Toward dual-action platinum(IV) complexes with bioactive ligands as potent anticancer agents

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Thesis for the Degree of PhD Chemistry

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Submitted October 2019

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Research carried out at the School of Chemistry
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&

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Declaration

I declare that the current study is my own work and has not been submitted for a degree to this or any other academic institution, previously.

AWATIF ALMOTAIRY
October 2019

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Acknowledgement

The vast field of my project has introduced me to new areas of chemistry and biology, that can be inspired on my future career. There are many challenges and unforgettable experiences in my study. This PhD has been done by a fantastic supervisory team and an amazing family.

First and foremost, I would like to express my sincere gratitude to my supervisor Dr. Andrea Erxleben for her expertise, support and direction during my research study in inorganic chemistry. It was my honour to be in her research group.

Secondly, I would like to express my sincere gratitude to my supervisor Prof. Orla Howe for her expertise, support and direction during my research study in biology. It was my honour to be in her research group.

Thirdly, I would also like to thanks to Dr. Diego Montagner and Prof. Michael Devereux for their discussions and support throughout the project.

Fourth, my sincere thanks also go to all the staff and the technical staff at the chemistry department in NUI Galway, FOCAS Research Institute and the RESC group.

Fifth, I am grateful to acknowledge the Ministry of Education for a Saudi Arabia Government Scholarship (King Abdullah Scholarship Program) and Taibah University Scholarship.

Sixth, Special thanks to my best friend Anna for being fantastic support.

Finally, I would like to express my deepest gratitude to my parents, sisters and brothers for their support, love and encouragement in my whole life. I would especially like to the thank ,Hana, for being a bedrock of support in my life.

Conferences & Presentations

1- **Advances in the achievement of a synergistic effect between ligands and anti-tumour platinum(IV) based on cisplatin complexes as anticancer drugs.** (5th Annual Postgraduate Research Day at NUI Galway)- (Poster).

2- **Advances in the achievement of a synergistic effect between ligands and anti-tumour platinum(IV) based on cisplatin complexes as anticancer drugs.** (13th International Symposium on Applied Bioinorganic Chemistry at NUI Galway (ISABC13) –(Poster).

3- **Platinum(IV) complexes with a histone deacetylase inhibitor in axial position as potent antitumor agents 2016.** (The 68th Irish Universities Chemistry Research Colloquium -Cork -Ireland) –(Poster). **(Got the 2nd best poster and research)**.

4- **Cellular effects of novel dual-acting platinum(IV) complexes based on cisplatin and phenylbutyrate 2016** (IBICS Irish Biological Inorganic Chemistry Society) Technological University Dublin, City Campus, Dublin, Ireland) -(Oral presentation-poster).

5- **Antitumor platinum(IV) derivatives of carboplatin and the histone deacetylase inhibitor 4-phenylbutyric acid 2017** (Annual School of Biological Sciences Research -Technological University Dublin, City Campus, Dublin, Ireland) (Poster).

6- **Antitumor platinum(IV) derivatives of carboplatin and the histone deacetylase inhibitor 4-phenylbutyric acid 2017** (IBICS Irish Biological Inorganic Chemistry Society- Maynooth University, Maynooth, Ireland) (Oral presentation).

7- Workshop: **Flow Cytometry Seminar**- May 2018 at University College Cork.

Some contributions in chemistry school at nuigalway

1-Teaching assistants (2015-2017).

2-I was involved in *An Cumann Ceimice* to design and print posters for all talks (2016).

Scientific dissemination

PhD thesis is based on three papers, which are presented in the original format:

1- Antitumor Platinum(IV) Derivatives of Carboplatin and the Histone Deacetylase Inhibitor 4-Phenylbutyric Acid.
A. R. Z. Almotairy, V. Gandin; L. Morrison; C. Marzano; D. Montagner; A. Erxleben, *J. Inorg. Biochem.* 177, (2017), 1–7.

2- Platinum(IV) Pro-Drugs with an Axial HDAC Inhibitor Demonstrate Multimodal Mechanisms Involving DNA Damage and Apoptosis Independent of Cisplatin Resistance. (**Submitted**)
Awatif Rashed Z. Almotairy, Diego Montagner, Liam Morrisson, Michael Devereux, Orla Howe, Andrea Erxleben.

3- Cytotoxicity and ROS Production of Novel Platinum(IV) Oxaliplatin Derivatives with Indole Propionic Acid.
D. Tolan;* **A. R. Z. Almotairy**;* O. Howe; M. Devereux; D. Montagner; A. Erxleben, *Inorg. Chim. Acta*. 492, (2019), 262-267.* These authors contributed equally to this manuscript

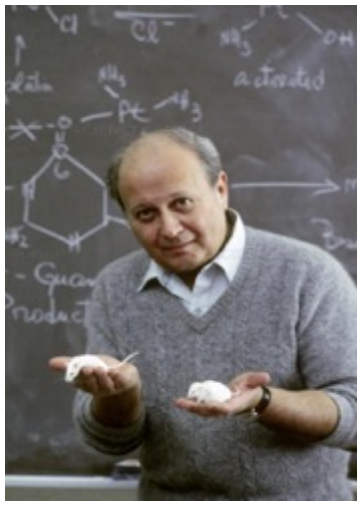
Statement of Ethical Approval

No ethical approval was required to undertake any part of this project

List of abbreviations:

AA	ascorbic acid
Ac	acetate
CBDCA	1,1'-cyclobutanedicarboxylic acid
CDDP	*cis*-diamminedichloridoplatinum(II)
DACH	1R,2R-1,2-diaminocyclohexane
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
DSB	double strand break
ESI-MS	electrospray ionisation mass spectrometry
FDA	Food and Drug Administration
GIT	gastrointestinal tract
GSH	glutathione
GST	glutathione-S-transferase
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HPLC	high-performance liquid chromatography
IC50	half-maximum inhibitory concentration
ICP-MS	inductively coupled plasma mass spectrometry
IR	infrared (spectroscopy)
MCFA	short/medium-chain fatty acids
MMR	mismatch repair
MW	molecular weight
NER	nucleotide-excision repair
NMR	nuclear magnetic resonance
NSAIDs	non-steroidal anti-inflammatory drugs
OCT	organic cation transporter
PDK	pyruvate dehydrogenase kinase
PBA	phenylbutyrate
QSAR	quantitative structure activity relationships
RF	resistance factor
ROS	reactive oxygen species
RT	room temperature
SAHA	suberoyl anilide hydroxamic acid
SAR	structure-activity relationships
List of cell lines	
A2780	ovarian endometroid adenocarcinoma
A2780cisR	ovarian endometroid adenocarcinoma, cisplatin-resistant subline
A375	melanoma cancer cell line
A431	cervical cancer cell line
BxPC3	human pancreatic cancer cell line
LoVo	colon cancer cell line

“For years I have been saying that cisplatin is the first platinum-based drug we discovered. It cannot be the best one!!! It’s disappointing that the scientific community has not been able to find better ones”



Barnett Rosenberg

Abstract

Platinum agents (cisplatin, carboplatin and oxaliplatin) have a crucial role in the treatment of many cancers for several decades. However, they have severe side effects and a high number of patients have intrinsic or acquired resistance to platinum-based chemotherapy. The need for new platinum agents with better patient tolerability and improved anti-tumoral efficacy is necessary in order to overcome the side effects of those drugs.

Platinum(IV) anticancer drugs are prodrugs, which can not only overcome side effects and the resistance to platinum-based chemotherapy but also lead to enhanced tolerability in patients. It is highlighted that only four platinum(IV) complexes have made it into clinical trials, and three out of these four have failed to show clinical efficacy. The efficacy of platinum(IV) agents depends on the stability, reduction rate, degree of cellular accumulation and which enzymes are targeted. Platinum(II) drugs and bioactive ligands (particularly the ones that have epigenetic activity) can act on various targets with various cellular mechanisms and it would be more appropriate to design them as multi-action platinum(IV) prodrugs.

This thesis focuses on the synthesis and characterisation of novel platinum(IV) complexes derived from FDA-approved platinum(II) complexes and bioactive, axial carboxylate ligands. Also explored here are the biological activity, cellular effects and reactions with cellular targets.

Chapter one provides an overview of platinum(IV) complexes and examples of various dual-action agents with the history of platinum anticancer therapy.

Chapter two examines the design and synthetic strategies employed for the development of novel platinum(IV) prodrugs for carboplatin with the histone deacetylase inhibitor (HDACi) 4-phenylbutyrate (PBA) and three different biologically inactive carboxylic acids. Lower accumulation levels and lower cytotoxicity were observed for the carboplatin platinum(IV) complexes compared to cisplatin. The carboplatin platinum(IV) derivative with PBA and benzoate had a higher cytotoxic activity and HDAC inhibition potency than carboplatin.

Chapter three investigates platinum(IV) complexes based on the cisplatin scaffold and PBA in cisplatin-sensitive and cisplatin-resistant ovarian cancer cells (A2780 and A2780cis). Three complexes in this study were more cytotoxic in A2780 and A2790cis ovarian cancer cells than cisplatin and induced cell death pathways leading to apoptosis.

In **Chapter four** new platinum(IV) derivatives of oxaliplatin are described which induced oxidative stress and cytotoxicity. Novel platinum(IV) oxaliplatin derivatives with indole propionic acid were synthesised and studied by various techniques. The monocarboxylated complex with an hydroxido ligand in the second axial position was the most cytotoxic and potent ROS inducer compared with cisplatin and oxaliplatin.

Chapter five provides overall conclusions and identifies key areas for future work.

Keywords: cancer, chemotherapy, cisplatin, carboplatin, oxaliplatin, platinum(IV), 4-phenylbutyrate, indole propionic acid.

Chapter 1：Introduction

1.1. Cancer

Cancer, which is regarded as being similar to a neoplasm, is a disease that results due to the uncontrolled proliferation of mutated cells or an autonomous growth of tissues[1]. Basically, this term describes several diseases that develop due to the inability to control cell growth, division and proliferation. Some forms of cancer lead to an encapsulated benign tumours that remain confined into their original area and do not invade and destroy nearby normal tissues nor do it spread to distant body areas, such as a common skin wart[2].

Non-encapsulated malignant tumours, however, can invade surrounding healthy tissue and spread throughout the body. They tend to develop at a swift pace to become metastasis.

Metastasis of cancer cells to secondary sites underlies 90% of deaths resulting from cancer. It involves secondary growth which has emerged at a site far from the initial tumour. (**Fig. 1**)

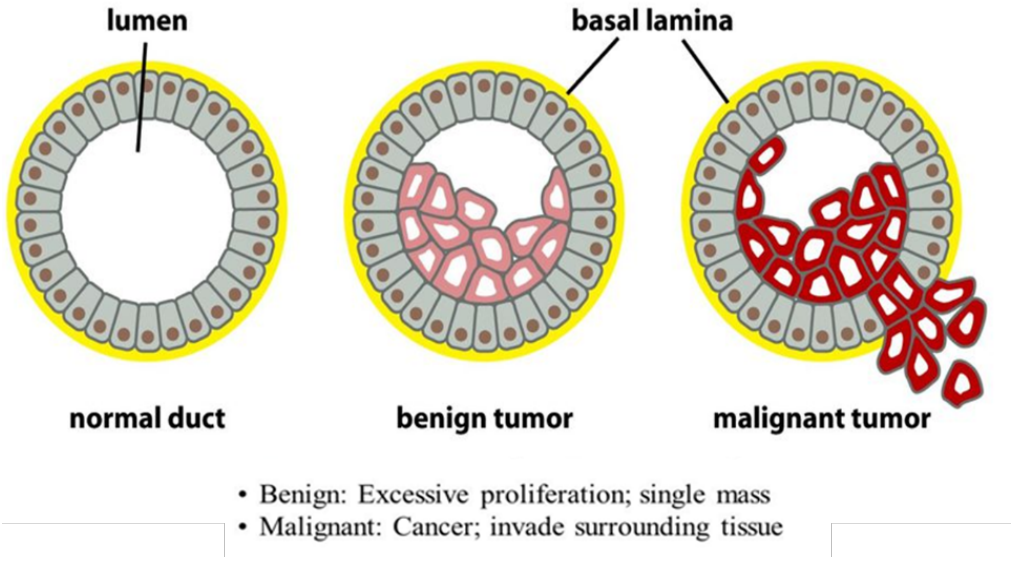


Figure 1: Benign versus malignant tumours. A benign glandular tumour (pink cells; an adenoma) remains inside the basal lamina (yellow) which marks the boundary of the normal structure. Whereas, a malignant glandular tumour (red cells; an adenocarcinoma) destroys the integrity of the tissue. Image adapted from reference [3]

According to GLOBOCAN 2012, a study conducted by the International Agency for Research on Cancer (IARC), there were 8.2 million deaths and 14.1 million new cases of cancer worldwide in 2012, making it one that leading causes of death globally.[4] It is estimated that by 2030, the incidence of cancer cases could reach a staggering 26.4 million, with deaths climbing to 17 million on economic development.[5, 6] (**Fig. 2)**

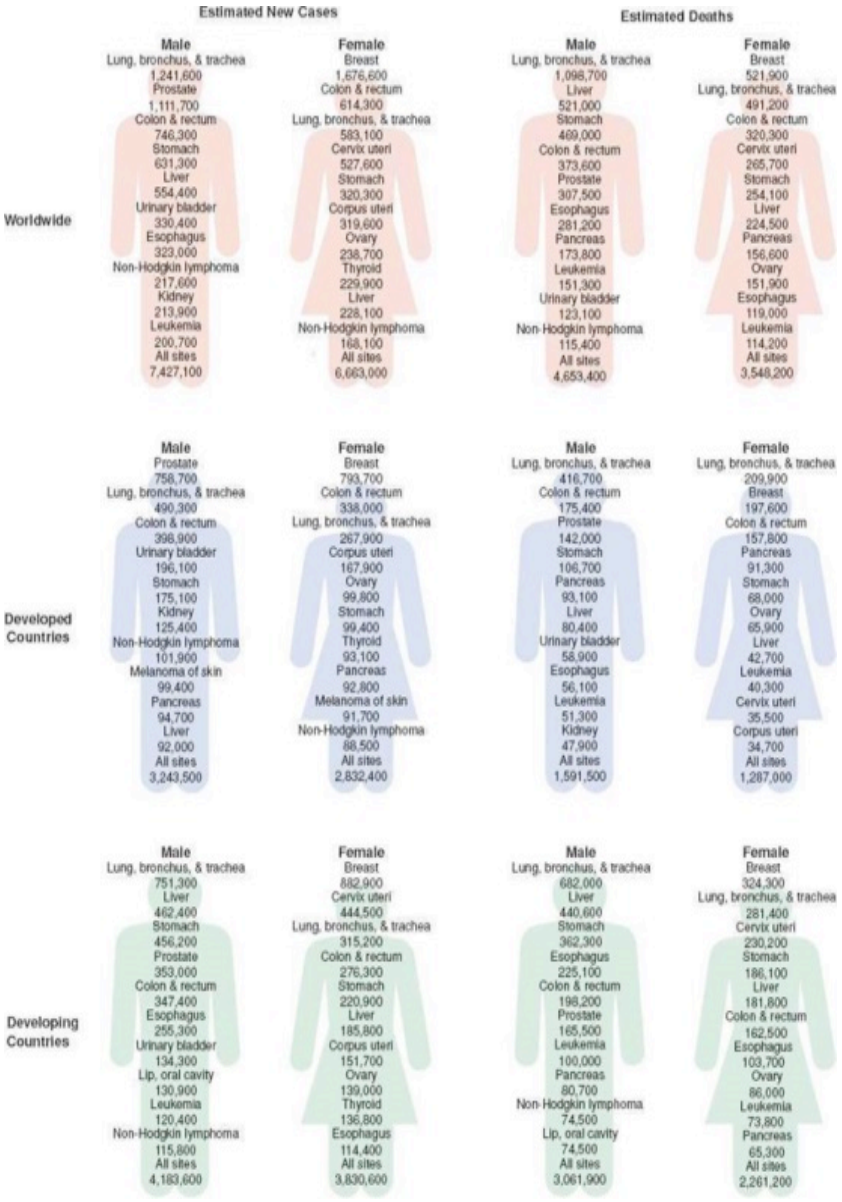


Figure 2: Estimated new cancer cases and deaths worldwide for leading cancer sites by level of economic development, 2012. Image adapted from reference [4]

1.2. Treatment of cancer

In the effort to address the multitude of cancers, many forms of treatment can be adopted, whether via monotherapy involving either surgery, radiotherapy or chemotherapy and a combination of the three. Targeted chemotherapeutics are aimed at killing malignant tumour cells selectively. Some of these include small molecule drugs such as doxorubicin and mitoxantrone, which are mainly targeted at controlling cell division and growth[7]. In addition to this, transition metal complexes are also vital in antitumor therapy, especially drugs based on platinum, copper and ruthenium that have received attention due to their clinical efficacy, (**Table 1)**. Since the discovery of the anti-cancer properties of cisplatin five decades ago, interest in the area of metal complex-based chemotherapy has grown steadily.

Table 1 : Metal complexes in clinical use.

Drug	Status in clinical use	Mode of actions	Indications
Platinum(II) 1-Cisplatin 2-Carboplatin 3-Oxaliplatin	Worldwide use	Known as DNA cross-linkers Well established co-treatment regiments.	**Cisplatin**: testicular cancer, ovarian cancer, head and neck cancer, gastric cancer, bladder cancer.[8] **Carboplatin**: melanoma cancer, ovarian cancer, breast cancer, head and neck cancer.[9, 10] **Oxaliplatin**: colorectal cancer.[11 12]
Casiopeinas family	Phase I	Well known targeting effects (DNA and ROS) High activity against cisplatin-resistant cancer.	Cytotoxic activity against medulloblastoma, glioma and colorectal adenocarcinoma cell lines.[13 14]
Ruthenium NAMI-A KP1019	Phase I Phase II	Overcoming therapeutic resistance in-vitro	**NAMI-A**: patients with progressive or relapsed cancer (colorectal, melanoma, lung and pancreatic).[15] **KP1019**: Advanced colorectal cancer.[15]

Recent extensive research into the medicinal inorganic chemistry field in the development of metal complexes as potential therapeutics with the discovery of cisplatin metal-based drug 54 years ago have contributed to the successful discovery of new metal-based anticancer drugs.[16, 17]

Platinum complexes remain the most widely used anticancer chemotherapeutics. The estimated 95% cure rate of testicular cancer patients is a good indicator of the effectiveness of cisplatin anticancer agent[18]. It is estimated by the National Cancer Institute and American Cancer Society that the number of cancer survivors will rise to more than 22.1 million by January 1, 2030, compared to this year with a history of 16.9 million Americans (8.1 million males and 8.8 million females) having survived cancer [19] (**Fig. 3)**. There is a need for an anticancer drug with broad specificity to treat different types of cancer and the success recorded for platinum-based drugs has resulted in many investigations into their potential as chemotherapeutics in different cancers.

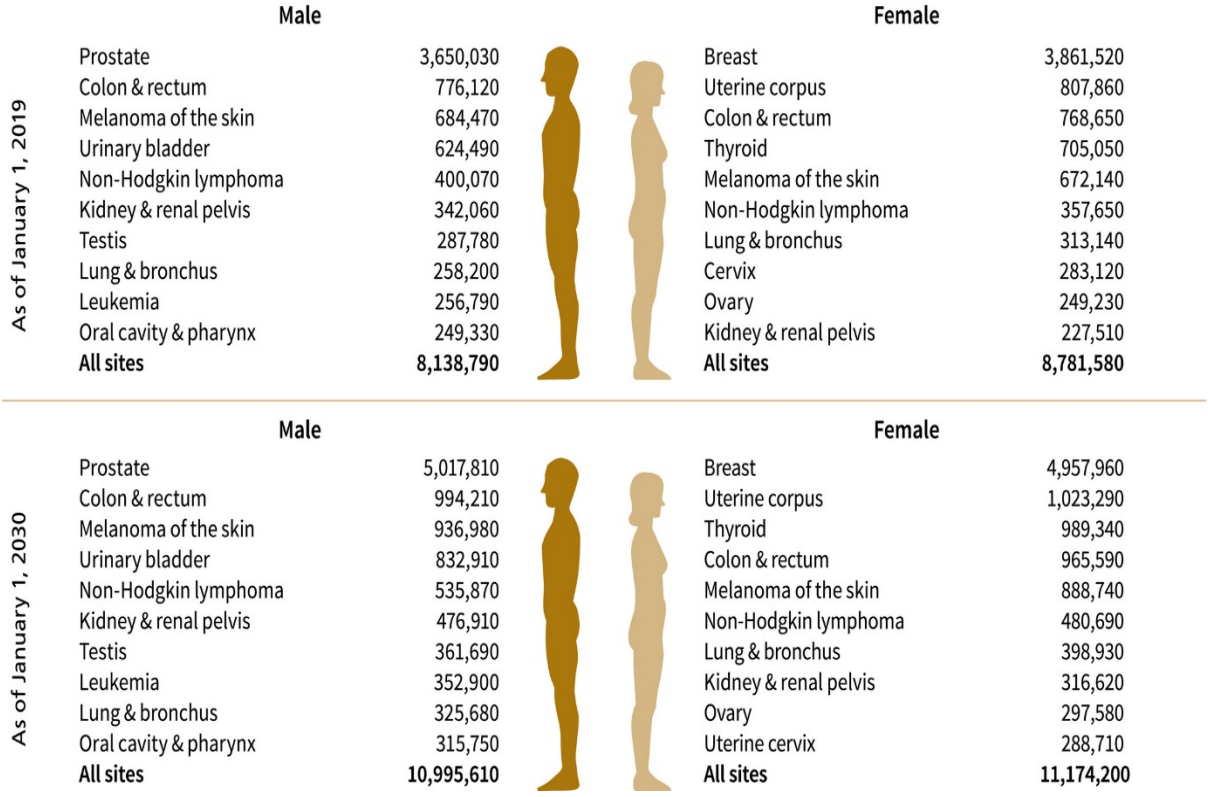
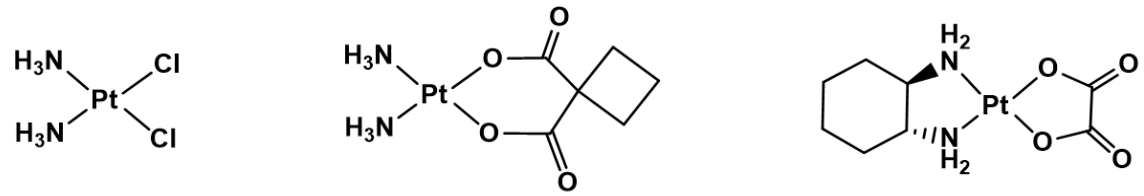


Figure 3: Estimated number of US cancer survivors. Image adapted from reference [19]

1.3. Platinum(II)-based anticancer drugs

Platinum-based drugs have, over time, evolved to become ubiquitous in cancer treatment. More than half of all patients who undergo chemotherapy are given platinum drugs.[20] The Food and Drug Administration (FDA)-approved platinum(II) based drugs which are the most frequently used drugs for cancer include cisplatin, carboplatin and oxaliplatin (**Fig. 4)**.



A **B** **C**

Figure 4: The FDA approved platinum(II) complexes for use in the world. A (cisplatin), B (carboplatin) and C (oxaliplatin).

1.3.1. Cisplatin

Cis-[$Pt(NH_3)_2Cl_2$], cis-diamminedichloridoplatinum(II), CDDP) is a chemotherapeutic drug frequently used to treat many forms of cancer. The widespread use of platinum compounds for cancer treatment began in the 1960s with the discovery of the antineoplastic activity of cisplatin by Barnett Rosenberg.[21] Cisplatin gained approval for clinical treatment by the FDA in the late 1970s and has been used as a chemotherapeutic drug since 1978, specifically in the treatment of testicular, head and neck, bladder and ovarian carcinomas.[22] Cisplatin is commonly used as a platinum-based cytotoxic and the first option for solid tumours treatment (breast and prostate). It is able to alter dramatically the effectiveness of therapy for testicular cancer, from 5-10 % to over 90%.[18]

Structurally, it is a square-planar platinum(II) compound with two coordinated ammonia and two chlorido ligands in the *cis* configuration. The generally accepted mode of action of cisplatin involves covalent binding of the *cis*-$[Pt(NH_3)_2]^{2+}$ entity to adjacent guanine bases in DNA following the loss of chloride leaving group ligands.

1.3.2. Carboplatin

Cis-diammine(cyclobutanedicarboxylato) platinum(II) is a second-generation platinum-based cytostatic drug that was approved by the FDA as Paraplatin in 1986. It is used in the treatment of ovarian cancer, retinoblastomas, neuroblastomas, nephroblastomas, brain, head and neck, cervical, testicular, breast, lung and bladder cancers, and presents a more favourable toxicity profile compared to cisplatin and is generally better tolerated. [23] The carboplatin drug of the platinum(II) series is very efficient in the treatment of some cancers that were initially known to be treated with cisplatin such as ovarian carcinoma.[24]

In addition to this, it also has lower levels of nephrotoxicity, neuropathy, and less of an adverse effect on the gastrointestinal and causes less ototoxicity as it binds to a lesser extent covalently and irreversibly to plasma proteins (30 % vs 90% for cisplatin). [25] The chelating dicarboxylate leaving the group is hydrolysed more slowly than the monodentate chloride ligands in cisplatin, thus reducing off-target binding to biomolecules before DNA coordination. It is reported that carboplatin is much less reactive with DNA than cisplatin, and the concentration of carboplatin required to bind and twist DNA is 100 times higher than for cisplatin.[26]

1.3.3. Oxaliplatin

((1R,2R)-1,2-diaminocyclohexane)oxalato) platinum(II)) is a third-generation platinum(II) drug that gained approval for clinical use by the FDA in 2002. It approved for the treatment of metastatic colorectal cancer and is also effective against lung and ovarian cancer cell lines.[27] Oxaliplatin contains a more stable leaving group compared to cisplatin and the *R,R*-diaminocyclohexane (DACH) chelating ligand which is responsible for overcoming resistance.[28]

It is the first platinum(II) anticancer drug approved that overcomes cisplatin and carboplatin resistance by inhibiting the activities of DNA damage repair proteins. Oxaliplatin leads to a spectrum of activity that is different from that of cisplatin. It also shows a different resistance profile and is usually better tolerated than cisplatin.[24, 29] The combination of 5-fluorouracil-folinic acid and oxaliplatin has shown remarkable clinical activity in patients with advanced colorectal cancer.[30]

1.4. Other platinum(II) drugs with regional approval:

Cisplatin, carboplatin and oxaliplatin have world-wide approval. However, other platinum(II) anticancer agents that have gained approval only for a specific region, and they are listed below.

Nedaplatin: (SP-4-3)-diamine(glycolato) platinum(II). **Table 2**

Nedaplatin is the second generation of cisplatin analogue and was approved as a platinum-based anticancer drug and for clinical use in 1995 in Japan. [31, 32] The anticancer activity of nedaplatin is similar to cisplatin and carboplatin. The latter has a cyclobutandicarboxylato ligand as the leaving group that but nedaplatin has glycolate making it an analogue of carboplatin. It is used to treat neck, head, NLCS and LSC cancers.[33] Common limitations of nedaplatin are myelosuppression, leucopenia, and anemia.[34]

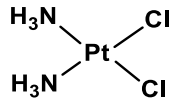
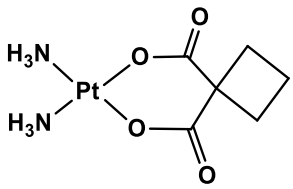
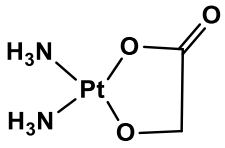
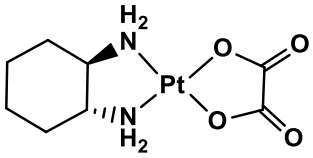
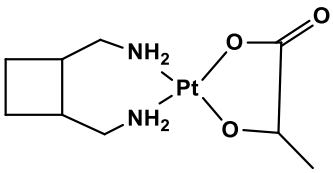
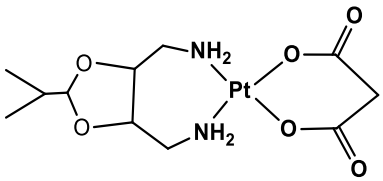
Heptaplatin:(SP-4-2)-(malonato)((4R,5R)-2-(1-methylethyl)-1,3-dioxolane-4,5-dimethylamine) platinum(II). **Table 2**

Heptaplatin is the third generation of cisplatin analogue and was approved in South Korea in 1999. [35, 36] It is highly stable in solution and is designed for gastric cancer therapy. It has fewer and milder side effects than cisplatin. Side effects of heptaplatin, include are nephrotoxicity, hepatotoxicity and myelosuppression.[33]

Lobaplatin: (SP-4-3)-(1,2-cyclobutanedimethanamine)(L-lactate) platinum(II).**Table2**

Another third-generation cisplatin analogue is Lobaplatin, which was approved in 2010 in China[32] as a platinum(II) anticancer drug in order to treat chronic myelogenous leukaemia (CML) and inoperable, metastatic breast and small cell lung cancer.[37] It was shown that lobaplatin is less active than cisplatin and carboplatin. A common problem with lobaplatin is thrombocytopenia.[38].

Table 2: Platinum complexes in current clinical use

Platinum(II)	carrier ligand(s)	leaving group(s)	chemical structure
First Generation			
Cisplatin	NH_3	Cl	
Second Generation			
Carboplatin	NH_3	CBDCA	
Nedaplatin	NH_3	Glycolate	
Third Generation			
Oxaliplatin	*1R*,*2R*-DACH	Oxalate	
Lobaplatin	1,2-cyclobutanedimethanamine	L-lactate	
Heptaplatin	4R,5R-2-(1-methylethyl)- 1,3-dioxolane-4,5-dimethanamine	Malonate (2-Dioatopropanoate)	

1.5. Pharmacology and structure-activity relationships (SAR) of platinum-based anticancer agents.

All platinum compounds in clinical use:

1. are neutral square-planar platinum(II) complexes with cis geometry.
2. their ligand coordination sphere is represented by two (or one bidentate) am(m)ines, called carrier groups and two chlorides or a chelating carboxylate called leaving groups (**Fig. 5**).

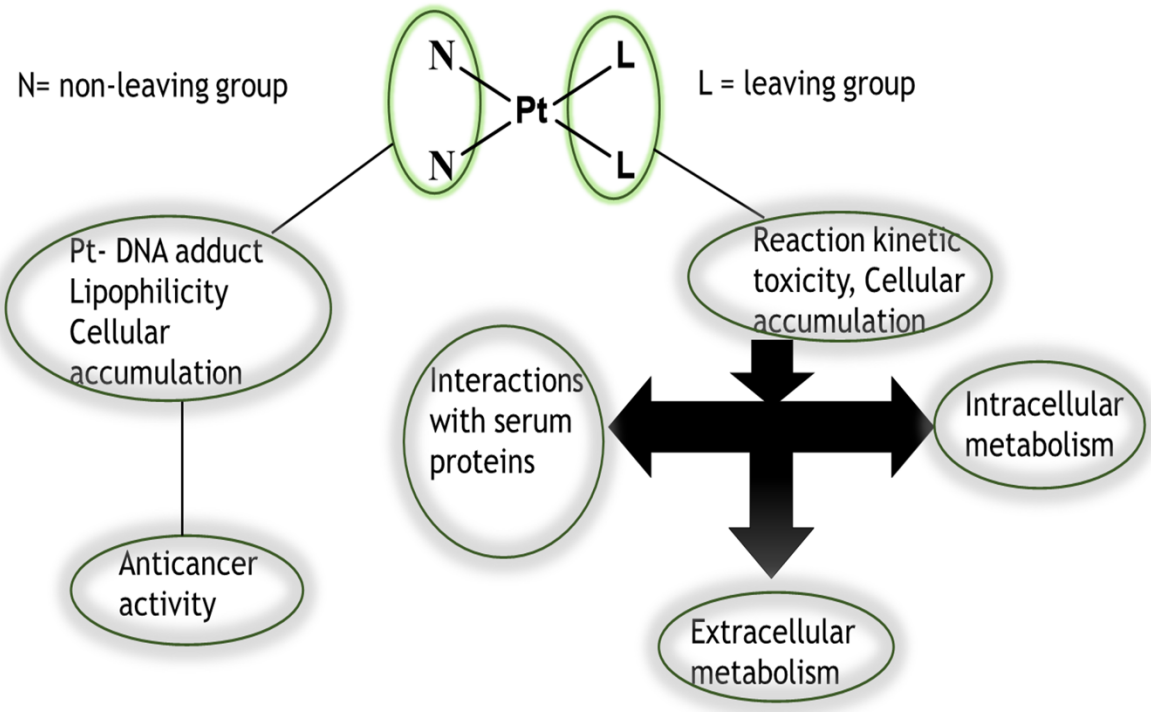


Figure 5: Structural features of platinum(II) anticancer agents

The accepted mechanism of action of platinum(II) involves activation by aquation to displace leaving groups using water and covalent binding to DNA to form DNA adducts. This has been achieved by exchanging the two chloride leaving groups in cisplatin with more stable chelating carboxylates (second generation platinum drugs) or the carrier ligands (NH_3) with bidentate amine ligands (third generation, oxaliplatin). Cisplatin transfers across cell membranes due to high chloride concentration. Intracellularly, the concentration of chloride ions is lower than in the plasma, and the chloride ligands on the cisplatin complex are displaced by water. The result is the formation of positively charged platinum complexes $[Pt(H_2O)Cl\ (NH_3)_2]^+$ and $[Pt(H_2O)_2(NH_3)_2]^{2+}$ that are toxic to cells.[39] The cis-$[Pt(NH_3)_2]^{2+}$

entity binds to the DNA molecule at the guanine bases, and thus inhibits DNA synthesis.[40] The drug forms intrastrand cross-links in the DNA molecule, and Pt-DNA adducts correlate with the cytotoxicity of the platinum(II) drug, which eventually leads to the cell's death.[40]

Platinum(II) drugs are administered to patients by intravenous infusion. Platinum anticancer complexes distribute more in cancer cells in the skin, liver, prostate, kidney and ovaries. The major route of platinum(II) elimination is renal excretion.

1.6. Mechanism of Action of Cisplatin

1.6.1. Cellular uptake

Despite the fact that identification of the antitumor activity of cisplatin was achieved half a century ago, there is no consensus regarding the mechanism of its intracellular uptake. Initial research indicated that cisplatin is internalised by traversing the cell membrane lipid bilayer by simple passive diffusion (**Fig. 6**). In recent years, mounting evidence has suggested that the accumulation of cisplatin can additionally be achieved via different membrane transporters, including through active transport using a copper transporter (Ctr1) [41,42] (**Fig. 6**).

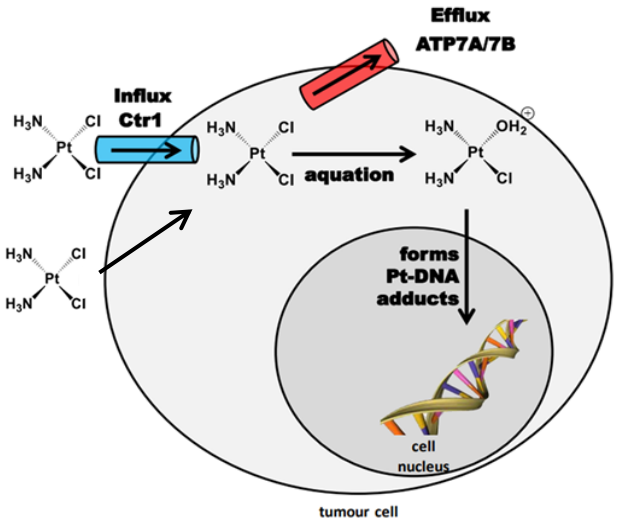


Figure 6: The uptake of cisplatin in the cells by passive diffusion and active uptake through the copper transporter (Ctr1) in order to form Pt-DNA. Image adapted from reference [41]

Cisplatin binds to the methionine-rich motifs of the extracellular domains of Ctr1, a transmembrane-bound transporter for monovalent copper, which is expressed ubiquitously. The downregulation of Ctr1 in yeast and fibroblast cells is correlated to a lower uptake of cisplatin. Correspondingly, increased expression of Ctr1 is linked with the efficacy of cisplatin impact on cancer cells, whereas a relationship has been found between Ctr1 mutations and cisplatin resistance.[43]

It has been proposed that a different group of transporters, ATP7A and ATP7B (which additionally have involvement in cisplatin efflux), act as mediators in the sub-cellular compartmentalisation of cisplatin.[44] It has been indicated that the organic cation transporter (OCT1/2) facilitates the uptake of oxaliplatin, which potentially reflects its natural organic characteristics as a result of its cyclohexane moiety.[44]

1.6.2. Cisplatin binds DNA

Upon entering the cell, cisplatin is hydrolysed due to sharply lowered chloride concentrations of between 2 – 30 mM in intracellular regions as compared to extracellular chloride concentrations 100 mM.[39] As a result, a very reactive positively charged $[Pt(H_2O)(Cl)(NH_3)_2]^+$ complex is formed, whose reactivity is due to H_2O is a good leaving group. [45,46] **(Fig. 7).**

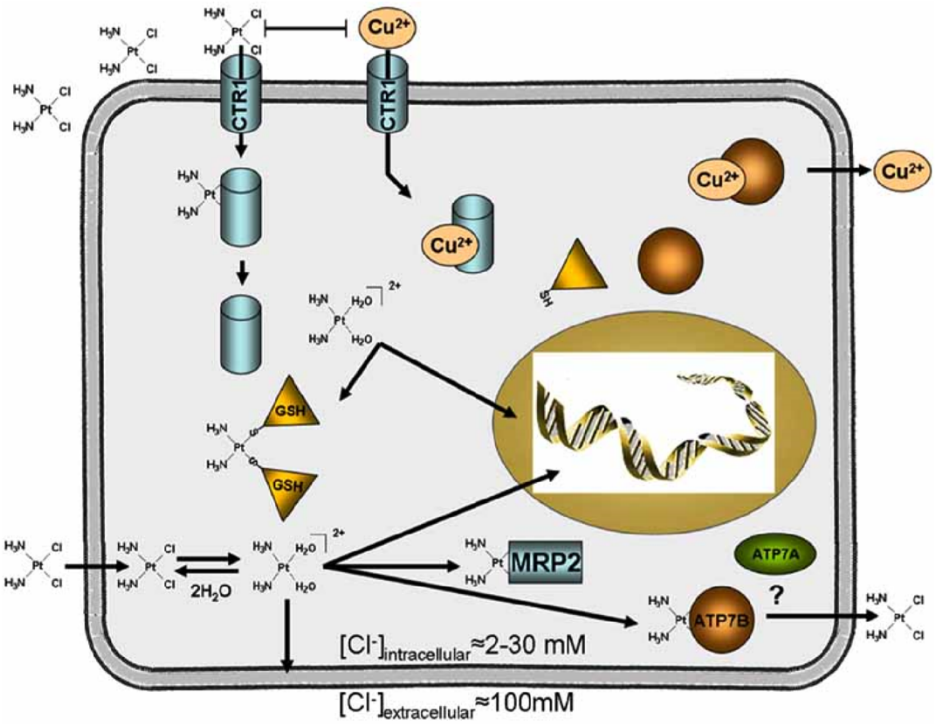


Figure 7: Mechanism of action of cisplatin entering the cells and binding to DNA. Image adapted from reference [39]

DNA platination predominantly happens via binding to the N7 atom of guanine or adenine base. Platinum(II) complexes that have a cis configuration mostly generate bifunctional intrastrand cross-links. The primary adducts observed for drugs based on platinum include 65% intrastrand G-G, 25% intrastrand A-G, 5-10% intrastrand G-N-G and 1-3% interstrand adduct formations.[47] (**Fig. 8**)

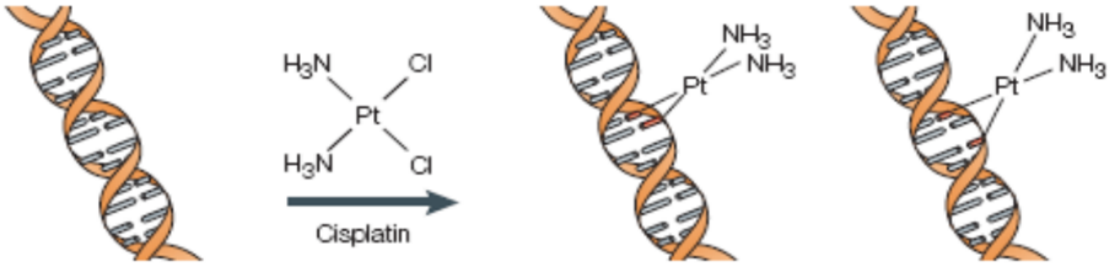


Figure 8: 1,2 intrastrand cross-linking and 1,3 intrastrand cross-linking. Image adapted from reference [46]

In addition to this, it has been demonstrated that distinct interstrand cross-link adducts are formed with DNA by cisplatin and oxaliplatin. These distinctions, resulting from the replacement of ammonia with the bidentate DACH ligand, partially explain why there is a broad range of activity of oxaliplatin and other platinum(II) drugs. It is generalised that DACH-Pt carrier ligand's structure has a considerable impact on the nature of the DNA interactions and the associated cytotoxic activity. DACH-Pt-DNA ligand is bulkier and more hydrophobic than cis-diammine-Pt-DNA which is effective to inhibit DNA synthesis.[30]

1.6.3. Cisplatin damages DNA

DNA integrity is a critical aspect of cellular homeostasis. Its management is achieved by a complicated molecular organisation involving the interaction between enzymes that react to different forms of DNA damage. DNA represents a core target in the mechanism of action of numerous different drugs.

The main cellular target of cisplatin is DNA, in a signalling process that starts with DNA damage and ends with cell death. [48] The identification of the DNA interaction of cisplatin has paved the way for investigating the utilisation of alternative coordination compounds based on transition metals for targeting DNA integrity and disrupting cellular survival (**Fig. 9**).

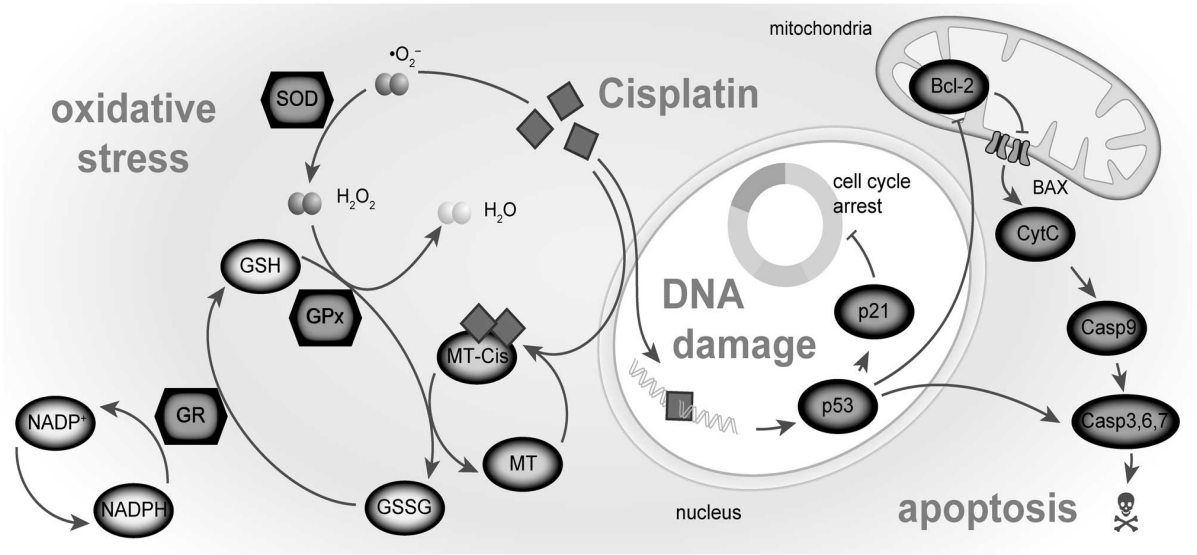


Figure 9: Cisplatin-induced DNA damage, oxidative stress and apoptosis. Image adapted from reference [48]

When DNA is damaged, the activation of cell cycle checkpoints occurs, thereby delaying cell cycle progression to repair or induce cell death. The cell's response to cisplatin toxicity and DNA damage can decide if it lives or dies. There is genetic evidence that cisplatin-induced DNA damage leads to the formation of double-strand breaks (DSBs) and gaps.[49] Studies have since shown that gaps and DSBs were the most likely substrates for recombination repair mechanisms.[50] Therefore, suggesting that cisplatin is an efficient inducer of recombination and the proposed molecular mechanism is achieved through the formation of DSBs. Thus,

Cisplatin is a DNA cross-linker that is known to be a highly efficient inducer of recombination.[51]

It has been demonstrated that platinum(II) complexes have the ability to bind and twist DNA. Due to the fact that this process causes the formation of double strand DNA damage, this leads to activation of the DNA repair machinery. As such the identification of DSB formation is a crucial measurement in terms of the efficacy of cisplatin and other DNA targeting therapeutics.

1.6.4. Cisplatin induces Reactive oxygen species

Reactive oxygen species (ROS) are categorised as a heterogeneous collection of molecules derived from diatomic oxygen from both free and non-free radical species. It has been determined that the functionality of numerous drugs based on metals involves the generation of ROS. Hence, the capacity of cisplatin to cause nuclear DNA damage in itself is not enough to provide an explanation as to why it is highly effective in causing cancer cell death. ROS is induced mainly in the mitochondria as well as via nicotinamide adenine dinucleotide phosphate (NADPH) oxidases.

The actions of antioxidant enzyme-catalysed reactions play a significant role in the existence of ROS. Through this process, antioxidant enzymes like catalase, glutathione, and superoxide dismutase counteract the physiological generation of ROS. The generation of ROS is an independent pathway from nuclear DNA damage induced by cisplatin and is realised in mitochondria due to loss of enzyme activity, inhibition of protein synthesis, and protein cross-linking DNA damage.[52]

There is a link between cisplatin toxicity and oxidative stress. The primary target for cisplatin to induce oxidative stress is via the mitochondrion. As result of this ROS stress, there is a reduction of mitochondrial membrane potential, a loss of mitochondrial proteins and a disruption in regular biological functioning. Cisplatin induces reactive oxygen species, leading to cell death, and DNA damage may also occur (**Fig. 10**). Numerous signalling pathways of cell death are activated, and the production of reactive oxygen species depends on the concentration of platinum(II) cisplatin and the length of exposure.[53]

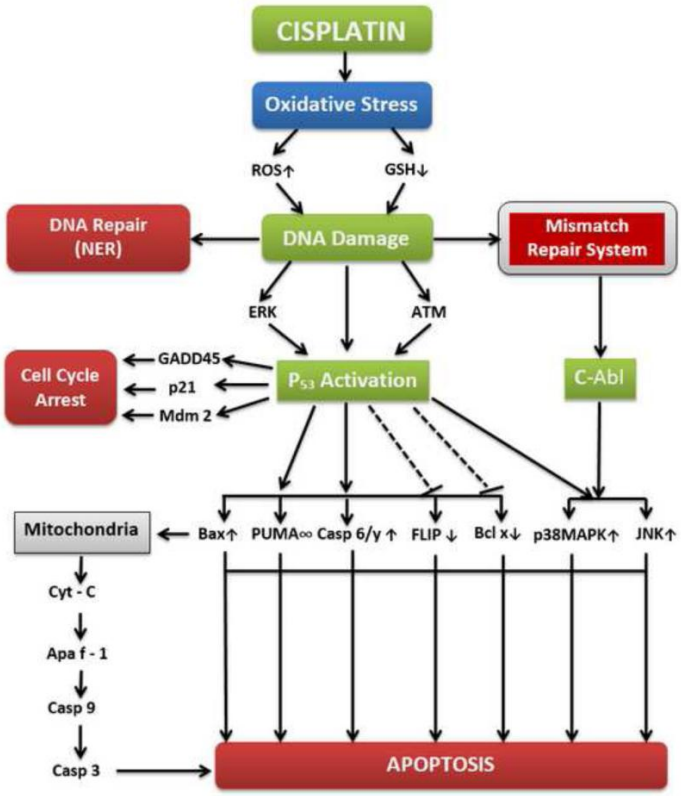


Figure 10: Several molecular mechanisms of cisplatin started with increase ROS production and leading to apoptosis. Image adapted from reference [24]

1.6.5. Cisplatin induces apoptosis

ROS play an essential role in cell signalling and regulation of apoptotic pathways either through the intrinsic or extrinsic pathway. The mechanism of apoptosis has various connections to pathogenic processes and has consequences for the actions of drugs and therapeutic reactions. Apoptosis is known programmed cell death process generally occurs via the extrinsic pathway involving death receptors or the intrinsic pathways involving the mitochondria. [54] (**Fig. 11**).

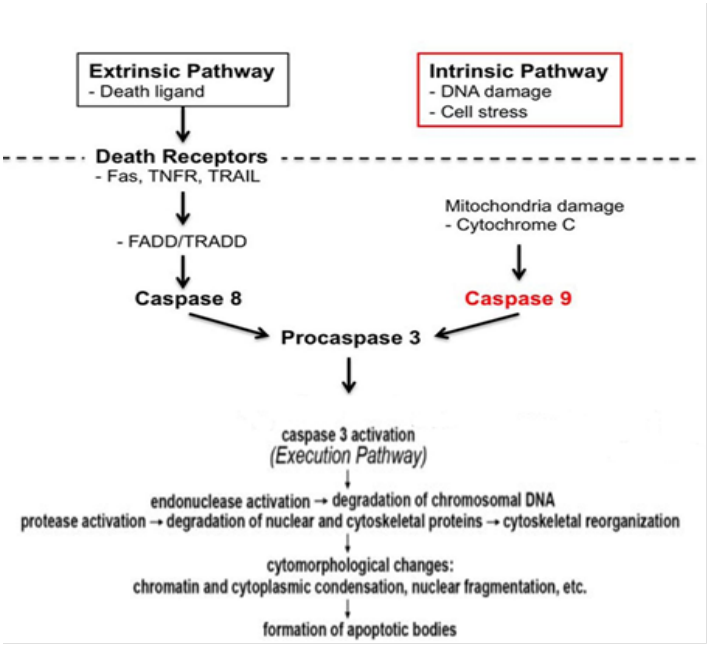


Figure 11: Intrinsic and extrinsic apoptosis pathways. Image adapted from reference [54]

These two pathways are the main medium through which cell death occurs within the cell. Hence, cisplatin reactivates these pathways, the formation of DNA adducts of cisplatin leads to the development of apoptosis mechanism as a trigger of death in cells, and this is the primary research objective in the field of drug development.

The extrinsic apoptosis pathway functions via activation of a cell surface receptor. Both the Tumour Necrosis Factor-1 (TNF-1) and Fas receptor have the ability to activate apoptosis via binding the ligands, and the resulting cleavage of pro-caspase 8 subsequently activates the effector caspases such as caspase 3 and 7, ultimately causing apoptosis. [24]

Numerous therapeutics based on metals as well as small molecules being evaluated have the capacity to generate ROS that eventually cause cell death and it is important that they are differentiated, particularly in regard to the treatment of cancer. The mitochondrial activity is a significant factor in the creation of oxidative radicals and plays a vital role in intrinsic apoptosis. ROS induction can activate pro-apoptotic Bax proteins and inhibit the functions of anti-apoptotic proteins. Hence, the cytotoxic effects of DNA-damaging agents or exposure to chemotherapeutic drugs can also trigger apoptosis, which is mediated by ROS as a result of impaired mitochondrial function.

ROS through their activity on Bax and Bcl2 cause mitochondrial membrane depolarisation followed by the release of cytochrome c into the cytosol. Cytochrome c plays an essential role in the formation of the apoptosome complex in the cytosol when it binds with Apaf-1. The apoptosome then interacts with procaspase-9, cleaving it to release the initiator caspase 9, and eventually resulting in caspase 9 activation of the effector caspases, caspase 3 and 7.

1.7. Limitations of platinum(II) anticancer agents

1.7.1. Toxicity

Some patients experience toxicities ranging from mild to severe due to cisplatin treatment. Some of these include ototoxicity, neurotoxicity and nephrotoxicity. Ototoxicity, which is defined as toxicity to an individual's hearing or ear, is reported for 75% to 100% of patients undergoing cisplatin treatment. Patients frequently complain of a loss of hearing at higher frequencies as well as nephrotoxicity. Cisplatin is predominantly cleared through the kidneys, where uptake is performed by the proximal tubule cells. When exposed to increased concentrations of cisplatin for extended periods, this can lead to kidney damage and ultimately, nephrotoxicity.[55] The main side effect of carboplatin is its myelosuppressive activity, while the primary side-effect of oxaliplatin is neurotoxicity.[56]

1.7.2. Resistance

Treatments involving cisplatin and other platinum drugs have reportedly been highly successful and cisplatin treatment has shown increased levels of effectiveness against testicular germ cell cancer. For example, a remission rate of higher than 90% has been recorded.[57] However, numerous studies have demonstrated resistance of cancer cells to cisplatin and that there are variety of different resistance mechanisms proposed.[58]

Levels of resistance to cisplatin treatments are significantly elevated among patients with ovarian, prostate, lung and colorectal cancers. The cellular resistance to cisplatin has a multi-factorial nature and result in severe limitation in clinical use (**Table 3)**:

Table 3: Drug resistance mechanisms

Multi-factorial nature of resistance mechanism	linked to…
DNA repair or removal of platinum adducts.	downregulating the mismatch repair (MMR) .[59] increased nucleotide excision repair (NER) .[60, 61]
reduced intracellular accumulation of cisplatin.[62]	overexpression of the copper efflux transporters ATP7A and ATP7B.[63] the lower expression level of the copper transporter 1 (CTR1) resulting in lower intracellular accumulation of cisplatin.[64]
inactivation of Pt complexes before they react with DNA via thiol including molecules like glutathione and metallothionein.[65]	more glutathione levels which can be as high as 10 mM inside resistant cells.
the lack of functional p53 to enhance apoptosis pathways.[58]	mutated p53 tumour suppressor gene as well as the overexpression in anti-apoptotic BCL-2 .[66 67]

Platinum-based drugs exhibit specific vulnerability to irreversible sequestration through macromolecular plasma proteins like albumin, which causes the reduction of the concentration affecting the therapeutic efficacy of the drug and has additionally been connected to various serious treatment side effects. This may have led to an increase in

cisplatin dosage for specific regimens creating selective pressure on cancer cells further driving the development of resistance to the drug. Furthermore, it is widely acknowledged that the lack of success of numerous chemotherapeutics including platinum agents is as a result of the inability, at the cellular level, to induce apoptosis (**Fig. 12**).

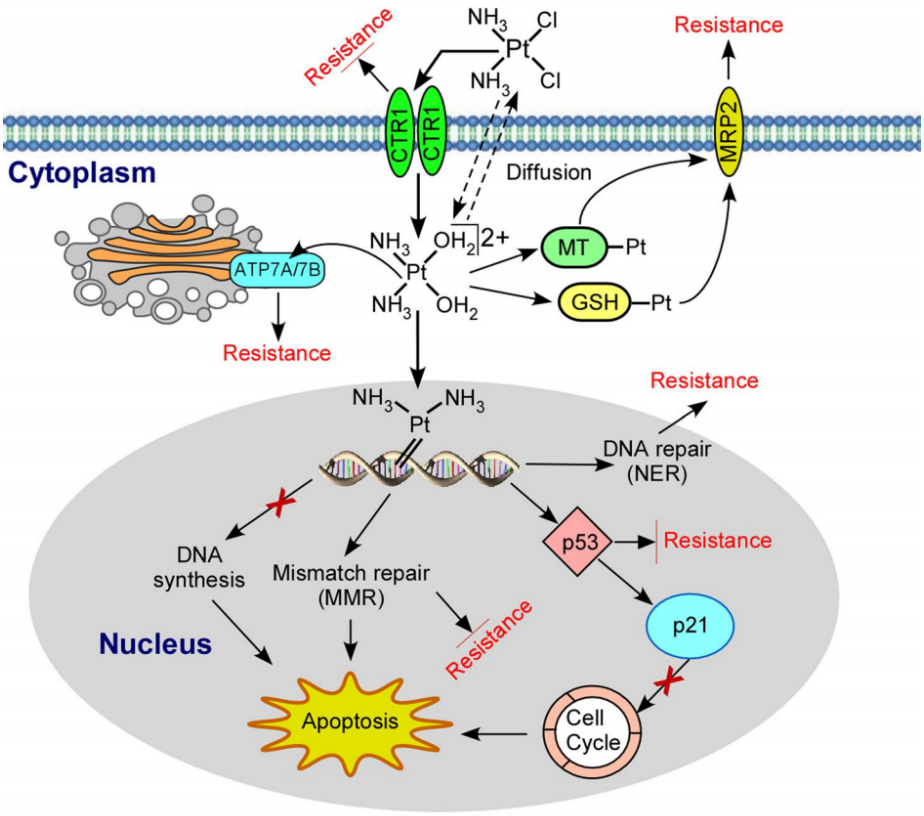


Figure 12: Resistance mechanisms. Image adapted from reference [68]

Significant attention has been focused on comprehending the mechanisms of natural resistance and developing platinum analogues to cisplatin that do not cause cross-resistance. In this regard, the emergence of different drugs based on metals presents the most feasible option for resolving the intrinsic deficiencies of platinum therapies.

1.8. Platinum(IV) drugs as a strategy to overcome the side effects of platinum(II)

Until now, researchers have synthesised and evaluated more than 6,000 platinum compounds, motivated by the necessity to find alternatives to the current platinum(II)-based drugs, which have inherent shortcomings. Unfortunately, less than 30 possible candidates have reached the clinical trial stage, and since the FDA approved carboplatin in 1986, only oxaliplatin subsequently received approval in 2002.

Although the majority of such compounds based on platinum have exhibited significant potential *in vitro,* in clinical terms, the outcomes have been similar to previous drugs, and they failed to offer specific clinical benefits in comparison to cisplatin. It is less widely recognised that the initial compound that was identified to trigger filamentous growth of *E. coli* was in fact a platinum(IV) complex, specifically ammonium hexachloroplatinate $(NH_4)_2PtCl_6$, which was generated from Pt electrodes via electrolysis.[69]

It has been determined that cell division *in vitro* was inhibited by cisplatin and platinum(IV) $[Pt(NH_3)_2Cl_4]$ subsequently, testing was conducted *in vivo*, which revealed that although both complexes were discovered to be active, the cytotoxic activity of cisplatin was higher.[70-72]

Platinum(IV) prodrugs represent an appealing option in comparison to their platinum(II) congeners in terms of mitigating the previously mentioned shortcomings of platinum(II) anticancer complexes.

Platinum(IV) anticancer prodrugs have the octahedral $[Pt(N_2)(L_2)(A_2)]$ motif, in which L and N denote the leaving groups and equatorial ammine ligands, respectively, whereas A ligands are the axial ligands (**Fig. 13**)

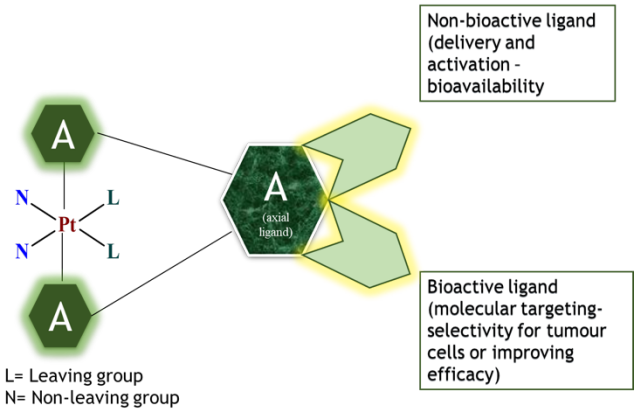


Figure 13: General structure of platinum(IV) octahedral geometry prodrugs

A particularly interesting action of the platinum(IV) structure is that the release of these axial ligands occurs simultaneously with the two-electron-reduction of the platinum(IV) prodrug to its active platinum(II) congener, which offers the potential to conjugate targeting groups or bioactive co-drugs, thus enabling the synergistic enhancement of the cytotoxicity on cancer cells (**Fig. 14**).

Evidence has shown that platinum(IV) complexes are capable of slowly binding to DNA *in vitro*.[22, 73, 74] There is a general consensus that platinum(IV) complexes are prodrugs that should be activated by reducing them to platinum(II) agents due to the fact that platinum(IV) complexes have minimal and slow reactivity.

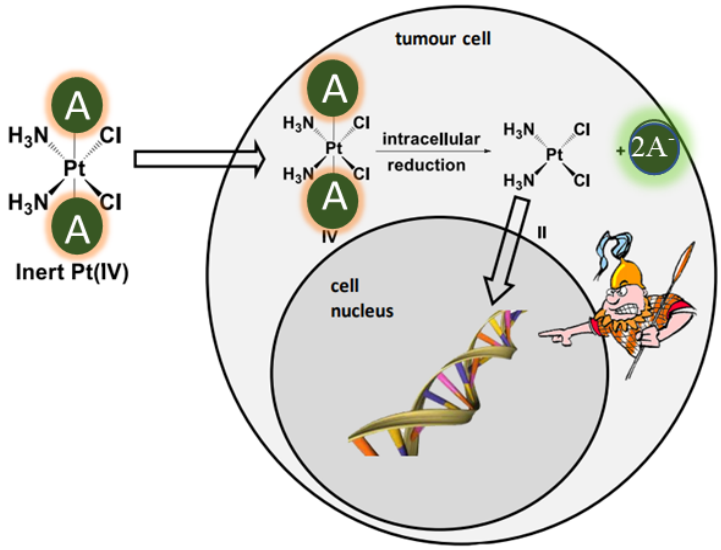


Figure 14: Platinum(IV) prodrugs reduce and release the active platinum(II) core as well as both axial ligands. Image adapted from reference [41]

It is essential to understand that the kinetic inertness of platinum(IV) complexes is due to the low spin d^6 configuration, which diminishes undesired side reactions before arriving at the desired target site, hence lowering toxicity and related side effects. Dissimilar to their square-planar platinum(II) equivalents, platinum(IV) complexes assume octahedral geometries (**Table 4**).

Table 4: Comparison of platinum(II) and platinum(IV) prodrugs anticancer agents

Drugs	Platinum(II)	Platinum(IV)
Oxidation State	+2	+4
Electronic configuration	$[Xe]4f^{14}5d^{8}$	$[Xe]4f^{14}5d^{6}$
Geometry of the complexes	Square planar	Octahedral
Coordination number	4	6
Intracellular activation	By ligand-exchange	By reduction
Administration	Intravenous	Oral
Pharmacokinetics.	Low stability in the gastrointestinal tract.	Sufficient stability in GIT.
Type of drugs	Anticancer drug	Anticancer prodrug concept (Selective tumour accumulation and/or selective activation in a tumour)
Side Effects	Severe toxicity to all healthy organs and cells. Acquired resistance	Fewer side effects due to lower reactivity
Cellular Accumulation	Less	Can be enhanced by receptor-specific axial ligands
Pharmacological properties	Low solubility, lipophilicity and cellular accumulation difficulty	Adequate solubility, optimal lipophilicity (log Po/w 0.5-3.5)
Inside cells	More reactive	Stable, more resistant to substitution
Reduction potential	Reversible	Irreversible
approved by FDA or other agencies and clinical entry examples for anticancer drugs	Cisplatin, carboplatin and oxaliplatin. Nedaplatin, lobaplatin and heptaplatin in Japan, China and Korea, respectively.	Tetraplatin, iproplatin and satraplatin failed in clinical trials in phase II- III.

1.8.1. Examples of common platinum(IV) prodrugs

The three major platinum(IV) prodrugs that have entered the clinical trial stage are tetraplatin, iproplatin and satraplatin (**Fig. 15**). The concept behind the development of these platinum(IV) prodrugs is that when entering the reducing conditions within the tumour cells, activation of the non-toxic platinum(IV) prodrugs would occur via $2e^-$ reduction, thus releasing their cytotoxic square planar platinum(II) congeners and simultaneously losing their two axial ligands.

As a result of its increased toxicity, **Tetraplatin** (OC-6-22)-tetrachlorido-(cyclohexane-1R,2R-diamine) platinum(IV) has not advanced to the phase II trial stage[22], which can likely be attributed to the fact that it is rapidly reduced to its platinum(II) congener via biological reducing agents.

Iproplatin (OC-6–33)-bis(hydroxido)dichlorido-diisopropyl-amine) platinum(IV) has greater water solubility than cisplatin and carboplatin. However, the activity of iproplatin was not better than cisplatin or carboplatin.[33]

A platinum(IV) prodrug that is orally active, **satraplatin** ((OC-6-43)-bis(acetato)ammine-dichloridocyclohexylamine) platinum(IV) has also undergone the I-III clinical trial stages as a single agent or combined with different drugs. It functions in combination with prednisone as an immunosuppressant[75] and also with bevacizumab as an angiogenesis inhibitor. It has been demonstrated that satraplatin delays the progression of the disease, but no benefits were seen in terms of overall survival.[76]

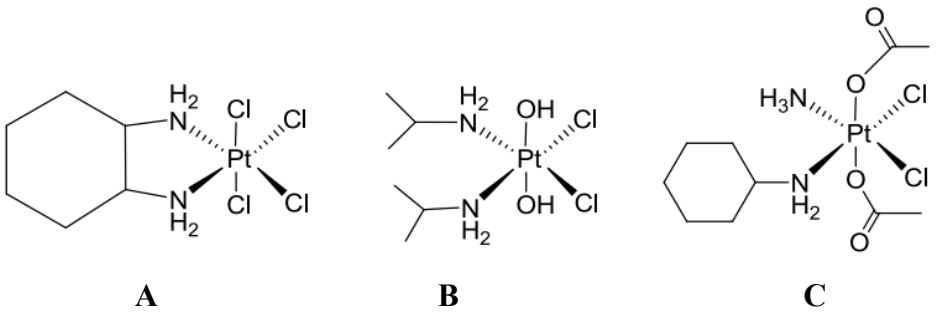


Figure 15: Three significant platinum(IV) prodrugs that have entered the clinical trial stage (A) Tetraplatin, (B) Iproplatin and (C) Satraplatin.

1.8.2. Activation and reactivity of platinum(IV) prodrugs

Various experimental and theoretical researchers have revealed that certain platinum(IV) complexes exhibit less inertness to ligand exchange reactions than anticipated under biologically relevant conditions and thus hydrolysis can occur.[77] This is dependent on the type of substituents surrounding the platinum(IV) core, i.e. derivatives carrying *fac*-trichlorido ligands [78]or axial halocarboxylato substituents.[79, 80]

It is expected that platinum(IV) complexes behave as prodrugs; activation of the compounds is achieved via two-electron reduction to their active platinum(II) analogues in the reducing tumour environment (activation via reduction). The primary outcome of this reduction is normally the active platinum(II) complex, which remains the equatorial ligands, whereas the two axial substituents are released. In certain rare instances, reorganisations may occur that generate different platinum(II) homologues, although carrier groups are maintained.[80, 81]

It appears to be evident that a connection must exist between the reduction potential and biological activity. It was demonstrated that the reduction potential of basic platinum(IV) complexes is predominantly impacted by the properties of the axial ligands, as well as to a lesser degree by the equatorial ligands.[82, 83]

Nevertheless, the simplicity of reduction is not consistently advantageous; normally, it is possible to reduce tetraplatin easily in the blood prior to being taken up by the cell, thus reducing its level of effectiveness as a prodrug.[84, 85] Conversely when the reduction process is complex, this does not automatically hinder biological activity. For instance, while the majority of iproplatin was excreted without changes in the urine and plasma of patients who were administered this drug, certain active platinum(II) metabolites were discovered, implying that the reduction process can be realised in vivo.[86] Platinum(IV) prodrugs as anticancer agents are an attractive area in chemotherapy, the benefits of platinum(IV) outweigh some of their drawbacks.

1.8.3. Synthetic strategies to platinum(IV)

The most frequent synthetic routes to the preparation of platinum(IV) complexes are oxidation and carboxylation.[87]

The main and most common approach used for synthesising platinum(IV) agents is via coordination with the axial carboxylate ligand, which involves **oxidation** of platinum(II) complexes by chlorine or hydrogen peroxide in water to obtain symmetrical trans-dihydroxido or dichlorido platinum(IV) species (**Fig. 16**).

Additionally, the synthesis of monocarboxylated (hydroxido-carboxylato) complexes can be accomplished through oxidation in a large volume of carboxylic acid anhydride combined with a stoichiometric volume of hydrogen peroxide. This produces successful results due to the fact that the oxidation of platinum(II) via peroxide functions through a process in which the axial hydroxide ligand is formed from H_2O_2.[88]

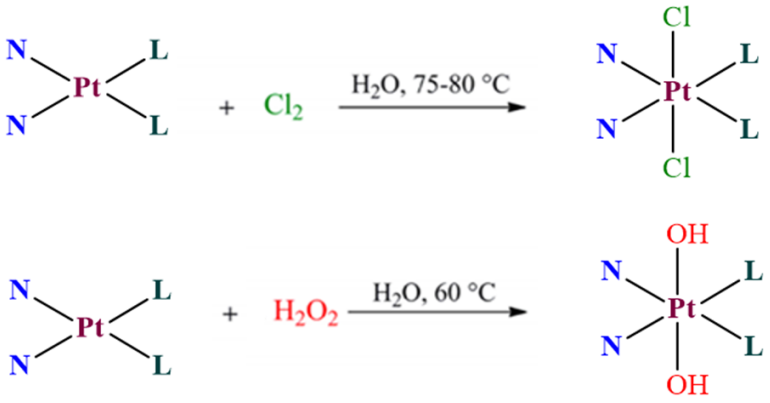


Figure 16: Oxidation of platinum(II) to platinum(IV) complexes by chlorine or hydrogen peroxide in the water.

It is then possible to derivatise these complexes further to produce a mixed dicarboxylate complex. Similarly, the facile synthesis of cis,cis,trans-$[Pt(N)_2(L)_2(OH)(OR)]$ alkoxyl platinum(IV) complexes could be accomplished through oxidation using hydrogen peroxide in alcohol (**Fig. 17),** which could subsequently be carboxylated to yield cis,cis,trans-$[Pt(N)_2(L)_2(OCOR)(OR)]$.[89]

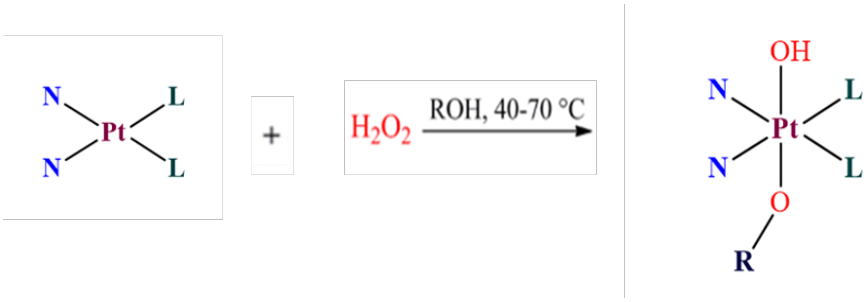


Figure 17: Oxidation of platinum(II) to platinum(IV) complexes by using hydrogen peroxide in alcohol.

It is then possible to react the nucleophilic hydroxido axial ligands within a general **carboxylation** scheme using a variety of electrophiles like anhydrides or acid chloride and isocyanates in a mild condition to produce dicarboxylate platinum(IV) complexes. The most frequent and synthetically available kind of platinum(IV) complexes are symmetrical axial dicarboxylates. Aside from basic anhydrides and acid chlorides, the process of carboxylation using anhydrides of dicarboxylic acids (succinic, glutaric, phthalic and maleic anhydrides) produces platinum(IV) carboxylates[90, 91] (**Fig. 18)**, which can be conjugated further through normal amide or ester coupling chemistry.

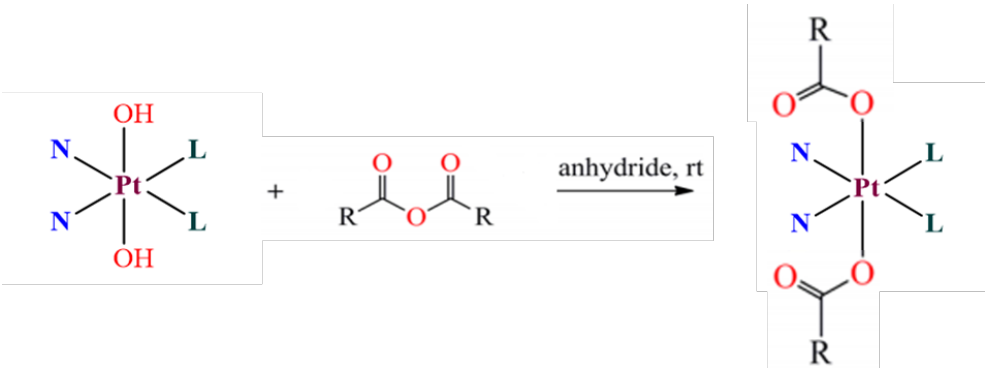


Figure 18: Synthesis of platinum(IV) carboxylates

Non-symmetrical or symmetric platinum(IV) derivatives can be synthesised through reactions N-hydroxysuccinimide (NHS) carboxylic acids or anhydride acids with oxoplatinum(IV) complexes (**Fig. 19**). It has been discovered that (NHS)-ester activated carboxylic acids with the coupling reagent dicyclohexylcarbodiimide (DCC) [87], can be utilised in significant excess in order to obtain symmetric platinum(IV) complexes and tend towards the generation of the monocarboxylate product.[92]

The assumption is made that the NHS-activated acid has insufficient reactivity to initiate a reaction with the second hydroxide group, which has lower nucleophilic properties in comparison with the first.[41] The second hydroxide group can be reacted with anhydride acids to obtain non-symmetrical platinum(IV) prodrugs (**Fig. 19**).

Complexes with an axial chloride or hydroxide trans to a carboxylate ligand are more water soluble and have higher reduction rates compared to their related dicarboxylated complexes. Conversely, the symmetric dicarboxylated complexes have two lipophilic axial ligands, and this can enhance the level of activity due to increased cellular uptake.

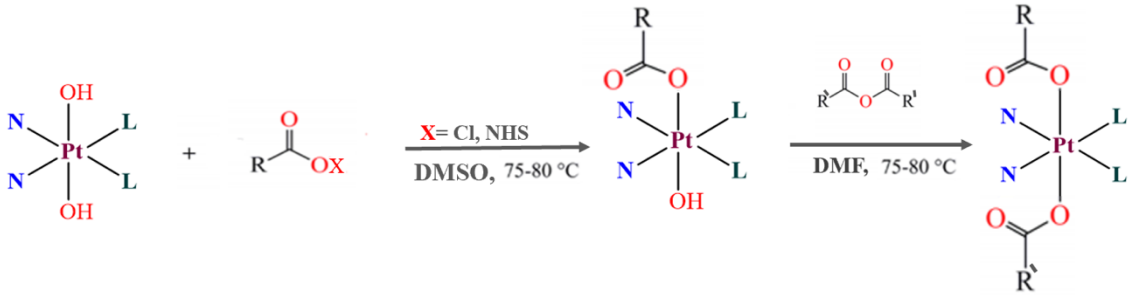


Figure 19: Synthesis of non-symmetrical of platinum(IV) complexes

Although platinum(IV) dihydroxido complexes are inert, the hydroxide oxygen atoms have strong nucleophilic properties and willingly attack applicable electrophiles, [93] thus enabling the formation of platinum(IV) complexes with varied axial carboxylate ligands.

The metal-ligand bonds of platinum(IV) complexes have kinetic inertness, a characteristic that researchers have taken advantage of in the chemical modification and the design of axial ligands.[82]

Furthermore, when two axial ligands are introduced into an octahedral geometry, this enables the pharmacological characteristics of the prodrug to be refined, including its specificity, activity and lipophilicity.

The ability to make structural changes to the six ligands surrounding the metal core means there is significant potential to create a number of different platinum(IV) prodrugs. The selection of the synthetic process is dependent on the intended lipophilicity and/or the ease at which the resulting complex can be reduced. It is possible to select the axial ligand to:

1. modify the electronic/lipophilic/kinetic characteristics.
2. create a linkage between the metal complex and a carrier to facilitate active or passive drug targeting in addition to strategies for delivery.

The axial ligands are predominantly responsible for modulating the lipophilicity (the capacity to enter tumour cells via passive diffusion) as well as the optimal redox attributes (the tendency for reduction in the hypoxic tumour, as opposed to the extracellular medium).

Quantitative structure-activity relationship (QSAR) models designed on the basis of in vitro data have indicated that both electronic and lipophilic attributes are essential for the determination of the complexes' activities.[94, 95] Although various researchers have focused on the reduction kinetics of platinum(IV) complexes (**Fig. 20**), the underlying mechanism remains only partially understood. A variety of distinct species can be acquired by reducing a platinum(IV) prodrug, based on both how the complex is configured and reducing agent selected, and this generates conflicting information.

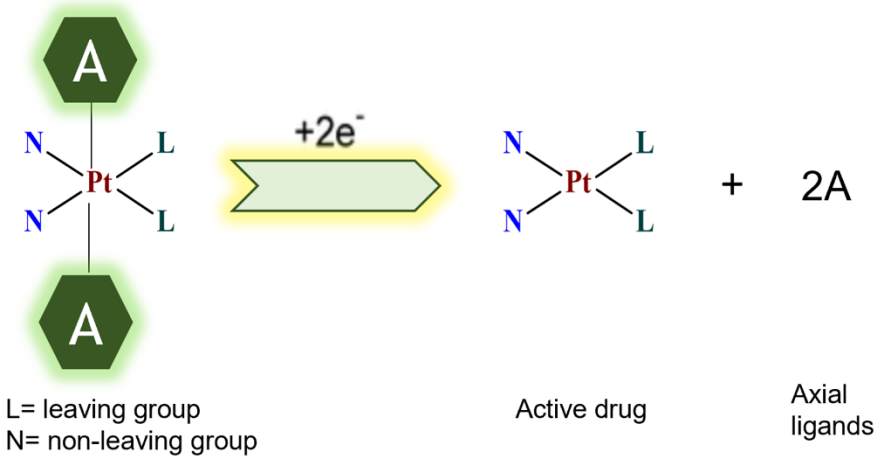


Figure 20: Reduction process of platinum(IV)

It is thought that glutathione (GSH) and ascorbic acid (AA) are the primary bio-reductants that have the capability to activate platinum(IV) prodrugs within cells, and have therefore been utilised as basic models for the purposes of in vitro reduction testing.[96, 97]

The reduction potential of cis,cis,trans-[Pt(ammine)$_2$L$_2$A$_2$] complexes is primarily dependent on the axial ligands and typically follows the order A = Cl > COOH > OH. It is found that there is no correlation between the reduction rate and the reduction potential of platinum(IV) complexes.

Chloride and hydroxide ligands can serve as bridging ligands in an inner-sphere mechanism facilitating the faster electron transfer between reducing agents and the metal complex. In contrast, platinum(IV) carboxylate complexes are generally reduced more slowly due to the poor ability of carboxylates to act as bridging group between ascorbic acid and Pt centre.[98, 99]

Numerous different reducing agents that exist within a cell could be responsible for activation of the platinum(IV) prodrugs while the rate at which the reduction occurs could be dependent on the reductive capabilities of each cell line. Specifically, intracellular high molecular mass biomolecules (e.g., cytochrome c in the presence of NADH) have the capability to reduce platinum(IV) complexes with axial carboxylate ligands in an efficient manner, even though this cannot be accomplished by ascorbic acid and/or glutathione.[100-102]

Various experimental techniques have been used to monitor reduction processes of platinum(IV) in order to investigate the reduction process (e.g., multinuclear NMR, HPLC and fluorescence).[78, 103-107]

While various clinical studies have been performed that have presented interesting findings, no platinum(IV) prodrug has reached clinical effectiveness. Researchers in this area have turned their attention to the targeted design of drugs and the action-refining of what were previously axial ligands to the introduction of designer ligands that offer extra desired multi-biological functionalities.

1.9. Dual-action platinum(IV) prodrugs

The majority of therapeutic treatments comprise at least two drugs which act synergistically or at least additively (combination therapy). An anticancer agent with various chemically-bonded and bioactive drugs is usually referred to as “combo” could represent a better strategy for chemotherapy drugs.[108]

The platinum(IV) derivatives are greatly appropriate for combination therapy as they delay the development of chemoresistance due to the fact that there are differences in terms of the mechanisms of action of the drugs used and they lower the impact of possible side effects.

The rational design of bifunctional platinum(IV) prodrugs that are conjugated with a single type of biologically active ligand include the release, subsequent to reduction, of the cytotoxic platinum(II) species as well as the ligands, thus enhancing the accumulation and the potency of the conjugate (**Fig. 21**).

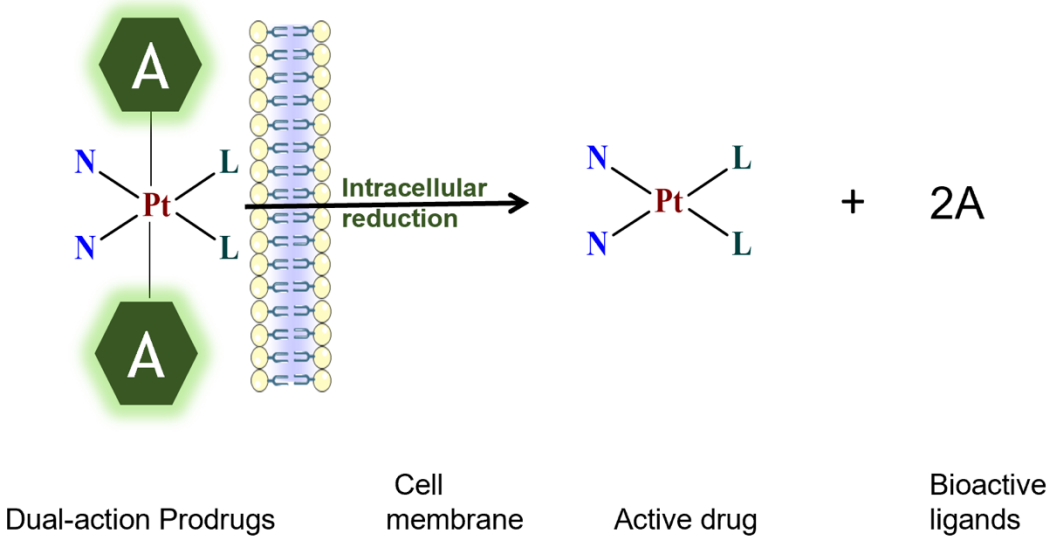


Figure 21: Reduction of dual-action platinum(IV) prodrugs.

Cisplatin itself could be regarded as a multi-targeted drug as it has been revealed by studies focused on the pharmacological activity that it has various molecular mechanisms of action, such as DNA binding and the ability to produce ROS.[78, 108, 109]

The platinum(IV) prodrugs may function as oxidising agents, with the ability to modify the cellular redox equilibrium, increase the reactive oxygen species (ROS) level and lower the potential of the mitochondrial membrane, consequently producing oxidative stress and facilitating the process through which cells induce apoptosis. Platinum(IV) prodrugs have been synthesised that contain efficient ROS-amplifying modulators, such as indole carboxylic acids.[110, 111]

It is generally reported that indole carboxylic acids can produce cytotoxic radicals on oxidation. Indole-3-acetic acid acts as non-toxic pro-drug that can form cytotoxic radicals in the presence of redox catalysts such as horseradish peroxidase.[112] As a result of this, cellular oxidative stress is produced. Therefore, IAA is used as oxidation therapy for cancer.

The equilibrium between production and removal of ROS is perturbed under several stressful conditions such as cancer pathogens, UV light and toxic metals and others. The enhanced level of ROS leads to damage to biomolecules such as DNA, proteins and lipids. This results in the loss of the mitochondrial membrane potential, loss of enzyme activity, inhibition of protein synthesis, protein cross-linking DNA damage, and eventually cell death.[113]

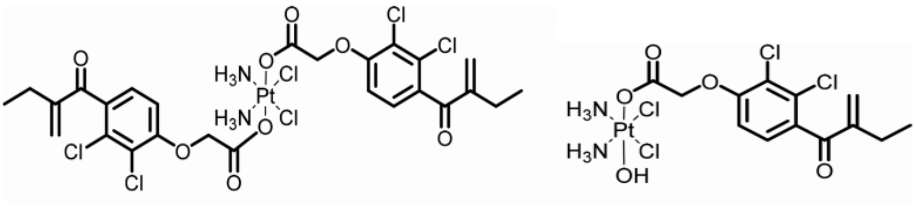
However, indole carboxylic acids as an example for redox chemotherapeutics also have antioxidant and radical scavenging properties. It is known that indole-3-propionic acid is a potent neuroprotective against oxidative toxic and protects against the oxidation of lipids and reduces the autoxidation and iron-induced lipid peroxidation in brain homogenates.[112]

Numerous examples of “dual action” compounds exist, making it impossible to consider or define them all; consequently, we have selected some examples for discussion to demonstrate the logic behind their design as well as to compare the experimental outcomes with the expectations. The most often-cited examples of platinum(IV) dual-action drugs are: Ethacraplatin, mitaplatin, chalcoplatin and asplatin/platin-A. Moreover, the platinum(IV) derivatives of cisplatin, carboplatin and oxaliplatin with ibuprofen, indomethacin, valproate or phenylbutyrate as axial ligands have been widely studied.

1.9.1 Ethacraplatin as platinum(IV) dual-action drug (GSH-S-Transferase-targeted)

Ethacraplatin, cis,trans,cis-$[Pt(NH_3)_2(EA)_2Cl_2]$ is an early attempt to design a platinum(IV) prodrug rationally with the purpose of overcoming resistance to cisplatin (**Fig. 22A**). It releases two equivalents of ethacrynic acid (EA) into the cell.

The resistance mechanisms of cisplatin are the intracellular deactivation of cisplatin by a thiol containing tri-peptide (glutathione- GSH) which exists in cells in mM concentrations and covalently binds to cisplatin, thereby hindering it from binding to the DNA and facilitating its excretion from the cell.[114] The binding of GSH to cisplatin is catalysed by glutathione-S-transferase (GST). Consequently, repressing GST may help to overcome this resistance mechanism.[115] During the testing for growth repression on a series of cisplatin-resistant breast MCF7 and T47D, lung A549 and colon HT29 carcinomas, it became evident that ethacraplatin had significantly lower IC50 values than did cisplatin.[115] Furthermore, the studies conducted by Dyson's group showed the ability of ethacraplatin to reverse cisplatin resistance in MCF-7 cancer cells.



A **B**

Figure 22: Chemical structures of two platinum(IV) complexes with bioactive ligands A ethacraplatin and B mono ethacraplatin.

In more recent times, Ang et al. prepared the monosubstituted platinum(IV)-EA (**Fig. 22B**) complex and discovered that it is more cytotoxic than ethacraplatin (**Fig. 22A**) although monoplatinum(IV) EA is a considerably weaker inhibitor of GST. Both mono-ethacrynate and diethacrynato complexes show better cytotoxic activity than cisplatin against the ovarian endometroid adenocarcinoma A2780 cell line and its cisplatin-resistant A2780cis[116] (**Fig. 22B**).

Another prominent resistance mechanism of platinum drugs is referred to as the nuclear excision repair (NER) system, which eliminates the platinum-DNA lesions formed by cisplatin.[117] Zhu et al. designed a platinum(IV) derivative of cisplatin with an inhibitor of the NER (**Fig. 23**) which blocks the interaction between the DNA excision repair proteins such as ERCC1 (excision repair cross-complementation group1) and XPF (xeroderma pigmentosum, complementation group F). Platinum(IV) complex with NERi targets the DNA repair in addition to platinating the nuclear DNA. It has been found that the platinum(IV) complex (**Fig. 23**) was more cytotoxic than cisplatin and that it also showed reduced DNA damage repair when compared with cisplatin.[118]

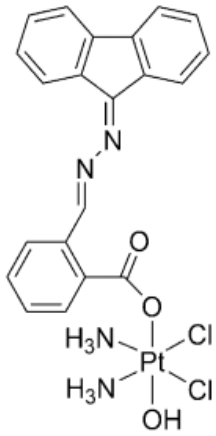


Figure 23: Chemical structure of a platinum(IV) complex targeting the nucleotide excision repair system.

1.9.2. Mitaplatin as platinum(IV) complex inhibiting pyruvate dehydrogenase kinase (PDK-targeted)

Lippard attached dichloroacetate (DCA) which is a pyruvate dehydrogenase kinase (PDK) inhibitor to cisplatin which forms mitaplatin cis,trans,cis-$[Pt(NH_3)_2(DCA)_2Cl_2]$ as "dual action" platinum(IV) complex (**Fig. 24**). Upon reduction in the cell, DCA targets the mitochondria and at the same time the released cisplatin binds to DNA.[119] A considerable amount of evidence is available from preclinical trials that confirms that DCA induces apoptosis selectively in cancer cells by reversing the Warburg effect.[120]

It has been frequently observed that cancer cells predominately produce energy by glycolysis (anaerobic respiration) instead of by oxidative phosphorylation (aerobic respiration) in mitochondria. This process disrupts apoptosis because the mitochondria initiate apoptosis by releasing proapoptotic elements like cytochrome C and apoptosis-inducing factor. DCA induces apoptosis selectively in cancer cells by inhibiting PDK, moving cellular metabolism from glycolysis to oxidative phosphorylation as well as reactivating the mitochondrial pathway of apoptosis. [121]

The cytotoxicity of mitaplatin was better or comparable to that of cisplatin in many sensitive and its cisplatin-resistant human cancer cells lines such as breast adenocarcinoma MCF-7, lung carcinomaA549, ovarian endometroid adenocarcinoma A2780 and A2780cis and osteosarcoma U-2 OS.[119]

It is noteworthy that not only did mitaplatin exhibit a high cytotoxicity, but it was also more selective towards cancer cells. In co-culture of cancer cells with normal fibroblasts, cisplatin and the mixture of cisplatin and DCA killed healthy fibroblasts as well as cancer cells. However, it is found that mitaplatin only killed cancer cells.[41]

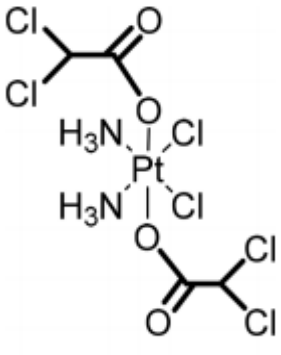


Figure 24: Chemical structure of mitaplatin.

1.9.3. Chalcoplatin as dual-action platinum(IV) prodrug (p53 activator targeted)

Chalcoplatin was obtained by the reaction of cis,cis,trans-[$Pt(NH_3)_2Cl_2(OH)_2$] with the acyl chloride form of chalcon[122] (**Fig. 25**). Moreover, it was accepted that promoting p53 activation could be used as a strategy in order to increase cisplatin's anticancer activity. The protein itself is now an emanating and attractive drug target because of its tumour-suppressor function. Chalcone inhibits the MDM2-p53 interaction, where MDM2 interacts and degrades p53, inhibiting p53 from sending apoptotic signal to downstream effectors.[123] It has been discovered that repressing the interaction between p53 and its negative regulator, human murine double minute 2 (P53-MDM2), showed a significantly greater cytotoxicity in p53 wild-type, than in p53 null cells, showing a synergistic effect of conjugating chalcone to cisplatin, and also that the compound was less active in normal cells.[122]

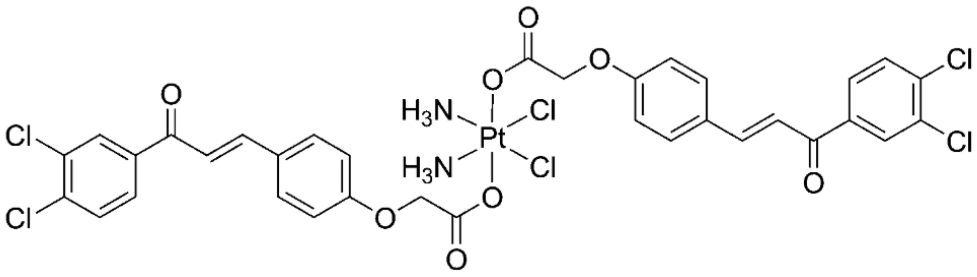


Figure 25 : Chemical structure of chalcoplatin

Such outcomes implied that, in comparison with cisplatin, chalcoplatin has a distinct mechanism of action that can kill cancer cells. The unique effect of chalcoplatin on cell cycle arrest and the p53 expression outcome distinctly demonstrated that the chalcone moiety has a major function in the cytotoxicity of chalcoplatin, not only by facilitating the cell entrance.[122] However, this study emphasises the benefits of conjugating cisplatin with a p53 activator as one anticancer agent and prepares for further development of chalcoplatin as a more active and cancer-cell specific anticancer drug candidate that overcomes resistance in cancer cells.[122]

1.9.4. Asplatin as dual-action platinum(IV) prodrug (cyclooxygenase inhibitor targeted)

Upon activation, asplatin (or platin-A), cis,trans,cis-[Pt $(NH_3)_2$(aspirin)$(OH)Cl_2$], releases one equivalent of cisplatin and one of aspirin (**Fig. 26)**. This was more potent than cisplatin in MCF- 7, HepG2, A549, and A549R cancer cells.[124] Nevertheless, it did not show any specific advantage over cisplatin in cytotoxicity studies against PC3, DU145 and in LaNCap cancer cells.[125] It is known that one of the features of cancer is the tumour tissue developing inflammations. Hence, non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, ibuprofen, indomethacin, are potential anticancer agents. The majority of these NSAIDs supress cyclooxygenase-2 (COX-2) which converts arachidonic acid to prostaglandins causing inflammation.

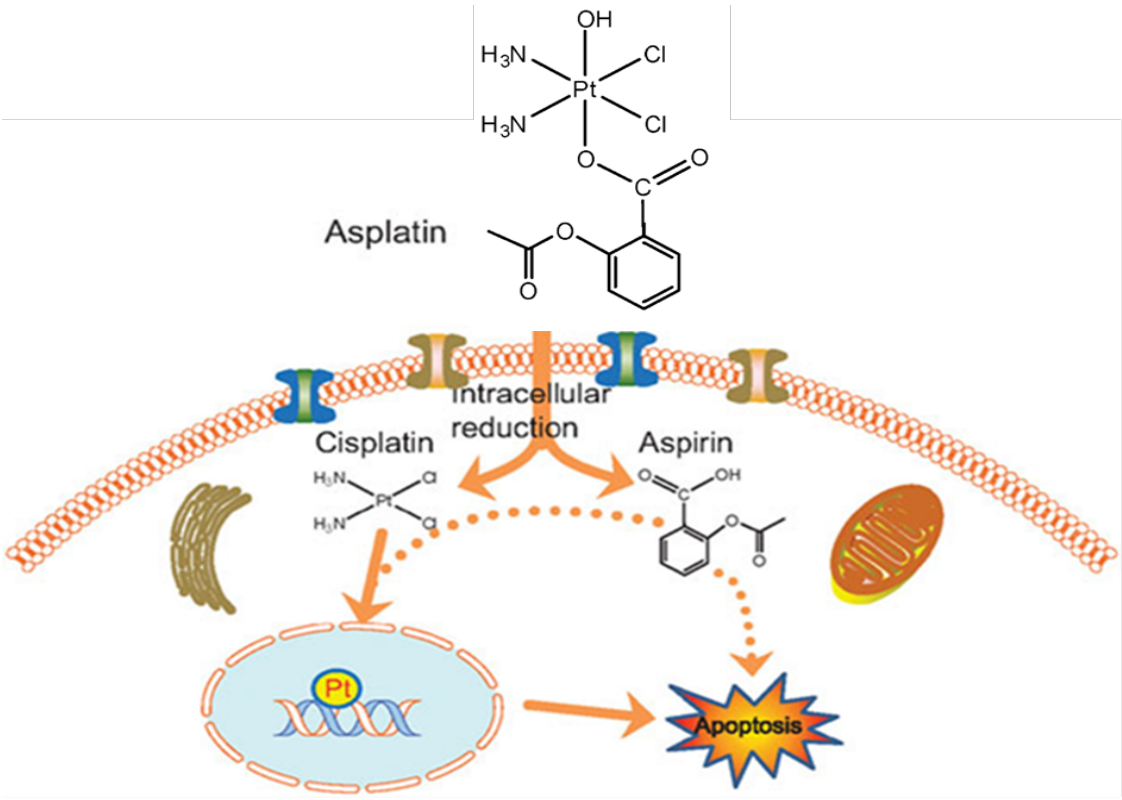


Figure 26: Mechanism of action of asplatin. Image adapted from reference [124]

Hey-Hawkins et al. studied the platinum(IV) derivatives of cisplatin or oxaliplatin with COX inhibitors like ibuprofen or indomethacin (**Fig. 27**). Intriguingly, no association was found between the ability to inhibit COX-1 or COX-2 and the cytotoxicity.[126] The ibuprofen derivatives are considerably more cytotoxic than the indomethacin derivatives against HCT-116 and MDA-MB-231 cancer cell lines although they are considerably weaker inhibitors of COX-1 and COX-2 in comparison with their indomethacin analogues. It is intriguing that the cisplatin derivative with ibuprofen was almost five times more potent than its oxaliplatin analogue.[126-128]

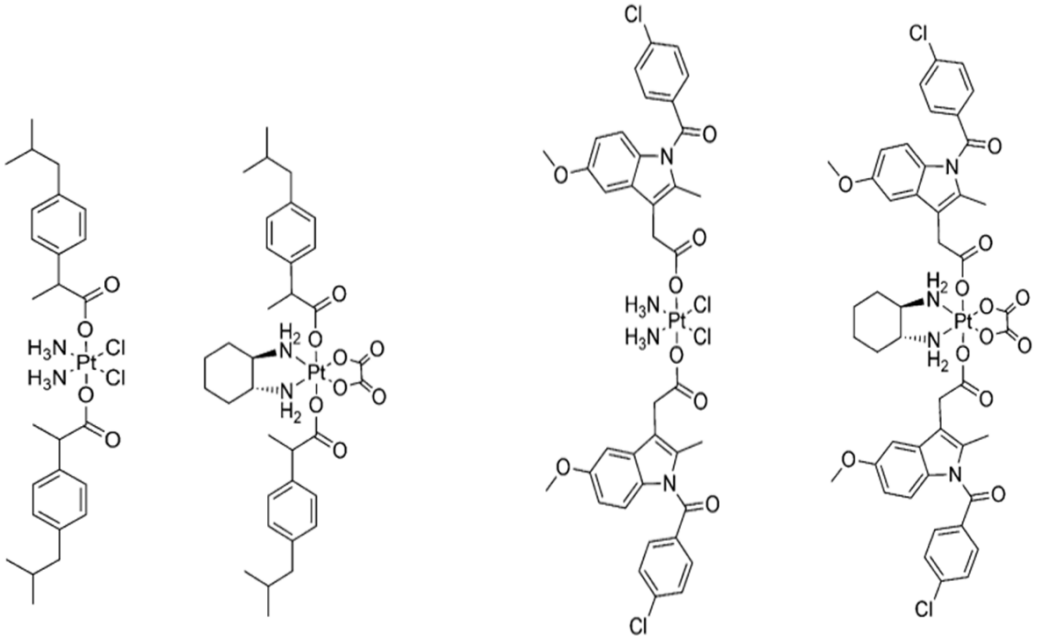


Figure 27: Chemical structures of platinum(IV) complexes A (ibuprofen attached to the cisplatin scaffold), B (ibuprofen attached to the oxaliplatin scaffold), C (indomethacin attached to the cisplatin scaffold) and D (indomethacin attached to the oxaliplatin scaffold)[126]

A targeted dual-action platinum(IV) prodrug was prepared in which the platinum(IV) derivative of cisplatin had an axial indomethacin ligand as well as a targeting biotin ligand [129] (**Fig. 28**). Despite the fact that the compound repressed the activity of both COX-1 and COX-2, its IC_{50} values against six cancer lines were particularly inferior to those of cisplatin. Moreover, this confirms the findings of Hey-Hawkins who demonstrated no correlation between cytotoxicity and COX inhibitor activity.[126, 129]

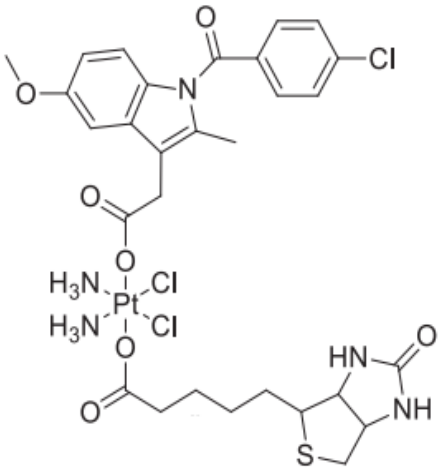


Figure 28: Chemical structures of biotin-platinum(IV)-indomethacin[129]

1.9.5. Platinum(IV) complexes with histone deacetylase inhibitors (epigenetic effects)

There is a close connection between development and progression of cancer and epigenetics.[130] Epigenetic mechanisms are capable of disabling tumour suppression genes or activate oncogenes in cancer cells which leads to the advancement of cancer progression. DNA methylation and histone modification are two of the principal epigenetic mechanisms.[131] DNA is wrapped around histones, and this action forms the nucleosomes which constitute chromatin. The positively charged amino acids (mostly lysines) of histones are bound electrostatically to the negatively charged DNA structure. Histone acetylation and histone deacetylation are regulated by two groups of enzymes, histone acetyl tranferases (HAT) and histone deacetylase (HDAC). The balance between these two procedures enables the reversible regulation of transcription and several other nuclear events.[132]

HDAC inhibition causes hyperacetylation of nucleosomal histones and other proteins, thereby leading to the expression of repressed genes and control of transcription factors which avert tight association with the nuclear DNA. Consequently, the DNA remains in an open form which facilitates transcription, but also causes it to be more susceptible to anticancer agents, produce cell growth arrest, terminal differentiation, cell death and/or inhibition of angiogenesis in neoplastic cells.[133]

It is recognised that healthy cells are resistant to HDAC-inhibitor (HDACi) induced cell death, which makes HDAC inhibitors attractive weapons in cancer therapy.[134] HDACi comprises two principal chemical families: hydroxamic acids and short/medium chain fatty acids (MCFA). Furthermore, benzamides, electrophilic ketones and macrocyclic peptides are able to occupy a function in the repression of such an enzyme.

Up to the present time, the FDA has approved four HDACi of the hydroxamic acid family namely, vorinostat and romidepsin to treat cutaneous T-cell lymphoma; belinostat and panobinostat against peripheral T-cell lymphoma and multiple myeloma, respectively.[135] (**Fig. 29**).

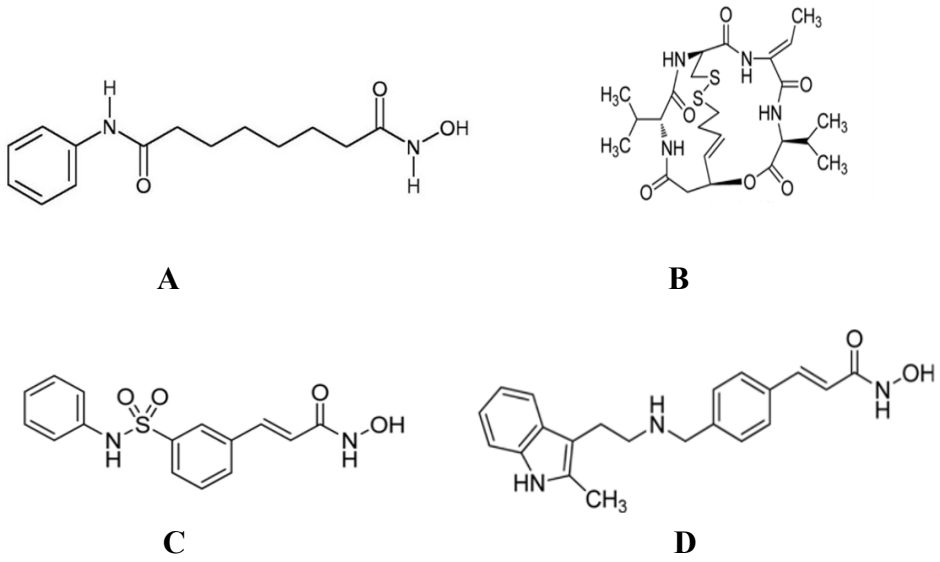


Figure 29: Chemical structures of A (vorinostat), B (romidepsin), C (belinostat) and D (panobinostat)**.**

HDAC inhibitors serve as epigenetic agents and play an important role in gene expression, can promote cellular differentiation and can induce apoptosis.[136] For example, vorinostat, belinostat and 4-phenylbutyric acid (PBA) were accepted as anticancer agents.
Contrastingly to vorinostat or belinostat whose IC_{50} values for cellular inhibition of HDAC activity are in the nanomolar range, the IC_{50} of valproate (VPA) and PBA were in the mM range making VPA and PBA exceptionally weak extracellular HDAC inhibitors.

The objective and logic for this combination of HDACi with anticancer agents are that hyperacetylation of lysines in nucleosomal histones establishes chromatin relaxation and subsequently increases the accessibility of nucleobases to the DNA-damaging agents. Therefore, the combination of HDACi with conventional cytotoxic drugs has attracted considerable attention with regard to cancer treatment.[137, 138]

Platinum(II) complexes with HDACi incorporate SAHA[139, 140], belinostat[141] and valproic acid[142]. Some of these bifunctional molecules display evident selectivity for tumour cells over non-tumorigenic cells but are not so potent as anticipated. It is certain that these platinum(II) conjugates have two disadvantages.

1) the HDACi must frequently be chemically modified in order to coordinate the platinum(II) metal center which may adjust the biological properties of the released moieties.[78]
2) the chelate impact in the resulting dicarboxylates slows down the kinetics of hydrolysis, and consequently the activation, as in carboplatin.[78]

Furthermore, numerous research groups applied various strategies; for example, platinum(IV) prodrugs of cisplatin, carboplatin and oxaliplatin with axial HDACis as prodrugs.

Shen et al. prepared the platinum(IV) derivative of cisplatin with two valproate ligands in the axial positions. They also contended that cis,trans,cis-[$Pt(NH_3)_2(VPA)_2Cl_2$] (**Fig. 30**) has a high level of activity as a result of the synergy of cisplatin and valproate metabolites and as a result of the HDAC inhibitory impact of the latter.[143,144] Gibson showed that VPA ligands influenced several other cellular procedures, interrupting enzymes such as GST.[145] This study generally deduced that the presence of the VPA axial ligands certainly results in an enhanced cytotoxicity profile, probably as a result of the added lipophilicity given to the complexes in the presence of the VPA ligands in addition to a reduction in HDAC expression instead of direct inhibition.[145] Osella et al. deduced that the observed outcome of VAAP complex is principally caused by the improved effectiveness of cisplatin as a result of its increased cellular accumulation as a result of increased passive diffusion of the more lipophilic platinum(IV) assembly.[144]

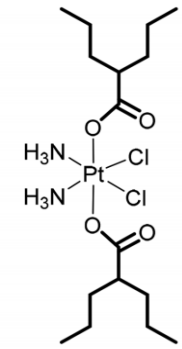
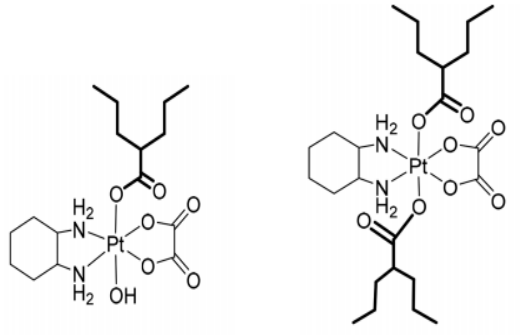


Figure 30: Chemical structures of platinum(IV) VAAP complex.

VPA units were added to one or both of the axial positions of a platinum(IV) oxaliplatin structure to afford the platinum(IV) complexes shown in (**Fig. 31 A** and **31 B**) and the activities were subsequently compared with that of the platinum(IV) oxaliplatin derivative bearing two axial hydroxido ligands. The oxaliplatin structure was used on the basis that oxaliplatin has enhanced cytotoxicity towards cisplatin-resistant cancers.[146]



A **B**

Figure 31: Chemical structures of platinum(IV) A (mono-VPA-oxaliplatin) and B (bis-VPA-oxaliplatin)

Previous studies on the topic of oxaliplatin in combination with the HDAC inhibitor trichostatin A showed an additive cytotoxicity impact towards gastric tumour cells.[146,147]
The findings revealed that the platinum(IV)-oxaliplatin derivatives with VPA as axial ligands had enhanced cytotoxicity across cisplatin-sensitive and cisplatin-resistant cell lines over their platinum(IV) analogues without any biologically active axial ligands.[147] Furthermore, it is stated that the platinum(IV)-VPA complexes led to a marked downregulation of HDAC, thereby causing a reduction in the level of HDAC in cells.[146,147] This was considered to be a powerful factor that has an impact on the enhanced cytotoxicity observed for these complexes.

PBA ligands and platinum(IV) prodrug containing PBA represent an excellent example of "drug repositioning", being a strategy that attracts greater attention in anticancer pharmacology; therefore, they have a potential in the clinic.[148] Consequently, the medium-chain fatty acids produced reasonable effective repression of HDAC, namely phenylbutyrate (PBA). Furthermore, cisplatin or oxaliplatin-based platinum(IV) complexes containing PBA, presented antiproliferative activity and have recently been reported in the literature and patented by Gibson.[78] It has been shown that cis,trans,cis-$[Pt(NH_3)_2(PBA)_2Cl_2]$ (**Fig. 32**) was noticeably more potent than cis,trans,cis-$[Pt(NH_3)_2(VPA)_2Cl_2]$ since it has sub-micromolar IC_{50} values against a panel of cancer cells.[148]

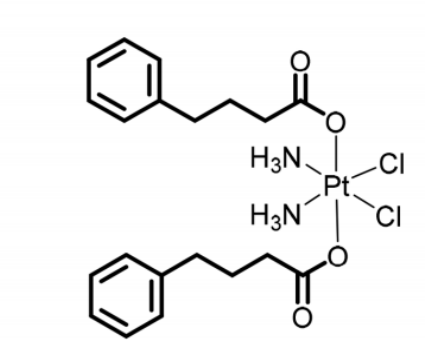


Figure 32: Chemical structures of platinum(IV)-PBA

1.9.6. Triple-action platinum(IV) prodrugs

PBA dual-action platinum(IV) and triple action platinum(IV) prodrugs with various bioactive axial ligands were all particularly potent against a panel of cancer cells from different sources.[149] This implies that attacking numerous intracellular targets simultaneously is an effective technique of killing the cancer cells without being dependent on a particular target or pathway and is a type of combination chemotherapy in one molecule.

Novel, triple-action cisplatin-based platinum(IV) complexes which contained various combinations of inhibitors of cyclooxygenase (aspirin or ibuprofen), histone deacetylase (VPA or PBA) or pyruvate dehydrogenase kinase (DCA) as axial ligands were prepared [149] (**Fig. 33**).

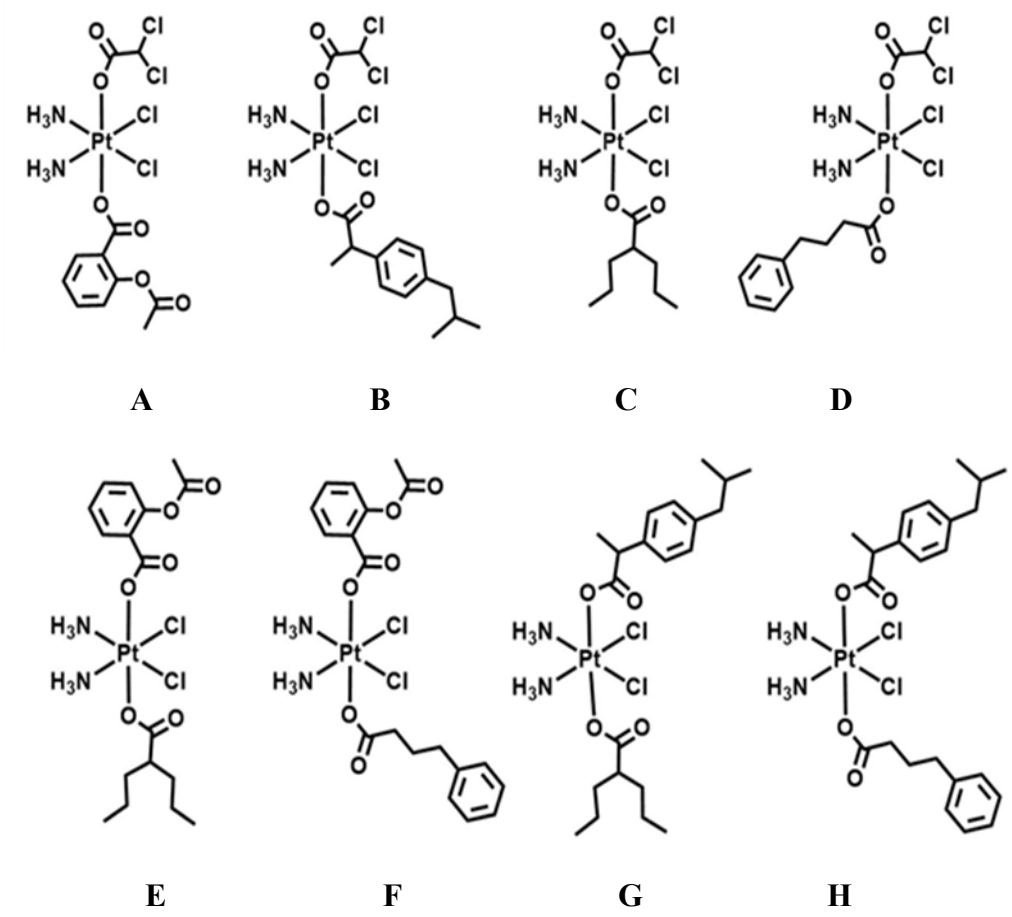


Figure 33: Chemical structures of multi-action platinum(IV) prodrugs based on cisplatin; A (DCA- Aspirin) complex, B (DCA- ibuprofen) complex, C (DCA-VPA) complex, D (DCA-PBA) complex, E (Aspirin-VPA) complex, F (Aspirin-PBA) complex, G (Ibuprofen-VPA) complex, H (Ibuprofen-PBA) complex. DCA is a DPK inhibitor, VPA or PBA are HDAC inhibitors and aspirin or ibuprofen are COX inhibitors attached to the cisplatin scaffold.[149]

These “triple-action” compounds are cis,trans,cis-[$Pt(NH_3)_2(COXi)(PDKi)Cl_2$], cis,trans,cis-[$Pt(NH_3)_2(COXi)$ $(HDACi)Cl_2$] and cis,trans,cis-[$Pt(NH_3)_2(HDACi)(PDKi)Cl_2$], where COXi = aspirin or ibuprofen, PDKi = dichloroacetate and HDACi = valproate or phenylbutyrate. It has been discovered that all complexes were noticeably more potent than cisplatin against a panel of human cancer cells. There is no connection between potency and cellular uptake, DNA platination or inhibition of HDAC, or COX or changes in mitochondrial parameters.

It is significant that all eight complexes, although they have axial ligands with very different biological properties, were noticeably more potent than cisplatin. Although it is assumed that each classification of compounds works by a combination of various mechanisms, and irrespective of the combination, they are particularly efficient.

It is interesting to note that platinum(IV) drugs with bioactive ligands are able to act on various targets as well as with multiple cellular mechanisms. This is specifically true for Gibson’s triple or tetra-functional platinum(IV) conjugates which contain many active components, often known as “anticancer cluster bombs”.[78, 149]

10.1. Objectives:

There are thousands of platinum(IV) agents, which have been synthesised and evaluated in order to overcome the drawbacks of existing clinically evaluated platinum(IV) complexes. The current platinum(IV) strategy focuses mainly on improving cytotoxicity, improving the accumulation and the potency of the complexes. The rational design of bifunctional platinum(IV) prodrugs conjugated with one or more type of biologically active ligand, upon activation by reduction, release the corresponding cytotoxic platinum(II) species and the ligand(s),

The main aim of this PhD project is to synthesise and study the biological and chemical properties of platinum(IV) complexes based on cisplatin and its analogues with HDACis and redox modulators ligands which are essential and relevant for their clinical application.

1-The apparent lack of studies on the functionalisation of platinum(IV) derivatives of carboplatin with bioactive ligands encouraged us to prepare five platinum(IV) complexes of type cis,cis,trans [Pt(CBDCA)$(NH_3)_2$(HDACi) L] with an HDAC inhibitory ligand based on the carboplatin scaffold, (**Fig. 34)**. The complexes have been studied in four cell lines with regard to:

- Cytotoxicity
- Cellular uptake
- HDAC inhibition
- Induction of apoptosis

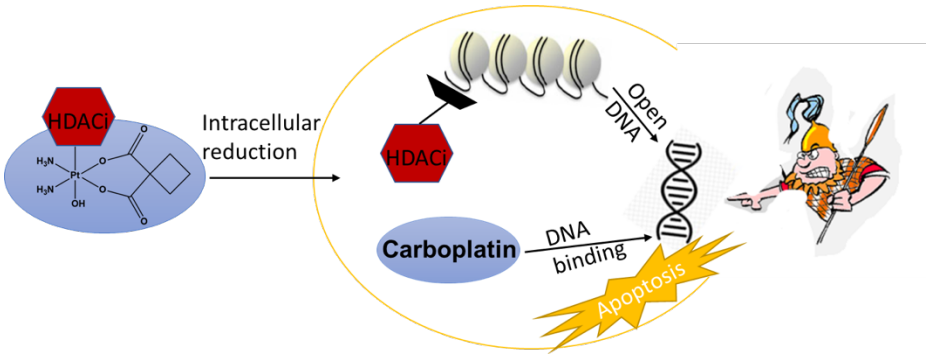


Figure 34: Mechanism of action of cis,cis,trans -[Pt(CBDCA)$(NH_3)_2$(HDACi)(OH)]

2- To gain more insight into the ability of platinum(IV) PBA pro-drugs to overcome cisplatin resistance, four complexes in this present study were compared with regard to the cellular responses of cisplatin-sensitive A2780 and cisplatin-resistant A2780cis cells (**Fig. 35)**. The cellular effects of platinum(IV) focus on:

- Induction of ROS
- DNA-platination and cellular uptake
- DNA damage (*in-vitro* γ-H2AX immunofluorescent detection)
- Induction of apoptosis
- HDAC inhibition

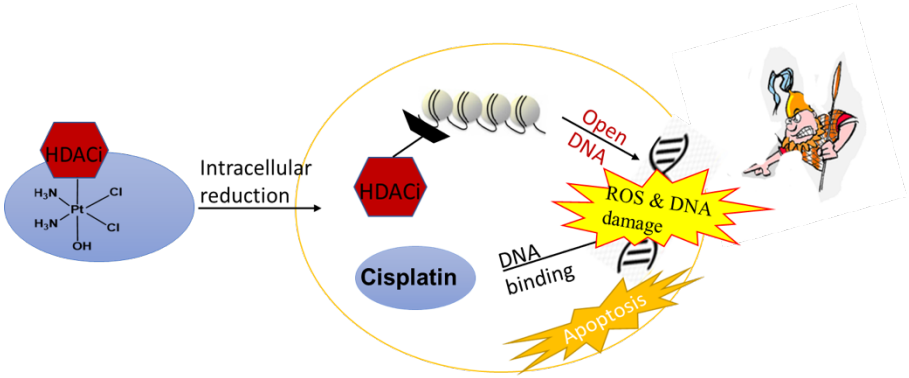


Figure 35: Mechanism of action of cis,cis,trans -[Pt $(NH_3)_2(Cl)_2$ (HDACi) (OH)]

3- Previous work prompted us to synthesise five oxaliplatin analogues of the cis,cis,trans [Pt(DACH)(ox)(IPA)(OH)] family, (**Fig. 36)**. The complexes have been investigated in cisplatin-sensitive and cisplatin-resistant ovarian cancer cells with regard to:

- Cytotoxicity
- ROS generation

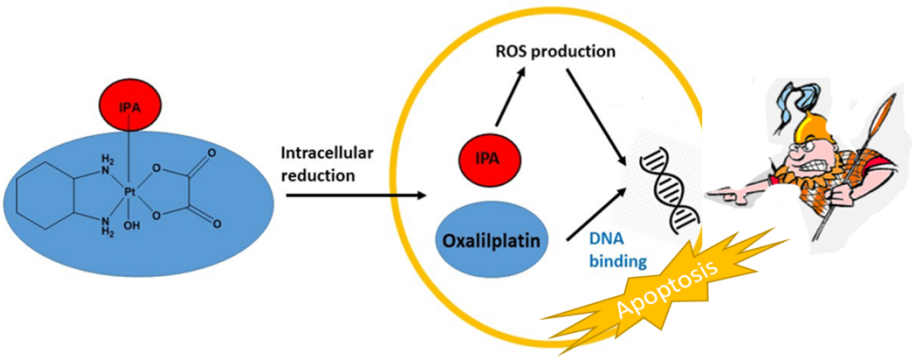


Figure 36: Mechanism of action of cis,cis,trans -[Pt(DACH)(ox)(IPA)(OH)]

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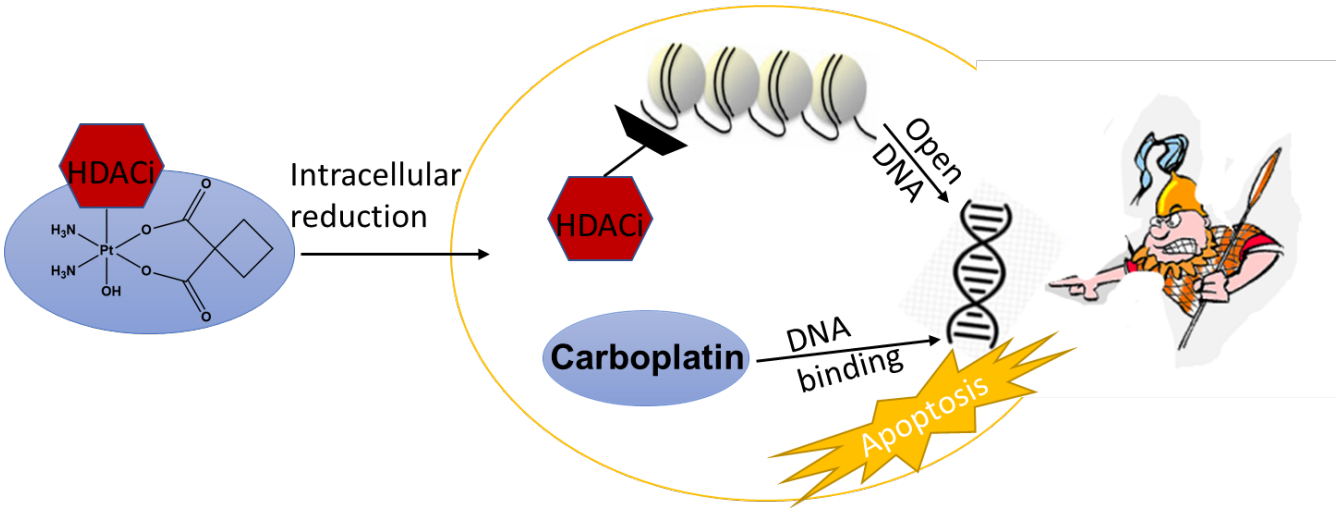
Results:

Chapter 2: Antitumor platinum(IV) derivatives of carboplatin and the histone deacetylase inhibitor 4-phenylbutyric acid



2. Brief description of the paper

Five novel platinum(IV) derivatives of carboplatin with a histone deacetylase inhibitor in axial position were synthesised. *cis*,*trans*-[Pt(CBDCA)$(NH_3)_2$(PBA)(bz)] was the most active complex and up to 10 times more cytotoxic than carboplatin. The complex showed HDACi activity as well.

Journal of Inorganic Biochemistry 177 (2017) 1–7

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

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Antitumor platinum(IV) derivatives of carboplatin and the histone deacetylase inhibitor 4-phenylbutyric acid

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ARTICLE INFO

Keywords:
Anticancer
Carboplatin
Histone deacetylase inhibitor
4-Phenylbutyric acid
Platinum(IV)

ABSTRACT

Five new platinum(IV) derivatives of carboplatin each incorporating the histone deacetylase inhibitor 4-phenylbutyrate in axial position were synthesized and characterized by ^{1}H and ^{195}Pt NMR spectroscopy, electrospray ionization mass spectrometry and elemental analysis, namely *cis*,*trans*-[Pt(CBDCA)$(NH_3)_2$(PBA)(OH)] (**1**), *cis*,*trans*-[Pt(CBDCA)$(NH_3)_2(PBA)_2$] (**2**), *cis*,*trans*-[Pt(CBDCA)$(NH_3)_2$(PBA)(bz)] (**3**), *cis*,*trans*-[Pt(CBDCA)$(NH_3)_2$(PBA)(suc)] (**4**) and *cis*,*trans*-[Pt(CBDCA)$(NH_3)_2$(PBA)(ac)] (**5**) (PBA = 4-phenylbutyrate, CBDCA = 1,1-cyclobutane dicarboxylate, bz = benzoate, suc = succinate and ac = acetate). The reduction behavior in the presence of ascorbic acid was studied by high performance liquid chromatography. The cytotoxicity against a panel of human tumor cell lines, histone deacetylase (HDAC) inhibitory activity, cellular accumulation and the ability to induce apoptosis were evaluated. The most effective complex, compound **3**, was found to be up to ten times more effective than carboplatin and to decrease cellular basal HDAC activity by approximately 18% in A431 human cervical cancer cells.

Authors	Contributions
Awatif Rashed Z .Almotairy	Synthesised and characterised five complexes
Valentina Gandin Cristina Marzano	Obtained all biological data and collected biological results; reviewed manuscript.
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Antitumor platinum(IV) derivatives of carboplatin and the histone deacetylase inhibitor 4-phenylbutyric acid

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Abstract

Five new platinum(IV) derivatives of carboplatin each incorporating the histone deacetylase inhibitor 4-phenylbutyrate in axial position were synthesized and characterized by ^{1}H and ^{195}Pt NMR spectroscopy, electrospray ionization mass spectrometry and elemental analysis, namely *cis,trans*-[Pt(CBDCA)$(NH_3)_2$(PBA)(OH)] (**1**), *cis,trans*-[Pt(CBDCA)$(NH_3)_2$$(PBA)_2$] (**2**), *cis,trans*-[Pt(CBDCA)$(NH_3)_2$(PBA)(bz)] (**3**), *cis,trans*-[Pt(CBDCA)$(NH_3)_2$(PBA)(suc)] (**4**) and *cis,trans*-[Pt(CBDCA)$(NH_3)_2$)(PBA)(ac)] (**5**) (PBA = 4-phenylbutyrate, CBDCA = 1,1-cyclobutane dicarboxylate, bz = benzoate, suc = succinate and ac = acetate). The reduction behavior in the presence of ascorbic acid was studied by high performance liquid chromatography. The cytotoxicity against a panel of human tumor cell lines, histone deacetylase (HDAC) inhibitory activity, cellular accumulation and the ability to induce apoptosis were evaluated. The most effective complex, compound **3**, was found to be up to ten times more effective than carboplatin and to decrease cellular basal HDAC activity by approximately 18 % in A431 human cervical cancer cells.

Keywords: Anticancer, Carboplatin, Histone deacetylase inhibitor, 4-Phenylbutyric acid, Platinum(IV)

Introduction

Platinum(IV) prodrugs are platinum(IV) complexes that on intracellular reduction release the axial ligands and the active square-planar platinum(II) species. Thus, conjugating a biologically active ligand to one or both axial positions of a platinum(IV) derivative of an anticancer platinum(II) drug is widely studied as a strategy for dual-action agents that target two distinct and/or independent cellular mechanisms [1]. Prominent examples are the platinum(IV) analogs of cisplatin ethacraplatin [2,3], chalcoplatin [4], aplatin [5,6] and mitaplatin [7] with the glutathione-S-transferase inhibitor ethacrynic acid, a chalcone derivative acting as a p53 inhibitor, the COX inhibitor acetylsalicylate and the apoptosis sensitizer dichloroacetate in axial position, respectively (Fig.1). Furthermore, phosphatase 2A inhibitors [8], the vitamin E analog α-tocopheryl succinate [9], carbohydrates [10,11], nucleotide excision repair inhibitors [12], alkylating agents [13] as well as redox modulators [14] have been attached to the cisplatin and oxaliplatin scaffold.

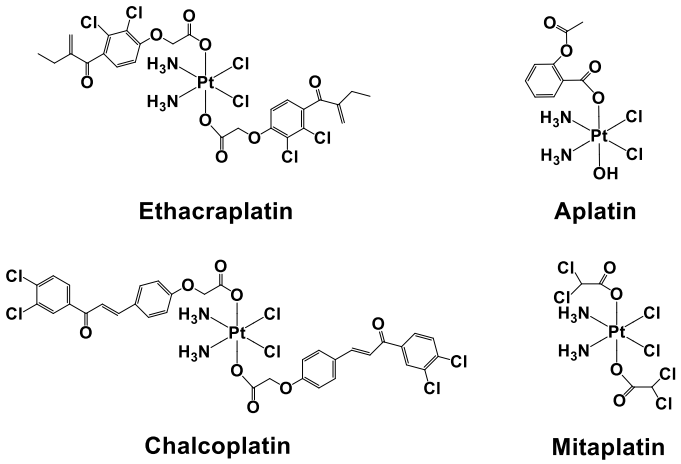


Figure 1: Examples for dual-action anticancer platinum(IV) complexes reported in the literature.

Carboplatin (*cis*-diammine-1,1-cyclobutanedicarboxylatoplatinum(II)), a second generation platinum(II) drug used in the treatment of ovarian cancer, retinoblastomas, neuroblastomas, nephroblastomas, brain, head and neck, cervical, testicular, breast, lung and bladder cancers, shows a more favorable toxicity profile compared to cisplatin and is generally better tolerated

[15]. The chelating dicarboxylate leaving group is hydrolyzed more slowly than the monodentate chlorido ligands in cisplatin, thus reducing off-target binding to biomolecules prior to DNA coordination. Platinum(IV) complexes based on carboplatin have been synthesized with various biologically inactive axial ligands and their pharmacokinetic properties and structure-activity relationship have been studied [16-20]. A platinum(IV) prodrug of carboplatin has also been modified with a polymeric nanodelivery carrier [21]. However, to the best of our knowledge, in contrast to dual-action prodrugs of cis- and oxaliplatin, platinum(IV) complexes with bioactive ligands attached to the carboplatin platform seem to have remained unexplored so far.

Histone deacetylase inhibitors (HDACi) are known to enhance the effect of DNA damaging agents [22]. Belinostat, for example, reverses platinum resistance in platinum-resistant lung cancer cells when co-administered with cisplatin [23] and Trichostatin A has an additive effect with oxaliplatin in gastric tumor cells [24]. Histone deacetylase (HDAC) regulates chromatin structure and transcription [25]. Its inhibition induces hyperacetylation of histone proteins around which DNA coils, making DNA more accessible within chromatin [26]. HDACi have also been shown to interact with non-histone proteins, including proteins that play a key role in cellular proliferation, migration, cell death, DNA repair, immune response, angiogenesis and inflammation [27]. Several HDACi are effective anticancer agents and have entered clinical trials [28]. They generally have few adverse effects on healthy cells.

Marmion and coworkers developed platinum(II) complexes with HDAC inhibitory ligands. They reported enhanced selectivity for cisplatin complexes of functionalized suberoylanilide hydroxamic acid [29,30] and belinostat [31] and enhanced cytotoxicity of transplatin complexes of valproate compared to cisplatin [32]. Brabec and coworkers studied platinum(IV) prodrugs of oxaliplatin with axial valproate ligands [33]. While displaying lower cytotoxicity than oxaliplatin in cell culture, the complexes showed enhanced accumulation in tumor cells and were active in cisplatin-resistant cells. The platinum(IV) complex *cis,cis,trans*-[$Pt(NH_3)_2Cl_2(valproate)_2$] was shown to exert significant cytotoxicity in a range of human carcinoma cell lines, including malignant pleural mesothelioma cells [34-36]. A larger fraction of total platinum taken up by cells was found to bind to DNA, when platinum(IV) complexes containing biologically active axial ligands were used compared to platinum(IV) complexes with biologically inactive axial ligands [34]. Kasparkova *et al*. recently reported a photoactivatable platinum(IV) complex with axial suberoylanilide hydroxamic acid ligands [37]. Gibson and coworkers attached the HDACi 4-phenylbutyric

acid (PBA) and valproate to the axial positions of platinum(IV) derivatives of cisplatin and oxaliplatin [38,39]. *Cis,trans,cis*-[$Pt(NH_3)_2(PBA)_2Cl_2$] was found to be up to 100-fold more potent than cisplatin [38]. Inhibition of HDAC activity by 60 – 70 % in cancer cells at concentrations lower than the IC_{50} value of PBA suggested a synergism between platinum and PBA.

The apparent lack of studies on the functionalization of platinum(IV) derivatives of carboplatin with bioactive ligands prompted us to prepare platinum(IV) complexes with HDAC inhibitory ligands based on the carboplatin scaffold. Here we report the synthesis, characterization, cytotoxicity and HDAC inhibitory activity of a series of platinum(IV) prodrugs of carboplatin with PBA as an axial ligand.

Experimental

Materials and methods

Reactions were performed under normal atmospheric conditions using as-received analytical or HPLC grade solvents and reagents from commercial suppliers. K_2PtCl_4 was purchased from Acros Organics, 4-phenylbutyric acid, acetic anhydride and succinic anhydride were obtained from Sigma-Aldrich and benzoic acid, *N*-hydroxysuccinimide and dicyclohexylcarbodiimide from TCI Europe. The ultra-pure water used was purified with a Milli-Q UV purification system. Carboplatin and oxo-carboplatin (*trans,cis*-[Pt(CBDCA)$(OH)_2(NH_3)_2$]) were synthesized according to the previously reported procedures [40].

Syntheses

NHS ester of 4-phenylbutyric acid

4-Phenylbutyric acid (2.0 g, 12.2 mmol) and *N*-hydroxysuccinimide (1.4 g, 12.2 mmol) were dissolved in tetrahydrofuran (20 mL), followed by dropwise addition of a solution of dicyclohexylcarbodiimide (2.5 g, 12.2 mmol) in tetrahydrofuran (12 mL). The mixture was stirred at room temperature for 7 h and then filtered to remove the dicyclohexylurea precipitate. The filtrate was concentrated under reduced pressure. The residue was dissolved in ethyl acetate (60 mL) and left at 4 °C overnight. After filtration to remove the insoluble residue the organic phase was washed with $NaHCO_3$ solution (6%, 120 mL) and water (60 mL) and dried over Na_2SO_4. Petroleum ether (60 mL) was then added to the ethyl acetate solution to precipitate the 4-PBA-NHS ester which was filtered off, washed with petroleum ether, dried, and purified by column chromatography (silica gel; 9 : 1 petroleum ether : ethyl acetate). Yield: 3.1 g (96 %); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.93 - 2.01 (m, 2H, $CH_2CH_2CH_2$), 2.58 (t, J = 7.5 Hz, 2H, $CH_2CH_2CH_2$), 2.73 (t, J = 7.4 Hz, 2H, $CH_2CH_2CH_2$), 2.80 (s, 4H, $COCH_2CH_2CO$), 7.20 - 7.29 (m, 5H, H_{ar}); ESI-MS: m/z = 261 $[M-H]^-$.

cis,trans-[Pt(CBDCA)$(NH_3)_2$(PBA)(OH)] (1)

The 4-PBA-NHS ester (87.2 mg, 0.34 mmol) was added to *cis,trans*-[Pt(CBDCA)$(NH_3)_2(OH)_2$] (150 mg, 0.37 mmol) in dimethyl sulfoxide (17 mL) and the mixture was stirred for 48 h at 65 °C. Unreacted *cis,trans*-[Pt(CBDCA)$(NH_3)_2(OH)_2$] was removed by filtration, the solvent was evaporated using a freeze-dryer and the residue was

dissolved in dimethylformamide (4 mL), followed by precipitation of the desired product with diethyl ether. The precipitate was isolated by centrifugation, washed several times with dichloromethane (2 mL) and diethyl ether (20 mL) and dried under vacuum. Yield: 144 mg (71%). Anal. Calcd (%) for $C_{16}H_{24}N_2O_7Pt$ (551.45): C, 34.84; H, 4.35; N, 5.08. Found: C, 34.63; H, 4.28; N, 5.09. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 1.6 - 1.7 (m, 4H, CCH_2CH_2, CBDCA and $CH_2CH_2CH_2$, PBA), 2.13 (t, J = 7.4 Hz, 2H, $CH_2CH_2CH_2$), 2.42 (t, J = 8.1 Hz, 2H, CCH_2CH_2, CBDCA), 2.56 (m, 4H, CCH_2CH_2, CBDCA, $CH_2CH_2CH_2$, PBA), 5.97 (m, br., 6H, NH_3), 7.13-7.25 (m, 5H, H_{ar}); ^{195}Pt NMR (107.6 MHz, DMF/D_2O): δ 1741 (quin, J_{PtN} = 322 Hz). ESI-MS: m/z = 550.45.0 $[M-H]^-$.

cis,trans-[Pt(CBDCA)(NH_3)$_2$(PBA)]·0.5H_2O (2)

The 4-PBA-NHS ester (1.546 g, 5.9 mmol) was added to *cis,trans*-[Pt(CBDCA)(NH_3)$_2$(OH)$_2$] (120 mg, 0.29 mmol) in dimethyl sulfoxide (20 mL). After stirring for 72 h at 80 °C the mixture was concentrated to a 2 mL volume and the desired product was precipitated by addition of water (20 mL). The solid was isolated by centrifugation, washed several times with dichloromethane (4 mL) and diethyl ether (40 mL) to remove excess ligand and dried under vacuum. Yield: 130 mg (64 %). Anal. Calcd (%) for $C_{26}H_{35}N_2O_{8.5}Pt$ (706.66): C, 44.19; H, 4.99; N, 3.96. Found: C, 44.18; H, 4.85; N, 4.01. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 1.6 - 1.7 (m, 2H, $CH_2CH_2CH_2$, PBA), 1.8 (m, 2H, CCH_2CH_2, CBDCA), 2.18 (t, J = 7.4 Hz, 2H, $CH_2CH_2CH_2$), 2.43 (t, J = 8.1 Hz, 2H, CCH_2CH_2, CBDCA), 2.50 (t, J = 7.4 Hz, 2H, $CH_2CH_2CH_2$), 2.64 (t, J = 8.0 Hz, 2H, CCH_2CH_2, CBDCA), 6.34 (m, br., 6H, NH_3), 7.19 - 7.29 (m, 10H, H_{ar}); ^{195}Pt{^{1}H} NMR (107.6 MHz, DMF/D_2O): δ 1929 (quin, J_{PtN} = 215 Hz). ESI-MS: m/z = 696.15 $[M-H]^-$.

cis,trans-[Pt(CBDCA)(NH_3)$_2$(PBA)(bz)] (3) and cis,trans-[Pt(CBDCA)(NH_3)$_2$(PBA)(suc)] (4)

The unsymmetrically substituted complexes **3** and **4** were synthesized by reaction of **1** with the respective carboxylic acid anhydride. Complex **1** and 10 equivalents benzoic or succinic acid anhydride were suspended in dimethylformamide and the mixture was stirred for 2 d at 80 °C. The solution was concentrated under reduced pressure and diethyl ether (40 mL) was added to precipitate the desired product which was isolated by centrifugation and washed with dichloromethane (4 mL) and diethyl ether (4 mL) to remove residual dimethylformamide.

Complex **3**: Yield: 100 mg (61 %) from **1** (140 mg, 0.25 mmol) and benzoic anhydride (574 mg, 2.5 mmol) in 20 mL dimethylformamide. Anal. Calcd (%) for: $C_{23}H_{28}N_2O_8Pt$ (655.56): C, 42.14; H, 4.31; N, 4.27. Found: C, 42.01; H, 4.11; N, 4.09. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 1.7 - 1.8 (m, 4H, CCH_2CH_2, CBDCA and $CH_2CH_2CH_2$, PBA), 2.22 (t, J = 7.4 Hz, 2H, $CH_2CH_2CH_2$), 2.45 (m, 2H, $CH_2CH_2CH_2$), 2.53 (t, J = 8.0 Hz, 4H, CCH_2CH_2, CBDCA), 6.4 (m, br., 6H, NH_3), 7.10 - 7.23 (m, 5H, H_{ar}, PBA), 7.37 (t, J = 8.0, 2H, H_{ar}), 7.46 (t, J = 8.0, 1H, H_{ar}), 7.85 (d, J = 6.9 Hz, 2H, H_{ar}); ^{195}Pt{^{1}H} NMR (107.6 MHz, DMF/D_2O): δ 1931 (quin, J_{PtN} = 206 Hz). ESI-MS: m/z = 654.14 $[M-H]^-$.

Complex **4**: Yield: 90 mg (77 %) from **1** (100 mg, 0.18 mmol) and succinic anhydride (181 mg, 0.81 mmol) in 20 mL dimethylformamide. Anal. Calcd (%) for: $C_{20}H_{28}N_2O_{10}Pt$ (651.53): C, 36.87; H, 4.33; N, 4.30. Found: C, 36.74; H, 4.23; N, 4.20. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 1.7 - 1.8 (m, 4H, CCH_2CH_2, CBDCA and $CH_2CH_2CH_2$, PBA), 2.20 (t, J = 7.4 Hz, 2H, $CH_2CH_2CH_2$), 2.32 - 2.59 (m, 10H, $CH_2CH_2CH_2$, CCH_2CH_2, CBDCA, CH_2, suc), 6.3 (m, br., 6H, NH_3), 7.13 - 7.24 (m, 5H, H_{ar}); ^{195}Pt{^{1}H} NMR (107.6 MHz, DMF/D_2O): δ 1934 (quin, J_{PtN} = 210 Hz). ESI-MS: m/z = 650.52 $[M-H]^-$.

cis,trans-[Pt(CBDCA)(NH_3)$_2$)(PBA)(ac)] (5)

Complex **1** (56 mg, 0.10 mmol) was stirred for 20 h in acetic anhydride (5 mL) at room temperature. The mixture was then lyophilized and washed with diethyl ether (2 x 5 mL) to isolate the desired product. Yield: 35 mg (60 %). Anal. Calcd (%) for $C_{18}H_{26}N_2O_8Pt$ (593.13): C, 36.43; H, 4.42; N, 4.72. Found: C, 36.34; H, 4.36; N, 4.60. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 1.7 - 1.8 (m, 4H, CCH_2CH_2, CBDCA and $CH_2CH_2CH_2$, PBA), 1.96 (s, 3H, CH_3COO), 2.20 (t, J = 7.4 Hz, 2H, $CH_2CH_2CH_2$), 2.4 - 2.6 (m, 6H, $CH_2CH_2CH_2$, PBA, CCH_2CH_2, CBDCA), 6.3 (m, br., 6H, NH_3), 7.11 - 7.24 (m, 5H, H_{ar}); ^{195}Pt{^{1}H} NMR (107.6 MHz, DMF/D_2O): δ 1935 (quin, J_{PtN} = 208 Hz). m/z = 592.17 $[M-H]^-$.

Measurements and instrumentation

^{1}H, ^{13}C and ^{195}Pt NMR spectra were acquired with a Varian 500 AR spectrometer. Chemical shifts are given in parts per million (ppm) with tetramethylsilane as the reference (δ (^{1}H NMR) = 0 ppm). Coupling constants (J) are given in Hertz (Hz). ^{195}Pt NMR spectra were acquired in dimethylformamide with an inserted tube containing D_2O. K_2PtCl_6 in D_2O was used as an external reference. Mass spectra were obtained with a Waters LCT Premiere XE

with electron spray ionization and time of flight mass analyzer. Elemental analyses (carbon, nitrogen and hydrogen) were carried out with a PerkinElmer 2400 series II analyzer.

Platinum concentrations were determined using inductively coupled plasma mass spectrometry (ICP-MS, ELAN DRCe, PerkinElmer, Waltham, USA) in a Class 1000 cleanroom [41-44]. Instrumental operating conditions included: ICP RF Power 1150 W: plasma gas flow 15 L min^{-1}; auxiliary gas flow 1 L min^{-1}; nebuliser gas flow 0.93 L min^{-1}; observed isotopes; ^{192}Pt, ^{194}Pt, ^{195}Pt, ^{196}Pt and ^{198}Pt. Platinum calibration standards were prepared using a single element Pt standard (SPEX CertiPrep, 1000 µg mL^{-1}) and Milli-Q™ water (18.3 mΩ) (Millipore, Bedford, USA) with 1 % HNO_3 (Trace Metal Grade, 67-69 %, Fisher, UK). Iridium (^{191}Ir) and indium (^{115}In) were used as internal standards to account for instrumental drift and matrix effects.

Reduction of the platinum(IV) complexes

The reduction of complexes **1, 2** and **3** was monitored by high performance liquid chromatography (HPLC) using an Agilent 1200 series DAD analytical HPLC instrument. The compounds were dissolved in 0.5 mL dimethylformamide, added to a 7 mM solution of ascorbic acid in 4 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (pH 7) and diluted with acetonitrile to a final concentration of 0.5 mM. The reduction was monitored at 37 °C until completion. The samples were analyzed using a Phenomenex Luna C18 column (5 µm, 100 Å, 250 mm × 4.60 mm i.d.). The mobile phase was 90 : 10 acetonitrile (1 % trifluoroacetic acid) : water (1 % trifluoroacetic acid). The flow rate was 1.0 mL min^{-1} and the fractions were detected at 254 nm.

Determination of lipophilicity

Octanol-water partition coefficients (log $P_{o/w}$) of the platinum(IV) compounds were determined by the shake flask method [45]. All experiments were done in duplicate. The respective platinum(IV) complex was mixed with 0.9 % NaCl (w/v) in ultrapure water that was presaturated with n-octanol for 3 d. All solutions were sonicated and filtered to remove undissolved platinum(IV) compound. The initial Pt concentrations were measured by ICP-MS (ELAN DRCe, Perkin Elmer, Waltham, USA) as described above. Subsequently, the platinum(IV) solution was added to an equal volume of n-octanol that was presaturated with 0.9 % NaCl (w/v) in ultrapure water. The heterogeneous mixture was shaken vigorously for 1 h before centrifuging at 4400 rpm for 1 h to achieve phase separation. The final Pt

concentration in the aqueous phase was measured again by ICP-MS. Log $P_{o/w}$ values were calculated as the ratio of Pt concentrations in the organic and aqueous phases.

Experiments with cultured human cells

The platinum(IV) complexes and the corresponding uncoordinated ligands were dissolved in dimethyl sulfoxide just before the experiment and a calculated amount of drug solution was added to the cell growth medium to a final solvent concentration of 0.5 %, which had no detectable effect on cell killing. Cisplatin and carboplatin were dissolved in 0.9 % sodium chloride solution. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), carboplatin and cisplatin were obtained from Sigma Chemical Co, St. Louis, USA.

Cell cultures

The human pancreatic (BxPC3) and colon (LoVo) carcinoma and melanoma (A375) cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). The human squamous cervical cancer cell line (A431) was kindly provided by Prof. G. Zunino (Division of Experimental Oncology B, Istituto Nazionale dei Tumori, Milan, Italy).

Cell lines were maintained in the logarithmic phase at 37 °C in a 5 % carbon dioxide atmosphere using the following culture media containing 10 % fetal calf serum (Euroclone, Milan, Italy), antibiotics (50 units/mL penicillin and 50 µg/mL streptomycin), and 2 mM L-glutamine: (i) RPMI-1640 medium (Euroclone) for BxPC3 and A431 cells; (ii) DMEM for A375 cells; (iii) F-12 HAM'S (Sigma Chemical Co.) for LoVo cells.

MTT assay

The growth inhibitory effect toward tumor cells was evaluated by means of MTT assay. Briefly, (3–8) × 10^3 cells/well, dependent upon the growth characteristics of the cell line, were seeded in 96-well microplates in growth medium (100 µL). After 24 h, the medium was removed and replaced with fresh medium containing the compound to be studied at the appropriate concentration. Triplicate cultures were established for each treatment. After 72 h, each well was treated with 10 µL of a 5 mg/mL MTT saline solution and following 5 h of incubation, 100 µL of a sodium dodecylsulfate (SDS) solution in HCl (0.01 M) was added. After an overnight incubation, cell growth inhibition was detected by measuring the absorbance of each well at 570 nm using a Bio-Rad 680 microplate reader. The mean absorbance for each drug dose was expressed as a percentage of the untreated control well

absorbance and plotted vs. drug concentration. IC_{50} values, the drug concentrations that reduce the mean absorbance at 570 nm to 50 % of those in the untreated control wells, were calculated by a four parameter logistic (4-PL) model. All of the values are the means ± SD of not less than five independent experiments.

Cellular accumulation

A431 cells (2×10^6) were seeded in 75 cm^2 flasks in growth medium (20 mL). After overnight incubation, the medium was replaced and the cells were treated with the tested compounds for 24 h. Cell monolayers were washed twice with cold PBS, harvested and counted. Samples were subjected to three freezing/thawing cycles at -80 °C and then vigorously vortexed. The samples were treated with highly pure nitric acid (Pt: ≤0.01 μg kg^{-1}, TraceSELECT® Ultra, Sigma Chemical Co.) and transferred into a microwave teflon vessel. Subsequently, samples were submitted to standard procedures using a speed wave MWS-3 Berghof instrument (Eningen, Germany). After cooling, each mineralized sample was analyzed for platinum by using a Varian AA Duo graphite furnace atomic absorption spectrometer (Varian, Palo Alto, CA; USA) at the wavelength of 324 nm. The calibration curve was obtained using known concentrations of standard solutions purchased from Sigma Chemical Co.

Histone deacetylase assay

Histone deacetylase activity was determined using Fluor-de-Lys®HDAC fluorometric activity assay kit (Enzo Life Sciences International, Inc., Plymount Meeting, PA, U.S.A.). A431 cells (5×10^4 seeded in 96-well microplates) were treated for 24 h with 5 μM tested complexes, then processed as reported by the manufacturer's instructions. Fluorescence was measured using a Fluoroskan Ascent FL (Labsystem, Finland) plate reader, with excitation at 360 nm and emission at 460 nm.

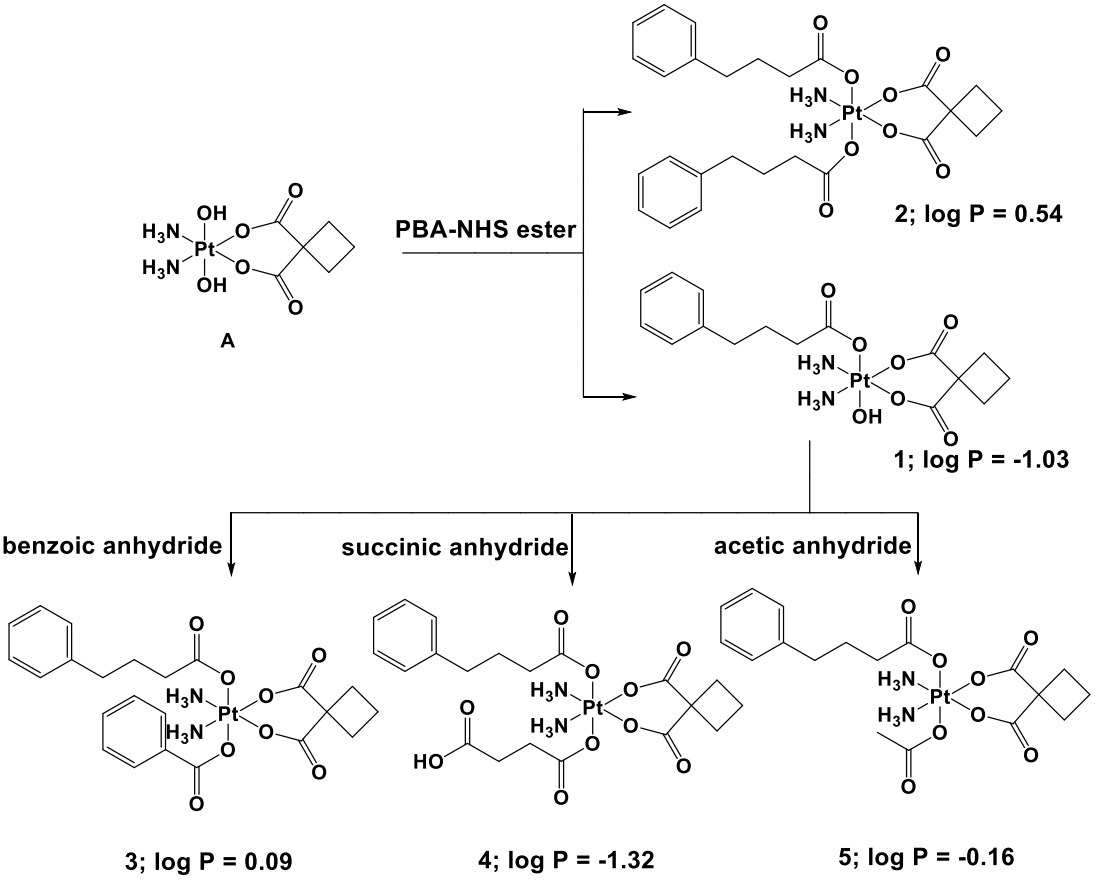
Hoechst 33258 staining

A431 cells were seeded into 8-well tissue-culture slides (BD Falcon, Bedford, MA, USA) at 5×10^4 cells/well (0.8 cm^2). After 24 h, the cells were washed twice with PBS and following 48 h of treatment with IC_{50} concentration of tested complexes, the cells were stained for 5 min with 1 mg/mL Hoechst 33258 (Sigma–Aldrich) in PBS before being examined by fluorescence microscopy (Olympus BX41, Cell F software, Olympus, Munster, Germany).

Results and discussion

Synthesis and characterisation

The chemical structures of the platinum(IV) complexes investigated in this paper are depicted in Scheme 1. The dihydroxoplatinum(IV) derivative of carboplatin (**A**) was obtained by oxidation of carboplatin with H_2O_2 in aqueous solution. The mono-substituted complex **1** could be synthesized by reaction of the activated NHS-ester of PBA (see experimental) and **A** (0.9 : 1 ratio) in dimethyl sulfoxide at 65 °C with a yield of 71 %. The bis-substituted complex **2** was isolated after reaction of **A** with a large excess of the activated NHS-ester of PBA. All the other complexes were synthesized by reaction of the mono-PBA complex **1** with the corresponding anhydride (benzoic anhydride, **3**; succinic anhydride, **4** and acetic anhydride, **5**) in dimethylformamide with yields in the range of 49 – 75 % (Scheme 1).



Scheme 1: Synthesis of the platinum(IV) complexes **1** – **5**.

Each complex was characterized by ESI-mass spectrometry, ^{1}H and ^{195}Pt NMR spectroscopy and the purity was assessed by elemental analysis and HPLC. The ^{1}H NMR spectra in DMSO-d_6 displayed the typical signal of the NH_3 protons of the carboplatin scaffold as a broad multiplet around 6 ppm in the case of mono-substituted **1** and as a broad signal around 6.5 ppm in the case of the bis-substituted complexes **2**–**5** (Supplementary Figs. S1 – S5). The ^{195}Pt NMR spectra of the bis-substituted complexes (Supplementary Figs. S6 – S10) showed a quintet around 1900 ppm while **1** gave a quintet at 1741 ppm in line with chemical shift data previously reported for related platinum(IV) complexes [14]. The appearance of the signals as quintets is due to spin-to-spin coupling between ^{195}Pt and two ^{14}N nuclei. The mass spectra of the complexes displayed a peak in the negative mode with the typical platinum isotope pattern corresponding to the respective $[M-H]^-$ species (Supplementary Figs. S11 – S15).

The different axial carboxylate ligands tune the lipophilicity of the complexes; the log $P_{o/w}$ values, determined for each complex using the shake flask method, are reported in Scheme 1 and indicate the order of lipophilicity as **2** > **3** > **5** > **1** > **4** as expected. platinum(IV) complexes enter the cell by passive diffusion and the lipophilicity index plays a major role in cellular Pt accumulation [34]. The mechanism of action of platinum(IV) complexes has been investigated in detail. The most important step is the reduction of the complexes inside the cell environment to produce the active platinum(II) species (carboplatin in this case) with the release of the axial ligands [46] and the pharmacological profiles of platinum(IV) complexes are greatly affected by their redox behavior. High reduction rates might lead to high systemic toxicity and to deactivation of the resulting platinum(II) form before the DNA is reached, whereas particularly low reduction rates might result in the absence of antitumor activity. While no direct correlation between the reduction potential and prodrug efficacy has been established in the literature, a reduction potential in the mid-range as observed for complexes with two axial carboxylate ligands (~ -600 mV) seems to be a good guideline for an effective platinum(IV) prodrug [1]. The reduction of platinum(IV) complexes has been investigated in the past with different techniques and under different conditions [47-51]. It is generally presumed that under physiological conditions platinum(IV) is reduced by low-molecular-weight reducing agents such as ascorbic acid or glutathione, although recently evidence has been obtained that higher molecular weight biomolecules such as metallothioneines can also play a major role in the reduction and activation of platinum(IV) prodrugs [52]. Until recently the reduction rate - and thus the biological activity - was assumed to directly correlate with

the platinum(IV)/platinum(II) redox potential. The latter is mainly determined by the nature of the axial ligands. The redox potentials of platinum(IV) complexes of general formula cis,trans,cis-[$PtCl_2L_2(Am)_2$] decrease in the order L = OH^- (~ -900 mV) < $RCOO^-$ (~ -600 mV) < Cl^- (~ -250 mV) [53]. Some exceptions have recently been reported where the reduction rate does not correlate with the redox potential of the platinum(IV) complex. In particular, it was found that mono-substituted species with one axial OH group are reduced faster than bis-carboxylated complexes due to the ability of the OH group to act as a bridging ligands in an inner-sphere electron transfer mechanism [54,55]. In summary, it is quite hard to predict and estimate the reduction rate of a platinum(IV) complex, as many factors may come into play (e.g. platinum(II) scaffold, nature of the axial ligands, reducing agent) [56,57]. The reduction with ascorbic acid and the release of the PBA ligands under physiological conditions (37 °C, pH 7) of the monocarboxylated complex **1**, the bis-PBA complex **2** and **3** as a representative of the mixed carboxylic acid complexes, was monitored by HPLC (Fig. 2 and Supplementary Figs. S16 and S17). Comparison of the HPLC chromatograms obtained during the reduction reaction with that of the pure PBA ligand confirmed that the reduction of platinum(IV) to platinum(II) leads to the release of the axial ligands. Incubation with 10 eq. of ascorbic acid for 5 h resulted in the reduction of 75% of complex **2** and 50% of complex **3**. The complexes were reduced completely after 12 h (**2**), 36 h (**3**) and 72 h (**1**). Noteworthy, for the reduction of complex **3** both released axial ligands (PBA and benzoic acid) could be identified in the chromatogram.

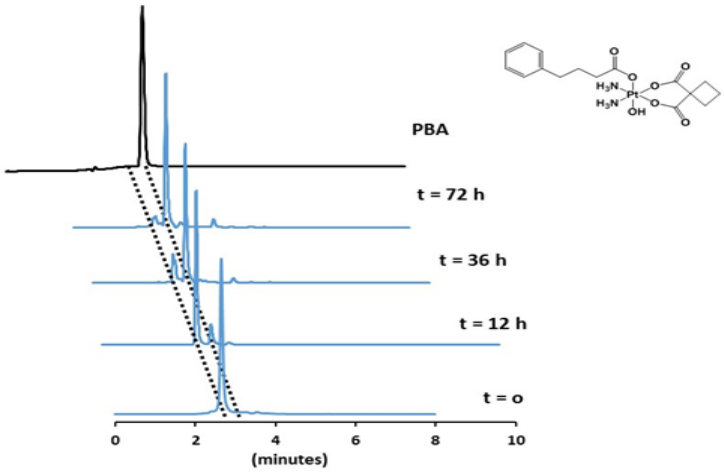


Figure 2: HPLC chromatogram of the reaction of **1** with 10 eq. ascorbic acid at 37 ° C and pH 7. The chromatogram of the free ligand PBA is shown for comparison purposes.

Cytotoxicity

The ability of the platinum(IV) complexes **1–5** to promote cell death was evaluated in several human cancer cell lines derived from solid tumors (A431 cervical, LoVo colon, BxPC3 pancreatic and A375 melanoma cells). The cytotoxicity parameters, in terms of IC_{50} values obtained after 72 h of exposure to the MTT assay, are listed in Table 1. For comparison purposes, the cytotoxicities of cisplatin and carboplatin were assessed under the same experimental conditions. Cancer cell lines included in the screen were endowed with different degrees of sensitivity to cisplatin and carboplatin and showed a good concentration-dependent response to treatment with the newly synthesized complexes. The uncoordinated HDACi ligand PBA displayed very low cytotoxic activities, with IC_{50} values mainly in the millimolar range, in agreement with the literature [38].

Table 1. IC_{50} values of **1 - 5** in different cancer cell lines [a]

	IC_{50} (μM)±S.D.			
	A375	BxPC3	LoVo	A431
1	18.5±5.9	36.2±8.3	13.5±3.9	16.8±3.6
2	16.6±3.5	24.2±3.2	13.5±2.5	15.9±3.7
3	11.1±2.9	6.2±0.3	5.1±1.3	5±2
4	24.2±3.5	36.3±3.2	18.4±2.9	19.7±5.1
5	23.3±6.2	34.6±3.1	39.3±4.2	25.3±2.3
carboplatin	13.5±3.0	30.9±4.2	49.3±7.1	12.3±3.3
PBA	39.4±4.1	1523±36	1075±44	1115±31
cisplatin	3.3±1.0	11.1±1.7	9.1±2.1	1.7±0.3

[a] Cells (3-5 × 10^4 mL^{-1}) were treated for 72 h with increasing concentrations of the tested compounds. The cytotoxicity was assessed by the MTT test. IC_{50} values were calculated by a four parameter logistic model ($P < 0.05$). S.D.= standard deviation.

Complexes **1–4** were found to be more effective than the reference metallodrug carboplatin, but only derivative **3** possessed an in vitro antitumor activity comparable to that of cisplatin, with the average IC_{50} values being 6.8 and 6.0 μM for **3** and cisplatin, respectively. Actually, **3** emerged as the most cytotoxic derivative, eliciting mean IC_{50} values from 3- to 5-fold lower

than those calculated for the other complexes of the series. It is noteworthy that complex **3** was roughly 1.8-fold more effective than cisplatin against human BxPC3 pancreatic and LoVo colon cancer cells and on average 5 and 10 times more effective than carboplatin.

Platinum cellular accumulation

In an attempt to correlate the cytotoxic activity with cellular accumulation, the platinum content was evaluated in A431 cells treated for 24 h with 3.125 and 6.25 μM solutions of the platinum(IV)-complexes. The intracellular platinum amount was quantified by means of GF-AAS analysis and the results, expressed as pg metal per 10^6 cells, are shown in Fig. 3. Although to a different extent, all complexes accumulated proportionally to drug concentration into A431 cancer cells. The cellular accumulation of **3** was greater compared to that of the other derivatives in the series and was comparable to that of cisplatin at both tested concentrations. These results allow us to correlate the cytotoxic activity with the ability of the platinum(IV) complexes to enter and accumulate into cancer cells. Interestingly, there is no correlation between lipophilicity and cellular internalization. Although this is somewhat unexpected, since, unlike platinum(II) complexes, platinum(IV) complexes are believed to enter cells solely by passive diffusion, a similar lack of correlation between log*P* values and cellular Pt accumulation has been reported for other platinum(IV) systems [14,38,58,59].

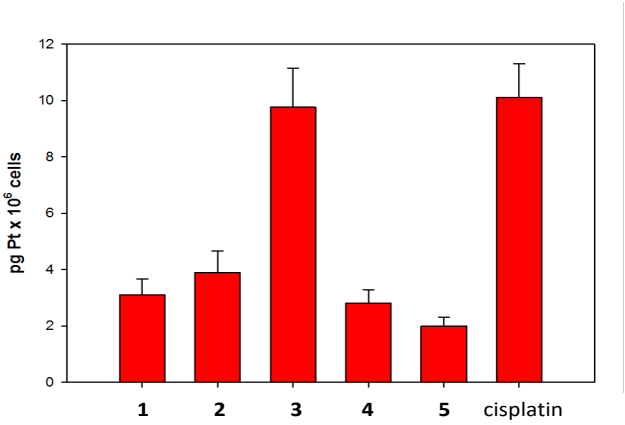


Figure 3: Cellular accumulation of **1** – **5** and cisplatin into A431 cervical cancer cells. A431 cells were incubated for 24 h with the complexes at 3.125 and 6.25 μM concentrations. The intracellular platinum content was determined by GF-AAS.

HDAC inhibition

As the newly synthesized complexes described here were designed to release the PBA moiety after reduction once inside the cells, we measured the ability of these prodrugs to inhibit HDAC activity in cells. A431 cells were incubated for 24 h with cytotoxic concentrations of the tested compounds (5 μM) and their HDAC activity was determined. The trend of HDAC inhibition resembles that obtained in the cytotoxicity studies. As expected, cisplatin and carboplatin were not effective in modifying HDAC activity (Fig. 4). Among all, compound **3** was the most effective in hampering HDAC activity, being able to decrease the cellular basal activity by roughly 18 %.

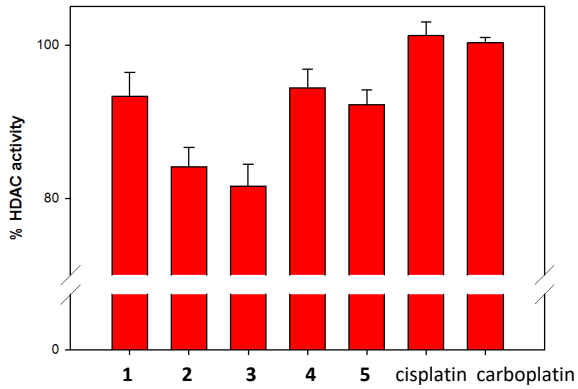


Figure 4: Effects on HDAC activity induced by **1** – **5**, cisplatin and carboplatin in A431 cervical cancer cells. A431 cells were incubated for 24 h with the complexes at 5 μM concentration. The HDAC activity was determined by FLUOR DE LYS® HDAC fluorometric activity assay kit (Enzo Life Sciences).

Induction of apoptosis

To gain more insight into the mode of action of the newly synthesized platinum(IV) complexes, we evaluated the capacity of the most effective derivative **3** to induce apoptosis in human cervical cancer cells. In particular, apoptotic cell death induction was confirmed through a Hoechst 33342 staining assay. Fig. 5 shows Hoechst 33342 stained A431 cells after 48 h treatment with IC_{50} concentrations of **3** or carboplatin. For both, compound **3** and carboplatin, treated cells as compared with untreated cells, displayed chromatin condensation and fragmentation as well as pyknotic nuclei, typical features of apoptosis.

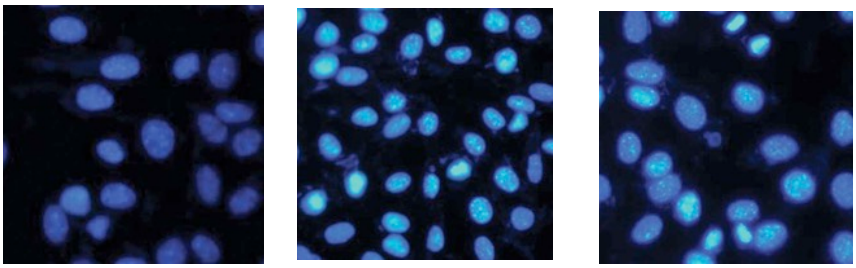


Figure 5: Apoptosis induction. A431 cells were treated with IC_{50} concentrations of **3** and carboplatin for 48 h and stained with the fluorescent dye Hoechst 33258. Ctr = untreated control.

Conclusions

A new series of platinum(IV) complexes based on the carboplatin scaffold and the HDAC inhibitor PBA as an axial ligand has been synthesized, characterized and tested on a panel of four different cancer cell lines. The lower cytotoxic activity with respect to the reference drug cisplatin is not unexpected and has been previously seen in other carboplatin derivatives since carboplatin in general requires a higher dose to show a significant anticancer activity [40]. Platinum accumulation studies revealed that complex **3** shows an intracellular accumulation comparable with that of cisplatin and indeed it possesses the highest cytotoxic activity and HDAC inhibition potency. Lower accumulation levels and consequently a lower cytotoxicity was observed for the other complexes. The slow reduction kinetics of the platinum(IV) prodrugs of carboplatin should also contribute to the relatively moderate cytotoxicity.

Carboplatin is generally better tolerated than cisplatin [60] and *in vivo* studies could demonstrate the benefit of the carboplatin scaffold with respect to cisplatin which, however, is beyond the aim of this paper.

Acknowledgement

A.A. acknowledges the Royal Embassy of Saudi Arabia Ministry of Education for a Saudi Arabia Government Scholarship. V.G. and C.M. acknowledge the University of Padova (grants 60A04-0443, 60A04-3189 and 60A04-4015/15, DOR2016).

Appendix A. Supplementary data

Supplementary data to this article can be found online at

(http://dx. doi.org/10.1016/j.jinorgbio.2017.09.009.)

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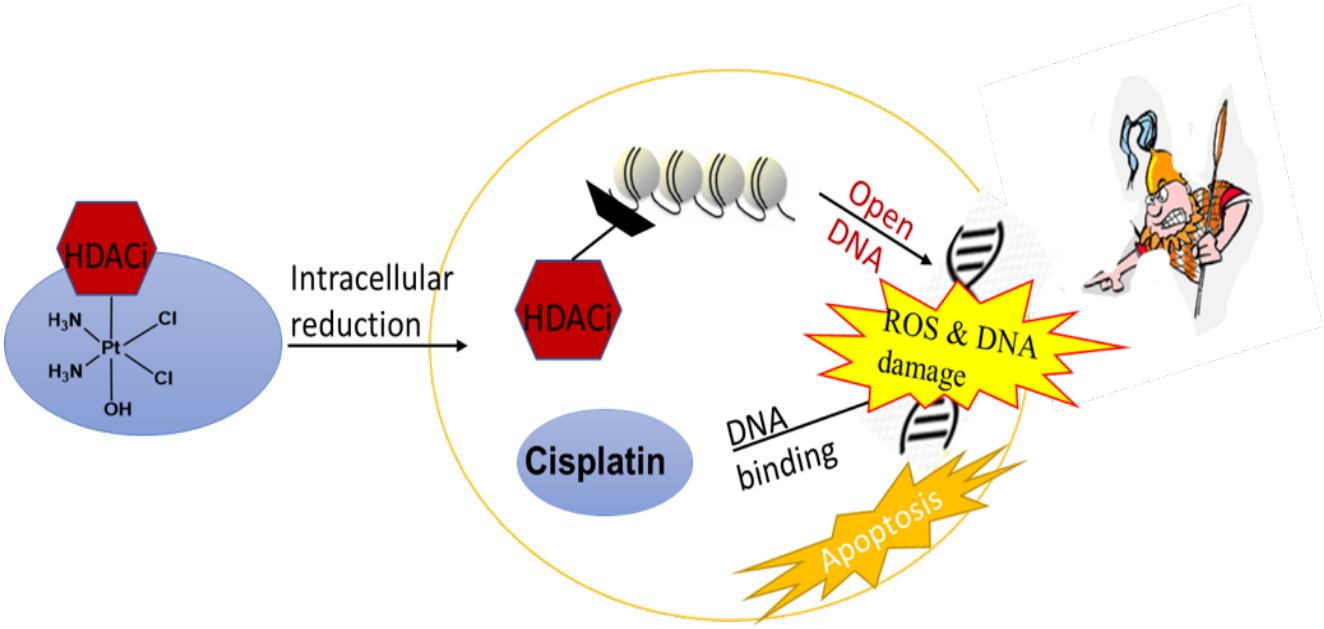
Results :

Chapter 3: Platinum(IV) pro-drugs with an axial HDAC inhibitor demonstrate multimodal mechanisms involving DNA damage and apoptosis independent of cisplatin resistance



3. Brief description of the paper

Four platinum(IV) derivatives of cisplatin with a histone deacetylase inhibitor in axial position were synthesised and their cellular effects were compared in cisplatin-sensitive and cisplatin-resistant ovarian cancer cells. *cis*,*cis*,*trans*-[$Pt(NH_3)_2Cl_2$(PBA)(Bz)] was the most cytotoxic complex and led to markedly increased cellular ROS levels which can mediate apoptosis independent of DNA damage.

Platinum(IV) pro-drugs with an axial HDAC inhibitor demonstrate multimodal mechanisms involving DNA damage and apoptosis independent of cisplatin resistance

Awatif Rashed Z. Almotairy,[1,2] Diego Montagner,[3] Liam Morrison,[4] Michael Devereux,[2] Orla Howe,*[2] Andrea Erxleben*[1]

Authors	Contributions
Awatif Rashed Z .Almotairy	Synthesised and characterised the complexes; obtained and collected all biological results
Orla Howe	Managed the project and reviewed manuscript
Liam Morrison	Obtained ICP-MS data
Michael Devereux	Reviewed manuscript
Diego Montagner	Managed the project
Andrea Erxleben	Managed the project and reviewed manuscript prior to and post the review process

Platinum(IV) pro-drugs with an axial HDAC inhibitor demonstrate multimodal mechanisms involving DNA damage and apoptosis independent of cisplatin resistance

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Abstract

Epigenetic agents such as HDAC inhibitors are widely investigated for use in combined anticancer therapy and the co-administration of Pt drugs with HDAC inhibitors has shown promise for the treatment of resistant cancers. Coordination of an HDAC inhibitor to an axial position of a platinum(IV) derivative of cisplatin allows the combination of the epigenetic drug and the Pt chemotherapeutic into a single molecule. In this work we carry out mechanistic studies on the known platinum(IV) complex *cis*,*cis*,*trans*-[$Pt(NH_3)_2Cl_2(PBA)_2$] (**B**) with the HDAC inhibitor 4-phenylbutyrate (PBA) and its derivatives *cis*,*cis*,*trans*-[$Pt(NH_3)_2Cl_2(PBA)(OH)$] (**A**), *cis*,*cis*,*trans*-[$Pt(NH_3)_2Cl_2(PBA)(Bz)$] (**C**), and *cis*,*cis*,*trans*-[$Pt(NH_3)_2Cl_2(PBA)(Suc)$] (**D**) (Bz = benzoate, Suc = succinate). The comparison of the cytotoxicity, effect on HDAC activity, ROS generation, γ-H2AX foci generation and induction of apoptosis in cisplatin-sensitive and cisplatin-resistant ovarian cancer cells shows that **A** – **C** exhibit multimodal mechanisms involving DNA damage and apoptosis independent of cisplatin resistance.

Introduction

Cisplatin (*cis*-[$Pt(NH_3)_2Cl_2$]) has been used as a chemotherapeutic cancer drug since 1978, specifically in the treatment of testicular, head and neck, bladder and ovarian carcinomas (Johnstone et al. 2016). The generally accepted mode of action of cisplatin involves covalent binding of the *cis*-$Pt(NH_3)_2^{2+}$ entity to adjacent guanine bases in DNA following loss of the chloride leaving group ligands. The formation of these DNA lesions triggers the DNA damage response by activating or silencing various genes. If the damage is beyond repair, mitochondrial apoptosis is initiated (Kohno et al. 2005; Torigoe et al. 2005).

Inherent and acquired resistance towards platinum drugs presents a major clinical challenge and a significant limitation of platinum-based chemotherapy (Housman et al. 2014). Cisplatin resistance is the consequence of genetic and epigenetic changes that lead to decreased cellular uptake, increased cellular efflux, detoxification due to increased glutathione or thioredoxin levels, enhanced DNA repair, enhanced DNA damage tolerance and late apoptotic response. The main types of epigenetic alterations in general are DNA methylation and histone methylation or acetylation (Kouzarides 2007). Histone acetylation and deacetylation regulate gene expression by opening (acetylation) and closing (deacetylation) the chromatin (Strahl et al. 2000). Histone acetylation has a critical regulatory function in DNA repair and recent studies suggest that it plays a role in the development of drug resistance (Housman et al. 2014). Histone acetylation and deacetylation are catalyzed by histone acetyltransferase (HAT) and histone deacetylase (HDAC) respectively.

The combination of epigenetic drugs with conventional chemotherapy shows significant promise as a strategy for the treatment of resistant cancers (Juergens et al. 2011). Several HDAC inhibitors (HDACis) have entered clinical trials (Lee et al. 2008; Leggat et al. 2011) and the first HDACi was approved by the FDA in 2006 (Mann 2007). Several studies have shown that the co-administration of cisplatin with an HDACi leads to enhanced therapeutic efficacies (Diyabalanage et al. 2013; Huang et al. 2014; Jin et al. 2010). Belinostat in combination with cisplatin reverses resistance in Pt-resistant lung cancer cells (To et al. 2017). Trichostatin A has an additive effect with oxaliplatin in gastric tumor cells (Zhang et al. 2006). Pt-resistant ovarian cancer cells are sensitized to cisplatin-mediated cell death when pre-treated with HDAC and methylation inhibitors (Cacan et al. 2014).

While different pharmacokinetics can be a problem in co-administration, coordination of the HDACi to the Pt centre ensures the simultaneous delivery of both drugs. Marmion and

coworkers developed platinum(II) complexes in which a functionalized HDAC inhibitor ligand is coordinated to the *cis*-$Pt(NH_3)_2^{2+}$ entity (Brabec et al. 2012; Griffith et al. 2009; Parker et al. 2013). Kasparkova et al. reported a photoactivatable platinum(IV) complex of the HDAC inhibitor suberoyl-bis-hydroxamic acid (Kasparkova et al. 2015). Gibson and coworkers and Brabec and coworkers attached the HDACis 4-phenylbutyrate (PBA) and valproate (VPA) to the axial positions of platinum(IV) derivatives of cisplatin, oxaliplatin and [Pt(1S,2Sdiaminocyclohexane)(5,6-dimethyl-1,10-phenanthroline)]$^{2+}$ (Harper et al. 2017; Kostrhunova et al. 2019b; Novohradsky et al. 2014 & 2015; Raveendran et al. 2016). Platinum(IV) complexes are pro-drugs that release the active platinum(II) drug and the axial ligands on intracellular reduction. The bis-PBA complex *cis,cis,trans*-$[Pt(NH_3)_2Cl_2(PBA)_2]$ showed an up to 100-fold higher cytotoxicity than cisplatin and significant HDAC inhibition activity suggesting a synergism between Pt and PBA (Raveendran et al. 2016). The platinum(IV) complex *cis,cis,trans*-$[Pt(NH_3)_2Cl_2(VPA)_2]$ with the HDACi valproate was studied by Osella and coworkers (Alessio et al. 2013), Shen and coworkers (Yang et al. 2012) and Gibson and coworkers (Raveendran et al. 2016). Very recently, the same researchers reported a triple-action platinum(IV) complex containing PBA and a second epigenetically acting ligand, octanoate (Kostrhunova et al. 2019a & 2019b). As part of our own work we have recently reported platinum(IV) derivatives of carboplatin with an axial PBA ligand and a second biologically inactive carboxylate ligand to modify the lipophilicity (Almotairy et al. 2017).

To provide more insight into the ability of platinum(IV) PBA pro-drugs to overcome cisplatin resistance, we compare here the cellular responses of cisplatin-sensitive A2780 and cisplatin-resistant A2780cis cells on treatment with the known platinum(IV) complex *cis*,*cis*,*trans*-$[Pt(NH_3)_2Cl_2(PBA)_2]$ and its derivatives *cis*,*cis*,*trans*-$[Pt(NH_3)_2Cl_2(PBA)(OH)]$, *cis*,*cis*,*trans*-$[Pt(NH_3)_2Cl_2(PBA)(Bz)]$, and *cis*,*cis*,*trans*-$[Pt(NH_3)_2Cl_2(PBA)(Suc)]$ (Bz = benzoate, Suc = succinate).

Materials and methods

Syntheses

The syntheses of the platinum(IV) complexes is described in the Supplementary Material.

Measurements and instrumentation

^{1}H and ^{195}Pt NMR spectra were recorded with a Varian 500 AR spectrometer. Chemical shifts are given in parts per million (ppm) with tetramethylsilane as the reference (δ (^{1}H NMR) = 0 ppm). Coupling constants (*J*) are given in Hertz (Hz). ^{195}Pt NMR spectra were acquired in dimethylformamide with an inserted tube containing D_2O. K_2PtCl_6 in D_2O was used as an external reference. Mass spectra were obtained with a Waters LCT Premiere XE with electron spray ionization (ESI) and time of flight mass analyzer. Elemental analyses (carbon, nitrogen and hydrogen) were carried out with a PerkinElmer 2400 series II analyzer. Pt concentrations were measured by ICP-MS (ELAN DRCe, PerkinElmer, Waltham, USA) in a class 1000 cleanroom (Crespo Alonso et al. 2015; Morrison et al. 2000; Ward et al. 2004; Whiteley et al. 2005). The calibration curve was obtained using known concentrations of platinum standard solutions (0 – 50 µM).

Monitoring of the reduction of the platinum(IV) complexes by HPLC

The reduction of complexes **A** and **C** was monitored by high-performance liquid chromatography (HPLC) using an Agilent 1200 series DAD analytical HPLC instrument. The compounds were dissolved in 0.5 mL dimethylformamide, added to a 7 mM solution of ascorbic acid in 4 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (pH 7) and diluted with acetonitrile to a final concentration of 0.5 mM. The reduction was monitored at 37 °C until completion. The samples were analyzed using a Phenomenex Luna C18 column (5 µm, 100 Å, 250 mm × 4.60 mm i.d.). The mobile phase was 90 : 10 acetonitrile (1% trifluoroacetic acid) : water (1% trifluoroacetic acid). The flow rate was 1.0 mL min^{-1} and the fractions were detected at 254 nm.

Determination of lipophilicity

Octanol-water partition coefficients ($\log P_{o/w}$) of the platinum(IV) compounds were determined by the shake flask method (Test No. 107: Partition Coefficient (n-Octanol/Water): Shake Flask Method; Organisation for Economic Co-operation and Development: Paris (1995)). All experiments were done in duplicate. The respective platinum(IV) complex was

mixed with 0.9% NaCl (*w*/*v*) in ultrapure water that was presaturated with n-octanol for 3 d. All solutions were sonicated and filtered to remove undissolved platinum(IV) compound. The initial Pt concentrations were measured by ICP-MS as described above. Subsequently, the platinum(IV) solution was added to an equal volume of n-octanol that was presaturated with 0.9% NaCl (w/v) in ultrapure water containing 1% HNO_3. The heterogeneous mixture was shaken vigorously for 1 h before centrifuging at 4400 rpm for 1 h to achieve phase separation. The final Pt concentration in the aqueous phase was measured again by ICP-MS. Log$P_{o/w}$ values were calculated as the ratio of Pt concentrations in the organic and aqueous phases.

Experiments with cultured human cells

Mammalian cell lines and culture conditions

The tested compounds (**A** – **D**) were dissolved in dimethyl sulfoxide (Merck, Germany) before the start of the experiment. The final concentration of DMSO was ≤0.3 %. A2780 and A2780cis cells were obtained commercially from European Collection of Authenticated Cell Cultures (ECACC) (Public Health England, UK). Cells were grown in Roswell Park Memorial Institute (RPMI - 1640) medium (Sigma, Ireland) supplemented with 12% Foetal Bovine Serum (Sigma, Ireland) and 5% L-glutamine (Sigma, Ireland) and incubated at 37 °C in 5% CO_2. A sub-lethal dose of 1 μM cisplatin was added to A2780cis cells to maintain cisplatin resistance. Stock cells were grown to 80 – 90 % confluence and were then trypsinised to generate a cell suspension for experimental use.

Cell viability

Cellular viability was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, Ireland) assay which is a colorimetric assay that measures the metabolic activity of cells (Mosmann 1983). The ovarian adenocarcinoma cell lines were plated in 96-well plates with a total number of 1×10^4 cells/well and 8×10^3 cells/well for 24 and 48 h, respectively for each cell line, and the plates were incubated at 37 °C for 24 h for attachment and growth. Cells were then treated with different concentrations of the test compounds (2.5 μM – 200 μM) in triplicate and a solvent control (0.3 % DMSO) for 24 and 48 h timepoints. The cell culture medium and test drug was discarded from the plate wells and 100 μL of a solution of MTT (5 mg / mL) was added to each well. The cells were incubated for 3 h and washed three times with sterilised PBS. 100 μL of DMSO was added to each well and plates were shaken gently for 15 min to dissolve the coloured

formazan crystals formed by cellular NAD(P)H dependent oxidoreductase enzymes. The absorbance at 595 nm of each well was measured using a microplate reader and a 1420 Multilabel Counter Victor3V spectrophotometer (PerkinElmer, USA). All MTT assays were performed in triplicate at three independent times with six replicate wells for each test compound. For statistical analysis, the mean and standard deviation was calculated using Microsoft® Excel (Microsoft Corporation, USA). The calculations of the IC_{50} concentrations for each cell line, timepoint, complex and control was performed using the statistical package GraphPad Prism (Ver. 6.0) (GraphPad, USA). The IC_{50} value is the concentration of the test compounds that reduces the cellular viability by 50 %.

Reactive oxygen species (ROS) assay

The induction of intracellular oxidative stress in A2780 and A2780cis cells was measured using a 2′,7′-dichlorofluorescein diacetate (DCFH-DA) dye which produces fluorescence on the production of reactive oxygen species. Briefly, cells were seeded into 96-well plates at a density of 1×10^4 cells/well and were incubated for 24 h at 37 °C. The medium was removed and 100 µL of a 20 µM DCFH-DA solution in PBS was added to each well and incubated at 37 °C and 5 % CO_2 for 60 min in darkness. Cells were washed twice with 100 µL PBS and then treated with 100 µL of 10 µM H_2O_2 in PBS (positive control), with PBS alone (untreated control) and a concentration range of 0.47 – 60 µM of each test compound. The fluorescence was measured at different time intervals between 15 and 120 min. ROS levels were determined by measuring the fluorescence of oxidized DCFH-DA using a spectramax M3 multiplate reader with excitation at 485 nm and emission at 530 nm. Three independent experiments were done with six replicate wells used for all test compounds, positive control and untreated control.

Cellular uptake

1×10^6 A2780 cells were seeded in T25 cm^2 flasks in growth medium (5 mL). After overnight incubation the medium was replaced and the cells were treated with IC_{50} concentrations of the tested compounds for 24 h. The cells were washed twice with cold PBS, harvested by trypsinization and counted using a hemocytometer. The cells were vortexed vigorously, frozen at −80 °C, treated with 200 µL of highly pure nitric acid (Trace Metal Grade, 67 – 69 %, Fisher, UK) and transferred into microwave tubes. The tubes were heated at 80 °C in order to digest the cells. After cooling, the samples were diluted with ultrapure water to a final concentration of 1 % HNO_3. Each mineralized sample was filtered and the

platinum content was analyzed by inductively coupled plasma mass spectrometry as described above.

Platination of cellular DNA

A2780 cells (0.8×10^6) were seeded in 9 cm^2 6-well culture plates in 5 mL of RPMI1640 cell culture medium. After overnight incubation, the medium was replaced, and the cells were treated with IC_{50} concentrations of the tested compounds for 24 h. The cells were washed twice with cold PBS, harvested by trypsinization and counted using a hemocytometer. The cells were stored at –80 °C until analysis. The DNA was extracted and purified by using a cell lysis buffer (2.5% Triton X-100, 100 mM Tris-HCl pH 8, 10 mM EDTA, 2% SDS, 200 mM NaCl, 200 µg mL^{-1} proteinase K) and incubated for 2 h at 56 °C. 6 µL of RNAse A solution (0.4 mg mL^{-1}) was added and samples were incubated at room temperature for 1 h. 1000 µL of 2-propanol was added to each sample in order to precipitate the DNA. The samples were stored overnight at –20 °C. The DNA was isolated by centrifugation at 18000 × g for 30 min at 4 °C. Then the DNA pellet was washed with 70 % ethanol. The DNA was dissolved in 200 µL of nuclease-free water and the DNA concentration was determined with a MaestroNano spectrophotometer. The samples were stored at –20 °C until analysis. The DNA samples were dried using a freeze dryer and 300 µL of high purity concentrated HNO_3 was added to all samples. The DNA samples were transferred to borosilicate glass microwave tubes. After heating at 80 °C for 2 h to digest the samples, they were diluted with 1% HNO_3 in ultrapure water. The samples were filtered and analyzed by ICP-MS as described above.

Determination of DNA double-strand breaks (DSBs)

The occurrence of DSBs in A2780 and A2780cis cells was quantified by using immunodetection of γ-H2AX foci by flow cytometry. Cells were seeded at different densities (1×10^4 cells/well and 8×10^3 cells/well for 24 and 48 h, respectively) and incubated at 37 °C in a humidified, 5 % CO_2 atmosphere for 24 h. The cells were exposed to IC_{50} concentrations of the Platinum(IV) complexes, cisplatin and PBA over the two incubation times, 24 h and 48 h. Then, the culture medium was removed and the cells were washed with 2 × 4 mL PBS. The cells were harvested and fixed in 200 µL 2% (v/v) paraformaldehyde in PBS for 10 min at room temperature. The solution was removed and 1 mL of cold 95 % ethanol was added to fix the cells which were then stored at 4 °C overnight. The ethanol was removed after centrifugation at 800 × g for 5 min and cells were washed once with PBS and resuspended in 1 mL of 0.25% (v/v) Triton X-100 in PBS for 5 min at RT to permeabilise the cells. This was

removed and cells were resuspended in 500 µL of blocking solution (PBS containing 4 % fetal bovine serum (FBS)) for 30 min at room temperature. The primary antibody (Anti-phospho-histone H2A.X (ser139) antibody, clone JBW301, Mouse, Millipore) was diluted 1:800 with blocking solution and was added to each sample and incubated overnight at 4 °C. The samples were washed with 500 µL of blocking solution and the secondary antibody (Alexa Fluor 488 goat anti-mouse immunoglobulin G (IgG, Invitrogen) was added to the samples for 1 h in the dark. Samples were washed with 500 µL PBS before analysis. The BD Accuri C6 (BD Biosciences, USA) flow cytometer was used to detect foci formation in cells staining positive for γ-H2AX fluorescence and was measured at excitation 495 nm and emission at 519 nm. At least three independent experiments were conducted for 1×10^4 cells per analysis.

Immunodetection of γ-H2AX foci with confocal microscopy

Fixed cells were stained with γ-H2AX and propidium iodide as described above. 100 µL of cells were aliquoted and deposited on glass slides by cytospin centrifugation at 1200 rpm for 15 min. Samples were dried quickly at room temperature for 30 min. Clover slips (0.085 - 0.13 mm thick) were mounted on the slides by using mounting medium and sealed with clear nail varnish. The LSM© 510 Meta Confocal Microscope (Zeiss, Germany) was used to capture images. The images were analysed using ImageJ software. All images were taken at 63× magnification with both argon and helium-neon lasers simultaneously scanning into its four component channels; green (γ-H2AX foci), red (propidium iodide), merged and brightfield.

Apoptosis

The induction of apoptosis in A2780 and A2780cis cells was quantified by flow cytometry using FITC-conjugated annexin V (BD Pharmingen™, UK) and propidium iodide (BD Pharmingen™, UK) staining. Both cell lines were seeded into T25 flasks at different densities for two different incubation times; 1×10^4 cells/well for 24 h and 8×10^3 cells/well for 48 h incubation. After 24 h new medium was added and the cells were incubated with IC_{50} doses of the test and reference compounds at 37 °C in a humidified, 5 % CO_2 atmosphere for 24 and 48 h. The cells were trypsinised, harvested and centrifuged (400 × g for 6 min). The cells were washed twice with cold PBS followed by 1X binding buffer (2 ×). The cells were resuspended in 1X binding buffer at a concentration of 1×10^6 cells/mL and stained with 5 µL of FITC Annexin V for 15 min. Then 5 µL propidium iodide was added

and incubated for 15 mins in the dark. 400 µL of 1X binding buffer was then added and each sample was analyzed by flow cytometry. At least three independent experiments were conducted for 1×10^4 cells per analysis.

HDAC activity/inhibition direct assay

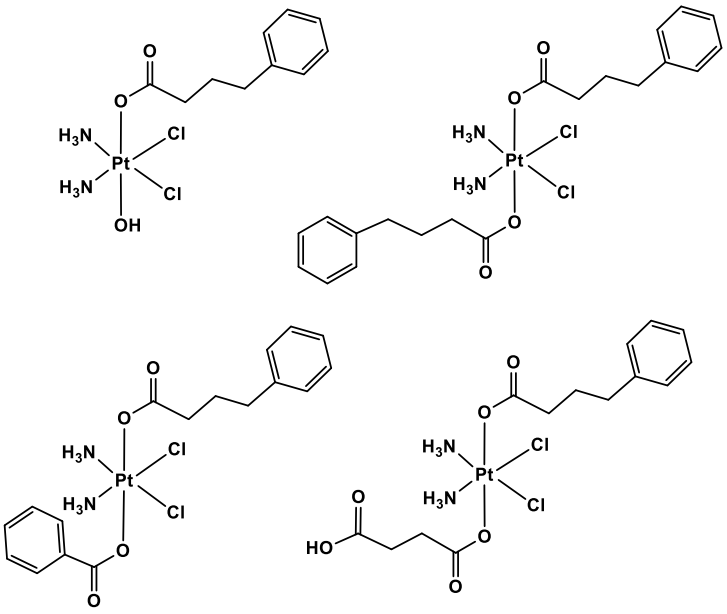
Histone deacetylase activity/inhibition was determined using the EpiQuik™ HDAC Activity/Inhibition Direct Assay Kit (Colorimetric; Farmingdale, NY). Briefly, A2780 and A2780cis cells were seeded (1×10^6) in T25 cm^2 flasks in growth medium. Cells were treated for 24 h with IC_{50} concentrations of the tested compounds and then processed according to the EpiGentek manufacturer's instructions. The measurement of HDAC activity was done in nuclear extracts according to the manufacturer instructions of a Nuclear Extraction Kit (Nucleic Acid-Free; EpiGentek, Farmingdale, NY). The absorbance was read on a spectraMax M3 multiplate reader at 450 nm within 2–15 min.

Results and discussion

Synthesis and characterization of the complexes

The chemical structures of the platinum(IV) complexes investigated in this study are shown in (**Scheme 1**). The monosubstituted complex *cis*,*cis*,*trans*-[$Pt(NH_3)_2Cl_2(PBA)(OH)$] (**A**) was synthesized by reacting *cis,cis,trans*-[$Pt(NH_3)_2Cl_2(OH)_2$] with 0.8 equivalents of the activated NHS-ester of PBA in dimethylsulfoxide at 70 °C for 48 h (Scheme S1, Supplementary Material). Reaction of **A** with benzoic and succinic anhydride in dimethylformamide gave the unsymmetric complexes *cis,cis,trans*-[$Pt(NH_3)_2Cl_2(PBA)(Bz)$] (**C**) and *cis,cis,trans*-[$Pt(NH_3)_2Cl_2(PBA)(Suc)$] (**D**). Benzoate and succinate were attached to the second axial position in order to vary the lipophilicity. The bis-substituted complex *cis*,*cis,trans*-[$Pt(NH_3)_2Cl_2(PBA)_2$] (**B**) was previously synthesized by Gibson and coworkers (Raveendran et al. 2016). The purity and composition of the complexes were confirmed by elemental analysis, HPLC, electrospray ionisation mass spectrometry, and ^1H and ^{195}Pt NMR spectroscopy.

The log $P_{o/w}$ values, determined using the shake-flask method, were found to be -0.12 ± 0.05 (**A**), 0.16 ± 0.07 (**B**), 0.08 ± 0.02 (**C**) and -0.14 ± 0.08 (**D**) and demonstrate that the complexes follow the expected order of lipophilicity of **B** > **C** > **A** > **D**.



Scheme 1: Chemical structures of complexes **A** – **D**.

As platinum(IV) pro-drugs require activation by biological reducing agents, the reduction of the monosubstituted complex (**A)** and of a representative biscarboxylated complex (**C**) was monitored under physiological conditions (37 °C, pH 7) in the presence of ascorbic acid using HPLC (**Figs. 1 and S1**). The chromatograms show the release of the axial PBA ligand that accompanies the conversion of the octahedral platinum(IV) complexes to square-planar cisplatin. In the case of **C** both, free PBA and free benzoate were identified in the chromatograms. **A** and **C** were completely reduced after 48 and 20 h, respectively. The reduction kinetics of platinum(IV) complexes depend on the nature of the axial ligands, the equatorial ligands and the reducing agent (Wexselblatt et al. 2012). Complexes with an axial hydroxido ligand are usually reduced faster by ascorbate than bis-carboxylated complexes, as the OH group can act as a bridging ligand and facilitate the electron transfer *via* an inner-sphere mechanism (Zhang et al. 2012). In the case of complexes of type *cis,cis,trans*-$[Pt(NH_3)_2Cl_2L_2]$ with two equatorial chlorido ligands, however, the rate of reduction by ascorbate often correlates with the reduction potential; L = OH (~ -900 mV) < L = $RCOO^-$ (~ -600 mV) (Graf et al. 2012; Johnstone et al. 2016) and the faster reduction kinetics of **C** compared to **A** are consistent with this.

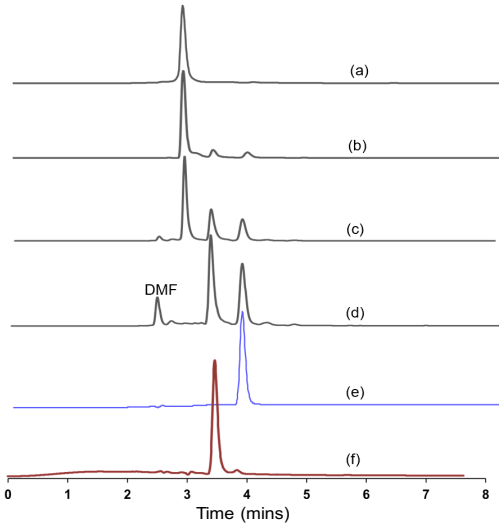


Figure 1: HPLC chromatograms of the reaction of **C** with 10 eq. ascorbic acid at 37 °C and pH 7. (a) t = 0, (b) 3 h, (c) 9 h, (d) 20 h, (e) the free ligand PBA and (f) the free ligand Bz.

Cytotoxicity

The cytotoxic activities of the complexes were evaluated in two human ovarian carcinoma cells lines, cisplatin-sensitive A2780 and cisplatin-resistant A2780cis. The cytotoxicity was determined after incubation for 24 and 48 h using the tetrazolium based MTT colorimetric assay. For comparison purposes, the cytotoxicities of cisplatin and of the uncoordinated HDACi PBA were assessed under the same experimental conditions. IC_{50} values were calculated from the growth inhibition curves and are shown in (**Table 1**). The platinum(IV) complexes displayed dose- and time-dependent cytotoxicities. The IC_{50} values of **A** – **C** are in the low micromolar range, while **D** is only modestly active. In the A2780 cell line, the cytotoxic activities follow the order **C** >> **B** ~ **A** > cisplatin >> **D**. **A** – **D** exhibit similar activities in cisplatin-sensitive and -resistant cells with resistance factors close to 1. The IC_{50} values of the most effective complex **C** were 16- and 37-fold lower than those of cisplatin in the two cell lines (48 h incubation). A comparison of the IC_{50} values at the 24 h and 48 h timepoints shows a decrease by a factor of 4.9 - 3.3 for the PBA complexes on prolonged exposure compared to a factor of about 2 for cisplatin. The delayed cytotoxicity of the platinum(IV) complexes can be attributed to the requirement for intracellular activation. The fact that the largest reduction in IC_{50} (4.9-fold) is observed for **A** correlates with the slow reduction kinetics of the monocarboxylated complex (complete reduction within 48 h (**A**) *vs*. 20 h (**C**)). In agreement with the literature (Raveendran et al. 2016), free PBA elicited no

cytotoxicity at micromolar concentrations. At physiological pH, the carboxyl group is deprotonated so that the IC_{50} value of PBA in the millimolar range may be a consequence of the hampered cellular uptake of its negatively charged form. It has been pointed out in the literature that platinum(IV) PBA complexes are not only pro-drugs of the platinum(II) agent but also present pro-drugs for PBA and this can involve synergistic accumulation (Raveendran et al. 2016).

Table 1: IC_{50} values of platinum(IV) complexes A – D, cisplatin and free ligand PBA in A2780 and A2780cis cells determined by the MTT test

	IC_{50} (μM) ± S.D.				R.F.[a]	
	A2 780		A2780cis			
	24 h	48 h	24 h	48 h	24 h	48 h
A	24.16 ± 7.1	4.93 ± 0.19	35.63 ± 11.6	6.33 ± 2.1	1.5	1.3
B	18.41 ± 4.3	4.59 ± 1.3	14.16 ± 2.2	5.09 ± 1.1	0.8	1.1
C	1.81 ± 0.1	0.51 ± 0.16	3.13 ± 1.4	0.71 ± 0.36	1.7	1.4
D	124.1 ± 26.9	38.10 ± 8.5	149.39 ± 36.8	43.24 ± 6.5	1.2	1.1
cisplatin	16.44 ± 5.2	8.27 ± 2.4	55.44 ± 14.1	26.54 ± 4.7	3.4	3.2
PBA	1945 ± 59.9	1587 ± 47.2	2076 ± 84.5	1970 ± 39.7	1.1	1.2

[a] R.F. = ratio of IC_{50} values for A2780 and A2780cis cells

Cellular uptake and DNA platination

(Fig. 2a) shows the Pt content in A2780 cells after exposure to IC_{50} doses of cisplatin and complexes **A** – **D** for 24 h. Except for **D**, the platinum(IV) complexes accumulate more efficiently than cisplatin in cells. The poor cellular uptake of **D** can explain the low cytotoxicity of this complex and may be attributed to the hydrophilic succinate ligand which would be negatively charged at pH 7.4. For **A** – **C**, however, the uptake (**B** < **C** < **A**) does not follow the order of increasing lipophilicity (**A** < **C** < **B**) which has also been reported for other platinum(IV) complexes (Raveendran et al. 2016; Tolan et al. 2016; Zajac et al. 2016).

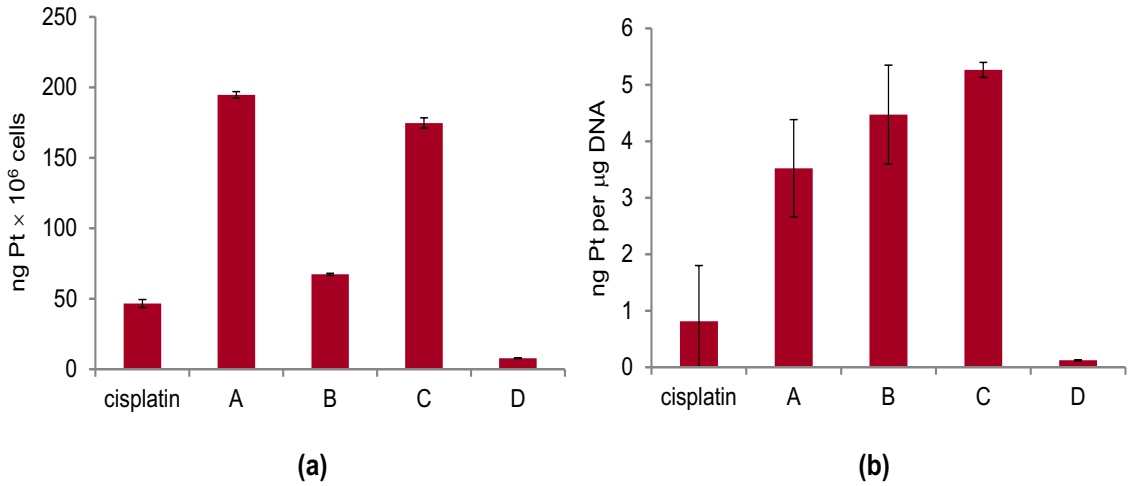


Figure 2: (a) Cellular uptake of cisplatin and complexes **A** – **D** into A2780 cells. (b) DNA platination in A2780 cells incubated with cisplatin and complexes **A** – **D.** Error bars represent standard deviations from three independent measurements. Cells were incubated with IC_{50} doses of the compounds (Table 1, 24 h timepoint) for 24 h.

As the binding to nuclear DNA is considered to be a crucial event in the cytotoxic action of platinum drugs, the Pt contents of the DNA from treated cells were also determined. A2780 cells were exposed to IC_{50} doses of **A** – **D** and cisplatin for 24 h and after extraction of the DNA the Pt concentration was measured by ICP-MS. The results, expressed as ng Pt per µg of DNA are shown in (**Fig. 2b)**. The platinum(IV) complexes gave higher levels of DNA platination than cisplatin, except for **D** that does not enter the cell effectively. It can be assumed that the cellular uptake and reduction kinetics are important for the DNA platination levels after 24 h incubation. The DNA platination in cells treated with **A** and **C** is coherent with this, while the high platination levels observed for complex **B** are somewhat surprising

given its relatively low cellular accumulation. It might be speculated that the low cellular uptake is compensated by the strong HDAC inhibitory activity of the complex (see below) that can lead to a greater accessibility of the DNA by keeping the chromatin structure open (Strahl et al. 2000). The IC_{50} values at the 48 h timepoint (**C** >> **B**, **A** > cisplatin > **D**) correlate with the Pt content in the DNA (**C** > **A** > **B** > cisplatin > **D**). However, at the 24 h timepoint, **A** and **B** have higher IC_{50} values than cisplatin despite their higher DNA platination levels. Apparently, the platinum(IV) complexes take longer to exert their cytotoxicity.

Cellular reactive oxygen species production

The induction of cellular reactive oxygen species (ROS) production by Pt drugs has been discussed in the literature (Pichler et al. 2015). Kaluderovic et al. reported enhanced cellular ROS levels in cells treated with tetrachlorido platinum(IV) complexes (Kaluderovic et al. 2002). The redox and mitochondrial stability can be stressed by treatment with cisplatin due to ROS production (Jungwirth et al. 2011; Pelicano et al. 2004). We therefore investigated the generation of ROS in A2780 and A2780cis cells after incubation with **A** – **D**, cisplatin and PBA. ROS levels were monitored over a 2 h period using 2',7'-dichlorofluorescein diacetate (H_2DCFDA) as a peroxide-sensitive fluorescent dye (**Figs. 3 and S2**). A2780 cells treated with cisplatin, PBA and the platinum(IV) complexes showed a time- and concentration-dependent increase in ROS production. However, while cisplatin, the free ligand and **A** and **D** had moderate effects only, concentrations of **C** in the IC_{50} range resulted in markedly enhanced ROS levels, comparable to those induced by the positive control H_2O_2. There were no significant differences in ROS generation on treatment with **C** between the sensitive and resistant cell line.

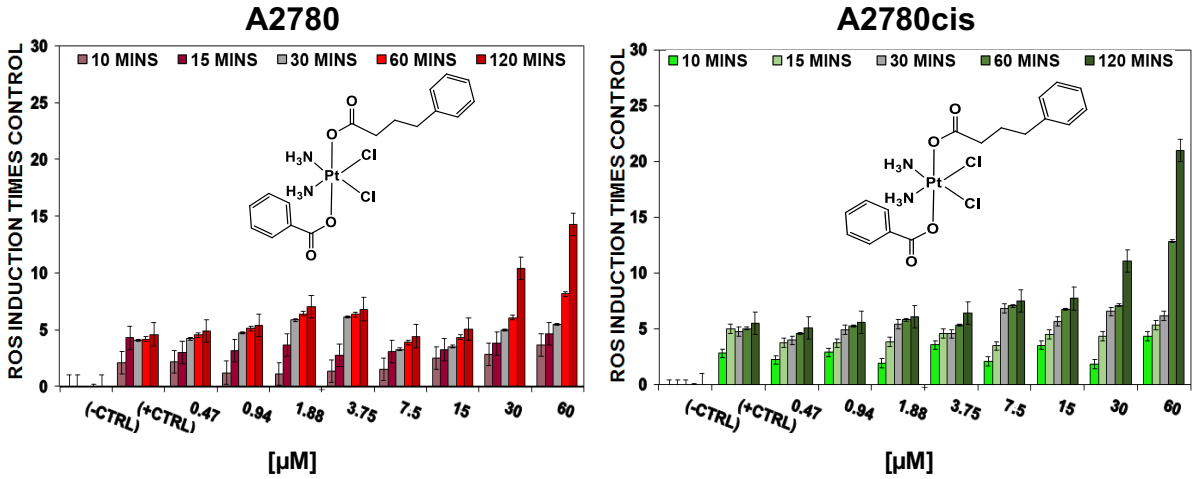


Figure 3: Fluorescent detection of ROS using DCFDA in A2780 and A2780cis cells after exposure to different concentrations of complex **C** over a period of 2 h. H_2O_2 (10 µM) was used as a positive control. Error bars represent standard deviations (n=3).

HDAC inhibition

The ability of the three most cytotoxic platinum(IV) pro-drugs to inhibit HDAC activity following activation and release of the HDACi PBA was measured in nuclear extracts of the cisplatin-sensitive and -resistant cell lines (**Fig. 4**). A2780 and A2780cis cells were incubated with IC_{50} doses (Table 1, 24 h timepoint) of **A** – **C** for 24 h. Cisplatin, the free ligand PBA and the known HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) were used as controls. All treated cells showed reduced HDAC activity. **B** and **C** had the strongest effect on HDAC activity and are more potent HDAC inhibitors than SAHA. The decrease in HDAC activity in A2780 and A2780cis cells treated with cisplatin is noteworthy, as this is in contrast to the study by Raveendran *et al*. who observed no HDAC inhibitory activity in MCF-7 cells exposed to cisplatin (Raveendran et al. 2016).

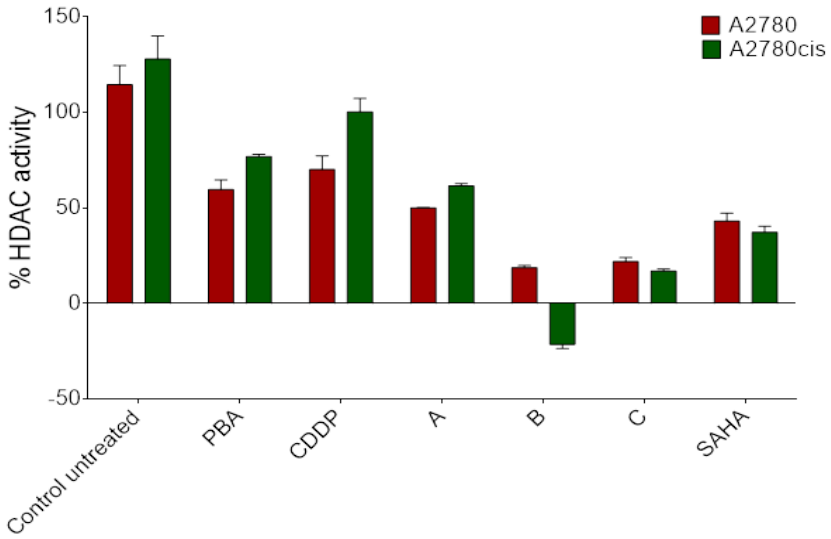


Figure 4: HDAC activity measured in nuclear extracts of A2780 and A2780cis cells after exposure to IC_{50} concentrations of **A** – **C**, cisplatin, PBA (Table 1, IC_{50} at the 24 h timepoint) and SAHA (IC_{50} (24 h) determined by the MTT assay: 30.2 µM (A2780) and 60.8 µM (A2780cis)).

DNA damage studies by immuno-detection of γ-H2AX foci

DNA damage induced by the coordination of cisplatin to the *N7* position of guanine bases and the formation of cisplatin-DNA cross-links triggers various cellular mechanisms such as cell cycle arrest, activation of repair mechanisms, and cell death pathways (Johnstone et al. 2016; Wang et al. 2005). When the DNA damage leads to double strand breaks (DSBs),

Ser-139 of the histone H2AX is rapidly phosphorylated at the site of the DSB resulting in the formation of discrete foci. The γ-H2AX foci can be detected and quantified by immunofluorescence (Ohnishi et al. 2009; Podhorecka et al. 2010; Takahashi et al. 2005). The number of γ-H2AX foci is directly proportional to the DSBs and is an indicator of DNA repair efficacy. H2AX phosphorylation has been observed on treatment with cisplatin (Huang et al. 2004; Pines et al. 2011). It is believed that the phosphorylation of H2AX plays a crucial role in the detection of DNA damage and in the activation of DNA repair pathways.

The immunofluorescence microscopy images of A2780 and A2780cis cells incubated with IC_{50} doses of complexes **A** – **C** for 24 and 48 h are depicted in **Fig. 5**. The formation of γ-H2AX foci is clearly visible. The images of cells treated with cisplatin and PBA are also shown for comparison.

γ-H2AX foci generation after 24 and 48 h exposure to IC_{50} concentrations of **A** – **C**, cisplatin and PBA was determined quantitatively by flow cytometry (**Fig. 6**). Compared to the untreated control, a significant increase in γ-H2AX foci was observed in A2780 cells treated with PBA, cisplatin, **A**, **B**, and **C** with the strongest response being elicited by **B**. Cells treated with the most potent complex **C** also show significant more DNA damage than cells treated with cisplatin. It has been reported that resistance towards cisplatin in A2780cis cells is caused by increased glutathione levels, enhanced DNA damage repair and/or enhanced DNA damage tolerance (Behrens et al. 1987). After 48 h, some decrease in γ-H2AX foci is observed in A2780 cells exposed to **B** and cisplatin indicating DNA repair. Overall, there is more DNA damage repair in the resistant A2780cis cells compared to the sensitive cell line as expected (**Fig. 6b**).

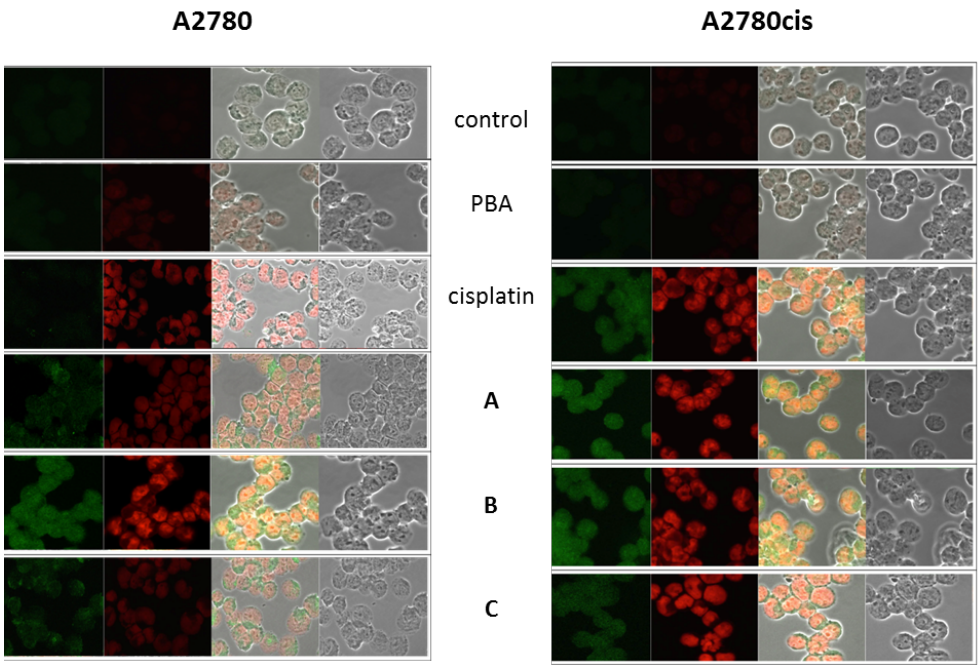


Figure 5: A2780 and A2780cis cells exposed to IC_{50} concentrations of PBA, cisplatin and complexes **A**, **B** and **C** (Table 1, IC_{50} at the 24 h timepoint) in comparison to the negative control. γ-H2AX foci were visualized using primary antibody staining with fluorescent FITC secondary label. Propidium iodide was used as a nuclear counter stain.

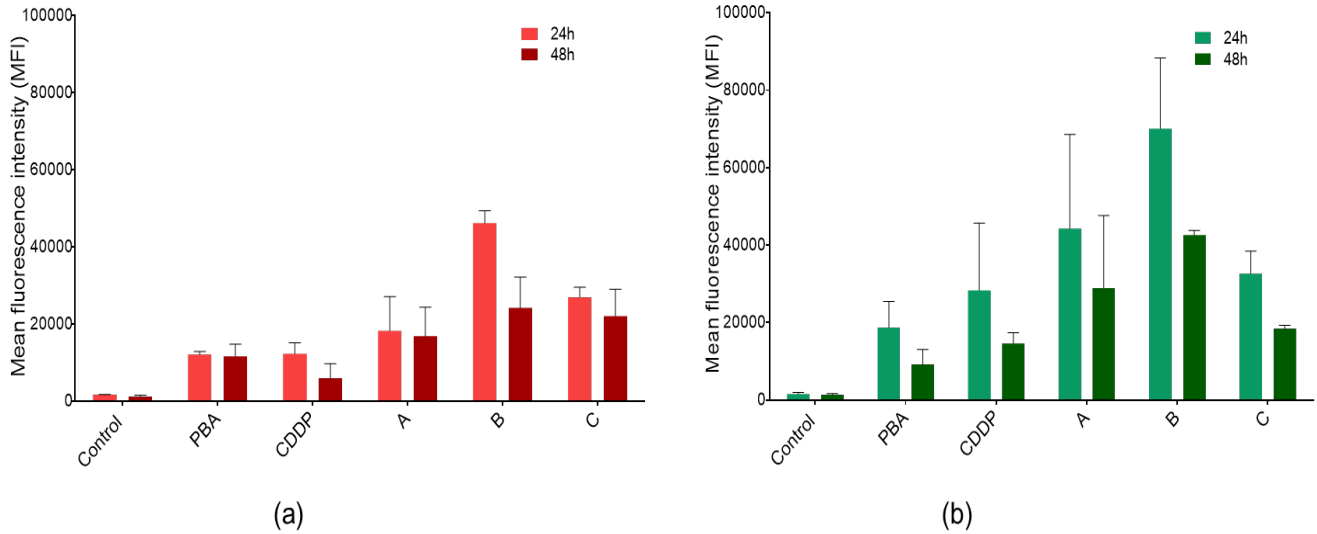


Figure 6: Immuno-detection of γ-H2AX foci post exposures in (a) A2780 and (b) A2780cis cells treated with IC_{50} doses of complexes **A** – **C**, cisplatin and PBA (Table 1, IC_{50} at the 24 h timepoint). Error bars represent standard deviations of three independent measurements.

Apoptosis

The induction of apoptosis in A2780 and A2780cis cells treated with **A**, **B**, **C**, cisplatin and PBA was studied using double staining with annexin V/propidium iodide (AV/PI) and flow cytometry (**Fig. 7**). Double staining allows for viable cells (AV-/PI-) to be distinguished in early apoptosis (AV+/PI-), late apoptosis (AV+/PI+) and necrosis (AV-/PI+). Treatment of A2780 and A2780cis cells with IC_{50} concentrations of the platinum(IV) complexes resulted in a significant percentage of late apoptotic cells after 24 h. The percentage of cells in late apoptosis are in the order **C** > **A** > cisplatin > **B** >> PBA and is similar in both cell lines. The percentage of late apoptotic cells decreases after 48 h. The observation that complex **C** leads to the highest percentage of late apoptotic cells correlates with its low IC_{50} value. Unlike cisplatin, for which a clear difference in the percentage of late apoptotic A2780 and A2780cis cells is observed, a similarly high percentage of cells of both cell lines enter the late apoptotic state on exposure to complex **C** confirming that the platinum(IV) complex is less discriminative, in line with its RF value of 1.4. Apparently, the mechanism of cisplatin-resistance in A2780cis cells does not interfere with the ability of **C** to elicit its cytotoxic effect after activation and release of PBA and cisplatin.

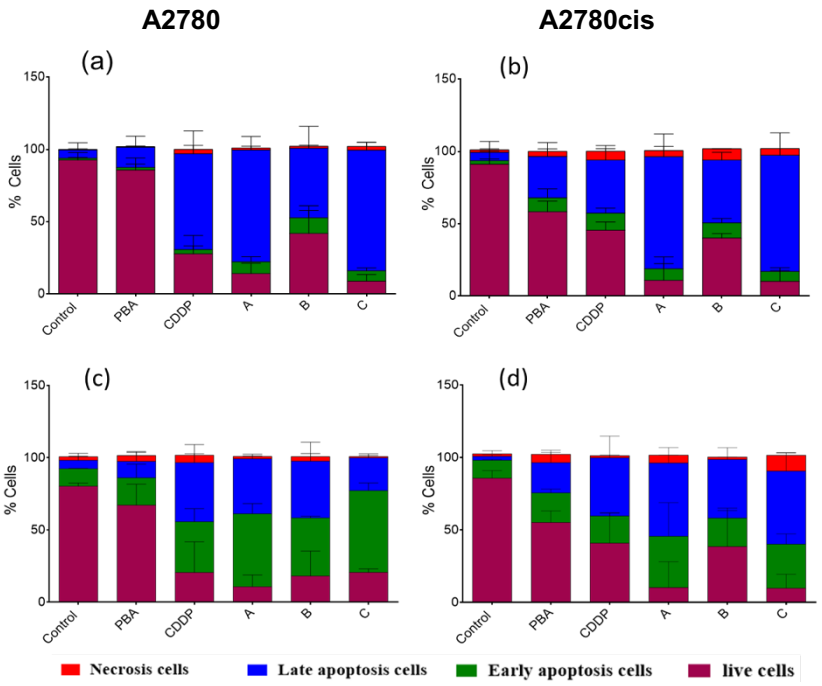


Figure 7: Annexin V/PI staining and flow cytometry analysis of A2780 and A2780cis cells after24 h (a, b) and after 48 h (c, d) exposure to IC_{50} concentrations of **A**, **B**, **C**, cisplatin and PBA (Table 1, IC_{50} at the 24 h timepoint).

Conclusions

The monosubstituted complex *cis*,*cis*,*trans*-[$Pt(NH_3)_2Cl_2(PBA)(OH)$], the symmetrically disubstituted complex *cis*,*cis*,*trans*-[$Pt(NH_3)_2Cl_2(PBA)_2$] and the unsymmetrically disubstituted complex *cis*,*cis*,*trans*-[$Pt(NH_3)_2Cl_2(PBA)(Bz)$] overcome cisplatin resistance, are more cytotoxic in A2780 and A2790cis ovarian cancer cells and induce cell death pathways leading to apoptosis in both cell lines.

While our study confirms that the three platinum(IV) complexes interact with their intended targets, DNA and HDAC, it has to be kept in mind that their mode(s) of action may be more complex. PBA is also known to activate transcription factors that upregulate genes for lipid metabolizing enzymes (Pineau et al. 1996) and to reverse the Warburg effect by inhibiting pyruvate dehydrogenase kinase (Ferriero et al. 2015). The suggestion that many platinum(IV) complexes with a bioactive axial ligand designed as dual-action pro-drugs may in fact be multi-action pro-drugs was discussed in a recent review article (Gibson 2019). The ROS data seem to support a multimodal mechanism for the PBA complexes. ROS play a role in the activation of signalling pathways and transcription factors that induce cell death processes including apoptosis. At concentrations close to its IC_{50} value the most cytotoxic complex **C** leads to markedly increased cellular ROS levels which can mediate apoptosis independent of DNA damage. Consistently, the γ-H2AX foci generation data confirm that the platinum(IV) PBA complexes kill cancer cells only to a certain extent by DNA damage and that DNA damage is only one of their cytotoxic effects.

In summary, *cis*,*cis*,*trans*-[$Pt(NH_3)_2Cl_2(PBA)(OH)$], *cis*,*cis*,*trans*-[$Pt(NH_3)_2Cl_2(PBA)_2$] and *cis*,*cis*,*trans*-[$Pt(NH_3)_2Cl_2(PBA)(Bz)$] are potent cytotoxins that act via multimodal mechanisms involving DNA damage *via* γ-H2AX foci production and cell death mechanisms independent of cisplatin resistance.

Acknowledgements

Taibah University, Saudi Arabia is acknowledged for a PhD scholarship to A.R.Z.A

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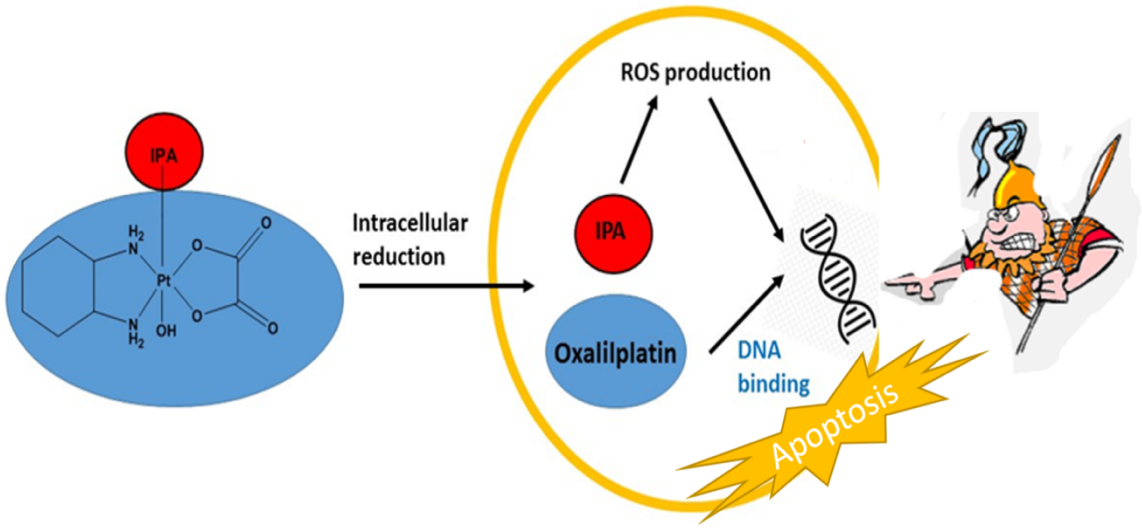
Result:

Chapter 4: Cytotoxicity and ROS Production of Novel Platinum(IV) Oxaliplatin Derivatives with Indole Propionic Acid



4. Brief description of the paper

Five new platinum(IV) derivatives of oxaliplatin with a redox modulator in axial position were synthesised. *trans*-[Pt(DACH)(ox)(IPA)(OH)] has the highest cytotoxicity and is the most potent ROS producer in A2780 and A2780cis cells.

Inorganica Chimica Acta 492 (2019) 262–267

Contents lists available at ScienceDirect

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Cytotoxicity and ROS production of novel Pt(IV) oxaliplatin derivatives with indole propionic acid

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ARTICLE INFO

Keywords:
Oxaliplatin
Pt(IV) prodrugs
Redox stress
Indole propionic acid
Cytotoxicity

ABSTRACT

The coordination of biologically active moieties to the axial positions of Pt(IV) derivatives of Pt(II) anticancer drugs allows the co-delivery and simultaneous activation of two pro-drugs for combination therapy. Pt(IV) complexes with a redox modulator as an axial ligand can kill cancer cells by a mechanism combining DNA platination and generation of oxidative stress. In this study we evaluated the cytotoxicity of Pt(IV) complexes based on the oxaliplatin scaffold and the pro-oxidant indole-3-propionate in cisplatin-sensitive and cisplatin-resistant ovarian cancer cells. A series of five complexes was synthesized and characterized by ^{1}H and ^{195}Pt NMR spectroscopy, IR spectroscopy, mass spectrometry and elemental analysis; *trans*-[Pt(DACH)(ox)(IPA)(OH)] (**1**), *trans*-[Pt(DACH)(ox)(IPA)$_2$] (**2**), *trans*-[Pt(DACH)(ox)(IPA)(bz)] (**3**), *trans*-[Pt(DACH)(ox)(IPA)(suc)] (**4**), and *trans*-[Pt(DACH)(ox)(IPA)(ac)] (**5**) (DACH = 1,2-diaminocyclohexane (1R,2R)-(−), ox = oxalate, IPA = indole-3-propionate, bz = benzoate, suc = succinate and ac = acetate). The complexes were shown to produce cellular reactive oxygen species (ROS) in a time-dependent manner. The most potent ROS producer, complex **1**, also elicited the highest cytotoxicity. Complex **1** was shown to form the mono- and bis-adducts [Pt(DACH)(guanosine)(OH)]$^{+}$ and [Pt(DACH)(guanosine)$_2$]$^{2+}$ in the presence of ascorbic acid, suggesting that on activation the released oxaliplatin will interact with DNA.

Authors	Contributions
Dina Tolan	Synthesised and characterised the complexes
Awatif Rashed Z .Almotairy	Carried out all biological studies
Orla Howe	Managed the project and reviewed manuscript
Michael Devereux	Reviewed manuscript
Diego Montagner	Managed the project and reviewed manuscript
Andrea Erxleben	Managed the project, reviewed manuscript prior to and post the review process

Cytotoxicity and ROS Production of Novel Platinum(IV) Oxaliplatin Derivatives with Indole Propionic Acid

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Abstract

The coordination of biologically active moieties to the axial positions of platinum(IV) derivatives of platinum(II) anticancer drugs allows the co-delivery and simultaneous activation of two pro-drugs for combination therapy. Platinum(IV) complexes with a redox modulator as an axial ligand can kill cancer cells by a mechanism combining DNA platination and generation of oxidative stress. In this study we evaluated the cytotoxicity of platinum(IV) complexes based on the oxaliplatin scaffold and the pro-oxidant indole-3-propionate in cisplatin-sensitive and cisplatin-resistant ovarian cancer cells. A series of five complexes was synthesized and characterized by ^{1}H and ^{195}Pt NMR spectroscopy, IR spectroscopy, mass spectrometry and elemental analysis; *trans*-[Pt(DACH)(ox)(IPA)(OH)] (**1**), *trans*-[Pt(DACH)(ox)$(IPA)_2$] (**2**), *trans*-[Pt(DACH)(ox)(IPA)(bz)] (**3**), *trans*-[Pt(DACH)(ox)(IPA)(suc)] (**4**), and *trans*-[Pt(DACH)(ox)(IPA)(ac)] (**5**) (DACH = 1,2-diaminocyclohexane (1R,2R)-(−), ox = oxalate, IPA = indole-3-propionate, bz = benzoate, suc = succinate and ac = acetate). The complexes were shown to produce cellular reactive oxygen species (ROS) in a time-dependent manner. The most potent ROS producer, complex **1**, also elicited the highest cytotoxicity. Complex **1** was shown to form the mono- and bis-adducts [Pt(DACH)(guanosine)(OH)]$^{+}$ and [Pt(DACH)$(guanosine)_2]^{2+}$ in the presence of ascorbic acid, suggesting that on activation the released oxaliplatin will interact with DNA.

Keywords: Oxaliplatin, Platinum(IV) prodrugs, Redox Stress, Indole Propionic Acid, Cytotoxicity

Introduction

About 50 % of cancer patients who undergo chemotherapy receive one of the three worldwide approved platinum(II) complexes, cisplatin, carboplatin and oxaliplatin, either alone or in combination with other anticancer drugs [1]. Co-administration of two or more chemotherapeutics with independent modes of action is widely used to reduce the risk of resistance development and to enhance the anticancer efficacy through synergistic effects [2]. However, different selectivities and pharmacokinetics of the individual drugs can pose significant challenges in the application of combination therapy regimens. Oxidation of platinum(II) to platinum(IV) allows the covalent attachment of a second biologically active entity to the axial positions of the cisplatin-, carboplatin- or oxaliplatin-scaffold [3-20]. In addition to the simultaneous delivery of the Pt drug and the co-administered drug, platinum(IV) complexes have the advantages of a higher stability, lower toxicity, reduced side effects and potential for oral administration [3,4]. Dual-action platinum(IV) compounds are pro-drugs that require activation by intracellular reducing agents to release the active platinum(II) species and the axial ligands (Chart 1) [3,21].

We have recently reported that the coordination of indole carboxylic acids to the axial positions of the platinum(IV) derivative of cisplatin leads to potent anticancer agents that overcome cisplatin resistance [22]. Indole carboxylic acids are redox modulators that can act as pro-oxidants [23-27]. Indole acetic acid, for example, is used in the oxidation therapy of cancer [23]. We showed that the platinum(IV) indole-3-propionic acid complexes *cis,cis,trans*-[$Pt(NH_3)_2Cl_2(IPA)(OH)$], *cis,cis,trans*-[$Pt(NH_3)_2Cl_2(IPA)_2$], *cis,cis,trans*-[$Pt(NH_3)_2Cl_2(IPA)(Bz)$] and *cis,cis,trans*-[$Pt(NH_3)_2Cl_2(IPA)(Ac)$] (IPA = indole-3-propionate, Bz = benzoate, Ac = acetate) exert their cytotoxic activity by a dual-action mechanism combining DNA platination and the generation of reactive oxygen species (ROS). *cis,cis,trans*-[$Pt(NH_3)_2Cl_2(IPA)(OH)$] was found to be up to four times more effective than cisplatin [22].

Oxaliplatin (**Chart 1**) is a third-generation platinum(II) drug that is approved for the treatment of metastatic colorectal cancer and is also active against lung and ovarian cancer cell lines [28,29]. It contains a more stable leaving group compared to cisplatin and the *R,R*-diaminocyclohexane (DACH) chelating ligand [30]. The latter leads to a spectrum of activity that is different from that of cisplatin and carboplatin. Oxaliplatin also shows a different resistance profile and is usually better tolerated than cisplatin [31,32]. In continuation of our previous work we therefore synthesized the oxaliplatin analogues of the *cis,cis,trans*-

$[Pt(NH_3)_2Cl_2(IPA)Y]$ complexes and investigated their cytotoxicity and ROS generating capability in cisplatin-sensitive and cisplatin-resistant ovarian cancer cells.

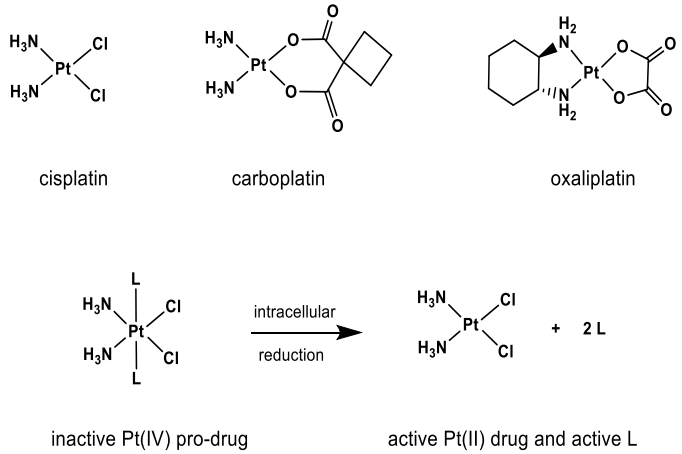


Chart 1: The three FDA-approved platinum(II) anticancer drugs, general structure of platinum(IV) pro-drugs for cisplatin and pro-drug activation by reduction.

Results and Discussion

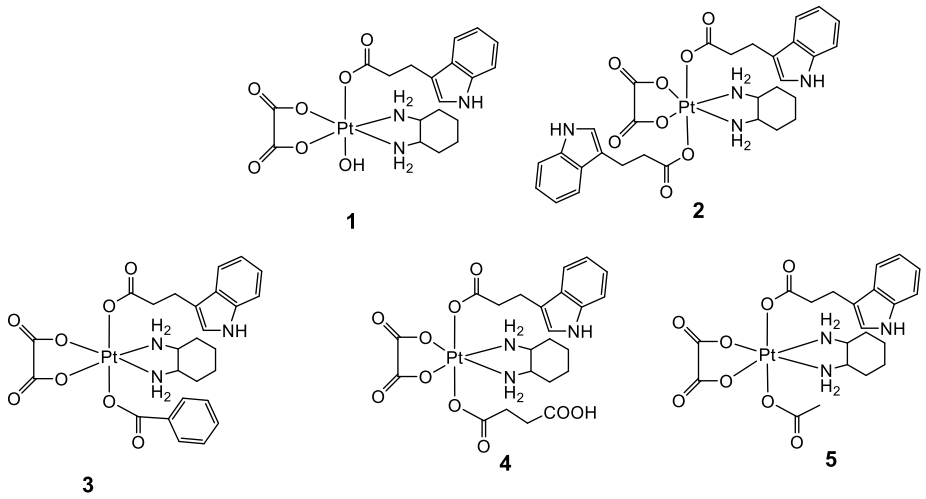


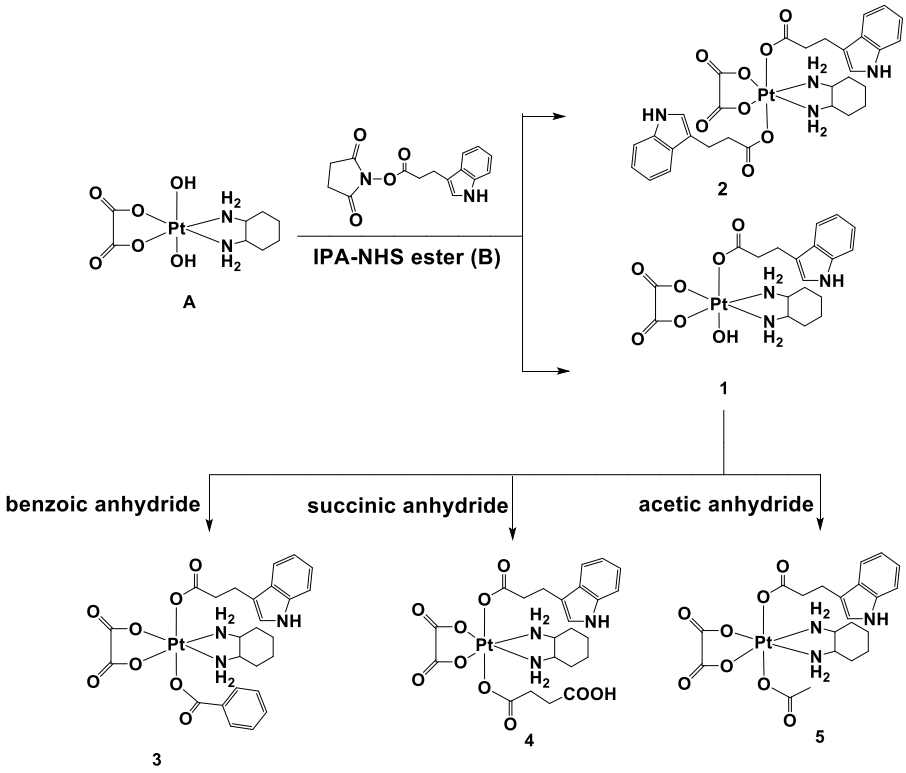
Chart 2: Structures of complexes **1** – **5**

The five complexes based on oxaliplatin (**Chart 2**) were synthesised as shown in Scheme 1 and in the Experimental Section in the Supporting Information.

Briefly, oxaliplatin was oxidised to the corresponding di-hydroxido platinum(IV) complex (**A**) by H_2O_2 in aqueous solution (**Scheme 1**). **A** was reacted with the activated *N*-hydroxysuccinimide (NHS) ester of indole-3-propionic acid (**B**) in DMSO solution to obtain complex **1**. This complex was the precursor to obtain complexes **3** - **5** that could be isolated by precipitation with diethyl ether from a DMF solution containing **1** and the corresponding anhydride; benzoic (**3**), succinic (**4**) and acetic (**5**) anhydride, respectively. **2** was obtained by reaction of **A** with a large excess of **B** in DMSO.

The complexes were characterized by ^{1}H and ^{195}Pt-NMR spectroscopy, infra-red spectroscopy, elemental analysis and mass spectrometry (Supporting Information, Figures 1S –12S). The purity and the stability of the complexes for 36 h have been evaluated in HEPES buffer solution by HPLC (Figure 13S). Elemental analyses are in good agreement with the expected values for the complexes. In the ESI-MS spectra the $[M - H]^-$ or $[M + Cl]^-$ parent ions of the platinum(IV) complexes were readily observed with the typical isotope pattern of Pt. The IR spectra displayed characteristic C=O stretching vibrations around 1710 cm^{-1} and 1650 cm^{-1} for all the complexes while the symmetric and antisymmetric N-H stretching bands of the amine ligands were found between 3325 and 3400 cm^{-1} and between 3150 and 3190 cm^{-1}.

The proton resonances of the coordinated amine ligands in the ^{1}H NMR spectrum of **1** appear as multiplet at *ca*. 7.08 - 8.52-ppm. This multiplet is broadened and downfield shifted to *ca.* 8.12 - 8.55 ppm in complexes **2** - **5**. The chemical shift values of the amine protons are well in line with those of previously reported platinum(IV) complexes based on oxaliplatin [33,34]. The ^{195}Pt NMR spectrum of **1** in DMF (insert D_2O) shows a single resonance at 1389 ppm that is shifted downfield to *ca*. 1590 ppm in the di-carboxylato complexes **2** - **5**. The presence of an additional electron withdrawing carboxylate ligand in **2** - **5** causes a de-shielding effect of about 200 ppm on the platinum signal with respect to the mono-carboxylated complex **1**.



Scheme 1: Syntheses of complexes **1** – **5**.

The anticancer activities of the complexes were evaluated in two human ovarian carcinoma cells lines, cisplatin-sensitive A2780 and cisplatin-resistant A2780cis. The cytotoxicity was determined after incubation for 24 and 48 h using the colorimetric MTT assay and IC_{50} values are reported in (**Table 1**). For comparison purposes, the cytotoxicities of cisplatin, oxaliplatin and of the free IPA ligand were assessed under the same experimental conditions. The complexes are less active than cisplatin, in particular in the sensitive A2780 cell line, whereas they show higher activity than the reference drug oxaliplatin. The IC_{50} values of **1** - **5** in the A2780 cells are about 3 to 6 times lower at the 48 h timepoint than at the 24 h timepoint. The decrease in the IC_{50} value with prolonged exposure time is less pronounced for cisplatin. That is, the oxaliplatin derivatives take longer to exert their cytotoxic effects. After 48 h, the activity of **1** is comparable to that of cisplatin (IC_{50}(48 h) = 12.6 (**1**) *vs.* 10.5 (cisplatin)). The complexes show cross-resistance with cisplatin.

Table 1: IC_{50} values of the platinum(IV) complexes **1** - **5**, cisplatin, oxaliplatin and the free ligand IPA in A2780 and A2780cis*R* cells determined by the MTT test.

Complex	**IC_{50} (µM) ± S.D.**			
	A2780		**A2780cis**	
	24 h	**48 h**	**24 h**	**48 h**
1	74.40 ± 8.1	12.62 ± 0.02	66.91 ± 1.8	55.68 ± 1.7
2	53.32 ± 1.8	15.47 ± 1.72	70.48 ± 1.83	46.17 ± 1.61
3	54.52 ± 1.7	16.10 ± 1.8	55.26 ± 2.30	54.59 ± 2.5
4	58.39 ± 8.7	16.55 ± 8.5	132.52 ± 2.7	63.69 ± 7.4
5	70.10 ± 4.65	21.45 ±2.7	103.01 ± 6.1	47.56 ± 0.68
Cisplatin	20.10 ± 4.65	10.46 ± 2.1	60.80 ± 8.2	29.04 ± 6.7
Oxaliplatin	83.25 ± 5.7	19.80 ± 1.10	110.68 ± 16.8	86.37 ± 2.9
IPA	> 200	> 200	> 200	> 200

In the A2780 cell line the most active complex at the 48 h timepoint is the mono-carboxylated complex **1** with one axial OH group. The bis-substituted complex **2** with two indole acids in both the axial positions is slightly more active in the resistant cells (48 h timepoint). The finding that the mono- and the bis-PBA complex exert the strongest cytotoxic activity is in line with the cytotoxicity data observed for the analogous cisplatin-based complexes [22].

The generally not exceptional activity of these complexes may be ascribed to the reduction rate of these complexes under physiological conditions. As mentioned in the introduction, platinum(IV) complexes are pro-drugs and must be reduced to active platinum(II) agents. The reduction of **1** was studied *via* HPLC in the presence of an excess of ascorbic acid in HEPES buffer at pH 7 and 37 °C. As shown in **Figure 1**, the complex releases the axial IPA ligand and is reduced to the active oxaliplatin(II) species. However, the reduction kinetics are much slower compared to the analogous platinum(IV) compound based on cisplatin [22]. After 72 h there is still 19 % of the starting platinum(IV) complex **1**. Under the same conditions, *cis,cis,trans*-[$Pt(NH_3)_2Cl_2(IPA)(OH)$] was completely reduced within 24 h [22]. A lower reduction rate corresponds to a lower cytotoxicity because the active oxaliplatin is not completely released. In general, a low cytotoxic activity can also be due to a low lipophilicity and poor cellular uptake. However, we did not further investigate this possibility, as for the analogous cisplatin derivatives no correlation between lipophilicity, cellular uptake and IC_{50}

value was observed. The slow pro-drug activation is consistent with the delayed cytotoxicity of **1** whose IC_{50} value becomes comparable to that of cisplatin at the 48 h timepoint.

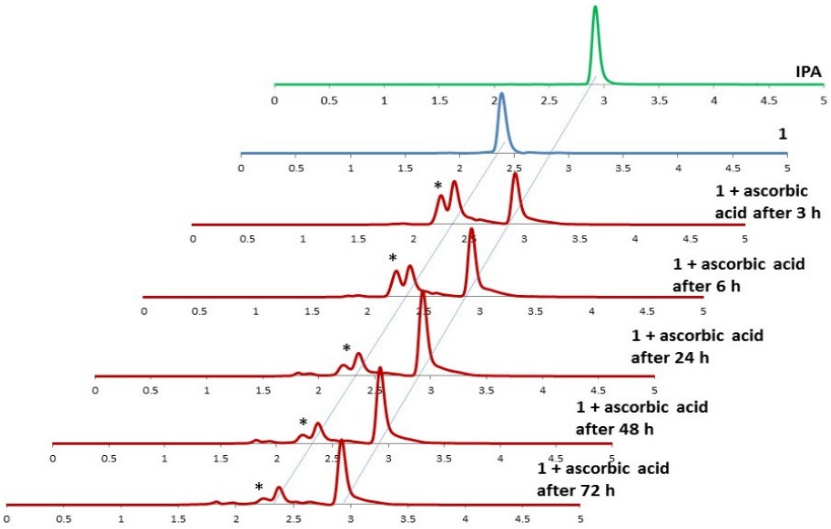


Figure 1: HPLC analysis of the reaction of **1** with ascorbic acid at 37 °C and pH 7 at different times. ∗ = ascorbic acid.

To further confirm the activation mechanism, **1** was incubated with guanosine (G) in the presence of sodium ascorbate to simulate the interaction of the platinum drug with the nucleobases of DNA. The mass spectrum of the reaction mixture (**Figure 2**) clearly shows the presence of two peaks, corresponding to the species $[Pt(DACH)(G)_2]^{2+}$ (m/z 875.25; $\{M-H\}^+$) and $[Pt(DACH)(OH)(G)]^+$ (m/z 609.16) which suggests that **1** can bind to the guanosine residues of DNA upon reduction. (Chart S1). The peak observed at m/z = 742.22 can be assigned to [Pt(DACH)(guanosine)(guanine)-H]$^+$ resulting from the loss of the sugar entity of one guanosine ligand. It is worthy of note that the incubation of **1** with guanosine without the reducing agent ascorbic acid does not give any Pt-guanosine adducts.

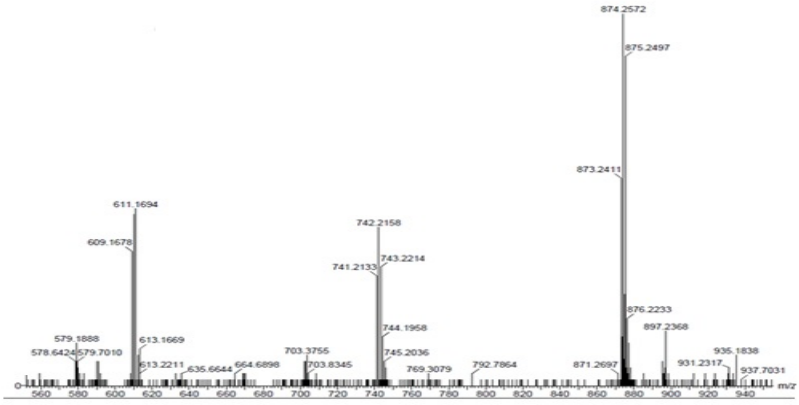


Figure 2: ESI-MS spectrum of the reaction between **1** and guanosine in presence of sodium ascorbate.

Indole carboxylic acids are agents that can alter and modify the cellular redox state and the cisplatin analogues of **1** - **5** induced a significant increase in ROS production. We speculated that the oxaliplatin-based platinum(IV) complexes with axial indole propionate ligands could also generate cellular oxidative stress. The generation of ROS in A2780 and A2780cis cells after incubation with **1** – **5**, cisplatin, oxaliplatin and IPA was measured. ROS levels were monitored over a 2 h period using 2′,7′-dichlorofluorescein diacetate (H_2DCFDA) as a peroxide-sensitive fluorescent dye and the results are reported in **Figure 3** and **Figure 14S** in the Supporting Information. All the complexes were able to stimulate the production of ROS in a time-dependent manner and the increased oxidative stress follows the order **1** > **4** > **3** > **2** > **5**. Complex **1** is the most efficient ROS generator and is significantly more potent than the free IPA ligand and the reference drugs cisplatin and oxaliplatin. The higher cytotoxic activity of complex **1** with respect to the other complexes is probably due to a synergistic effect of the two active species released upon intracellular reduction, oxaliplatin and IPA. As known, while oxaliplatin will interact with DNA as its cellular target, IPA will increase the ROS production with these two mechanisms operating simultaneously.

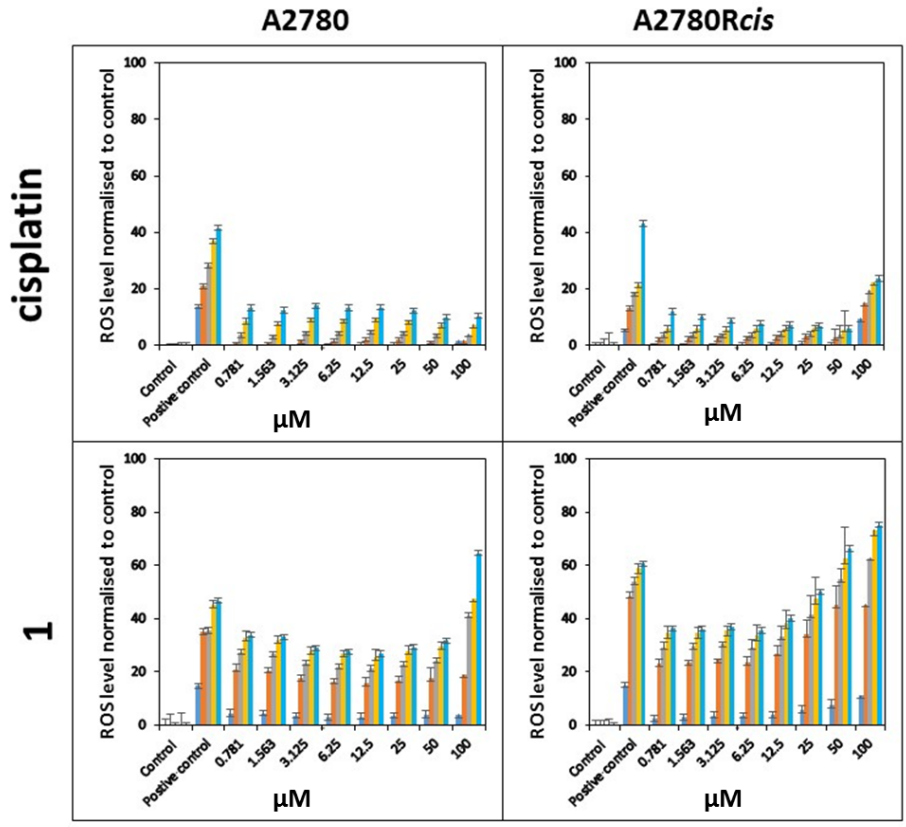


Figure 3: Fluorescent detection of ROS using DCFDA in A2780 and A2780cis cells after exposure to cisplatin (top) and **1** (bottom) over a period of 2 h. H_2O_2 was used as a positive control. ● 10 min; ● 15 min; ● 30 min; ● 1 h; ● 2 h.

Conclusions

The most active complex of the newly synthesized platinum(IV) derivatives of oxaliplatin, *cis,cis,trans*-[Pt(DACH)(ox)(IPA)(OH)] is more potent than oxaliplatin in A2780 ovarian cancer cells. Overall, compared to their cisplatin-based counterparts reported earlier by us [22], **1** – **5** are only moderately cytotoxic. This is in line with reports on other oxaliplatin/cisplatin pro-drugs [17] and can be attributed to the relatively slow reduction kinetics of platinum(IV) derivatives of oxaliplatin. **1** – **5** are more efficient ROS producers in A2780 and A2780cis cells than the free indole carboxylic acid.

Acknowledgement

A.A. acknowledges a PhD scholarship from Taibah University. D.T. acknowledges the Egyptian Ministry of Higher Education (MoHE) for funding her research stay at the National University of Ireland Galway.

Appendix A. Supplementary data

Supplementary data to this article can be found online at
(https:// doi.org/10.1016/j.ica.2019.04.038)

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Chapter 5: Conclusion and future directions

5.1 Conclusion

Platinum-based drugs are currently used worldwide for the treatment of various cancers; however, there are severe side effects and intrinsic or acquired resistance that limits their clinical utility. Recently, much attention is being paid to platinum(IV) prodrugs in order to reduce the side effects of platinum(II) complexes. In this study, novel series of platinum(IV) complexes based on carboplatin, oxaliplatin and cisplatin have been investigated.

The complexes in **Chapter 2** and **Chapter 3** contain the histone deacetylase inhibitor (HDACi) PBA and three different biologically inactive carboxylate ligands. In **Chapter 4**, the axial ligands of platinum(IV) compounds consist of indole propionic acid and three carboxylates.

This thesis describes the synthesis, characterisation as well as chemical and biological studies of PBA and IPA platinum(IV) complexes as potential anticancer agents for chemotherapy. The first series that was prepared consisted of five novel and dual-action platinum(IV) complexes namely, cis,cis,trans,-[Pt(CBDCA)$(NH_3)_2$(PBA)(OH)] (**1**),cis,cis,trans-[Pt(CBDCA)$(NH3)_2(PBA)_2$](**2**), cis,cis,trans-[Pt(CBDCA)$(NH_3)_2$(PBA)(benzoate)] (**3**), cis,cis,trans-[Pt(CBDCA)$(NH_3)_2$(PBA)(succinate)] (**4**) and cis,cis,trans-[Pt(CBDCA)$(NH_3)_2$)(PBA)(ac)] (**5**) (PBA = 4-phenylbutyrate, CBDCA = 1,1- cyclobutane dicarboxylate). The anti-proliferative activities of these complexes were examined in a panel of four human cancer cell lines (A431 cervical, LoVo colon, BxPC3 pancreatic and A375 melanoma cells). Although the effects of these platinum(IV) complexes based on carboplatin varies between the different cell lines, it can be generalised that all of the novel platinum(IV) complexes are more effective than the reference metallodrug carboplatin. platinum(IV) complex **3** shows superior activity against human BxPC3 pancreatic and LoVo colon cancer cells compared to cisplatin.

The cellular accumulation of **3** was higher compared to that of the other and carboplatin, but it is comparable to cisplatin. The HDAC inhibition potency and apoptosis induction by **3** was higher than control in these studies. It is noted in this research that slow reduction kinetics of the platinum(IV) prodrugs of carboplatin lead to moderate cytotoxicity.

The second series was based on cisplatin and the HDACi ligand PBA, which resulted in four dual-action platinum(IV) complexes, namely; cis,cis,trans-[Pt$(NH_3)_2Cl_2$(PBA)(OH)] (**A**),

cis,cis,trans-[Pt(NH_3)$_2$$Cl_2(PBA)_2$] (**B**), cis,cis,trans-[Pt(NH_3)$_2$$Cl_2$(PBA) (benzoate)] (**C**), and cis,cis,trans-[Pt(NH_3)$_2$$Cl_2$(PBA)(succinate)] (**D**),PBA = 4-phenylbutyrate. The anti-proliferative activities of these complexes were investigated in two cancer cell lines which are cisplatin sensitive and cisplatin-resistant in order to compare activity profiles, ROS production, DNA damage and types of cell death induced in cancer cells by these complexes. These platinum(IV) complexes are generally more effective than cisplatin except for complex D which was less active than cisplatin.

To examine other novel platinum(IV) compounds with bioactive ligands, the third series that was prepared consisted of cis,cis,trans-[Pt(DACH)(oxalate)(IPA)(OH)] (**1**), cis,cis,trans-[Pt(DACH)(oxalate)(IPA)$_2$] (**2**), cis,cis,trans-[Pt(DACH)(oxalate)(IPA)(benzoate)] (**3**), cis, cis,trans-[Pt(DACH)(oxalate)(IPA)(succinate)] (**4**), and cis,cis,trans [Pt(DACH)(oxalate)(IPA)(acetate)] (**5**) (DACH = 1,2- diaminocyclohexane (1R,2R).

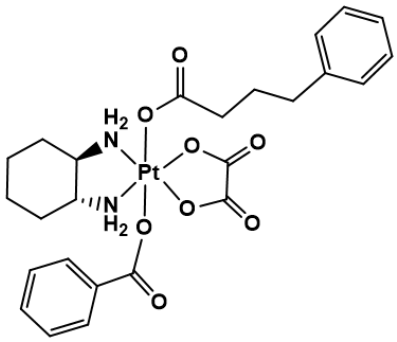
The anti-proliferative activities of these complexes were examined in two ovarian cancer cell lines. The effects of these complexes vary between the two cell lines; it can be generalised that all of the novel complexes are moderately cytotoxic compared to their cisplatin-based counterparts reported earlier. cis,trans,cis-[Pt(DACH)(ox)(IPA)(OH)] is more potent than oxaliplatin in A2780 ovarian cancer cells and is the most efficient ROS generator. It is significantly more potent than the free IPA ligand and the reference drugs cisplatin and oxaliplatin, suggesting that this novel platinum complexes may have a synergistic effect of the two active species released upon intracellular reduction, oxaliplatin and IPA. It is known that oxaliplatin will interact with DNA as its final cellular target, IPA will increase the ROS production with these two mechanisms operating simultaneously.

In summary it is possible to design and develop new platinum complexes which are even more cytotoxic than current platinum agents in clinical use. The anticancer complexes investigated in this thesis warrant further exploration.

To conclude, it is believed that this thesis might help other researchers to relook at platinum anticancer drug design and to develop the next generation of platinum-based anticancer agents in clinical use.

5.2 Future directions

1-This thesis highlighted the importance of cytotoxic activity as well as lipophilicity when designing clinically relevant platinum(IV) complexes. Looking forward, the same strategy can be applied for the design of platinum(IV) complexes with an oxaliplatin platform. It is reported that oxaliplatin exhibits a spectrum of activity different from that of cisplatin, with little to no cross resistance with cisplatin. Based on our findings presented about combing HDACi and benzoic acid as axial ligands in platinum(IV) complexes with an oxaliplatin core, such as the PBA benzoate complex shown below it is reasonable to suggest that platinum(IV) prodrugs with an oxaliplatin core could improve the efficacy currently seen with oxaliplatin treatment.



2-Lastly, instead of directly modifying the platinum(IV) complexes to improve their activity, it might be beneficial to explore delivery methods such as incorporation into nanomolecular delivery system.

Supplementary data & Appendix

Appendix I

Antitumor platinum(IV) derivatives of carboplatin and the histone deacetylase inhibitor 4-phenylbutyric acid

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Supplementary Material

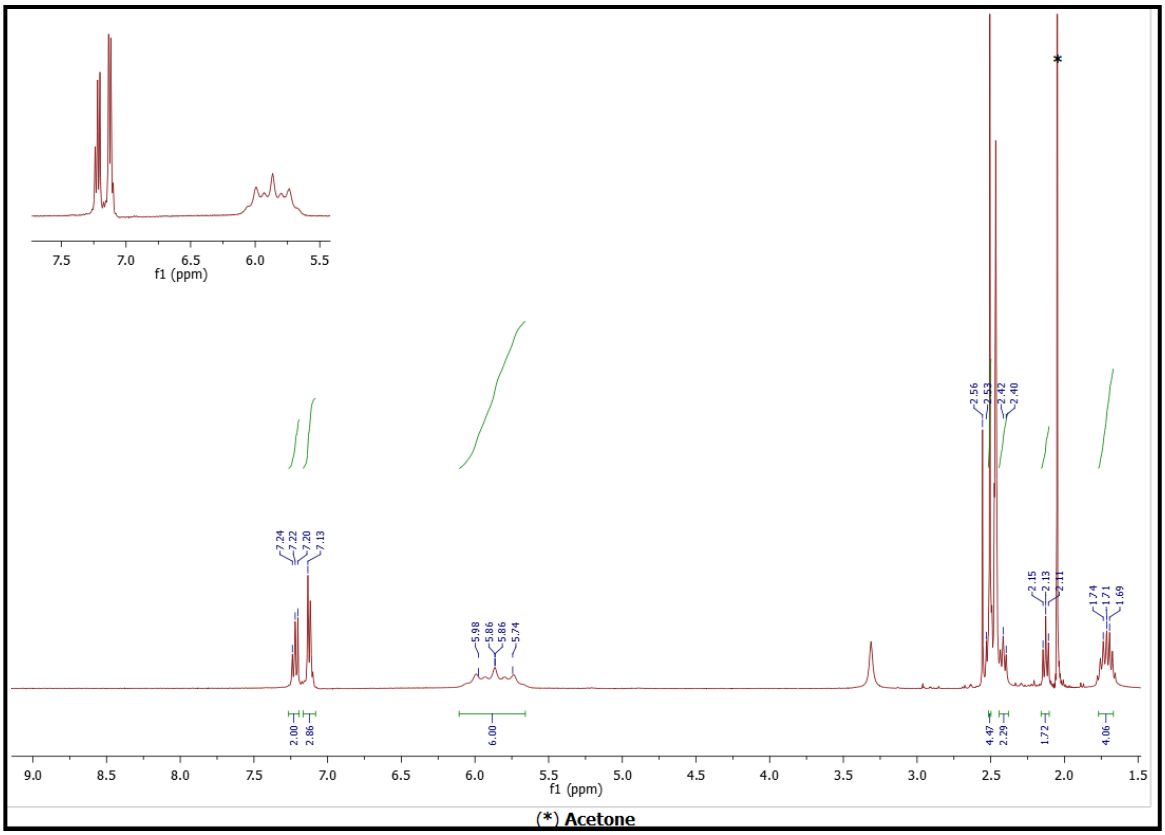

Fig. S1. ^{1}H NMR spectrum (DMSO-d_6) of *cis,trans*-[Pt(NH_3)$_2$(CBDCA)(OH)(PBA)] (**1**).

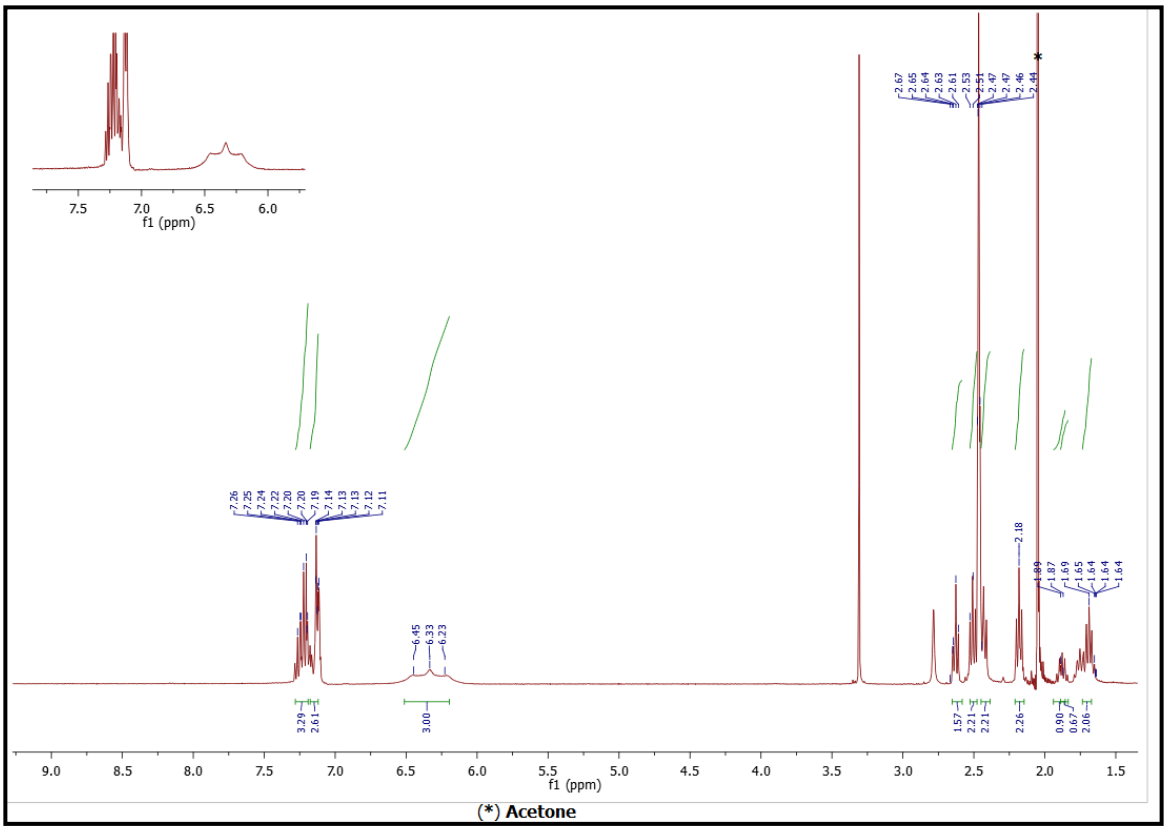

Fig. S2. ^{1}H NMR spectrum (DMSO-d_6) of *cis,trans*-[$Pt(NH_3)_2$(CBDCA)$(PBA)_2$] (**2**).

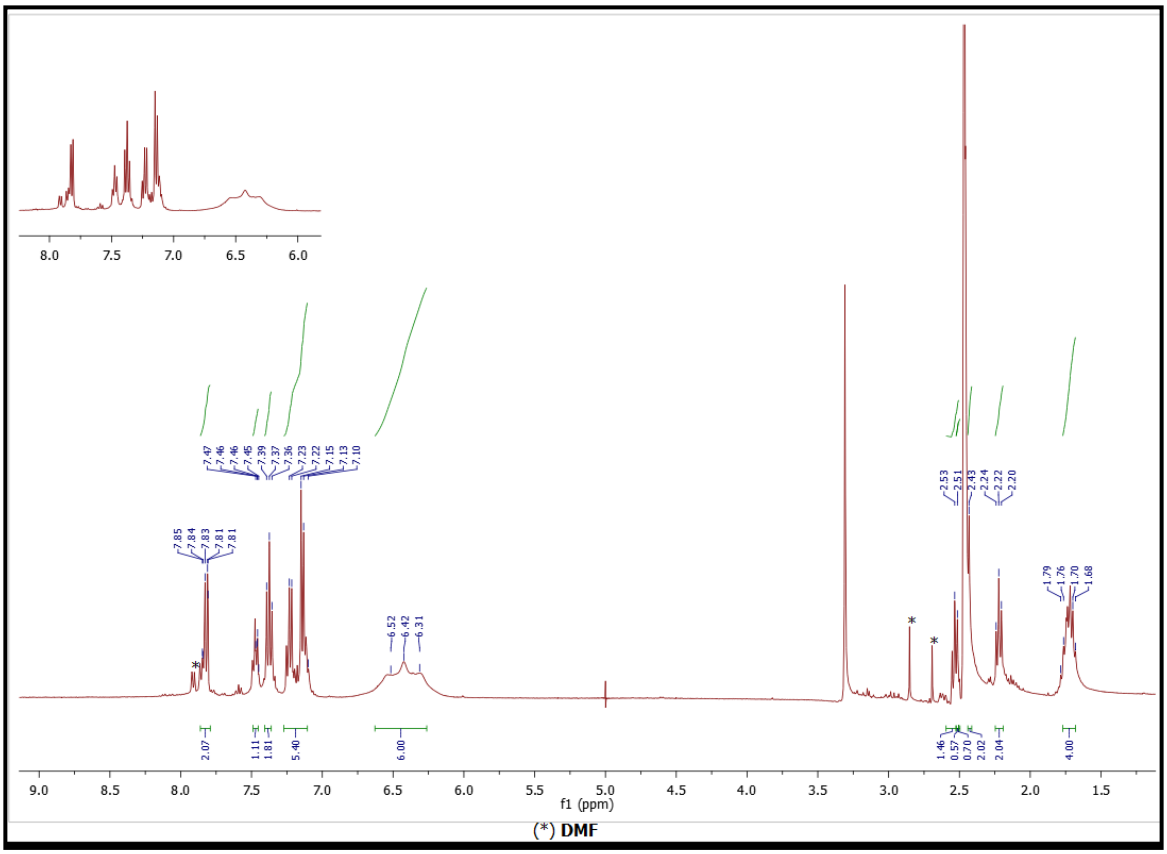

Fig. S3. ^{1}H NMR spectrum (DMSO-d_6) of *cis,trans*-[Pt(NH_3)$_2$(CBDCA)(PBA)(bz)] (**3**).

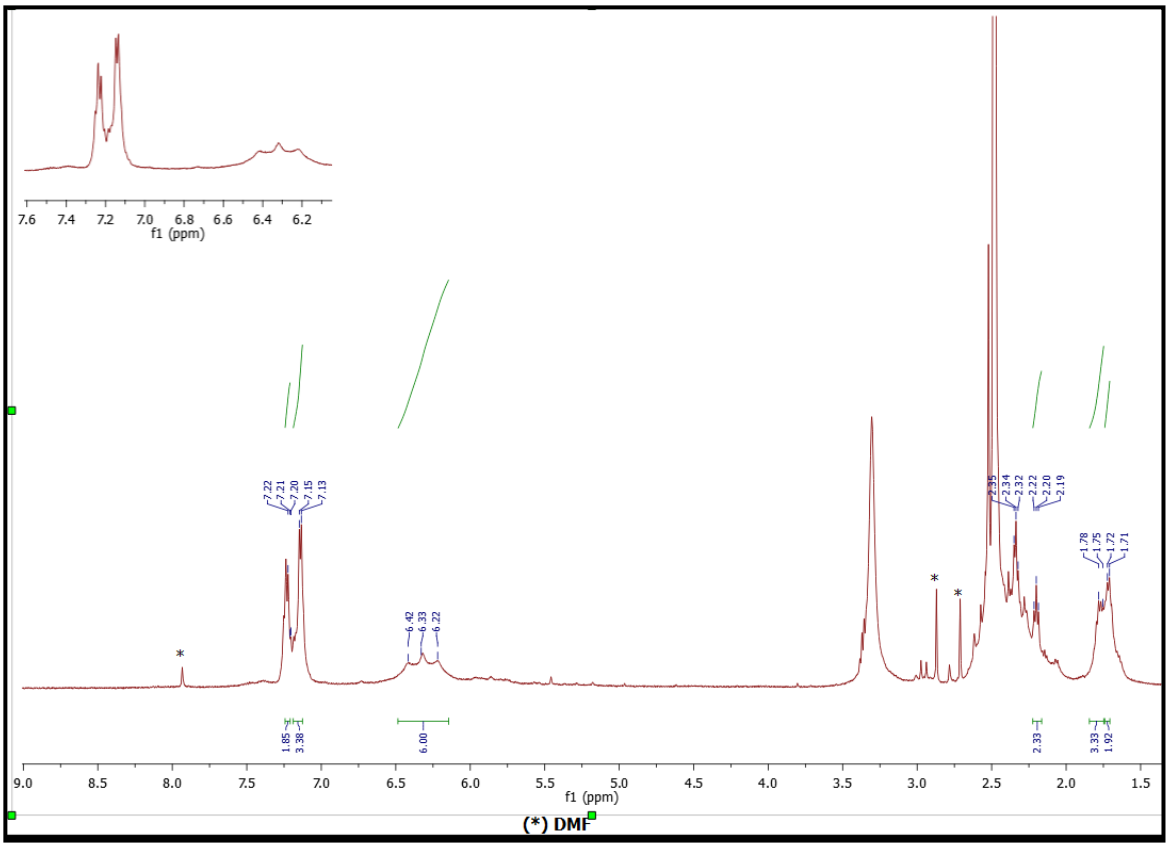

Fig. S4. ^{1}H NMR spectrum (DMSO-d_6) of *cis,trans*-[Pt(NH_3)$_2$(CBDCA)(PBA)(suc)] (**4**).

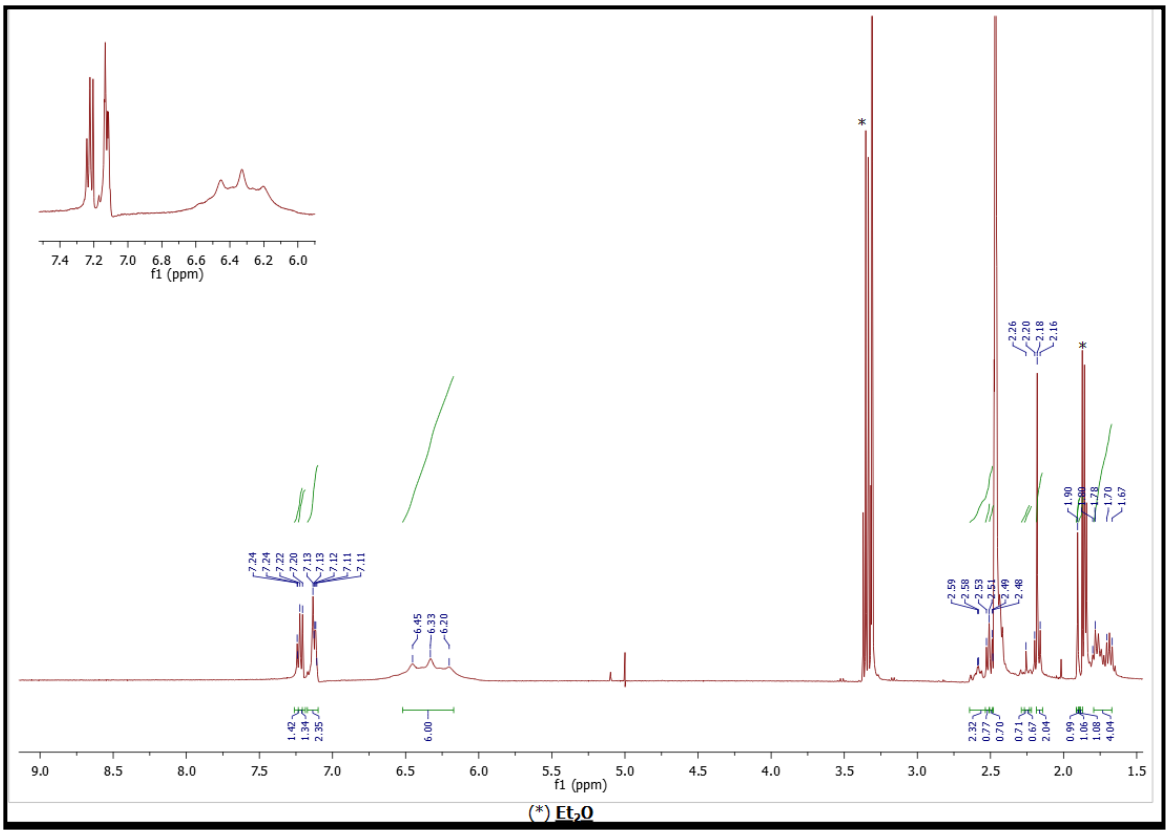

Fig. S5. ^{1}H NMR spectrum (DMSO-d_6) of *cis,trans*-[Pt(NH_3)$_2$(CBDCA)(PBA)(ac)] (**5**).

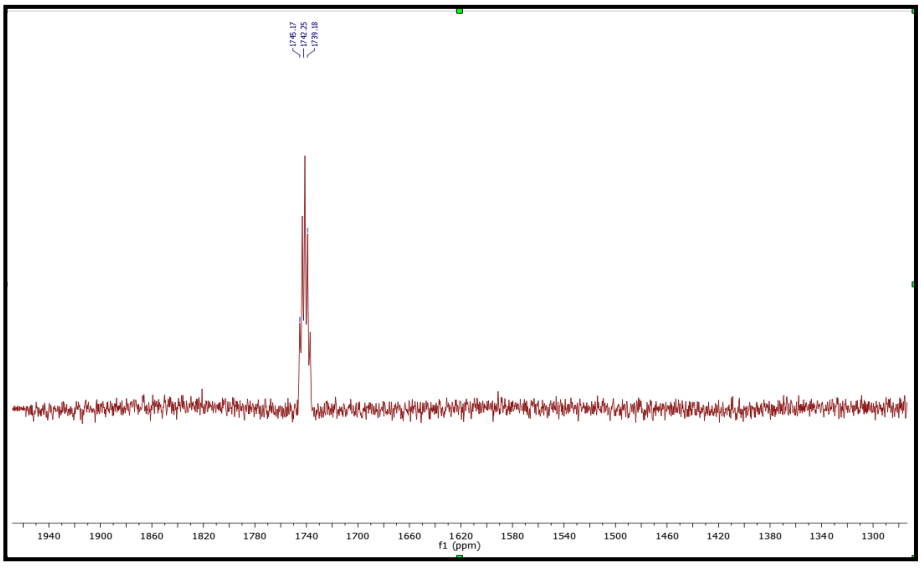

Fig. S6. ^{195}Pt NMR spectrum (DMF/D_2O) of *cis,trans*-[Pt(NH_3)$_2$(CBDCA)(OH)(PBA)] (**1**).

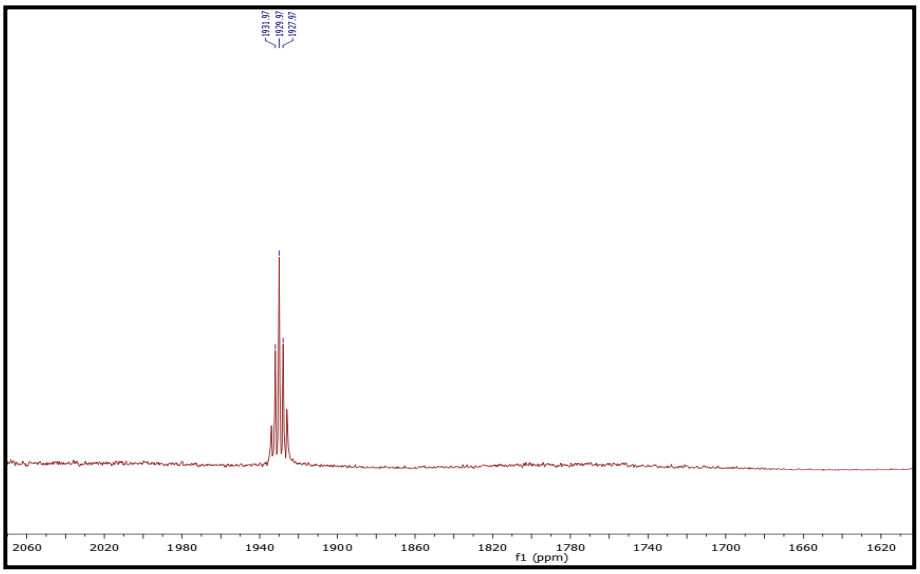

Fig. S7. ^{195}Pt NMR spectrum (DMF/D_2O) of *cis,trans*-[Pt(NH_3)$_2$(CBDCA)(PBA)$_2$] (**2**).

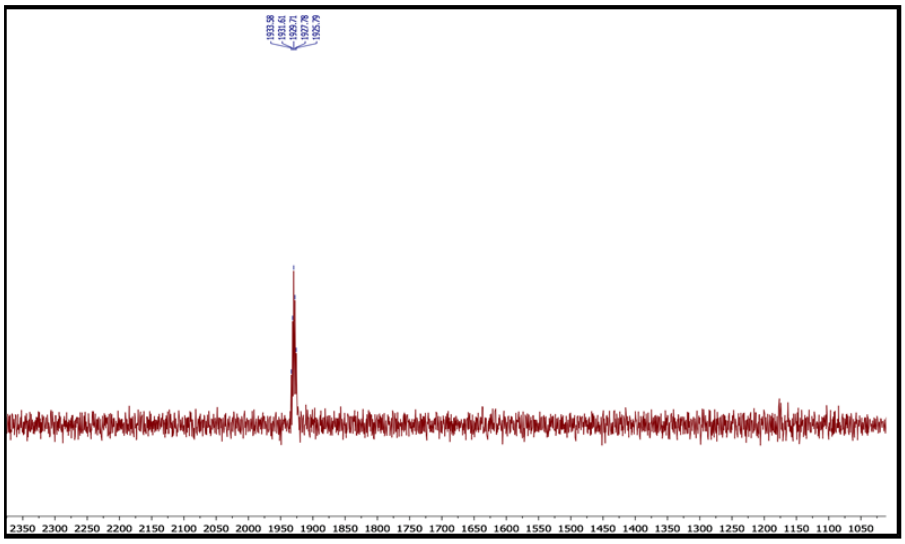

Fig. S8. ^{195}Pt NMR spectrum (DMF/D_2O) of *cis,trans*-[Pt(NH_3)$_2$(CBDCA)(PBA)(bz)] (**3**).

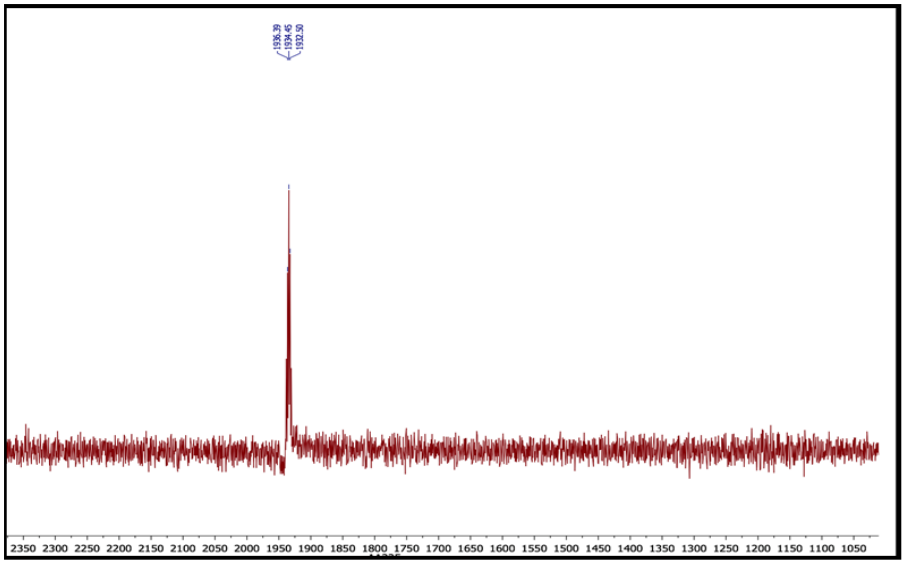

Fig. S9. ^{195}Pt NMR spectrum (DMF/D_2O) of *cis,trans*-[Pt(NH_3)$_2$(CBDCA)(PBA)(suc)] (**4**).

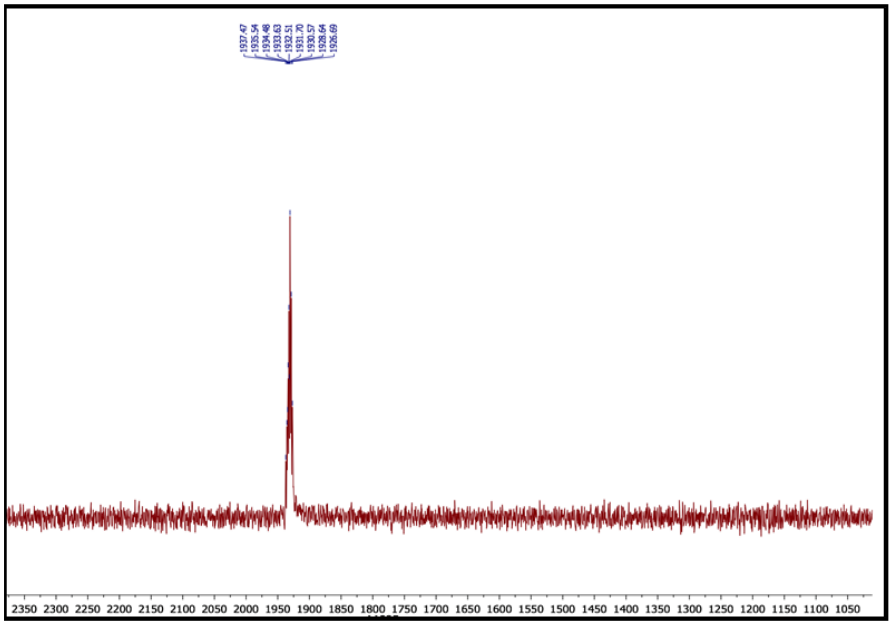

Fig. S10. ^{195}Pt NMR spectrum (DMF/D_2O) of *cis,trans*-[Pt(NH_3)$_2$(CBDCA)(PBA)(ac)] (**5**).

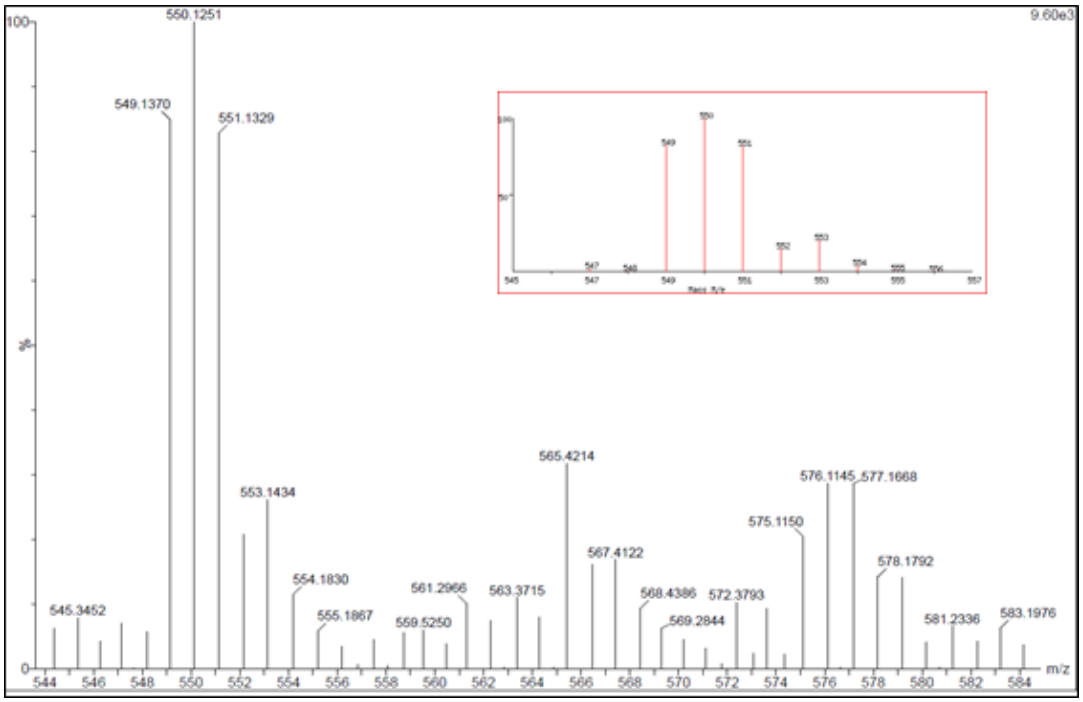

Fig. S11. ESI MS spectrum (negative mode) of *cis,trans*-[Pt(NH_3)$_2$(CBDCA)(OH)(PBA)] (**1**). Inset: Calculated isotope pattern for $[M-H]^-$

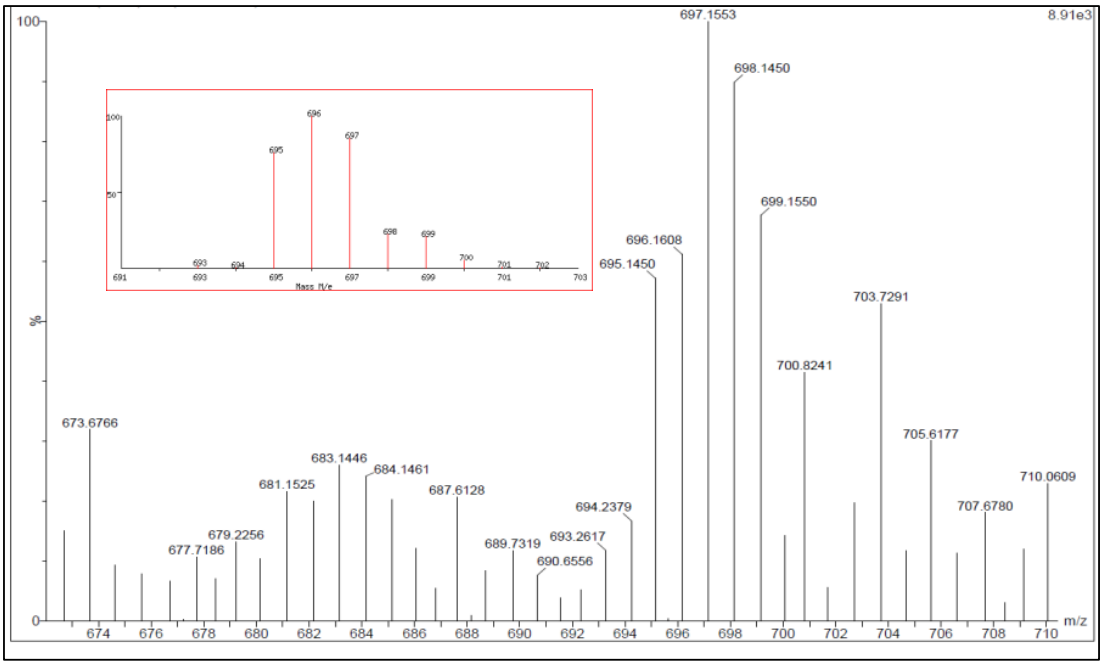

Fig. S12. ESI MS spectrum (negative mode) of *cis,trans*-[Pt(NH$_3$)$_2$(CBDCA)(PBA)$_2$] (**2**). Inset: Calculated isotope pattern for [M-H]$^-$

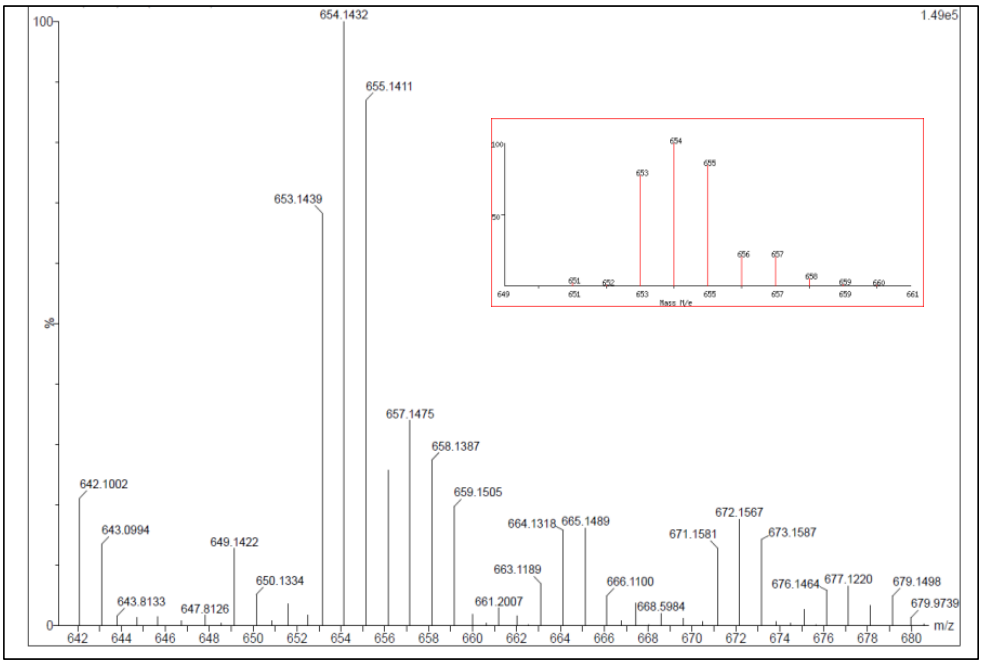

Fig. S13. ESI MS spectrum (negative mode) of *cis,trans*-[Pt(NH_3)$_2$(CBDCA)(PBA)(bz)] (**3**). Inset: Calculated isotope pattern for [M-H]$^-$

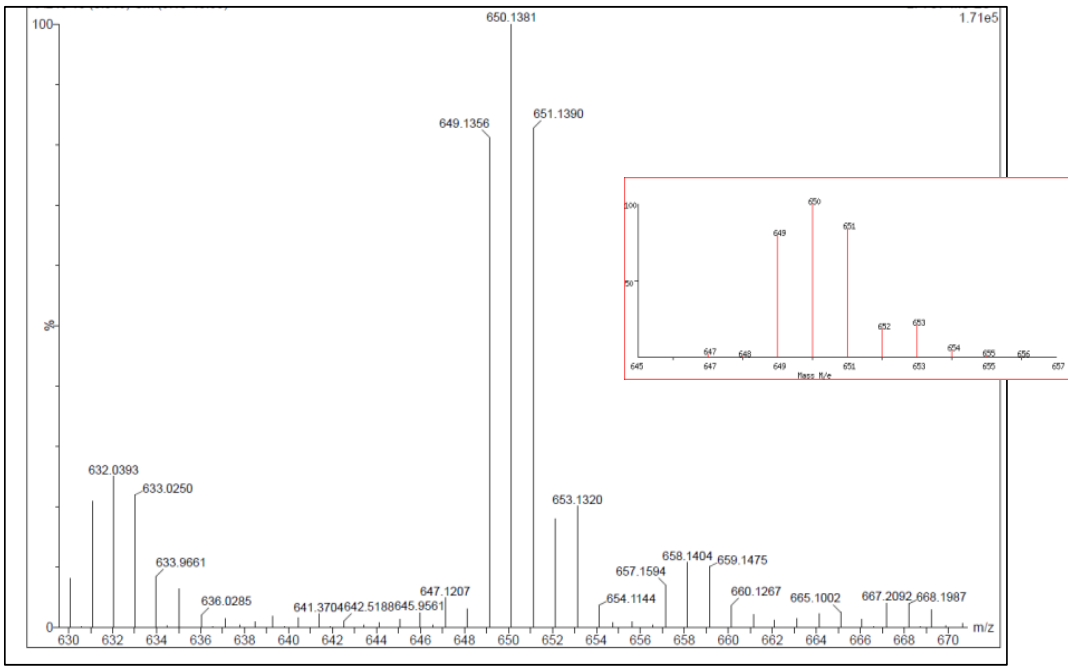

Fig. S14. ESI MS spectrum (negative mode) of *cis,trans*-[Pt(NH_3)$_2$(CBDCA)(PBA)(suc)] (**4**). Inset: Calculated isotope pattern for [M-H]$^-$

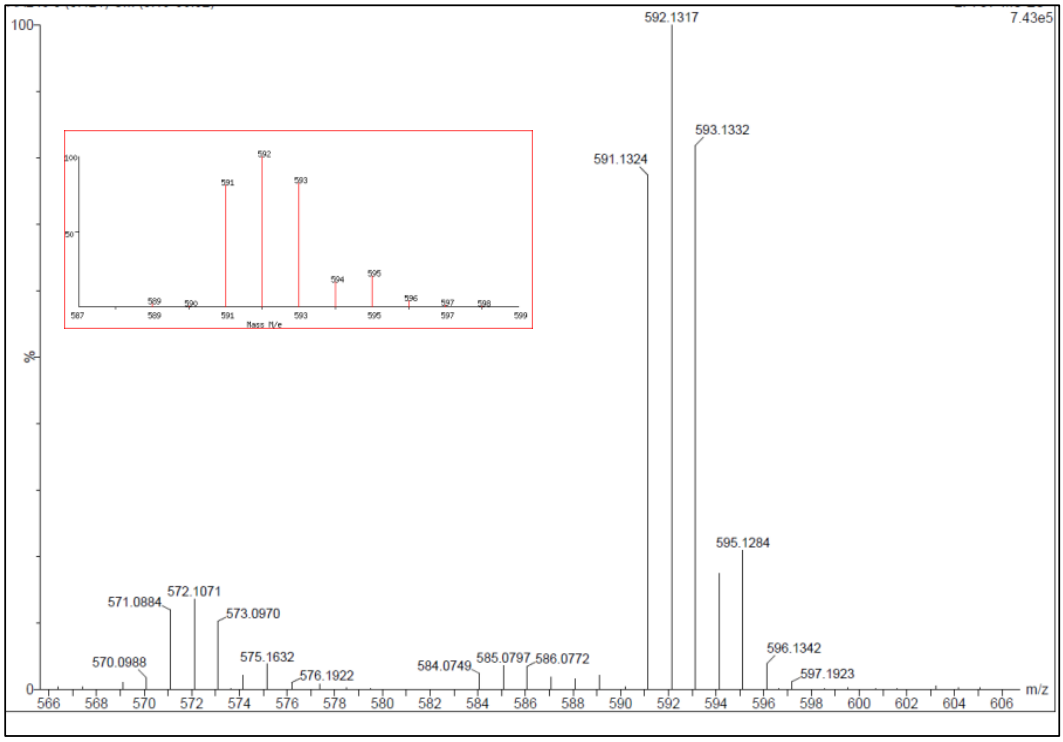

Fig. S15. ESI MS spectrum (negative mode) of *cis,trans*-[Pt(NH_3)$_2$(CBDCA)(PBA)(ac)] (**5**). Inset: Calculated isotope pattern for $[M-H]^-$

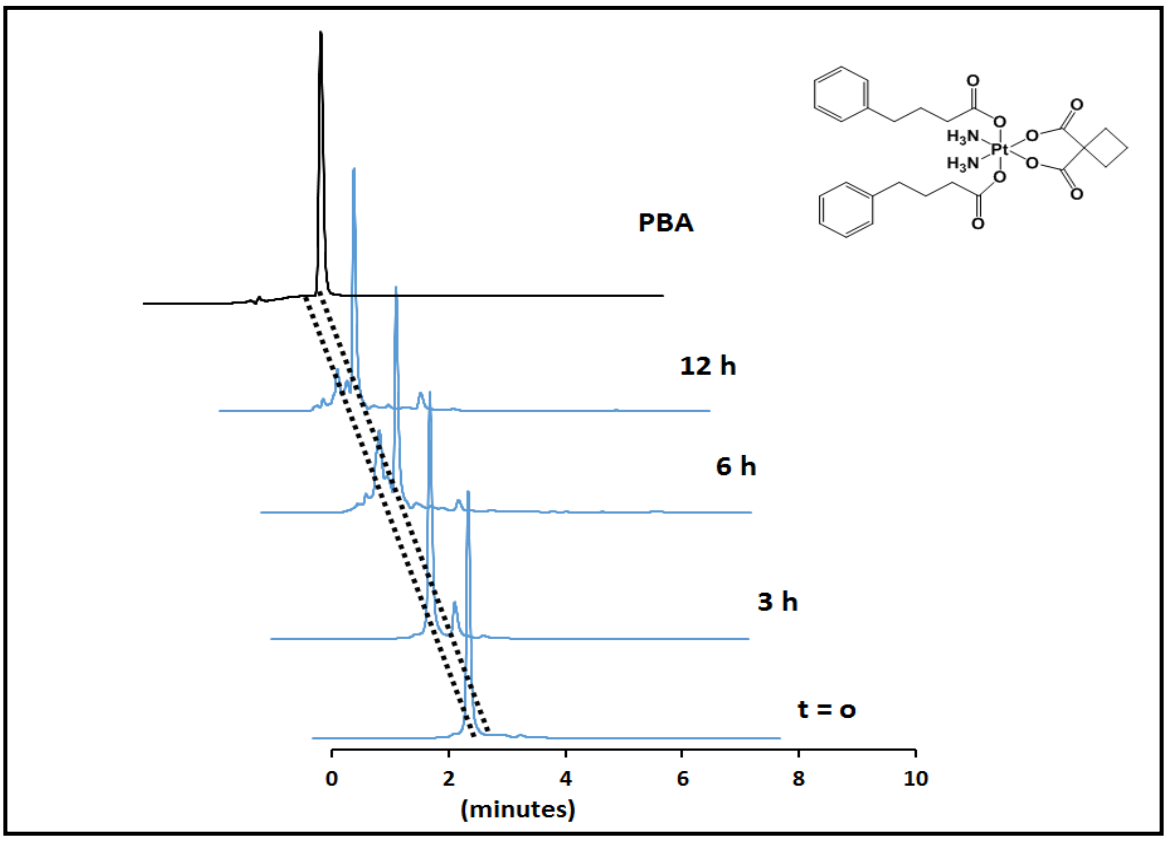

Fig. S16. HPLC chromatogram of the reaction of **2** with 10 eq. ascorbic acid at 37 °C and pH 7. The chromatograms of the free ligand PBA and benzoic acid are shown for comparison purposes.

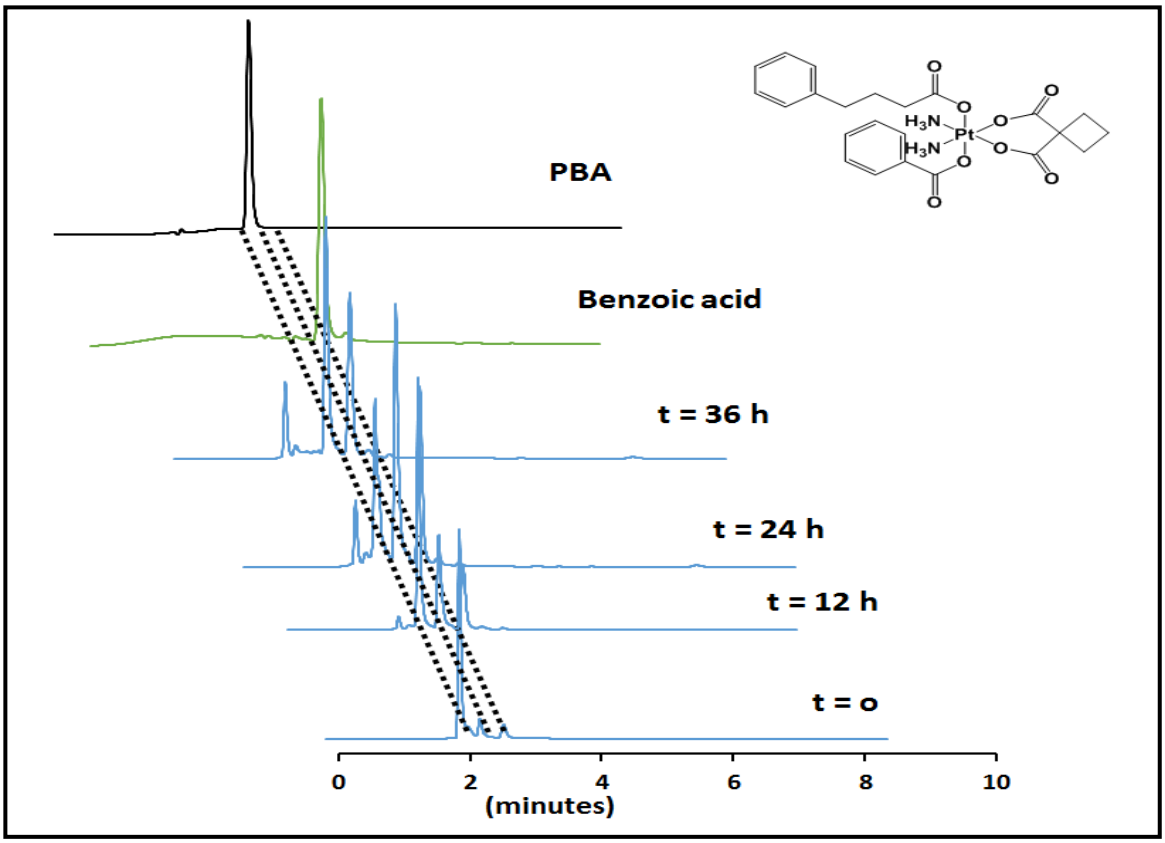

Fig. S17. HPLC chromatogram of the reaction of **3** with 10 eq. ascorbic acid at 37 °C and pH 7. The chromatogram of the free ligand PBA is shown for comparison purposes.

Appendix II

Platinum(IV) pro-drugs with an axial HDAC inhibitor demonstrate multimodal mechanisms involving DNA damage and apoptosis independent of cisplatin resistance

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Supplementary Material

Material and syntheses

Materials

Potassium tetrachloroplatinate was purchased from Acros Organics. 4-Phenylbutyric acid and succinic anhydride were acquired from Sigma-Aldrich and benzoic anhydride, *N*-hydroxysuccinimide and dicyclohexylcarbodiimide were acquired from TCI Europe. Chemical reagents and solvents were used as received (analytical or HPLC grade). All reactions were performed under normal atmospheric conditions. The ultra-pure water used was purified with a Milli-Q UV purification system. *Cis*-[$Pt(NH_3)_2Cl_2$] (Dhara 1970), *cis,cis,trans*-[$Pt(NH_3)_2Cl_2(OH)_2$] (Brandon et al. 1984) and the NHS ester of 4-phenylbutyric acid (Almotairy et al. 2017) were synthesized as previously reported.

Synthesis of *cis,cis,trans*-[$Pt(NH_3)_2Cl_2(PBA)(OH)$] (A)

cis,cis,trans-[$Pt(NH_3)_2Cl_2(OH)_2$] (150 mg, 0.45 mmol) and the NHS ester of 4-phenylbutyric acid (93 mg, 0.36 mmol) were reacted in 30 mL dimethyl sulfoxide. The mixture was stirred at 70 °C for 48 h. Unreacted *cis,cis,trans*-[$Pt(NH_3)_2Cl_2(OH)_2$] was filtered off and the yellow solution was evaporated using a freeze-dryer. The residual solid was dissolved in 10 mL dimethylformamide, followed by precipitation of the desired product with diethyl ether. The precipitate was isolated by centrifugation and washed several times with 5 mL dichloromethane, 5 mL acetonitrile and 20 mL diethyl ether. The yellow solid was dried under vacuum overnight to yield the product. Yield: 99 mg (57%). Anal. calcd (%) for $C_{10}H_{18}Cl_2N_2O_3Pt$ (480.25): C, 25.0; H, 3.8; N, 5.8. Found: C, 25.3; H, 4.0; N, 5.6. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 1.75 (m, 2H, $CH_2CH_2CH_2$), 2.16 (t, ^3J = 7.3 Hz, 2H, $CH_2CH_2CH_2$), 2.58 (t, ^3J = 7.4 Hz, 2H, $CH_2CH_2CH_2$), 6.00 (m, br., $^1J_{H\text{-}N}$ = 52.9 Hz, $^2J_{H\text{-}Pt}$ = 52.2 Hz, 6H, NH_3), 7.17–7.28 (m, 5H, H_{ar}).^{195}Pt{^{1}H} NMR (107.6 MHz, DMF/D_2O): δ 990. ESI-MS: *m/z* = 515.01 $[M+Cl]^-$.

Synthesis of *cis,cis,trans*-[$Pt(NH_3)_2Cl_2(PBA)_2$] (B)

cis,cis,trans-[$Pt(NH_3)_2Cl_2(OH)_2$] (100 mg, 0.30 mmol) was stirred in dimethyl sulfoxide (16 mL) and 4-phenylbutyric acid NHS ester was added (1.60 g, 6.1 mmol). After stirring for 72 h at 80 °C the mixture was concentrated to a 2 mL volume and the desired product was precipitated by addition of 30 mL water. The solid was isolated by centrifugation, washed with 8 mL dichloromethane and 60 mL diethyl ether to remove excess ligand and dried under vacuum. Yield: 145 mg, 77%. Anal. calcd (%) for $C_{20}H_{28}Cl_2N_2O_4Pt$ (626.43): C 38.35; H

4.51; N, 4.47; Found: C 38.70, H 4.77, N 4.60. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 1.72 (quint, ^{3}J = 7.86 Hz, 2H, $CH_2CH_2CH_2$), 2.18 (t, ^{3}J = 7.5 Hz, 2H, $CH_2CH_2CH_2$), 2.55 (t, ^{3}J = 7.5 Hz, 2H, $CH_2CH_2CH_2$), 6.50 (s, br, 6H, NH_3), 7.13–7.25 (m, 10H, H_{ar}). ^{195}Pt{^{1}H} NMR (107.6 MHz, DMF/D_2O): δ 1180. ESI-MS: *m/z* = 626.05 $[M]^-$.

Synthesis of *cis,cis,trans*-[Pt(NH_3)$_2$$Cl_2$(PBA)(Bz)] (C) and *cis,cis,trans*-[Pt(NH_3)$_2$$Cl_2$(PBA)(Suc)] (D)

The unsymmetrically substituted complexes **C** and **D** were synthesized by reaction of **A** with the respective carboxylic acid anhydride. Complex **A** and 12 equivalents benzoic anhydride and 10 equivalents succinic anhydride, respectively were reacted in dimethylformamide and the mixtures were stirred for 60 h at 80 °C. The solutions were concentrated under reduced pressure and diethyl ether (40 mL) was added to precipitate the desired product which was isolated by centrifugation and washed with dichloromethane (10 mL) and diethyl ether (20 mL) to remove residual dimethylformamide.

Complex **C**: Yield: 109 mg (88%) from **A** (100 mg, 0.21 mmol) and benzoic anhydride (565 mg, 2.6 mmol) in 30 mL dimethylformamide. Anal. calcd (%) for $C_{17}H_{22}N_2O_4Cl_2Pt$ (584.35): C, 34.94; H, 3.79; N, 4.79. Found: C, 35.01; H, 3.89; N, 4.90. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 1.71–1.79 (m, 2H, $CH_2CH_2CH_2$), 2.23 (t, ^{3}J = 7.4 Hz, 2H, $CH_2CH_2CH_2$), 2.57–2.60 (m, 2H, $CH_2CH_2CH_2$), 6.57 (m, br., 6H, NH_3), 7.18–7.25 (m, 5H, H_{ar}, PBA), 7.38–7.40 (m, 4H, $H_{ar,}$ Bz), 7.85 (t, ^{3}J = 8.0, 1H, $H_{ar,}$ Bz), ^{195}Pt{^{1}H} NMR (107.6 MHz, DMF/D_2O): δ 1187. ESI-MS: *m*/*z* = 585.29 $[M+H]^+$.

Complex **D**: Yield: 90 mg (74%) from **A** (100 mg, 0.21 mmol) and succinic anhydride (208 mg, 2.08 mmol) in 30 mL dimethylformamide. Anal. Calcd (%) for: $C_{14}H_{22}N_2O_6Cl_2Pt$ (580.32): C, 28.98; H, 3.82; N, 4.83. Found: C, 28.88; H, 3.96; N, 4.76. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 1.66–1.77 (m, 2H, $CH_2CH_2CH_2$), 2.18 (t, ^{3}J = 7.4 Hz, 2H, $CH_2CH_2CH_2$), 2.33–2.35 (m, 4H, $C(O)CH_2CH_2CO$), 2.53 (t, ^{3}J = 7.8 Hz, 2H, $CH_2CH_2CH_2$), 6.49 (s, br., 6H, NH_3), 7.13–7.24 (m, 5H, H_{ar}); ^{195}Pt{^{1}H} NMR (107.6 MHz, DMF/D_2O): δ 1179. ESI-MS: *m*/*z* = 579.04 $[M-H]^-$.

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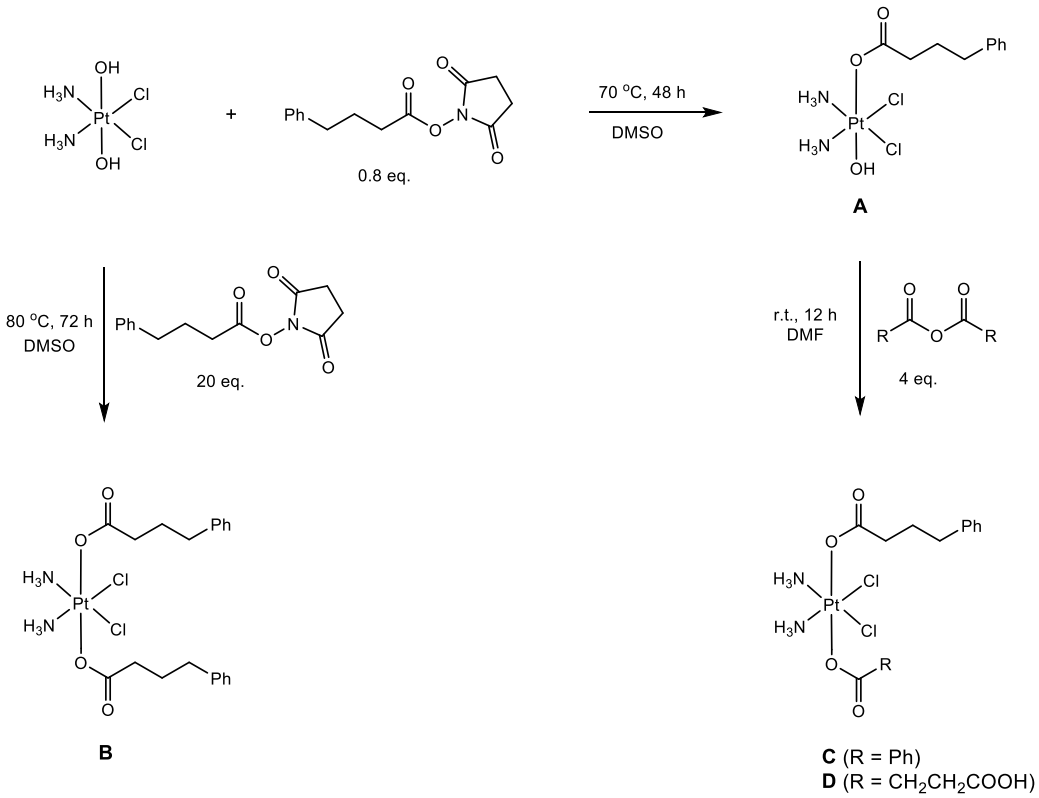
Brandon RJ, Dabrowiak JC (1984) Synthesis, characterization, and properties of a group of platinum(IV) complexes. J Med Chem 27:861–865

Dhara SCA (1970) Rapid method for the synthesis of cis-[$Pt(NH_3)_2Cl_2$]. Indian J Chem 8:193–194



Scheme S1. Synthesis of **A** – **D**.

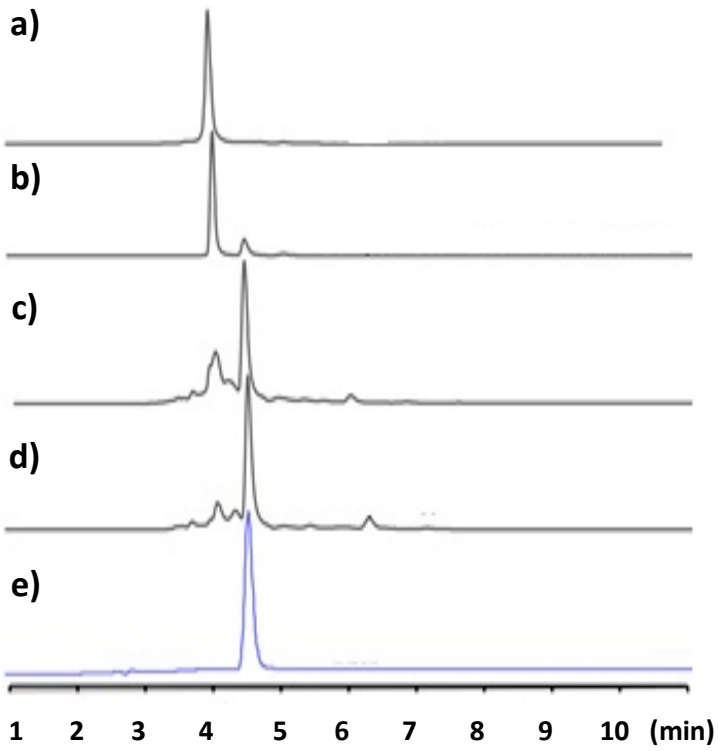


Fig. S1. HPLC chromatograms of the reaction of A with 10 eq. ascorbic acid at 37 °C and pH 7. (a) t = 0, (b) 12 h, (c) 24 h, (d) 48 h and (e) the free ligand PBA.

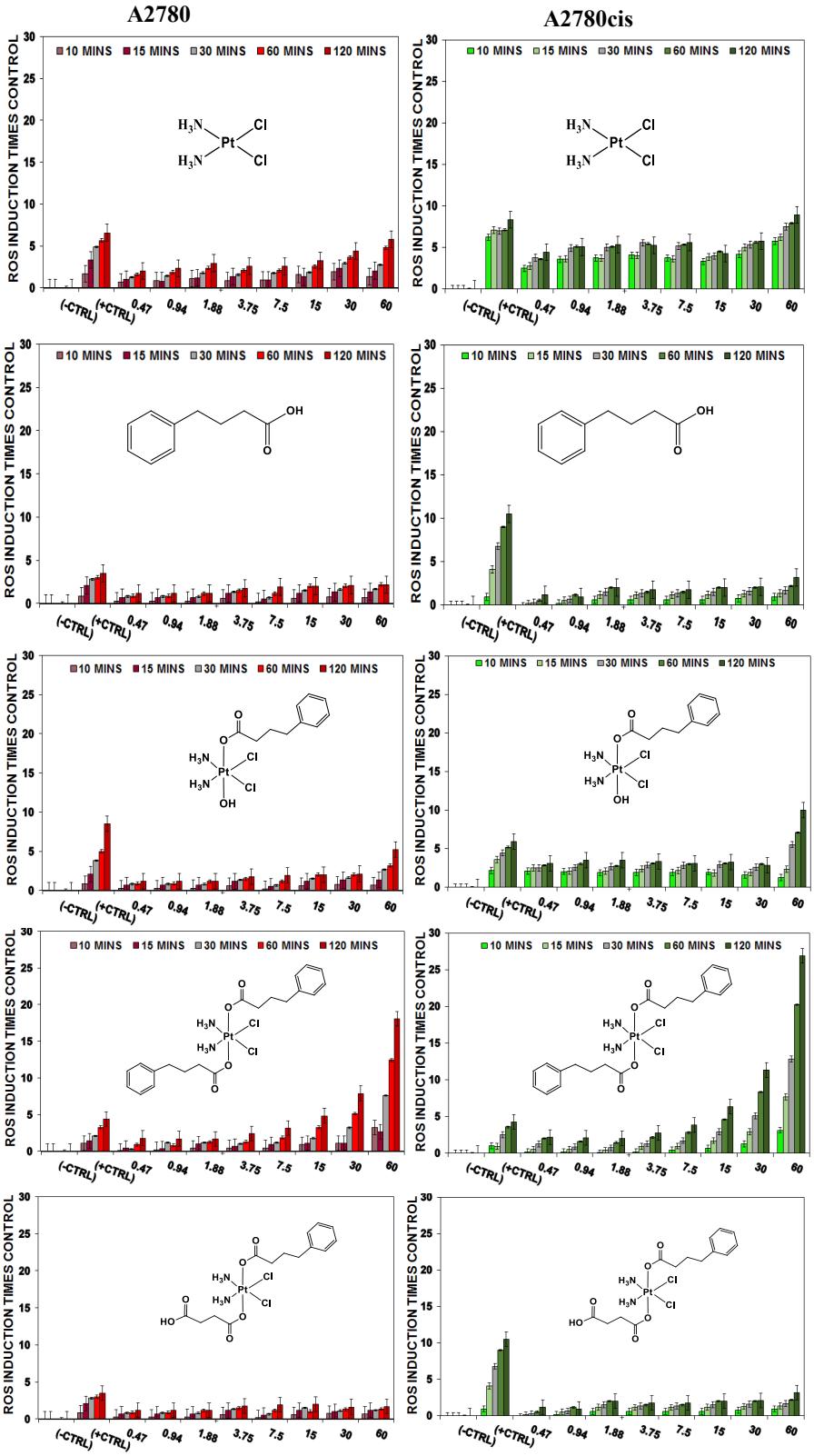


Fig. S2. Fluorescent detection of ROS using DCFDA in A2780 and A2780cis cells after exposure to different concentrations (μM) of cisplatin, PBA, **A**, **B** and **D** over a period of 2 h. H_2O_2 was used as a positive control.

Appendix III

Cytotoxicity and ROS Production of Novel Platinum(IV) Oxaliplatin Derivatives with Indole Propionic Acid

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[⊥] These authors contributed equally to this manuscript.

Supplementary Material

Materials and Methods

All reactions were carried out under normal atmospheric conditions. Solvents and chemicals were of analytical grade or HPLC grade and were obtained from commercial sources. The ultrapure water used was purified by a Milli-Q UV purification system. K_2PtCl_4 was obtained from Acros Organics. Indole-3-propionic acid, succinic anhydride and acetic anhydride were obtained from Sigma-Aldrich. Benzoic anhydride, *N*-hydroxysuccinimide and dicyclohexylcarbodiimide were purchased from TCI. All chemicals were used as received. [Pt(DACH(ox)(OH)$_2$] [1] and the NHS ester of indole-3-propionic acid (**B**) [2] were synthetized as previously reported.

^{1}H NMR spectra were recorded on a Jeol ECX-400. Chemical shifts (δ) are reported in parts per million (ppm) and referenced internally using the residual solvent signals relative to tetramethylsilane (δ (^{1}H NMR) = 0 ppm). One dimensional ^{195}Pt NMR spectra were recorded on a Varian 500 AR spectrometer in DMF with an inserted co-axial tube containing D_2O. K_2PtCl_6 in D_2O was used as an external standard. Mass spectra were measured using a Waters LCT Premiere XE with electron spray ionisation and time of flight mass analyser. Elemental analysis (carbon, nitrogen and hydrogen) were performed by an Exeter Analytical CE-440. FT-IR spectra were recorded on a PerkinElmer FT-IR spectrometer fitted with an ATR accessory. The HPLC studies were carried out using an Agilent 1200 series DAD analytical HPLC instrument.

Reduction reaction studied by HPLC. The reduction of complex **1** with ascorbic acid was followed by HPLC using a Phenomenex Luna C18 (5 µM, 100 Å, 250 mm × 4.60 mm i.d.) column at a flow rate of 1.0 mL/min with 280 nm UV detection at room temperature. The mobile phase was 80:20 acetonitrile (1% trifluoroacetic acid) : water (1% trifluoroacetic acid). The complex was dissolved in DMF (0.5 mL) and added to a 5 mM solution of ascorbic acid in 2 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (pH 7) and diluted to a final concentration of 0.5 mM using acetonitrile. The process was followed at 37 °C until complete reduction.

Reaction with guanosine studied by ESI-MS. Complex **1** (1.5 mg, 3 µmol) and guanosine (4.24 mg, 15 µmol) were dissolved in 1 mL DMF and a 15 mM aqueous solution of sodium ascorbate (1 mL) was added. The mixture was kept at 37 °C for 24 h. Thereafter, the reaction solution was analyzed by ESI-MS.

Experiments with Cultured Human Cells

Mammalian Cell Lines and Culture Conditions. A2780 and A2780cis are cisplatin-sensitive and cisplatin-resistant ovarian adenocarcinoma cell lines respectively and were obtained commercially from European Collection of Authenticated Cell Cultures (ECACC) (Public Health England, UK). Cells were grown in Roswell Park Memorial Institute (RPMI-1640) medium (Sigma, Ireland) supplemented with 12% Foetal Bovine Serum (Sigma, Ireland) and 12% L-glutamine (Sigma, Ireland) and incubated at 37 °C in 5% CO_2. A sub-lethal dose of 1 μM cisplatin was added to A2780cis cells to maintain cisplatin resistance. Stock cells were grown to 80-90% confluence and were then trypsinised to generate a cell suspension for experimental use.

The five tested compounds (**1**-**5**) were prepared prior to experimental use, and were dissolved in dimethyl sulfoxide (DMSO) (Merck, Germany). The final concentration of dimethyl sulfoxide was ≤0.3%.

Cell Viability. Cellular viability was evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay which is a colorimetric assay that measures the metabolic activity of cells [3]. The ovarian adenocarcinoma cell lines were plated in 96-well plates with a total number of 1×10^4 cells/well and 8×10^3 cells/well for 24 and 48 h, respectively for each cell line, and the plates were incubated at 37 °C for 24 h for attachment and growth. The test compounds were dissolved in DMSO and diluted with cell growth medium so that the final DMSO concentration did not exceed 0.5 %. Cells were then treated with different concentrations of the test compounds (2.5 μM – 200 μM) in triplicate and a solvent control (0.3% DMSO) for 24 and 48 h time-points. The cell culture medium and test drug was discarded from the plate wells and 100 μL of a solution of MTT (5 mg/mL) was added to each well. The cells were incubated for 3 h and washed three times with sterilised PBS. 100 μL of DMSO was added to each well and plates were shaken gently for 15 min to dissolve the coloured formazan crystals formed by cellular NAD(P)H dependent oxidoreductase enzymes. The absorbance at 595 nm of each well was measured using a microplate reader and a 1420 Multilabel Counter Victor3V spectrophotometer (PerkinElmer, USA). All MTT assays were performed in triplicate at three independent times with six replicate wells for each test compound. For statistical analysis, the mean and standard deviation was calculated using Microsoft® Excel (Microsoft Corporation, USA). The calculations of the IC_{50} concentrations for each cell line, time-point, complex and control was

performed using the statistical package GraphPad Prism (Ver. 6.0) (GraphPad, USA). The IC_{50} value is the concentration of the test compounds that reduces the cellular viability by 50 %.

Reactive Oxygen Species (ROS) Assay. The induction of intracellular oxidative stress in A2780 and A2780cis cells was measured using a 2′,7′-dichlorofluorescein diacetate (DCFH-DA) dye which produces fluorescence on the production of reactive oxygen species. Briefly, cells were seeded into 96-well plates at a density of 1×10^4 cells/well and were incubated for 24 h at 37 °C. The medium was removed and 100 µL of a 20 µM DCFH-DA solution in PBS was added to each well and incubated at 37 °C and 5% CO_2 for 60 min in darkness. Cells were washed twice with 100 µL PBS and then treated with 100 µL of 10 µM H_2O_2 in PBS (positive control), with PBS alone (untreated control) and a concentration range of each of the five test compounds and fluorescence was measured at different time intervals over 2 h. Reactive oxygen species levels were determined by measuring the fluorescence of oxidized DCFH-DA using a spectramax M3 multiplate reader with excitation at 485 nm and emission at 530 nm. Three independent experiments were done with six replicate wells used for all test compounds, positive control and untreated control.

Syntheses

Oxaliplatin. Oxaliplatin was synthesized as described in patent PCT/EP2010/003753 [4] with slight modifications. K_2PtCl_4 (2 g, 4.8 mmol) was dissolved in 40 mL of water. To this solution diaminocyclohexane (DACH) (5.4 g, 4.8 mmol) in 20 mL ethanol was added and the mixture was allowed to react for 6 h at rt. During the reaction, the product $(DACH)PtCl_2$ precipitated. Then $(DACH)PtCl_2$ was suspended in 60 mL of water, 1.43 g Ag_2SO_4 was added and the solution was stirred at room temperature for 20 h under light exclusion. This led to the formation of soluble $[Pt(DACH)(H_2O)_2]SO_4$ complex accompanied by the formation of poorly soluble AgCl, which was filtered off. To the filtrate 0.46 g (3.7 mmol) of oxalic acid dihydrate and 0.5 M NaOH were added until the pH reached 6. The mixture was stirred at r.t. for further 20 h. The solution was concentrated using a rotary evaporator, until the final product [Pt(DACH)(ox)] precipitated. This was filtered off, washed with water and acetone and dried under vacuum. Yield: 0.63 g (33 %). Purity was confirmed by IR-spectroscopy and elemental analysis. IR (cm^{-1}): 3211m, 3160 m, 3081 s (ν_{N-H}); 2960 w (ν_{C-H}); 2929 m; 2864 m; 1696 s, 1660 s, 1653 m ($\nu_{C=O}$); 1609 m; 1373 s; 1226 s; 1069 s; 808 s,

742 (γ_{C-H}). Anal. Calcd. for $C_8H_{14}N_2O_4Pt$: C 24.19, H 3.55, N 7.05 %. Found C 23.95, H 3.65, N 7.01 %.

trans-[Pt(DACH)(ox)(OH)$_2$], A.

Oxaliplatin (0.5 g, 1.25 mmol) was oxidised with 18 mL of 30 % H_2O_2 and 12 mL of water as previously reported [2]. The reaction mixture was stirred for 1 h at 50 °C. The clear solution was then concentrated using a rotary evaporator the desired product was precipitated by adding ethanol. The white precipitate formed was filtered off, washed with ethanol and diethyl ether and dried under vacuum. Yield 0.49 g, 90 %. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.2 (s, 2 H, OH), 7.62 (2 H, NH_2), 6.85 (2 H, NH_2), 1.95 (d, 2 H, DACH), 1.44 (m, 4 H, DACH), 1.04 (m, 4 H, DACH). IR (cm^{-1}): 3427 m (ν_{Pt-OH}); 3268 m, 3211m, 3150 br, 3056 br (ν_{N-H}); 2952 m (ν_{C-H}); 2816 m; 1716 s, 1655 s ($\nu_{C=O}$); 1609 m; 1554 m; 1449 m; 1390 s; 1221 s; 1065 m; 805 s, 770 (γ_{C-H}). ESI-MS (negative ion mode): *m/z* = 430.26 [M - H]$^-$.

trans-[Pt(DACH)(ox)(IPA)(OH)], IPA = indole-3-propionate (1)

B (26 mg, 0.092 mmol) was added to **A** (50 mg, 0.116 mmol) in DMSO (5 mL). The reaction mixture was stirred at 50 °C for 20 h. The solvent was evaporated and the residue was dissolved in DMF (2 mL). The solution was then centrifuged to remove the insoluble excess **A**. The desired product was precipitated by adding diethyl ether. The solid was collected by centrifugation, washed several times with dichloromethane and diethylether to remove the residual DMF, and finally dried under vacuum. Yield: 44 mg (63 %). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.72 (1 H, NH), 8.52 - 7.08 (m, 4 H, NH_2), 7.44 (d, 1 H, Ar-H), 7.27 (d, 1 H, Ar-H), 7.11 (s, 1 H, Ar-H), 7.01 (t, 1 H, Ar-H), 6.89 (t, 1 H, Ar-H), 2.84 (t, 2 H, CH_2), 2.55 (t, 2 H, CH_2), 2.2 – 1.05 (m, 10 H, DACH). ^{195}Pt NMR (107.6 MHz, DMF (D_2O)): δ 1390. IR (cm^{-1}): 3384 m, 3178 m (ν_{N-H}); 3051 m, 2940 w (ν_{C-H}); 1716 s, 1686 w, 1659 m ($\nu_{C=O}$); 1631 m ($\nu_{C=C}$); 1352 s; 1218 s; 806, 742(γ_{C-H}). ESI-MS (negative ion mode): *m/z* = 601.13 [M - H]$^-$, 636.10 [M + Cl]$^-$. Anal. Calcd. for **1**·H_2O ($C_{19}H_{27}N_3O_8Pt$): C 36.78, H 4.39, N 6.77 %. Found C 36.73, H 4.45, N 6.80 %.

trans-[Pt(DACH)(ox)(IPA)$_2$] (2).

B (660 mg, 2.3 mmol) was added to **A** (50 mg, 0.116 mmol) in DMSO (5 mL). The reaction mixture was stirred at 65 °C for 72 h. The solvent was evaporated and the residue was dissolved in 1 mL of DMF. The desired product was precipitated by adding distilled water

(20 mL). The solid was collected by centrifugation, washed several times with dichloromethane to remove excess ligand and the residual DMSO, washed with diethylether and finally dried under vacuum. Yield: 26.6 mg (30 %). ^{1}H NMR (400 MHz, DMSO-d_6): δ =10.75 (2 H, NH), 8.40 - 8.20 (m, 4 H, NH_2), 7.44 (d, 2 H, Ar-H), 7.28 (d, 2 H, Ar-H), 7.07 (s, 2 H, Ar-H), 7.02 (t, 2 H, Ar-H), 6.93 (t, 2 H, Ar-H), δ 2.89 (t, 4 H, CH_2), 2.61 (t, 4 H, CH_2), 2.2 - 1.05 (m, 10H, DACH). ^{195}Pt{^{1}H} NMR (107.6 MHz, DMF (D_2O)): δ1588. IR (cm^{-1}): 3404 m, 3190 m (ν_{N-H}); 3059 m, 2857 w (ν_{C-H}); 1718 s, 1651 m ($\nu_{C=O}$); 1635 m ($\nu_{C=C}$); 1341 s; 1207 s; 806 m, 741 s (γ_{C-H}). ESI-MS (negative ion mode): *m/z* = 772.20 [M - H]$^{-}$. Anal. Calcd. for $C_{30}H_{34}N_4O_8Pt$: C 46.57, H 4.43, N 7.24 %. Found C 46.69, H 4.36, N 7.27 %.

trans-[Pt(DACH)(ox)(IPA)(bz)] (3) and *trans*-[Pt(DACH)(ox)(IPA)(suc)] (4), bz = benzoate, suc = succinate

1 and an excess of the corresponding anhydride were suspended in DMF. The reaction mixture was stirred overnight at 60 °C. The DMF solution was then concentrated under reduced pressure. The desired product was precipitated by adding diethylether. The solid was collected by centrifugation, washed with dichloromethane and diethylether to remove the residual DMF.

3. Yield: 22.7 mg (44 %) ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.77 (1 H, NH), 8.55 - 8.10 (m, 4 H, NH_2), 7.83 - 7.40 (m, 6 H, Ar-H), 7.28 (d, 1 H, Ar-H), 7.10 (s, 1 H, Ar-H), 7.02 (t, 1 H, Ar-H), 6.93 (t, 1 H, Ar-H), 2.86 (t, 2 H, CH_2), 2.65 (t, 2 H, CH_2), 2.2 – 1.05 (m, 10 H, DACH). ^{195}Pt NMR (107.6 MHz, DMF (D_2O)): δ 1588. IR (cm^{-1}): 3404 m, 3190 m (ν_{N-H}); 3055 m, 2937 m (ν_{C-H}); 1720 s, 1690 w, 1641 m ($\nu_{C=O}$); 1345 m, 1318 s; 1290 s; 805, 715(γ_{C-H}). ESI-MS (negative ion mode): *m/z* = 705.16 [M - H]$^{-}$. Anal. Calcd. for $C_{26}H_{29}N_3O_8Pt$: C 44.19, H 4.14, N 5.95 %. Found C 44.03, H 4.36, N 5.81 %.

4. Yield: 85 mg (73.3 %) ^{1}H NMR (400 MHz, DMSO-d_6): δ = 12.04 (s, 1 H, COOH), 10.76 (1 H, NH), 8.37 - 8.11 (m, 4 H, NH_2), 7.43 (d, 1 H, Ar-H), 7.28 (d, 1 H, Ar-H), 7.07 (s, 1 H, Ar-H), 7.01 (t, 1 H, Ar-H), 6.92 (t, 1 H, Ar-H), 2.84 (t, 2 H, CH_2), 2.60 (t, 2 H, CH_2), 2.38 - 2.30 (m, 4 H, $COCH_2CH_2CO$), 2.25 - 1.05 (m, 10 H, DACH). ^{195}Pt NMR (107.6 MHz, D_2O): δ 1592. IR (cm^{-1}): 3392 m, 3174 m (ν_{N-H}); 3059 m, 2939 m (ν_{C-H}); 1718 s, 1657 m ($\nu_{C=O}$); 1347 s; 1242 m; 806, 743 (γ_{C-H}). ESI-MS (negative ion mode): *m/z* = 701.15 [M - H]$^{-}$. Anal. Calcd. for $C_{23}H_{29}N_3O_{10}Pt$: C 39.32, H 4.16, N 5.98 %. Found C 39.23, H 4.33, N 5.91 %.

***trans*-[Pt(DAC)(ox)(IPA)(ac)] (5), ac = acetate**

1 (72 mg) was stirred at r.t. in acetic anhydride (20 mL) for 20 h. The reaction mixture was lyophilized and washed with diethylether (2 x 5 mL) to yield complex **5** as a yellow precipitate. Yield: 50 mg (61 %) ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.76 (1 H, NH), 8.41 - 8.2 (m, 4 H, NH_2), 7.44 (d, 1 H, Ar-H), 7.28 (d, 1 H, Ar-H), 7.07 (s, 1 H, Ar-H), 7.01 (t, 1 H, Ar-H), 6.91 (t, 1 H, Ar-H), 2.84 (t, 2 H, CH_2), 2.60 (t, 2 H, CH_2), 2.2 - 1.05 (m, 10 H, DACH), 1.87 (s, 3 H, CH_3). ^{195}Pt NMR (107.6 MHz, DMF (D_2O)): δ 1593. IR (cm^{-1}): 3325 m, 3162 m (ν_{N-H}); 3055 m, 2935 m (ν_{C-H});1715 s, 1658 m ($\nu_{C=O}$); 1353 s; 1268 s; 807, 743 (γ_{C-H}). ESI-MS (negative ion mode): *m/z* = 643.13 $[M - H]^-$. Anal. Calcd. for $C_{21}H_{27}N_3O_8Pt$: C 39.13, H 4.22, N 6.52 %. Found C 39.07, H 4.33, N 6.41 %.

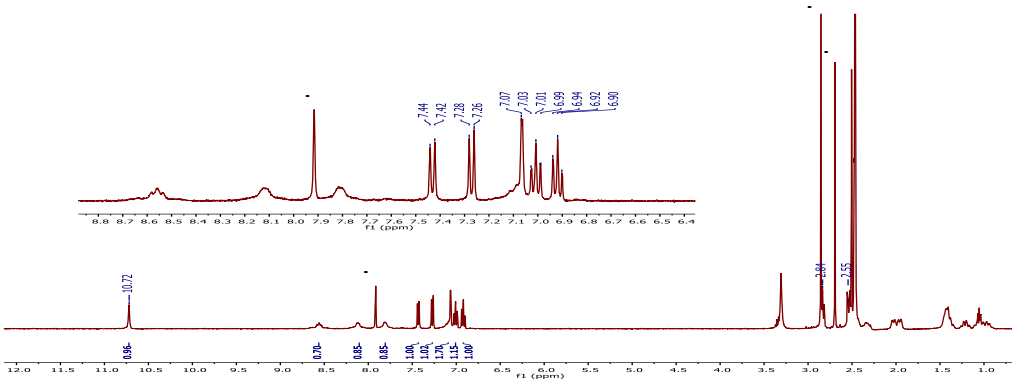


Figure 1S. ^{1}H NMR (DMSO-d_6) of **1** (* DMF).

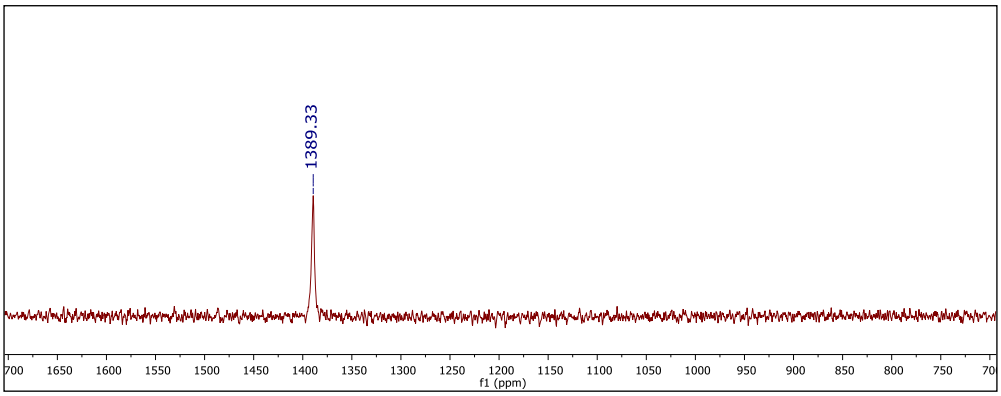


Figure 2S. ^{195}Pt NMR (DMF insert D_2O) of **1.**

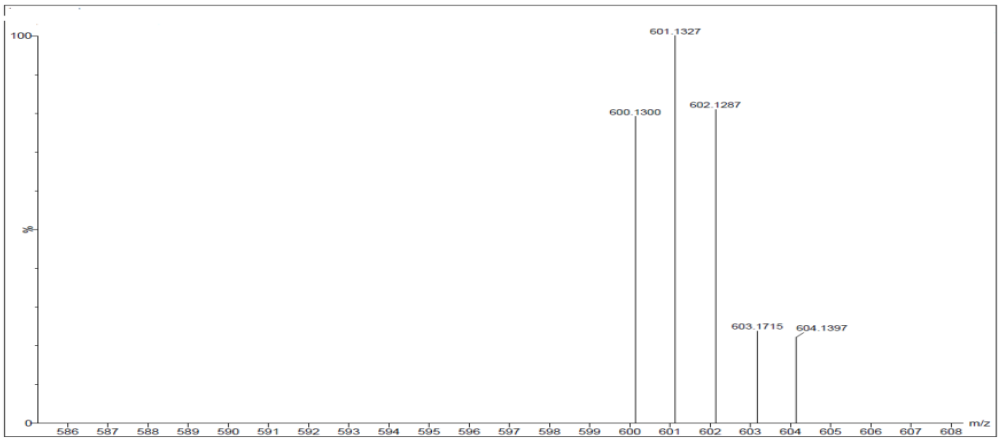


Figure 3S. ESI-MS spectrum (-) of **1**.

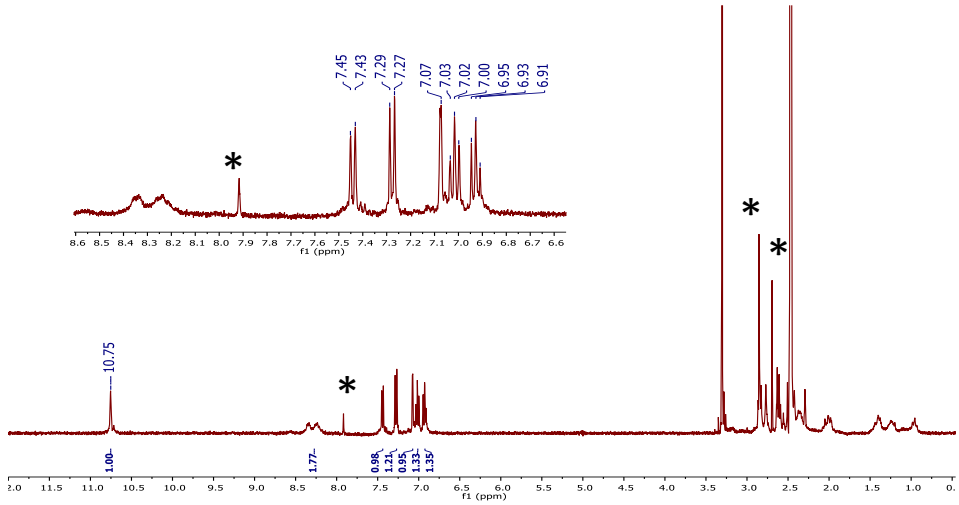


Figure 4S. ^{1}H NMR (DMSO-d_6) of **2** (* DMF).

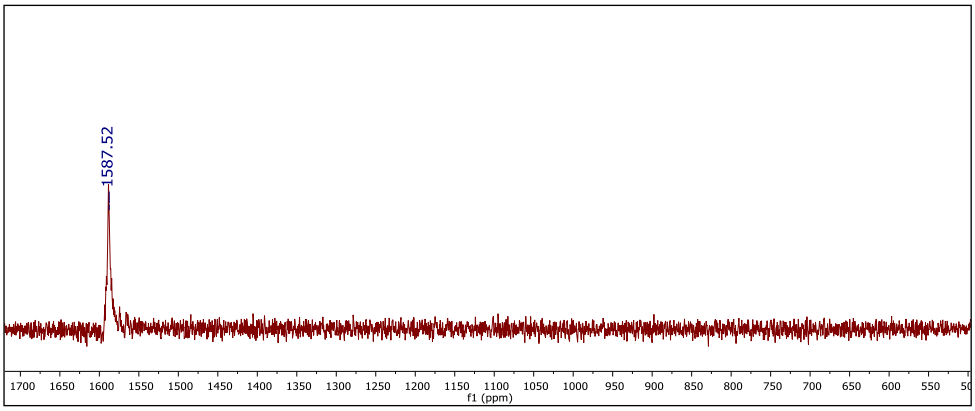


Figure 5S. ^{195}Pt NMR (DMF insert D_2O) of **2.**

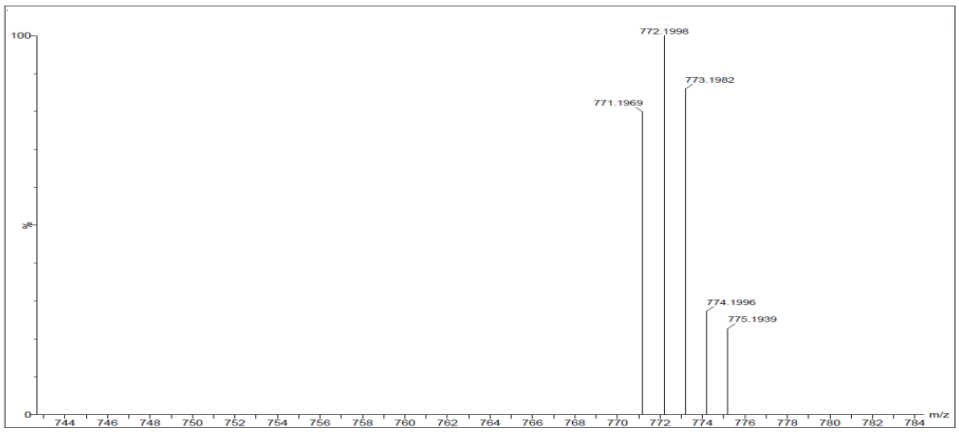


Figure 6S. ESI-MS spectrum (-) of **2**.

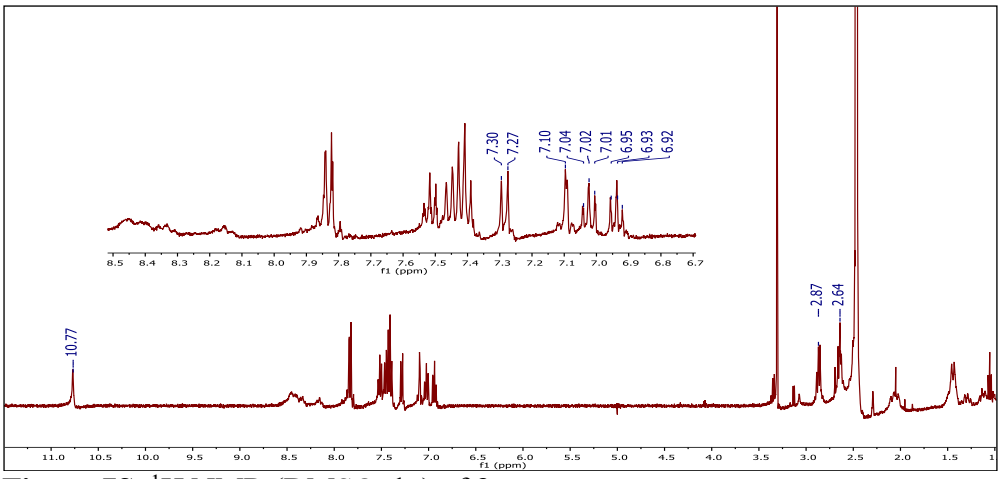


Figure 7S. ^{1}H NMR (DMSO-d_6) of **3.**

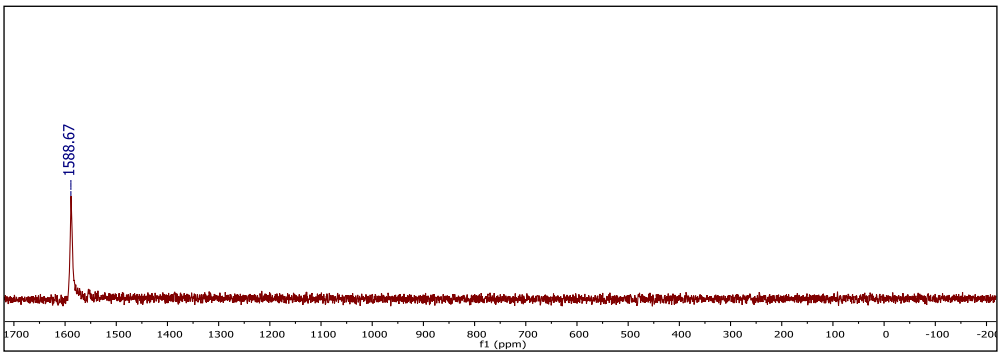


Figure 8S. ^{195}Pt NMR (DMF insert D_2O) of **3.**

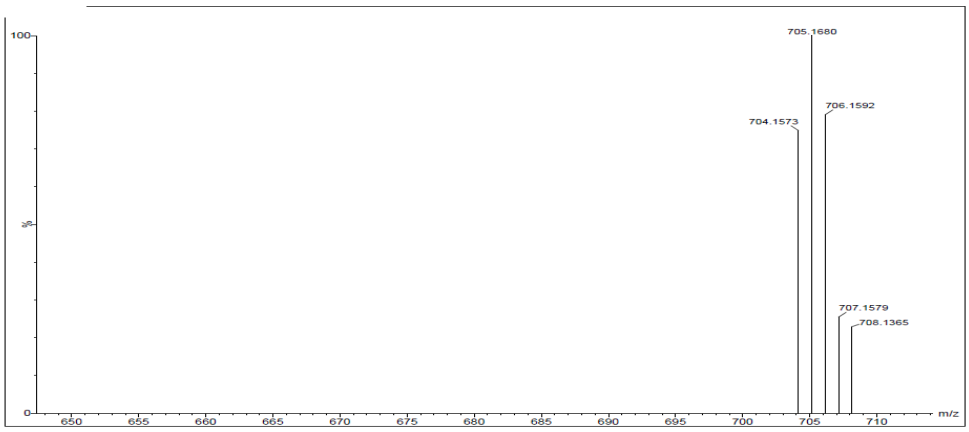


Figure 9S. ESI-MS spectrum (-) of **3**.

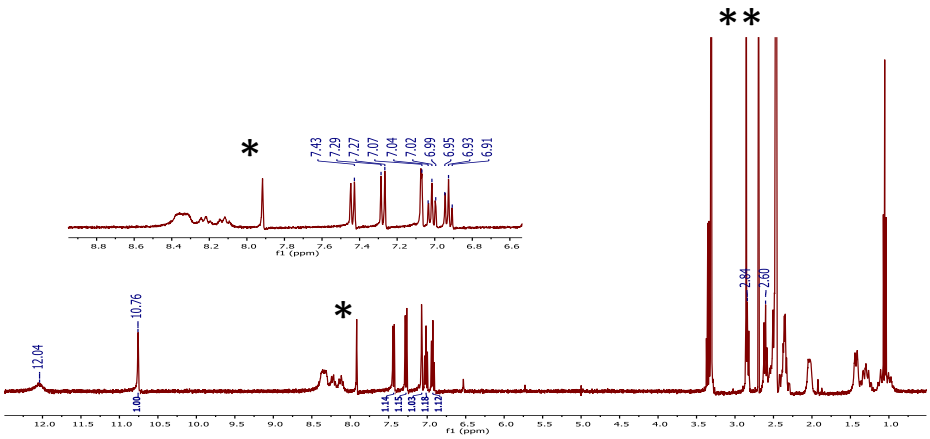


Figure 10S. ^{1}H NMR (DMSO-d_6) of **4** (* DMF).

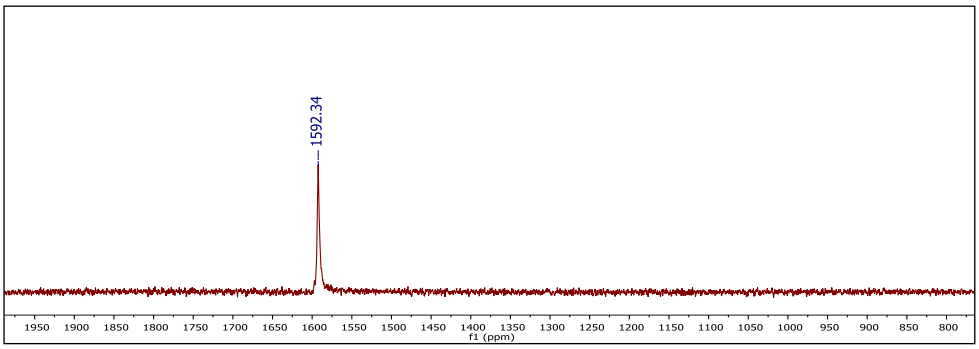


Figure 11S. ^{195}Pt NMR (DMF insert D_2O) of **4.**

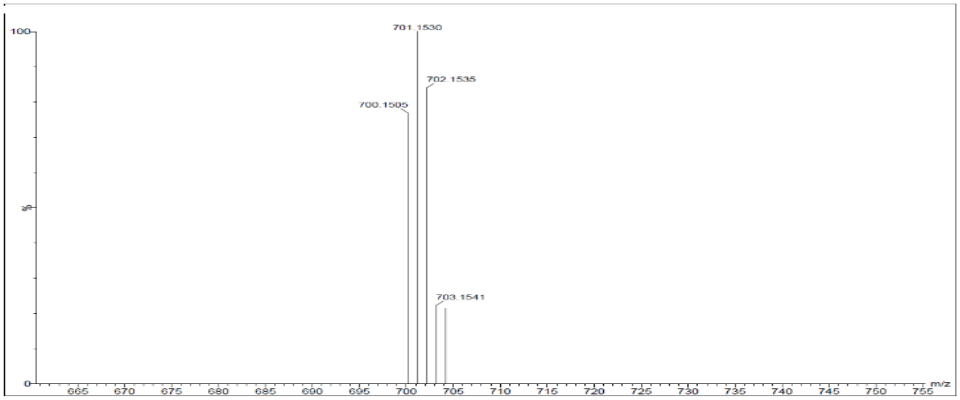


Figure 12S. ESI-MS spectrum (-) of **4**.

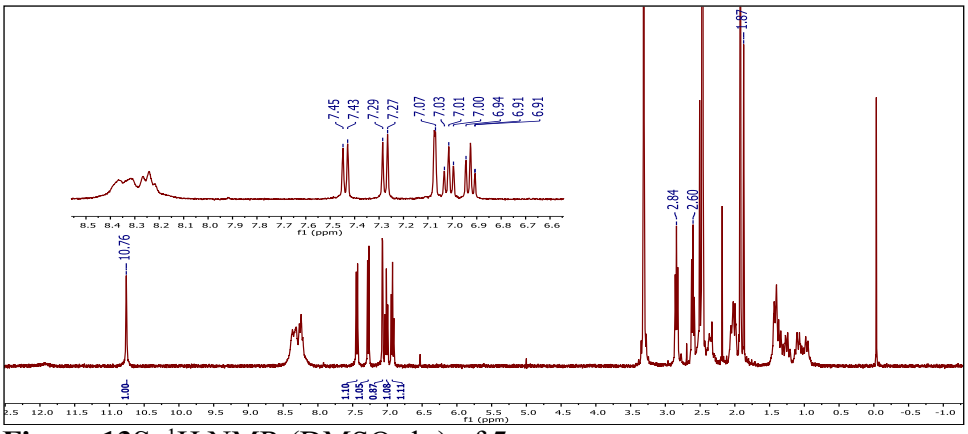


Figure 13S. ^{1}H NMR (DMSO-d_6) of **5.**

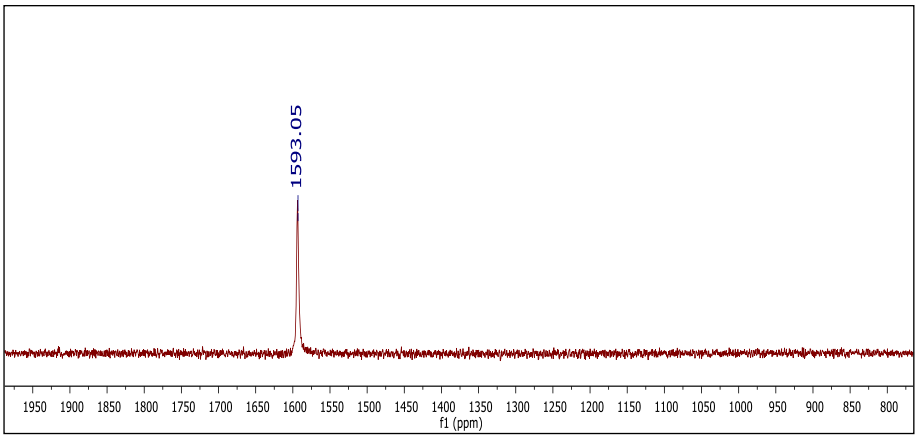


Figure 14S. ^{195}Pt NMR (DMF insert D_2O) of **5.**

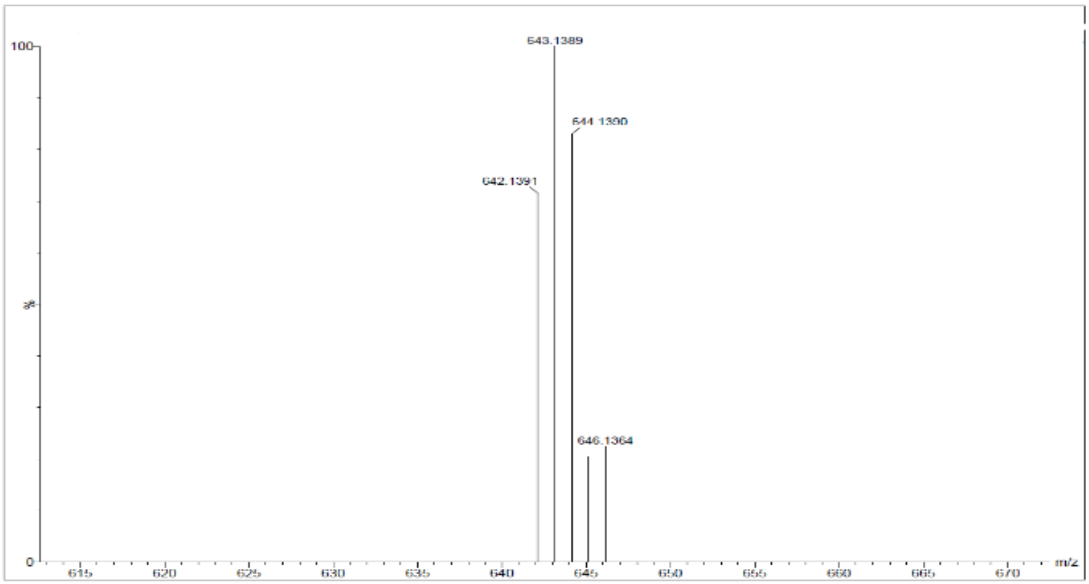


Figure 12S. ESI-MS spectrum (-) of **5**.

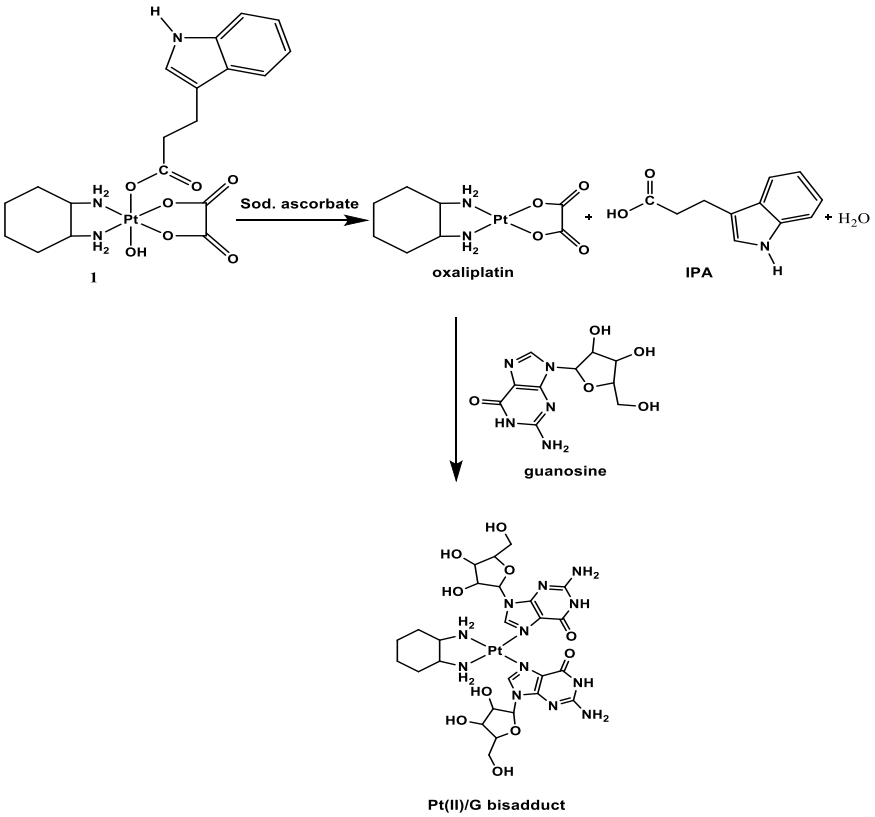


Chart 1S. Reduction of **1** and its interaction with guanosine.

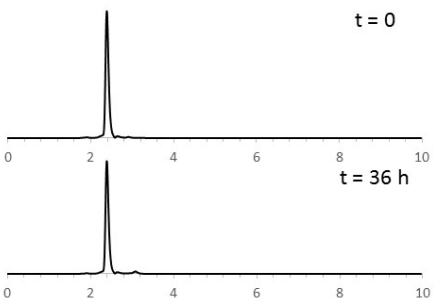
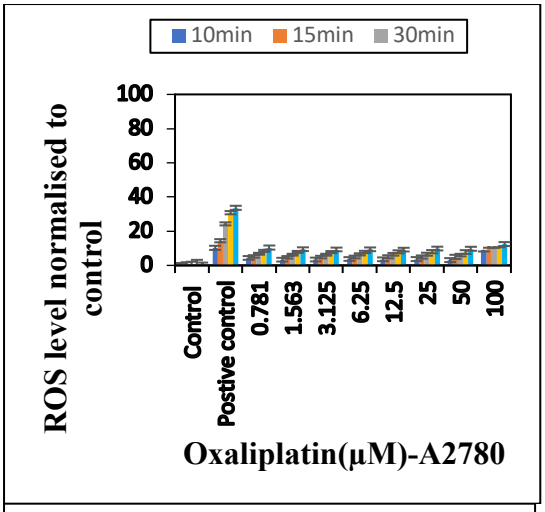
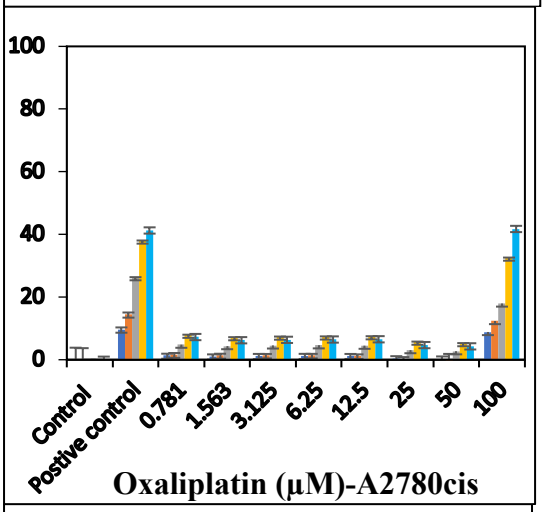
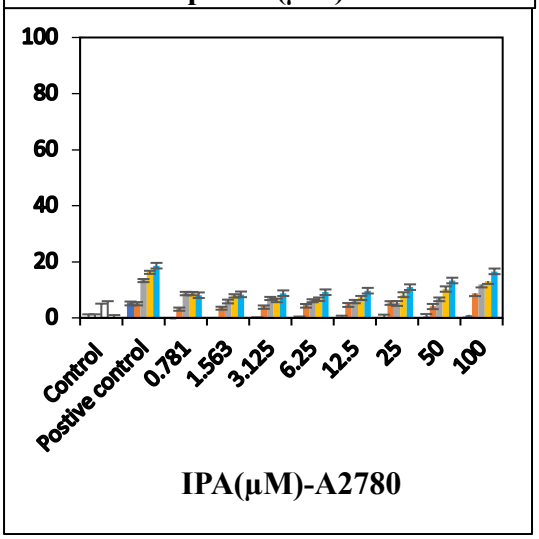
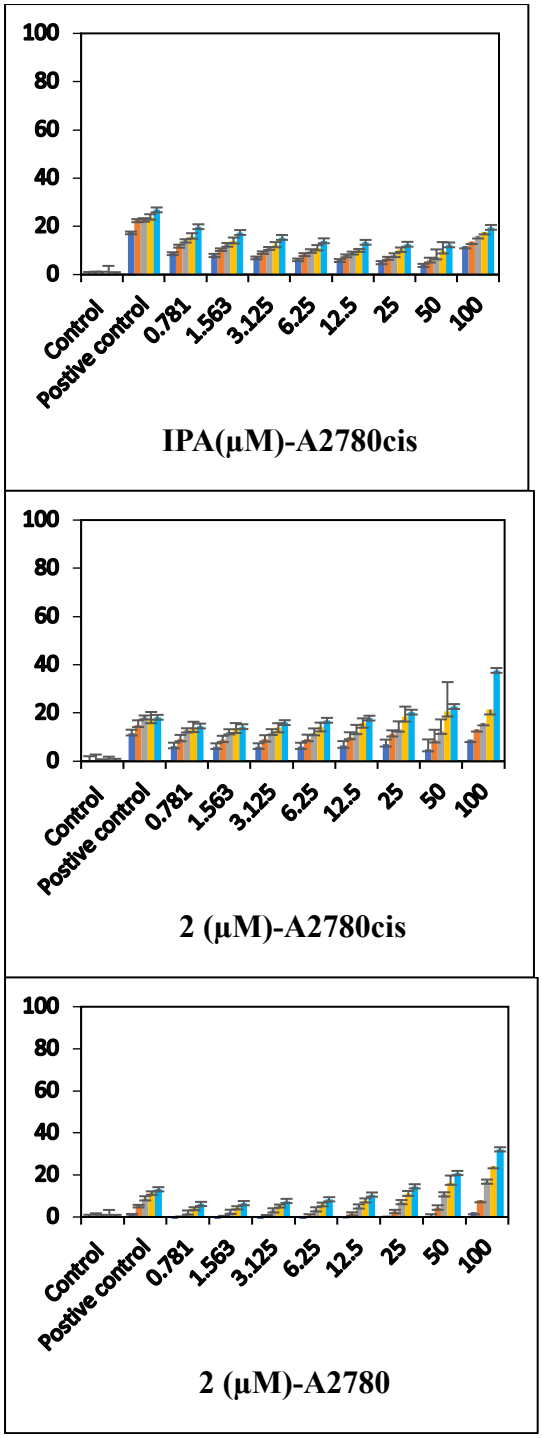


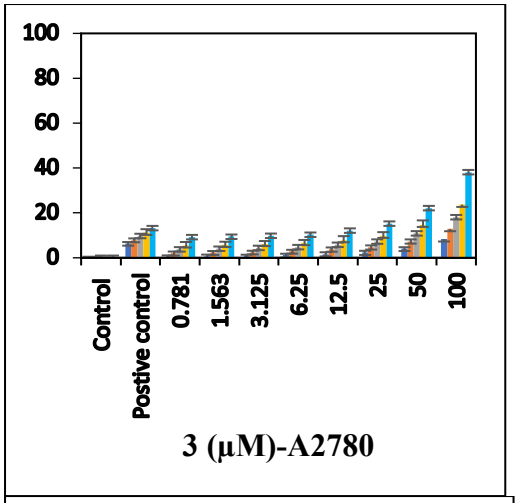
Figure 13S. Chromatograms of **1** in HEPES buffer.

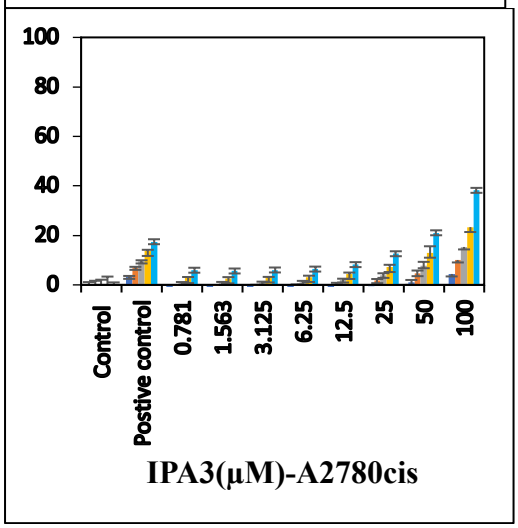

10min
15min
30min
ROS level normalised to control
100
80
60
40
20
0
Control
Postive control
0.781
1.563
3.125
6.25
12.5
25
50
100
Oxaliplatin(µM)-A2780

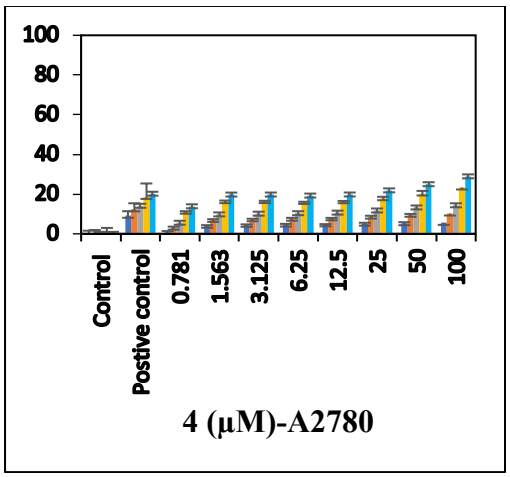

100
80
60
40
20
0
Control
Postive control
0.781
1.563
3.125
6.25
12.5
25
50
100
Oxaliplatin (µM)-A2780cis


100
80
60
40
20
0
Control
Postive control
0.781
1.563
3.125
6.25
12.5
25
50
100
IPA(µM)-A2780


100
80
60
40
20
0
Control
Postive control
0.781
1.563
3.125
6.25
12.5
25
50
100
IPA(μM)-A2780cis
2 (μM)-A2780cis
2 (μM)-A2780


100
80
60
40
20
0
Control
Postive control
0.781
1.563
3.125
6.25
12.5
25
50
100
3 (μM)-A2780


100
80
60
40
20
0
Control
Postive control
0.781
1.563
3.125
6.25
12.5
25
50
100
IPA3(μM)-A2780cis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80
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40
20
0
Control
Postive control
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3.125
6.25
12.5
25
50
100
4 (μM)-A2780

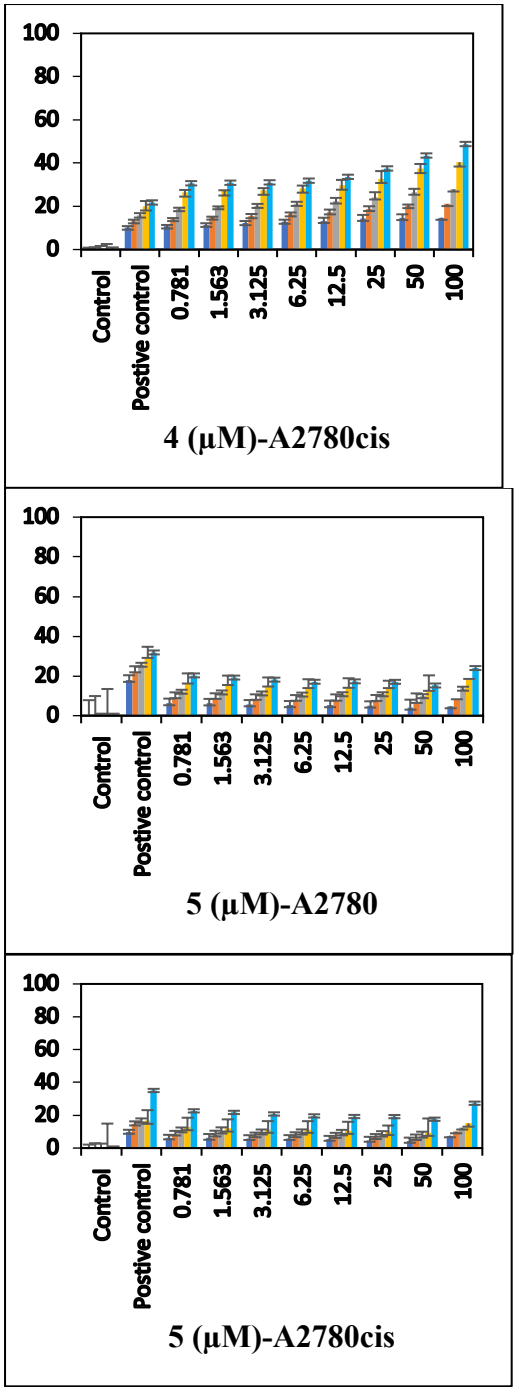

Figure 14 S. ROS production of the free ligand IPA, oxaliplatin and complexes **2-5** in A2780 and A270cis cells.

References

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[4] N. P. Cid, M. J. Novas, A. A. Tomei, Process for Preparation of 1,2-Diamino-Cyclohexane-Platinum(II) Complexes. U.S. Patent 8,637,692, issued January 28, 2014.