.0 (C4/C5), 23.9 (C4/C5), 23.4 (C10) ppm. ^{15}NNMR ([D$_6$]DMSO): δ = 82.7 (NH), –6.7 (NH_2) ppm. ^{195}Pt NMR ([D$_6$]DMSO): δ = 3238 ppm. MS (180 °C): m/z = 851 [M + Na$^+$].

Evaluation of Biological Activity

Cell Lines and Cultivation Conditions: The cytotoxicity tests were performed in three cancer cell lines: CH1 (ovarian carcinoma), SW480 (colon carcinoma), and A549 (non-small cell lung cancer). Adherent cell monolayer cultures were grown in 75 cm^2 culture flasks (CytoOne, Starlab, UK) in complete medium [i.e. minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 4 mM L-glutamine, and 1% nonessential amino acids from 100× ready-to-use stock (all purchased from Sigma–Aldrich, Austria)]. Cell cultures were incubated at 37 °C in a moist atmosphere containing 5% CO_2.

Cytotoxicity Tests in Cancer Cell Lines: The cytotoxic activity of the compounds was determined by means of the MTT-based colorimetric microculture assay [MTT = 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide]. The cells were harvested from culture flasks by trypsinization and passaged into 96-well microculture plates (CytoOne, Starlab, UK) in densities of 1×10^3 cells per well (CH1), 2×10^3 cells per well (SW480), and 3×10^3 cells per well (A549) in volumes of 100 µL per well. The cells were preincubated for 24 h before exposure to the drugs. Stock solutions of each complex were prepared in DMSO. These stock solutions were diluted in complete medium and then added in aliquots of 100 µL per well (final DMSO concentration 0.5%). After continuous exposure for 96 h, drug solutions were replaced with 100 µL of a RPMI/MTT mixture (6 parts RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine; 1 part MTT solution in phosphate-buffered saline [5 mg/mL]). After incubation for 4 h, the medium/MTT mixtures were removed, and the formazan crystals formed by viable cells were dissolved in 150 µL of DMSO per well. Optical densities at 550 nm were measured with a microplate reader (ELx808 Absorbance Microplate Reader, Bio-Tek, USA), by using a reference wavelength of 690 nm to correct for unspecific absorption. The quantities of viable cells were expressed in terms of T/C values by comparison to untreated control microcultures, and 50% inhibitory concentrations (IC_{50}) were calculated from concentration-effect curves by interpolation. Evaluation is based on means from at least three independent experiments, each comprising triplicates per concentration level.

Stability in DMSO/H_2O: Since the complexes had to be dissolved in DMSO and then diluted in aqueous medium for biological evaluation, stability under these conditions was addressed by NMR spectroscopy. In deuterated DMSO, all complexes were stable during the acquisition of the data (24–60 h), which is in accord with previous findings in the case of analogous platinum(IV) complexes. The stability of a representative complex (compound **3**) in a mixture of DMSO/D_2O was investigated by ^{1}H NMR spectroscopy. For this purpose, 0.5 mg of complex **3** was dissolved in 0.5 mL of DMSO mixed with 0.1 mL of D_2O. ^{1}H NMR spectra were measured from time to time. After sequential intervals of 24, 23.5, 8.5, and 14 h, 0.1 mL of D_2O was added, and the mixtures were investigated in the same way. The last mixture, consisting of 0.5 mL of DMSO and 0.5 mL of D_2O, was investigated for 48 h during which the signals broadened as a result of crystallization of **3**. However, a change in the NMR spectra could not be observed; in sum, **3** proved to be stable over 118 h under these conditions. In order to exclude precipitation of a decomposition product, 0.5 mL of the final suspension was mixed with 0.5 mL of DMSO. The clear solution was investigated by ^{1}H NMR spectroscopy, which proved the stability of **3** (significant amounts of hydrolysis products could not be detected).

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2.3. Coupling of Cytotoxic Platinum(IV) Prodrugs to Biodegradable Polyphosphazenes: Synthesis, Antiproliferative Activity, and Cellular Accumulation

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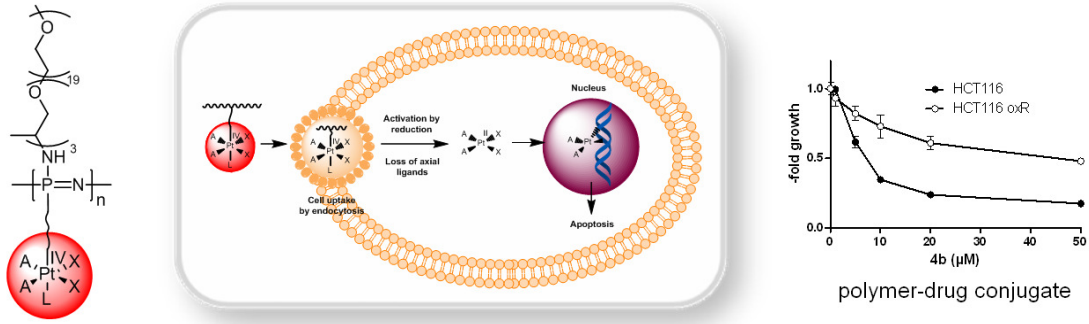

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J. Banfić synthesized and characterized platinum complexes and carried out coupling to the polyphosphazene. H. Henke synthesized and characterized the polymer and carried out GPC measurements. K. Kryeziu carried out cell culture experiments, P. Heffeter supervised and discussed results of the cell culture experiments. I. Teasdale supervised synthesis carried out be H. Henke, discussed results and finalized the manuscript. S. Theiner carried out ICP-MS of the final products, whereas W. Körner carried out ICP-MS measurements after cell accumulation studies. M. Galanski discussed results, carried out NMR measurements of the final products, and supervised the synthetic part at the Institute of Inorganic Chemistry. B. K. Keppler, W. Berger, and O. Brüggemann supervised the work at the prevailing institute, corrected the manuscript, and discussed results.

Coupling of cytotoxic platinum(IV) prodrugs to biodegradable polyphosphazenes: synthesis, antiproliferative activity, and cellular accumulation

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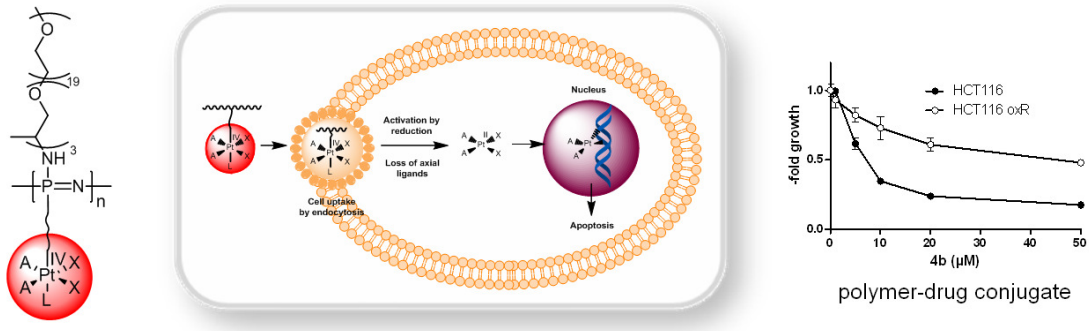
Graphical abstract and description



Platinum(IV) complexes, coupled to biodegradable polyphosphazenes, feature an enhanced cellular accumulation in conjunction with promising IC_{50} values in the micromolar range.

Abstract

Polymer therapeutics have proven valuable tools in recent years in order to improve drug solubility, extend plasma retention times and above all achieve passive tumor targeting via the enhanced permeation and retention (EPR) effect. In the present work, we report on novel drug-polymer conjugates in which cytotoxic platinum(IV) prodrugs were loaded onto water-soluble, biocompatible, degradable polyphosphazenes prepared with controlled molecular weights. The final products were characterized by ICP-MS, SEC and multinuclear (^{1}H and ^{31}P) NMR spectroscopy. Antiproliferative properties were investigated *in vitro* in the ovarian cancer cell line A2780, the colon carcinoma cell line HCT-116, and their corresponding cisplatin-resistant A2780/cis or oxaliplatin-resistant HCT-116 oxR sublines. High cellular accumulations were found for one platinum conjugate, as well as promising IC_{50} values in the low micromolar range. Impact of acquired oxaliplatin resistance on the novel conjugates were significantly smaller compared to clinically established platinum drugs, indicating that the platinum(IV) prodrug together with polymer coupling strategy might be able to overcome the acquired resistance against platinum(II) drugs.

Keywords: Polyphosphazenes, drug carrier, platinum(IV), anticancer activity, cytotoxicity, cell accumulation

Introduction

When Michele Peyrone synthesized cisplatin for the first time in the middle of the 19th century, he was not aware of the antiproliferative potential of his newly designed complex [1]. The discovery of its biological activity by Barnett Rosenberg in the 1960s led to further investigations until the drug was approved by the FDA (US Food and Drug Administration) as an anticancer agent in 1978 [2]. Since then, several efforts have been made to develop additional platinum-based drugs with antiproliferative potential in order to circumvent drug resistance and to minimize the severe adverse effects [3]. In addition, one second- and one third-generation cisplatin analogue, namely carboplatin and oxaliplatin, respectively, have gained worldwide approval as chemotherapeutics [4]. Furthermore, five additional platinum(II) drugs have been approved regionally: nedaplatin and miriplatin in Japan, dicycloplatin and lobaplatin in China, and heptaplatin in South Korea [5,6]. During the last decades, platinum(IV) complexes have also been considered as potential anticancer drugs, since they show some non-negligible advantages over platinum(II) complexes. Systemic toxicity is reduced since platinum(IV) complexes act as prodrugs and are reduced inside the cell by loss of their axial ligands (activation by reduction). Finally, their kinetic inertness enables the possibility of oral administration [7]. To date, several platinum(IV) complexes have been tested in clinical trials. Tetraplatin was abandoned due to its high toxicity while iproplatin lacked activity (Fig. 1). Satraplatin has not yet achieved approval by the FDA since the overall survival time of cancer patients could not be increased compared to the standard therapy [8,9]. Nevertheless, testing of satraplatin has been continued in several clinical trials [9,10,11]. The differences in biological activity of these platinum(IV) compounds can be explained by their deviant coordination sphere and consequently by their reduction potential: Tetraplatin, featuring two axial chlorido ligands is reduced rapidly in the organism in accordance with its highest reduction potential (E_p= -90 ± 19 mV), whereas iproplatin, bearing two hydroxido ligands in axial position, has a significantly lower reduction potential of Ep= -730 ± 100 mV vs. Ag/AgCl [9, 12]. Platinum(IV) analogues with two axial carboxylato ligands show reduction potentials resulting at around -600 mV vs. Ag/AgCl, which might be in an optimum range for anticancer prodrug chemotherapy [13]. Satraplatin, the most promising representative of this group, is characterized by moderate reduction potentials due to its two axial carboxylato ligands.

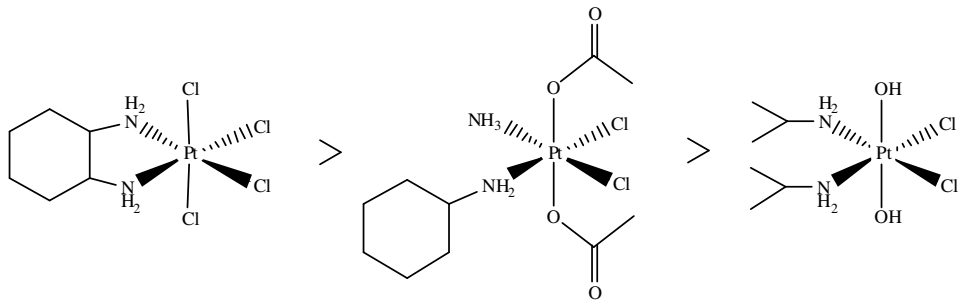


Figure 1. Chemical structures of platinum(IV) complexes. Chemical structures of tetraplatin, satraplatin, and iproplatin listed by magnitude of their reduction potential. Tetraplatin and iproplatin have been abandoned, whereas satraplatin is currently undergoing clinical studies.

Alongside activation by reduction, the malignant tissue can also be targeted by the enhanced permeability and retention effect (EPR effect) [14,15,16]. It is regarded as a key mechanism for selective accumulation of macromolecular anticancer drugs in tumor tissue. Since tumor cells are characterized by uncontrolled cell proliferation, formation of an own supporting vasculature is necessary in order to assure supply of the cancer tissue with the needed nutrients [17 , 18]. However, tumor-associated vessels are often dilated, leaky, show irregularities in shape, and, furthermore, the arrangement of endothelial cells of tumor blood vessels frequently shows gaps. As a result, the vascular permeability of the malignant tissue is enhanced and macromolecular agents can enter the tumor tissue via the interstitial space, which does not occur in healthy blood vessels. Additionally, lymph drainage is impaired leading to retention of the macromolecules [177]. Indeed, promising pre-clinical studies of a 25 KDa HPMA (poly(N-(2-hydroxypropyl) methacrylamide))-loaded DACH (diaminocyclohexane) platinum (II) conjugates (ProLindac) have shown superior efficacy compared to oxaliplatin, an effect attributed to distinctly enhanced delivery of platinum to the malignant tissue and especially to tumor DNA [19].

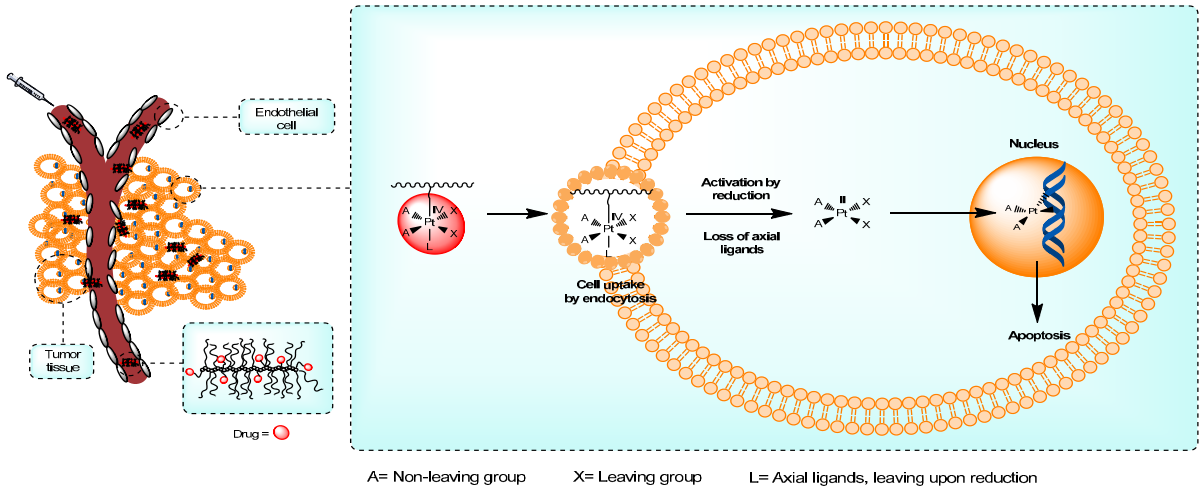


Figure 2. Proposed mode of action of polymer-loaded platinum(IV) anticancer drugs and the EPR effect. After intravenous administration, the loaded macromolecular agent circulates through the body via the blood vessel system. The disordered tumor vasculature allows passive tissue targeting since the leaky endothelial cell layer can be infiltrated by macromolecules [20]. Furthermore, since the platinum drug is coupled onto a macromolecular carrier, cell uptake by endocytosis is facilitated. Once inside the cell, the platinum(IV) drug is reduced to platinum(II) by loss of its axial ligands and parallel release from the macromolecular carrier. The reduced compound can now reach the nucleus, where it interacts with the DNA and induces apoptosis [21].

One current approach is the preparation of polymer therapeutics by coupling of platinum(IV) anticancer agents onto macromolecular carriers to take advantage of both the reduced renal clearance rates of macromolecules as well as the passive tumor-targeting via the EPR effect. Moreover, since platinum(IV) complexes are activated inside the cell by loss of their axial ligands, covalent coupling to macromolecular agents, such as polymers, could be the key to a targeted tumor therapy with reduced adverse effects. Furthermore, it is well known that such macromolecular carriers enter cells via the endocytic route [22], in which significantly lower pH values are encountered. The use of macromolecular, reducible platinum(IV) conjugates can thus exploit this pathway for a triggered intracellular release [23,24,25].

Inorganic-organic hybrid poly(organo)phosphazenes were chosen as carrier since they offer a unique combination of features which make them ideally suited as macromolecular drug carriers [26]. Essential for this is excellent water solubility for plasma dissolution and multifunctionality for the covalent loading of multiple drug moieties onto the carrier [27]. To this end, a number of Pt(II) conjugates have been prepared with polyphosphazenes showing promising properties [28,29,30]. Since biodistribution, renal clearance, endosomal cell uptake and not least the EPR effect are all size-controlled phenomena, the recently developed

methods to prepare polyphosphazenes with carefully controlled molecular dimensions [31,32] is also a vital feature. Furthermore, polyphosphazenes show a well-studied, tunable hydrolytic degradability due to their unique inorganic backbone [33] which, although hydrolytically instable, can be stabilized through careful choice of substituents to give hybrid polymers with defined degradation rates [34]. The use of polymers which are not just biodegradable, but degrade in a suitable time frame, is a critical feature for macromolecular drug delivery. The post-drug delivery fate of non-degradable synthetic polymers with molecular weights above the renal clearance limit is, although often ignored in novel preparations, regarded as a serious hindrance to the success of such polymer therapeutics [35, 36], especially with the relatively large, repetitive doses required for the chemotherapeutic application of metal-based therapeutics.

Experimental Section

Materials and methods

Chemicals were purchased from Sigma-Aldrich or Fisher Scientific and used without further purification, whereas $K_2[PtCl_4]$ was obtained from Johnson Matthey (Switzerland). Dialysis membranes (Spectra/Por 1, 6000-8000 Daltons) were purchased from SpectrumLabs. Reactions involving platinum(II) agents were performed with glass-coated magnetic stirring bars under light protection; doubly distilled osmosis water was used for reactions in water.

Platinum(IV) compounds (OC-6-54)-(3-carboxypropanoato)dichlorido(*N,N*-dimethylethane-*1,2*-diamine)hydroxidoplatinum(IV) **(2a)** and (OC-6-44)-acetato(3-carboxypropanoato)(*1R,2R*-diaminocyclohexane)oxalatoplatinum(IV) **(2b)** were synthesized according to literature methods [37,38].

The synthesis of the monomer, precursor and polymer was carried out under argon. Prior to use, the glassware was dried in an oven at 120 °C. The under vacuum sublimed PCl_5 was stored in the glove box under argon. Prior to use, Et_3N was distilled and dried over molecular sieves. The mono-boc protection of 2,2'-(ethylenedioxy)-bis-(ethylamine) [39] and the synthesis of the monomer $Cl_3PNSiMe_3$ [31] were carried out according to literature procedure. Jeffamine M-1000 is an amino capped statistical poly(ethylene oxide-co-propylene oxides), PEO-PPO-NH_2, donated by Huntsman Performance Products (Netherlands), with a nominal

molecular weight of 1000 g mol^{-1} and an ethylene oxide/propylene oxide ratio of 19/3. All other chemicals were purchased from Sigma Aldrich and used as received.

NMR spectra of the monomer, precursor and substituted polymer were measured on a Bruker 300 MHz spectrometer. The signal of $CDCl_3$ was used as internal reference for the ^{1}H NMR measurements. The ^{31}P NMR measurements were carried out at 121 MHz and 85 % phosphoric acid was used as external standard. NMR spectra of the final compounds and platinum precursors were carried out on a Bruker FT-NMR Avance III 500 MHz instrument at 500.32 (^{1}H) and 202.44 (^{31}P) MHz. External quantification of the average platinum content of the final products was performed by ICP-MS. The digestion of samples was performed with a microwave system Discover SP-D (CEM Microwave Technology, Germany) using 2 mL of 32.5% subboiled nitric acid (≥65%, p.a., Fluka, Buchs, Switzerland). Samples were diluted with Milli-Q water (18.2 MΩ cm, Milli-Q Advantage) resulting in nitric acid concentration lower than 3% and platinum concentrations lower than 15 µg g^{-1}. The total platinum content after accumulation studies was determined with an ICP-quadrupole MS instrument Agilent 7500ce (Agilent Technologies, Waldbronn, Germany) equipped with a CETAC ASX-520 autosampler (Nebraska, USA) and a MicroMist nebulizer at a sample uptake rate of approximately 0.25 mL min^{-1}. Platinum and rhenium standards for ICP-MS measurements were derived from CPI International (Amsterdam, The Netherlands). Rhenium served as internal standard for platinum and concentrations were determined via the isotopes ^{185}Re, ^{194}Pt and ^{195}Pt. The ICP-MS was operated with 15 L min^{-1} argon as plasma gas, 0.95 L min^{-1} argon as carrier gas and with a RF power of 1500 W. The size exclusion chromatography (SEC) measurements were carried out with a Viscothek GPCmax instrument equipped with a PFG column from PSS, (Mainz, Germany) 300 × 8 mm^2 , 5 µm particle size, and DMF containing 10 mM LiBr as eluent at a flow rate of 0.75 mL min^{-1} at 60 °C.

Synthesis

Platinum(IV) Compounds 2a and 2b

(OC-6-54)-(3-Carboxypropanoato)dichlorido(*N,N*-dimethylethane-*1,2*-diamine)hydroxidoplatinum(IV) (2a). A suspension of (OC-6-43)-dichlorido(*N,N*-dimethylethane-1,2-diamine)dihydroxidoplatinum(IV) **(1a)** (850 mg, 2.19 mmol) and succinic anhydride (440 mg, 4.4 mmol) was stirred in 12 mL of DMF abs. under an argon

atmosphere at 50°C for 1 hour. A clear solution indicated completion of the reaction. The solvent was removed under reduced pressure; the crude product was dissolved in 10 mL of acetone and the pale yellow solid was precipitated with diethyl ether. The product was dried *in vacuo*. Yield: 772.2 mg, 73 %. ^{1}H-NMR (DMSO-d_6): δ = 12.08 (bm, 1H, *OH*), 9.41 (bs, 1H, *NH_2*), 7.13 (bs, 1H, *NH_2*), 2.83 (bm, 4H, H3/H4), 2.69 (s+d, 3H, H2a/H2b), 2.61 (s+d, 3H, H2a/H2b), 2.42 (m, 4H, H6/H7).

(OC-6-44)-Acetato(3-carboxypropanoato)(*1R,2R*-diaminocyclohexane)oxalatoplatinum(IV) (2b). (OC-6-44)-Acetato(*1R,2R*-diaminocyclohexane)hydroxido(oxalato)platinum(IV) **(1b)** (1 g, 2.11 mmol) and succinic anhydride (634 mg, 6.33 mmol) were suspended in 14 mL of DMF abs. and stirred for 24 hours at 50 °C. After removal of DMF under reduced pressure, the residue was suspended in 15 mL of acetone. Methanol was added slowly until no more substance could be dissolved. The insoluble substance was filtered off; the filtrate was evaporated and the crude product was dissolved again in acetone. Precipitation of the product was performed with ethyl acetate and the white product was filtered, washed with diethyl ether, and dried *in vacuo*. Yield: 943.22 mg, 78%. ^{1}H-NMR (DMSO-d_6): δ = 12.12 (bm, 1H, *OH*), 8.10-8.53 (bm, 4H, *NH_2*), 2.58 (m, H1/H2), 2.41 (m, 2H, H12/H13), 2.12 (m, 2H, H3/H4/H5/H6), 1.97 (s, 3H, H10), 1.51 (m, 2H, H3/H4/H5/H6), 1.41 (m, 2H, H3/H4/H5/H6), 1.16 (m, 2H, H3/H4/H5/H6) ppm.

Polymer (3)

The poly(dichloro)phosphazene precursor was synthesized in the glove box at room temperature. The initiator PCl_5 (37.1 mg, 0.18 mmol) and the monomer $Cl_3PNSiMe_3$ (1.00 g, 4.46 mmol) were dissolved separately, each in anhydrous CH_2Cl_2 (5 mL). The monomer was then added to the initiator in solution, stirred overnight and the solvent was removed under vacuum. Yield: quantitative. ^{31}P NMR (121 MHz, $CDCl_3$, δ): -18.13 ppm.

The poly(dichloro)phosphazene precursor was then dissolved in THF abs., tertbutyl-2-(2-(2-aminoethoxy)ethoxy)ethylcarbamate (0.89 g, 3.56 mmol) in THF abs. and Et_3N (1 eq., 0.36 g) was added, and the solution stirred for 24 hours. Jeffamine M-1000 (7.57 g, 7.57 mmol) in excess was dissolved in THF and Et_3N (1 eq., 0.77 g), added to the partially substituted precursor in THF and stirred in the glove box at room temperature for 24 hours. After removal of the solvent under vacuum, the polymer was purified by dialysis (12 kDa cut-off) in H_2O for 24 hours followed by 72 hours in EtOH. Removal of the solvent under vacuum gave a waxy solid.

Yield: 3.13 g, 49 %

^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.05 (br, 6H), 1.36 (s, 9H), 3.30 (s, 3H), 3.57 (m, 79H) ppm; ^{31}P NMR (121 MHz, $CDCl_3$, δ): 0.94 ppm.

General procedure for synthesis of conjugates 4a and 4b.

Polymer **3** was dissolved in CH_2Cl_2 (100 mg polymer in 2 mL), trifluoroacetic acid was added (CH_2Cl_2/TFA 2:1 ratio) and stirred for 3 hours. The solvent was removed under reduced pressure and the deprotected polyphosphazene was stored under vacuum until further use. *1,1'*-carbonyldiimidazole and the respective complex (**2a** or **2b**) were dissolved in DMF abs. and stirred for 10 minutes at 60°C under an argon atmosphere. After cooling down to room temperature, the reaction mixture was flushed with argon in order to remove CO_2. Deprotected compound **3** was dissolved in DMF under absolute conditions and added to the solution. After addition of triethylamine, the reaction mixture was stirred for 24 hours. The solvent was removed under reduced pressure; the product was dialyzed against methanol for five days and the obtained product was dried *in vacuo*.

Conjugate 4a. *1,1'*-Carbonyldiimidazole (73 mg, 0.44 mmol), **2a** (193 mg, 0.40 mmol), **3** (400 mg, 0.29 mmol), triethylamine (73 µL, 0.53 mmol). Yield: 117.7 mg, 29 %. ICP-MS: 36934.46 ng/mg Pt, amount of **3** bound to amine groups: 30 %. ^{31}P-NMR (DMSO-d_6): δ = 0.88 ppm.

Conjugate 4b. *1,1'*-Carbonyldiimidazole (36 mg, 0.22 mmol), **2b** (114 mg, 0.20 mmol), **3** (200 mg, 0.15 mmol), triethylamine (36 µL, 0.26 mmol). Yield: 37.3 mg, 18 %. ICP-MS: 29348.88 ng/mg Pt, amount of **3** bound to amine groups: 21 %. ^{31}P-NMR (DMSO-d_6): δ = 0.44 ppm.

Cell culture. The ovarian carcinoma A2780 cell line and its cisplatin-resistant subline A2780/cis were purchased from Sigma and cultivated in RPMI-1680 cell media with 10% fetal bovine serum. The colorectal carcinoma cell line HCT-116 was donated by Dr. B. Vogelstein (John Hopkins University, Baltimore, MD), whereas oxaliplatin-resistant subline HCT-116 oxR was established by stepwise exposure of HCT-116 cells to the drug as previously reported [[40]]. Both the parental and oxaliplatin-resistant cell lines were grown in McCoy cell media with 10% fetal bovine serum. Cell cultures were maintained at 37°C in humidified atmosphere containing 5% CO_2. Cultures were regularly checked for *Mycoplasma* contamination.

Cytotoxicity assays. Cells were plated (2-4x10^3 cells/well) in 100 µL per well in 96-well plates. After a recovery period of 24 h, drugs and polymers were added in another 100 µL growth medium and cells were exposed for 72 hours. The proportion of viable cells was determined by MTT assay following the manufacturer's recommendations (EZ4U, Biomedica, Vienna, Austria). Cytotoxicity was calculated using the Graph Pad Prism software (La Jolla, USA) (using a point-to-point function) and was expressed as IC_{50} values calculated from full dose-response curves (drug concentrations inducing a 50% reduction of cell number in comparison to untreated control cells cultured in parallel).

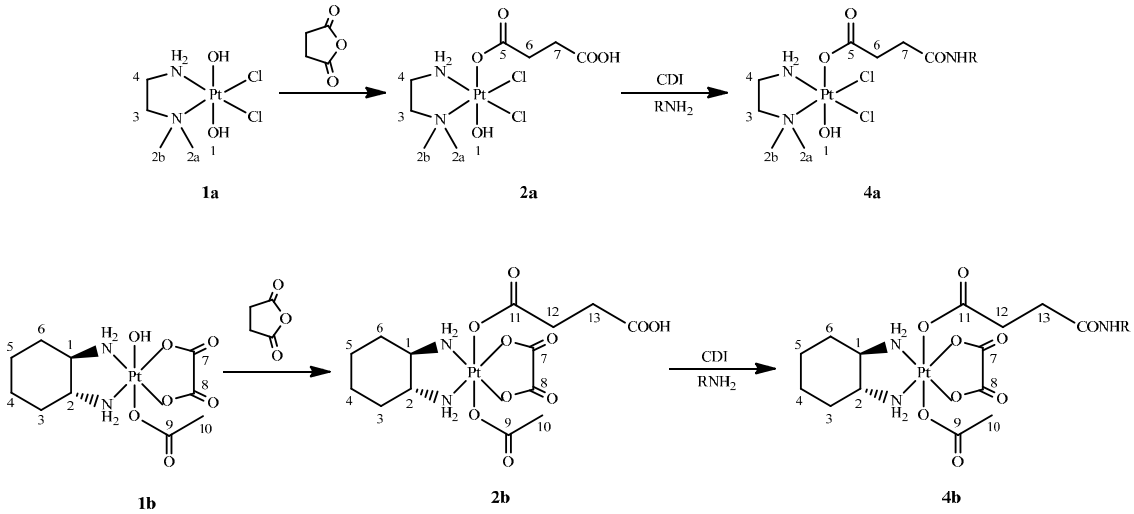
Platinum accumulation. A2780 and A2780/cis cells (1.5 x 10^5) were exposed to 10 µM cisplatin, **2a**, and **4a** at 37°C for 3 hours, respectively. Cell samples were lysed in tetramethylammonium hydroxide and diluted in 0.4 N HNO_3. Total cytosolic platinum accumulation concentrations were determined by inductively coupled plasma mass spectrometry (Agilent Technologies, 7700 Series). The system used for these measurements was equipped with an octopole reaction cell using helium as inert collision gas [41]. Values represent means of at least three independent experiments. To avoid unspecific binding to cell culture plastic, results were corrected for Pt levels of a blank well containing no cells.

Results and Discussion

Synthesis

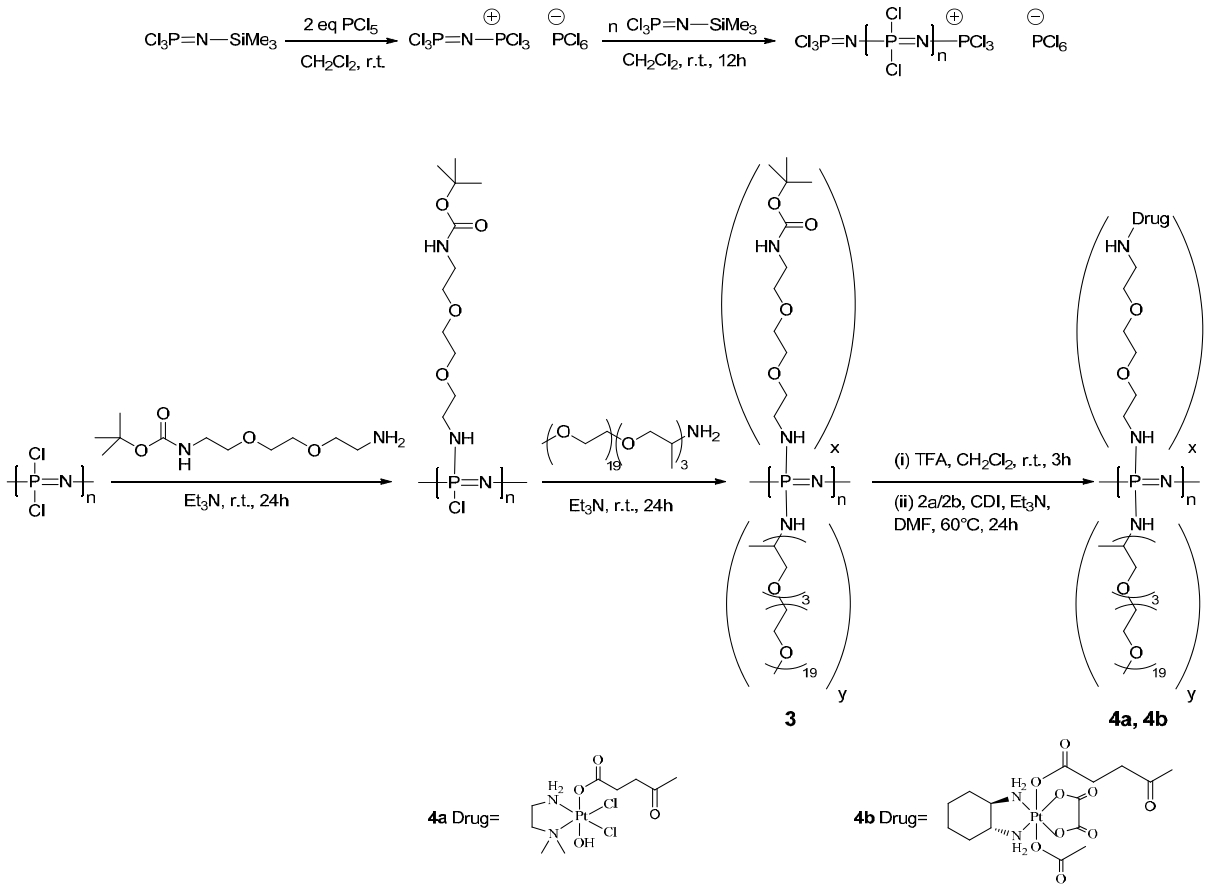
For coupling onto the polyphosphazene carrier, two platinum(IV) compounds, each carrying only one free carboxylic acid in the axial position, were chosen in order to avoid polymer cross-linking. By addition of *N,N*-dimethylethane-1,2-diamine, $K_2[PtCl_4]$ was converted into (SP-4-3)-dichlorido(*N,N*-dimethylethane-1,2-diamine)platinum(II) (supporting information, Scheme S1). Subsequent oxidation was performed with H_2O_2 in order to transform the platinum(II) complex into (OC-6-43)-dichlorido(*N,N*-dimethyethane-*1,2*-diamine)dihydroxidoplatinum(IV) (**1a**). The compound was brought to reaction with succinic anhydride under absolute conditions and yielded the monocarboxylated compound **2a** (Scheme 1) due to steric hindrance of the two methyl groups as could be shown recently [37]. In case of complex **2b**, $K_2[PtCl_4]$ was reacted with 1 eq. of (*1R,2R*)-diaminocyclohexane and subsequently with sodium oxalate in order to generate the well-known antineoplastic agent oxaliplatin (supporting information, Scheme S1). Peracetic acid in acetic acid was used as

oxidizing agent to obtain (OC-6-44)-acetato(*1R,2R*-diaminocyclohexane)hydroxido(oxalato)platinum(IV) (**1b**). The monocarboxylated complex **2b** was produced by carboxylation with succinic anhydride (Scheme 1).



Scheme 1. Synthesis of platinum(IV)-loaded polyphosphazenes (RNH_2 = polymer with deprotected NH_2 groups).

Living cationic polymerization was applied to synthesize the poly(dichloro)phosphazene precursor due to its ability to yield polymers with controlled chain lengths [42]. The total consumption of the monomer was confirmed by ^{31}P NMR spectroscopy. In a successive two-step nucleophilic substitution, the chlorine atoms of the poly(dichloro)phosphazene precursor were then completely substituted (Scheme 2). Firstly, mono-boc protected *2,2'*-(ethylenedioxy)-bis(ethylamine) was attached to the polymer backbone to provide subsequent reaction sites for the conjugates. The remaining chlorine atoms were then replaced by Jeffamine, a mono-amine PPO-PEO oligomer (M_n: 1000) to give the highly branched, water soluble poly(organo)phosphazene (**3**). Jeffamine was used for its excellent aqueous solubility and superior cell uptake kinetics [43]. The resulting polymer was characterized by ^{1}H and ^{31}P NMR spectroscopy, and GPC analysis. Integration of the boc groups allowed the calculation of 40 % reactive sites.



Scheme 2. Synthetic pathway leading to conjugates 4a and 4b. Synthesis of conjugate **4a** and **4b** with polymer backbone chain length n=50 and carbamate (x) to PEO/PPO (y) ratio of x:y = 40% : 60%.

The poly(organo)phosphazene (**3**) was deprotected by stirring in a 2:1 solution of CH_2Cl_2 and TFA for 3 hours. The solvent was removed under reduced pressure and the residue kept under vacuum until it was brought to reaction with the prevailing carboxylic acid. Activation of complex **2a** or **2b** was accomplished with the peptide coupling agent *1,1'*-carbonyldiimidazole (CDI) in DMF under absolute conditions to form platinum(IV) imidazolides. The deprotected polymer **3** (not shown) was dissolved in DMF abs. under an argon atmosphere and brought to reaction with the activated platinum(IV) species for 24 hours at room temperature. Additionally, triethylamine was added to the reaction mixture since the remaining TFA could not easily be removed by extraction. After completion of the reaction, the solvent was removed *in vacuo* and the crude product was dissolved in methanol. The conjugated polyphosphazenes were dialyzed against methanol for five days and dried *in vacuo* before characterization and biological testing was carried out. The final conjugation step was performed with yields of 29% and 18%, respectively. Calculation of the percentage of platinum loading was determined by the amount of platinum found in a weighted sample of conjugates **4a** or **4b** measured with ICP-MS (see supporting information, example for

calculation of polymer loading). In case of conjugate **4a**, 30% of the free amine groups were coupled to the cytotoxic platinum(IV) complex. However, loading of conjugate **4b** was found to be 21%. A summary of the polymer conjugate characterization is given in table 1.

Table 1. Characterization of the polymer drug conjugates

Conjugate	Polymer	Drug	Reactive sites [%]	M_n [GPC a)] (kg mol^{-1}) (M_w/M_n)	R_h [b)] [nm]	^{31}P NMR	% Amine conversion	Total amount of Pt (µg mg^{-1})
4a	3	2a	45	15.9 (1.85)	4.1	0.88	30	36.93
4b	3	2b	45	13.2 (1.36)	3.9	0.44	21	29.35

[a)] Molecular weight as determined by GPC in DMF versus linear polystyrene standards (N.B. calibrated against linear polystyrene standards and thus highly underestimated)

[b)] Hydrodynamic radius measured by GPC in DMF

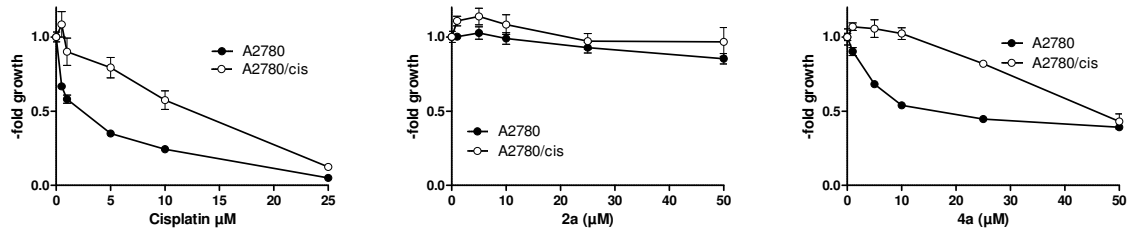
Cytotoxicity

For cytotoxicity experiments in cell culture, the ovarian cancer cell line A2780 and colon carcinoma cell line HCT-116 together with their corresponding cisplatin-resistant (A2780/cis) or oxaliplatin-resistant (HCT-116 oxR) sublines were used. Cisplatin, complex **2a,** and conjugate **4a** were tested in A2780 and A2780/cis cells, whereas oxaliplatin, complex **2b**, and conjugate **4b** were tested in HCT-116 and HCT-116 oxR cell lines (Figure 3).

In general, cisplatin and oxaliplatin were distinctly more potent than their respective platinum(IV) complexes **2a** and **2b** (Table 2 and 3 as well as Figure 3), which is in good agreement with the platinum(IV) prodrug concept. The activity of the platinum(IV) drugs increased upon conjugation to the polymers. Notably, this was also associated with enhanced efficacy against the platinum-resistant sublines. Thus, the resistance factors (IC_{50} of a given drug in resistant cancer subline divided by the IC_{50} of the same drug in the parental cell line) of the cisplatin-resistant A2780 model was reduced from 5.2-fold to 2.8-fold in case of polymer **4a** and from more than 22-fold to 5.9-fold in case of polymer **4b** in the oxaliplatin-

resistant HCT-116 model. This indicates that the use of a platinum(IV) prodrug strategy together with polymer conjugation might be able to overcome, at least partially, the acquired resistance against platinum(II) drugs.

A



B

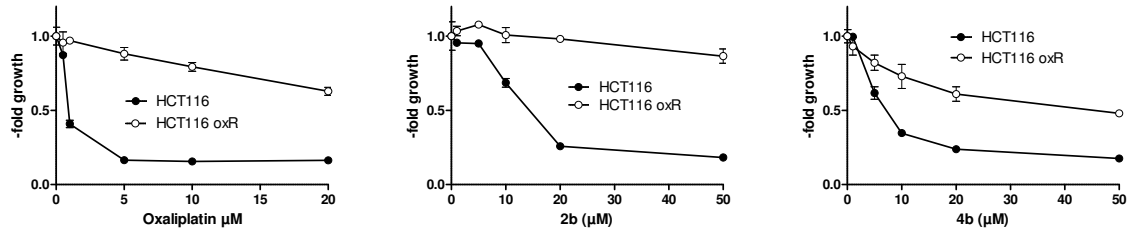


Figure 3. Viability of cancer cells after treatment with novel complexes and conjugates. A.) A2780 ovarian cancer cells and the respective cisplatin-resistant subline A2780/cis were treated with cisplatin, **2a**, and **4a**. B.) HCT-116 colon carcinoma cells and the respective oxaliplatin-resistant subline HCT-116 oxR were treated with oxaliplatin, **2b**, and **4b**. Viability changes as compared with the untreated control were measured by MTT assay after 72 hours incubation with drugs. Values given are means ± SD of three experiments performed in triplicate.

Table 2. Cytotoxicity of **2a** and **4a** vs. cisplatin in cisplatin-sensitive vs. -resistant cancer cells.

	Cisplatin		**2a**		**4a**	
cell line	IC_{50} (µM)	RF	IC_{50} (µM)	RF	IC_{50} (µM)	RF
A2780	**2.4 ± 0.1**		**> 50**		**16.3 ± 1.9**	
A2780/cis	**12.5 ± 0.5**	5.2	**> 50**	**-**	**45.6 ± 1.7**	2.8

RF – resistance factor

Table 3. Cytotoxicity of **2b** and **4b** vs. oxaliplatin in oxaliplatin-sensitive vs. -resistant cancer cells

	Oxaliplatin		**2b**		**4b**	
cell line	IC_{50} (µM)	RF	IC_{50} (µM)	RF	IC_{50} (µM)	RF
HCT-116	**0.9 ± 0.1**		**14.3 ± 0.1**		**7.2 ± 0.1**	
HCT-116 oxR	**> 20**	> 22	**> 50**	> 3.5	**42.4 ± 1.3**	5.9

RF – resistance factor

In order to test, whether increased drug uptake was underlying the enhanced activity of the polymers, A2780 and A2780/cis cells were treated with cisplatin, complex **2a**, and polymer conjugate **4a** for 3 hours and the total cellular platinum content was analyzed by ICP-MS. The highest platinum levels were detected in both cell lines treated with conjugate **4a**, whereas uptake of cisplatin and complex **2a** was 30-fold lower or even below the limit of detection (LOD) (Figure 4). Thus, these preliminary results indicate that conjugation to the new polymers results in distinctly enhanced cellular uptake, thus explaining the higher activity of polymer **4a** in comparison to the free complex **2a**.

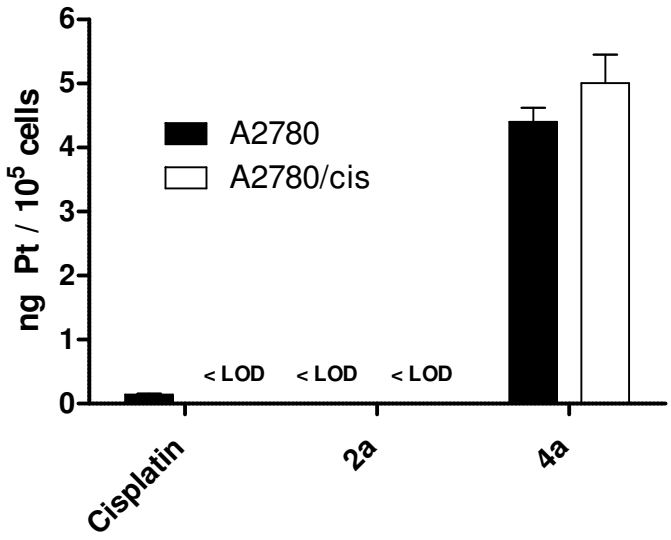


Figure 4. Platinum accumulation *in vitro*. Treatment of A2780 and A2780/cis cells with 10 µM of cisplatin, complex **2a**, and polymer conjugate **4a** for 3 hours, respectively. Values are expressed in nanograms of platinum per 10^5 cells ± SD. (<LOD; below limit of detection).

Conclusion

Two cytotoxic platinum(IV) complexes were successfully coupled to a biodegradable polyphosphazene. Cell culture studies revealed promising antiproliferative activity both in A2780 and HCT-116 cancer cells, presumably as a result of enhanced drug accumulation. Furthermore, the relative impact of cis- and oxaliplatin-resistance on the activity of the new conjugates was distinctly weaker as compared to the respective platinum(II) drugs. This might be attributed, at least in part, to the significantly enhanced cellular accumulation of the macromolecular conjugate, probably accompanied by changes in the drug uptake route. Macromolecules are known for their dependence on endocytosis, in contrast to uptake via passive diffusion as is case of unbound platinum drugs. Together, the unique combination of a Pt(IV) prodrug strategy with a biodegradable polymer potentially allows the safe use of higher molecular weight polymer carriers, above the renal clearance limit. Thus, further evaluation of this novel type of drug-polymer conjugates to explore their potential for *in vivo* tumor targeted delivery is warranted.

Acknowledgements

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Supporting Information

Coupling of cytotoxic platinum(IV) prodrugs to biodegradable polyphosphazenes: synthesis, antiproliferative activity, and cellular accumulation

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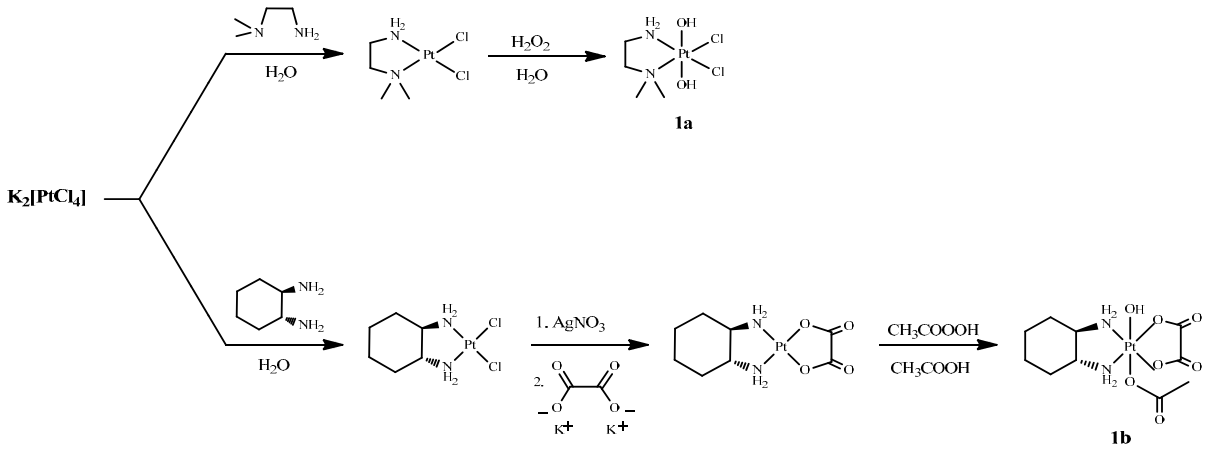
Contents



Scheme S1. Synthetic pathway leading to precursors **1a** and **1b**.

Example for Calculation of Polymer Loading (Conjugate 4a)

Results of ICP-MS measurements:

Conjugate **4a**	Pt [ng/mg]
Measurement 1	36348.20
Measurement 2	37520.72
Arithmetric mean	36934.46

M [Pt] = 195.1 g/mol

M [Complex **2a**] = 488.2 g/mol

M [one unit of polymer **3**] = 1366 g/mol (45% of linker loaded with n-boc protected amine groups)

M [one unit of deprotected polymer **3**] = 1277 g/mol

Mass of complex 2a in the polymer

36934.46 [ng] /195.1*10^9 [ng/mol] = *1.893*10^{-7} mol* Pt in 1 mg of loaded polymer

1.893*10^{-7} [mol] * 488.2 [g/mol] = *9.2*10^{-5} g* (= 0.092 mg) of complex **2a** in 1 mg of loaded polymer **4a**.

Polymer loading

The amount of complex **2a** in 1 mg of loaded polymer **4a** has to be subtracted:

1 [mg] – 0.092 [mg] = *0.908 mg* of deprotected polymer **3** in 1 mg of **4a**.

0.908 [mg]/1277*10^3 [mg/mol] * 2 * 0.45 = *6.3994*10^{-7} mmol* of amines per mg of deprotected polymer **3**.

Loading is equal to ratio of complex **2a** to mol of amines per mg of deprotected polymer **3**:

1.893*10^{-7} [mol] */6.3994*10^{-7}* [mol] = *0.2958* = ***29.6 %*** of amine groups are loaded with complex **2a**.

3. Conclusion

During this PhD work, synthesis, characterization, and first in vitro studies of several novel antitumor platinum(IV) compounds could be realized. The results have been published in two peer-reviewed publications and one further manuscript has been submitted on April 23rd, 2014.

The first part of this work was dedicated to investigations of four different ^{13}C- and ^{15}N-labelled platinum(IV) complexes bearing ester and amide moieties in cancer cell lysates. First, the chlorido ligands of $K_2[PtCl_4]$ were replaced by iodido ligands upon reaction with KI. $K_2[PtI_4]$ was converted to bis{(^{15}N)ammine}diiodidoplatinum(II) by addition of $^{15}NH_4Cl$ in the presence of NaOH. Subsequent reactions according to literature methods yielded the desired ^{15}N-labelled well-known cytostatics cisplatin and carboplatin. Both precursors were oxidized with H_2O_2 and brought to reaction with (2,5-$^{13}C_2$) succinic anhydride under absolute conditions. The obtained platinum(IV) compounds, (*OC*-6-33)-bis((^{15}N)ammine)bis[(3-(^{13}C)carboxy(1-^{13}C)propanoato]-dichloridoplatinum(IV) and (*OC*-6-33)-bis((^{15}N)ammine)bis[(3-(^{13}C)carboxy(1-^{13}C)propanato](cyclobutane-1,1-dicarboxylato)platinum(IV), were converted into amides and esters, respectively. The free carboxylic acid groups of both substances were activated with *1,1'*-carbonyldiimidazole and subsequently each reacted with NaOMe and propylamine. The four final products were fully characterized by ESI-MS, elemental analysis, and multinuclear (^1H, ^{13}C, ^{15}N, ^{195}Pt) NMR spectroscopy. Each of the synthesized complexes was incubated with SW480 cell lysates in the same complex to cell number ratio, and their reduction times were evaluated by ^1H, ^{13}C HMBC NMR spectroscopy. It was found that the cisplatin-derived complex (*OC*-6-33)-bis((^{15}N)ammine)dichloridobis{(4-methoxy)-4-oxo(1,4-$^{13}C_2$)butanoato}platinum(IV) was reduced much faster than its carboplatin analogue. Unfortunately, reduction times of the prevailing amides could not be detected due to overlapping of product and starting material cross peaks.

The aim of the second project was to synthesize and fully characterize the first anticancer platinum(IV) complexes bearing ferrocenyl moieties. In a first step, five different ferrocenyl compounds bearing either an alcohol or an amine moiety were synthesized starting from ferrocenecarboxylic acid. Five novel complexes could be produced by coupling of two different platinum(IV) precursors each bearing two carboxyl groups in axial position with the

synthesized ferrocenyl compounds. Since these five compounds were not soluble in water, one further platinum(IV) precursor, deriving from (*1R,2R*-diaminocyclohexane)(oxalato)platinum(II) and containing only one carboxyl group in axial position, was synthesized; by reaction with three of the initially prepared ferrocene moieties, further ferrocenyl-platinum(IV) systems could be designed. All of the synthesized complexes were characterized by elemental analysis, ESI-MS, and multinuclear (^{1}H, ^{13}C) NMR spectroscopy. Furthermore, all of them were tested in terms of their cytotoxic activity in three different human cancer cell lines, showing promising IC_{50} values in the low micromolar range.

In the last project, anticancer platinum(IV) compounds were coupled to polyphosphazenes and investigated *in vitro*. Since polyphosphazenes are non-toxic and biodegradable, they are suitable macromolecular drug carrier which may increase passive tumor targeting due to the well-known EPR effect. For coupling reactions, two platinum(IV) agents bearing only one carboxyl group were chosen in order to prevent cross-linking with the polymer. The final products were characterized by ^{31}P NMR and SEC; the amount of loaded platinum was determined by ICP-MS. Cytotoxicity of both platinum(IV)-polyphosphazenes could be determined in three different human cancer cell lines, displaying IC_{50} values in the low micromolar range. Additionally, high cell accumulation of one conjugate in the A2780 human ovarian carcinoma cell line as well as in the corresponding cisplatin-resistant cell line could be found. This project was carried out in cooperation with the Institute of Polymer Chemistry (Johannes Kepler University Linz) and the Institute for Cancer Research (Medical University of Vienna). Poly(organo)phosphazenes were synthesized and delivered by Helena Henke of the research group of Prof. O. Brüggemann at the Johannes Kepler University Linz, whereas in vitro investigations were carried out by Kushtrim Kryeziu from the group of Prof. W. Berger at the Medical University of Vienna.

4. Curriculum Vitae

Personal data

Full name: Jelena Banfić, BSc MSc
Date of birth: July 14th 1987
Nationality: Croatia

Education

2011 – to date	University of Vienna, PhD studies in chemistry Research area: Bioinorganic chemistry, development of platinum(IV) complexes as potential chemotherapeutics
2009 – to date	Vienna University of Economics and Business, Business studies
2005 – 2011	University of Vienna, Studies in chemistry – Graduation: M.Sc.
1997 – 2005	Akademisches Gymnasium Salzburg (High School)
1993 – 1997	Elementary School Mülln, Salzburg (Elementary School)

Language Skills

Croatian	Native language
German	Native language
English	Fluent (speaking, reading, writing)
Spanish	Intermediate (speaking, reading, writing)
French	Intermediate (speaking, reading, writing)
Latin	7 years at school

Employment History

2012 Feb – to date	University of Vienna, Institute of Inorganic Chemistry - Teaching in undergraduate inorganic lab courses
2013 May – to date	University of Vienna, Institute of Organic Chemistry - Teaching in undergraduate organic lab courses
2011 Oct – to date	University of Vienna, Institute of Inorganic Chemistry - PhD student, Project employee
2009 Aug	Akzo Nobel Coatings GmbH - Production department

2008 Aug	Akzo Nobel Coatings GmbH - Production department
2007 Aug	Akzo Nobel Coatings GmbH - Production department
2006 Jul	Akzo Nobel Coatings GmbH - Production department
2005 Jul	Grundfos Pumpen GmbH - Marketing department
2004 Jul	Grundfos Pumpen GmbH - Marketing department

International Experience

2012 June – Sep	Université du Québec à Trios-Rivières, QC, Canada, Département de Chimie-Biologie (Depatment of Chemistry and Biology) – Research field: Steroids, coupling to platinum(IV) agents Supervison: Prof. Gervais Bérubé, PhD

Peer-reviewed Publications

J. Banfić, A. A. Legin, M. A. Jakupec, M. Galanski, and B. K. Keppler: **"Platinum(IV) Complexes Featuring One or Two Axial Ferrocene Bearing Ligands – Synthesis, Characterization, and Cytotoxicity"**, Eur. J. Inorg. Chem., 3, 484-492, 2014.

J. Banfić, M. S. Adib-Razavi, M. Galanski, and B. K. Keppler: **"Platinum(IV) Complexes Featuring Axial (1, 4–13C2)Succinato Ligands – Synthesis, Characterization, and Preliminary Investigations in Cancer Cell Lysates"**, Z. anorg. Allg. Chem. 639 (8-9), 1613-1620, 2013.

Conferences

J. Banfić, A. A. Legin, M. A. Jakupec, M. Galanski, and B. K. Keppler: **"Synthesis, characterization and cytotoxicity of bis- and tetrakis(carboxylated) platinum(IV) compounds featuring ferrocene-ligands",** International Conference on Bioinorganic Chemistry 16, Grenoble, France, July 22-26th, **2013**.

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Jelena Banfić, April 2014