



The Development of Trackable Polynuclear Platinum(II) Compounds for Targeted Anticancer Applications

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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List of Abbreviations

ACN	Acetonitrile
BCN	Bicyclononyne
BspQI	<i>Bacillus stearothermophilus</i>
DNA	Deoxyribonucleic acid
DDD	Dickerson-Drew dodecamer
DIPEA	Diisopropylethylamine
DMF	Dimethyl formamide
DNMT	DNA methyltransferase
dsDNA	double-stranded DNA
EcoRI	Restriction endonuclease from <i>Escherichia coli</i> RY-13 vector
FDA	Food and Drug Administration
FPX	Fondaparinux
FTIR	Fourier transform infrared spectroscopy
GSH	Glutathione
HMG1	High-mobility group 1
HpaII	<i>Haemophilus aphrophilus</i> II
HPLC	High performance liquid chromatography
HSPGs	Heparan sulfate proteoglycans
HSQC	Heteronuclear single quantum coherence
L	Linear
LOD	Limit of detection
MET	Metallothionein
MMR	Mismatch repair
MSPI	<i>Moxarella</i> species plasmid from <i>Escherichia coli</i>
MTD	Maximum tolerated dose
NCI	National cancer institute
NER	Nucleotide excision repair
NMR	Nuclear magnetic resonance
OC	Open circular
PNA	Peptide nucleic acid
PPC	Polynuclear platinum(II) complex
pUC19	Plasmid – University of California 19
RNA	Ribonucleic acid
ROS	reactive oxygen species
SAM	S-adenosyl methionine
SC	Supercoiled
ssDNA	single-stranded DNA
TBP	TATA binding protein

TEA	Triethylamine
TFO	Triplex-forming oligonucleotide
TNBC	Triple negative breast cancer
TO	Thiazole orange

Abstract

Sinéad O'Carroll, B.Sc

The Development of Trackable Polynuclear Platinum(II) Compounds for Targeted Anticancer Applications

Recently, there has been major interest in the design and development of therapeutic polynuclear platinum(II) complexes (PPCs). The potential use for these complexes has been epitomised by BBR3464—a trinuclear platinum(II) complex featuring *trans* geometry with platinum(II) centres separated by 1,6-hexanediamine linkers and terminal *trans* labile chloride ligands—which was the first and only platinum(II) drug to enter clinical trials not based on the conventional mononuclear *cis*-chemotype. While this drug class has excellent therapeutic potential, issues regarding off-target toxicity cannot be overlooked. This thesis presents the synthesis, characterisation, cytotoxic analyses, and DNA binding studies of a novel azide-appended polynuclear platinum(II) complex series (N₃-PPCs). These agents feature fully *trans*-symmetric geometry, vary in nuclearity, and contain a terminal azide to enable biological tracking. Furthermore, although each platinum(II) centre is separated by a 1,6-hexanediamine linker similar to BBR3464, this series lack a labile chloride ligand and bind with DNA through a non-covalent mechanism called the phosphate clamp. The stand-out complex in this series, N₃-TriplatinNC, has demonstrated excellent anticancer properties *in-vitro* in MDA-MB-231 triple negative breast cancer cells and displays impressive *in-vivo* tumour regression in mice bearing MDA-MB-231 xenografts. Click chemistry labelling studies were conducted to investigate the cellular localisation and accumulation properties of the N₃-PPC series in MDA-MB-231 triple negative breast cancer cell lines. This method of post-exposure 'click' modification facilitates accurate detection of the localisation and accumulation properties of the native drug alone. Finally, azide-appended PPCs were functionalised with a thiazole orange (TO) reporter molecule to create the N₃-TO-PPC series. These TO-appended N₃-PPCs were studied for their DNA binding properties and results were compared to the aforementioned N₃-PPCs and earlier reported PPCs. Future work on the TO appended PPCs entails cellular tracking within MDA-MB-231 cell lines and compared to non-TO modified congeners.

Chapter I

Platinum(II) Drugs as Anticancer Agents

1. Introduction

1.1. Mononuclear platinum(II) drugs as anticancer agents: discovery and development

Platinum(II)-based anticancer drugs have held an essential role in chemotherapy since the fortuitous discovery of the biological activity of cisplatin (*cis*-[Pt(NH₃)₂Cl₂]) by Barnett Rosenberg in 1965 (Figure 1). During an investigation of the potential impact of magnetic dipoles and electric fields on bacterial cell growth, Rosenberg observed complete inhibition of cell division in the presence of platinum electrodes. It was hypothesized that the current applied to the system was generating platinum complexes within the culture medium and that these species were interfering with and inhibiting the growth of *E. Coli*. Comprehensive chemical analysis identified *cis*-diamminedichloro-platinum(II) (cisplatin) as the dominant biologically active species present.¹ The *trans* isomer was significantly less active.² It was immediately realised that a platinum complex with the ability to inhibit cell growth would be useful for treatment of human cancer.

Rosenberg and co-workers began to study the effects of platinum complexes on mice bearing different types of cancerous tumours. Cisplatin, as well as other analogues, were tested and it was observed that cisplatin inhibited tumour growth in mice infected with leukaemia L1210 and sarcoma 180.³ This discovery drove much interest toward the potential medicinal use of platinum coordination complexes and other inorganic materials for the treatment of cancer. Previous to these discoveries, identifying new compounds for cancer treatment was exclusive to the field of organic chemistry. Further studies conducted at the National Cancer Institute (NCI) on the efficacy of cisplatin for clinical use revealed that the treatment proved extremely effective, notably on patients who had not responded to other forms of chemotherapy.⁴ Cisplatin received FDA clinical approval in 1978 and since then has been used either in combination with other chemotherapeutic agents, or by itself, for the treatment of breast, colon, melanoma, ovarian, testicular, lung, oesophageal, bladder, head and neck, gastric and prostate cancer.⁵⁻⁸

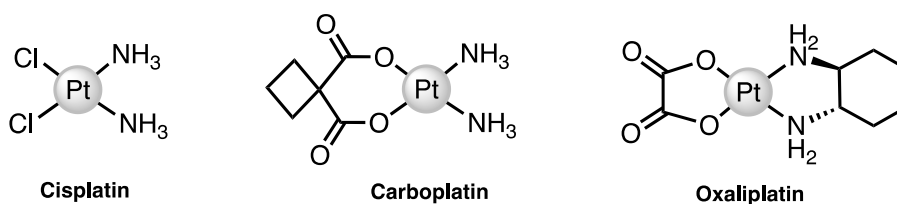


Figure 1. Structures of cisplatin, carboplatin and oxaliplatin; the only platinum(II)-based drugs to gain FDA approval for the treatment of various cancers.

The effectiveness and success of cisplatin, however, cannot overshadow its clinical shortcomings with regard to severe dose-limiting toxicity and the development of cellular resistance. Efforts have been made to circumvent these limitations by developing analogous *cis*-chemotype platinum(II) drugs that maintain a comparable cytotoxic effect but with slightly altered DNA binding kinetics. Carboplatin and oxaliplatin (Figure 1) were developed to combat cisplatin resistance within some cancer cell lines, as well as reduce the level of ototoxicity and nephrotoxicity induced by cisplatin. The key difference in molecular structure between cisplatin and carboplatin is the substitution of labile chloride ligands for a bidentate oxygen donor chelating ligand. This alters the chemical reactivity of carboplatin and makes it less reactive than cisplatin. The aquation kinetics for a bidentate ligand inside the cell is slower, which minimises interactions of the aquated species with intracellular nucleophiles, lowering the chances of complex deactivation. Carboplatin is used to treat a number of cancer types such as ovarian, small cell lung, breast and brain tumours either by itself, or in combination with other treatments. It is much less nephrotoxic and ototoxic than cisplatin, however it is myelosuppressive, and can potentially compromise the patients' immune system by reducing the number of white blood cells produced in the bone marrow.

Oxaliplatin gained clinical approval in the European Union and the United States in 1999 and 2002, respectively. Its structure incorporates two bidentate chelating ligands: (i) an oxalate (good leaving group), and (ii) a 1,2-diaminocyclohexane (inert leaving group). The success of oxaliplatin is attributed to its lower ototoxicity and nephrotoxicity in comparison to that of cisplatin, as well as being less myelosuppressive than carboplatin. Unfortunately, oxaliplatin displays a different spectrum of side-effects to that of cisplatin and carboplatin including peripheral neuropathy. Effects of peripheral neuropathy are

usually reversible after acute exposure to the drug, however after prolonged exposure the effects of the drug-induced neuropathy may not be entirely reversible. Oxaliplatin is regularly used to treat cisplatin-resistant tumours and is particularly effective in combination with 5-fluorouracil for the treatment of metastatic colorectal cancer.⁹

Although cisplatin, carboplatin, and oxaliplatin have gained approval for clinical use world-wide, more mononuclear *cis*-platinum(II) anticancer agents have gained regional approval and some are still currently undergoing clinical trials. These include (Figure 2): nedaplatin which has gained clinical approval in Japan for the treatment of ovarian and cervical cancer as well as oesophageal and bladder cancer¹⁰; lobaplatin which gained regional approval for clinical use in China for the treatment of breast tumours, non-small cell lung cancer and specific forms of leukemia¹¹; heptaplatin gained clinical approval in South Korea for the treatment of gastric cancer and has been found particularly effective in combination with paclitaxel for the treatment of head and neck squamous cancers¹²; satraplatin (Figure 2) is yet to gain FDA approval and is effective for the treatment of hormone-refractory prostate cancers¹³; picoplatin which is still undergoing clinical trials (Phase III) for the treatment of metastatic colorectal cancer. Picoplatin and phenanthriplatin (Figure 2) are other *cis*-chemotype complexes that are currently undergoing pre-clinical investigation.

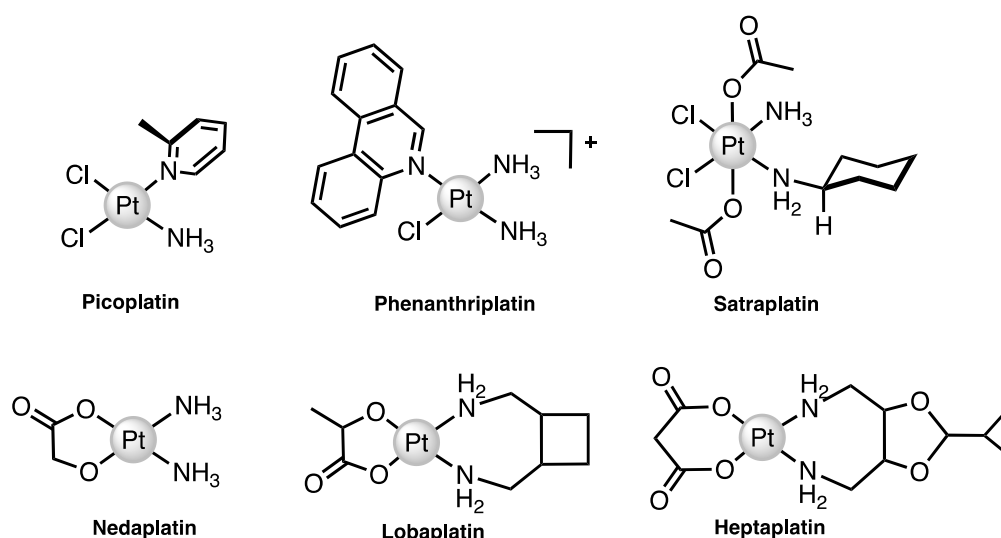


Figure 2. Structures of more platinum(II) anticancer agents that have gained regional clinical approval, are currently undergoing clinical trials, or are undergoing preclinical investigation.

Next, topics related to *cis*-chemotype platinum(II) complex DNA binding, their modes of action, and the development of resistance will be described. Later, the invention of the polynuclear platinum complex series (PPCs) will be detailed with emphasis on their unique, modular DNA-binding profiles. The invention of the PPC series challenged the mononuclear *cis*-chemotype structure-activity relationship paradigm associated with platinum(II) anticancer agents and present promising new avenues in the realm of platinum(II) anticancer drug discovery.

1.2. Cisplatin and its analogues and their interactions with genomic DNA

While there is an abundance of evidence to suggest that the primary cellular target of cisplatin and platinum(II) anticancer drugs is genomic deoxyribose nucleic acid (DNA), the chemical reactivity of platinum(II) complexes is largely controlled by the displacement reactions of the various ligands surrounding the metal centre. With this in mind, for platinum-DNA interactions to occur, cisplatin must first become activated via spontaneous sequential intracellular aquation of the labile *cis*-chloride ligands¹⁴ before covalently binding to the N-7 position of the heterocyclic base of DNA (Figure 3).¹⁵ This leads to the formation of predominantly 1,2-intrastrand GpG crosslinks, and to a lesser extent 1,2-intrastrand ApG crosslinks. The consecutive hydrolysis of chloride ligands allows for a mono-aquated species to firstly platinate the heterocyclic base of DNA before the hydrolysis of the second chloride ligand occurs. The mono-aquated species carries a charge of +1 which also enhances the inherent binding affinity of the drug to the polyanionic DNA (Figure 3).

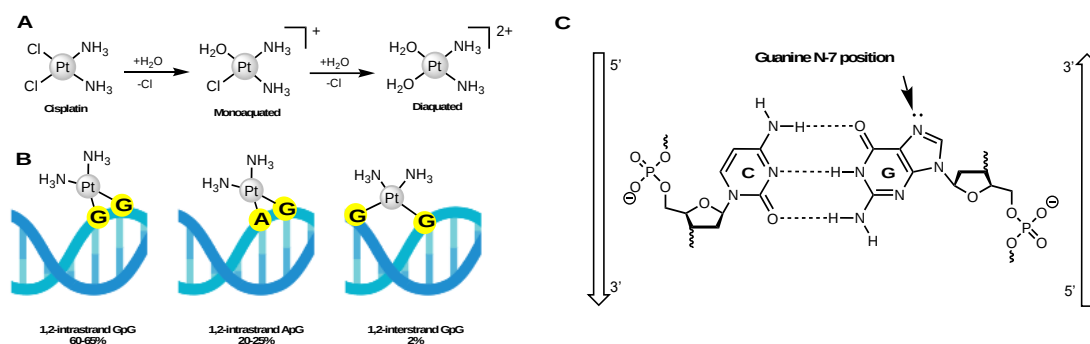


Figure 3. A. Mechanism of sequential hydrolysis of neutral cisplatin to mono-aqua and di-aqua species; B. Predominant types of cisplatin-DNA intra- and interstrand crosslinks formed; C. Nitrogen-7 platination site on a guanine nucleobase.

The mechanism of DNA binding is primarily driven by the *trans*-effect, which can be defined as the ability of a ligand to direct substitution opposite (*trans*) to itself in square-planar complexes. The formation of these platinated DNA adducts results in duplex unwinding and directional bending in the major groove of the DNA, which consequently triggers the activation of various signal transduction pathways and ultimately results in apoptosis (Figure 4). This process is initiated by the binding of approximately 20 individual proteins to locations on the DNA that have been distorted due to the formation of the intrastrand platinum-DNA adducts.¹⁶ Interestingly, it was discovered that some of these proteins, such as high-mobility group 1 (HMG1) and mismatch repair (MMR), show preferential binding to cisplatin-DNA adducts over other platinum(II) drugs, satraplatin and oxaliplatin.^{17, 18} Although the suspected role of these DNA recognition proteins is to transmit DNA damage signals and trigger downstream effects, these proteins may also function to enhance the toxicity of the active drug. For example, it was demonstrated that HMG1 firstly interacts with platinated DNA before shielding the adduct from repair by human excision nuclease.¹⁹

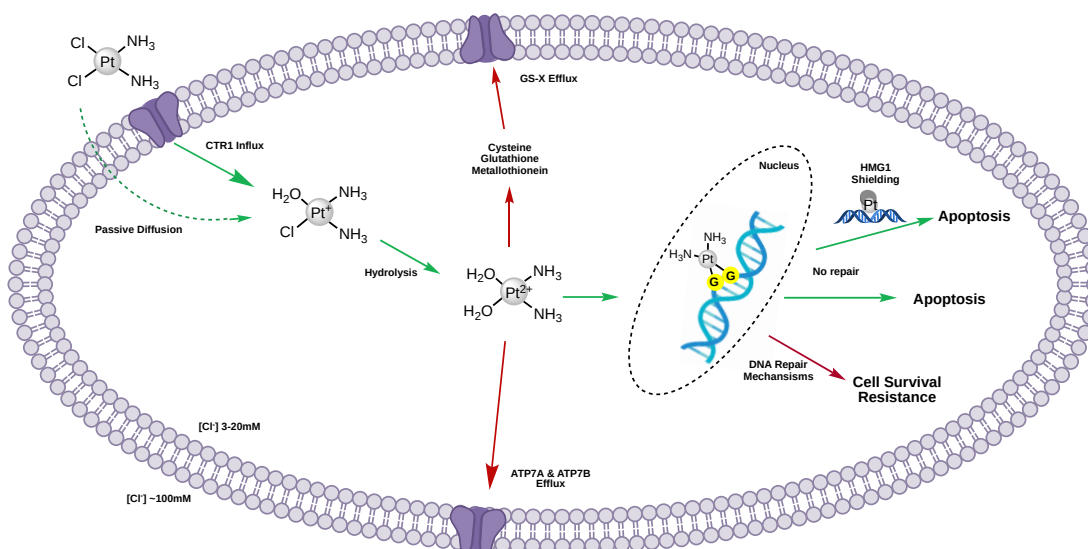


Figure 4. Cellular uptake, mode of action and mechanisms of resistance to cisplatin. Cisplatin is taken up into the cell by the CTR1 copper transporter protein and can also enter the cell by passive diffusion. Passive diffusion is facilitated by the difference in chloride ion concentration inside and outside the cell. Once inside the cell, the labile chlorides are sequentially aquated. Both the mono- and di-aqua forms of cisplatin are susceptible to reaction with sulphur-containing nucleophiles, and these adducts can then be removed from the cell by copper efflux proteins ATP7A and ATP7B or GS-X pumps. Once the fully aquated form enters the nucleus, it covalently binds to the N-7 position of the nitrogenous bases of DNA forming platinum(II)-DNA adducts. The platinum(II)-DNA adducts are then either repaired by nuclear DNA repair mechanisms, which can lead to the development of resistance, or they're not repaired and the adduct formation triggers downstream effects which eventually results in apoptosis.

1.3. Mechanisms of platinum(II) resistance: intrinsic and acquired

While the discovery and use of cisplatin for medicinal purposes has revolutionised cancer treatment in the clinic and dramatically reduced mortality rates on an international scale, significant issues regarding the development of acquired and intrinsic resistance cannot be ignored. As is common with many chemotherapeutics, cells can either acquire platinum resistance upon prolonged, chronic exposure to cisplatin, or it can reveal itself as an intrinsic phenomenon. Acquired resistance mechanisms are multifactorial and resistant cells can experience several independent resistance mechanisms simultaneously, such as reduced drug accumulation, deactivation by sulfur-containing nucleophilic proteins and an increase in platinum-DNA adduct repair by nucleotide excision repair enzymes.^{20,21} Each of these mechanisms contributes significantly to the development of acquired platinum(II) resistance and is the reason platinum anticancer drug discovery remains an exciting and important area of research today.

1.3.1. Reduced intracellular drug accumulation

Platinum(II) *cis*-chemotype drugs are believed to enter the cell by the combined effects of passive diffusion and active transport. Due to the extracellular high chloride concentration (~100 mM), cisplatin is transported across the lipophilic cellular membrane as the charge neutral dichloro-species, before the labile chloride ligands are sequentially displaced by H₂O ligands inside the cell.²² It has been suggested that a number of membrane-bound transporter proteins may facilitate the transport of cisplatin inside the cell. In 2000, the relationship between copper regulatory-transporter proteins and cisplatin cell accumulation was investigated. Komatsu *et al.* demonstrated that cisplatin-resistant cells express a higher level of the ATP7A and ATP7B copper efflux proteins than cisplatin-sensitive cells, suggesting that these copper efflux proteins may also sequester and remove platinum from the cell.²³ Moreover, Atox1, a copper metallochaperone protein that directly transports copper ions to ATP7A and ATP7B, was heavily implicated in the transportation of cisplatin into the cell, as well as in the mechanism of resistance to platinum(II) drugs.^{24,25} While there is no question that cisplatin and oxaliplatin interact with Atox1,²⁶ and the platinum(II) metal-centres can bind in the sulfur-rich copper-binding sites of the protein,²⁷ the transport of [Pt(NH₃)₂]²⁺ was not directly observed and remains open to interpretation. Despite this, it is reasonable to believe that cisplatin may take advantage of this transport system. Recent studies demonstrated that simultaneous cisplatin and copper binding to Atox1 is possible. The binding of both metal-centres to Atox1 is glutathione-mediated and results in the formation of a copper/platinum/sulfur cluster which sterically facilitates the platinum(II) centre to retaining a square-planar geometry.²⁸

1.3.2. Reactions with sulfur-containing biomolecules

In 2003, kinetic reactions between cisplatin and GSH or MET under physiological conditions were studied using UV-visible spectroscopy and high performance liquid chromatography (HPLC). It was discovered that MET binds to cisplatin *ca.* 50 times more effectively than GSH, indicating that MET may be a crucial protein target for combating cisplatin resistance.²⁹

A significant trend has been identified for the concentration difference of the thiol-containing tripeptide glutathione (GSH) and cysteine-rich metallothionein (MET) proteins present in cisplatin-resistant and non-resistant cells. Cells typically contain between 0.7 – 1.4 mM of GSH or MET, however, cisplatin-resistant cells were found to have significantly higher levels. It is believed that these sulfur-containing nucleophiles bind the platinum drug, forming strong Pt – S bonds, and essentially deactivate the complex before it can reach its nuclear target of DNA. Cytoplasmic detoxification of platinum(II) drugs can occur through covalent binding of GSH or MET and although the exact fate of platinum-GSH or platinum-MET adducts is not entirely understood, there is evidence to suggest that these adducts are excised from the cell by glutathione S-conjugate efflux pumps.³⁰

1.3.3. DNA repair mechanisms: nucleotide excision repair and mismatch repair

Acquired resistance to cisplatin and platinum(II) *cis*-chemotype drugs can occur due to accelerated removal of platinum-DNA adducts by DNA repair mechanisms within the cell. Nucleotide excision repair (NER) is a mechanism which utilises enzymes to identify and repair segments of DNA that have undergone chemical modification, such as platination. NER enzymes recognise the modified DNA, remove the fragment of damaged DNA, and replaces it with a correct nucleotide sequence by reading the complementary strand opposite it. It has been shown that cells with lower expression levels of NER enzymes are more sensitive to platinum(II) drugs than cells with a higher level of expression of these proteins. For example, *Xeroderma pigmentosum* cells possess decreased NER mechanisms and were shown to be 5-10 times more sensitive to cisplatin than healthy cells.³¹ Furthermore, studies have shown that the mismatch repair (MMR) mechanism is associated with the DNA damage response process to cisplatin. The MMR mechanism corrects mutations in the DNA double helix including base-base mismatches, insertions, and deletion mutations. Loss of MMR enzymes has been shown to contribute to the development of acquired resistance.³²

To circumvent issues associated with conventional *cis*-platinum(II)-like chemotype drugs such as dose limiting toxicity and the development of acquired and intrinsic resistance, researchers were forced to think beyond the known structure-activity relationship of the mononuclear *cis*-platinum(II) drugs. Indeed, the development of the polynuclear platinum(II) complex series challenged the deeply rooted paradigm that has been long accepted within the field of platinum(II) anticancer agents: the *cis* geometry is a prerequisite for the anticancer activity of platinum(II) drugs. Next, the polynuclear platinum(II) complex series will be discussed in detail, with a focus on their DNA binding profiles, ability to combat cisplatin resistance, cytotoxicity properties, and their interactions with intracellular proteins.

2. Discovery and development of polynuclear platinum(II) complexes as pre-clinical anticancer agents

The advent of the polynuclear platinum(II) complex (PPC) series presented a unique class of anticancer agents structurally distinct from the aforementioned mononuclear *cis*-chemotype. It was hypothesised that structurally different platinum(II) complexes would produce different types of platinum–DNA adducts, and potentially exhibit a broader spectrum of cytotoxic activity.³³ Following this, many novel dinuclear and trinuclear platinum(II) agents were developed and their potential was highlighted by the trinuclear bifunctional platinum(II) complex, BBR3464.³⁴

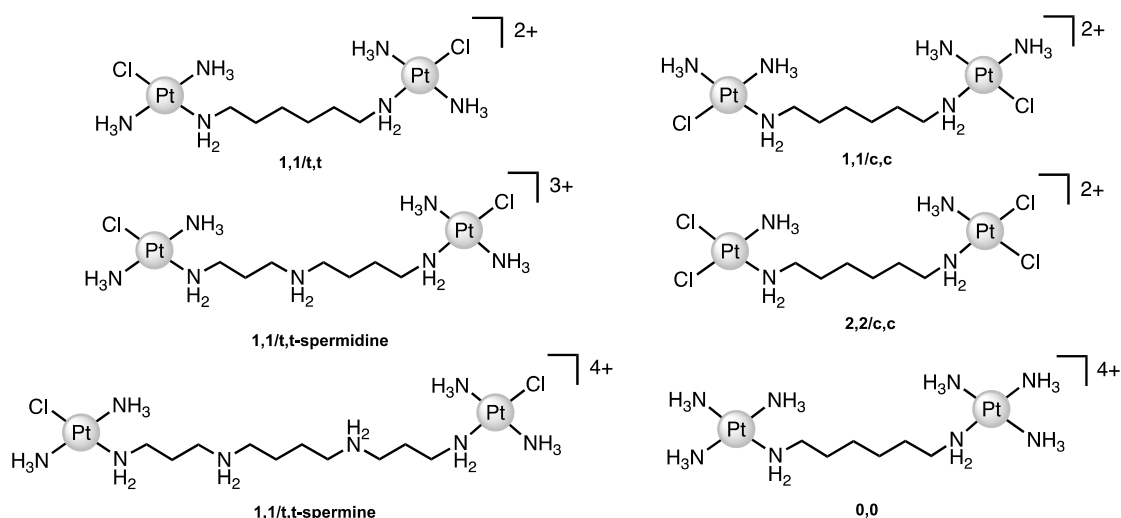


Figure 5. Structures of dinuclear platinum(II) complexes with varying charge, geometry and bidentate linker.

2.1. Dinuclear platinum(II) anticancer agents

When discussing the *trans*-symmetric dinuclear platinum(II) complex series, a unique notation was developed to describe the number of chloride ligands per platinum(II) centre and their geometry in relation to the alkyl-diamine linker. For example, “1,1/t,t” translates to one chloride coordinated to each platinum(II) centre with both of their orientations are *trans* to the diamine bridge. The first dinuclear complex, 2,2/c,c (Figure 5) was reported in 1988 by Farrell and co-workers. It can be described as two *cis*-[PtCl₂(NH₃)] units connected by an alkyl-diamine chain.³⁵ The predominant goal of its development was to demonstrate that changing the nature of the platinum(II) drug promotes an alternative DNA binding motif with the ability to form a unique platinum(II)-DNA lesion with consequential broad-spectrum activity against cisplatin-resistant cell lines. Indeed, it was discovered that 2,2/c,c produced a large amount of dinuclear platinum interstrand crosslinks, and did not exhibit cross-resistance in cisplatin-resistant cells. However, the 2,2/t,t analogue did not display any enhancement in cytotoxic activity or DNA binding when compared to its *trans*-mononuclear counterpart.³⁶ The DNA binding of the 2,2/c,c complex is tetrafunctional corresponding to four substitution sites on the complex. This is facilitated by two labile *cis*-chloride ligands on each platinum(II) centre, however, it is axiomatic that the formation of crosslinks by dinuclear platinum(II) complexes only requires a single Pt–Cl bond per platinum(II) centre. This prompted the development of the geometric isomers, 1,1/c,c and 1,1/t,t (Figure 5).³⁷ This generation of novel dinuclear platinum(II) complexes with equivalent coordination spheres and terminal labile chloride ligands were linked together by a variable-length alkyl-diamine chain. The previously established mononuclear *cis*-chemotype structure-activity-relationship, as well as the structure-activity relationship of the 2,2/c,c and 2,2/t,t series, would reason that 1,1/t,t would not display antitumour activity. Unexpectedly, further investigations demonstrated that this bifunctional dinuclear *trans*-drug possessed significant in vivo and in vitro cytotoxic activity—comparable to that of cisplatin—and efficiently overcomes cisplatin resistance more effectively than its 1,1/c,c isomer. This discovery represents a fundamental difference between the chemical and biological principles and mechanisms of mononuclear and dinuclear platinum(II) anticancer drugs. Therefore, *cis*-

geometry is not a prerequisite for cytotoxic activity in dinuclear platinum(II) anticancer agents. These structurally different, *trans*-symmetric, platinum(II) drugs are distinct in their own right, and with this discovery the structure-activity-relationship paradigm of platinum(II) anticancer agents was challenged.

2.1.1. Dinuclear platinum(II) DNA binding

Dinuclear platinum(II) complexes covalently bind to the heterocyclic base of DNA and inhibit intracellular processes such as DNA replication and transcription. This indicates that the nature of the platinum(II)-DNA adduct formed plays a crucial role in their mechanism of action. Early studies showed the distinct differences in DNA modification between the dinuclear complexes and cisplatin. 1,1/*t,t* and 1,1/*c,c* both induce helical unwinding of duplex DNA by 10-12°, reminiscent of the duplex DNA unwinding of cisplatin. However, both dinuclear isomers induce B→Z DNA conformational changes in poly(dG-dC).poly(dG-dC) predominantly forming interstrand crosslinks with DNA (Figure 6).^{38, 39} It was observed that 1,1/*t,t* produces a minor number of 1,2-GpG intrastrand crosslinks while the 1,1/*c,c* complex does not. ¹H NMR spectroscopic studies then revealed a restricted rotation around the 3' guanine base in single-stranded 1,2-GpG intrastrand crosslinks of 1,1/*c,c*. This restricted rotation would make it physically more difficult for the complex to form 1,2-GpG intrastrand crosslinks on duplex DNA and was thought to be responsible for the inability of 1,1/*c,c* to form 1,2-GpG intrastrand crosslinks on duplex DNA.⁴⁰

Studies of dinuclear platinum(II) complexes have largely focused on structural and mechanistic differences and similarities between 1,1/*t,t* and 1,1/*c,c* DNA adducts. Notably, these complexes are both capable of predominantly forming 1,2 interstrand crosslinks, and to a lesser extent 1,3 and 1,4 interstrand crosslinks. 1,3 and 1,4 interstrand crosslinked platination sites are separated by 1 or 2 intervening base pairs and show preferential directional DNA binding in the 5'→5' direction (Figure 6).⁴¹

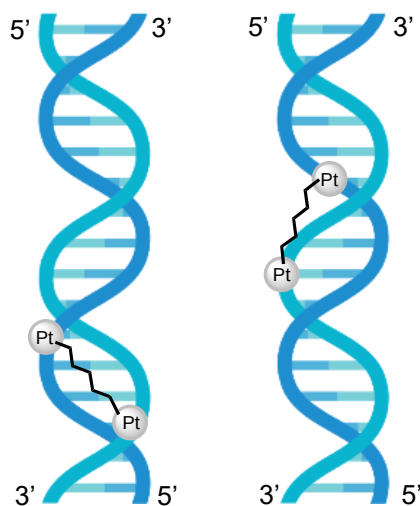


Figure 6. Schematic diagram showing the directional isomers of $5' \rightarrow 5'$ (left) and $3' \rightarrow 3'$ (right) 1,4 interstrand crosslinks. Same applies to trinuclear trans-symmetric platinum(II) complexes.

2.1.2. Cytotoxicity of dinuclear platinum(II) complexes

Cytotoxicity studies were carried out on L1210 murine cancer cell lines that were either sensitive or resistant to cisplatin. 2,2/c,c exhibited cytotoxicity that was consistent with previous results, however 1,1/t,t demonstrates a notably low cytotoxic activity in cisplatin-sensitive cells. This can be attributed to its significantly lower resistance factor, which is defined as the $IC_{50}(\text{resistant})/IC_{50}(\text{sensitive})$, than the 2,2/c,c derivative.⁴² To support the viability of these new agents as antitumour agents, the results were corroborated by a cytotoxicity assay on a panel of human ovarian carcinoma cell lines. Results from this study demonstrated similar behaviour patterns to what was observed in the L1210 murine cells; 2,2/c,c and 1,1/t,t showed similar sensitivity to the cisplatin-sensitive cells but surprisingly 1,1/t,t displayed significantly enhanced activity in cisplatin-resistant cell lines when compared to the 2,2/c,c complex.⁴³ Together, these data validate the activity of the *trans*-polynuclear platinum(II) complexes as a unique class of anticancer agent.

2.2. Trinuclear platinum(II) complexes as anticancer agents

Following the body of research described above, and exciting potential of the 1,1/t,t dinuclear complex to overcome cisplatin resistance, it was believed that the addition of another platinum(II) centre could increase the overall cytotoxicity of the complex while maintaining favourable long-range binding modes. With this

came the development of a novel trinuclear *trans*-symmetric platinum(II) complex known as BBR3464 (Figure 7). The complex features *trans*-labile chloride ligands at either end that enables formation of long-range inter- and intra-strand crosslinks.

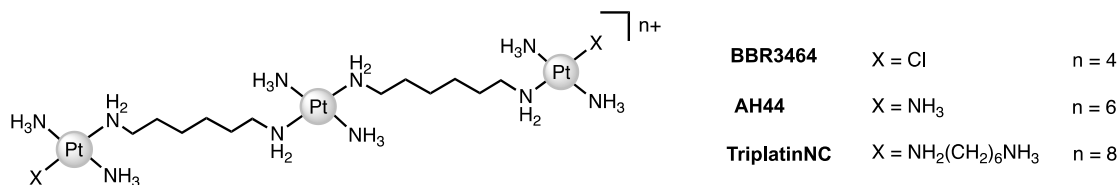


Figure 7. Chemical structures of trinuclear platinum(II) complexes BBR3464, AH44 and TriplatinNC.

2.2.1. BBR3464 – a covalent DNA binder

BBR3464 contains three platinum(II) centres separated by two 1,6-hexane diamine flexible linkers with *trans*-symmetric substitution/platination sites at either end (Figure 7). The C₆ linkers facilitates long-range DNA binding with platination sites separated by up to four intervening nucleobases. BBR3464 is inherently electrostatically attracted to the polyanionic backbone of DNA due to its polycationic nature with a 4+ charge. The central platinum(II) nucleus does not participate in covalent DNA binding, however its 2+ charge gives it a hydrogen-bonding functionality which stabilizes and enhances preassociation of the drug to DNA.⁴⁴ The directionality of the crosslink is dependent on the nature of the crosslink, that is 1,2-interstrand crosslinks occur 3'→3'; 1,4-interstrand crosslinks occur in both 5'→5' and 3'→3' directions; and 1,6-interstrand crosslinks (Figure 8) occur only in 5'→5' direction. Both directional isomers induce similar unwinding of the DNA duplex however the 3'→3' crosslink has a notably increased ability at bending the DNA towards the major groove than its 5'→5' counterpart.⁴⁵ It has also been reported that the formation of the 1,4-interstrand crosslink is largely regulated by the initial electrostatic preassociation of the central platinum(II) unit into the minor groove of the phosphate backbone.^{44, 46, 47}

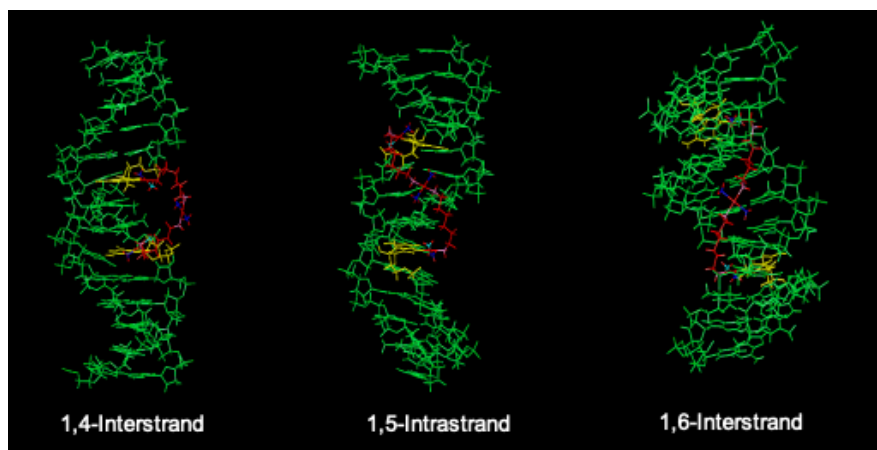


Figure 8. Molecular models of BBR3464 bound to DNA forming 1,4-interstrand, 1,5-intrastrand and 1,6-interstrand crosslinks.

BBR3464 was the first and only platinum(II) anticancer agent to reach clinical trials that did not adhere to the classical mononuclear *cis*-chemotype. This trinuclear complex effectively induces apoptosis and exhibits IC₅₀ values 20 times lower than that of cisplatin in neuroblastoma cell lines SK-N-DZ, BE(2)M17 and LAN-1, and retains potent cytotoxicity in mice bearing human neuroblastoma xenografts.⁴⁸ The effectiveness and efficiency of BBR3464 was exemplified by demonstrating complete tumour regression in lung tumours.⁴⁹ When screened against the NCI-60 cancer cell lines, it was identified that breast, renal, and colon cancer were more susceptible and sensitive to BBR3464 than cisplatin.⁵⁰ It was then suggested that this overall effect could be due to the enhanced cellular accumulation of BBR3464 relative to cisplatin, which can be attributed to its overall +4 charge. The pre-clinical data ensured that BBR3464 was a viable anticancer drug candidate, with a distinct activity profile from that of the mononuclear *cis*-chemotype platinum(II) drugs. The distinguished findings of the clinical trials were as follows:

- i. Phase I showed promise, with virtually no nephrotoxicity and neurotoxicity. Dose-limiting toxicity was identified as diarrhoea, which can be treated with loperamide, and the maximum tolerated dose was defined as 0.9 – 1.1 mg m⁻².⁵¹
- ii. Phase I demonstrated partial responses in metastatic pancreatic cancer, metastatic melanoma and bronchioloalveolar carcinoma. A combination of 5-fluorouracil with BBR3464 gave positive response to breast cancer.⁵²

- iii. Pharmacokinetic studies during phase II demonstrated drug dissociation/decomposition under physiological conditions. The trinuclear drug decomposes to produce essentially inactive mononuclear and dinuclear counterparts. This was identified to occur three times quicker in human plasma than what was observed in murine plasma. The products of the dissociation are equivalent to that produced by reaction with sulfur-containing nucleophiles.⁴⁹

Unfortunately, BBR3464 did not progress beyond phase II clinical trials, however its unique profile of activity and DNA binding cannot be denied. Further studies continued to investigate the contribution of the non-covalent interactions of the inert platinum(II) centre to the pre-association kinetics to DNA. A new trinuclear library was then developed and the labile chloride ligands were replaced with either inert NH_3 (AH44) or a 'dangling' hexyl-chain amine (TriplatinNC), now known as the substitution-inert polynuclear platinum(II) complex (Figure 7).

2.2.2. AH44 & TriplatinNC non-covalent DNA binding properties

To investigate the potential effects of the non-covalent electrostatic central platinum(II) nucleus of BBR3464 and its significance to the preassociation process of the drug to DNA, Farrell and co-workers developed two 'substitution-inert' trinuclear platinum(II) complexes known now as AH44 and TriplatinNC (Figure 7)⁵³. These complexes can only participate in electrostatic hydrogen-bonding interactions with DNA because they lack labile ligands which behave as substitution sites and are replaced by inert am(m)ine ligands for the former, or 'dangling' amines for the latter.

In 2006, the X-ray crystal structure of TriplatinNC bound to the Dickerson-Drew Dodecamer (DDD) revealed a unique, entirely electrostatic mode of DNA binding, discrete from the well-studied intercalation and minor groove binding modes.⁵⁴ Strong electrostatic interactions results in the formation of 'phosphate clamps' (Figure 9B) which can be described as selective bidentate hydrogen-bonding between am(m)ines coordinated to each platinum(II) centre and oxygens in the phosphate backbone of DNA.⁵⁵ The PPCs can bind to DNA and

form phosphate clamps either by groove-spanning within the minor groove of DNA (Figure 9D) or backbone tracking along the polyanionic backbone of DNA (Figure 9C). This new DNA-binding motif for substitution-inert PPCs was supported by a second X-ray crystal structure of AH44 with the DDD.⁵⁶ AH44 and TriplatinNC both induce similar yet significant conformational changes to DNA. TriplatinNC-DNA phosphate clamp interactions have been likened to those of the arginine fork (Figure 9A), whereby the guanidino group of arginine has a clamping ability to phosphate backbone of DNA.

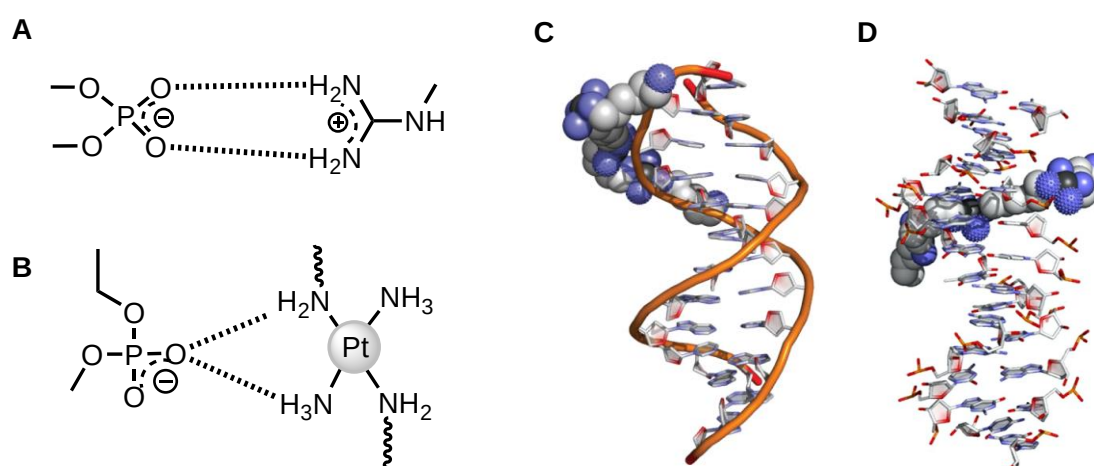


Figure 9A. Arginine fork showing selective hydrogen-bonding of nitrogens for phosphate oxygens; B. The phosphate clamp showing selective bidentate hydrogen-bonding between nitrogens surrounding the platinum(II) nucleus and phosphate oxygens forming an N-O-N motif; C. Backbone tracking of TriplatinNC along the anionic phosphate backbone of DNA; D. Groove-spanning of TriplatinNC into the minor groove of DNA.

Two DNA binding motifs of TriplatinNC were identified using X-ray crystallography: backbone tracking and groove-spanning and 2D NMR solution studies have supported this finding.⁵⁷ HSQC spectroscopy with fully labelled ¹⁵N TriplatinNC and the DDD demonstrated how the drug interacts differently with DNA at varying pH. At physiological pH, the non-covalent binding of TriplatinNC to the phosphate backbone is likely mediated by water, an effect which is observed for other well-known minor groove binding agents.^{56, 58} Backbone tracking and groove-spanning binding motifs of the substitution-inert polynuclear platinum(II) complexes were also investigated for their base-specific interactions. In these experiments, different DNA co-polymers containing varying A·T/G·C content were treated with TriplatinNC. It was elucidated that the percentage A·T content was directly proportional to the stabilizing effect of TriplatinNC, i.e.

increasing A·T content resulted in increased stabilization. This effect was reflected by a dramatic increase the thermal melting temperature of TriplatinNC bound DNA. Furthermore, comparison of biophysical and NMR studies demonstrated that TriplatinNC predominantly binds by backbone tracking to G·C rich DNA.⁵⁹

A separate study then examined the binding mode interactions of TriplatinNC and AH44 were determined using a variety of biophysical techniques including a fluorescence quenching technique of limited bound ethidium bromide (EtBr), a known DNA intercalator, along with Hoechst 33258, a minor-groove binder, to calf-thymus DNA. Results were compared to that of well-studied DNA minor-groove binders netropsin, pentamidine and $[\text{Co}(\text{NH}_3)_6]^{3+}$. In the fluorescence quenching assay, as expected, classical minor-groove binders all displaced Hoechst 33258 with greater specificity than EtBr. Conversely, the PPCs demonstrated unique profiles from the classical minor groove binders. Here, the PPCs demonstrated effective quenching of EtBr with quenching values of approximately 5 μM but they also facilitated cooperative binding of Hoechst 33258 and provided fluorescence enhancement of this classical minor-groove binder by up to 25%.⁶⁰

2.2.2.1 Structure-activity relationship study of varying linker length of PPCs

In 2014, a structure-activity-relationship (SAR) study was conducted to examine the effect of linker chain length on DNA conformational changes induced by the PPCs. Three different complexes were prepared and examined: AH78P containing a pentane linker; AH78 otherwise known as TriplatinNC containing a hexane linker; and AH78H containing a heptane linker (Figure 10A). Agarose gel electrophoresis was implemented to examine the influence of these PPCs with varying linker length on supercoiled pUC19 plasmid migration (Figure 10B). AH78P was found to be the most potent inducer of plasmid DNA condensation and began inhibiting plasmid migration at 0.5 μM with total inhibition achieved thereafter. AH78 and AH78H both required higher drug concentrations to

instigate plasmid condensation (~3 μ M) with the free ligand alone lacking condensation effects.⁶¹

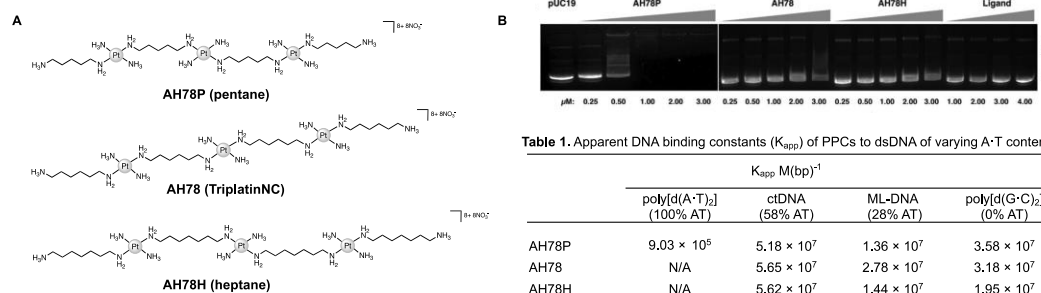


Table 1. Apparent DNA binding constants (K_{app}) of PPCs to dsDNA of varying A-T content

	K_{app} M(bp) ⁻¹			
	poly[d(A·T) ₂] (100% AT)	ctDNA (58% AT)	ML-DNA (28% AT)	poly[d(G·C) ₂] (0% AT)
AH78P	9.03×10^5	5.18×10^7	1.36×10^7	3.58×10^7
AH78	N/A	5.65×10^7	2.78×10^7	3.18×10^7
AH78H	N/A	5.62×10^7	1.44×10^7	1.95×10^7

Figure 10A. Molecular structures of PPCs with varying linker length; **B.** influence of PPCs with varying linker length on supercoiled pUC19 migration investigated by agarose gel electrophoresis. Adapted from ref. 56.

An EtBr competitive displacement assay was next employed to determine the apparent DNA binding constants (K_{app}) of the three PPCs. A selection of dsDNA nucleic acids with varying A·T content was utilised to probe potential base-specific binding constants of the drugs (Table 1). Results were then compared to that of well-studied minor-groove binder netropsin and intercalator actinomycin D. These classical DNA-binders demonstrated predictable behaviour with netropsin favouring A·T-rich sequences while actinomycin D favoured G·C-rich sequences. TriplatinNC-DNA binding for poly[d(A·T)₂] was unexpectedly undetectable up to 500 μ M of drug exposure. AH78P was the only congener to yield a detectable binding constant for poly[d(A·T)₂] with a low K_{app} value of 9.03×10^5 M(bp)⁻¹ (Figure 10, Table 1).⁶¹ These findings are in contrast to what was observed for AH44 (Figure 7), which exhibits slight preferential binding to A·T-rich sequences.⁶² The highest K_{app} values for the three PPCs tested in this study, AH78P, TriplatinNC, and AH78H was observed on ctDNA (58% AT) with K_{app} values of 5.18×10^7 M(bp)⁻¹, 5.65×10^7 M(bp)⁻¹, and 5.62×10^7 M(bp)⁻¹ respectively. K_{app} values determined for the three PPCs studied here on ML-DNA (28% AT) and poly[d(G·C)₂] (0% AT) highlight that these complexes have a higher apparent binding affinity for GC-rich nucleic acids than AT-rich nucleic acids (Figure 10, Table 1.), however optimum K_{app} values were observed on all three test complexes for the mixed ctDNA sequence.

2.2.2.2 Condensation effects

It has been identified by atomic force microscopy (AFM), gel electrophoresis, dynamic light scattering, and UV-visible spectroscopy that TriplatinNC is a potent

DNA condensation agent and is approximately 27 times more effective at condensing DNA than spermine. In 2014, TriplatinNC was shown to produce significantly large aggregates when exposed to linearized plasmid pSP73, even at micromolar concentrations.⁶² When the drug concentration was increased, the topology of the aggregates became larger and more compact. Furthermore, TriplatinNC was found to condense tRNA while comparatively spermine could not. However, condensation effects are reversible, and can be controlled by varying the ionic strength of the solution. It was further demonstrated that by increasing NaCl concentration of the solution up to approximately ~600 mM, TriplatinNC was inhibited from condensing the DNA.^{62, 63, 64}

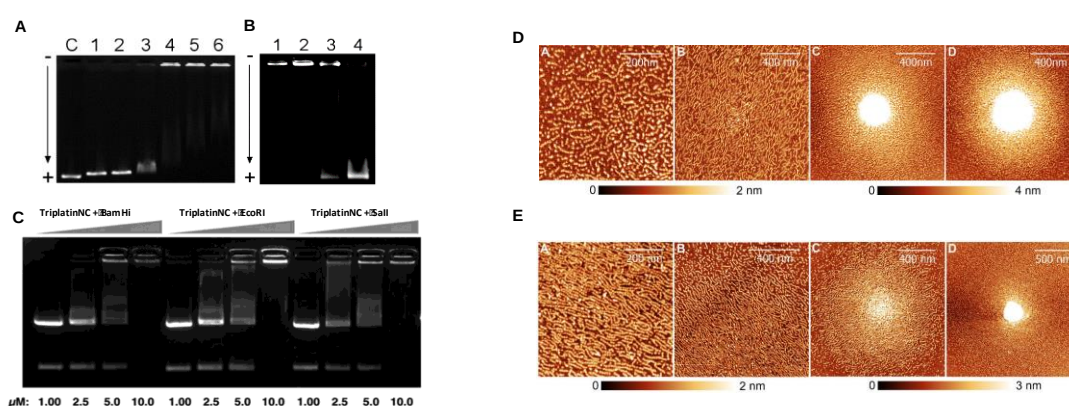


Figure 11A. Native agarose gel electrophoresis demonstrating the condensation effect of TriplatinNC on the migration of negatively supercoiled plasmid SP73 (30 μ M) with increasing drug concentration. Lanes C: control (no drug); lanes 1-6: 0.75, 1.5, 3.0, 6.0, 9.0 and 12.0 μ M TriplatinNC. Adapted from reference 61; B. Native agarose gel electrophoresis demonstrating condensation inhibition of AH44 (12 μ M) on SP73 plasmid (30 μ M) by increasing ionic strength in Tris-HCl buffer (10 mM, pH 7.4). Lane 1: control (0mM NaCl); lanes 2-4: 200, 400 and 650 mM NaCl. Adapted from reference 61; C. Type II endonuclease cleavage experiments on linearized pUC19 (400 ng) with TriplatinNC (1-10 μ M) by BamHI, EcoRI and Sall. Adapted from reference 62; D. AFM imaging of a 45 pyrimidine/20 purine DNA construct in the presence of 2 μ M TriplatinNC (A,B) and 4 μ M TriplatinNC (C,D); E. AFM imaging of a 45 pyrimidine/20 purine DNA construct in the presence of 4 μ M AH44 (A,B) and 8 μ M AH44 (C,D) Adapted from reference 63.

2.2.2.3. Inhibiting protein-DNA recognition

It is well understood that strong drug-DNA binding can inhibit and disrupt protein-DNA recognition processes. The modular nature of the phosphate clamp, together with the unparalleled condensation ability of the PPCs, significantly contributes to their ability to inhibit protein-DNA recognition and interactions. The effectiveness of PPCs at preventing protein recognition has been demonstrated using endonuclease inhibition, topoisomerase I-mediated relaxation inhibition, and TATA binding protein inhibition.^{61,62,65} The effect can also be visualised using plasmid DNA (pUC19) and gel electrophoretic techniques. To demonstrate the

effectiveness of endonuclease inhibition of the PPCs, pUC19 was linearised using the *AatII* restriction enzyme, treated with either AH78P, TriplatinNC, or AH78H, and then exposed to the endonucleases that only have a single recognition site on the pUC19 vector: *Bam*HI; *Eco*Ri; and *Sal*I. Gel electrophoresis revealed that complete endonuclease inhibition by AH78P, TriplatinNC, and AH78H is concentration dependent and that these PPCs can protect pUC19 from site-specific cleavage by the aforementioned endonucleases (Figure 11C). These experiments were verified using a microfluidic chip method The Bioanalyzer 2100 operates through capillary electrophoresis and offers advantages over other electrophoretic methodologies due to its ability to quantitatively detect DNA fragments of varying size precisely, with high-resolution, at extraordinarily low concentrations.⁶¹

Topoisomerase enzymes contribute significantly to important metabolic events associated with DNA manipulation such as transcription and replication, and so are important anticancer drug targets. Topoisomerase I-mediated DNA relaxation assays have been developed to probe the DNA binding effects of different drugs on supercoiled plasmid DNA. The assay is typically monitored by agarose gel electrophoresis as the DNA isoforms migrate through the agarose medium according to their size and charge. Strong drug-DNA binding results in the relaxation of negatively supercoiled plasmid DNA to open circular plasmid DNA. When the plasmid is exposed to high concentrations of a drug—particularly a DNA intercalator—it can become further unwound in the opposite direction forming positively supercoiled plasmid DNA that is unrecognisable by topoisomerases. With this in mind, TriplatinNC was shown to completely inhibit topoisomerase I-mediated relaxation of plasmid DNA at concentrations approximately 250 times lower than that of the endogenous condensation agent spermine.⁶⁴

To investigate the durability of the phosphate clamp in comparison to the arginine fork, it was later demonstrated that TriplatinNC effectively inhibits the recognition ability of the TATA binding protein (TBP) towards an AT-rich duplex sequence.⁶⁵ TBP is an important transcription factor that binds to the TATA box sequence of DNA. It is involved in the preinitiation transcription complex of all

eukaryotic RNA polymerases. Using PAGE analysis, it was observed that TBP inhibition begins at TriplatinNC concentrations as low as 0.63 μM and is completely inhibited at 1.25 μM . Considering the AT-rich sequence has a high affinity for minor groove binders such as netropsin, it is worth noting that netropsin is comparably less efficient at inhibiting TBP binding. A fundamental component of TBP-DNA binding is the formation of arginine fork motifs, and so it appears TriplatinNC directly competes with multiple arginine residues of TBP for binding sites on the DNA backbone.⁶⁵

More recently, it was reported that PPCs function as metalloshielding agents for heparan sulfate. Heparan sulfate proteoglycans (HSPGs) are carbohydrate-modified proteins which are involved in critical cellular functions. HSPGs are cleaved by an enzyme known as heparanase, and this cleavage activity modulates processes related to tumours such as metastasis and angiogenesis. A library of PPCs differing in charge and nuclearity was developed and studied for their ability to inhibit the cleavage activity of bacterial heparinase II on Fondaparinux (FPX). Competition assays with EtBr and methylene blue were used to examine the charge-dependent affinity of the PPCs for FPX. Furthermore, competitive inhibition of TAMRA-R₉ internalization in human carcinoma cells along with studies in HCT116 with and lacking in heparan sulfate demonstrated that HSPG interactions hold an important role in cellular uptake of PPCs.⁶⁶

Finally, in a recent publication from Farrell and co-workers, PPCs were investigated for their use as effective stabilizers of DNA triplexes generated by triplex forming oligonucleotides (TFOs). The formation of DNA triplexes is heavily implicated in the modulation of gene expression, however, DNA triplexes are unstable under physiological conditions due to the electrostatic repulsion between phosphate backbones and the requirement for acidic conditions to protonate cytosine groups engaged in Hoogsteen bonding. When the PPCs were bound to a triplex-forming DNA template, DNA synthesis was efficiently inhibited by DNA polymerase and, moreover, such a high degree of stabilization was observed that the PPC-bound TFO template could form triple helical structures

with duplexes that were not fully complementary to the TFO. This evidence further supports the PPCs as strong stabilising agents of high-order structures that leads to the inhibition of protein recognition and processing.⁶⁷

2.2.2.4. TriplatinNC cytotoxicity

TriplatinNC has shown unparalleled *in vitro* efficacy as a chemotherapeutic agent displaying potent cytotoxic activity across a broad range of tumour cell lines, both sensitive and resistant to cisplatin, at micromolar concentrations.⁵⁷ It is well known that most cancers exhibit some genetic defects that inhibit the tumour-suppressive function of the p53 gene. It was previously shown that the activity of cisplatin is significantly dependent on the presence of p53 mutations. For example, testicular cancers tend to respond well to cisplatin therapy due to the low frequency of p53 mutations found in this tumour type. Subsequently it was uncovered that the antiproliferative activity of cisplatin is particularly limited in the absence of p53, and occurs largely by two mechanisms: p53-dependent apoptosis or p53-independent cell cycle arrest.⁶⁸ Conversely, it was identified that the absence of p53 does not affect the cytotoxic activity of TriplatinNC. Studies revealed that there are two predominant antiproliferative mechanisms of TriplatinNC, and they are distinguished depending on cell-cycle stage; cells in the G₁ stage undergo cell-cycle arrest when treated with TriplatinNC, while cells in the S-G₂ stage tend to undergo DNA condensation.⁶⁵

The cytotoxicity of TriplatinNC can be further distinguished from covalent-binding platinum(II) drugs like BBR3464 and cisplatin, as the activity of this trinuclear electrostatically binding drug is independent of glutathione concentration in the cell. In 2011, a study was conducted to investigate the effect of cellular glutathione status on the ability of TriplatinNC, BBR3464, and cisplatin to induce apoptosis. Cells were treated with buthionine sulfoximine to reduce glutathione levels prior to treatment with one of the above platinum(II) drugs. The results of this study indicated the antiproliferative activity of cisplatin and BBR3464 were compromised while the activity of TriplatinNC remained unchanged. Furthermore, A2780 ovarian carcinoma cells were treated with human serum albumin (HSA) bound to each of the platinum(II) drugs.

HSA/BBR3464 and HSA/cisplatin were shown to exhibit decreased cellular accumulation, cytotoxic activity, and platinum-DNA adduct formation. Conversely, the TriplatinNC/HSA presented almost identical cytotoxic profiles as TriplatinNC alone. The overall results of this study indicate the ability of TriplatinNC to avoid intracellular metabolic deactivation by GSH and sulfur-containing nucleophiles which covalent platinum(II) DNA binding agents are susceptible toward.⁶⁹

3. Platinum(II) Bioconjugates

3.1. Platinum(II) fluorophore conjugates

Despite the vast amount of research continuously carried out on platinum(II) complexes, they are still quite poorly understood with regard to their localisation properties in cells, reactions with non-DNA targets, and their exact mechanism of action. In order to obtain a more comprehensive understanding of this class of drugs, fluorescent-tagging has been employed to shed light on the cellular fate of platinum(II) drugs. Methods have been established to conjugate fluorophores to biologically relevant platinum(II) drugs for a number of reasons including: (i) fluorescent detection to understand cellular localisation patterns; (ii) optimised delivery and transport; and (iii) gaining a synergistic therapeutic effects. Most commonly, platinum(II)-fluorophore ligation is achieved by direct covalent modification to the ligands coordinated to the platinum(II) centre. While the conjugation of a fluorescent probe to platinum(II) drugs has certainly improved our understanding of the cellular fate of these drugs, the assumption that the fluorophore-platinum(II) conjugate remains intact while monitoring fluorescence detection cannot be overlooked. As cellular localisation studies are monitored by the detection of the emission of the fluorescent probe, this does not necessarily mean that the fluorescent detection corresponds to the intact platinum(II)-fluorophore conjugate. Probes conjugated to a monodentate ligand could be susceptible to dissociation in the cell. For this reason, both 'pre-tethering' and 'post-tethering' of fluorescent probes to platinum(II) drugs will be discussed below.

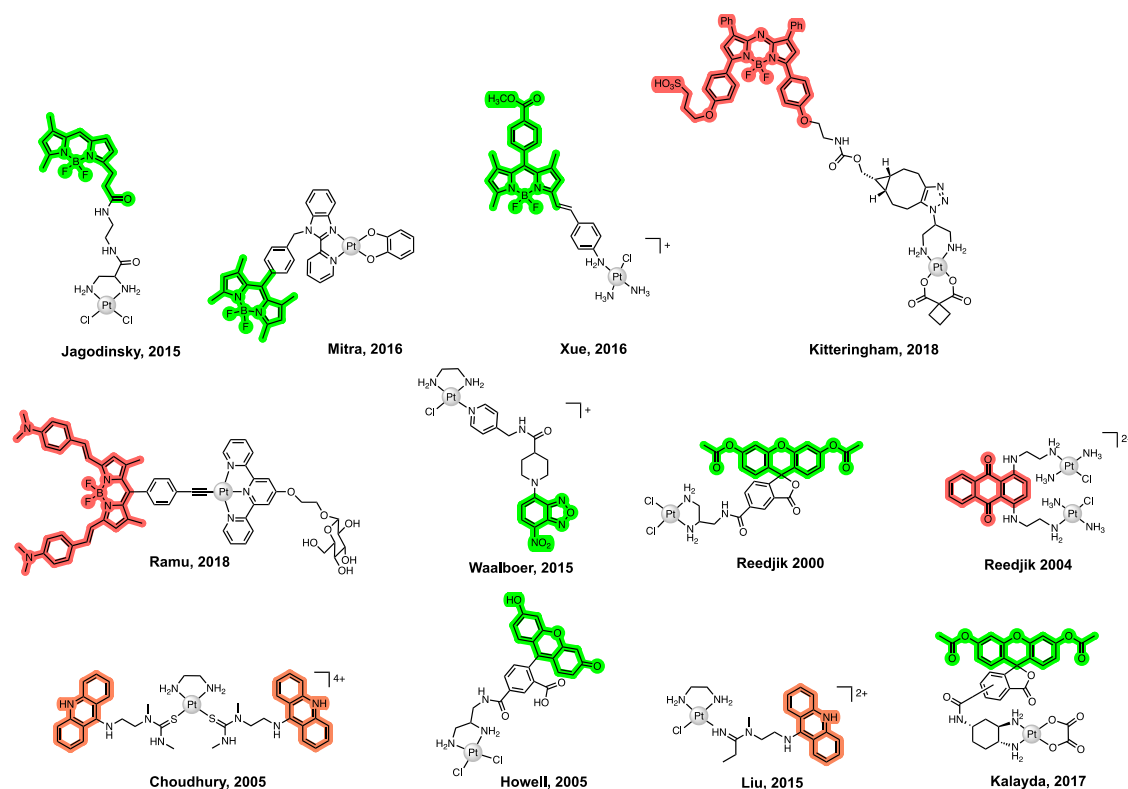


Figure 12. Structures of 'pre-tethered' platinum(II)-fluorophore conjugates developed in recent years.

3.1.1. 'Pre-tethered' platinum-fluorophore conjugates

In 2000, Reedijk and co-workers developed a platinum(II)-fluorophore conjugate consisting of a fluoresceindiacetate moiety (Figure 12) and studied its properties using fluorescence microscopy for its cellular localization properties in live U2-OS human osteosarcoma cells. Nuclear accumulation occurred after 2 – 3 hours and fluorescence diminished completely in the nucleus after 24 hours.⁷⁰ While this study certainly highlighted the potential of using fluorescent-conjugates to track the cellular fate of platinum(II) drugs, this cannot be considered a cisplatin mimetic—as was intended—as the cytotoxicity of the complex alone was not studied, cellular mapping studies were not conducted of the fluorophore alone, and no differences in localization patterns were observed for cisplatin sensitive and resistant cells.

In 2005, a similar compound was developed by Howell *et al.* (Figure 12) and was monitored in ovarian carcinoma cells stained with organelle-specific markers.⁷¹ The predominant advantage of using this complex for cellular mapping studies of *cis*-platinum(II) drugs is that it imitates the inherent cytotoxic and cellular accumulation properties of cisplatin and most importantly, the

platinum(II)-fluorophore conjugate displays a distinctly different cellular localization pattern than the fluorophore alone. In more recent years and in a similar manner, Kitteringham and co-workers have developed a carboplatin-fluorophore conjugate (Figure 12) via strain promoted click chemistry,⁷² that mimics the cellular properties of carboplatin itself and was tracked in both cisplatin resistant and sensitive cell lines. In 2017, Kalayda *et al.* developed an oxaliplatin-fluorescein conjugate that imitates the cellular properties of oxaliplatin alone and has been used to study the mechanisms of oxaliplatin resistance.⁷³ Indeed, the development of these mimetic agents has greatly contributed to the understanding of resistance mechanisms as well as the localization properties of platinum(II) drugs.

While the development of platinum-fluorophore mimics has certainly helped in elucidating the mechanisms of cellular localization of these drugs in live cells, many conjugates have also been designed for use as anticancer agents in their own right. Well-studied DNA intercalators ethidium bromide, proflavine and acridine are known to enhance cisplatin-DNA binding when co-administered with cisplatin, a synergistic effect between the high affinity intercalating moiety and the cytotoxic platinum(II) centre is achieved. This type of approach was demonstrated by Reedijk and co-workers in 2004 (Figure 12).^{74,75} Here, a dinuclear platinum(II) complex linked by a fluorescent anthraquinone intercalator was developed. This compound displayed rapid nuclear accumulation in U2-OS human osteosarcoma cells. Cellular localisation studies of the complex compared with studies of the anthraquinone ligand alone showed distinctly different localization profiles, eliminating the possibility of fluorophore dissociation.

Recent advances in the development of platinum(II)-fluorophore conjugates feature platinum(II)-acridine conjugates (Figure 12) which account for some of the most potent platinum(II) anticancer drugs reported to date.^{76, 77} As has been seen for many other platinum(II) chemotherapeutics, these platinum(II)-acridine conjugates have exhibited adverse pharmacokinetics and dose-limiting toxicity. Efforts have been concentrated on developing these platinum(II)-acridine conjugates to enhance their target specificity. Following

this, a cysteine-directed platinum(II)-acridine conjugate was developed whereby attachment of the platinum(II)-acridine conjugate to a carrier protein was shown to enhance the selectivity of this class of drug.⁷⁸

When designing platinum(II)-fluorophore conjugates the choice of the fluorophore used is important. Previous work has demonstrated that conjugation of bidentate or large bulky fluorophores to cisplatin has resulted in the cisplatin-fluorophore conjugate behaving more like the fluorophore than the platinum(II) drug itself.⁷⁹ It was discovered that conjugation of an NBD fluorophore directly through the primary amine generated platinum(II)-fluorophore conjugates that behave more like the platinum(II) parent drug than the fluorescent tag.⁷⁹ For this reason, in 2011, Farrell and co-workers prepared a fluorescent polynuclear platinum(II) complex which functioned as a probe for investigating cellular localization patterns of this class of platinum(II) drugs using an NBD fluorophore. Confocal microscopy of the fluorescent conjugate (TriplatinNC-NBD) and a TriplatinNC-NBD (Figure 13) bound to human serum albumin (TriplatinNC-NBD/HSA) revealed nucleolar localization patterns which were distinctly different from the lysosomal localization patterns observed for HSA alone.⁶⁹

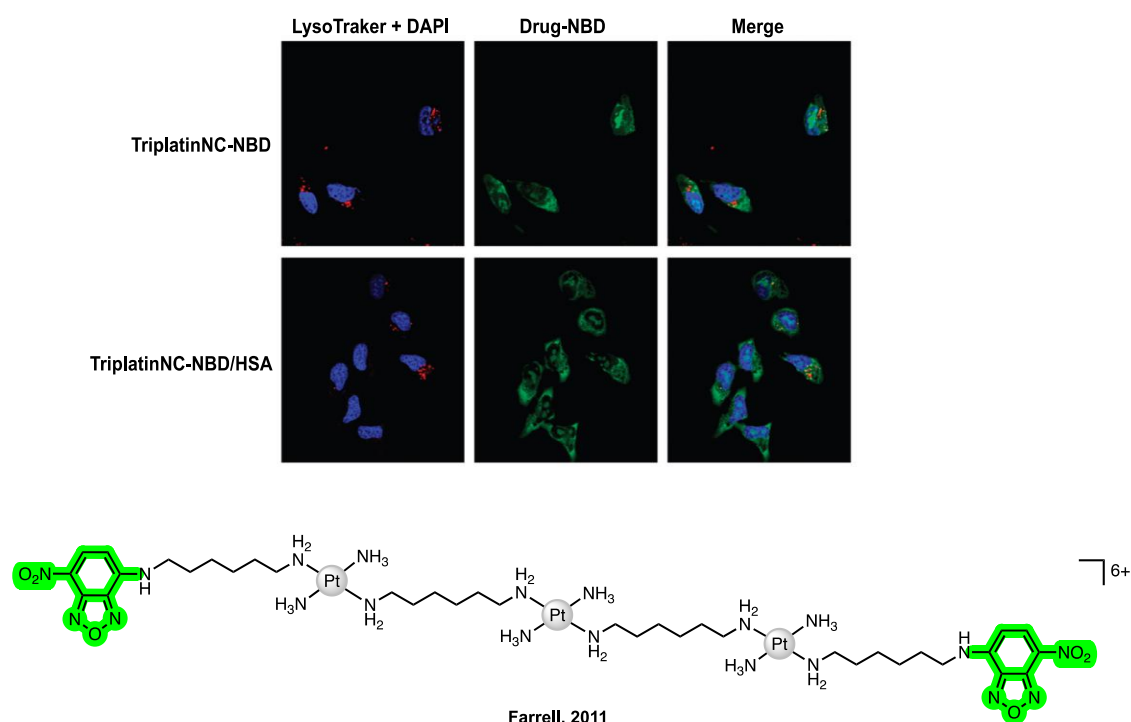


Figure 13. Confocal images of TriplatinNC-fluorophore conjugate in cells:(top row) TriplatinNC-NBD incubated with A2780 cells for 6 hr and LysoTracker Red for final 30 minutes of drug incubation. (bottom row) TriplatinNC-NBD/HSA incubated with A2780 cells for 6 hr and LysoTracker Red for final 30 minutes of drug incubation. Images adapted from ref. 57.

3.1.2. 'Post-tethered' platinum(II)-fluorophore conjugates

It is axiomatic that the incorporation of a bulky fluorophore to a platinum(II) drug can influence the interactions that guide their localisation in cellular environments. Therefore, an attractive approach to observe platinum(II) drugs for their cellular localisation properties is by introducing the fluorophore through post-labelling. This 'post-tethered' strategy has seen a large focus on using the bio-orthogonal click reaction due to its broad scope, high selectivity, and efficiency.^{80, 81} While this new post-labelling click strategy for the detection of platinum drugs in cells is still in its infancy, it is believed that it will inform our understanding of platinum uptake, delivery, and transport in cellular environments and, moreover, will allow for more accurate and precise characterisation of platinum(II) drugs *in vitro*.

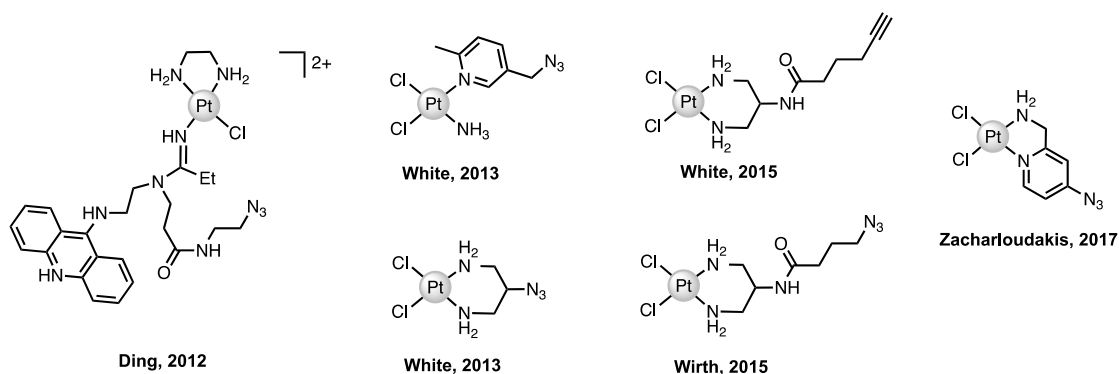


Figure 14. Chemical structures of 'post-tethered' platinum(II) complexes that have the ability to undergo click ligation to an alkyne-fluorophore for fluorescent cellular imaging.

The azide moiety can be easily attached to terminal amine-containing ligands that are coordinated to platinum(II) centres by nucleophilic substitution. While alkyne-modified platinum(II) complexes are usually quite unstable and participate in unwanted side-reactions, in 2015 an alkyne-appended platinum(II) complex (Figure 14) was successfully used in post-tethering click reactions with dansyl azide-modified fluorophores.⁸² An alkyne functionalised platinum(II) drug may provide some favourable properties over their azide equivalent such as improved solubility as well as larger substrate availability.

The platinum(II)-acridine conjugates prepared by Bierbach and co-workers account for some of the most cytotoxic platinum(II) drugs ever observed in non-small cell lung cancers.⁸³ To gain a more accurate understanding of the cellular localisation of these drugs, an azide handle was incorporated into the

platinum-acridine complex (Figure 14). Human non-small cell lung carcinoma cells were treated with the azide-modified platinum(II) acridine conjugate, fixed, and then reacted with an alkyne-modified fluorescein dye, Alexa Fluor 488, to undergo copper(I) catalysed click chemistry.⁸⁴ Results and images from this study validated the effectiveness of this ‘post-tethering’ click chemistry approach to observe platinum(II) accumulation in cells by fluorescence detection.

The DeRose research group has also implemented this ‘post-tethering’ click chemistry approach to gain a more accurate understanding of the cellular localisation of platinum(II) drugs. In 2013, an azide derivative of the sterically hindered picoplatin, now known as picazoplatin (Figure 14) was prepared for ‘post-tethering’ click labelling studies of *S. cerevisiae* tRNA and rRNA using strain promoted click chemistry. Results of this study revealed significant labelling of all rRNA bands in the PAGE gel image, and was the first demonstration of *in vivo* platinum(II) tRNA accumulation.⁸⁵ Furthermore, the same approach was used for a sterically unhindered platinum(II) complex, investigated by Moghaddam in 2015. Here, an alkyne-appended Alexa Fluor 488 was clicked to the N₃-DAP cisplatin derivative to determine the longevity of the platinum(II)-rRNA lesion in *S. cerevisiae* cells.⁸⁶ Finally, in 2017, ‘post-tethering’ strain promoted click chemistry was used to visualise platinum(II)-DNA lesions produced by a 2-aminomethylpyridine (dichloro) platinum(II) azide complex (Figure 14) in cells at high resolution and to investigate the effect of platinum on gene expression.⁸⁷

3.2. Platinum(IV)-fluorophore conjugates

Platinum(IV) prodrugs have become an area of interest within the field of platinum drug discovery due to their improved efficacy when compared to the conventional cis-chemotype platinum(II) anticancer agents. Platinum(IV) prodrugs become ‘activated’ either intracellularly or extracellularly to their platinum(II) congeners by the loss of axial ligands upon reduction.⁸⁸ The therapeutic potential for the use of platinum(IV) prodrugs as anticancer agents is exemplified by satraplatin (Figure 2), a platinum(IV) prodrug which has reached stage III clinical trials for the treatment of advanced prostate cancer.⁸⁹ Satraplatin demonstrates potent cytotoxicity against cisplatin-resistant tumours as well as

improved oral bioavailability, however, a question still remains with regard to exactly how and when platinum(IV) is reduced to the 'active' platinum(II) species.¹³ Fluorescent molecules have been employed to visualise this reductive activation mechanism in cells. In 2009, Hambley *et. al.* coordinated a coumarin fluorophore to a platinum(IV) complex in a non-leaving group position (Figure 15).⁹⁰ It was demonstrated that using a coumarin fluorophore exhibiting coordination-sensitive fluorescence is an effective technique for visualising platinum(II)/(IV) metabolic events. Here, the platinum(II)-coumarin complex displays luminescence, but when oxidised to platinum(IV) the intensity is significantly diminished and this probe activity is therefore indicative of platinum(IV) activation to platinum(II).⁹⁰

In 2012, Lippard and co-workers synthesised both a platinum(IV) prodrug and a platinum(II) complex bearing a chelating dansyl fluorophore in non-leaving group positions on the platinum centre (Figure 15).⁹¹ Photophysical studies on these complexes show a significant difference in fluorescence signal between the platinum(IV) and platinum(II) compounds. Moreover, it was shown that reduction of the platinum(IV)-dansyl fluorophore complex to platinum(II) triggers a dramatic 6-fold enhancement in the fluorescent signal. This work further supports the idea that platinum(IV)-fluorophore conjugates can be used as mechanistic probes for monitoring the reduction of platinum(IV) in biological systems.⁹¹

Other work has demonstrated that locating a fluorogen in an axial position on the platinum metal-centre can provide insight into the reductive activation mechanism of platinum(IV)→platinum(II).⁹² Systems based on aggregation induced emission (AIE) have been developed for the in situ monitoring of platinum(IV) reductive activation mechanism in live cell systems. A platinum(IV) prodrug containing a tetraphenylethene pyridinium (PyTPE) unit in the axial position is non-fluorescent but becomes highly emissive when reduced to platinum(II) (Figure 15).⁹²

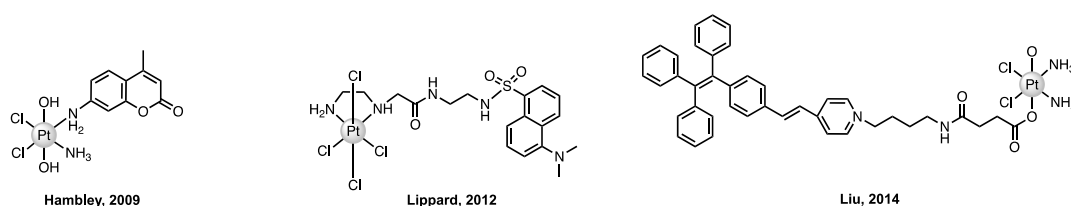


Figure 15. Structures of some platinum(IV)-fluorophore conjugates developed in recent years.

3.3. Thiazole orange

Thiazole orange (TO) is an asymmetrical cyanine dye consisting of a benzothiazole moiety and quinoline moiety linked together by a methine bridge. TO is a weakly fluorescent fluorophore in solution due to intramolecular rotation of the two heterocyclic systems around the methine bridge. This freely rotating and twisting system results in rapid non-radiative decay of the excited state. However, upon dsDNA or dsRNA intercalation, the two rotating aromatic systems become locked in place in a planar conformation and rotation around the methine bridge is restricted which in turn results in a large fluorescent enhancement.⁹³ Due to this unique fluorescent property of TO, it is commonly used for the detection of DNA (Figure 16).^{94, 95}

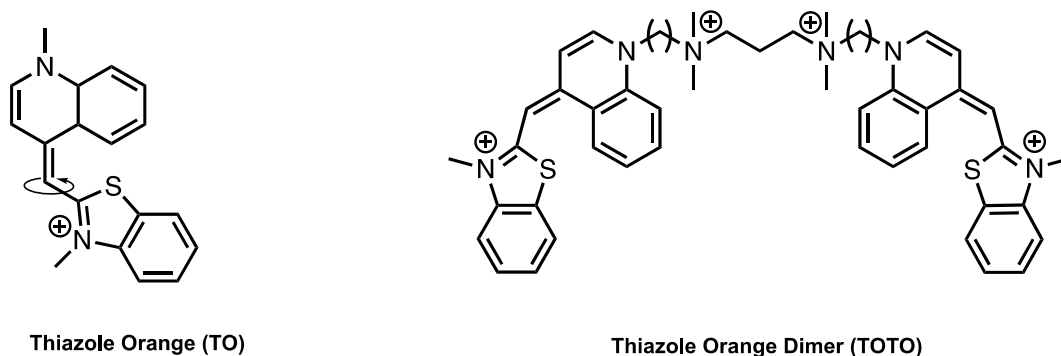


Figure 16. Structures of thiazole orange and thiazole orange dimer TOTO. The arrow indicates the rotation around the methine bridge.

While the detection of nucleic acids is undoubtedly one of most obvious uses of TO, this fluorophore was first employed for the detection of reticulocytes using flow cytometry.⁹⁶ The outstanding sensitivity of this 'light-switch' organic dye demonstrated a 3000-fold increase in fluorescence signal upon DNA binding with a high signal:noise ratio which ultimately establishes TO as a superior reporter molecule with improved applicability over weaker and less sensitive fluorophores such as thioflavin and acridine.⁹³

Efforts have shifted towards the design and development of TO probes with improved sequence-targeting properties. Such work began with the development of TO-appended peptide nucleic acid (PNA) probes.⁹⁷ These probes contained a sequence of nucleobases on a PNA backbone with a TO molecule conjugated at one end through a flexible linker. Once these probes

were introduced to their complementary target these probes were shown to demonstrate a 50-fold increase in fluorescence.⁹⁸ These PNA-TO probes have found applications in areas such as real-time PCR⁹⁹ and as a FRET-pair for sensing RNA maturation.¹⁰⁰ While the PNA-TO probes described above demonstrates their potential for sensing DNA, limitations such as high background signals and false positives cannot be overlooked. Moreover, any changes to the sequence length, AT/GC base composition, and linker can cause unpredictable changes to the TO fluorescence quantum yield.¹⁰¹

To address the issues of false positives with TO due to non-specific intramolecular activation, Seitz and co-workers began using TO as a surrogate base within the probe sequence.¹⁰² When TO is used as a surrogate base it is linked to the PNA backbone through a rigid, short carboxymethylene linker which does not allow for freely rotating flexibility, and forces intercalation between neighbouring nucleobases which in turn minimises self-activation and false positives which was previously seen for the TO-appended PNA probes. Along with increased sensitivity, these probes, now termed 'FIT probes' (forced intercalation probes), also exhibit an enhanced sensitivity to mismatches within the target sequence. If a single mismatch was located adjacent to the TO surrogate base, the fluorescent signal was dramatically diminished in comparison to the fully complementary DNA target.¹⁰²

While recent efforts for the development of platinum(II)-fluorophore conjugates has largely focused on generating conjugates using fluorophores such as BODIPY, acridine, xanthene, and NBD, TO—to the best of our knowledge—has never been conjugated to a platinum(II) drug before. If this could be achieved, a TO-platinum(II) conjugate might exhibit enhanced DNA binding properties when compared to the parent platinum(II) drug due to the intercalation of TO, and furthermore, contain an “on-switch” once TO has intercalated between the DNA. As such, TO-appended platinum(II) complexes would advance the field of platinum(II)-fluorophores for the direction and detection of platinum(II) drugs in cells.

3.4. Platinum(II) oligonucleotide conjugates

Advances in platinum(II) drug discovery has progressed and much research has been driven towards the discovery and development of platinum(II) bioconjugates for antisense therapeutic application. Antisense therapy is a gene silencing technology whereby a 'foreign' oligonucleotide is designed to target and bind a specific mRNA sequence which subsequently inhibits the mRNA from passing through the ribosome and producing an undesired protein (Figure 17).¹⁰³ Nucleic acid targeting vectors such as triplex-forming oligonucleotides (TFOs) and peptide nucleic acids (PNAs) have been conjugated to a platinum(II) complex and the resulting platinum(II)-oligonucleotide hybrids were found to effectively bind specific oligonucleotide targets.^{104, 105} A level of symbiosis is achieved between the platinum(II) moiety and oligonucleotide moiety of these hybrid drugs, whereby the oligonucleotide lends a sequence-specific targeting element to the platinum(II) drug and the platinum(II) can stabilize the formation of the resulting antisense adduct.

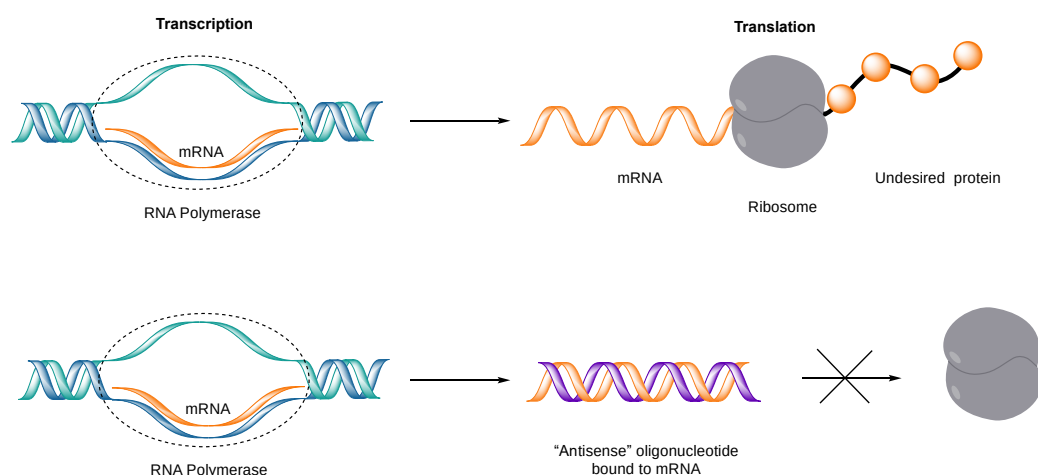
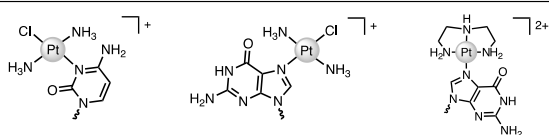
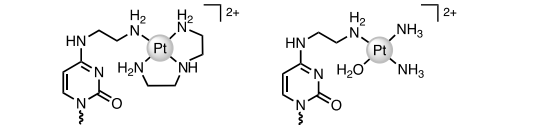
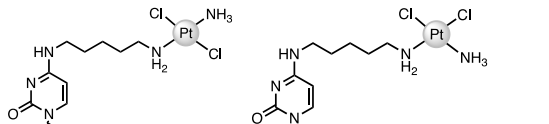
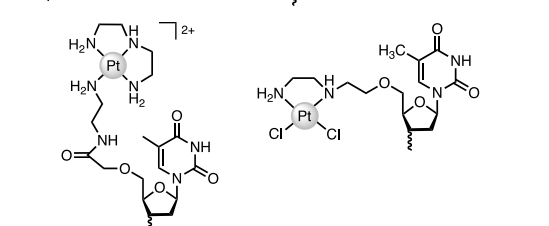


Figure 17. Transcription and translation processes can lead to mutations which contribute to the development of genetic diseases. Antisense therapy presents an effective way of targeting mutated mRNA at the transcriptional level by using an antisense oligonucleotide to bind the mRNA to prevent it being translated in the ribosome to the undesired protein.

Mononuclear platinum(II) metal complexes, such as transplatin, have been known for their crosslinking abilities for some time, and have been shown to bind with specific targets.¹⁰⁶ Their geometry (square planar) and coordination behaviour make them ideal candidates for crosslinking two oligonucleotides specifically, without giving rise to any large local steric distortions. Early work shows efficient methods developed for the sequence-specific crosslinking of

complementary oligonucleotides using dinuclear platinum complexes.¹⁰⁷ Following this, Leng and co-workers began studying whether monofunctional transplatin-adducts within a TFO sequence could form interstrand crosslinks between the transplatin-TFO adduct and complementary duplex sequence. Transplatin modifications were positioned either at the 5'-end or in the middle of the Hoogsteen binding strand, and indeed all triplexes studied exhibited specific interstrand crosslinking between the Hoogsteen (polypyrimidine) strand and purine-rich strand of the duplex. Interstrand crosslinking was not observed between the transplatin-TFO adduct and non-complementary duplex DNA.^{108, 109} The formation of a triplex, while usually unstable, has been shown to be stabilized by the formation of transplatin crosslinks.¹¹⁰

Table 2. Pt(II) DNA hybrids and their binding modality.

Binding Mode	Motif	Reference
Direct platination to base		Leng 1996, 1998 ^{108,109} Müller 2000 ¹¹⁰ Miller 2009, 2015 ^{111,112}
N-4 Aminoethyl linker		Miller 2007 ¹¹³
N-4 Aminopentyl linker		Miller 2009 ¹¹¹
C-5 ribose linker		Miller 2007 ¹¹³ McLaughlin 2002 ¹¹⁴

Further studies support the efficacy of these hybrid drugs and show inhibition of translation *in vitro* within E. Coli cells.¹¹¹ More recently, Miller and co-workers designed transplatin-TFO hybrids to specifically target a purine tract on the human androgen receptor gene. Indeed, this work showed that the platinum(II)-TFO hybrids specifically bound to their genomic targets after transfection into human prostate cancer cells and subsequently resulted in reduced expression of human androgen receptor mRNA and protein.^{112, 113} These observations supported the hypothesis that platinum-bound

oligonucleotides could play a vital role in the modulation of gene expression, and that this type of hybrid drug could advance the therapeutic development of antisense and antigene technologies.¹¹⁴

Further to this work, recent advancements in the field of platinum(II)-oligonucleotide bioconjugates has resulted in the development of a modular click chemistry protocol to generate platinum(II)-TFO hybrid materials. These *cis*-platinum(II)-TFO hybrids exhibit site specific recognition, targeting, and crosslinking which is facilitated by the formation of a triplex structure with the duplex DNA target.¹⁰⁵ Novel constructs such as these could hold a source of untapped biological potential for the development of platinum(II)-oligonucleotide with applications in the realm of antigene therapy for the targeting of oncogenes to achieve enhanced selectivity.

3.5. References

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Chapter II

Project Rationale

2. Project Rationale

2.1. Project overview

Platinum(II) anticancer drugs comprise of one of the most widely used drug classes in clinical use today, and are used in ~60% of all chemotherapeutic regimes for the treatment of solid epithelial cancers.¹ However, the success and effectiveness of these platinum(II) anticancer drugs does not overshadow their clinical shortcomings, specifically with regard to their dose-limiting toxicity and lack of target nucleic acid specificity. These limitations give rise to significant issues such as off-target toxicity and the development of platinum resistance. In recent years, the development of the polynuclear platinum(II) complex (PPC) series by the Farrell group presented a novel, unique class of platinum(II) drugs distinctly different from the conventional mononuclear, *cis*-chemotype platinum(II) drugs. The PPC series feature *trans*-geometry and bind DNA using a distinct electrostatic interaction mode now commonly known as the phosphate clamp. It was hypothesised that platinum(II) drugs with an alternative mode of DNA binding could display anticancer activity against a broader spectrum of human cancers. It is believed that PPC series could overcome issues regarding resistance and have a broader spectrum of activity due to their inherently different mode of action. In 2014 Kellett and co-workers elucidated a specific sequence-dependent DNA binding mode of TriplatinNC, of which two major non-covalent interactions were observed: backbone tracking and groove spanning.²

While the PPC series have exceptional clinical potential for the treatment of cancers, and despite impressive advances within the field of platinum(II) drug discovery to date, questions regarding their localisation and targeting properties remain unanswered. Methods have been established to conjugate fluorophores to biologically relevant platinum(II) drugs with direct covalent modification to the platinum(II) centre being the most common. However, recent advances in the use of click chemistry now provides an alternative strategy whereby a bio-orthogonal handle—such as an azide group—can be reliably introduced to enable post-tethering to an alkyne fluorophore. This strategy has several advantages, not least an ability to study the drug molecule in a close to native environment. In this work, two polynuclear platinum(II) complex series will be

developed—one series with a “turn-on” reporter molecule (thiazole orange), and one series capable of click ligation with an alkyne appended fluorophore. This will allow for direct comparison of the ‘pre-tethered’ and ‘post-tethered’ strategies for the use of fluorescent tagging to shed light on the cellular fate of this drug class.

Both the TO-modified PPCs and the azide-appended PPCs will be studied for their DNA binding properties and interactions with DNA. Furthermore, both complex series will undergo cytotoxic screening against a variety of cancer cell lines and compared to the classical platinum(II) chemotherapeutics. Finally, fluorescent-tagging and cellular localisation studies of the intrinsically fluorescent PPCs and azide-appended PPCs will be performed and images will be obtained by confocal microscopy.

2.2. Project objectives

The first objective (**Objective 1**) of this project is the development of a range of novel azide-functionalised polynuclear platinum(II) complexes (N_3 -PPCs) materials (Figure 18). The platinum(II) centres will be complexed with organic alkyl diamine chains and contain a single azide group at their terminus that can provide ‘click’ ligation functionality to an alkyne-modified fluorophore. The novel complex series will vary in nuclearity comprising of a mononuclear, a dinuclear, and a trinuclear complex. A non-functionalised series will also be prepared as a control and all complexes will be characterised by methods previously reported by Farrell and co-worker (Figure 18).³

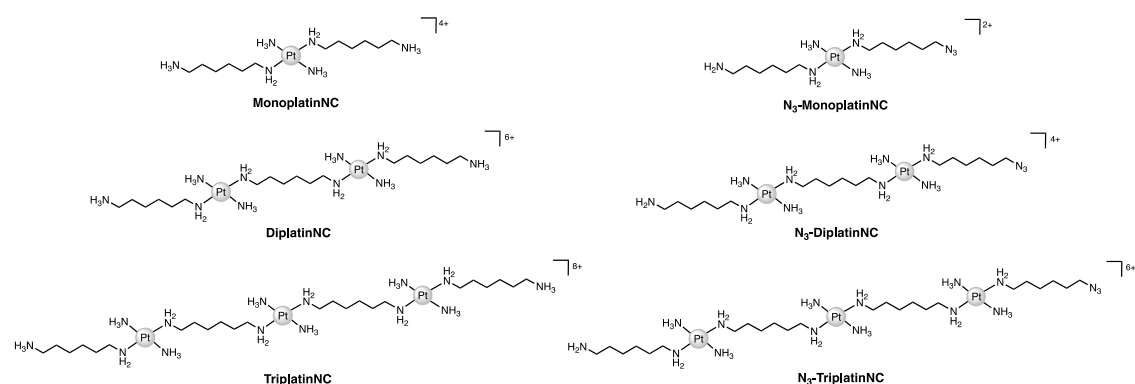


Figure 18. Solid state structures of non-functionalised and novel azide-functionalised polynuclear platinum(II) complexes.

The second objective (**Objective 2**) of this project is the development of a novel azido-polynuclear platinum(II) complex series conjugated to TO—a known DNA intercalating fluorophore (Figure 19). Similar to the series in **Objective 1**, these complexes will be linked by an alkyl diamine chain, vary in nuclearity, contain a single azide group at one of their terminus, and also contain a thiazole orange (TO) fluorescent dye at the other terminus. TO is an asymmetric cyanine dye that becomes highly fluorescent when it binds to DNA. The increase in fluorescence upon DNA intercalation is due to the restriction of rotation of the bridge that connects the two aromatic systems.⁴ Conjugation of TO to the azido-functionalised polynuclear platinum(II) complex series should enhance the inherent DNA binding affinity of the overall complex, as well as facilitate fluorescence detection of the complex series in cells. The novel N₃-TO-PPC series will be characterised in a similar manner to the N₃-functionalised and non-functionalised PPC series.

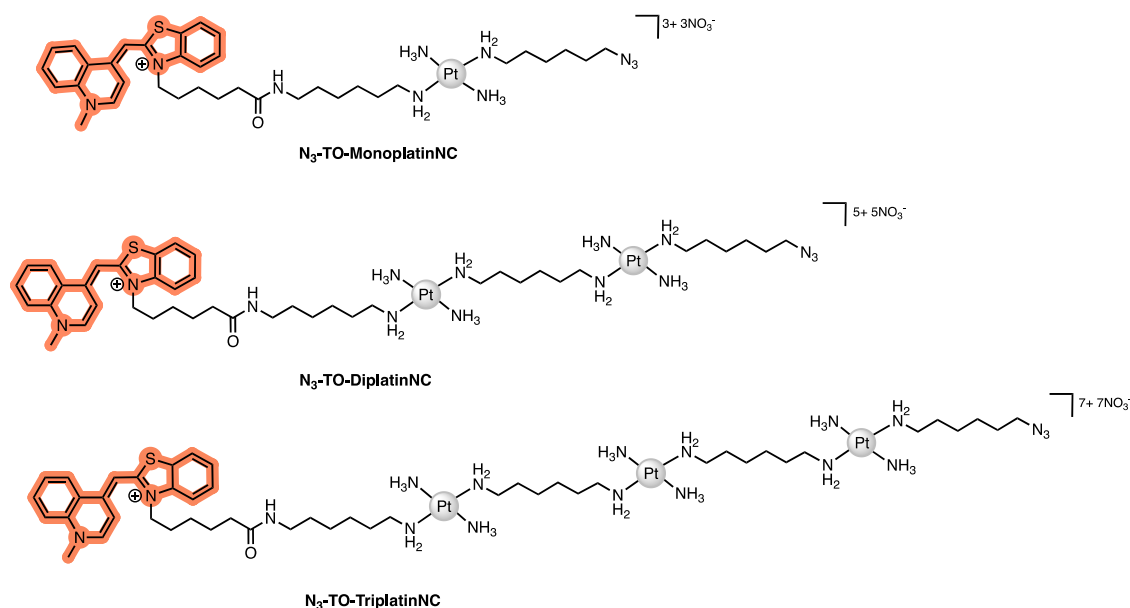


Figure 19. Solid state structures of the novel N₃-TO-PPC series.

The third objective (**Objective 3**) of this project will be to study the PPCs, N₃-PPCs, and N₃-TO-PPCs for their DNA condensation properties, DNA binding profiles, and cytotoxic activity. A variety of biophysical methods previously established by Kellett and co-workers will be used.² These include gel electrophoresis studies, thermal melting analysis, fluorometric assays, and 'lab-on-chip' epigenetic microfluidic studies. The use of a wide variety of biophysical

assays will inform our understanding of the biophysical properties of these novel drugs and their interactions with nucleic acids.

The fourth and final objective (**Objective 4**) of this research project is to study the N₃-PPCs for their *in vitro* and *in vivo* cytotoxicity properties, along with probing their cellular localisation properties. Fluorescent tagging will be employed to study the cellular accumulation profiles of the N₃-PPC series. An alkyne appended fluorophore will be 'clicked' to the N₃-PPCs *in situ* post exposure to human cancer cells. Finally, future work involving cellular analysis of the N₃-TO-PPC series will be described.

2.3. References

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Chapter III

Development of an Azide-modified Polynuclear Platinum(II) Complex Series

3. Results and Discussion

3.1. General introduction to the preparation of PPCs

A library of PPC and N₃-PPC complexes was prepared. The azide functionality is favourable in the realm of medicinal chemistry, offering a bioorthogonal “clickable” handle to a drug, whereby an alkyne-modified vector can be easily conjugated to a complex. Such vectors can possess properties to enhance the therapeutic activity, selectivity, solubility, stability and pharmacokinetics of the overall drug. Three novel complexes N₃-MonoplatinNC, N₃-DiplatinNC and N₃-TriplatinNC (Figure 20) all feature *trans*-symmetric platinum(II) centres. Non-functionalised derivatives of the PPC series were prepared according to procedures previously developed by Farrell *et al.* with some modification.¹ All organic ligands and platinum(II) complexes were characterized by ¹H NMR and ¹⁹⁵Pt NMR and where applicable, Fourier transform infrared (FTIR) spectroscopy. The purity of PPC and N₃-PPC compounds was confirmed by HPLC and elemental analysis. Transplatin, (*trans*-[PtCl₂(NH₃)₂]), silver nitrate, *N*-Boc-1,6-hexane diamine, hexamethylene diamine, and anhydrous DMF were purchased from Sigma Aldrich. 1-amino-6-azido-hexane was synthesized by reacting *N*-Boc-1,6-hexane diamine with equimolar amounts of the hydrochloride salt of the diazotransfer reagent, 1-imidazole-sulfonyl-azide according to methods developed by Goddard-Borger *et al.* with slight modification.² The *tert*-butoxycarbonyl protecting group was cleaved using 6 M hydrochloric acid to afford the free base azide ligand. **Important note:** Azides are hazardous materials and are known to be heat- and shock-sensitive. Explosive decomposition can potentially occur with very little energy input. To ensure safe manipulation and non-explosiveness for organic azides, the rule is that the number of nitrogen atoms must not exceed that of carbon, where $(N_C + N_O)/N_N \geq 3$. (N = number of atoms).³

3.1.1 Synthetic method development of the N₃-PPC library

The synthesis of any platinum(II) complex is dominated by a phenomenon known as the *trans*-effect, which is defined as the ability of a ligand to direct substitution *trans* to itself in a square planar complex. The *trans* series (Table 3) consists of common ligands ranked according to their ability to direct a ligand to substitute

at the coordination site opposite to itself, and is critical to directing the position of coordination of a ligand to a platinum(II) metal centre.⁴

Table 3. The *trans* effect series from strongest *trans* directing ligand (left) to weakest (right).

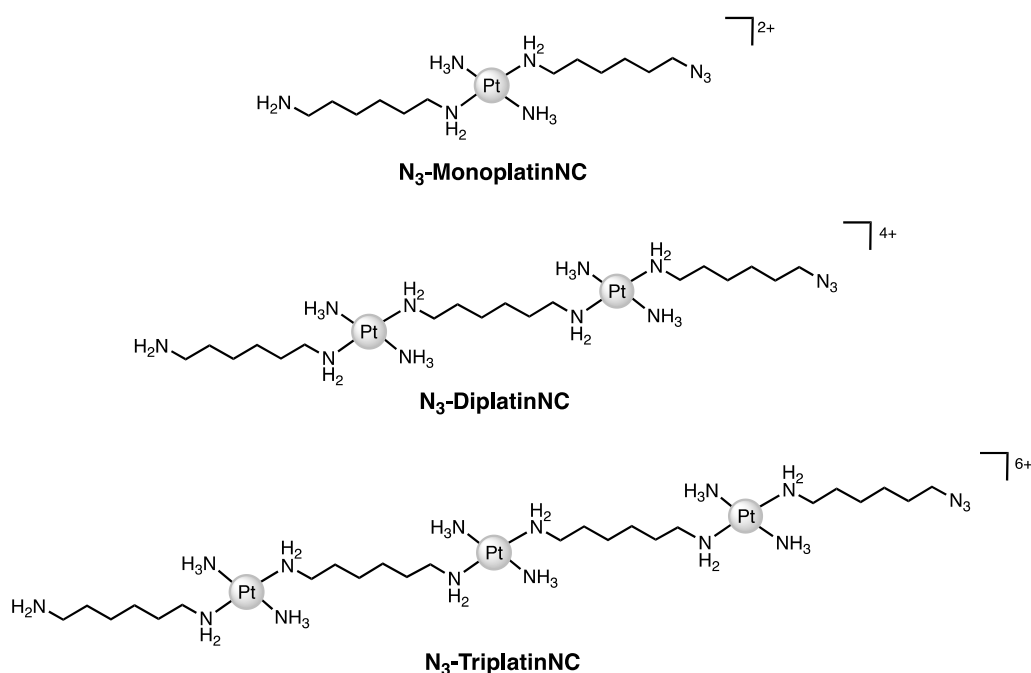
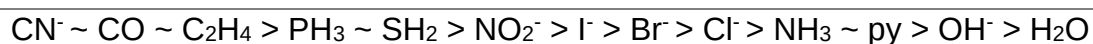


Figure 20. Molecular structures of novel N₃-PPC complexes. Complexes were synthesized as the nitrate salt.

The azide moiety is commonly known to be light-, heat- and shock-sensitive which results in the degradation of azides. With this in mind, all of the routes for the development of the N₃-PPC series were originally designed to minimise the number of synthetic steps the azide moiety would have to survive. Figure 21A shows the first attempted synthetic route of N₃-MonoplatinNC. Transplatin was double-activated using two equivalents of silver nitrate to precipitate labile chloride ligands from the complex, and the silver chloride precipitate was readily removed by filtration. The double-activated transplatin in DMF appeared colourless and the temperature of the reaction was reduced to -20 °C. A solution containing one equivalent of 1,6-hexane diamine in DMF was then added dropwise to the solution over 1 h. Subsequently, the reaction was brought to room temperature and allowed to stir before being left to stand

overnight. A solution containing one molar equivalent of 1-amino-6-azido-hexane was then added dropwise to the reaction mixture and allowed stir overnight shielded from light. The product was then isolated by precipitation using diethyl ether (Et₂O). This synthetic route failed as it was discovered that the 1,6-hexane diamine ligand was coordinating both sides of the platinum(II) centre, resulting in a cyclic-type structure. To address this problem, a BOC-protected ligand, *N*-BOC-hexane diamine was used to prevent coordination of the ligand on both sides of the platinum(II) centre. This new approach is shown in Figure 21B. After several attempts, this route was abandoned due to extremely poor yields.

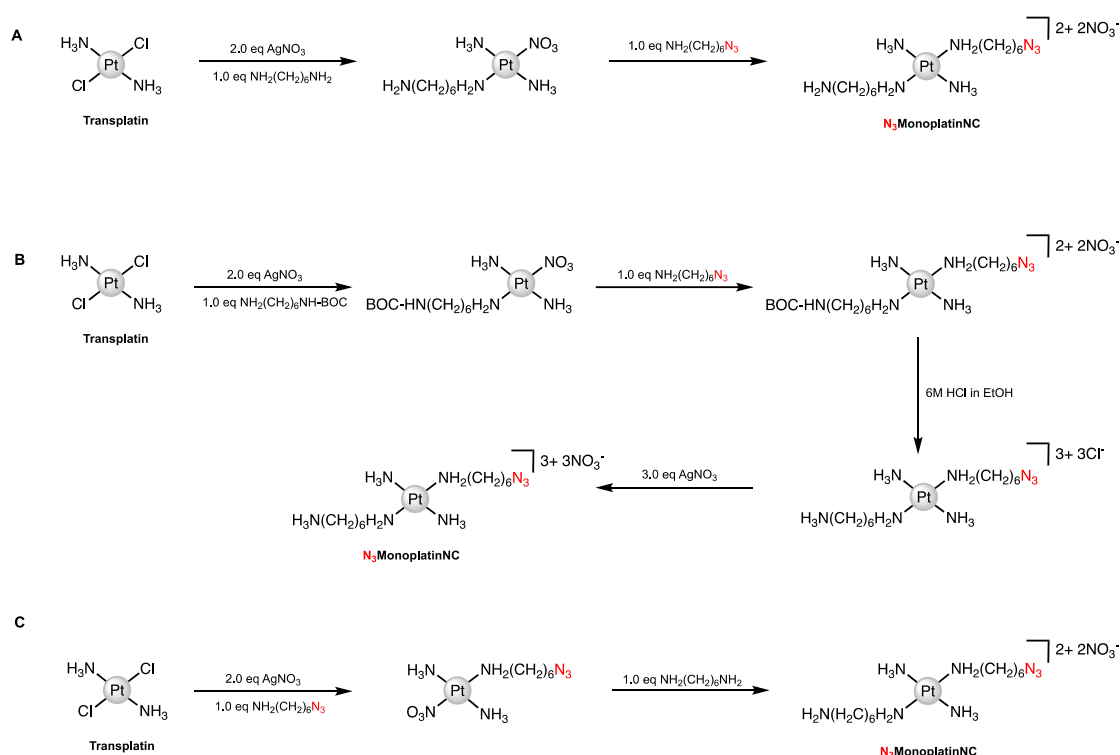


Figure 21A. First attempted synthetic route of *N*₃-MonoplatinNC; B. Second attempted synthetic route of *N*₃-MonoplatinNC; C. Successful synthetic route of *N*₃-MonoplatinNC.

A simple and more direct approach was then designed in order to prepare the desired *N*₃-MonoplatinNC (Figure 21C). Here, the azide ligand is firstly coordinated to the platinum(II) centre followed by 1,6-hexane diamine. By coordinating the azide ligand first, a single coordination site on the platinum(II) centre is then available for the 1,6-hexane diamine ligand to occupy. This synthetic route therefore removes the need for BOC-protection/deprotection, decreases the number of steps required, and increases the overall yield of the mono-functional complex.

The first attempted synthetic routes for preparing N₃-DiplatinNC and N₃-TriplatinNC is shown in Figure 22A and Figure 22B, respectively. Transplatin was double-activated for ligand substitution by the treatment of two equivalents of silver nitrate which functioned to remove both labile chloride ligands from the complex and deposit them as silver chloride, an insoluble white precipitate readily removed by filtration. The double-activated transplatin complex appeared colourless in DMF and the temperature was reduced to -20 °C. The dropwise addition of one equivalent of 1-amino-6-azido-hexane in DMF over 2 h then initiated a colour change from colourless to orange-brown indicating the coordination of the azide ligand. The reaction mixture was stirred for 2 days at room temperature to ensure complete substitution of the azide ligand. A solution of one equivalent of MonoplatinNC, or DiplatinNC, (prepared according to literature methods¹) pre-mixed with one equivalent of DIPEA in DMF was added dropwise to the reaction mixture and allowed stir for 4 days before the potential product was isolated by precipitation with Et₂O as a nitrate salt without need of further purification.

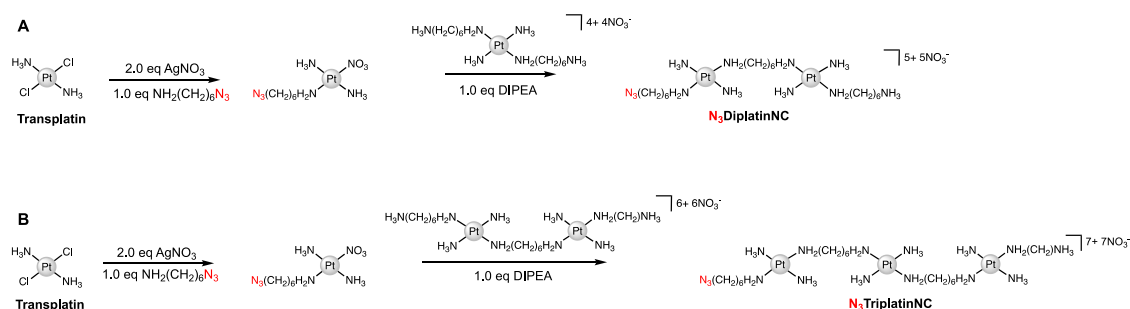


Figure 22A. First proposed synthetic route for N₃-DiplatinNC; B. First proposed synthetic route for N₃-TriplatinNC.

Unfortunately, both synthetic routes failed and the essential azide peak at ~2100 cm⁻¹ was not observed in the IR spectra. A thorough investigation of each step of the syntheses was then carried out to identify the exact point at which the azide was degrading. It was uncovered that DIPEA was reducing the azide to an amine group. DIPEA is a non-nucleophilic base that is frequently used as a proton scavenger, and in this synthesis it was intended to be used to deprotonate one of the charged 'dangling' amines of MonoplatinNC or DiplatinNC thereby rendering a primary amine available to coordinate a vacant site on the incoming platinum(II) ion. Following this observation, it was then understood that N₃-

DiplatinNC and N₃-TriplatinNC needed to be prepared in the absence of DIPEA for the deprotonation of 'dangling' amines of MonoplatinNC or DiplatinNC.

Given the effectiveness of the successful finalised synthetic route developed for N₃-MonoplatinNC, a similar approach was adopted for N₃-DiplatinNC and N₃-TriplatinNC, and is described in Figure 23. This route employs 1,1/t,t as a common precursor to both N₃-DiplatinNC and N₃-TriplatinNC. Thus, 1,1/t,t was prepared by activating one coordination site of transplatin using one equivalent of silver nitrate. Next, 0.5 eq. of 1,6-hexane diamine in DMF was titrated into the reaction mixture over 1 h and the product was precipitated from solution using Et₂O as an off-white powder. 1,1/t,t was then activated with silver nitrate to produce the dinuclear complex for coordination of either the 1-amino-6-azido-hexane ligand to afford N₃-DiplatinNC (Route Y, green), or N₃-MonoplatinNC to afford N₃-TriplatinNC (Route Z, blue). Overall, it appears that there are several plausible synthetic routes are possible for the construction of these complexes, however, not all of them are feasible and the routes outlined in Figure 23 appear the most efficient in terms of modularity and yield for each of the target complexes.

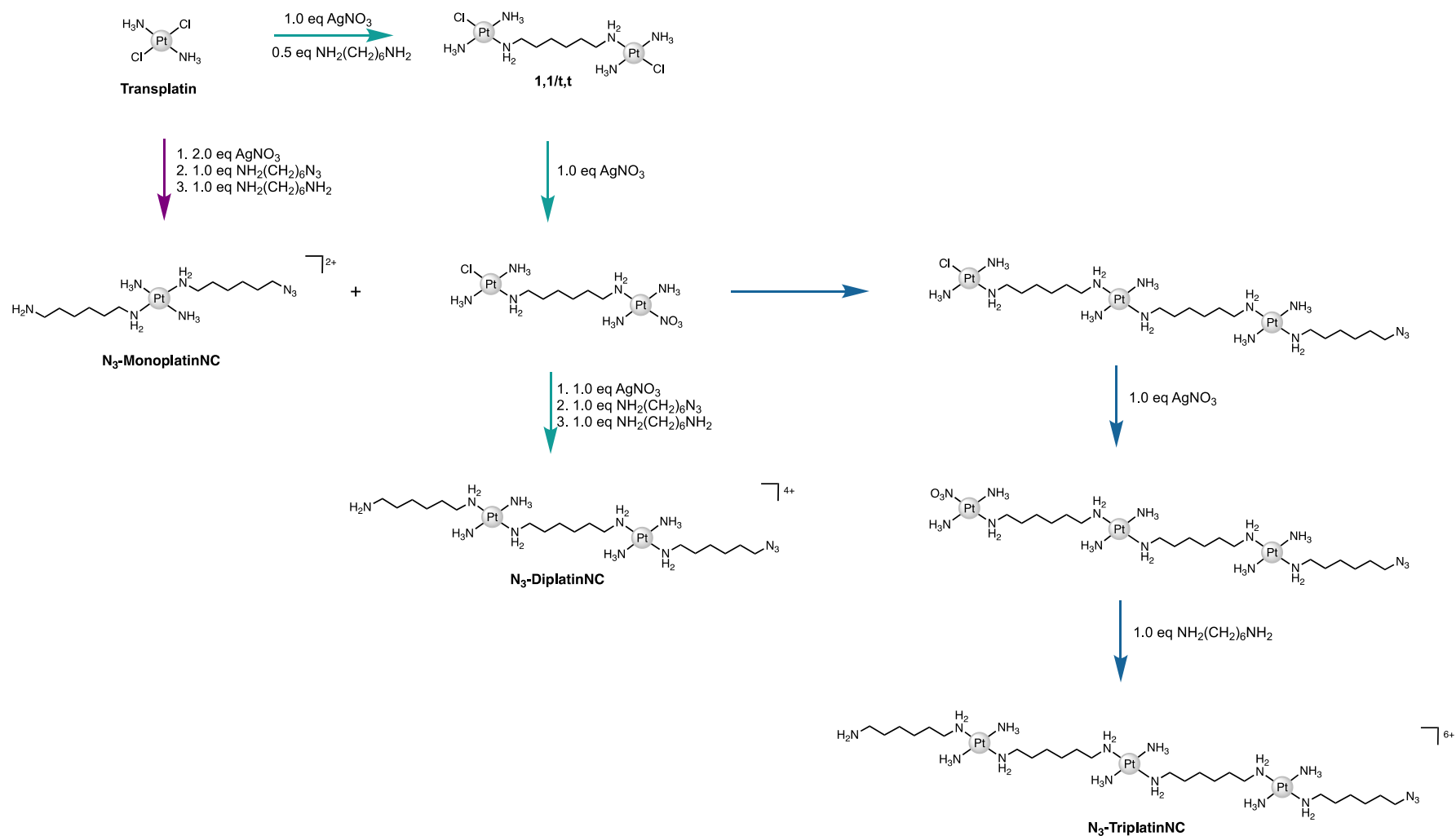


Figure 23. Synthetic routes for N_3 -PPC series: Route X (purple) for the preparation of N_3 -MonoplatinNC; Route Y (green) for the preparation of N_3 -DiplatinNC; Route Z (blue) for N_3 -TriplatinNC

3.2. Characterisation - nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is an analytical technique used to characterise organic and diamagnetic inorganic compounds. When a sample is exposed to an external magnetic field, the NMR-active nuclei align with the magnetic field and are excited by radiowaves which causes a nuclear spin flip from the α spin state to the β spin state. The relaxation of the NMR-active nuclei back down to the α spin state releases energy which is detected by the NMR instrument. This excitation and relaxation of the NMR-active nucleus is known as its resonance frequency. The intramolecular magnetic field in a compound is contingent on the local electron density surrounding the NMR-active nucleus, which thus influences its resonance frequency. The changes in resonance frequency give an insight into the electronic structure of a compound. In order for an atom to be considered NMR-active the spin quantum number must be greater than zero.⁵

In relation to diamagnetic inorganic complexes, the identification of ligand coordination can often be detected by an anisotropic downfield shift in NMR signals corresponding to the ligand. The electron density of the ligand is pulled towards the metal centre causing an overall deshielding effect of the ligand NMR signals. For this reason, NMR can be a useful technique for determining ligand coordination in metal complexes.⁶

3.2.1. ^1H NMR spectroscopy

Fortunately, platinum(II) is a diamagnetic, d^8 transition metal which allows for coordinated ligands to be suitably analysed by ^1H NMR spectroscopy. ^1H NMR spectra of the $\text{N}_3\text{-PPC}$ complexes are nearly identical, as they all share equivalent proton environments with the only significant difference in their spectra being the integrals of the peaks. As there are no aromatic protons present in these complexes—only aliphatic protons—the only peaks present are in the aliphatic region between 0-4 ppm.

chain and have multiplet splitting patterns as they are adjacent to protons in unequal environments.

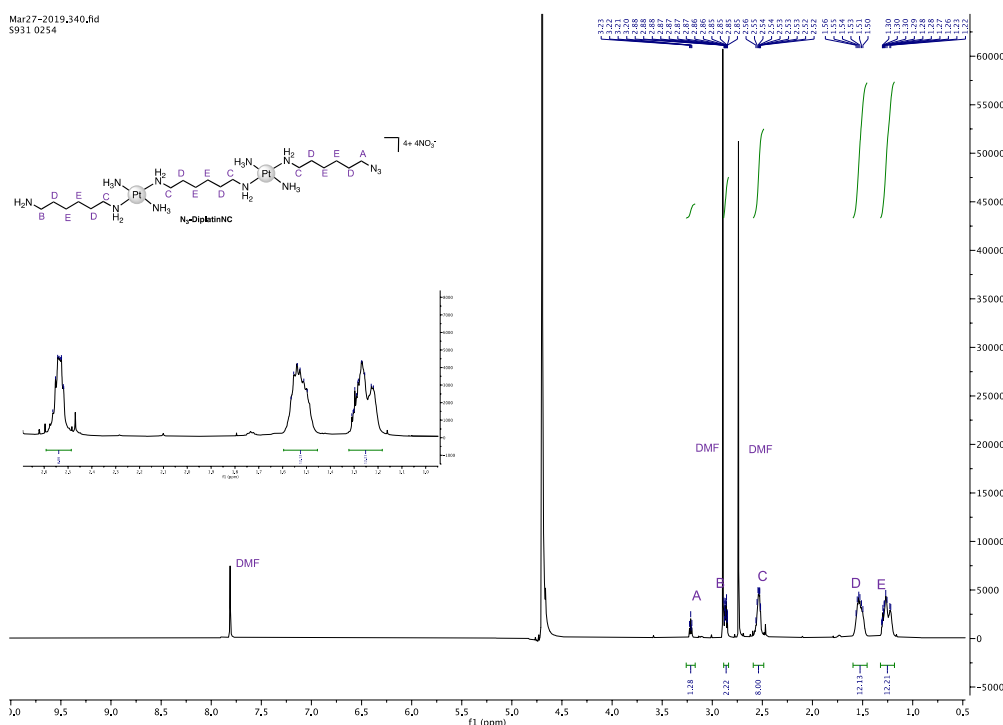


Figure 25. ^1H NMR spectrum obtained on a Bruker Avance Ultrashield 600 MHz spectrometer of N_3 -DiplatinNC in D_2O . ^1H NMR spectra were processed using MestReNova Mestrelab Research chemistry software solutions.

It is clear from the spectrum of N_3 -DiplatinNC (Figure 25) has an almost identical ^1H NMR spectrum to N_3 -MonoplatinNC. As identified previously, there appears to be 5 proton environments in this complex and each proton environment is accounted for using reasons identical to earlier N_3 -MonoplatinNC analysis. Thus, the protons on the CH_2 moiety next to the azide appear at an identical ppm as N_3 -MonoplatinNC. However, there is an increase in the integration for C, D and E which is accounted for due to the additional aliphatic diamine linker present in this complex.

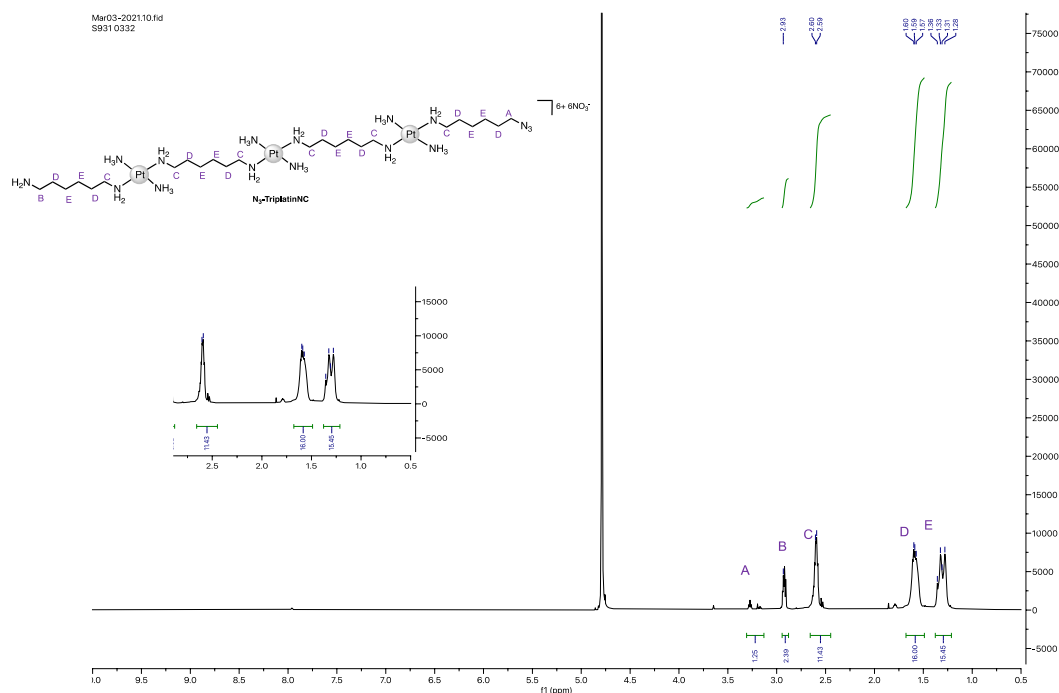


Figure 26. ^1H NMR spectrum obtained on a Bruker Avance Ultrashield 600 MHz spectrometer of N_3 -TriplatinNC in D_2O . ^1H NMR spectra were processed using MestReNova Mestrelab Research chemistry software solutions.

The ^1H NMR spectrum of N_3 -TriplatinNC is shown in Figure 26 and again shows the same five proton environments that are present in the earlier described N_3 -MonoplatinNC and N_3 -DiplatinNC complexes. Protons labelled C which integrate for 12 protons—corresponding to those on the CH_2 adjacent to the amine coordinated to the platinum(II) centre—confirms the addition of the third platinum(II) nucleus to the complex. The increased number of aliphatic protons at peaks labelled E and D are a consequence of the additional 1,6-hexane diamine chain.

3.2.2. ^{195}Pt NMR spectroscopy

^{195}Pt NMR spectroscopy is an analytical tool that allows for the characterization of platinum complexes in solution. While 32 isotopes of platinum exist, only 3 isotopes have a natural abundance of substantial proportion, and only one of these is NMR compatible with a quantum spin number = 1/2, platinum-195. The natural abundance of the platinum-195 isotope is relatively similar to that of carbon-13 at 33.8%, and so samples for NMR analysis are required to be quite concentrated in order to obtain sufficient spectra. As with ^1H NMR spectroscopy, ^{195}Pt NMR spectroscopy is used to identify different platinum environments

present in a sample, where coupling can be observed in multinuclear complexes between platinum centres occupying different environments.⁷ ¹⁹⁵Pt NMR analysis of the N₃-PPC series shows almost identical ¹⁹⁵Pt NMR spectra for each of the three complexes (Figure 27). While the number of platinum(II) nuclei increases, the environment of each of the platinum centres does not change through the series, therefore it is axiomatic that the chemical shift of the peaks are almost identical.

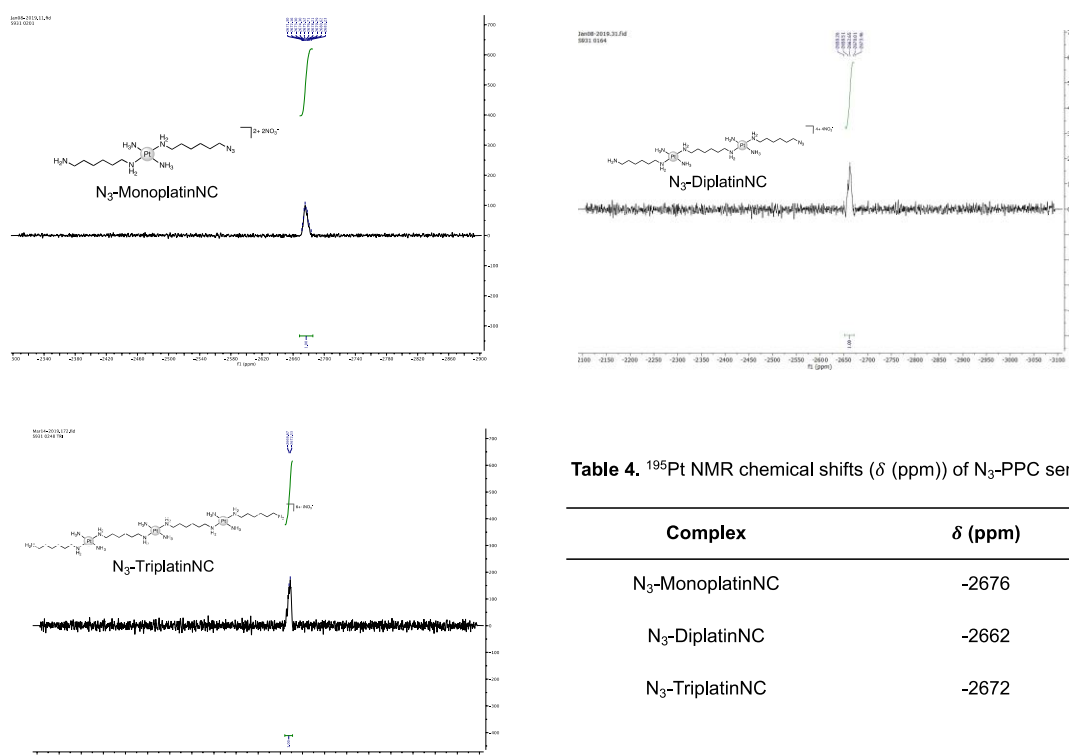


Table 4. ¹⁹⁵Pt NMR chemical shifts (δ (ppm)) of N₃-PPC series.

Complex	δ (ppm)
N ₃ -MonoplatinNC	-2676
N ₃ -DiplatinNC	-2662
N ₃ -TriplatinNC	-2672

Figure 27. ¹⁹⁵Pt NMR spectra of N₃-PPC series. Samples were dissolved in D₂O and spectra were obtained on a Bruker Avance Ultrashield 600 MHz spectrometer. ¹⁹⁵Pt NMR spectra were processed using MestReNova Mestrelab Research chemistry software solutions.

3.3. High performance liquid chromatography

High performance liquid chromatography (HPLC) is a technique that is widely used to measure the purity of a sample. Analyses were carried out according to previously established methods by Farrell and co-workers.⁸ A gradient elution method was used with a solvent system of solvent A: H₂O/ACN 60/40; solvent B: 95/5. Samples were prepared as 1 mg/mL solutions in HPLC grade H₂O. The injection volume was 50 μ L and flow rate was kept constant at 1.5 mL/min for 60 mins.

Peak purity analysis revealed that complexes were synthesised to a high degree of purity: N₃-MonoplatinNC, N₃-DiplatinNC and N₃-TriplatinNC peak purity of 99%, 96% and 98% respectively.

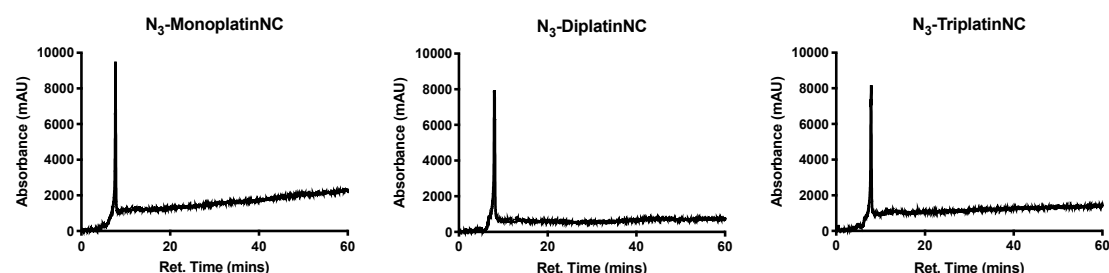


Figure 28. HPLC chromatograms of N₃-PPC series with detection at 254 nm. HPLC was performed on a Shimadzu Prominence system with Nexera X2 photo-diode array detector module. HPLC chromatograms were processed using Prism 8 GraphPad software and percentage peak purity was calculated as the percentage area under the curve.

3.4. Characterisation by Fourier transform infra-red spectroscopy (FT-IR)

Infra-red (IR) spectroscopy is a qualitative technique used widely to detect the presence of functional groups within molecules. This analytical technique exploits the fact that different functional groups have different characteristic vibrational energies that correspond to their absorption frequencies. As for the N₃-PPC series, the most distinctive characteristic feature of these complexes is the azide moiety, usually showing a strong sharp peak at $\sim 2100\text{ cm}^{-1}$. Although IR spectroscopy is primarily a qualitative rather than quantitative method, this technique has been a useful indicator to confirm the increasing number of platinum(II) nuclei in the complex series. In N₃-MonoplatinNC (Figure 29), a large sharp peak at 2103 cm^{-1} is representative of the presence of the azide; N₃-DiplatinNC (Figure 29) shows a smaller peak at 2102.3 cm^{-1} corresponding to the azide and N₃-TriplatinNC Figure 29 shows an even lower intensity for the azide peak at 2100.7 cm^{-1} . As N₃-MonoplatinNC has the lowest molecular weight of the three compounds, this complex has the highest concentration of azide relative to the overall complex, resulting in the mononuclear derivative showing the highest peak intensity for the azide moiety. The curtailing of the intensity of the azide peak with increasing platinum(II) nuclei is symptomatic of the increasing size of the N₃-PPC series and strongly suggests that the number of platinum(II) nuclei is increasing in the complex series.⁹

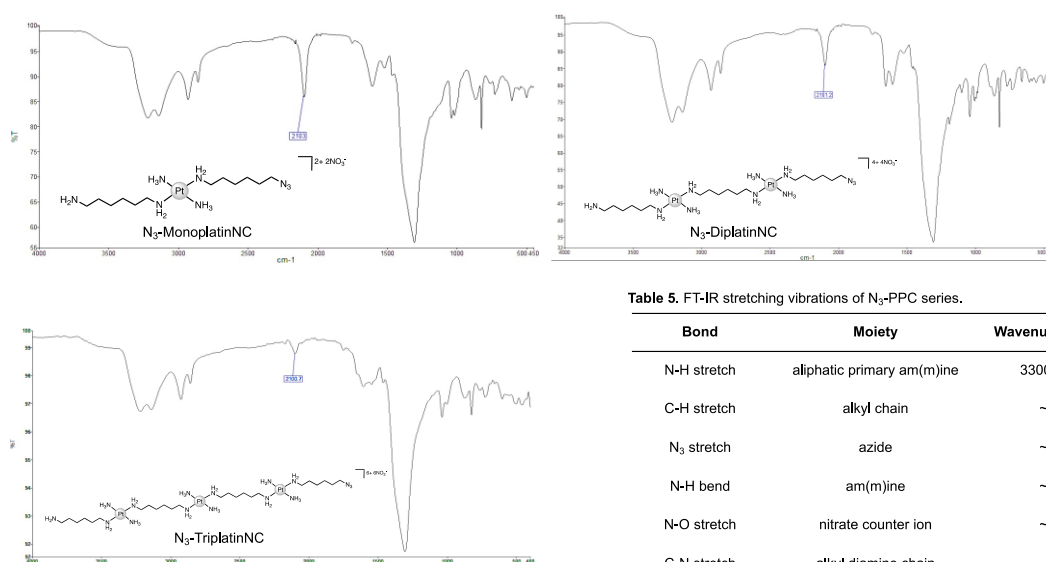


Table 5. FT-IR stretching vibrations of N₃-PPC series.

Bond	Moiety	Wavenumber (cm ⁻¹)
N-H stretch	aliphatic primary am(m)ine	3300 – 3200
C-H stretch	alkyl chain	~2900
N ₃ stretch	azide	~2100
N-H bend	am(m)ine	~1600
N-O stretch	nitrate counter ion	~1400
C-N stretch	alkyl diamine chain	~1050

Figure 29. FT-IR spectrum and characteristic peaks of N₃-PPC series.

3.5. PPC characterisation by elemental analysis

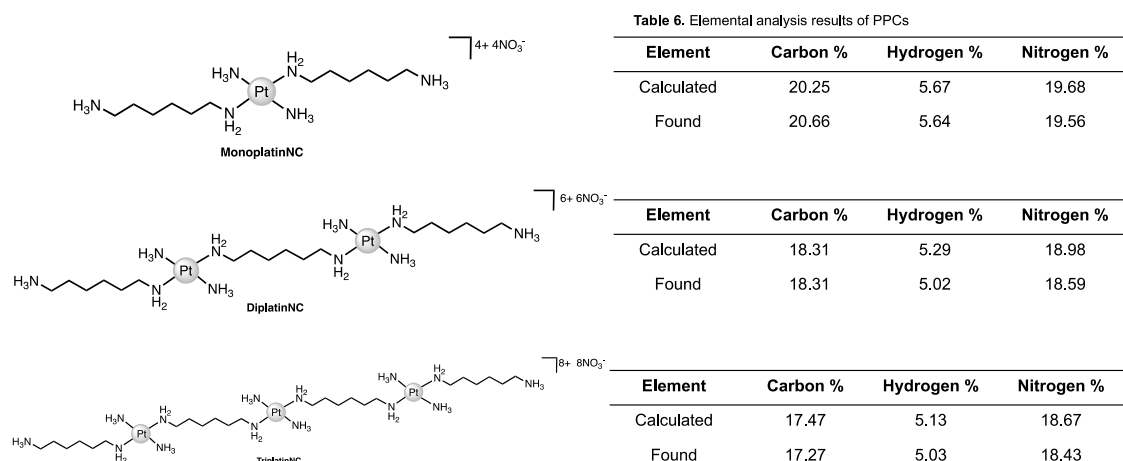
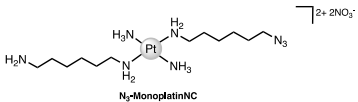
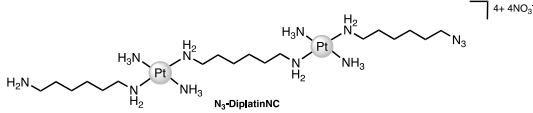


Figure 30. Elemental analysis results of MonoplatinNC, DiplatinNC, and TriplatinNC.

Elemental analysis is a quantitative analytical technique used to determine the elemental composition of different compounds or materials, independent of geometry and structure. Figure 30 shows the elemental analysis of the PPCs. CHN results from this study show that each of the PPCs were synthesized to a high degree of purity with percentage carbon, hydrogen, and nitrogen in agreement with the theoretically calculated results. In the case of the N₃-PPC series, the percentage of carbon, hydrogen and nitrogen were determined for each and compared with the theoretically calculated results. From the data provided in Table 7, it is clear that the percentage carbon and hydrogen found

during elemental analysis agrees with that of the calculated theoretical results. However, the percentage nitrogen is out by 3-4% out for all three of the compounds. This can be attributed to the inherent instability and volatility of the azide moiety itself. It is well known that azides are not stable under harsh conditions, such as the combustion technique used for elemental analysis.¹⁰ When the elemental analysis results of the PPCs and N₃-PPCs are compared, it is clear that the discrepancy in results for the percentage nitrogen of the N₃-PPCs is a direct consequence of the instability of the azide moiety under the harsh conditions required for elemental analysis. The azide moiety is the only difference between each of the complex series so therefore must be the reason for this disparity.

 N₃-MonoplatinNC		Table 7. Elemental analysis results of N₃-PPCs		
Element	Carbon %	Hydrogen %	Nitrogen %	
Calculated	23.57	5.93	22.90	
Found	22.48	5.23	18.75	

 N₃-DiplatinNC				
Element	Carbon %	Hydrogen %	Nitrogen %	
Calculated	20.00	5.41	20.73	
Found	20.77	5.06	16.63	

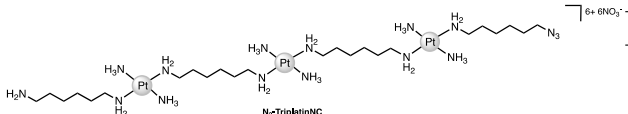
 N₃-TriplatinNC				
Element	Carbon %	Hydrogen %	Nitrogen %	
Calculated	18.59	5.20	19.88	
Found	19.14	5.13	16.21	

Figure 31. Elemental analysis results of N₃-PPC series.

3.6. Conclusion

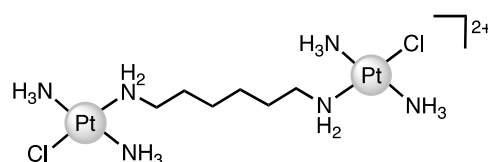
This work reports the synthesis and characterisation of a novel series of azide-functionalised polynuclear platinum(II) complexes, meeting the aims of the first objective—to prepare a family of azide-modified PPCs—outlined in Chapter 2. A modular, stepwise synthetic protocol was developed for the preparation of this novel series of drugs. The novel complex series varies in nuclearity comprising of a mononuclear, a dinuclear, and a trinuclear complex. Comprehensive characterisation was conducted to verify that each complex was successfully generated to a high degree of purity using a variety of analytical techniques including NMR spectroscopy (¹H, ¹⁹⁵Pt), FT-IR spectroscopy, elemental analysis, and HPLC.

3.7. Experimental

Chemicals, reagents and high-performance liquid chromatography (HPLC) solvents including CHCl_3 , H_2O and MeOH of analytical grade were purchased from Sigma-Aldrich (Ireland) or Tokyo Chemical Industries (TCI, UK Ltd) and used for the synthesis and preparation of organic ligands and metal complexes without purification. All platinum(II) complexes were synthesized as a nitrate salt.

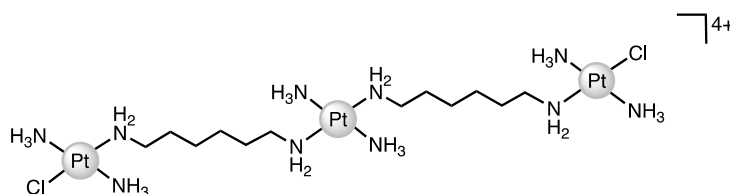
3.7.1. Synthesis and Characterisation of non-functionalised PPC series

1,1/t,t



Transplatin (0.100 g, 0.33 mmol) was dissolved in DMF (8 mL) and added with a portion of silver nitrate (0.056 g, 0.33 mmol) and stirred overnight, shielded from light, at ambient temperature. The precipitated silver chloride was removed by filtration to leave a clear colourless liquid. Subsequently, the temperature of the reaction was brought to $-20\text{ }^{\circ}\text{C}$ before a dropwise addition of hexamethylene diamine was performed steadily over 3 hrs. The reaction mixture was stirred for 5 days at ambient temperature before the product was precipitated out of solution using diethyl ether (400 mL). The product was allowed dry under nitrogen. Yield: 0.152 g (60%). ^1H NMR (600 MHz, D_2O): δ = 2.63 ppm (m, 4H), 1.65 ppm (m, 4H), 1.31 ppm (m, 4H). IR (ATR, cm^{-1}): 3262, 3073, 1594, 1536, 1315, 1041, 900, 825, 770, 716.

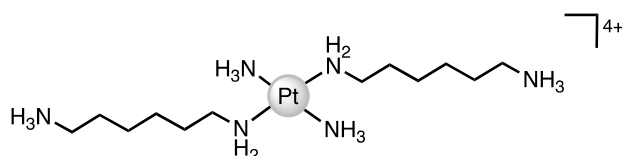
BBR3464



MonoplatinNC (0.200 g, 0.28 mmol) was dissolved in DMF (15 mL) and treated with DIPEA (2 eq) and stirred for 20 mins at ambient temperature shielded from

light. Separately, transplatin (0.168 g, 0.56 mmol) was dissolved in DMF (12 mL) and added with silver nitrate (0.095 g, 0.56 mmol). The reaction mixture was allowed stir overnight shielded from light. The precipitated silver chloride was removed from the reaction mixture by filtration. The mono-activated transplatin solution was then added dropwise into the MonoplatinMC mixture dropwise over 2 hrs shielded from light. Once the entire reaction mixture was added to the other, the reaction was allowed stir for 7 days, shielded from light, at room temperature. The product was precipitated out of solution using diethyl ether, and product was firstly dried under vacuum and subsequently dried under nitrogen. Yield: 0.152 g (60%). ^1H NMR (600 MHz, D_2O): δ = 2.56 ppm (m, 8H), 1.58 ppm (m 8H), 1.24 ppm (m 8H). IR (ATR cm^{-1}): 3261, 2931, 1536, 1314, 1041, 825, 770, 730, 716, 600

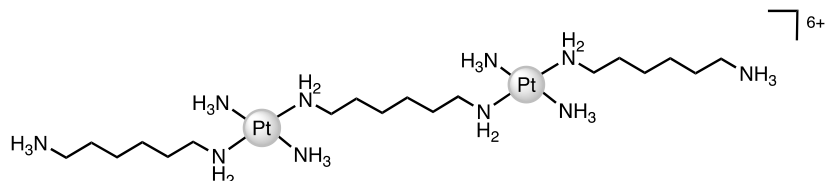
MonoplatinNC



Transplatin (0.200 g, 0.66 mmol) was dissolved in DMF (15 mL) and added with silver nitrate (0.244 g, 1.32 mmol) in one portion. The reaction mixture was allowed stir for 24 h, at ambient temperature, shielded from light. The precipitated silver chloride was filtered off and the clear filtrate was cooled to $-20\text{ }^{\circ}\text{C}$. A solution of N-BOC-1,6-hexane diamine (0.314 g, 1.45 mmol) in DMF (5 mL) was added dropwise to the cooled filtrate over 3 hrs and subsequently left stir for 2 days at $20\text{ }^{\circ}\text{C}$, shielded from light. The solvent volume was reduced to $\sim 5\text{ mL}$, diluted with Et_2O (200 mL) and allowed stir for 30 mins on an ice bath to precipitate product. The reaction mixture was filtered and allowed dry under vacuum overnight. The filter cake was dissolved in MeOH (50 mL) and treated with a solution of 8M HCl in EtOH and allowed stir for 7 days. The precipitated product was filtered, washed using ice-cold Et_2O and dried under vacuum for 1 h. The solid was dissolved in H_2O (20 mL), treated with silver nitrate (0.205 g, 1.21 mmol) and allowed stir for 24 hrs shielded from light. The silver chloride precipitate was filtered off and the product as a nitrate salt was precipitated out

of solution using acetone (500 mL), collected by filtration and dried under vacuum. Yield: 0.183 g (70%). ^1H NMR (600 MHz, D_2O): δ = 2.94 ppm (t, J = 7.6 Hz, 4H), 2.62 ppm (t, J = 7.74 Hz, 4H), 1.62 ppm (m, 8H), 1.34 ppm (m, 8H); ^{195}Pt NMR (600 MHz, D_2O): δ = -2674 ppm. Elemental analysis calculated (%) for $\text{C}_{12}\text{H}_{40}\text{N}_{10}\text{O}_{12}\text{Pt}$: C 20.25, H 5.67, N 19.68; found: C 21.52, H 5.48, N 19.24. IR (ATR, cm^{-1}): 3262, 3073, 1594, 1536, 1315, 1041, 900, 825, 770, 716.

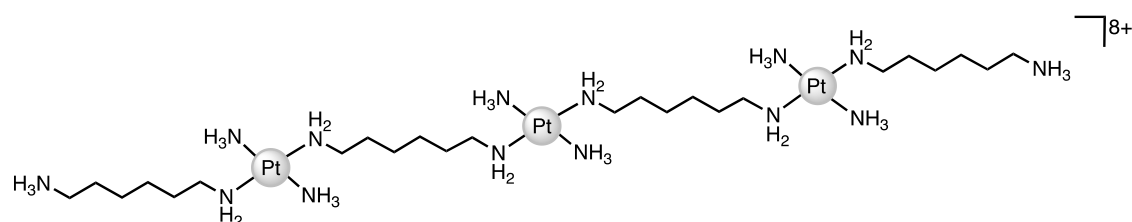
DiplatinNC



Transplatin (0.200 g, 0.66 mmol) was dissolved in DMF (15 mL), added with silver nitrate (0.101 g, 0.59 mmol) in one portion and allowed stir for 24 h at room temperature, shielded from light. The silver chloride precipitate was filtered off and the clear yellow filtrate was cooled to $-20\text{ }^{\circ}\text{C}$. A solution of hexamethylenediamine (0.038 g, 0.33 mmol) in DMF (5 mL) was added dropwise to the filtrate, with vigorous stirring over 2 h. The reaction mixture was allowed stir for 2 days at ambient temperature. The solvent volume was reduced under vacuum to ~ 5 mL, diluted with Et_2O (200 mL) and allowed stir for 30 min. The reaction mixture was filtered, washed with ice-cold Et_2O and allowed dry overnight. The filter cake was dissolved in DMF (15 mL) and treated with silver nitrate (0.095 g, 0.56 mmol) and allowed stir for 24 h, shielded from light. The precipitated silver chloride was filtered off and the clear filtrate cooled to $-20\text{ }^{\circ}\text{C}$. A solution of *N*-BOC-hexamethylenediamine (0.121 g, 0.56 mmol) in DMF (2 mL) was added dropwise to the cooled filtrate over 2 h, and subsequently allowed stir for 2 days at ambient temperature. The solvent volume was reduced under vacuum, diluted with Et_2O (300 mL) and allowed stir for 30 min. The precipitate was collected by filtration, dissolved in MeOH (50 mL), treated with a solution of 8 M HCl in EtOH and stirred for 10 days. The chloride salt was collected by filtration, dissolved in H_2O (30 mL), treated with silver nitrate (0.180 g, 1.47 mmol) and allowed stir for 20 h shielded from light. Precipitated silver chloride was filtered off and the filtrate diluted with acetone (600 mL) and left stir for 1 h. The product was collected by filtration, washed using ice-cold acetone and dried under vacuum to yield the product as

a nitrate salt. Yield: 0.202 g (26%). ^1H NMR (600 MHz, D_2O): δ = 2.92 ppm (t, J = 7.62 Hz, 4H), 2.59 ppm (m, 8H), 1.58 ppm (m, 12H), 1.29 ppm (m, 12H). ^{195}Pt NMR (600 MHz, D_2O): δ = -2662 pm. Elemental analysis calculated (%) for $\text{C}_{18}\text{H}_{62}\text{N}_{16}\text{O}_{18}\text{Pt}_2$: C 18.31, H 5.29, N 18.98; found: C 18.39, H 5.02, N 18.21. IR (ATR cm^{-1}): 3261, 2931, 1536, 1314, 1041, 825, 770, 730, 716, 600.

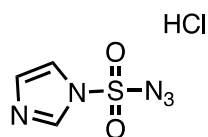
TriplatinNC



Transplatin (0.300 g, 0.99 mmol) was dissolved in DMF (20 mL) and added with silver nitrate (0.168 g, 0.99 mmol) and stirred overnight shielded from light. The silver chloride precipitate was filtered off and the clear yellow filtrate was cooled to $-20\text{ }^{\circ}\text{C}$. A solution of N-BOC-hexane diamine (0.214 g, 0.99 mmol) in DMF (4 mL) was added dropwise to the reaction mixture over 1 h. The reaction was allowed stir overnight, shielded from light. The Boc-protected $\text{Pt(II)(NH}_3)_2\text{Cl}$ was precipitated out of solution using diethyl ether. This platinum(II) unit was then activated using 1 eq of silver nitrate. MonoplatinNC (0.352 g, 0.495 mmol) was dissolved in DMF and added with 2 eq DIPEA and stirred for 10 min. Subsequently, the activated Boc-protected platinum unit was added to the monoplatinNC reaction mixture dropwise over 3 h. The reaction was allowed stir for 7 days, at ambient temperature shielded from light. Boc deprotection was performed for 2 months using 9 M HCl. The product precipitated out as TriplatinNC as a chloride salt, which was subsequently converted to the nitrate salt by reaction with 8 equivalence of silver nitrate. Yield: 0.104 g (9%). ^1H NMR (600 MHz, D_2O): δ = 2.92 ppm (t, J = 7.62 Hz, 4H), 2.59 ppm (m, 12H), 1.58 ppm (m, 16H), 1.29 ppm (m, 16H). ^{195}Pt NMR (600 MHz, D_2O): δ = -2661 pm. Elemental analysis calculated (%) for $\text{C}_{18}\text{H}_{62}\text{N}_{16}\text{O}_{18}\text{Pt}_2$: C 18.31, H 5.29, N 18.98; found: C 18.39, H 5.02, N 18.21. IR (ATR cm^{-1}): 3261, 2931, 1536, 1314, 1041, 825, 770, 730, 716, 600

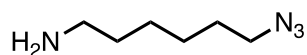
3.7.2. Synthesis and characterisation of imidazole-sulfonyl-azide hydrochloride salt and 1-amino-6-azido-hexane

Imidazole-sulfonyl-azide.HCl



Sulfuryl chloride (2.496 mL, 30.8 mmol) was slowly added to a cooled solution of NaN_3 (2.000 g, 30.8 mmol) in ACN (50 mL) and stirred for 18 h at room temperature, shielded from light. Imidazole (2.097 g, 46.2 mmol) was added to the slurry over 30 min and subsequently allowed stir for 18 h at room temperature. The mixture was diluted with EtOAc (150 mL), washed with H_2O (2×200 mL), washed with a saturated aqueous solution of NaHCO_3 (2×100 mL) and dried using magnesium sulfate. Solvent was removed under reduced pressure to ~15 mL and a solution of HCl in EtOH was added dropwise to the cooled solution with vigorous stirring to precipitate out product. The mixture was filtered and filter cake washed with ice-cold EtOAc (2×50 mL) to give product as white fluffy needles. Yield: 4.179 g (65%). ^1H NMR (600 MHz, CDCl_3): δ = 8.50 ppm (dd, 1H), 7.95 ppm (dd, 1H), 7.29 ppm (dd, 1H). Elemental analysis calculated (%) for $\text{C}_3\text{H}_4\text{N}_5\text{SO}_2\text{Cl}$: C 17.19, H 1.92, N 33.41; found: C 17.97, H 2.00, N 31.79. IR (ATR cm^{-1}): 3105, 2169, 1913, 1425, 1298, 1229, 1189, 1159, 1137, 1103.

1-amino-6-azido hexane

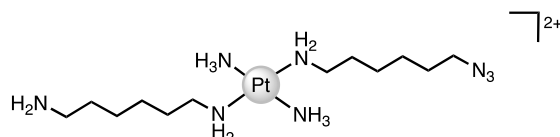


Imidazole-1-sulfonyl-azide hydrochloride (0.683 g, 3.30 mmol) was added to a solution of *N*-BOC-hexane diamine (0.595 g, 2.75 mmol), potassium carbonate (0.663 g, 4.80 mmol), and copper sulfate (3 mg, 18.78 μmol) in MeOH (50 mL) and allowed stir for 36 h at ambient temperature, shielded from light. The mixture was concentrated to ~2 mL, diluted with H_2O (30 mL), acidified to pH 1 using conc. HCl and extracted with EtOAc (5×50 mL). The organic phases were combined, dried using magnesium sulfate, filtered and solvent removed to yield

the BOC-protected product as a yellow oil. The yellow oil was dissolved in MeOH (50 mL) and treated with a solution of 5 M HCl in EtOH and stirred vigorously for 48 h shielded from light. The reaction mixture was basified to pH 10 using 1 M NaOH before extracting with DCM (5 × 50 mL). Organics were combined, dried using magnesium sulfate and solvent removed under vacuum. Yield: 0.141 g (36%). ¹H NMR (600 MHz, CDCl₃): δ = 3.23 ppm (t, *J* = 6.78 Hz, 2H), 2.65 ppm (t, 2H), 1.57 ppm (p, 2H), 1.42 ppm (m, 2H), 1.33 ppm (m, 6H). IR (ATR cm⁻¹): 2932, 2859, 2090, 1463, 1349, 1244, 1175, 1038, 728, 665.

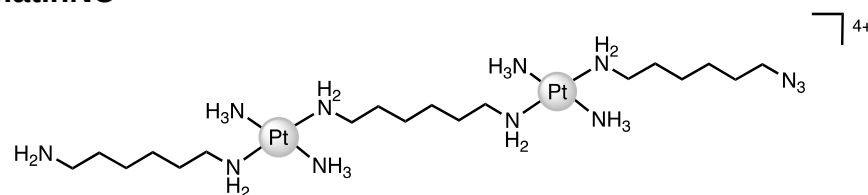
3.7.3. Synthesis and characterisation of N₃-PPC series

N₃-MonoplatinNC



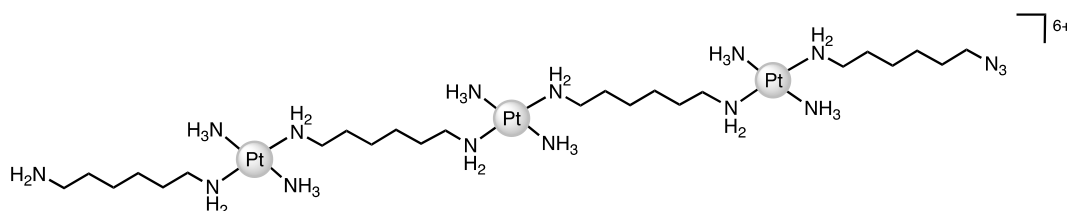
Transplatin (0.210 g, 0.70 mmol) was dissolved in anhydrous DMF (20 mL), treated with silver nitrate (0.239 g, 1.40 mmol) and stirred for 18 hrs, at room temperature, shielded from light. The precipitated silver chloride was filtered off and filtrate cooled to -20 °C. A solution of 1-amino-6-azido hexane (0.100 g, 0.70 mmol) in DMF (15 mL) was added dropwise to the cooled filtrate over 3 h and allowed stir for 48 h, at room temperature, shielded from light. The reaction mixture was cooled to -20 °C and added with a solution of 1,6-hexane diamine (0.082 g, 0.70 mmol) in DMF (15 mL) over 3 h and allowed stir for 18 h shielded from light. Volume of solvent was reduced to ~5 mL, diluted with Et₂O (500 mL) and allowed stir for 30 mins before filtering. The filter cake was washed with ice-cold Et₂O. Yield 0.272 g (68%). ¹H NMR (600 MHz, D₂O): δ = 3.28 ppm (t, 2H), 2.94 ppm (m, 2H), 2.62 ppm (m, 4H), 1.62 ppm (m, 8H), 1.35 ppm (m, 8H). ¹⁹⁵Pt NMR (600 MHz, D₂O): δ = -2664 ppm. Elemental analysis calculated (%) for C₁₂H₃₆N₁₀O₆Pt: C 23.57, H 5.93, N 22.90; found: C 22.48, H 5.23, N 18.75. IR (ATR cm⁻¹): 3220, 3140, 2931, 2860, 2097, 1606, 1307, 1041, 825.

N₃-DiplatinNC



Transplatin (0.025 g, 0.82 mmol) was dissolved in DMF (10 mL), activated using silver nitrate (0.014 g, 0.80 mmol) and stirred for 18 h at room temperature, shielded from light. The precipitated silver chloride was filtered off and the filtrate cooled to -20 °C. A solution of azido-monoplatinNC (0.050 g, 0.82 mmol) in DMF (15 mL) was added dropwise to the filtrate over 3 h and stirred for 72 h at room temperature. Silver nitrate (0.014 g, 0.80 mmol) was added to the reaction mixture and stirred for 12 h at room temperature, shielded from light. Precipitated silver chloride was filtered away and a solution of 1,6-hexane diamine (0.010 g, 0.81 mmol) in DMF (5 mL) was added dropwise to the cooled filtrate over 2 h. The reaction was stirred for 48 h and volume of solvent reduced to ~10 mL. The solution was diluted with 500 mL Et₂O, filtered and filter cake washed with ice-cold Et₂O. Yield: 0.032 g (35%). ¹H NMR (600 MHz, D₂O): δ = 3.28 ppm (t, 2H), 2.85 ppm (m, 2H), 2.53 ppm (m, 8H), 1.53 ppm (m, 12H), 1.27 ppm (m, 12H). ¹⁹⁵Pt NMR (600 MHz, D₂O): δ = -2678 ppm. Elemental analysis calculated (%) for C₁₈H₅₈N₁₆O₁₂Pt₂: C 20.00, H 5.41, N 20.73; found: C 20.77, H 5.06, N 16.63. IR (ATR cm⁻¹): 3226, 2930, 2863, 2097, 1656, 1310, 1097, 1038, 825.

N₃-TriplatinNC



Intermediate 1,1/t,t (0.080 g, 0.11 mmol) was dissolved in anhydrous DMF (15 mL), treated with silver nitrate (0.018 g, 0.10 mmol) and stirred at room temperature overnight shielded from light. The precipitated silver chloride was filtered off and the filtrate cooled to -20 °C. A solution of azido-monoplatinNC (0.064 g, 0.11 mmol) in DMF (15 mL) was added dropwise to the filtrate over 3 hrs and stirred for 72 hours at room temperature. The solution was filtered to remove any traces of reduced platinum. The filtrate was treated with silver nitrate

(0.018 g, 0.10 mmol) and stirred over night at room temperature, shielded from light. The precipitated silver chloride was filtered off and the filtrate cooled to -20 °C. A solution of hexamethylene diamine (0.012 g, 0.11 mmol) in DMF (5 mL) was added dropwise to the filtrate over 1 h and subsequently stirred for 2 days at room temp. The product was precipitated out of solution using Et₂O (400 mL), filtered and the filter cake washed with ice-cold Et₂O. Yield: 0.104 g (63%). ¹H NMR (600 MHz, D₂O) δ 3.24 – 3.19 (m, 2H), 2.85 – 2.81 (m, 2H), 2.58 – 2.49 (m, 12H), 1.59 – 1.50 (m, 16H), 1.27 (td, *J* = 10.2, 3.5 Hz, 16H). ¹⁹⁵Pt NMR (600 MHz, D₂O): δ = -2672 ppm. Elemental analysis calculated (%) for C₂₄H₈₀N₂₂O₁₈Pt₃: C 18.59, H 5.20, N 19.88; found: C 19.14, H 5.13, N 16.21. IR (ATR cm⁻¹): 3213, 3153, 2933, 2863, 2099, 1655, 1603, 1307, 1038, 826.

3.7.4. HPLC

High pressure liquid chromatography (HPLC) analyses were carried out according to previously reported methods by Farrell *et al.* with slight modifications.⁸ Analyses were carried out on a Shimadzu Prominence system with Nexera X2 photo-diode array detector module. A Bio-Rad Bio-Sil Sec 400-5, 300 mm x 7.8 mm was used with a gradient elution method solvent A: H₂O/ACN 60/40; solvent B: 95/5. Samples were prepared as 1 mg/mL solutions in HPLC grade H₂O. The injection volume was 50 µL and flow rate was kept constant at 1.5 mL/min for 60 min.

3.8. References

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Chapter IV

Development of a Thiazole Orange labelled Azide-Polynuclear Platinum(II) Complex Series

4. Results and Discussion

4.1. General introduction to the preparation of N₃-TO-PPCs

A library of covalently linked thiazole orange labelled N₃-PPCs was generated to facilitate fluorescent detection of these drugs in biological environments. Thiazole orange (TO) is an asymmetrical cyanine dye consisting of a benzothiazole moiety and quinoline moiety linked together by a methine bridge. TO is a weakly fluorescent fluorophore in solution due to intramolecular rotation of the two heterocyclic systems around the methine bridge.¹ This freely rotating and twisting system results in rapid non-radiative decay of the excited state. However, upon dsDNA or dsRNA intercalation, the two rotating aromatic systems become locked in place and rotation around the methine bridge is restricted which in turn results in a large fluorescent enhancement.² The intercalation of TO is stabilised by π - π stacking between the nucleobases of dsDNA or dsRNA. Due to this unique 'turn-on' characteristic, TO has been incorporated into systems as fluorescent probes with the aim of sensing or tracking targets in biological environments.³ Construction of this TO-labelled library uses similar synthetic strategies as was developed for the N₃-PPCs in Chapter 3, as well as previous work conducted by Farrell and co-workers.^{4, 5} A key advantage of developing N₃-TO-PPCs is the probe functionality of TO where it is anticipated that enhanced DNA binding and luminescence will occur upon intercalation.

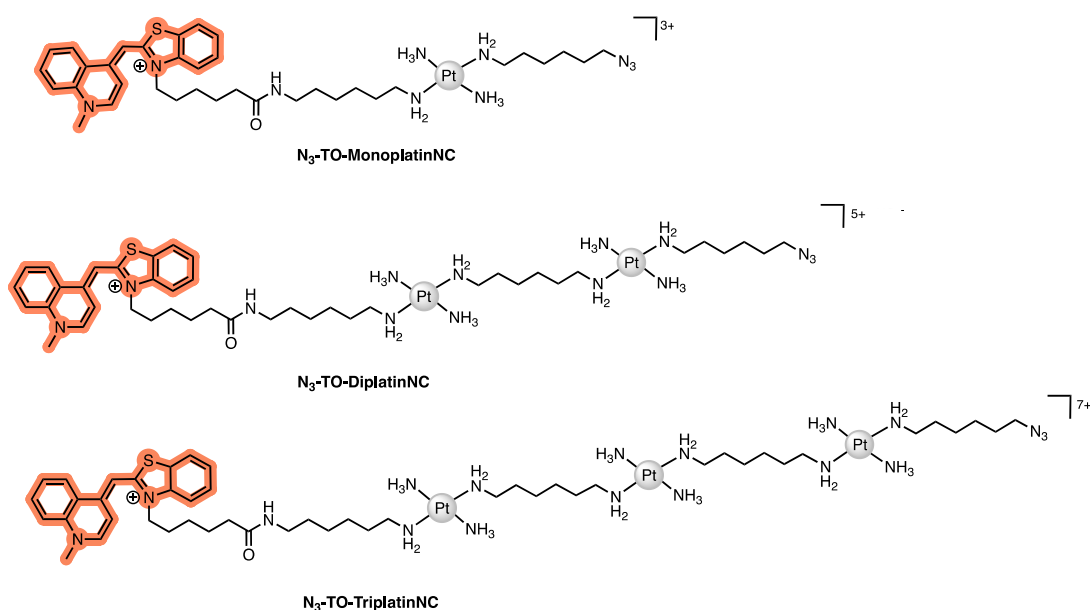


Figure 32. Structures of the novel N₃-TO-PPC series.

4.1.1. Synthetic method development of the N₃-TO-PPC library

The N₃-TO-PPC series consists of N₃-TO-MonoplatinNC, N₃-TO-DiplatinNC, and N₃-TO-TriplatinNC and features a terminal azide handle at one end and a thiazole orange fluorophore at the other. All platinum(II) centres are *trans*-symmetric and the platinum(II) centres are separated by intervening 1,6-hexane diamine linkers. The development of this novel complex series began with the design of a TO ligand that would be compatible with platinum(II) coordination chemistry. The TO fluorophore was synthesized according to procedures previously reported with slight modification.^{6, 7} The 3-(5-carboxypentyl)-2-methylbenzothiazol-3-ium bromide precursor was generated using a nucleophilic substitution reaction between 2-methylbenzylthiazole and 6-bromohexanoic acid under inert conditions (Figure 33A). 4-chloroquinoline was reacted with methyl trifluoromethanesulfonate to afford the second TO precursor 4-chloro-1-methylquinolin-1-ium trifluoromethanesulfonate (Figure 33B). Both precursors were then reacted together under inert conditions in the presence of triethylamine and subsequent salt metathesis was employed to produce the TO fluorophore as a hexafluorophosphate salt (Figure 33C).

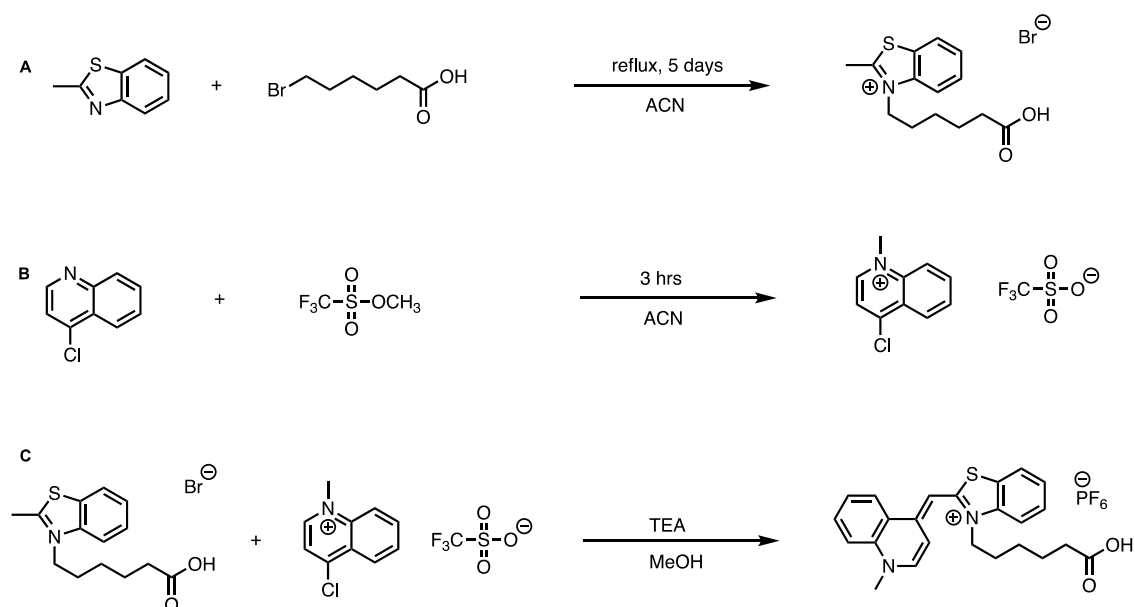


Figure 33A. Nucleophilic substitution reaction between 2-methylbenzylthiazole and 6-bromohexanoic acid to generate 3-(5-carboxypentyl)-2-methylbenzothiazol-3-ium bromide; B. Synthesis of 4-chloro-1-methylquinolin-1-ium trifluoromethanesulfonate from 4-chloroquinoline and methyl trifluoromethanesulfonate; C. Final step in the synthesis of thiazole orange.

N-Boc-1,6-hexane diamine was conjugated to the free hydroxyl group through an amide bond coupling reaction using *N,N,N',N'*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uromium (HBTU) to produce the BOC-protected TO ligand. BOC deprotection was achieved by reaction with concentrated HCl, followed by salt metathesis of $\text{Cl}^- \rightarrow \text{NO}_3^-$ using silver nitrate to afford the TO ligand as a nitrate salt that is compatible with platinum(II) coordination chemistry. The free terminal 'dangling' amine that is generated as a result of BOC-deprotection was identified as a coordination site to the platinum(II) centre.

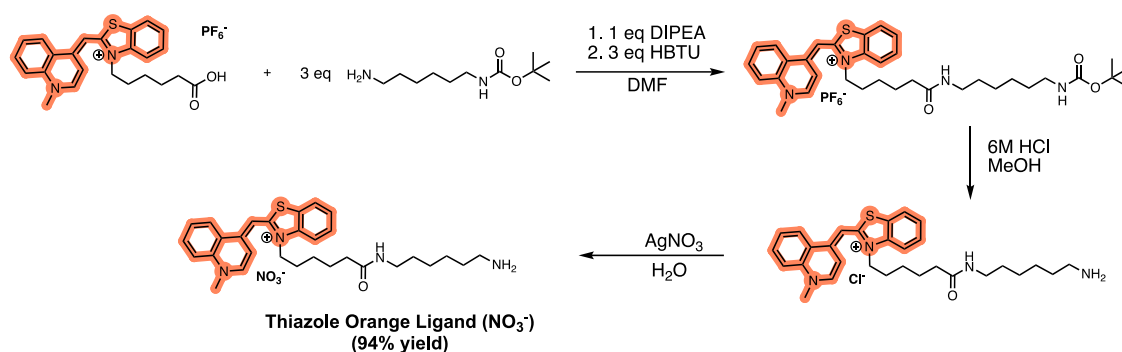


Figure 34. Reaction scheme for the synthesis of thiazole orange ligand for platinum(II) coordination.

The assembly of the N_3 -TO-PPCs follows a similar step-wise approach that was developed for the N_3 -PPCs. For N_3 -TO-MonoplatinNC, transplatin was first dissolved in DMF and double-activated using two equivalence of silver nitrate to precipitate labile chloride ligands from the platinum(II) centre, and the precipitated silver chloride was readily removed by filtration. A solution of one equivalent of 1-amino-6-azido-hexane in DMF was added dropwise to the reaction mixture and allowed stir for 2 days shielded from light. A colour change from colourless $\rightarrow$ orange/brown was observed. Subsequently, a solution of one equivalent of TO ligand in DMF was added dropwise to the reaction mixture and stirred for 2 days shielded from light. N_3 -TO-MonoplatinNC was then precipitated out of solution using diethyl ether (Et_2O) as a nitrate salt and isolated by filtration without need for further purification (Figure 35A).

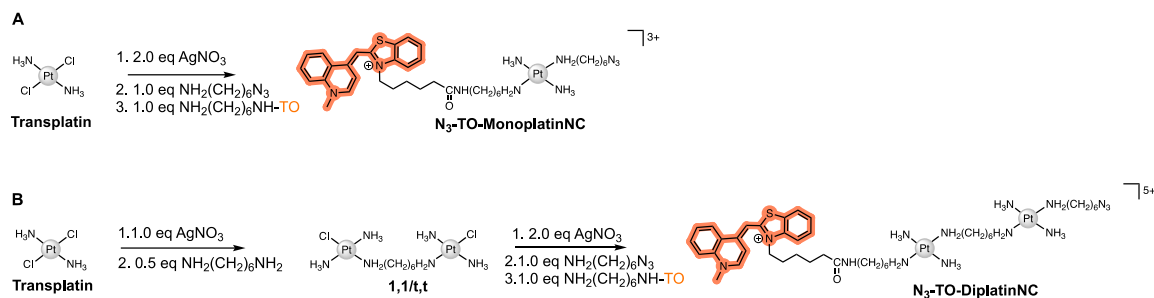


Figure 35A. Synthetic route of N₃-TO-MonoplatinNC; B. Synthetic route of N₃-TO-DiplatinNC.

The synthesis of N₃-TO-DiplatinNC utilises the aforementioned 1,1/t,t complex as a dinuclear intermediate. Thus, 1,1/t,t was prepared by activating one coordination site of transplatin using one equivalent of silver nitrate. After filtration of the precipitated silver chloride, 0.5 molar equivalent of the 1,6-hexane diamine linker in DMF was slowly titrated into the reaction mixture over 1 h and stirred overnight at room temperature shielded from light. Each terminal amine on the 1,6-hexane diamine linker should coordinate to two different $[\text{Pt}(\text{NH}_3)_2\text{Cl}]^+$ units and link them together. The 1,1/t,t complex was precipitated out of solution using diethyl ether (Et_2O) and dried under vacuum without further need for purification. The 1,1/t,t complex was then double-activated using two equivalents of silver nitrate. The precipitated silver chloride was removed by filtration and a solution of 1 equivalent of 1-amino-6-azido hexane in DMF was added dropwise to the reaction mixture over 1 hr and then subsequently allowed stir for 2 days at room temperature shielded from light. A solution of one equivalent of TO ligand in DMF was added dropwise to the reaction mixture for 1 h and allowed stir for 2 days. The reaction volume was reduced to ~5 mL and product was precipitated out of solution using diethyl ether. Product was collected by filtration and dried under vacuum without need for further purification (Figure 35B).

N₃-TO-TriplatinNC was prepared using the BBR3464 trinuclear platinum(II) complex with terminal labile chloride ligands. BBR3464 was prepared according to published methods with slight modification.⁸ A solution of MonoplatinNC in DMF was treated with 2 equivalents of *N,N*-diisopropylethylamine (DIPEA) and stirred for 10 mins at room temperature shielded from light. DIPEA is a non-nucleophilic base that is readily used to deprotonate hydroxyl groups and primary am(m)ines. Subsequently, a solution of 2 equivalents a mono-activated transplatin was added dropwise to the reaction

mixture over 2 h and stirred for 4 days at room temperature. BBR3464 was isolated by precipitation using Et₂O and dried under vacuum without need for further purification. BBR3464 was then redissolved in DMF and treated with 2 equivalents of silver nitrate. The precipitated silver chloride was removed by filtration. A solution of 1 equivalent of 1-amino-6-azido-hexane in DMF was added dropwise to the reaction mixture over 1 h and stirred for 2 days at room temperature shielded from light. A colour change of colourless → orange/brown was observed. This indicates coordination of the 1-amino-6-azido-hexane ligand to a platinum(II) centre. A solution of 1 equivalent of TO ligand in DMF was added dropwise to the reaction mixture over 1 hr and subsequently stirred for 2 days at room temperature shielded from light. N₃-TO-TriplatinNC was isolated from the reaction mixture by precipitation using Et₂O, collected by filtration and dried under vacuum without need for further purification (Figure 36).

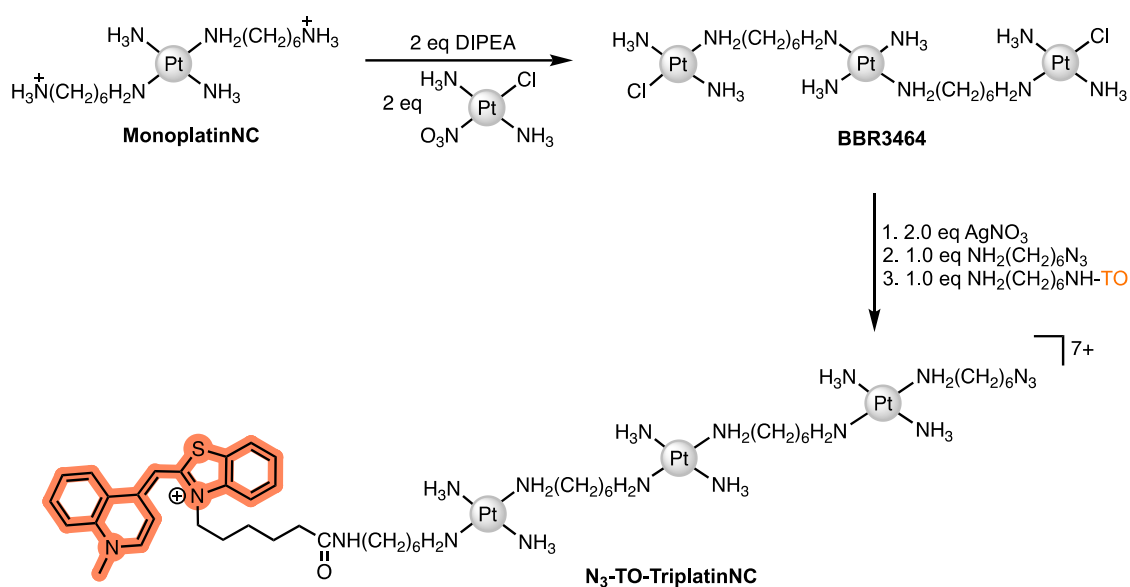


Figure 36. Synthetic route for the isolation of N₃-TO-TriplatinNC.

4.2. Characterisation via nuclear magnetic resonance spectroscopy

Characterisation of the N₃-TO-PPCs was performed in a similar manner to the N₃-PPCs. Concentrated samples of the thiazole orange ligand were dissolved in deuterated acetonitrile and thiazole orange-labelled azide-polynuclear platinum(II) complexes were dissolved in deuterium oxide. All NMR experiments were performed on a Bruker Avance Ultrashield 600 MHz spectrometer and spectra were processed using MestReNova Mestrelab Research chemistry software.

4.2.1. ¹H NMR spectroscopy

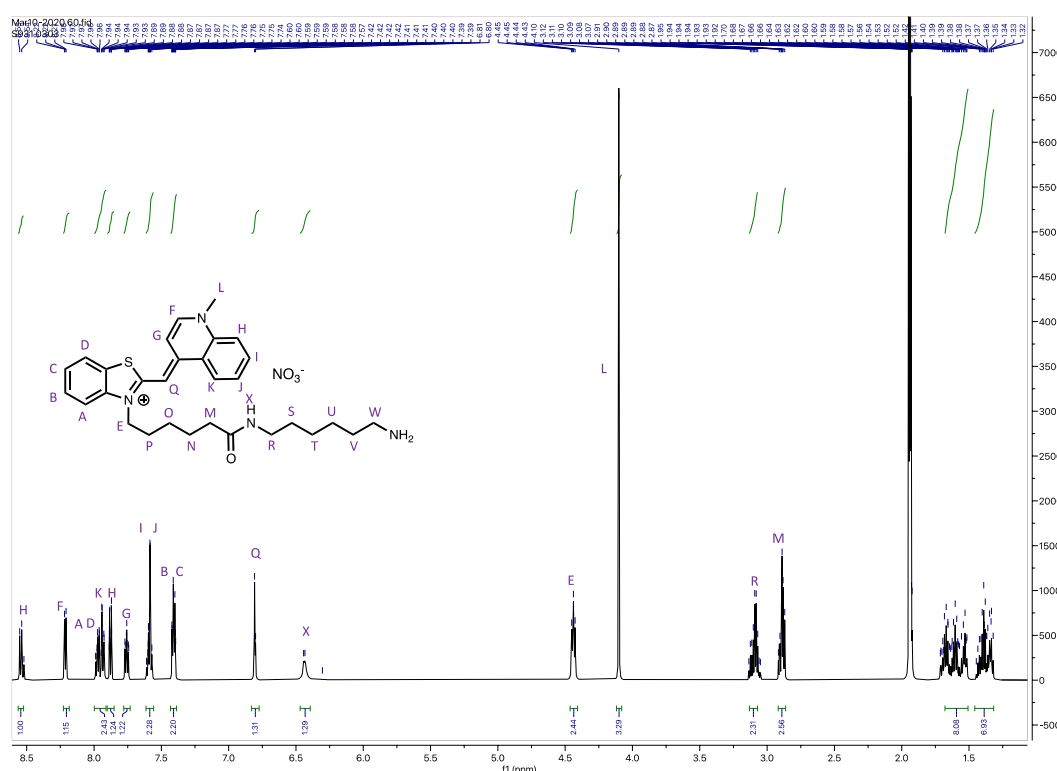


Figure 37. ¹H NMR spectrum of the thiazole ligand (NO₃⁻) in ACN-d₃. Spectrum obtained on a Bruker Avance Ultrashield 600 MHz spectrometer.

Figure 37 shows the ¹H NMR spectrum obtained for the thiazole orange ligand as a nitrate salt. All 14 shielded aliphatic protons can be seen upfield in the aliphatic region at 1.22 – 1.73 ppm. Signals appearing at approximately 2.80 ppm and 3.17 ppm correspond to the protons labelled M and R respectively. These protons, while still aliphatic, are significantly deshielded due to their proximities to the amide bond. The singlet appearing at 4.10 ppm which integrates for 3 protons corresponds to the methyl group protons labelled L. These protons are deshielded as they are attached directly to the quinoline moiety and appear as a

singlet as they are not coupling with any neighbouring protons. The triplet appearing at ~4.44 ppm corresponds to the protons labelled E. These protons appear as a triplet as they are directly next to a carbon with 2 protons attached. E protons experience intense anisotropic deshielding as they are directly conjugated to the charged nitrogen on the thiazole moiety. The sharp singlet appearing at ~6.82 ppm corresponds to the alkenyl proton, Q, and the small broad singlet labelled X at 6.45 ppm corresponds to the proton of the secondary amine. All 10 aromatic protons corresponding the fluorescent thiazole orange moiety of the ligand are present in the aromatic region at ~7.33 – 8.53 ppm. The integration and coupling pattern of the thiazole orange moiety was validated with literature values previously reported. ⁶

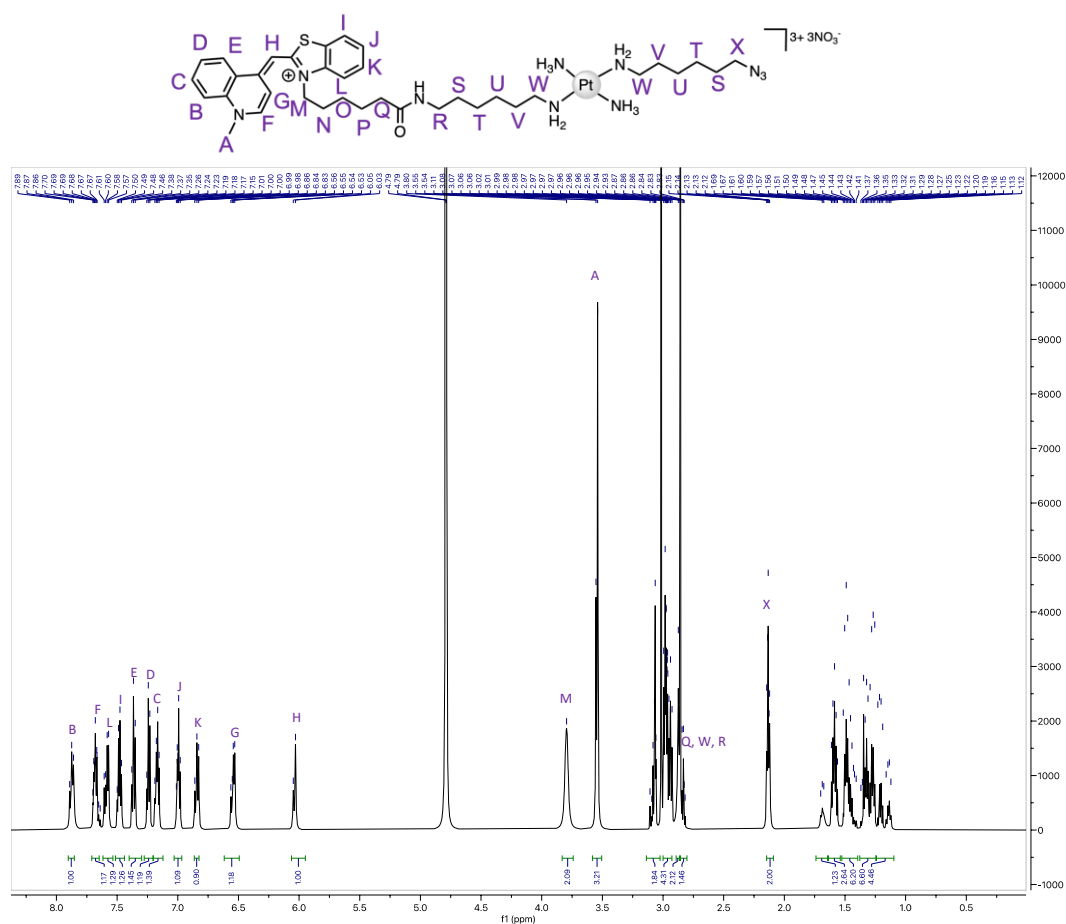


Figure 38. ^1H NMR spectrum of N_3 -TO-MonoplatinNC obtained in D_2O . The spectrum was obtained on a Bruker Avance Ultrashield 600 MHz spectrometer. The spectrum was processed using MestReNova.

The proton NMR spectra for the N₃-TO-PPCs follows a similar pattern to what was previously observed for the N₃-PPCs. ¹H NMR spectra of the N₃-PPC complexes are nearly identical, as they all share equivalent proton environments with the only significant difference in their spectra being the integrals of the peaks. The significant difference between these two complex series is that the N₃-TO-PPCs will have 10 protons in the aromatic region corresponding to the fluorescent thiazole orange moiety. Figure 38 shows the ¹H NMR spectrum for N₃-TO-MonoplatinNC in deuterium oxide. Characteristic peaks of the aromatic thiazole orange moiety appear ~6.6 – 7.9 ppm. Protons labelled X correspond to the protons next to the azide moiety. Q and R protons are corresponding to the CH₂ groups either side of the amide group and appear at ~3 ppm and overlap with the protons that correspond to the CH₂ groups that are next to the primary amines which are coordinated directly to the platinum(II) centre.

Figure 39 displays the proton NMR spectrum for N₃-TO-DiplatinNC. When compared to the proton NMR spectrum of N₃-TO-MonoplatinNC, it is clear that the only significant difference in the spectra corresponds to the increased number of aliphatic protons due to the increased number of 1,6-hexane diamine linkers in the complex. Protons labelled W appear just below 3 ppm correspond to the aliphatic protons of the CH₂ directly beside the amines which are coordinated to the platinum(II) centres. A significant anisotropic shift downfield is observed due to their proximity to the highly electron dense platinum(II) centres.

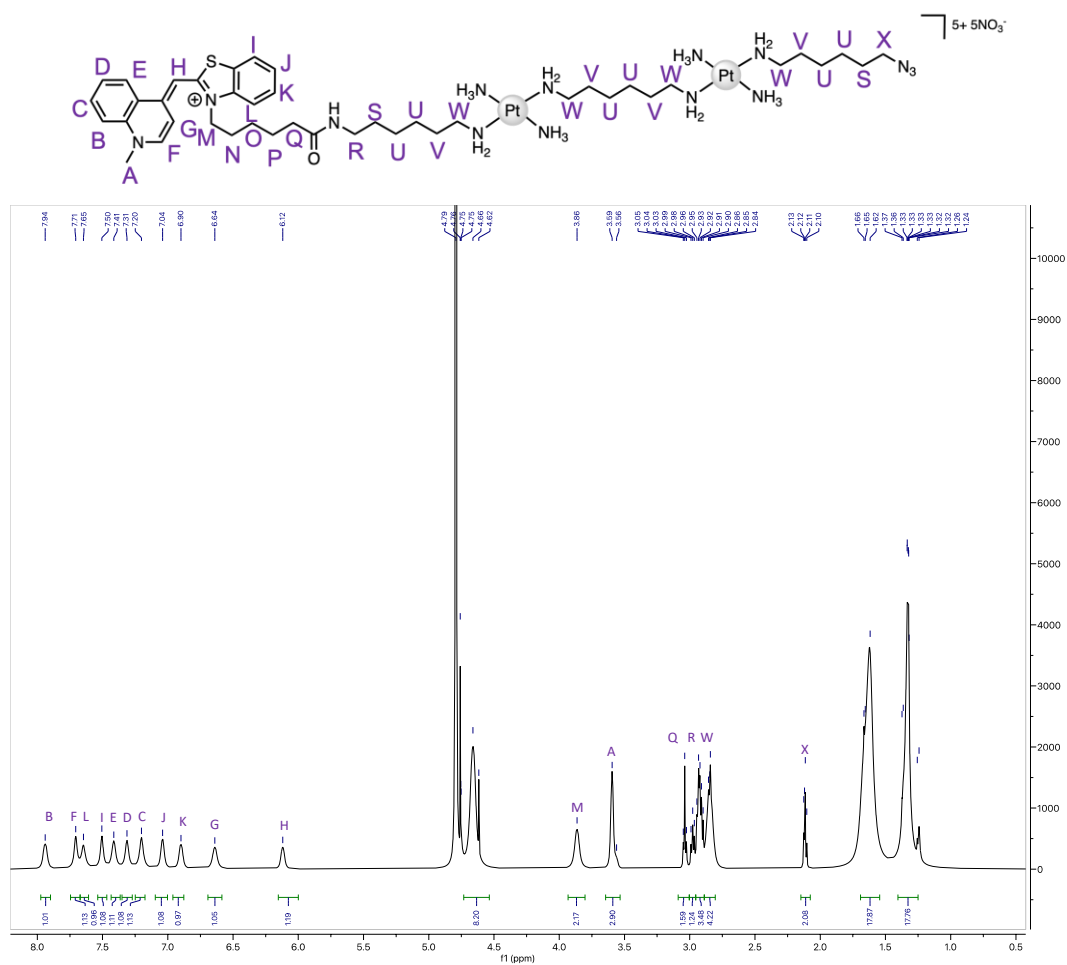


Figure 39. ^1H NMR spectrum of N_3 -TO-DiplatinNC obtained in D_2O . Spectrum was obtained on a Bruker Avance Ultrashield 600 MHz spectrometer. Solvent peaks of DMF at 2.7 ppm, 2.9 ppm, and 8.1 ppm were suppressed for clarity.

Figure 40 shows the proton NMR spectrum of N₃-TO-TriplatinNC. This spectrum appears almost identical to both the mononuclear and dinuclear congeners with the only difference being the integration numbers of the aliphatic protons as the number of 1,6-hexane diamine linkers increases. Protons labelled W at ~ 2.6 ppm which integrates for 12 protons corresponds to the protons of the CH₂ groups that are directly next to the amines coordinated to the platinum(II) centres. As was observed for both N₃-TO-MonoplatinNC and N₃-TO-DiplatinNC, a significant downfield anisotropic shift is observed for these protons due to their close proximity to the electron-dense platinum(II) centres. 10 aromatic protons correspond to the 10 protons in the aromatic systems of the thiazole orange moiety. The alkenyl proton that connects the two aromatic systems in thiazole orange labelled H, appears at ~ 6.4 ppm. Characteristic protons of the CH₃ group labelled A integrates for 3 protons and appears at ~3.75 ppm. These protons experience a significant anisotropic deshielding effect due to its direct conjugation to the nitrogen of the quinoline moiety of thiazole orange.

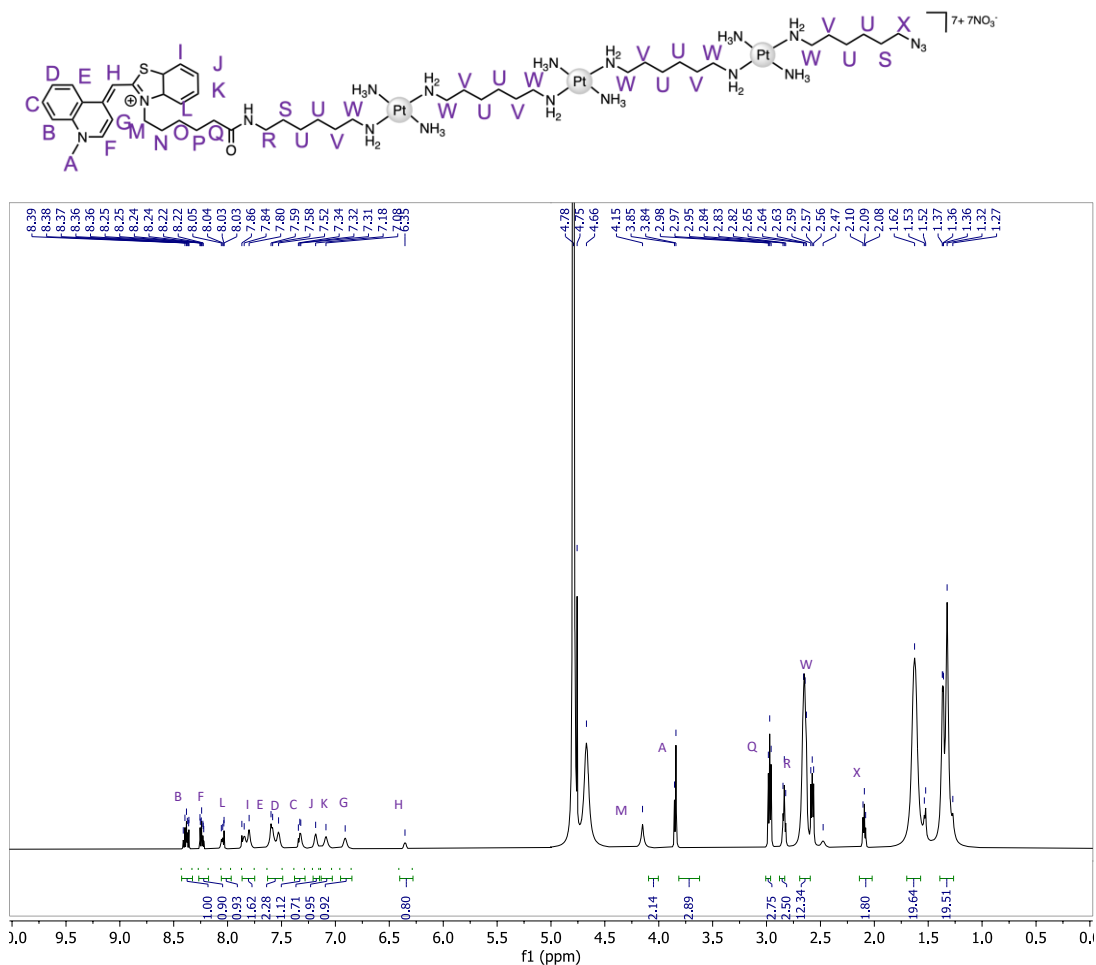


Figure 40. ^1H NMR spectrum of N_3 -TO-TriplatinNC obtained in D_2O . Spectrum was obtained on a Bruker Avance Ultrashield 600 MHz spectrometer.

4.2.2. ^{195}Pt NMR spectroscopy

The ^{195}Pt NMR spectra obtained for the N_3 -TO-PPC series follows a similar trend to what was observed for the N_3 -PPC series. All platinum(II) nuclei in the complex series inhabit the same environment and a single peak is observed for each complex. While the number of platinum(II) nuclei in the complex series increases, the environment of the platinum(II) nuclei does not change, hence a single signal is observed for each complex. The introduction of the thiazole orange moiety to the complex series does not impact the chemical shift of the signal observed in the ^{195}Pt NMR spectra as it is too far away to induce an anisotropic shift. The chemical shift for the platinum(II) environment for N_3 -TO-MonoplatinNC, N_3 -TO-DiplatinNC, and N_3 -TO-TriplatinNC is -2662 ppm, -2659 ppm, and -2664 ppm

respectively (Table 8.) This is in line with what is expected for a $[\text{Pt}(\text{NX})_4]^{2+}$ coordination sphere.⁹

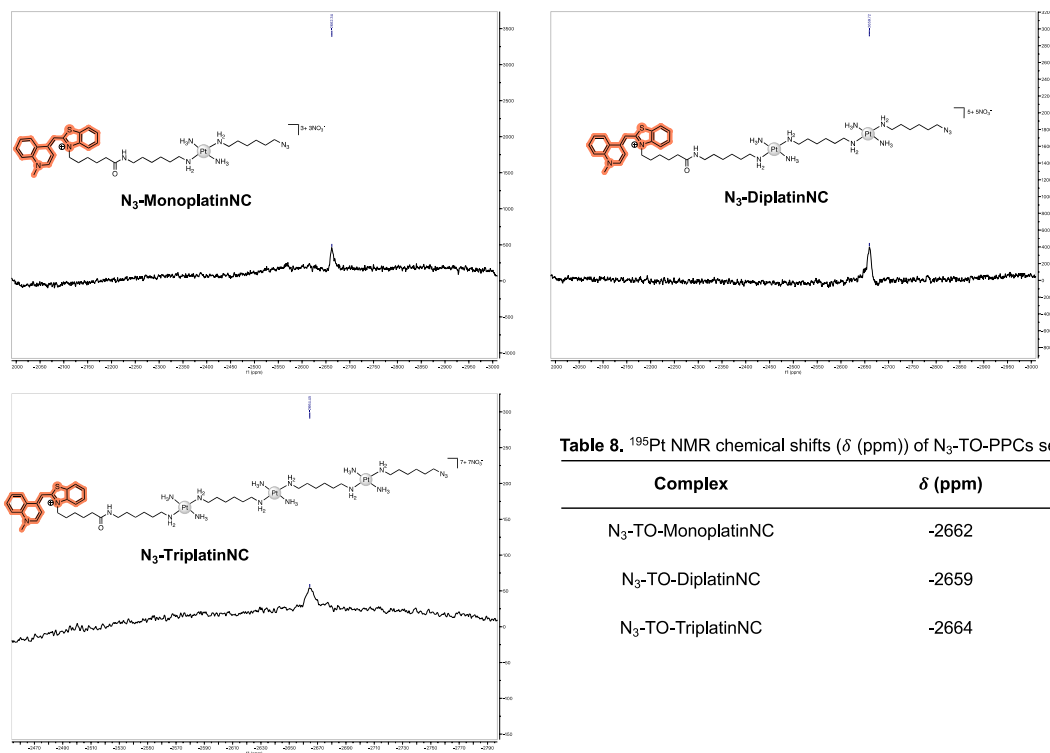


Table 8. ^{195}Pt NMR chemical shifts (δ (ppm)) of N₃-TO-PPCs series.

Complex	δ (ppm)
N ₃ -TO-MonoplatinNC	-2662
N ₃ -TO-DiplatinNC	-2659
N ₃ -TO-TriplatinNC	-2664

Figure 41. ^{195}Pt NMR spectra of N₃-TO-PPC series. Spectra were obtained on a Bruker Avance Ultrashield 600 MHz spectrometer. ^{195}Pt NMR spectra were processed using MestReNova Mestrelab Research chemistry software solutions.

4.3. Characterisation by fourier transform infra-red spectroscopy (FT-IR)

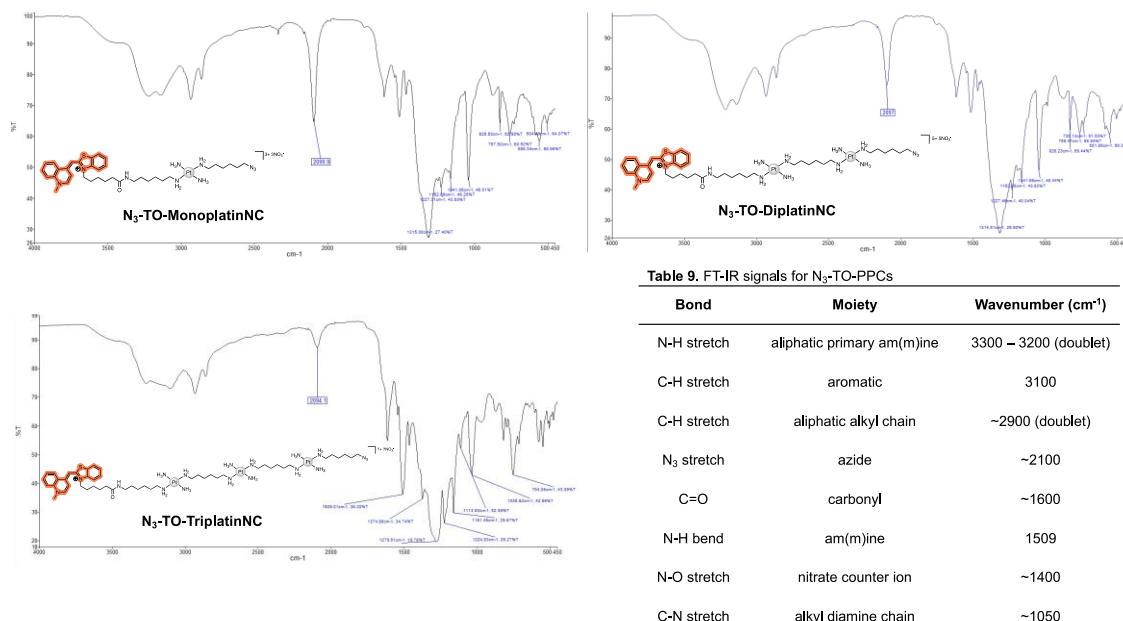


Figure 42. FT-IR spectrum and characteristic peaks of N₃-TO-PPC series

The infra-red spectra for the N₃-TO-PPCs are shown in Figure 42 and vibrational peaks corresponding to the various functional groups are displayed on Table 9. In all three spectra above the distinctive azide peak is observed at ~2100 cm⁻¹. A similar trend is observed for the N₃-TO-PPCs as was observed for the N₃-PPCs—the intensity of the azide peak diminishes as the overall size and molecular weight of the complex increases i.e. N₃-TO-MonoplatinNC has the most intense azide peak; N₃-TO-DiplatinNC has a diminished azide peak relative to N₃-TO-MonoplatinNC; and N₃-TO-TriplatinNC has the least intense azide peak in the complex series. The curtailing of the azide peak is a symptom of the increasing molecular weight as more platinum(II) nuclei are added to the complex and is direct evidence of the increasing number of platinum(II) nuclei in the complex series. Another key feature of the IR spectra of the N₃-TO-PPCs is the complex fingerprint region observed at 400 cm⁻¹–1500 cm⁻¹. This is a feature of molecules with aromatic systems. Conversely, the IR spectra of PPCs and N₃-PPCs, which only containing aliphatic alkyl linkers, contains a less complex the fingerprint region.

4.4. Characterisation by elemental analysis

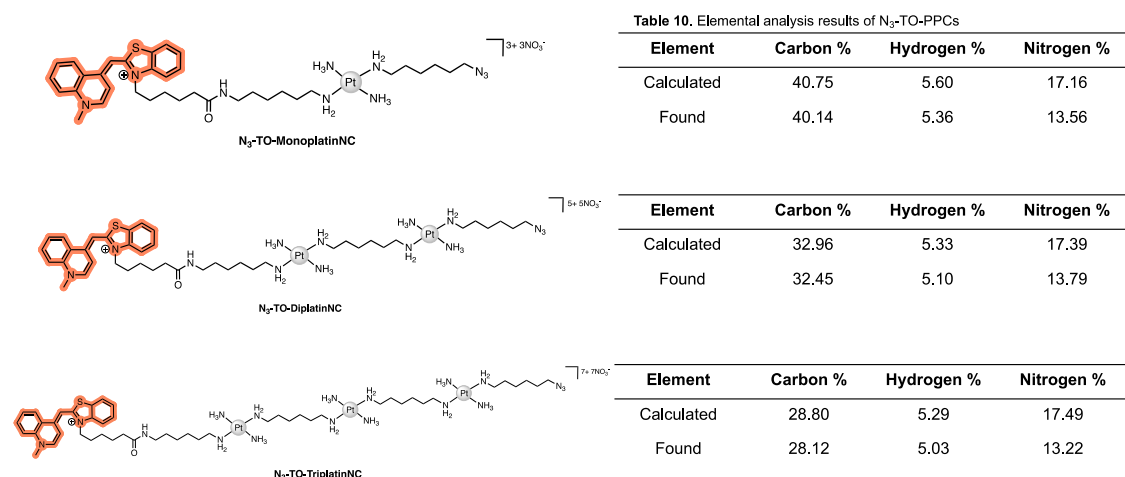


Figure 43. Elemental analysis results of N_3 -TO-PPCs.

Elemental analysis of the N_3 -TO-PPCs was performed and results are displayed in Figure 43. The percentage of carbon, hydrogen, and nitrogen was determined for N_3 -TO-MonoplatinNC, N_3 -TO-DiplatinNC, and N_3 -TO-TriplatinNC and compared with the theoretically calculated results. For all three complexes, the percentage carbon and hydrogen found during analysis is in agreement with the calculated theoretical results. However, the percentage nitrogen found during analysis is off from the calculated theoretical results by 3-4%. This discrepancy may be a symptom of the intrinsic instability of the azide moiety itself. It is long since known that azides have a tendency to be quite unstable and can be incapable of surviving harsh conditions. The elemental analysis technique used here requires the combustion of a 5 mg sample of each drug tested. The combustion of carbon results in the formation of carbon dioxide which directly detected by the elemental analyser, the burning of hydrogen results in the generation of water which is detected by the elemental analyser, and combustion of nitrogen results in the formation of various nitric oxides.¹⁰ The discrepancy in the percentage nitrogen found for the N_3 -TO-PPCs is similar to what was observed for the N_3 -PPCs (Figure 31). When observing elemental analysis results of the PPCs, N_3 -PPCs, and N_3 -TO-PPCs altogether it is clear that the azide moiety is the reason for the difference in percentage nitrogen of the N_3 -TO-PPCs.

4.5. HPLC

HPLC analyses were carried out according to previously established methods by Farrell and co-workers and was previously used to measure the purity of the N₃-PPCs in Chapter 3.¹¹ Peak purity analysis revealed that complexes were synthesised to a high degree of purity; N₃-MonoplatinNC, N₃-DiplatinNC and N₃-TriplatinNC peak purity of 96%, 97% and 95% respectively.

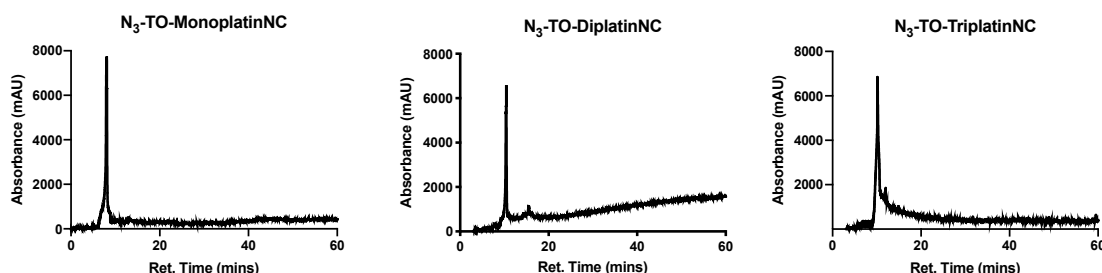


Figure 44. HPLC chromatograms of N₃-PPC series with detection at 254 nm. HPLC was performed on a Shimadzu Prominence system with Nexera X2 photo-diode array detector module. HPLC chromatograms were processed using Prism 8 GraphPad software and percentage peak purity was calculated as the percentage area under the curve.

4.6. Conclusion

This work reports the synthesis and characterisation of a novel series of thiazole orange-labelled N₃-polynuclear platinum(II) complexes with intrinsic fluorescent properties, meeting the aims of the second objective as outlined in **Chapter 2**. These complexes are linked by an alkyl diamine chain, vary in nuclearity, contain a single azido group at one of their terminus, and also contain a thiazole orange fluorescent dye at the other terminus. Comprehensive characterisation was conducted to verify that each complex was successfully generated to a high degree of purity using a variety of analytical techniques including NMR spectroscopy (¹H, ¹⁹⁵Pt), FT-IR spectroscopy, elemental analysis, and HPLC. Conjugation of thiazole orange to the azido-functionalised polynuclear platinum(II) complex series should enhance the inherent DNA binding properties of the complex series, as well as facilitate in-situ fluorescence detection of the complex series in cells. Furthermore, the thiazole orange “light-switch” moiety will act as a direct “turn-on” reporter molecule when the complex binds to its DNA target.

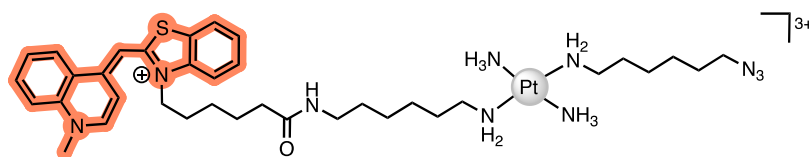
4.7. Experimental

4.7.1. Synthesis and characterisation of the TO-ligand

Thiazole orange (TO) was synthesized as a PF₆ salt by previously reported methods with slight modifications.⁶ TO (0.500 g, 0.91 mmol) was dissolved in DMF (12 mL), treated with DIPEA (316 μ L, 1.82 mmol) and stirred for 5 min at room temperature. Subsequently, HBTU (1.033 g, 2.73 mmol) and *N*-BOC-hexane diamine (0.393 g, 1.82 mmol) was added to the reaction mixture and stirred at room temperature for 5 days. The reaction volume was reduced to ~5 mL before diluting with H₂O (100 mL). The BOC-protected product was isolated by filtration. The crude red product was redissolved in MeOH (15 mL) and treated with a solution of 6M HCl in EtOH (12 mL) and stirred vigorously for 48 h shielded from light. The reaction mixture was basified to pH 9 using NaOH before extracting with DCM (5 $\times$ 75 mL). The organics were combined, dried using magnesium sulfate and solvent was removed under vacuum. The product was redissolved in acetone and treated with silver nitrate. The precipitated silver chloride was removed by filtration and solvent was removed in vacuo. The final product was dried under N₂. Yield: 0.480 g (94%). ¹H NMR (600 MHz, ACN-d₃): δ = 8.54 ppm (d, *J* = 8.7 Hz, 1H), 8.23 – 8.18 ppm (m, 1H), 8.00 – 7.91 ppm (m, 2H), 7.88 ppm (dq, *J* = 7.9, 0.9 Hz, 1H), 7.76 ppm (ddt, *J* = 8.4, 6.8, 1.7 Hz, 1H), 7.61 – 7.56 ppm (m, 2H), 7.43 – 7.39 ppm (m, 2H), 6.80 ppm (d, *J* = 4.1 Hz, 1H), 6.44 ppm (d, *J* = 6.0 Hz, 1H), 4.44 ppm (t, *J* = 7.7 Hz, 2H), 4.10 ppm (s, 3H), 3.13 – 3.05 ppm (m, 3H), 2.92 – 2.86 ppm (m, 3H), 1.73 – 1.64 ppm (m, 3H), 1.64 – 1.57 ppm (m, 3H), 1.53 ppm (p, *J* = 7.8 Hz, 3H), 1.44 – 1.31 ppm (m, 6H), 1.30 – 1.21 ppm (m, 6H). MS (ESI): *m/z* calculated for C₃₀H₃₉N₄OS + NO₃⁻: 565.27 [M + NO₃]⁺; found: 565.2717. IR (ATR cm⁻¹): 3288, 2932, 2872, 1505, 1464, 1374, 1311, 1225, 1161, 1114.

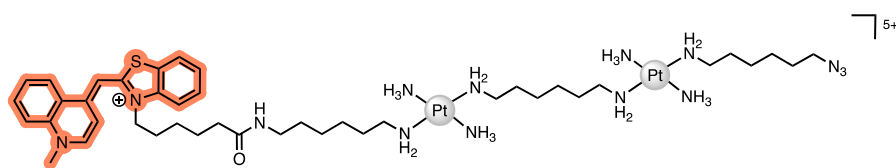
4.7.2. Synthesis and characterisation of N₃-TO-PPCs

N₃-TO-MonoplatinNC



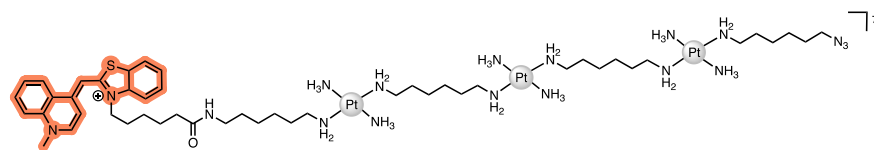
Transplatin (0.100 g, 0.33 mmol) was dissolved in DMF (10 mL) and added with silver nitrate (0.113 g, 0.66 mmol) in one portion. The reaction mixture was stirred for 24 h, at ambient temperature, shielded from light. The white precipitated silver chloride was removed by filtration and the clear pale yellow filtrate was cooled to -20 °C. A solution of 1-amino-6-azido-hexane (0.047 g, 0.33 mmol) in DMF (8 mL) was added dropwise to the cooled filtrate over 3 h and subsequently left stir for 2 days at room temperature. The reaction mixture was then cooled to -20° C. A solution of thiazole orange ligand (0.188 g, 0.33 mmol) in DMF (20 mL) added dropwise to the cooled reaction mixture over 3 h and the solution was left stir for 4 days. The reaction volume was reduced to ~10 mL and diluted with Et₂O (400 mL) to precipitate out the product. The red solid product was collected by filtration and dried under vacuum. Yield: 0.068 g (20%). ¹H NMR (600 MHz, D₂O) δ 7.88 (t, *J* = 9.6 Hz, 1H), 7.71 – 7.65 (m, 1H), 7.59 (dd, *J* = 14.7, 7.0 Hz, 1H), 7.48 (q, *J* = 7.0 Hz, 1H), 7.36 (t, *J* = 8.3 Hz, 1H), 7.24 (t, *J* = 7.4 Hz, 1H), 7.17 (q, *J* = 7.1 Hz, 1H), 7.03 – 6.97 (m, 1H), 6.84 (t, *J* = 9.7 Hz, 1H), 6.55 (dd, *J* = 12.2, 6.9 Hz, 1H), 6.04 (d, *J* = 10.9 Hz, 1H), 3.80 (s, 2H), 3.55 (d, *J* = 8.4 Hz, 3H), 3.14 – 3.02 (m, 2H), 3.00 – 2.93 (m, 4H), 2.87 (s, 2H), 2.86 – 2.80 (m, 1H), 2.15 – 2.09 (m, 2H), 1.74 – 1.64 (m, 1H), 1.59 (p, *J* = 7.6 Hz, 3H), 1.53 – 1.39 (m, 6H), 1.38 – 1.25 (m, 7H), 1.24 – 1.10 (m, 4H). ¹⁹⁵Pt NMR (600 MHz, D₂O): δ = -2662. Elemental analysis calculated (%) for C₃₆H₅₉N₁₃O₁₀SPt: C 40.75, H 5.60, N 17.16; found: C 40.14, H 5.36. N 13.56. IR (ATR cm⁻¹): 3385, 3263, 2977, 2849, 2099, 1315, 1227, 1162, 1041, 826.

N₃-TO-DiplatinNC



Transplatin (0.150 g, 0.49 mmol) was dissolved in DMF (10 mL) and added with silver nitrate (0.083 g, 0.49 mmol) and stirred for 18 h shielded from light. The white precipitated silver chloride was filtered off and filtrate was cooled to -20 °C. A solution of 1,6-hexane diamine (0.029 g, 0.25 mmol) in DMF (5 mL) was added dropwise to the cooled filtrate over 3 hrs and subsequently stirred for 4 days at ambient temperature. The reaction mixture was diluted with Et₂O (400 mL) and the precipitate was isolated by filtration and washed with ice-cold Et₂O. The 1,1/t,t product (0.110 g, 0.14 mmol) was dissolved in DMF (10 mL) and added with silver nitrate (0.049 g, 0.29 mmol) in one portion and stirred at room temperature for 48 h protected from light. The white silver chloride precipitate was removed from the solution by filtration and filtrate was cooled to -20 °C. A solution of 1-amino-6-azido-hexane (0.020 g, 0.14 mmol) in DMF (5 mL) was added dropwise to the cooled filtrate over 3 h. The reaction mixture was allowed return to room temperature and stirred for 2 days. The reaction mixture was cooled to -20 °C and a solution of thiazole orange ligand (0.080 g, 0.14 mmol) in DMF (5 mL) was added dropwise to the cooled reaction mixture over 3 h and then stirred for 2 days at room temperature. Reaction volume was reduced under vacuum to ~5 mL before diluting with Et₂O (400 mL). The precipitated product was collected by filtration, washed with ice-cold Et₂O and dried under vacuum. Yield: 0.048 g (7%). ¹H NMR (600 MHz, D₂O) δ 7.94 (s, 1H), 7.71 (s, 1H), 7.65 (s, 1H), 7.50 (s, 1H), 7.41 (s, 1H), 7.31 (s, 1H), 7.20 (s, 1H), 7.04 (s, 1H), 6.90 (s, 1H), 6.64 (s, 1H), 6.12 (s, 1H), 4.98 (s, 1H), 4.73 – 4.54 (m, 8H), 4.13 (s, 1H), 3.86 (s, 2H), 3.59 (s, 3H), 3.09 – 3.00 (m, 2H), 2.98 (t, *J* = 7.7 Hz, 1H), 2.92 (dt, *J* = 14.4, 7.7 Hz, 3H), 2.89 – 2.80 (m, 4H), 2.15 – 2.08 (m, 2H), 1.69 – 1.54 (m, 18H), 1.40 – 1.25 (m, 18H). ¹⁹⁵Pt NMR (600 MHz, D₂O): δ = -2659. Elemental analysis calculated (%) for C₄₂H₈₁N₁₉O₁₆SPt₂: C 32.96, H 5.33, N 17.39; found: C 32.45, H 5.10, N 13.79. IR (ATR cm⁻¹): 3258, 3109, 2945, 2889, 2097, 1314, 1227, 1162, 1041, 826.

N₃-TO-TriplatinNC



Transplatin (0.088 g, 0.29 mmol) was dissolved in DMF (8 mL) and added with silver nitrate (0.050 g, 0.29 mmol) was added in one portion and stirred at ambient temperature for 24 h shielded from light. The white precipitated silver chloride was removed from the reaction mixture by filtration and the filtrate was cooled to -20 °C. A separate solution of monoplatinNC nitrate (0.100 g, 0.14 mmol) and DIPEA (0.036 g, 0.28 mmol) in MeOH (10 mL) was stirred for 30 min at room temperature. The methanolic solution was added to the cooled filtrate over 3 h and subsequently allowed stir for 5 days at ambient temperature shielded from light. The reaction volume was reduced to ~5 mL and diluted with Et₂O (400 mL). The precipitated BBR3464 product was isolated by filtration, washed with ice-cold Et₂O and dried under vacuum. BBR3464 (0.060 g, 0.05 mmol) was dissolved in DMF (10 mL) and added to silver nitrate (0.017 g, 0.10 mmol) and stirred for 48 hrs at room temperature shielded from light. The precipitated silver chloride was removed from the reaction mixture by filtration and the filtrate was cooled to -20 °C. A solution of 1-amino-6-azido hexane (0.007 g, 0.05 mmol) in DMF (5 mL) was added dropwise to the cooled filtrate over 3 h and subsequently allowed stir for 2 days at room temperature. The reaction mixture was cooled to -20 °C and a solution of thiazole orange ligand (0.027 g, 0.05 mmol) was added dropwise to the reaction mixture over 3 h and subsequently allowed stir for 2 days at room temperature shielded from light. The reaction volume was reduced under vacuum to ~5 mL before diluting with Et₂O (400 mL). The precipitated product was collect by filtration, washed with ice-cold Et₂O and dried under vacuum. Yield: 0.021 g (4%). ¹H NMR (600 MHz, D₂O) δ 8.18 – 8.09 (m, 1H), 8.01 – 7.91 (m, 1H), 7.86 (dd, *J* = 14.6, 7.2 Hz, 1H), 7.82 – 7.71 (m, 2H), 7.60 – 7.49 (m, 2H), 7.29 (t, *J* = 7.3 Hz, 1H), 7.20 – 7.05 (m, 2H), 6.39 (d, *J* = 8.5 Hz, 1H), 4.06 (s, 2H), 3.76 (d, *J* = 8.2 Hz, 2H), 2.88 (t, *J* = 7.7 Hz, 2H), 2.75 (t, *J* = 7.6 Hz, 2H), 2.63 – 2.44 (m, 11H), 2.01 (t, *J* = 7.4 Hz, 2H), 1.73 – 1.38 (m, 18H), 1.34 – 0.99 (m, 19H). ¹⁹⁵Pt NMR (600 MHz, D₂O): δ = -2664. Elemental analysis calculated (%) for C₄₈H₁₀₅N₂₅O₂₂SPt₃: C 28.80, H 5.29,

N 17.49; found: C 28.12, H 5.03, N 13.22. IR (ATR cm^{-1}): 3301, 3121, 2989, 2789, 2094, 1509, 1374, 1279, 1224, 1151.

4.8. References

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Chapter V

Electrophoretic Studies of the N₃-PPCs and N₃-TO-PPCs with Nucleic Acids

5. Results and Discussion

5.1. General introduction to gel electrophoresis

Gel electrophoresis is an important analytical technique that is commonly used to study and purify nucleic acid samples. It involves the migration of DNA through a porous medium, typically agarose or polyacrylamide. The physical movement of negatively charged DNA samples through the porous medium is pushed by an external electric current and thus the DNA samples migrate from the cathode to the anode. The rate at which the DNA samples migrate and separate in the gel is dependent on DNA size, conformation, and length—i.e. extended DNA fragments with a large mass experience a greater amount of resistance in the porous medium than tightly compact large DNA fragments with a small mass-to-charge ratio. Supercoiled DNA migrates fastest through a gel because it is densely packed and experiences the least resistance. When a single strand break occurs on supercoiled DNA and forms open circular DNA, the topology of the DNA is such that it becomes more loosely packed and experiences the most amount of resistance of supercoiled (SC), open circular (OC), and linear (L) DNA (Figure 45). External parameters such as voltage, percentage agarose/polyacrylamide, run time, and gel length can be varied to optimize the resolution of the gel image. Frequently, plasmid DNA is used for electrophoresis. Since plasmid DNA has been extracted from bacterial cells it is usually isolated in a supercoiled state. Due to the densely packed state of supercoiled DNA, with a low mass-to-charge ratio, supercoiled DNA can migrate through the agarose gel with relative ease and experiences minimal resistance in the porous medium.

1, 2

Covalent and non-covalent metallodrug-DNA interactions can be monitored and visualised using the electrophoretic mobility shift assay. The nature of the activity of the metallodrug dictates the pattern that is visualised on the gel. For example, if a drug exhibits nuclease activity and has the ability to induce single-strand and/or double-strand breaks on supercoiled DNA, a series of bands is observed corresponding to the three types of DNA; linear, open-circular, and supercoiled (Figure 45).³

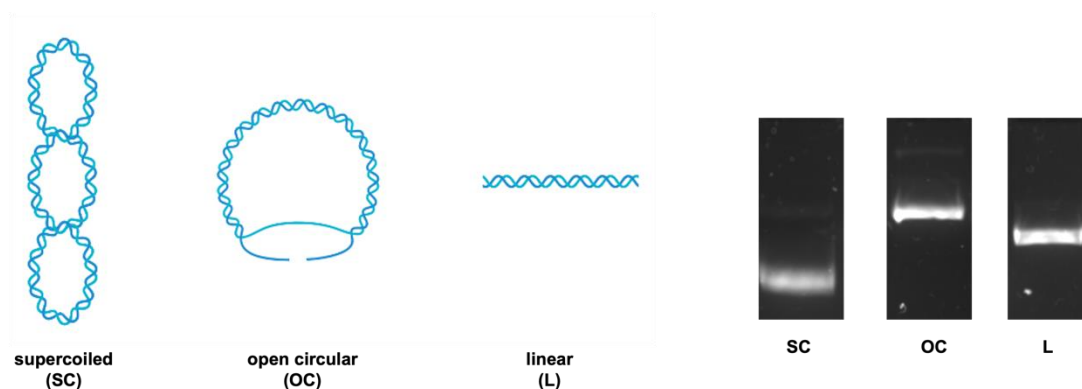


Figure 45. Diagram of supercoiled (SC), open circular (OC), and linear (L) DNA and agarose gel depicting the difference in migration of the three different forms of DNA.

In contrast, if a drug has an alternative DNA-damage mechanism, such as condensation or aggregation, a concentration-dependent disappearance of the DNA band is observed. The PPCs, N₃-PPCs, and N₃-TO-PPCs, all exhibit condensation activity with the exception of MonoplatinNC, therefore the concentration-dependent disappearance of the plasmid DNA band on the agarose gel was investigated.

5.1.1. DNA Condensation assay

A condensation assay was employed to determine the concentration at which complete pUC19 plasmid condensation was achieved by the PPCs, N₃-PPCs, and N₃-TO-PPCs. All complexes were incubated with 400 ng pUC19 for 24 hrs at 37 °C. Samples were treated with 4 µL of 6x loading dye before loaded onto a 1.2% agarose gel. The agarose gel was stained with SYBR safe and electrophoresis was performed at 70 v for 60 min. Results from this study revealed that MonoplatinNC displays no condensation activity up to 200 µM, N₃-MonoplatinNC demonstrates complete DNA condensation at 50 µM, and N₃-TO-MonoplatinNC achieves total DNA condensation at 80 µM (Appendix Figure B-9). The condensation activity of the series is dramatically enhanced by addition of the second platinum(II) nucleus. Here, DiplatinNC achieves total DNA condensation at 8 µM drug concentration, N₃-DiplatinNC accomplishes condensation at 15 µM, and N₃-TO-DiplatinNC demonstrates condensation at an intermediate concentration of 12.5 µM (Figure 46). The condensation activity of the trinuclear complexes is enhanced further and appears commensurate with the addition of a third platinum(II) nucleus to this chemotype. TriplatinNC exhibits

complete DNA condensation at 5 μM , N₃-TriplatinNC at 10 μM , and N₃-TO-TriplatinNC at 9 μM (Figure 46B).

Overall, the non-modified complexes DiplatinNC and TriplatinNC retain the most potent condensation activity within both the dinuclear and trinuclear complex series. The replacement of a singular dangling amine unit with an azide handle ultimately results in the loss of a singular positive charge, which may be the cause of the diminished activity within the N₃-PPC series. Interestingly, the activity of the dinuclear and trinuclear N₃-TO-PPCs is slightly enhanced with the introduction of a terminal thiazole orange fluorophore ligand. This observation supports the hypothesis that the introduction of an intercalating unit to a platinum(II) drug can facilitate synergistic therapeutic activity. Notably, there appears to be a cooperative DNA-unwinding and condensation activity observed for N₃-TO-TriplatinNC.

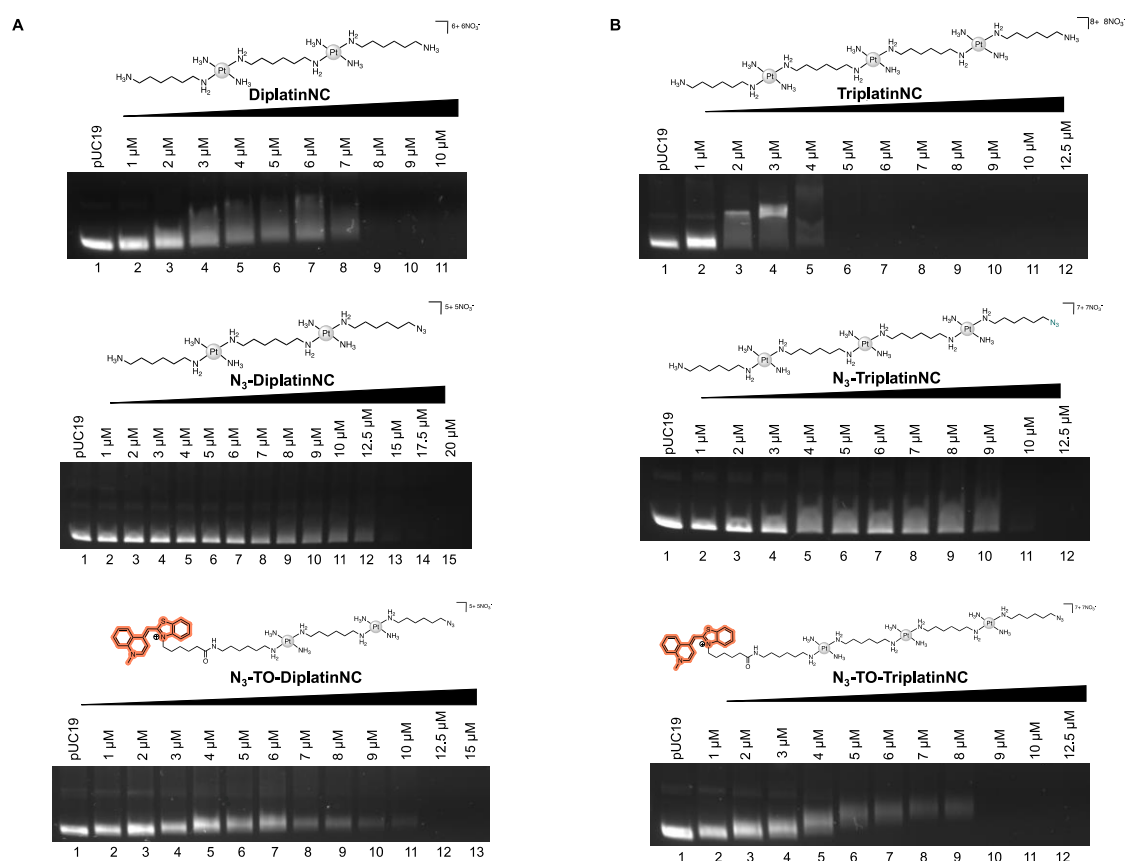


Figure 46A. Condensation assay of dinuclear platinum(II) complexes analysed by agarose gel electrophoresis; B. Condensation assay of trinuclear platinum(II) complexes analysed by agarose gel electrophoresis.

5.1.2. Groove blocking electrophoretic assay

A groove blocking assay was employed to indirectly determine if the PPCs, N₃-PPCs, and N₃-TO-PPCs preferentially bind in either the major groove, minor groove, or by intercalation. Here, 400 ng pUC19 was preincubated with either methyl green (a major groove binder), netropsin (a minor groove binder), or actinomycin D. (a threading intercalator) for 30 minutes at 37 °C before drug exposure for 24 hours at 37 °C. Figure 47 displays the groove blocking results of the dinuclear complexes DiplatinNC, N₃-DiplatinNC, and N₃-TO-DiplatinNC. DiplatinNC did not exhibit any preferential binding in the major groove, minor groove, or by intercalation. Similarly, N₃-DiplatinNC also does not discriminate between binding in the major groove, minor groove, or by intercalation. However, a significant difference is observed for N₃-TO-DiplatinNC (Figure 47C). When 400 ng of pUC19 is preincubated with netropsin or methyl green followed by exposure to N₃-TO-DiplatinNC, near identical gel profiles are observed indicating that N₃-TO-DiplatinNC binding does not discriminate between the major and minor grooves. However, when the plasmid is preincubated with actinomycin D., a threading intercalator, followed by exposure to N₃-TO-DiplatinNC, the plasmid is protected from the condensation activity at 5 µM when compared to the preincubations with netropsin and methyl green. This suggests that the thiazole orange moiety plays a clear role in the DNA binding affinity of N₃-TO-DiplatinNC and, when inhibited, the condensation activity of N₃-TO-DiplatinNC diminishes. Furthermore, as no differences were observed for DiplatinNC and N₃-DiplatinNC, it is reasonable to suggest that these drugs bind to the polyanionic phosphate backbone of DNA.

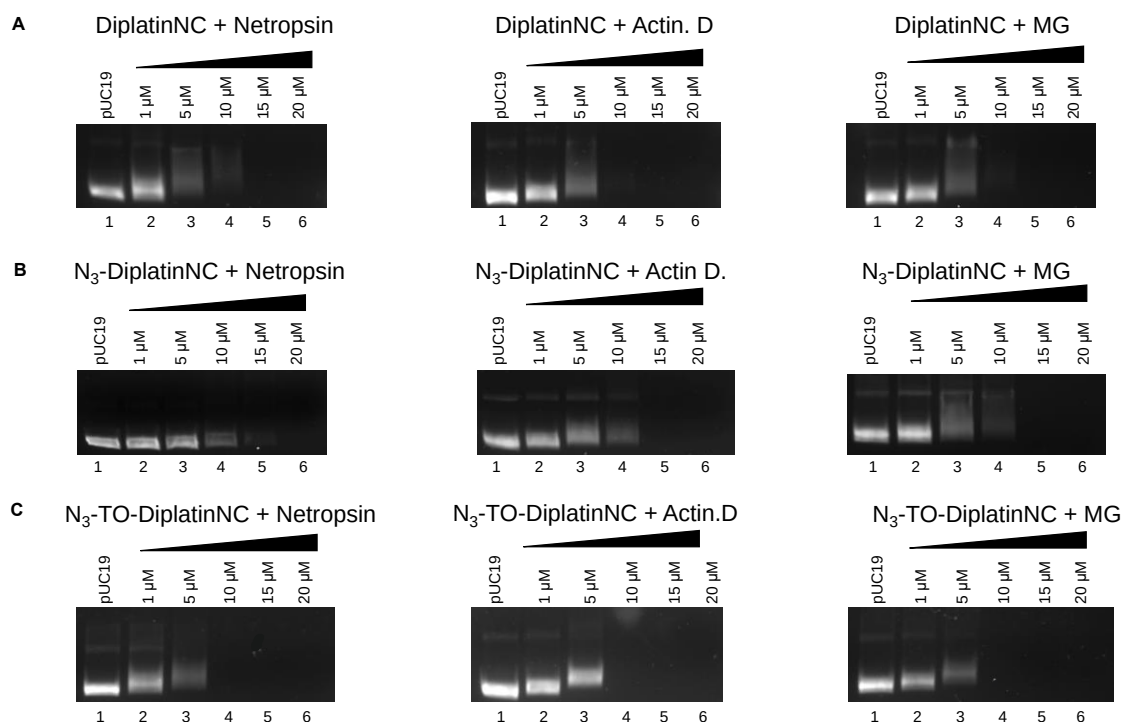


Figure 47A. Groove blocking studies *DiplatinNC*; B. Groove blocking studies of *N₃-DiplatinNC*; C. Groove blocking studies of *N₃-TO-DiplatinNC*.

The groove blocking studies of *TriplatinNC*, *N₃-TriplatinNC*, and *N₃-TO-TriplatinNC* are shown in Figure 48. The results of this study follow a similar trend to what was observed for the dinuclear complexes. *TriplatinNC* and *N₃-TriplatinNC* do not demonstrate any discrimination in activity when pUC19 is preincubated with either methyl green, netropsin, or actinomycin D. This observation suggests that these complexes may preferentially bind purely via phosphate clamping. However, for *N₃-TO-TriplatinNC*, actinomycin D significantly protects the plasmid from drug-induced condensation activity when compared to methyl green and netropsin (Figure 48C). This further supports the theory that thiazole orange intercalation contributes to the condensation activity of *N₃-TO-TriplatinNC*.

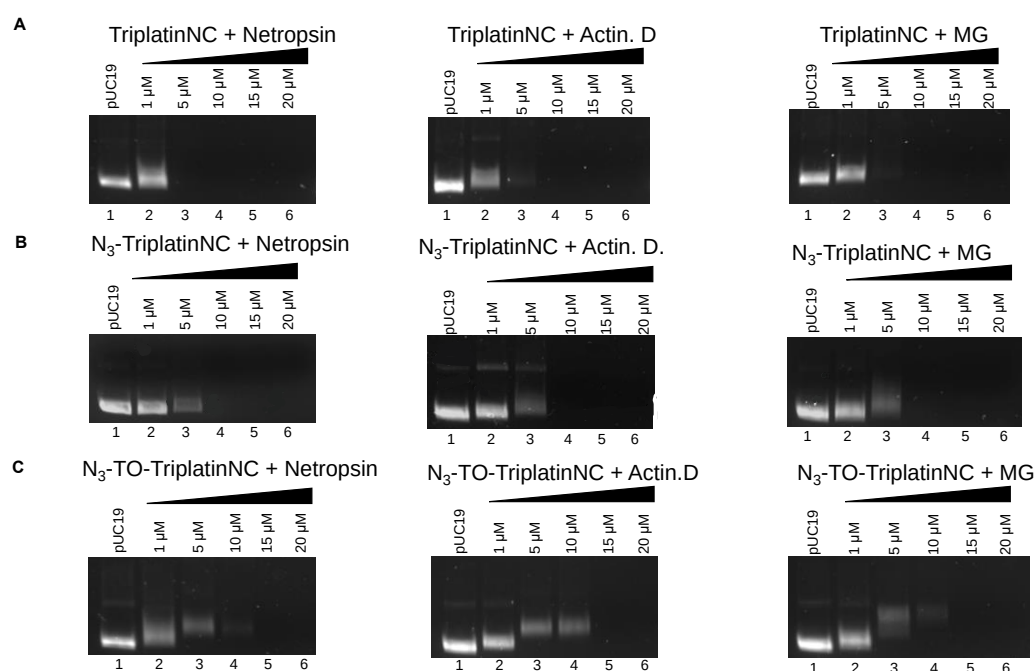


Figure 48A. Groove blocking studies of TriplatinNC; B. Groove blocking studies of N₃-TriplatinNC; C Groove blocking studies of N₃-TO-TriplatinNC.

5.1.3. Endonuclease recognition inhibition assay

An endonuclease inhibition assay was used to investigate the effects of the PPCs, N₃-PPCs, and N₃-TO-PPCs on site-specific endonuclease recognition. DiplatinNC, N₃-DiplatinNC, N₃-TO-DiplatinNC, along with TriplatinNC, N₃-TriplatinNC, and N₃-TO-TriplatinNC were incubated with 400 ng pUC19 at 37 °C for 24 hours before restriction endonuclease digestion reactions were performed using EcoRI and Nt. BspQI. EcoRI and Nt.BspQI are both type II endonucleases with one recognition sight on the pUC19 vector. EcoRI recognizes a palindromic GAATTC tract within the pUC19 vector and cleaves between both A↓G steps causing a double-strand break to release the linear DNA conformer. Nt. BspQI is a nicking enzyme which recognises and cuts at a single site 5'-GCTCTTCN↓-3' on the pUC19 vector thereby inducing a single strand break to form open circular DNA (Figure 49).

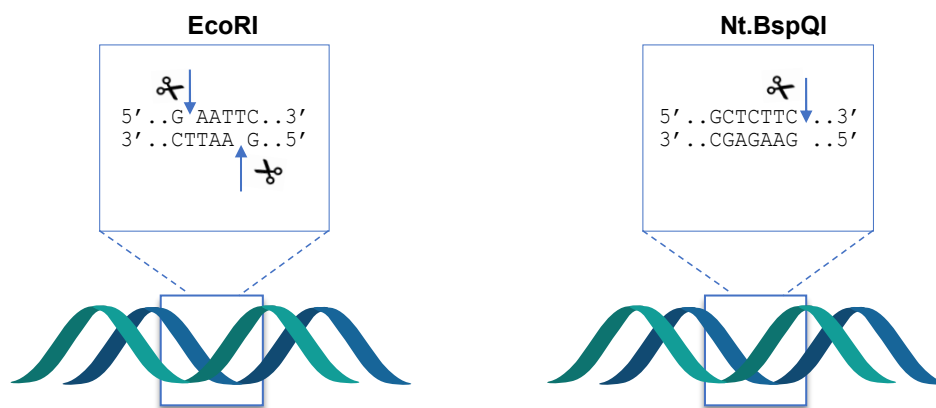


Figure 49. Schematic diagram of the recognition sites of restriction endonucleases *EcoRI* and *Nt.BspQI*.

Figure 50A displays the endonuclease inhibition results of the dinuclear complexes. All dinuclear complexes demonstrate endonuclease excision inhibition in a concentration dependent manner which is characterised by the disappearance of the DNA bands on the agarose gel. DiplatinNC demonstrates endonuclease inhibition of *Nt.BspQI* and *EcoRI* at 10 μM , N_3 -DiplatinNC inhibits *EcoRI* at 15 μM but does not achieve total inhibition of *Nt. BspQI* up to 20 μM , and N_3 -TO-DiplatinNC exhibits total endonuclease recognition inhibition of *EcoRI* and *Nt.BspQI* at 10 μM and 15 μM respectively. Figure 50B shows the endonuclease recognition inhibition studies of the trinuclear complexes TriplatinNC, N_3 -TriplatinNC, and N_3 -TO-TriplatinNC. The inhibitive activity of the three trinuclear complexes is comparable and shows endonuclease inhibition in a concentration-dependent manner, similar to what was observed for the dinuclear complexes. TriplatinNC induces total excision inhibition of *Nt. BspQI* and *EcoRI* at 10 μM and 5 μM , respectively. N_3 -TriplatinNC achieves total inhibition of both endonucleases at 10 μM , and N_3 -TO-TriplatinNC exhibits complete recognition inhibition for *EcoRI* and *Nt. BspQI* at 15 μM . The endonuclease inhibitory effect is greater for the trinuclear complexes than the dinuclear complexes and all complexes demonstrate an ability to condense all three forms of plasmid DNA: supercoiled, open circular, and linear. Across the complex library, it appears that there is slight preferential cleavage inhibition for *EcoRI* over *Nt.BspQI*.

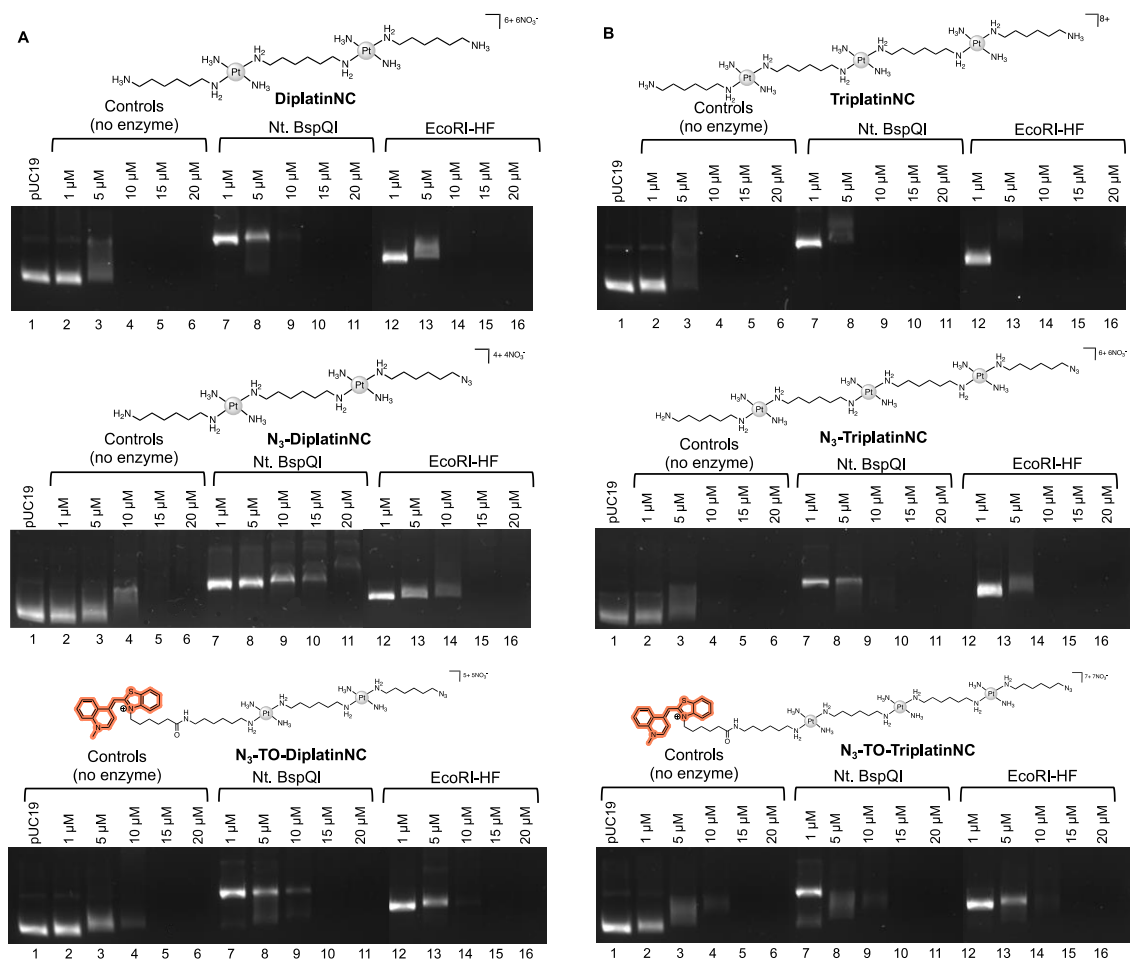


Figure 50A. Endonuclease inhibition studies using agarose gel electrophoresis of dinuclear PPCs, N₃-PPCs, and N₃-TO-PPCs and B. trinuclear PPCs, N₃-PPCs, and N₃-TO-PPCs.

5.1.4. Topoisomerase I mediated relaxation inhibition

Topoisomerase I is a ubiquitous enzyme that is involved in nearly all events associated with DNA metabolism including DNA replication, repair, and transcription. Many of these DNA processes are highly active within cancer cells, and these events can impart significant topological and torsional strain on DNA. With this in mind, the crucial role of topoisomerase I is to instigate the reversible cleavage of a single strand of duplex DNA in order to relieve this torsional stress.⁴ It is for this reason that topoisomerase I is considered an important therapeutic target and moreover, that topoisomerase I inhibitors have clear potential as anticancer therapeutics.⁵ The mechanism of action of topoisomerase I (Figure 51) begins with recognition and subsequent DNA binding of the enzyme forming a 1:1 enzyme:DNA complex. The formation of this enzyme-DNA complex facilitates a transesterification reaction which connects a tyrosine residue on the enzyme with the 3'-phosphate end of a DNA fragment via nucleophilic substitution. This reaction results in a single-strand break in the DNA duplex which facilitates the rotation of the nicked strand around the intact strand and thereby releases superhelical tension from the DNA. Finally, a second nucleophilic substitution reaction occurs between the 3'-phosphotyrosyl residue and the free 5'-hydroxyl group which religates the DNA strand without the loss of any genetic information and releases topoisomerase I, ready for another cycle.⁶

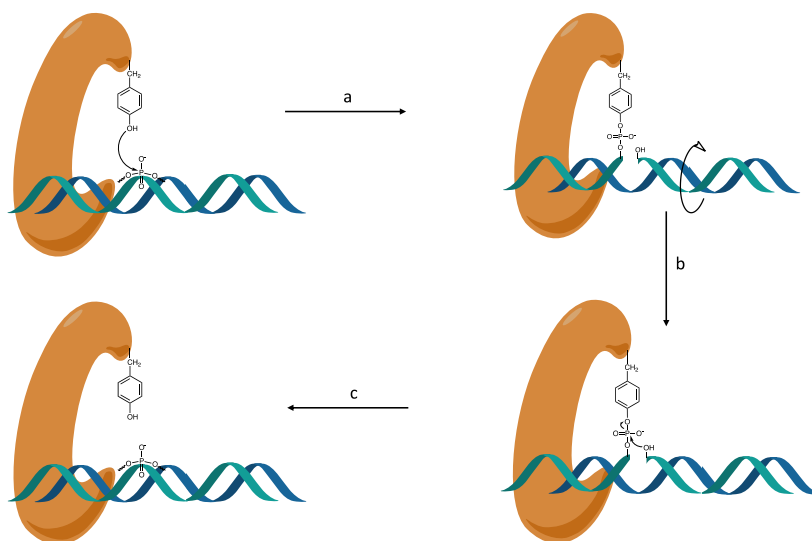


Figure 51. Schematic diagram of the mechanism of action of topoisomerase I with DNA. Enzyme-DNA binding facilitates the nicking of one strand of DNA via nucleophilic attack of tyrosine residue with 3'phosphate of DNA. The nicked strand can then rotate around the un-cut strand and thereby relieve torsional strain in the duplex. The nicked strand is then religated, releasing the topoisomerase I enzyme, without the loss of any genetic information.

Agarose gel electrophoresis was employed to investigate the inhibitive effects of the PPCs, N₃-PPCs, and N₃-TO-PPCs on topoisomerase I. Here, 400 ng of pUC19 was firstly incubated with each of the dinuclear and trinuclear complexes at varying concentrations, for 30 minutes at room temperature. 1 unit of topoisomerase I was then added and solutions were incubated at 37 °C for 20 minutes. The reaction was then stopped by addition of 0.25% sodium dodecyl sulphate and 250 µg/mL proteinase K. Gel electrophoresis was performed for 3 hours at 40 volts followed by 2.5 hours at 50 volts to allow for adequate separation of the topoisomers. Strong non-covalent drug-DNA binding can result in the unwinding of negatively supercoiled DNA (-) to open circular DNA (0). Figure 52 shows the topoisomerase I mediated relaxation inhibition studies of N₃-DiplatinNC and N₃-TO-DiplatinNC. Both N₃-DiplatinNC and N₃-TO-DiplatinNC were observed to unwind negatively supercoiled pUC19 plasmid DNA to open circular DNA upon 2.5 µM drug exposure. As the drug-loading concentration increases, a DNA condensation effect is also observed for both dinuclear complexes. N₃-DiplatinNC does not achieve total condensation up to 30 µM whereas N₃-TO-DiplatinNC exhibits complete condensation at 25 µM. This shows that the TO moiety contributes to the condensation activity of the complex. These profiles clearly display the effect that the TO moiety has in the topoisomerase activity.

Figure 53 shows the electrophoretic profiles of the topoisomerase-I mediated relaxation inhibition experiments of N₃-TriplatinNC and N₃-TO-TriplatinNC. N₃-TriplatinNC and N₃-TO-TriplatinNC exhibit enhanced inhibitive effect when compared to the dinuclear complexes discussed above. In this experiment, the control lane (lane 1) of N₃-TriplatinNC shows there is a small fraction of nicked pUC19 DNA in the stock but otherwise this does not have a bearing on the result. N₃-TriplatinNC releases the supercoiled plasmid DNA to open circular between 1 μ M and 2.5 μ M and accomplishes total condensation at 22.5 μ M. N₃-TO-TriplatinNC displays similar activity at equivalent drug concentrations however total plasmid condensation is achieved at 20 μ M. Similar to what was observed for N₃-TO-DiplatinNC, a series of tightly stacked topoisomers was observed for N₃-TO-TriplatinNC from 2 μ M to 10 μ M drug concentration. This again demonstrates the effect of the TO intercalating moiety on the topoisomerase-I activity. It is reasonable to suggest that as the drug concentration increases, and thus the concentration of the TO increases, it blocks the topoisomerase-I from its binding and subsequent cleavage of the pUC19 plasmid DNA. Moreover, topoisomerase I cannot recognize fully unwound plasmid DNA or positively supercoiled DNA and its activity is therefore inhibited. It appears that the DNA conformational changes induced by the complex renders the enzyme incapable of recognising the DNA substrate. Overall, results from this study further support these complexes as viable anticancer agents with potent topoisomerase-I inhibitive activity.

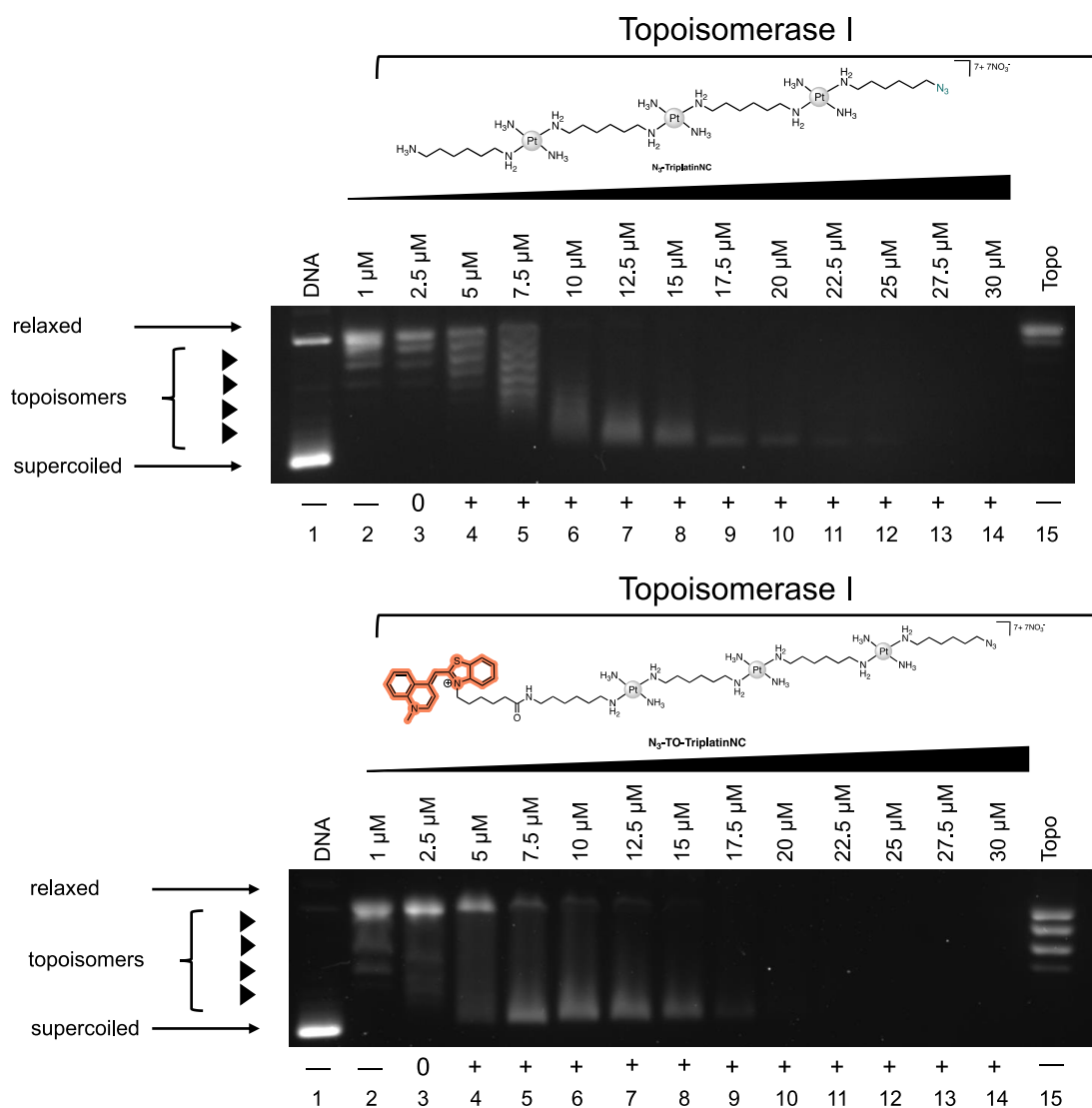


Figure 53 Topoisomerase I-mediated relaxation inhibition assay of *N*₃-TriplatinNC (top) and *N*₃-TO-TriplatinNC (bottom).

5.1.5. Epigenetic studies

Epigenetics is a term used to describe the study of inheritable changes that can occur in DNA without directly altering the DNA sequence and genetic information (i.e. DNA methylation).⁷ In recent years and as the relatively new field of epigenetic research has expanded, DNA methylation has been heavily implicated in the regulation of gene expression and a relationship between the development of cancer/tumour progression and DNA methylation has been observed.⁸ DNA methylation requires S-adenosyl methionine (SAM) as a methyl (CH_3) donor, a DNA methyltransferase enzyme (DNMT), and occurs primarily on the cytosine residue of 5'-CpG-3' islands.⁹ A general reaction scheme for cytosine methylation by DNMTs using SAM is shown in Figure 54. DNA methylation has been discovered to modulate gene expression by regulating chromatin structure by blocking transcription factors from binding to the DNA.¹⁰ Furthermore, overexpression of epigenetic enzymes has been correlated with tumorigenesis. With this in mind, compounds that inhibit epigenetic enzymes have been realised as important new therapeutic targets for the treatment of various cancers. To date, a number of small molecules have gained FDA approval as epigenetic inhibitors for the treatment of cancers such as 5-azacytidine for the treatment of acute lymphoblastic leukaemia,¹¹ and decitabine for the treatment of myelomonocytic leukaemia. In 2020 decitabine gained further FDA approval for the treatment of myelodysplastic syndromes in oral combination with cedazuridine.¹²

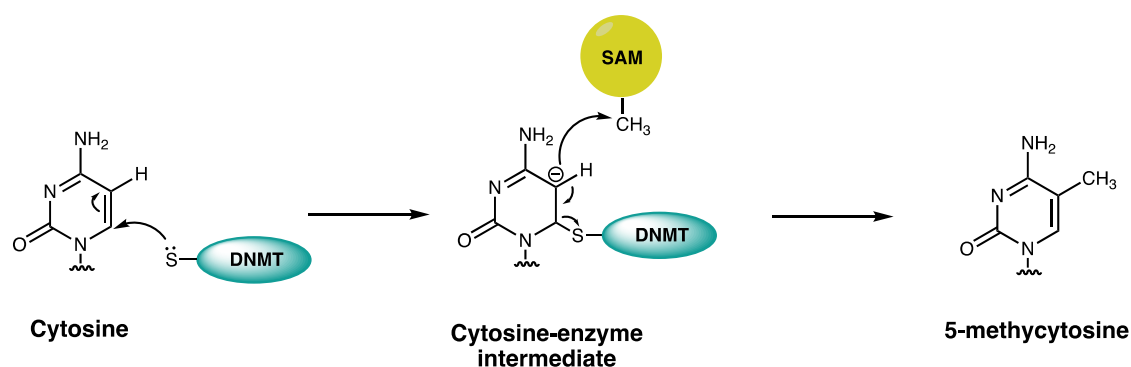


Figure 54. Schematic diagram for the reaction mechanism of cytosine methylation by DNMTs using SAM as a CH_3 donor to produce 5-methylcytosine.

An experimental protocol was designed to identify the effects of the PPCs, N₃-PPCs, and N₃-TO-PPCs on enzymes responsible for reading and writing epigenetic bases. A 798 base pair amplicon (54% GC) within the pBR322 plasmid vector was identified to contain four distinct 5'-CCGG-3' islands and was amplified using PCR (Figure 55A). Subsequently, the internal cytosine residues of each of the four 5'-CCGG-3' islands was methylated using the DNMT HpaII methyltransferase to produce an equivalent 798bp transcript with four discrete 5'-C5mCGG-3' tracts. To validate the presence of four individual 5'-CCGG-3' islands within the amplicon, the non-methylated transcript was treated with isoschimers HpaII and MSPI. HpaII is an enzyme that recognizes the 5'-CCGG-3' sequence and cleaves between both cytosine residues but is sensitive to DNA methylation. MSPI is an enzyme with the same nuclease activity as HpaII however, it is not sensitive to DNA methylation and can therefore cleave the cytosine and 5-methylcytosine stop of the methylated amplicon. While there are four distinct 5'-CCGG-3' islands within the 798 bp amplicon, and endonuclease restriction activity at each site generates five fragments of DNA in total, only four are observed on the agarose gel as the smallest fragment of 34 bases (Figure 55C) is too small to be directly visualised by agarose gel electrophoresis and four bands are observed (Lanes 3, 4, and 6 Figure 55B).

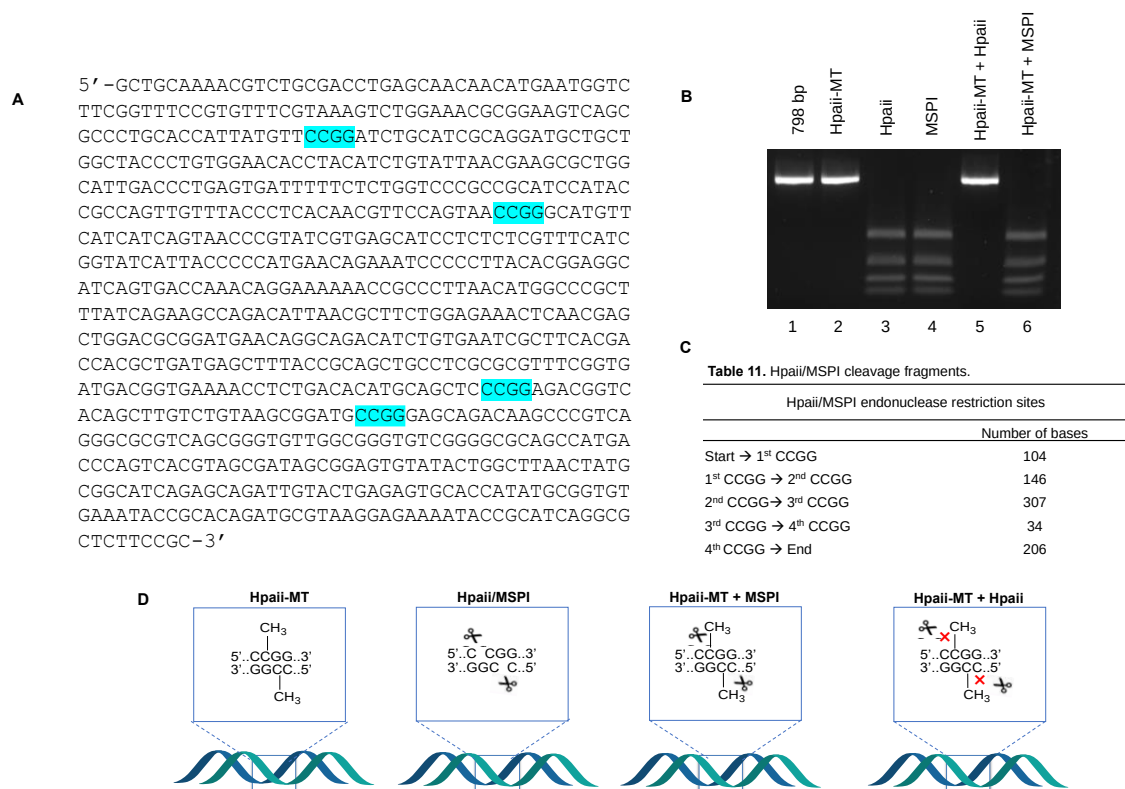


Figure 55A. 798 bp sequence amplified from the pBR322 plasmid with 4 distinct 5'-CCGG-3' islands highlighted in blue; B. Control agarose gel profile of the effect of HpaII and MSPI isozoschimers on the 798 bp amplicon in the presence and absence of the DNA methyltransferase HpaII-MT; C. Table of the number of base pairs per cleaved fragment of DNA generated from endonuclease activity of HpaII and MSPI; D. Schematic diagram of the activity of HpaII-MT, HpaII/MSPI in the absence of HpaII-MT, and in the presence of HpaII-MT.

5.1.5.1. Effects of cytosine methylation on condensation activity

The effects of DNA methylation on the condensation activity of dinuclear and trinuclear PPCs, N₃-PPCs, and N₃-PPCs was first investigated. Here, 400 ng of the 798 bp amplicon was incubated with HpaII-MT prior to drug incubations for 24 hours at 37 °C at concentrations of 1 μM, 5 μM, 10 μM, 15 μM, and 20 μM for all dinuclear and trinuclear complexes. The results of this study for dinuclear compound exposure are displayed in Figure 56A and the trinuclear complex results are shown in Figure 56B. Control experiments of the condensation activity of the dinuclear and trinuclear complexes on the non-methylated 798 bp amplicon are shown in lanes 2 – 6 of all gels. DiplatinNC achieved complete condensation for the unmethylated 798 bp sequence at 15 μM but did not completely condense the methylated amplicon up to 20 μM. N₃-DiplatinNC exhibits almost complete condensation at 20 μM for the non-methylated

transcript, however the methylated amplicon appears to be somewhat protected from the condensation activity as characterised by the more intense DNA band observed in lane 10 for N₃-DiplatinNC. N₃-TO-DiplatinNC condensed the non-methylated 798 bp amplicon at 10 µM, however when the transcript was pre-treated with HpaII-MT followed by drug incubation for 24 hr at 37 °C, it appears that 5mC bases shield the amplicon from DNA condensation up to 20 µM of drug exposure.

Similar findings were observed for the trinuclear complexes (displayed in Figure 56B). Enzyme incubation, drug incubation times and temperature, and electrophoresis experiments were performed in an identical manner to the earlier dinuclear experiments. TriplatinNC control experiments revealed both non-methylated and methylated amplicons were condensed at 10 µM. This is in stark contrast to what was observed for the dinuclear complexes where DNA methylation usually resulted in less potent condensation activity. N₃-TriplatinNC exhibits complete non-methylated amplicon condensation at 15 µM and almost complete condensation at 20 µM for the methylated transcript. N₃-TO-TriplatinNC follows a similar trend where total non-methylated amplicon condensation occurs at 10 µM, and occurs at 15 µM for the methylated transcript. All complexes tested in Figure 56 follow the trend that treatment of the 798 bp amplicon with HpaII-MT to generate the methylated transcript results in diminished condensation activity, except for TriplatinNC. TriplatinNC exhibits no discrimination in condensation activity for either the methylated or non-methylated amplicon. It is reasonable to suggest that the reason for this could be due to the remarkably high binding affinity of TriplatinNC for DNA.

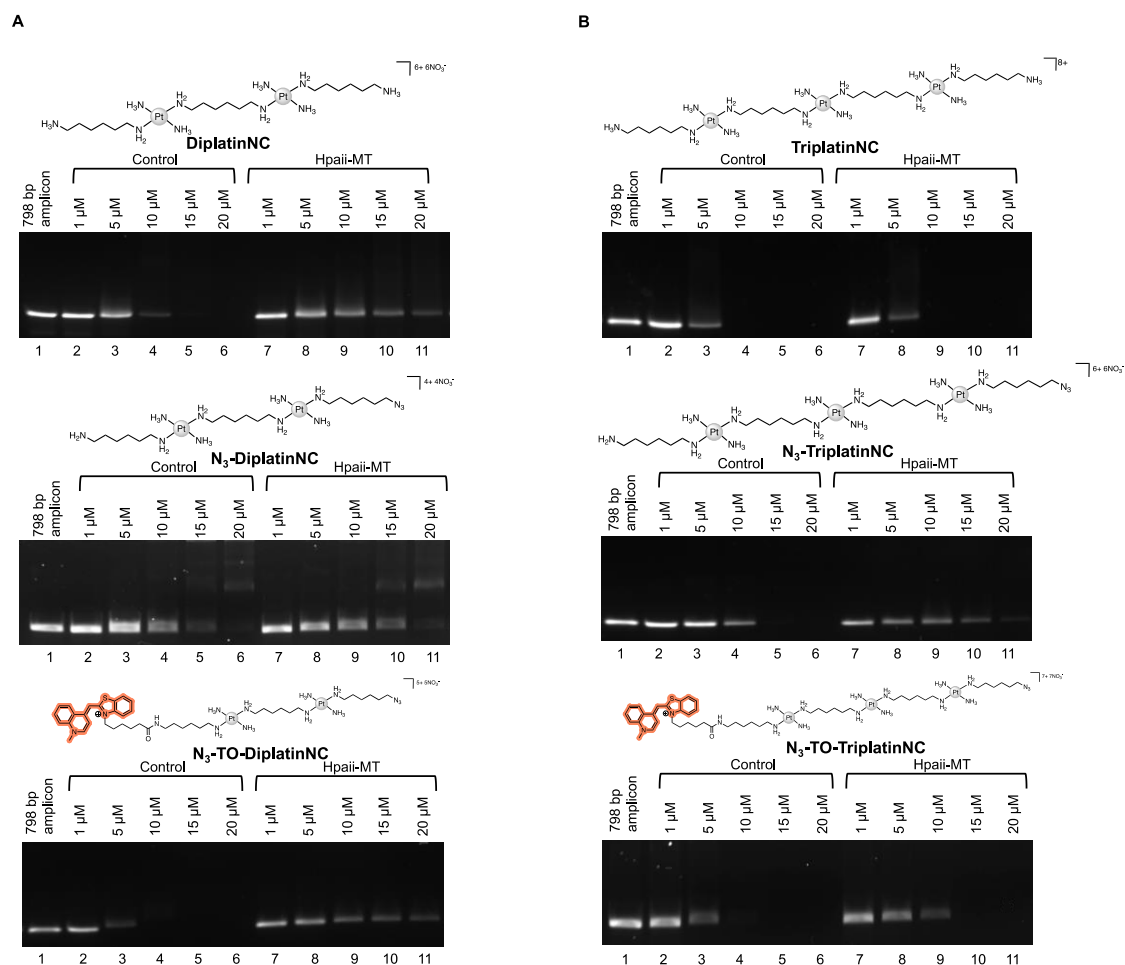


Figure 56A. Agarose gel electrophoresis experiments investigating the effects of DNA methylation on the condensation activity of the dinuclear and *B. trinu*uclear PPCs, N₃-PPCs, and N₃-TO-PPCs.

5.1.5.2. Polynuclear platinum(II) inhibition activity of “epigenetic-reader” MSPI

Next, the dinuclear and trinuclear PPCs, N₃-PPCs, and N₃-TO-PPCs were investigated for their ability to inhibit the recognition and subsequent cleavage of the epigenetic-reading enzyme MSPI. Here, the 798 bp amplicon was treated with HpaII-MT prior to drug incubation for 24 hrs at 37 °C, followed then by endonuclease digestion reactions using MSPI. Electrophoresis was performed with 2% agarose content at 60 V for 60 minutes. Figure 57A shows the profiles of this experiments for the dinuclear complexes. Lanes 1 – 4 are the control reactions and fragmentation patterns of the methylated and non-methylated amplicon as a result of MSPI digestion reaction. For DiplatinNC, MSPI cleavage inhibition is observed in a concentration-dependent manner as characterised by the lowest DNA bands in lanes 5 – 8 (1 µM – 5 µM) becoming fainter and the highest band—corresponding to the intact 798 bp amplicon—becoming visible. Thus, it appears a combined effect of MSPI cleavage inhibition and nucleic acid condensation occurs with complete DNA condensation identified at 9 µM. N₃-DiplatinNC does not exhibit MSPI endonuclease inhibition up to 14 µM. The intensity of the bands in the fragmentation pattern as a consequence of MSPI endonuclease digestion does not change in the way that was observed for DiplatinNC. Here, concentration-dependent condensation activity is observed for N₃-DiplatinNC as characterised by the fading of the DNA bands and almost complete condensation is achieved at 14 µM. N₃-TO-DiplatinNC demonstrates somewhat similar MSPI endonuclease inhibition activity to DiplatinNC but at higher drug-loading concentrations. From lanes 9–18 the highest DNA band, which correlates with the intact 798 bp amplicon, gradually becomes visible and more intense, which is indicative of Mspl restriction inhibition. The lower bands gradually become fainter and eventually disappear as the concentration of N₃-TO-DiplatinNC increases. This is a symptom, again, of the combined effect of MSPI cleavage inhibition as well as condensation activity by the complex.

Figure 57B shows the results obtained from the study of TriplatinNC, N₃-TriplatinNC, and N₃-TO-TriplatinNC. Firstly, concentration-dependent MSPI endonuclease inhibition and condensation activity was observed for TriplatinNC. MSPI cleavage inhibition appears to begin at 2 µM (lane 6) as the DNA band

appears to shift upwards and continues to do so as the drug concentration increases. The potent MSPI inhibitive activity as well as condensation activity of TriplatinNC is observed as evidenced by complete disappearance of a DNA band at 9 μ M. N₃-TriplatinNC displays concentration-dependent MSPI restriction inhibition but as its concentration effects are lower compared with TriplatinNC some interesting differences are observed. Firstly, the onset of inhibition begins at 5 μ M (lane 9) and progress as the drug concentration increases. Additionally, a combined condensation effect is observed with total DNA condensation reached at 13 μ M. N₃-TO-TriplatinNC demonstrates the most potent MSPI inhibition of all the complexes presented in this study. MSPI endonuclease inhibition appears to begin at concentrations as low as 1 μ M and is completely inhibited at 3 μ M. No MSPI cleavage is identified after 3 μ M as evidenced by the lack of fragmentation in the DNA band. Thereafter, nucleic acid condensation is achieved at 9 μ M of drug exposure.

Overall, the results of this study demonstrates that these dinuclear and trinuclear complexes, with the exception of N₃-DiplatinNC, are potent inhibitors of the epigenetic-reading enzyme MSPI, and to the best of our knowledge, represent the first example of any polynuclear platinum(II) complexes with direct application as epigenetic inhibitors. Moreover, these findings may further support the efficacy of polynuclear platinum(II) complexes as potent anticancer agents with new molecular targets not yet realised.

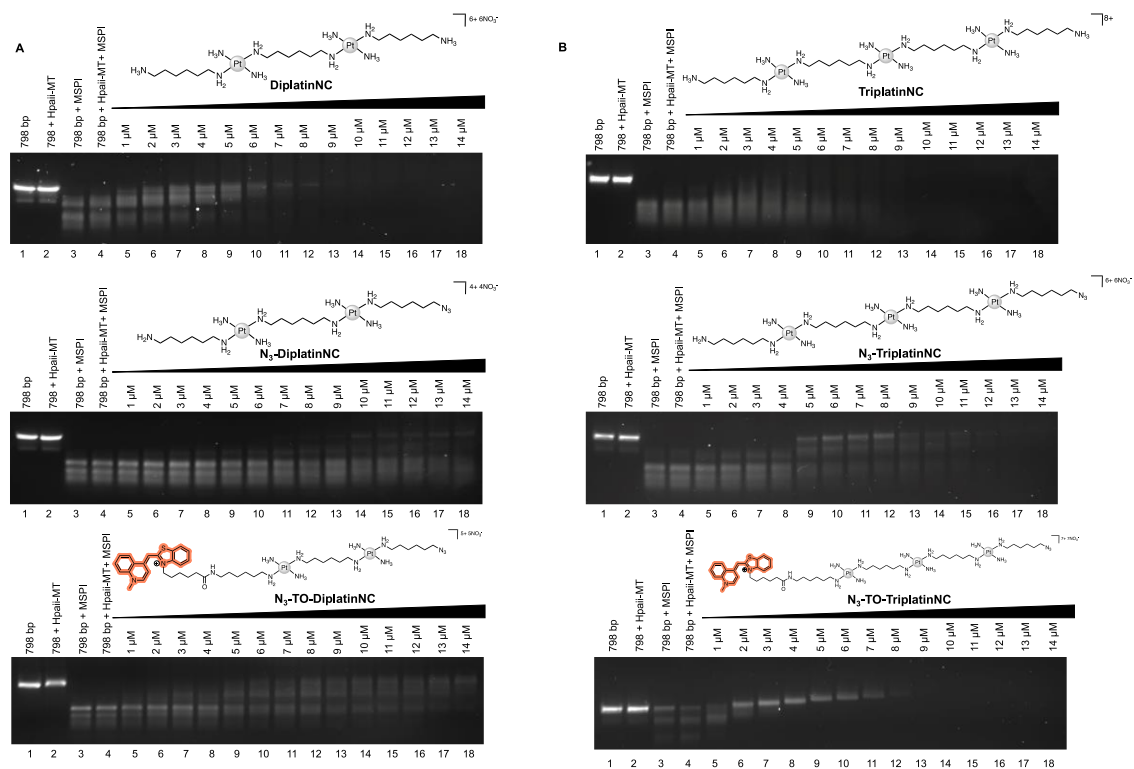


Figure 57A. Agarose gel electrophoresis studies of the dinuclear complexes on their ability to inhibit the reading of the epigenetic enzyme MSPI and B. agarose gel electrophoresis studies of the trinuclear PPCs on their ability to inhibit the reading of the epigenetic reading enzyme MSPI.

5.1.5.3. Microfluidic on-chip analysis of MSPI enzyme inhibition

An on-chip microfluidic method was developed for the Agilent Bioanalyzer 2100 for investigating the inhibition of the endonuclease activity of MSPI by the PPCs, N₃-PPCS, and N₃-TO-PPCs. The Bioanalyzer 2100 operates through capillary electrophoresis and offers advantages over other electrophoretic methodologies due to its ability to quantitatively detect DNA fragments of varying size precisely, with high-resolution. In this study, DNA 1000 microfluidic chips were employed which are designed to detect DNA fragments sized between 15 and 1500 bp. As highlighted earlier in Figure 55C, endonuclease excision of MSPI on the 798 bp amplicon generates five fragments of DNA of varying size, one of which (34 bp fragment) is not visualised on the agarose gel. In this work, the 798 bp amplicon was treated with HpaII-MT to generate the methylated 798 bp amplicon and subsequently incubated with dinuclear and trinuclear PPCs, N₃-PPCs, and N₃-TO-PPCs for 24 hours at 37 °C. Following this, MSPI digestion reactions were performed prior to loading the reaction samples on a DNA 1000 microfluidic chip and inserted into the Agilent Bioanalyzer 2100 for analysis.

Electropherograms for the microfluidic chip analysis of the methylated 798 bp amplicon and the MSPI treated methylated 798 bp sequence are displayed in Figure 58A. In each sample, internal DNA upper and lower markers are detected at 15 bp and 1500 bp. The methylated 798 bp amplicon generated a single peak and treatment of the methylated 798 bp amplicon with MSPI generated 5 DNA fragments as expected that correspond to fragments of 104 bp, 146 bp, 307 bp, 34 bp, and 206 bp.

Figure 58B-D shows the electrogram analysis produced by the Agilent Bioanalyzer 2100 for the study of DiplatinNC, N₃-DiplatinNC, and N₃-TO-DiplatinNC at 1 µM, 10 µM and 15 µM on the inhibition of endonuclease excision of MSPI on the methylated 798 bp amplicon. Here, a concentration-dependent inhibition is observed for all dinuclear complexes studied. Notably, the enhanced resolution of this technique can be seen due to the detection of the 34 bp fragment in the electrograms. DiplatinNC and N₃-DiplatinNC demonstrate similar concentration-dependent inhibition of MSPI excision, along with DNA condensation, and protection of the methylated 798 bp transcript is identified to

such an extent that at 15 μM drug-loading, the re-emergence of the 798 bp peak is observed. The effects of N₃-TO-DiplatinNC are more pronounced as characterised by the intensity of each of the DNA fragments diminishing as the concentration of N₃-TO-DiplatinNC increases and complete condensation is observed at 15 μM drug concentration.

The electrograms for the analysis of MSPI inhibition on the methylated 798 bp transcript by TriplatinNC, N₃-TriplatinNC, and N₃-TO-TriplatinNC are shown in Figure 59. As was observed for the dinuclear complexes, all three trinuclear compounds demonstrate a concentration-dependent inhibition of the MSPI endonuclease activity. Effects of the trinuclear series are more pronounced as is expected due to the inherent increased activity of the trinuclear complex series when compared to the dinuclear series. For TriplatinNC and N₃-TriplatinNC (Figure 59B and C) at concentrations as low as 1 μM , protection of one cleavage site is observed resulting in the appearance of a peak on the electrogram corresponding to ~500 bp. When the drug concentration was increased to 10 μM this effect becomes even more pronounced, and the re-emergence of a band at 811 bp for TriplatinNC—corresponding to the fully intact amplicon—is observed. At 15 μM drug exposure, more of the 798 bp amplicon remains intact and the quantity of the amplicon that is not protected from endonuclease excision is susceptible to cleavage by MSPI producing the five expected fragments. The effects of N₃-TriplatinNC are almost identical to those of TriplatinNC. N₃-TO-TriplatinNC exhibits remarkable MSPI inhibition where band broadening is observed as a consequence of the potent condensation and inhibition activity of the drug at concentrations as low as 1 μM , as characterised by a diminished intensity of the fragment peak. Overall, results of this study clearly illustrate combined endonuclease and condensation activity of both the dinuclear and trinuclear complexes. N₃-TO-DiplatinNC and N₃-TO-TriplatinNC are certainly most effective at inhibiting the epigenetic-reading enzyme MSPI. It is reasonable to suggest that this may be a symptom of the intercalative interaction of the thiazole orange moiety on these drugs with this 798 bp amplicon, and that thiazole orange intercalation may occupy the site of cleavage activity of MSPI.

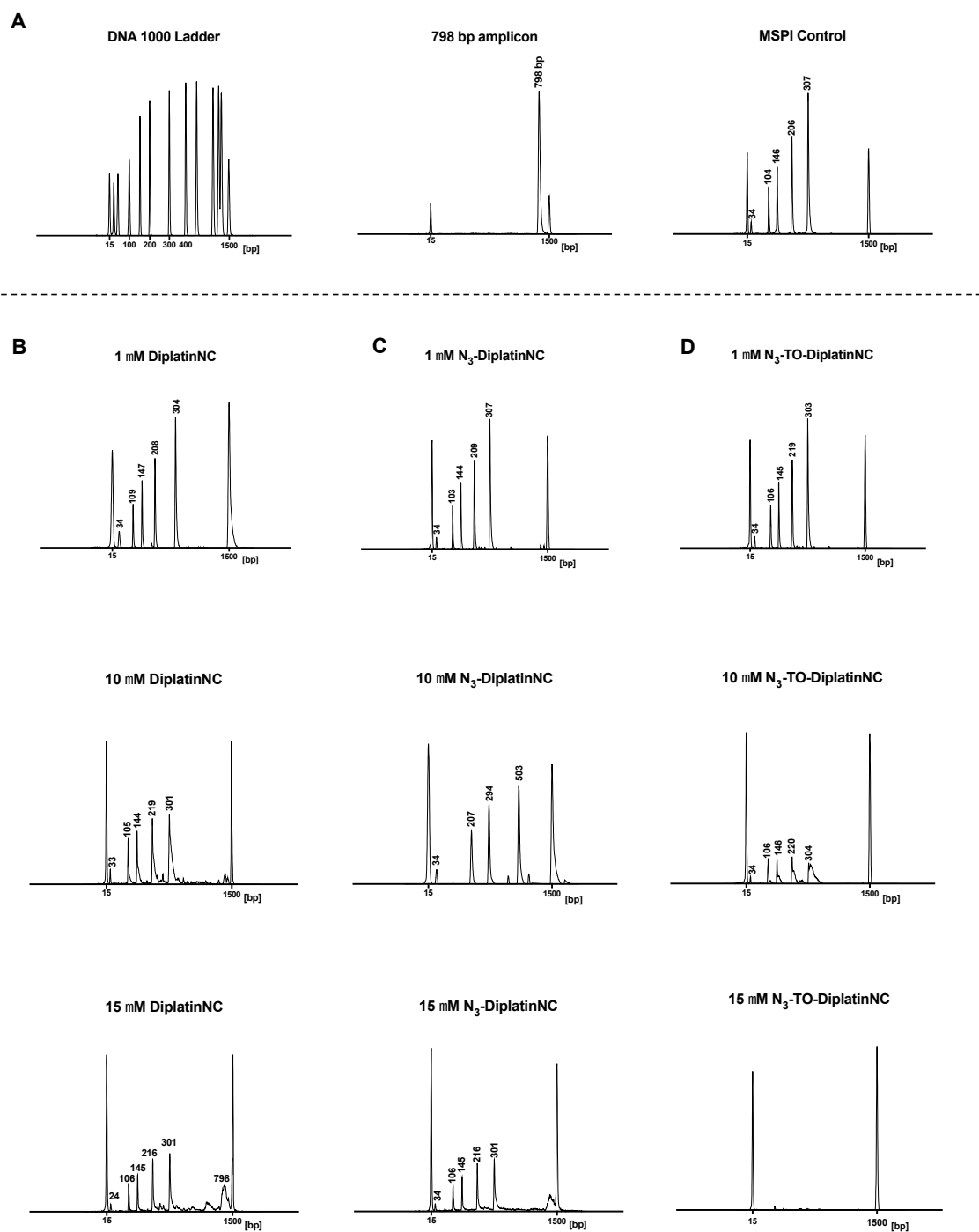


Figure 58A. Electrograms generated using the Agilent Bioanalyzer 2100 of the DNA 1000 ladder, methylated 798 bp amplicon, and excision activity of MSPI on the 798 bp amplicon; B-D. Electrograms of the inhibitive activity of DiplatinNC, N₃-DiplatinNC, and N₃-TO-DiplatinNC on the endonuclease activity of MSPI, respectively.

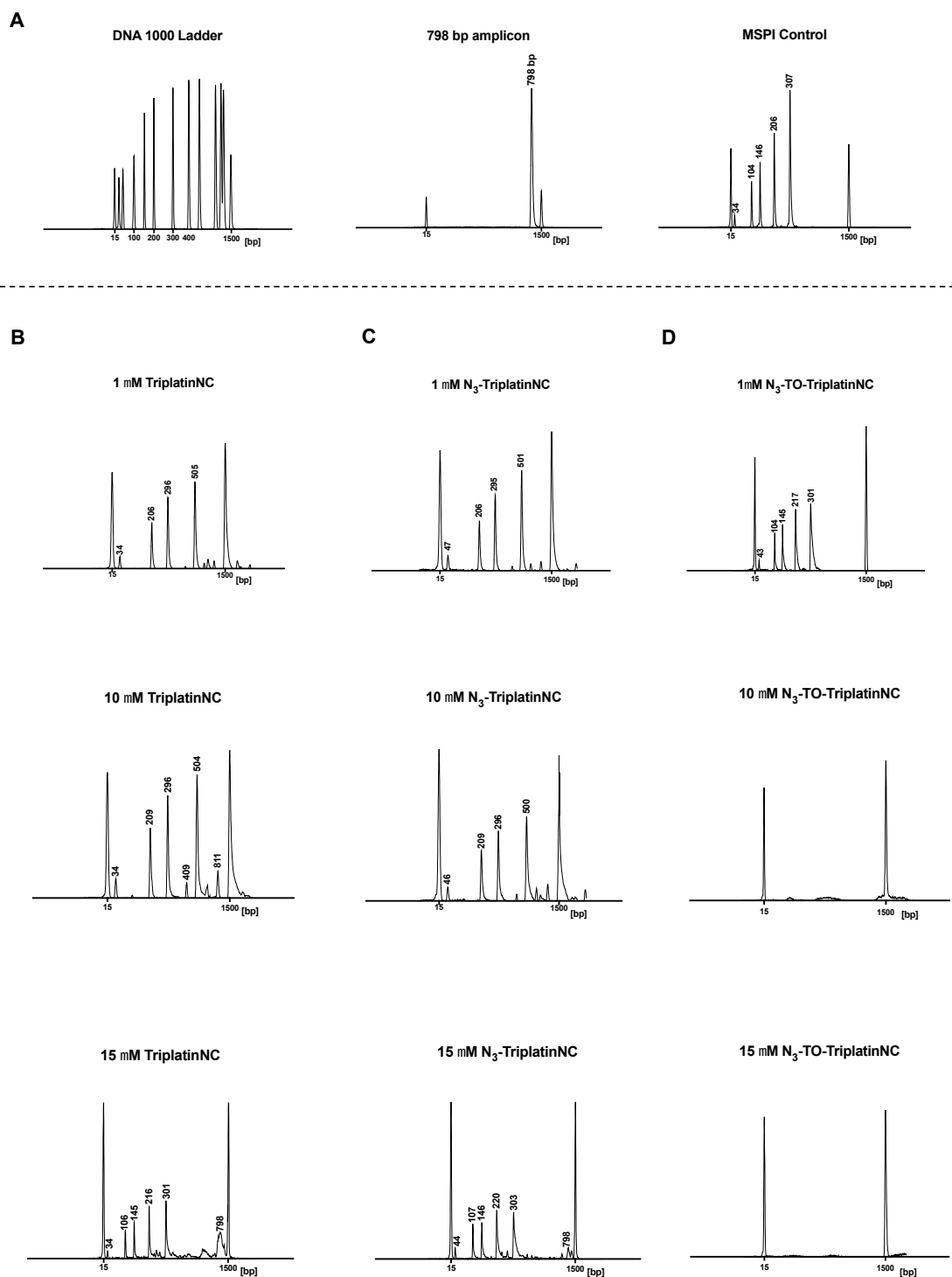


Figure 59A. Electrograms generated using the Agilent Bioanalyzer 2100 of the DNA 1000 ladder, methylated 798 bp amplicon, and excision activity of MSPI on the 798 bp amplicon; B-D. Electrograms of the inhibitive activity of TriplatinNC, N₃-TriplatinNC, and N₃-TO-TriplatinNC on the endonuclease activity of MSPI, respectively.

5.1.5.4. Enzymatic inhibition of HpaII-MT

To further probe the potential of the dinuclear and trinuclear PPCs, N₃-PPCs, and N₃-TO-PPCs as epigenetic inhibitors, an experiment was designed to investigate whether the complexes could not only inhibit the reading of epigenetic enzymes, but also the writing of epigenetic enzymes. With this in mind, and to precisely examine whether DiplatinNC, N₃-DiplatinNC, N₃-TO-DiplatinNC, TriplatinNC, N₃-TriplatinNC, and N₃-TO-TriplatinNC have the ability to inhibit the imprinting of cytosine methylation by HpaII methyltransferase, a new experimental protocol was designed. It was hypothesized that if the 798 bp amplicon used previously was first treated with one of the aforementioned PPCs, and subsequently treated with HpaII methyltransferase, and finally then treated with HpaII, the fragmentation of DNA or lack thereof would be indicative of whether cytosine methylation had occurred. HpaII cleavage only occurs on non-methylated sequences and so DNA fragmentation would indicate that cytosine methylation had not occurred and that the drug compound had successfully inhibited HpaII methyltransferase activity.

In this work, 400 ng of the 798 bp was first incubated with varying concentrations of either the dinuclear or trinuclear compounds for 24 hours at 37 °C. Subsequently, methylation of the reaction samples was carried out using HpaII methyltransferase. Endonuclease digestion reactions using HpaII were then performed and samples were finally loaded onto a 2% agarose gel and electrophoresis was performed at 60 V for 60 minutes.

The results of this study are displayed in Figure 60. Drug-loading concentrations were deliberately altered to have a lower and more narrow concentration range so that the condensation activity did not obstruct epigenetic inhibition activity from being observed. Lanes 1-4 of both gels display the enzymatic control studies and the effect of cytosine methylation on the activity of HpaII. None of the dinuclear and trinuclear complexes inhibit the writing of HpaII methyltransferase as characterised by the lack of any fragmentation observed after endonuclease digestion reactions were performed using HpaII. A concentration-dependent condensation effect is observed for all dinuclear and trinuclear on the methylated 798 bp amplicon. At 6 µM TriplatinNC (Figure 60,

bottom, Lane 7) there is still no enzyme induced fragmentation within the remaining faint DNA observed. If TriplatinNC had the ability to inhibit the epigenetic writing of HpaII methyltransferase it would most probably be at this point that this effect would be visualised. Overall, while results of this study demonstrate that the PPCs, N₃-PPCs, and N₃-TO-PPCs are not capable of inhibiting the epigenetic-writing of HpaII-MT, the results from the previous study indicates that these compounds may have utility in the realm of epigenetic processing enzymes and that they may be a source of untapped potential in this relatively new and promising field.

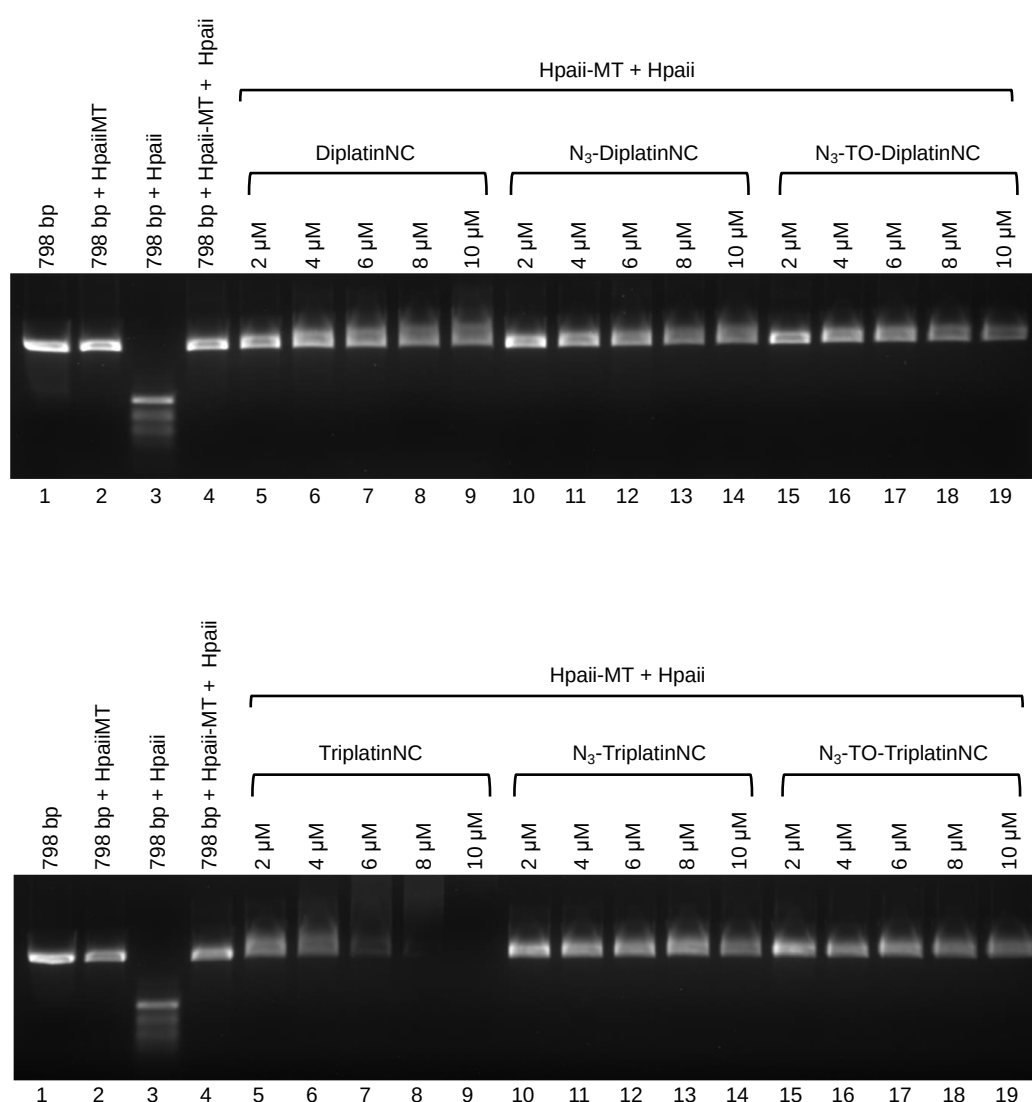


Figure 60. Agarose gel electrophoresis studies of the inhibition of the epigenetic writing activity of HpaII-MT by the dinuclear PPCs (top) and trinuclear PPCs (bottom).

5.2. Conclusion

Comprehensive electrophoretic studies with nucleic acids were conducted using the PPCs, N₃-PPCs, and N₃-TO-PPCs to: (i.) investigate their condensation activity; (ii.) probe their DNA binding sites using groove blocking assays; (iii.) study the potential inhibition activity of these drugs on a variety of enzymes including topoisomerase-I, nicking endonucleases, and cleavage endonucleases, and (iv.) explore the potential of the PPCs, N₃-PPCs, and N₃-TO-PPCs as inhibitors of epigenetic readers and writers. While it is clear that each dinuclear and trinuclear complex here displays excellent condensation effects, they are also all effective inhibitors of endonuclease excision recognition at micromolar concentrations. Each of the dinuclear and trinuclear complexes are also potent inhibitors of topoisomerase-I activity with the thiazole orange labelled complexes showing extraordinary inhibition activity at concentrations as low as 2.5 μ M for N₃-TO-TriplatinNC.

Although epigenetic inhibition studies have been studied for copper(II) phenazine complex,¹³ to the best of our knowledge, this is the first example of PPCs being studied for their potential as epigenetic enzyme inhibitors. Results from this study further validates the PPCs, as well as the N₃-PPCs and N₃-TO-PPCs, as a unique class of epigenetic inhibitors. Overall, the results presented here meet the electrophoretic based aims of **Objective 3** in Chapter II.

5.3. Experimental

DNA condensation assay

The ability of the N₃-PPC and N₃-TO-PPC series to condense supercoiled pUC19 plasmid DNA was determined using a method previously published by this laboratory with minor changes.¹⁴ Each of the N₃-PPC were initially prepared in DMF and further diluted in 80 mM HEPES buffer. Condensation reactions were carried out according to the following general procedure: in a total volume of 20 μ L using 80 mM HEPES buffer (pH 7.2), 400 ng pUC19 and varying concentrations of test compound (1-15 μ M), samples were incubated at 37 °C for 24 hrs. Reactions were quenched by adding 6x loading dye. Samples were loaded onto an agarose gel (1.2%) containing 3 μ L ethidium bromide. Electrophoresis was completed at 70 V for 1 hr in 1x TAE buffer.

Endonuclease enzyme inhibition assay

The ability of the N₃-PPC and N₃-TO-PPC series to inhibit endonucleases EcoRI-HF and Nt.BspQI was studied using methods previously reported by Kellett and co-workers, with slight modifications.¹⁵ The N₃-PPCs and N₃-TO-PPCs stock solutions were initially prepared in DMF and further diluted in 80 mM HEPES buffer (pH 7.2). In a typical endonuclease inhibition experiment, 400 ng of pUC19 was incubated with varying concentrations of the test compound (1, 5, 10, 15, 20 μ M) at 37 °C for 24 hrs. Subsequent endonuclease digestion reactions were performed by incubating the drug-treated and untreated pUC19 with EcoRI-HF or Nt.BspQI endonuclease restriction enzymes. This reaction was carried out by adding 1 μ L of EcoRI-HF restriction enzyme (20,000 U/mL), and 2 μ L of Cutsmart buffer or 2 μ L of Nt.BspQI restriction enzyme and 2 μ L of NEBuffer 3.1 to the reaction samples. The digestion reaction for EcoRI-HF was incubated for 1 hr at 37 °C, while the digestion reaction for Nt.BspQI was incubated for 1 hr at 50 °C, as per manufacturer's guidelines. The reaction products were examined using agarose gel electrophoresis. Reaction samples were added with 4 μ L of 6x loading dye before loaded onto an agarose gel (1.2%) containing 10 μ L of SYBR Safe DNA stain. Electrophoresis was carried out at 70 V for 1 hr in 1x TAE buffer.

Groove blocking assay

This assay was adapted from procedures previously reported by Kellett and co-workers.¹⁶ The groove blocking assay was employed to examine the activity of the N₃-PPC and N₃-TO-PPC series on supercoiled pUC19 in the presence of an excess of non-covalent DNA recognition agents methyl green (major groove binder), netropsin (minor groove binder) and actinomycin D. (threading intercalator). 400 ng of pUC19 was pre-treated with methyl green, netropsin or actinomycin D. (500 μ M) for 45 min at 37 °C. Subsequently, the test compounds were incubated with the pre-treated pUC19 at varying concentrations (1, 5, 10, 15, 20 μ M) for 24 hrs at 37 °C. The reaction products were examined using agarose gel electrophoresis. 4 μ L of 6x loading dye was added to each reaction

sample before loading onto an agarose gel (1.2%) containing 10 μ L of SYBR Safe DNA stain. Electrophoresis was carried out at 70 V for 1 hr in 1x TAE buffer.

Topoisomerase-I-mediated DNA relaxation inhibition assay

This experiment was carried out according to published procedure by Kellett and co-workers with slight modifications.^{17,18} In a final volume of 20 μ L containing 80 mM HEPES buffer (pH 7.2), CutSmart buffer and bovine serum albumin, 400 ng of supercoiled pUC19 plasmid DNA was exposed to varying concentrations of the N₃-PPC and N₃-TO-PPC series (1, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5, 25, 27.5, 30 μ M) for 30 min at ambient temperature. Subsequently, 1 unit of topoisomerase I was incubated with the reaction samples for 20 min at 37 °C. The reactions were quenched by the addition of 0.25% sodium dodecyl sulfate (SDS) and 250 μ g mL⁻¹ protein kinase (Sigma Aldrich) and incubating for 30 min at 50 °C. 4 μ L of 6x loading dye was added to each reaction sample before loading onto an agarose gel (1.2%). The DNA topoisomers were separated by electrophoresis in 1x TBE buffer for 3 hr at 40 V followed by 2.5 hr at 50 V. The agarose gel was post-stained using an EtBr bath (20 μ M) for 30 min. The gel was then soaked in deionised water overnight before imaging using a UV transilluminator.

Condensation sensitivity to DNA methylation by HpaII methyltransferase

This experiment was designed to investigate the effect of cytosine methylation on the condensation activity of the N₃-PPC and N₃-TO-PPC series. The pBR322 vector (4361 bp) was selected and 798 bp region on the vector was identified containing 54% GC content. A set of primers were designed (Forward: 5'-GCTGCAAAACGTCTGCGACC-3' and reverse: 5'-CGCGAAGAGCGCCTGATGCG-3') to generate the 798 bp amplicon. This amplicon was generated by employing PCR (35 cycles) with 1 ng of pBR322 vector with 2x MyTaq Red Mix and validated using agarose gel electrophoresis

with a 1 Kb DNA ladder as a control. 400 ng of the amplicon was then pre-treated with 1 unit of HpaII-methyltransferase, HpaII-methyltransferase buffer and S-adenosylmethionine (SAM) (1600 μ M) as per manufacturer's guidelines for 1 hr at 37 °C. The methylated amplicon was then incubated with varying concentrations of the N₃-PPC and N₃-TO-PPC complexes (1, 5, 10, 15, 20 μ M) for 24 hr at 37 °C. The reaction mixtures were added with 4 μ L 6x loading dye before loaded onto an agarose gel (1.2%) containing 10 μ L of SYBR Safe DNA stain. Electrophoresis was carried out at 60 V for 60 min in 1x TAE buffer solution.

MspI Inhibition—Inhibition of epigenetic reading

This experiment was performed according to the literature procedure by Kellett and co-workers with modifications.¹³ PCR: This experiment was designed to investigate the inhibitive effect that the N₃-PPC and N₃-TO-PPC series have on the reading of an epigenetic enzyme that recognizes and cleaves the internal methylated cytosine of a CCGG island. A 798 bp region of the pBR322 vector (4361 bp) was identified containing four CCGG tracts. A set of primers were designed (forward: 5'-GCTGCAAACGTCTGCGACC-3' and reverse: 5'-CGCGAAGAGCGCCTGATGCG-3') to produce the 798 bp transcript. The amplicon was generated using PCR (35 cycles) with 1 ng pBR322 plasmid using 2x MyTaq Red Mix at suitable annealing temperatures for the primer set. The amplicon was verified using agarose gel electrophoresis using a 1 Kb DNA ladder as a control.

Control: A 400 ng quantity of the amplicon was exposed to a range of enzymes including HpaII, MspI and HpaII-MT for 1 hr at 37 °C according to manufacturer's guidelines. Successful methylation of the internal cytosine within the CCGG islands by HpaII methyltransferase was verified by agarose gel electrophoresis when HpaII demonstrated no nuclease effect due to methylcytosine sensitivity. Electrophoresis experiments were carried out at 60 V for 60 min on a 2% agarose gel in 1x TAE buffer solution.

MspI Inhibition: A 400 ng aliquot of the 798 bp amplicon was exposed to 1 unit of HpaII methyltransferase, HpaII-methyltransferase buffer and S-

adenosylmethionine (SAM) (1600 μ M) at 37 °C for 1 hr followed by heat deactivation at 65 °C for 20 min as per manufacturer's guidelines. The methylated 798 bp amplicon was exposed to varying concentrations of the N₃-PPC and N₃-TO-PPC series for 24 hr at 37 °C. The drug-treated amplicon was exposed to 1 unit of MspI (20,000 U/mL) for 1 hr at 37 °C. 6x loading dye was added to each reaction mixture before loading onto an agarose (2%) gel containing 12 μ L SYBR safe DNA stain. Electrophoresis was performed at 60 V for 1 hr in 1x TAE buffer solution.

HpaII-methyltransferase inhibition—inhibition of epigenetic writing

A 400 ng aliquot of the 798 bp transcript was exposed to DiplatinNC, N₃-DiplatinNC, N₃-TO-DiplatinNC, TriplatinNC, N₃-TriplatinNC, and N₃-TO-TriplatinNC at drug concentrations of 2, 4, 6, 8, 10 μ M for 24 hours at 37 °C. Subsequently, cytosine methylation reactions were performed using HpaII-MT as per manufacturer's guidelines followed by heat deactivation for 20 min at 65 °C. Endonuclease digestion was then performed using HpaII for 1 hour at 37 °C as per manufacturer's guidelines. Samples were then added with 4 μ L 6x loading dye prior to loading onto an agarose gel (2%) containing 12 μ L SYBR safe DNA stain. Electrophoresis was performed at 60 V for 1 hour in 1x TAE buffer solution.

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Chapter VI

DNA Binding Studies of the PPCs, N₃-PPCs, and N₃-TO-PPCs

6. General introduction

A variety of biophysical techniques have been employed to probe the PPC-DNA binding interactions. These experiments inform our understanding of the strength of the drug-DNA interaction, as well as the stabilising effect that the drug molecule has on the nucleic acid. These include competitive displacement, fluorescent quenching, and thermal melting. These high-throughput techniques can provide valuable insight into potential base-specific binding interactions as well as the stability of the drug-DNA complex.

6.1. Ethidium bromide competitive displacement assay

The ethidium bromide competitive displacement assay is a widely employed high-throughput, fluorescence-based method used to indirectly probe the apparent DNA binding constants (K_{app}) of non-fluorescent molecules with different DNA polymers.^{1, 2} This technique involves the treatment of a DNA sample with an excess of the DNA intercalator ethidium bromide. Ethidium bromide is a well-studied reporter molecule that is weakly fluorescent in solution but becomes highly emissive when it binds DNA and intercalates between DNA base pairs. Once the DNA is saturated with ethidium bromide and all possible binding sites are occupied, a non-fluorescent DNA binder is titrated into the reaction sample and the K_{app} is determined by concentration of the DNA binder required to induce a 50% decrease in fluorescence detection (Figure 61). ($K_{app} = K_b \times 12.6/C_{50}$ where $K_b = 8.8 \times 10^6 \text{ M}(\text{bp})^{-1}$ and C_{50} = concentration of drug required to reduce fluorescence by 50%).³

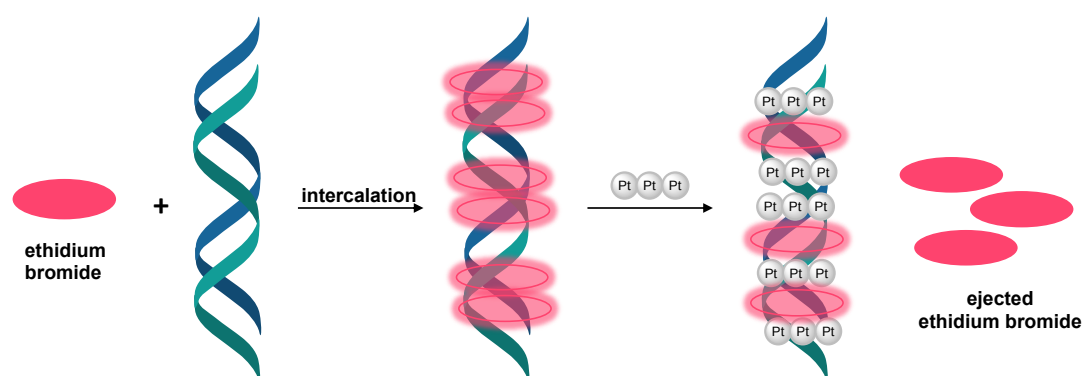


Figure 61. Schematic diagram of the competitive ethidium bromide displacement assay to determine the apparent DNA constants of polynuclear platinum(II) compounds.

The ethidium bromide competitive displacement assay was performed using three different DNA polymers of varying percentage AT content—poly[d(G-C)₂] (0%), poly[d(A-T)₂] (100%), and ctDNA (58%)—to determine the potential base-specific apparent DNA binding constants (K_{app}) of the PPCs, N₃-PPCs, and N₃-TO-PPCs. Figure 62 displays results for the dinuclear and trinuclear PPCs, N₃-PPCs, and N₃-TO-PPCs as well as the K_{app} constants calculated for the three different nucleic acid polymers used and C₅₀ value obtained for each complex. Results from the mononuclear complex series are not shown as they did not decrease 50% fluorescence up to a tested maximum of 275 μ M (Appendix Figure C- 1).

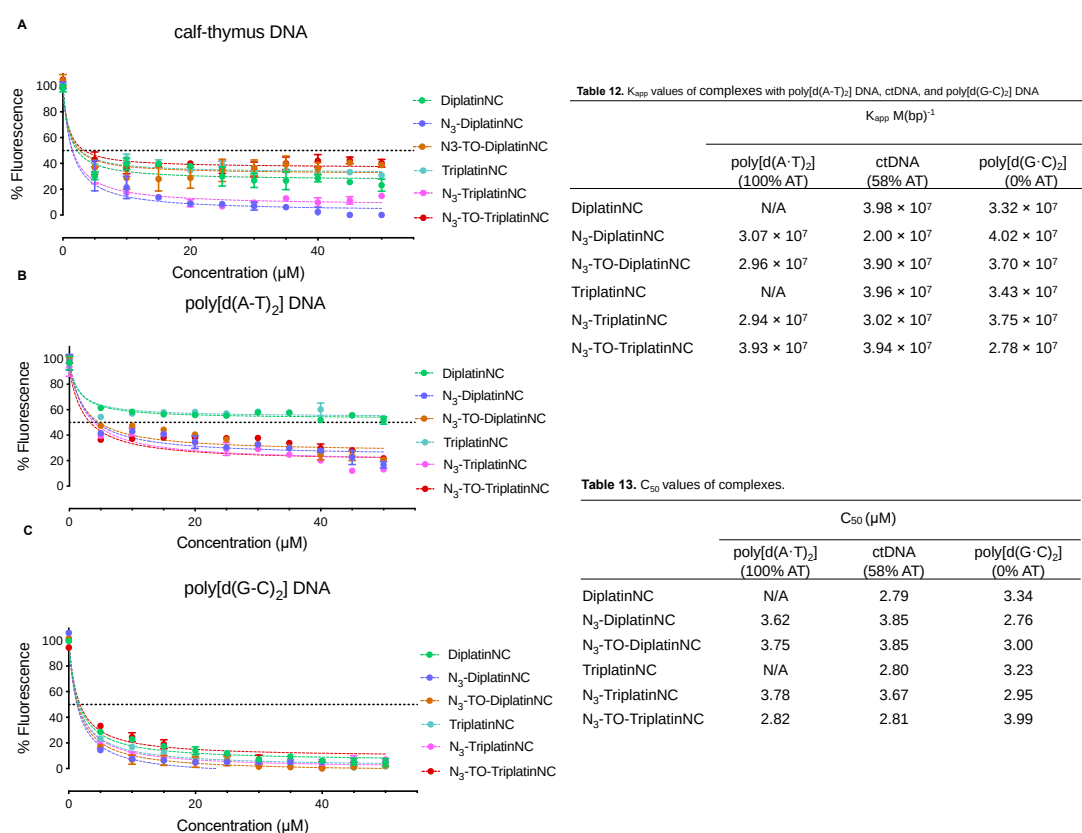


Figure 62. Competitive ethidium bromide displacement of dinuclear and trinuclear complexes with A. calf-thymus, B. poly[d(A-T)₂], and C. poly[d(G-C)₂]. Graphs were generated using GraphPad Prism 8. Data points presented here as an average of triplicate fluorescent measurements $\pm$ standard deviation.

All di- and tri-nuclear complexes in the series display a preferential binding affinity for the poly[d(G-C)₂] co-polymer (Figure 62C) when compared to the poly[d(A-T)₂] polymer (Figure 62B). This phenomenon is in agreement with what was investigated by Kellett and co-workers in 2014 for TriplatinNC.⁴ Thus, DiplatinNC and TriplatinNC do not have the ability to displace ethidium bromide up to 50 μ M drug loading to induce a 50% decrease in fluorescence for the

poly[d(A-T)₂] polymer. In contrast, the introduction of the azide moiety, or both the azide moiety and the thiazole orange reporter molecule appears to enhance the apparent binding affinity of the dinuclear and trinuclear complexes toward poly[d(A-T)₂]. All complexes in the above study were highly efficient at displacing ethidium bromide from calf-thymus DNA and show a high apparent binding with K_{app} values varying from $2.00 \times 10^7 - 3.98 \times 10^7 \text{ M(bp)}^{-1}$.

6.2. Fluorescence quenching assay

The fluorescence quenching assay employs a similar technique to the ethidium bromide competitive displacement assay however, the DNA sample is treated with a limited volume of ethidium bromide rather an excess of ethidium bromide prior to drug titrations. This method allows for the indirect identification of the preferred DNA binding sites and/or DNA base composition by assessing the fluorescence quenching efficiency of a DNA binding molecule on oligomers of varying AT/GC content. The fluorescence quenching efficiency of a molecule is dictated by the accessibility of the molecule for the ethidium bromide-bound DNA. Here, the data is presented as quenching values (Q), which can be defined as the concentration of a molecule required to induce 50% quenching of fluorescence when compared to non-drug treated DNA. Fluorescence quenching assays here have been performed according to literature methods reported by Kellett and co-workers with slight modification.^{1, 4, 5}

Results of the fluorescence quenching assay for the dinuclear and trinuclear PPCs, N₃-PPCs, and N₃-TO-PPCs using calf-thymus DNA, poly[d(G-C)₂], and poly[d(A-T)₂] are shown in Figure 63. The mononuclear complexes do not demonstrate fluorescence quenching of limited ethidium bromide by 50% up to 275 μM on any of the DNA polymers tested. Conversely, a clear preference for ethidium bromide fluorescence quenching on poly[d(A-T)₂] DNA is observed for all dinuclear and trinuclear complexes when compared to calf-thymus DNA and poly[d(G-C)₂] DNA (Figure 63). TriplatinNC retains the best fluorescent quenching efficiency of the complex series for poly[d(A-T)₂]. TriplatinNC displays 0% fluorescence at 5 μM drug concentration, meaning that TriplatinNC efficiently quenches ethidium bromide fluorescence within poly[d(A-T)₂] DNA almost completely at 5 μM . This would suggest that TriplatinNC has an unquestionable

binding preference for poly[d(A-T)₂] tracts of DNA and favours binding across the minor groove. The fluorescence quenching efficiency of the thiazole orange labelled drugs N₃-TO-DiplatinNC and N₃-TO-TriplatinNC favours poly[d(A-T)₂] DNA over poly[d(G-C)₂] DNA. This effect is more pronounced for the trinuclear complex than the dinuclear complex. Thiazole orange has an intrinsic binding preference for intercalation within the minor groove of poly[d(A-T)₂]⁶⁻⁸ which is evident here when comparing the fluorescence quenching efficiency of the dinuclear and trinuclear complexes on the difference DNA polymers.

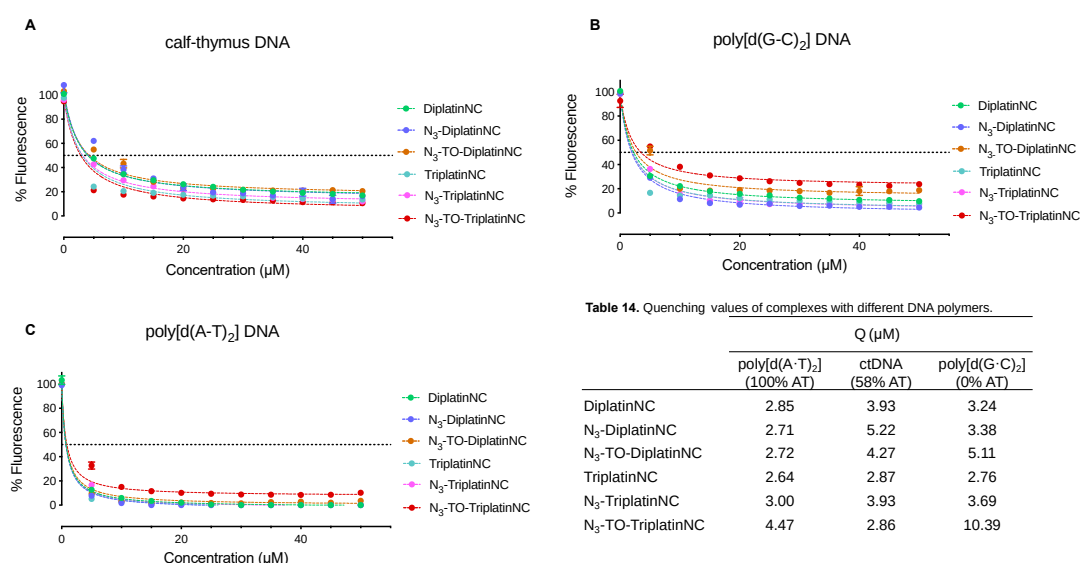


Figure 63. Fluorescence quenching assay of ethidium bromide by dinuclear and trinuclear complexes on A. calf-thymus DNA, B. poly[d(G-C)₂] and C. poly[d(A-T)₂]. Quenching values (Q) are displayed in the table above. Graphs were generated using GraphPad Prism 8. Data points are presented here as an average of triplicate fluorescent measurements $\pm$ standard deviation.

6.3. Thermal melting analysis (T_m)

Thermal melting analysis is a technique that is commonly used to explore the stabilization effects of a given DNA binder on a nucleic acids of varying AT/GC content. This analytical technique offers wide-range utility in studying the strength of the interaction between a DNA binding drug and a nucleic acid sequence.³ Typically, a stronger drug – DNA interaction would require more energy to denature. The thermal melting (T_m) marks the point at which a 50:50 equilibrium is achieved between duplex DNA and single-stranded DNA. AT-rich DNA has fewer hydrogen bonds and therefore will exhibit a lower T_m value than GC-rich DNA, which has more hydrogen bonds. Upon thermal

denaturation of DNA, secondary DNA structure is lost as a result of the destabilisation of π - π stacking interactions between the heterocycles of the DNA nucleobases, as well as the hydrogen bonds between the Watson-Crick base pairs. The thermal denaturation of DNA is highly sensitive to salt concentrations in the sample therefore this feature can be exploited to manipulate the thermal melting value of a DNA sequence so that the T_m is within the limit of detection (LOD) of the instrument used. For example, phosphate salts can destabilize the DNA backbone and lower the T_m values of DNA polymers with high GC content.^{3,9}

Fluorescence melting analyses were carried out on the dinuclear and trinuclear PPCs, N_3 -PPCs, and N_3 -TO-PPCs on nucleic acid sequences of varying AT content including poly[d(A-T)₂] DNA (100%), calf-thymus DNA (58%), and *Micrococcus lysodeikticus* DNA (ML-DNA) (28%) to investigate the potential stabilizing effects of the complexes on the nucleic acids mentioned. Here, high resolution fluorescence melting experiments were conducted on a LightCycler 480 Real-Time PCR Detection System (Roche) using LightCycler 480 software. Figure 64A displays the melting results of PPCs, N_3 -PPCs, and N_3 -TO-PPCs on poly[d(A-T)₂]. Poly[d(A-T)₂] DNA displays an intrinsic T_m value of 61.49 °C and a dramatic stabilisation effect is observed for DiplatinNC, TriplatinNC, N_3 -DiplatinNC, and N_3 -TriplatinNC on poly[d(A-T)₂] with ΔT_m values of + 12.45 °C, + 12.53 °C, + 11.41 °C, and + 10.95 °C respectively. This is in agreement with the data presented by Kellett and co-workers in 2014 for TriplatinNC.⁴ This indicates that the dinuclear and trinuclear PPCs and N_3 -PPCs have a strong binding interaction with AT-rich DNA sequences. Notably, N_3 -TO-DiplatinNC and N_3 -TO-TriplatinNC exhibit only half the degree of stabilisation as the PPCs and N_3 -PPCs with ΔT_m values of +5.01 °C and +6.82 °C on poly[d(A-T)₂]. Interestingly, when following the orange and red markers corresponding to the thermal melting of N_3 -TO-DiplatinNC and N_3 -TO-TriplatinNC respectively, in Figure 64, a secondary melting event is observed for these two complexes at 23 – 25 °C. This corresponds to the biphasic melting of the thiazole orange moiety from the nucleic acid.

The thermal melting studies of the dinuclear and trinuclear PPCs, N₃-PPCs, and N₃-TO-PPCs on calf-thymus DNA are shown in Figure 64B. Calf-thymus DNA has a much higher intrinsic thermal melting temperature (74.84 °C) than poly[d(A-T)₂] (of 61.49 °C) due to the increase of GC content (42%). N₃-DiplatinNC and N₃-TriplatinNC demonstrate high ΔT_m values of +15.42 °C and +14.64 °C respectively indicating that the azide-labelled series have stronger binding interactions DNA polymers with mixed AT/GC content. DiplatinNC, TriplatinNC, N₃-TO-DiplatinNC, and N₃-TO-TriplatinNC display high ΔT_m values of +14.62 °C, +9.05 °C, +10.54 °C, and +9.64 °C respectively. TriplatinNC displays the lowest ΔT_m value which highlights the effect of the azide modification, TO modification (or both) on the strength of the interaction of these drugs with mixed AT/GC polymers.

ML-DNA is DNA which has been extracted from bacteria with 72% GC content (28% AT). This GC-rich DNA has a high intrinsic thermal melting temperature of ~99.5 °C.¹⁰ In order to study the strength of the interactions of the dinuclear and trinuclear PPCs, N₃-PPCs, and N₃-TO-PPCs with ML-DNA, 350 mM sodium pyrophosphate (Na₄P₂O₇) was added to each sample prior to analysis. It was hypothesized that the addition of a bulky anion such as pyrophosphate would destabilize the anionic DNA phosphate backbone and thereby decrease the thermal melting temperature of GC-rich DNA. Addition of 350 mM sodium pyrophosphate resulted in the intrinsic thermal melting temperature of ML-DNA to reduce to 82.23 °C (Figure 64C). Thermal melting analyses of the PPCs, N₃-PPCs, and N₃-TO-PPCs on ML-DNA was then performed then using 350 mM sodium pyrophosphate (Figure 64D). Results from this study show that all dinuclear and trinuclear complexes have a modest stabilising effect on this GC-rich DNA. N₃-DiplatinNC demonstrates the greatest ΔT_m value of + 3.10 °C. DiplatinNC and TriplatinNC stabilize the ML-DNA with identical efficiency with ΔT_m values of + 2.48 °C.

Overall, results from this study clearly shows that these complexes all have strong DNA binding interactions with poly[d(A-T)₂] and calf-thymus DNA. None of the complexes presented here displayed high stabilising effects towards the GC-rich ML-DNA. In general, it appears a mixture of A-T and G-C bases are

required to achieve the highest interactions and this observation is in line with earlier work showing limited binding modes of TriplatinNC complexes (backbone tracking and groove spanning) is dependent on a mixture of tracts.⁴ Finally, the thiazole orange labelled complexes also display a distinct enhancement in their ability to stabilise the denaturation of calf-thymus DNA rather than either of the AT-rich or GC-rich sequences tested.

Table 15. Thermal melting temperatures of test compounds with dsDNA varying A-T content

	poly[d(A-T) ₂] (100% AT)		calf-thymus DNA (58% AT)		ML-DNA (28%% AT)	
	T _m	ΔT _m	T _m	ΔT _m	T _m	ΔT _m
DiplatinNC	73.94	+ 12.45	89.45	+ 14.62	85.71	+ 2.48
TriplatinNC	74.02	+ 12.53	83.88	+ 9.05	85.71	+ 2.48
N ₃ -DiplatinNC	72.90	+ 11.41	90.25	+ 15.42	86.33	+ 3.10
N ₃ -TriplatinNC	72.44	+ 10.95	89.47	+ 14.64	84.48	+ 1.25
N ₃ -TO-DiplatinNC	66.50	+ 5.01	85.37	+ 10.54	85.09	+ 1.86
N ₃ -TO-TriplatinNC	68.31	+ 6.82	84.47	+ 9.64	85.79	+ 2.56

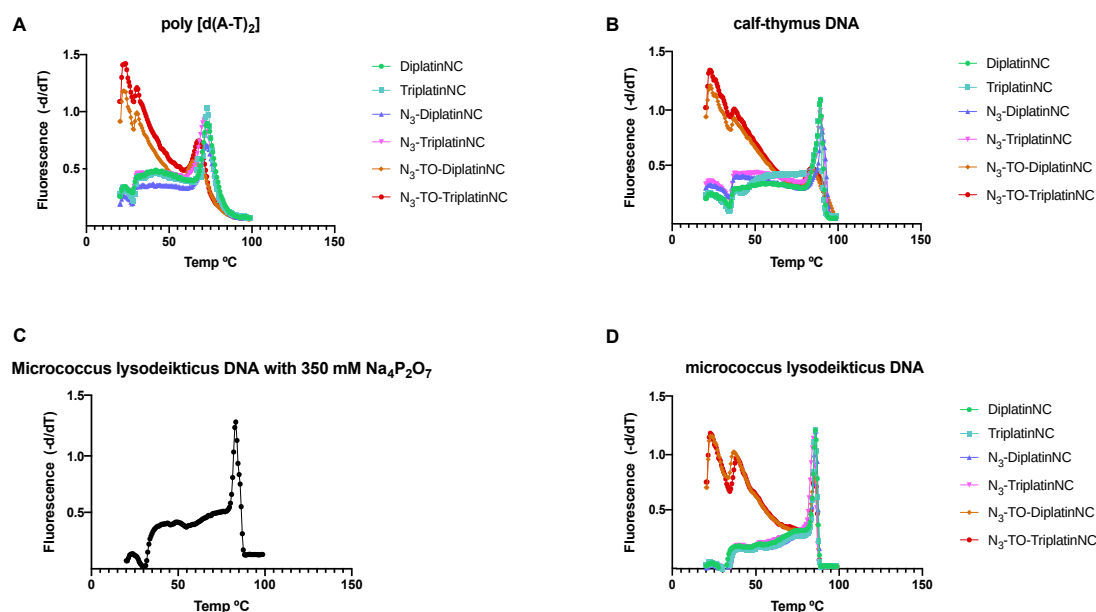


Figure 64. Thermal melting analysis of the dinuclear and trinuclear PPCs, N₃-PPCs, and N₃-TO-PPCs on A. poly[d(A-T)₂]; B. calf-thymus DNA; D ML-DNA. C. Shows the thermal melting profile of ML-DNA with 350 mM sodium pyrophosphate.

6.4. Conclusions

DNA binding studies of the PPCs, N₃-PPCs, and N₃-TO-PPCs have revealed that all dinuclear and trinuclear test compounds exhibit significant DNA binding affinity towards calf-thymus DNA with K_{app} values all in magnitude of $\sim 10^7$ M(bp)⁻¹. N₃-TO-DiplatinNC and N₃-TO-TriplatinNC display preferential binding for AT-

rich DNA which is in contrast to the non-functionalised PPCs—DiplatinNC and TriplatinNC—which show preferential binding to GC-rich DNA sequences in this assay. Thermal melting studies revealed that all test complexes demonstrate a strong nucleic acid binding interaction with poly[d(A-T)₂] DNA and calf-thymus DNA, however, this effect becomes less pronounced for the GC-rich ML-DNA. N₃-DiplatinNC and N₃-TriplatinNC have greater stabilising effects on calf-thymus DNA (58% AT content) compared to either the AT-rich polymer poly[d(A-T)₂] or the ML-DNA (28% AT content) demonstrating that these N₃-PPCs interact strongest with mixed base composition nucleic acids.

6.5. Experimental

Competitive Ethidium Bromide (EtBr) Displacement Assay

Experiments were performed according to literature methods reported by Kellett and co-workers with modifications.^{4, 5} A working solution of 20 µM calf thymus DNA (ctDNA, $\epsilon_{260} = 12,823 \text{ M}^{-1} (\text{bp})^{-1}$), poly[d(A-T)₂] DNA $\epsilon_{262} = 6650 \text{ M}^{-1} \text{ cm}^{-1}$) or poly[d(G-C)₂] DNA ($\epsilon_{254} = 8400 \text{ M}^{-1} \text{ cm}^{-1}$) along with 25.2 µM ethidium bromide, 40 mM NaCl in 80 mM HEPES buffer (pH 7.2) was prepared. Stock solutions of the platinum(II) complexes were initially dissolved in DMF and further diluted using HEPES buffer. 50 µL of the working solution was placed in each well of a 96 well microplate with the exception of blanks. Varying concentrations of the N₃-PPC and N₃-TO-PPC series were added to each well containing the working solution and the final volume was adjusted to 100 µL using HEPES buffer. The microplate was incubated for 1 hr at ambient temperature before analysed using a Bio-Tek synergy multi-mode microplate reader with the excitation and emission wavelengths set to 530 nm and 590 nm respectively. Each drug concentration was measured in triplicate, on at least two separate occasions and the apparent DNA binding (K_{app}) constants were calculated using the equation $K_{\text{app}} = K_{\text{b}} \times 12.6 / C_{50}$ where K_{b} is the intrinsic binding constant of ethidium bromide where $K_{\text{b}} = 8.80 \times 10^6 \text{ M}(\text{bp})^{-1}$.

Competitive DNA Fluorescence Quenching Assay

Experiments were performed in a similar manner to what was reported by Kellett and workers with slight modifications.^{4, 5} A working solution of 50 μM calf thymus DNA (ct-DNA, $\epsilon_{260} = 12,823 \text{ M (bp)}^{-1} \text{ cm}^{-1}$), poly[(dA-dT)₂] DNA or poly[(dG-dC)₂] DNA, along with 10 μM ethidium bromide and 40 mM NaCl in HEPES buffer (pH 7.2) was prepared. Stock solutions of the platinum(II) complexes were initially dissolved in DMF followed by further dilution in HEPES buffer. 50 μL of the working solution was added to each well in a 96 well microplate with the exception of the blanks. Varying concentrations of the platinum(II) complexes were added to each well containing the working solution and final volume was adjusted to 100 μL using 80 mM HEPES buffer. The microplate was incubated for 1 hr at ambient temperature before analysis using a Bio-Tek synergy HT multi-mode microplate reader with excitation and emission wavelengths being set to 530 and 590 nm respectively. Each drug concentration was measured in triplicate on at least two separate occasions. From a plot of percentage fluorescence versus drug added, the quenching value (Q) was given by the concentration required to effect 50% removal of the initial fluorescence of DNA bound dye.

Thermal Melting Analysis

Fluorescence melting experiments were conducted on a LightCycler 480 Real-Time PCR Detection System (Roche) using LightCycler 480 software. In a final volume of 20 μL , 100 μM ct-DNA, poly[d(A-T)₂] DNA or *Micrococcus lysodeikticus* DNA (ML-DNA) were incubated with 10 μM of the platinum(II) complexes and incubated for 1 hr at 37 °C. The samples were then added with a 1 μL aliquot of SYBR Green DNA stain. Samples were denatured by heating to 95 °C at a rate of 0.1 °C/s then held at 95 °C for 5 min before cooling to 25 °C at the same rate. Samples were held at 25 °C for 5 min then denatured by heating to 95 °C at a rate of 0.6 °C/min before holding at 95 °C for 5 min. The samples were then cooled to 25 °C at the same rate. The fluorescence melting curve data was converted to the first derivatives to give the melting temperatures ($-d(F)/dT$ where F is the fluorescence and T is the temperature in °C).

6.6. References

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Chapter VII

**In-vitro, in-vivo cytotoxic analysis, and cellular
localisation studies**

7. Results and Discussion

7.1. *In vitro* cytotoxic analysis of N₃-PPCs

N₃-MonoplatinNC, N₃-DiplatinNC and N₃-TriplatinNC were selected for in-vitro cytotoxicity screening. Cytotoxicity assays were performed in collaboration with Prof. Afshin Samali and Prof. Adrienne M. Gorman at the Apoptosis Research Centre, NUI Galway, Ireland. Cytotoxicity experiments were conducted by Dr. Creina Slator and Dr. Katarzyna Mnich.

Recalcitrant breast cancer cell lines MCF-7, T-47D, MDA-MB-231, MDA-MB-468 were selected for cytotoxic analysis. T-47D is a ductal carcinoma while all other cancer lines are adenocarcinomas. Each line is further categorised into molecular sub-types based expression levels, grade and prognosis (Table 7). All cancerous lines are metastatic, particularly in the build-up of fluid in the space that surrounds the lungs known as pleural effusion. The mutation status of other key biomarkers such as TP53, a tumour receptor protein that regulates DNA damage, are also listed, which can limit the use of combination therapies which hone these particular cellular targets. Triple negative breast cancer (TNBC) cells MDA-MB-231 and MDA-MB-468 lack expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER-2), three of the most common receptors that stimulate cancer growth. Of all breast cancer subtypes, TNBC demonstrates the most aggressive behaviour with the worst prognosis with limited treatment options as standardised targeted and immunotherapies rely on receptors that are not expressed in TNBC. An immortalised non-tumorigenic breast epithelial cell line, MCF-10a, was also selected and exposed to each of the N₃-PPC complexes as a control to determine the cytotoxic activity of the drugs in non-cancerous cells.

Table 6. Cell line characteristic, receptor expression and mutation status

Cell lines	ER	PR	HER2	Mutation status	Sub-type	Other mutations
MCF-7	+	+	-		Luminal A	CDKN2A, PIK3CA
T-47D	+	-	-		Luminal A	TP53, PIK3CA
MDA-MB-231	-	-	-	TNB	Mesenchymal	TP53, CDKN2A, BRAF, KRAS, NF2
MDA-MB-468	-	-	-	TNA	Basal-like 1	TP53, PTEN, RB1, SMAD4
MCF-10a	-	-	+			

All cell lines were exposed to each of the N₃-PPC complexes at concentrations between 1-100 μ M with cell viability and IC₅₀ values determined. Results from this study are displayed in Figure 65. After 48 hrs of continuous exposure, cisplatin displayed an IC₅₀ value of 18.5 μ M against MCF-7 cells, while N₃-DiplatinNC and N₃-TriplatinNC both show enhanced cytotoxicity with IC₅₀ values of 16.6 μ M and 9.4 μ M respectively. Similarly, N₃-DiplatinNC and N₃-TriplatinNC show increased cytotoxicity (9.2 μ M and 5.4 μ M, respectively) in MDA-MB-231 cells in comparison to cisplatin (61.7 μ M). N₃-MonoplatinNC does not display any notable cytotoxic activity against any of the other cell lines tested. This may be attributed to the lower ability of N₃-MonoplatinNC to bind with DNA and induce conformational changes such as condensation. Cisplatin was shown to retain the most effective antiproliferative activity in MDA-MB-468 cells with IC₅₀ of 3.5 μ M after 48 hrs. N₃-TriplatinNC exhibits impressive antiproliferative activity across all cell lines tested and is the most potent anticancer therapeutic of the N₃-PPC series. The remarkable cytotoxic activity of N₃-TriplatinNC may be ascribed to the increased number of platinum(II) metal centres and thus increased number of platinum(II)-tetra(m)mine DNA binding sites of the trinuclear complex when compared to N₃-MonoplatinNC and N₃-DiplatinNC. Additionally, polynuclear platinum(II) compounds of this nature have previously exhibited enhanced cellular uptake with increasing charge,¹ which indicated that the N₃-PPC series might follow a similar trend. Increased cellular uptake directly corresponds to increased complex activity, allowing more of the complex to reach the DNA target. The cytotoxicity of the N₃-PPC series follows the general

trend of N₃-TriplatinNC > N₃-DiplatinNC > N₃-MonoplatinNC which may also directly correlate to the solution charge trend in the series of 7+ > 5+ > 3+.

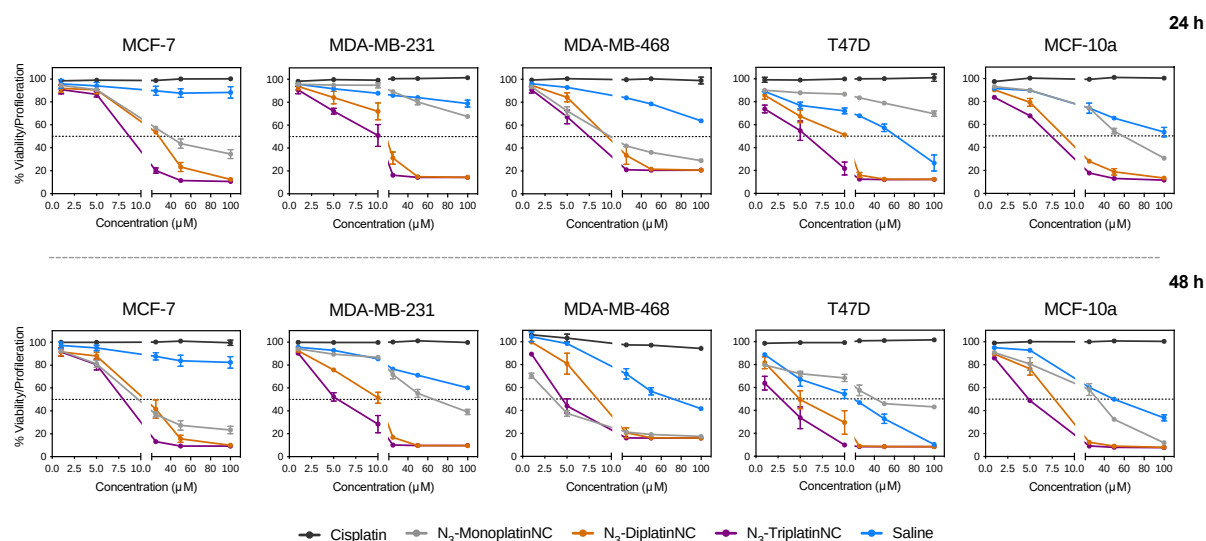


Figure 65. Cell viability expressed as normalized percent of viable cells relative to controls of cisplatin, NaCl and 1,10-phenanthroline. Top and bottom. Cell lines treated with N₃-PPC and control compounds over 24 hr and 48 hr incubations.

Table 7. IC₅₀ values for the N₃-PPC series.

IC ₅₀ (μM)	24 h				48 h			
	cisplatin	N ₃ -Mono	N ₃ -Di	N ₃ -Tri	cisplatin	N ₃ -Mono	N ₃ -Di	N ₃ -Tri
Cell line								
MCF-7	40.3	>100	22.7	11.5	18.5	>100	16.6	9.4
MDA-MB-231	>100	>100	14.1	9.2	61.7	>100	9.2	5.4
MDA-MB-468	22.2	>100	16.6	10.1	3.5	69.3	12.1	5.2
T-47D	>100	47.6	7.8	4.9	40.7	17.5	4.4	2.2
MCF-10a	56.1	95.4	12.8	8	25.1	46.5	8.5	4.6

7.2. N₃-PPC click labelling localisation studies

Click chemistry enabled intracellular tracking in MDA-MB-231 cells through N₃-appended PPCs. The click handle was identified to undergo copper azide-alkyne cycloadditions (CuAAC) with a fluorescent label to determine selective localisation in TNBC cells (Figure 66A). MDA-MB-231 cells were exposed to the most cytotoxic agent in the PPC series, N₃-TriplatinNC, for 24 h at 10 μM prior to fixation. Samples were then subjected to copper(I) catalysed click chemistry with a 1:1 ratio of alkyne fluorophore in the presence CuSO₄ and added

ascorbate. Distinct organelles such as nuclei and mitochondria were counterstained to distinguish the overall cell structure and compartmental accumulation of the trinuclear complex. Non-specific uptake of the unclicked dye was not observed in non-treated control cells as no fluorescence was detected (Figure 66B). There was little or no overlap of fluorophore-labelled N₃-TriplatinNC with nuclear chromatin and mitochondrial structures. Predominant and uniform accumulation was shown in non-DNA regions within the nucleus suggesting localisation in the nucleolus whose function is ribosomal RNA biogenesis and synthesis. This was validated through immunofluorescent staining of nucleophosmin (NPM) – also known as B23 – a protein nucleolar phosphoprotein involved in diverse cellular processes such as ribosome biogenesis. It shuttles between the nucleus and cytoplasm but predominately resides in the perimeter of the nucleoli. NPM was detected using a monoclonal mouse anti-NPM as the primary antibody which was subsequently stained with a goat anti-mouse IgG secondary antibody fragment conjugated to a complementary fluorophore. Clear co-staining of NPM displayed distinct sub-compartmental nucleolar accumulation of N₃-TriplatinNC. This is not surprising given the similarity to the non-functionalised parent compound, TriplatinNC. Work conducted by Peterson *et al.* found that PPC interacts with rRNA resulting in rapid uptake and accumulation in nucleolar compartments.² In samples presented in Figure 66B, morphological changes characteristic of apoptotic cell death is indicated with the formation of apoptotic bodies and blebbing caused by membrane degradation in which high concentrations of the click-labelled complex was observed. Evidence of distinct voids, most likely cytosolic vacuoles, suggests that selective organelle accumulation may depend on cationic complex charge. Further investigations are required to determine if nuclearity and overall cationic charge determine localisation properties of N₃-MonoplatinNC and N₃-DiplatinNC. If so the pharmacokinetics of cellular uptake should be directly compared to cytotoxicity given the varying activity profiles within the N₃-PPC series.

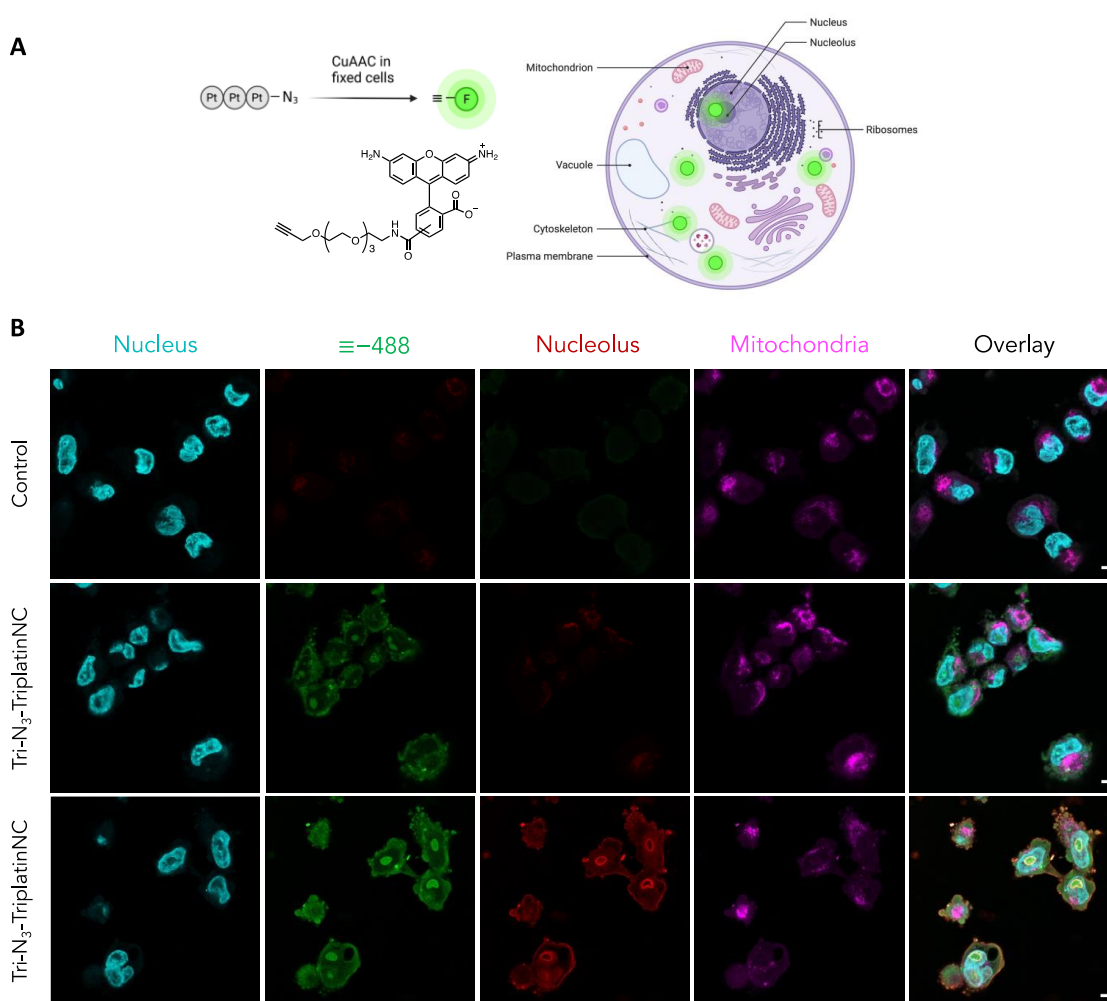


Figure 66A. Intracellular fluorescent labelling of N₃-PPC through CuAAC chemistry to examine cellular uptake and localisation; B. Confocal microscope images on 63x oil immersion lens of fixed MDA-MB-231 cells exposed to 10 μ M of N₃-TriplatinNC for 24 h and click to alkyne 488 dye (green). Nuclei were stained with NucBlue Fixed (blue), mitochondria were stained with MitoTracker Deep Red (magenta) and nucleoli were visualised by immunofluorescent staining of NPM (red). Scale bars indicate 10 μ m (overlay, bottom right corner).

7.3. *In vivo* cytotoxicity studies of N₃-TriplatinNC in mice bearing MDA-MB-231 xenografts

Following the impressive *in-vitro* cytotoxicity of N₃-TriplatinNC in the triple negative breast cancer cell line MDA-MB-231, *in vivo* cytotoxicity studies were conducted on mice bearing MDA-MB-231 breast cancer xenografts. MDA-MB-231 cells are commonly used to model triple negative breast cancer as this cell line is oestrogen receptor, progesterone receptor, and HER2 negative. This cancer cell line is particularly invasive *in-vitro* and when animal models are implanted with these cells to produce xenografts, the tumours undergo metastasis and can spread to the lymph nodes.³ *In-vivo* studies were conducted in collaboration with Prof. Nicholas Farrell at Virginia Commonwealth University.

In-vivo experiments were conducted by Dr. Creina Slator, Dr. Bin Hu, and Dr. Jennifer E. Koblinksi at The Department of Pathology, The Massey Centre, Virginia Commonwealth University.

7.3.1. Maximum Tolerated Dose (MTD)

In vivo preclinical analysis was conducted in NCG mice for the lead compound N₃-TriplatinNC. Initially acute toxicity analysis was used to determine the maximum tolerated dose for the drug which was administered *via* intraperitoneal injection on day 1, 5 and 9 of the study in 3 animals per group at 1, 5 and 10 mg/kg in saline (Figure 67A). Tolerance of dose and the health of the animals were continuously monitored for a further 15 days and represented as relative body weight (Figure 67B). Although no decrease in body weight was observed in animals administered with 10 mg/kg, behavioural changes were noted which resulted hypoactivity manifesting as slow locomotor activity and was classified as mildly severe. As such, a dose of 5 mg/kg was selected for further analysis given the additional burden of tumour implantation and growth in future studies.

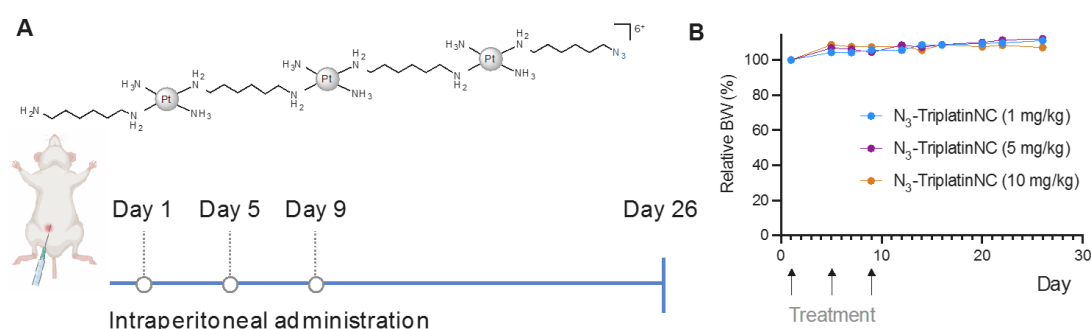


Figure 67A. Experimental plan of maximum tolerated dose study for N₃-TriplatinNC; B. Relative body weight (BW) expressed as percent.

7.3.2. Xenograft models

MDA-MB-231 *luc* cells (2×10^4) were implanted through intraductal (ID) injection into the mammary glands of NCG mice. Tumours started to develop and were allowed to grow to a minimum volume of 50 mm³ (Figure 68A). Tumour size was manually measured as volume and quantified using luciferin bioluminescence where MDA-MB-231 cells were transfected with a recombinant vector resulting in luciferase expression. Cells were then injected with D-luciferin which reacted with luciferase to generate a chemiluminescent reaction (Figure 68B). The

luminescent flux of photons generated in the tumour was detected through next-generation bioluminescence optical *in vivo* imaging known as IVIS. Mice were then randomised to groups of 5 and treated with saline vehicle in the negative control cohort, 5 mg/kg of N₃-TriplatinNC or 40 mg/kg of positive control carboplatin as an established clinical agent and then monitored for a further 20 days. Mice were euthanized when tumour size impeded the welfare of the animals (including drug treated mice).

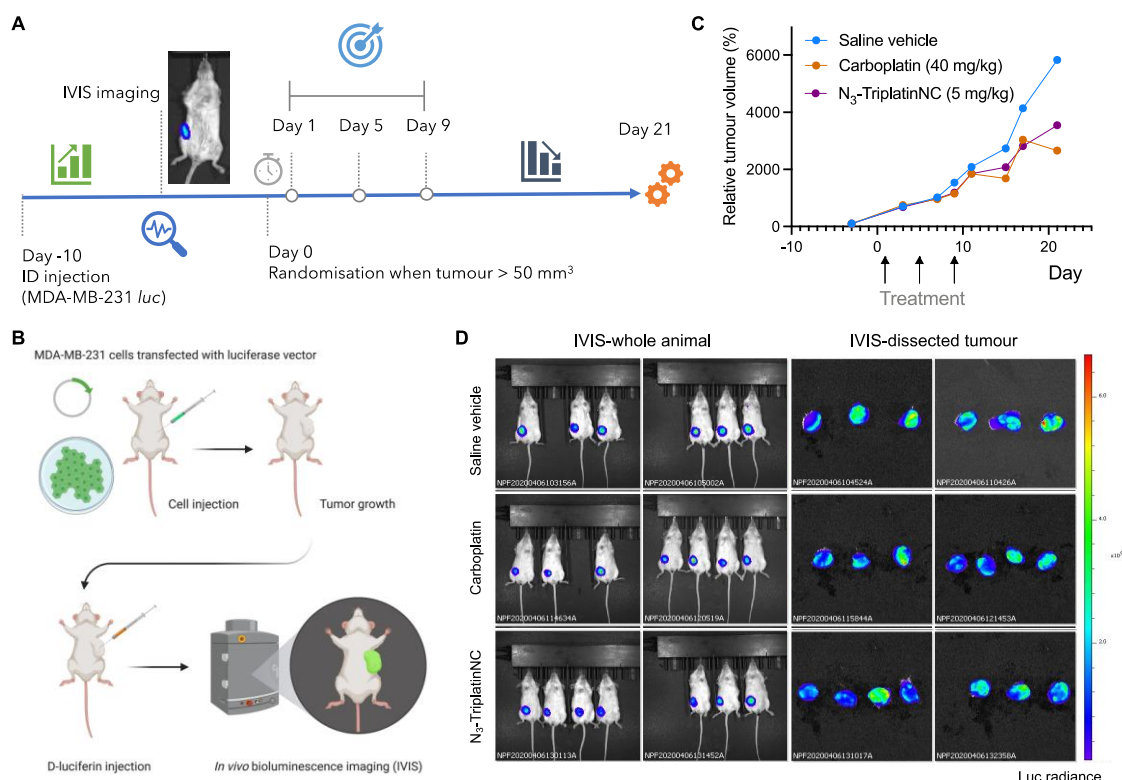


Figure 68A. Experimental set-up and timeline of xenograft work; B. Transfection, culture, and injection of MDA-MB-231 luciferase (luc) cells for inherent tumour detection; C. Relative tumour volume is represented as a percentage; D. Bioluminescent imaging of tumours in whole animals on day 21 (endpoint) and dissected samples.

Analysis of primary tumour growth showed regression and presented as relative tumour volume (Figure 68C). Luminescent detection of cancer cells similarly corroborate reduction in tumour size in drug-treated mice (Figure 68D). The trinuclear complex show comparable results with the clinical standard although at lower dose concentrations that could potential overcome common side effects such as nausea, pain or serve allergic reactions. Furthermore, carboplatin shares cross-resistance with cisplatin (both drugs are effective against the same population of solid epithelial tumours) limiting the efficacy of platinum drugs currently available in clinical treatment. Thus, N₃-TriplatinNC is

promising drug candidate that can mitigate acquired and innate chemoresistance and recurrence of TNBC which may not be sensitive to traditional platinum therapies.

7.4. Conclusion

From the high-throughput screen of mutated breast cancer cell lines, both N₃-DiplatinNC and N₃-TriplatinNC were identified as novel treatment avenues for TNBC. The trinuclear drug candidate has also demonstrated the potential to treat recurrent and metastatic cancers and those that have developed acquired cis-type platinum resistance, particularly in cancers with similar characteristics to MDA-MB-231 and T-47D. N₃-TriplatinNC can also be used as a frontline treatment of TNBC and chemoresistant breast cancers as shown in pre-clinical analysis of xenograft breast cancer models. Tumour regression was comparable to that of clinical standard carboplatin at significantly lower dose concentrations. Finally, N₃-TriplatinNC was identified to target nucleophosmin—a nucleolar phosphoprotein—within the nucleolus which can now be used to selectively target resistant cancers or those with mutated DNA damage repair pathways.

7.5. Experimental

Cytotoxicity assays were performed in collaboration with Prof. Afshin Samali and Prof. Adrienne M. Gorman at the Apoptosis Research Centre, NUI Galway, Ireland. Cell viability experiments were conducted by Dr. Creina Slator and Dr. Katarzyna Mnich. Preclinical *in vivo* studies including orthotopic breast cancer models were conducted in Virginia Commonwealth University (VCU) Massey Cancer Centre in collaboration with Prof. Nicholas Farrell and Prof. Jennifer Koblinski. All animal handling, experimental procedures and interventions, anaesthesia and euthanasia were performed by Dr. Bin Hu.

Cell culture and treatments

MCF-7, MDA-MB-231 and MDA-MB-468 cells were maintained in high glucose DMEM and T47D were maintained in RPMI-1640. All media were supplemented with 10% FBS, 1% L-glutamine and 50 units/mL penicillin and 50 mg/mL streptomycin. MCF-10a were cultured in DMEM/F-12 supplemented with 5% horse serum, 20 ng/mL epidermal growth factor, 100 ng/mL cholera toxin, 0.01 mg/mL insulin and 500 ng/mL hydrocortisone. Cells were maintained at 37 °C

and 5% CO₂ in a humidified atmosphere. Complex stock solutions were prepared in 0.9% saline (w/v) and stored at 4 °C for up to 1 week. Further dilutions were prepared in culture media daily.

Cell viability assay

Seeding densities and resazurin (Alamar Blue) incubation times were predetermined based on Z-score analysis (> 0.6). MCF-7, MDA-MB-231, MDA-MB-468 and MCF-10a cells were seeded at 5000 cells per well in a 96-well plate, while T47D cells were seeded at 7500 cells per well for 18 h prior to drug treatment. Cells were exposed to platinum complexes at 1-100 µM using an automated liquid handling system (JANUS Automated Workstation) and incubated for 24 or 48 h. A 1:10 dilution of resazurin reagent (stock concentration of 5.6 mM, Sigma R7017) was added and incubated for a further 4 h (MCF-7) or 6 h (MDA-MB-231, MDA-MB-468, T47D and MCF-10a). Fluorescence was measured on an integrated Victor X5 Multilabel Reader (ex: 530 nm, em: 620 nm). Cell viability was expressed as normalised percent of viable cells relative to non-treated control and IC₅₀ values were calculated in GraphPad Prism.

Confocal analysis for N₃-TriplatinNC localisation

MDA-MB-231 cells were treated with 10 µM N₃-TriplatinNC for 24 h then stained with 150 nM MitoTracker Deep Red for 20 min at 37 °C. Cells were fixed in 4% paraformaldehyde (30 mins, RT) and permeabilized with 0.25% Triton X-100. Samples were then blocked with 2% BSA for 30 mins at RT and clicked with 10 µM Fluor 488-alkyne in the presence of CuSO₄ (10 mM) and ascorbate (20 mM) for 30 min at RT and then counter-stained with NucBlue Fixed. Specific localisation protein, nucleophosmin (NPM), was stained to investigate nucleolar localisation. The primary antibody (anti-B23 antibody, mouse monoclonal antibody, B0556-25UL, Merck) was incubated at 1:500 dilution, RT for 2 h followed by secondary antibody (Alexa Fluor 594 goat anti-mouse IgG F(ab)₂ fragment, A11020, ThermoFisher Scientific) at 1:1000 dilution, RT for 1 h. Cells were washed twice with PBS in between each step. Samples were finally mounted in ProlongGold and allowed to cure.

Confocal samples were imaged on a STED-Leica DMI8 super resolution confocal microscope equipped with a CCD camera. NucBlue Fixed was excited

with a 405 nm picoquant laser unit and emission captured between 450 - 550 nm. Fluor 488-alkyne ($\equiv$ -488) was excited at 488 nm (laser power 1) with emission captured between 580 - 625 nm, and MitoTracker Deep Red was excited at 637 nm (laser power 3) with emission was captured at 650 - 700 nm. Alexa Flour 594 conjugated secondary antibody was excited at 594 nm (laser power 5) with emission was captured at 640 - 680 nm. Images were acquired whereby combinations of excitation and emission wavelengths for specific dyes were applied sequentially to avoid overlap between photophysical profiles (Figure 69).

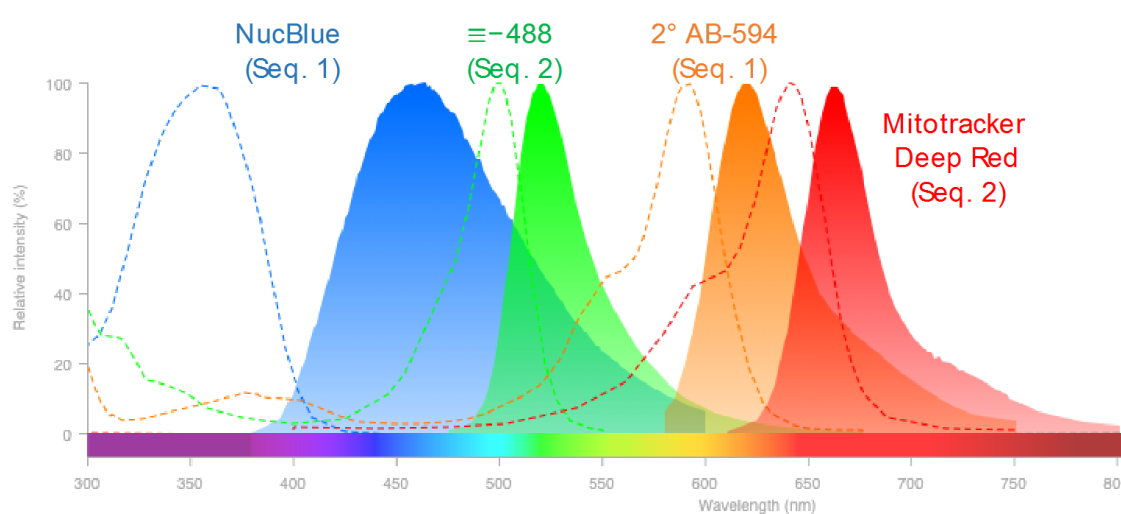


Figure 69. Image sequence (seq.) acquisition order. Dye combinations within seq. 1 or 2 were obtained in parallel.

Maximum tolerated dose (MTD)

Animal work carried out in Virginia Commonwealth University (VCU), USA. Both toxicity and xenograft studies were approved by VCU Institutional Animal Care and Use Committee (IACUC/ Animal Welfare License AD10000943). All research involving animals were conducted in strict accordance of the Animal Welfare Act and regulations as amended (Public Law 99-198 – The Improved Standard for Laboratory Animals Act and Public Law 103-43 – Plan for Use of Animals in Research). Female NCG mice (NOD CRISPR Prkdc Il2r gamma) of 6-8weeks old and weighing ~20 g were purchased from Charles Rivers Laboratories. NCGs are coisogenic immunodeficient models achieved through CRISPR knockout which can host xenograft cells as they lack proper T-cell and B-cell formation and NK cells. Animals were randomised based on body weight

and grouped per dosage with each group containing 3 animals. N₃-TriplatinNC was administered through IP injections in 100 µL volumes containing dose concentrations of 1, 5 and 10 mg/kg on day 1, 5 and 9 after randomisation. After administration the animals were observed for 2 h. After final dose injections, overall health and body weight was monitored for a further 15 days.

Xenograft studies

MDA-MB-231-*luc* cells were cultured in DMEM media supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. Cells already provided in VCU were generated by infection with lentiviruses containing the pFULT vector expressing fire-fly luciferase. MDA-MB-231-*luc* cells (2×10^4) in a PBS:matrigel mixture were injected into the forth right lactiferous duct of 6-8weeks old female mice. Animals were monitored for 1 week after which tumour growth was visualised *via* IVIS imaging by injecting 100 µL of luciferin subcutaneously, waiting 10 mins and using inhalant isoflurane to anesthetise the animals. Mice were randomised to 5 animals per group when tumour volumes reached 50 mm³ on day 0. Animals per group were randomisation on the basis of tumour volume (obtained using a digital calliper and IVIS imaging) and body weight through randomization Mluti-Taks program as described by Hampton *et al.*⁴ N₃-TriplatinNC [5 mg/kg], carboplatin [40 mg/kg] and vehicle [saline] treatments were administered through intraperitoneal (IP) injections on day 1, 5 and 9. All mice were continuously monitored for tumour size, weighed and imaged until day 21. At the endpoint, tumours were harvested, submerged in luciferin solution (0.3 mg/mL) for 5 mins and imaged *ex vivo*.

7.6. References

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Chapter VIII

Future work and outlook

8. Future Work

While the studies presented in this thesis offer good insight into the therapeutic activity and DNA binding properties of the PPCs, N₃-PPCs, and N₃-TO-PPCs, more biophysical experimental work would inform our understanding of exactly the effects that these complexes could have on global DNA structure and topology. Circular dichroism is a UV-based technique that can probe changes in secondary structure of DNA.¹ It is long-since known that many DNA binding agents can infer global changes to DNA conformation, which can have various downstream effects. Circular dichroism studies could be performed to investigate, identify, and interpret whether these complexes are effective at inducing B→Z DNA transitions or B→A DNA transitions with high sensitivity. Comparison of the circular dichroism studies between the PPCs, N₃-PPCs, and N₃-TO-PPCs could also help determine exactly which components of the drug series induce specific changes to the DNA secondary structure.

Future work including cellular accumulation studies of the TO-appended N₃-PPCs would allow for the *in situ* detection of these complexes within the cell—without the requirement for post-labelling covalent modification. The TO should become luminescent when it has reached, and intercalated with its nucleic acid target within the nucleus. Furthermore, comparison of these results with the click labelling studies of the N₃-PPCs would highlight which strategy (pre-labelling or post-labelling) is most efficient for the intracellular localisation of PPCs. Furthermore, this analysis would reveal whether the TO moiety influences the localisation properties of the PPCs in human cells.

While the PPCs, N₃-PPCs, and N₃-TO-PPCs have exceptional clinical potential for the treatment of cancers, this thesis has not addressed the critical issue of their inherent lack of nucleic acid target specificity causing off-target toxicity. With this in mind, gene therapies such as antisense therapy (Figure 70), are emerging as effective, highly specific, personalised treatments with vast therapeutic application. Antisense therapy targets protein production at the transcriptional level and typically involves introducing an oligonucleotide complementary to the target DNA/RNA to prevent the production of proteins involved in the development of genetic diseases.²

The development of polynuclear platinum(II) hybrid biomaterials for antisense therapeutic application could address the issue of off-target toxicity associated with this drug class. They would consist of N₃-functionalised polynuclear platinum(II) complexes conjugated to an alkyne-modified nucleic acid targeting vector such as a peptide nucleic acid (PNA) by 'click' chemistry (Figure 71). PNAs are nucleic acid analogues that feature an N-(2-aminoethyl) glycine peptide backbone in place of the phosphate linkage of DNA/RNA, and retain the ability to store genetic information and form base-pairs with complimentary oligonucleotides.³ PNAs are resistant to degradation by endogenous nucleases and proteases because these enzymes only recognize the phosphate backbone of DNA and do not recognize and bind the pseudo-peptide backbone of PNA.⁴ It is this critical feature which accounts for PNAs high metabolic stability in comparison to triplex-forming oligonucleotides (TFOs). The peptide backbone is neutral in charge and thereby prevents any "phosphate clamp" interactions occurring between the polycationic platinum(II) moiety and PNA targeting vector. While PNAs have great clinical potential, they have been associated with limitations such as poor cellular uptake, poor water solubility as well as high sensitivity to local ionic concentrations which can impact their ability to hybridise with target oligonucleotides.⁵⁻⁸ Development of PPC-PNA hybrid biomaterials should address these limitations because (i) PPCs have previously demonstrated enhanced cellular uptake with increasing charge⁹ therefore the overall PPC-PNA hybrid should possess greater cell penetrating abilities (ii) the PPCs are inherently water soluble so overall hybrid solubility should be enhanced and (iii) PPC-PNA hybrids should demonstrate enhanced binding affinity due to the intrinsic affinity of the PPCs for anionic DNA and RNA.

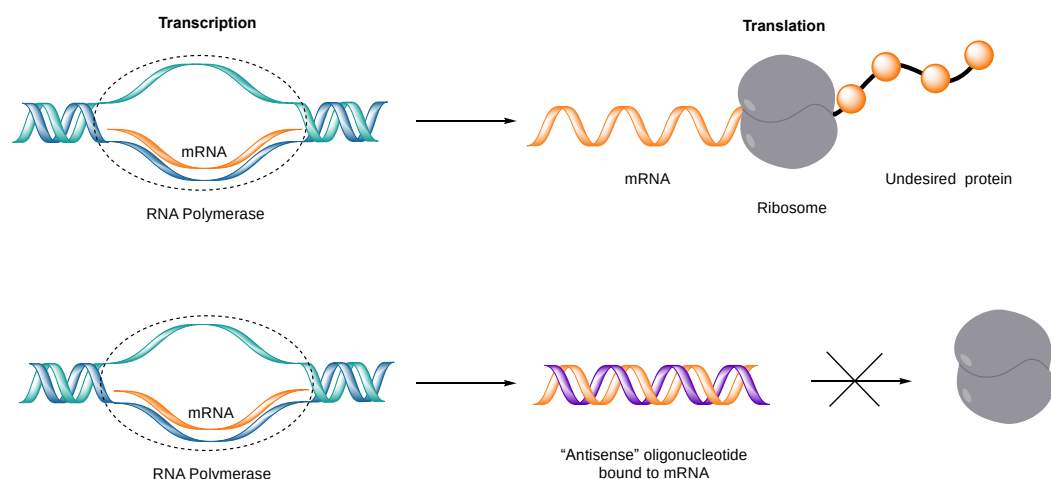


Figure 70. Transcription and translation processes can lead to mutations which contribute to the development of genetic diseases. Antisense therapy presents an effective way of targeting mutated mRNA at the transcriptional level by using an antisense oligonucleotide to bind the mRNA to prevent it being translated in the ribosome to the undesired protein.

Futhermore, by generating PPC-PNA hybrid biomaterials using the N₃-TO-PPCs described in this thesis, the hybrid biomaterials would have a built-in diagnostic probe functionality. These hybrid biomaterials could have vast therapeutic applications for the targeted treatment of genetic diseases.

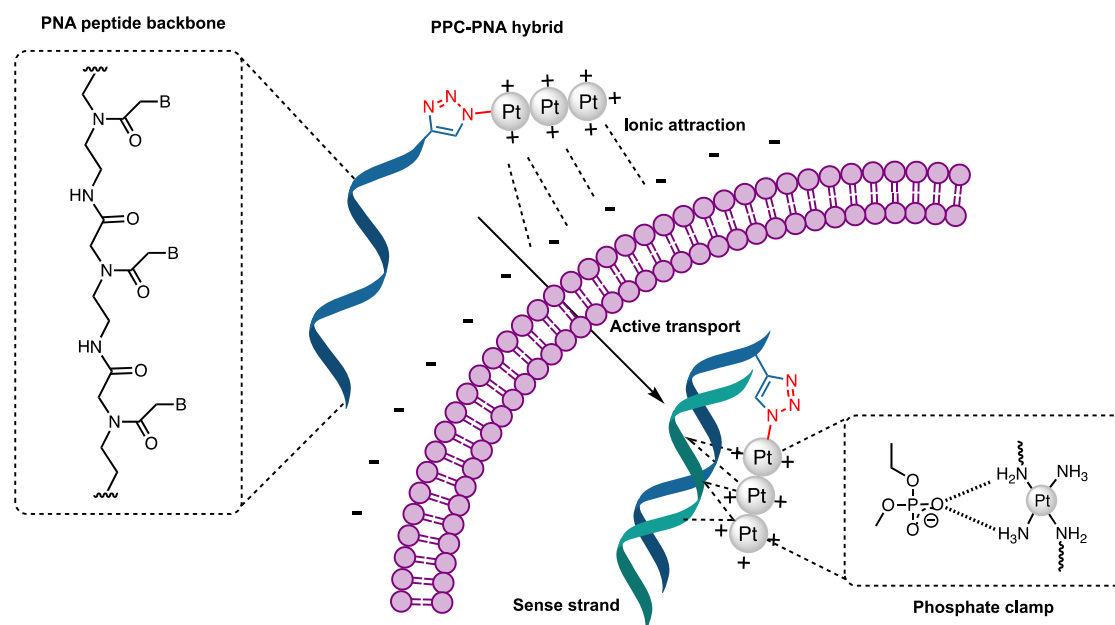


Figure 71. PPC-PNA hybrid biomaterial showing neutral peptide backbone of PNA. The PPC-PNA hybrid should exhibit enhanced cell penetrating ability and thus enhanced cellular uptake due to the electrostatic attraction between the cationic platinum and anionic cell membrane. Once inside the nucleus, the PNA should act as a homing-device and only bind to its complimentary sense strand thereby minimising off-target toxicity. The polynuclear platinum(II) moiety of the hybrid will then be in able to phosphate clamp to the sense strand's phosphate backbone thereby genetically silenced transcripts.

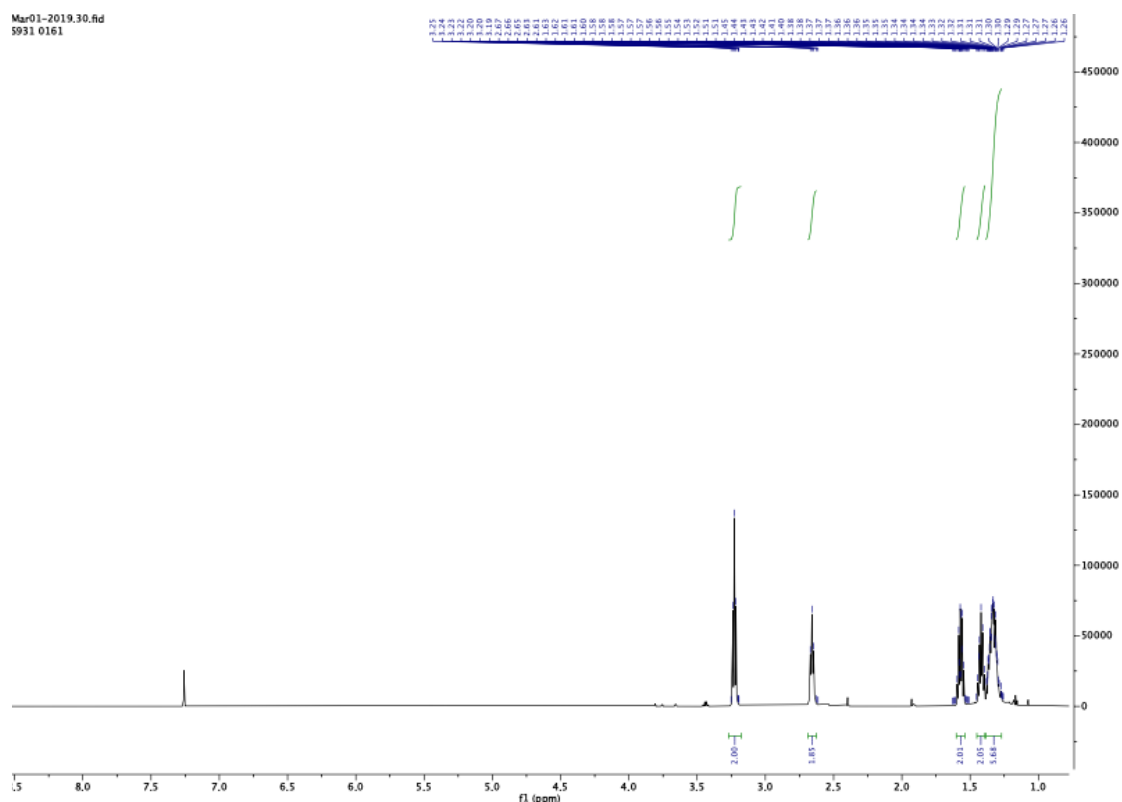
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Appendix A

^1H NMR, ^{195}Pt NMR and FTIR spectra of PPCs, N_3 -PPCs, N_3 -TO-PPCs, and ligands.

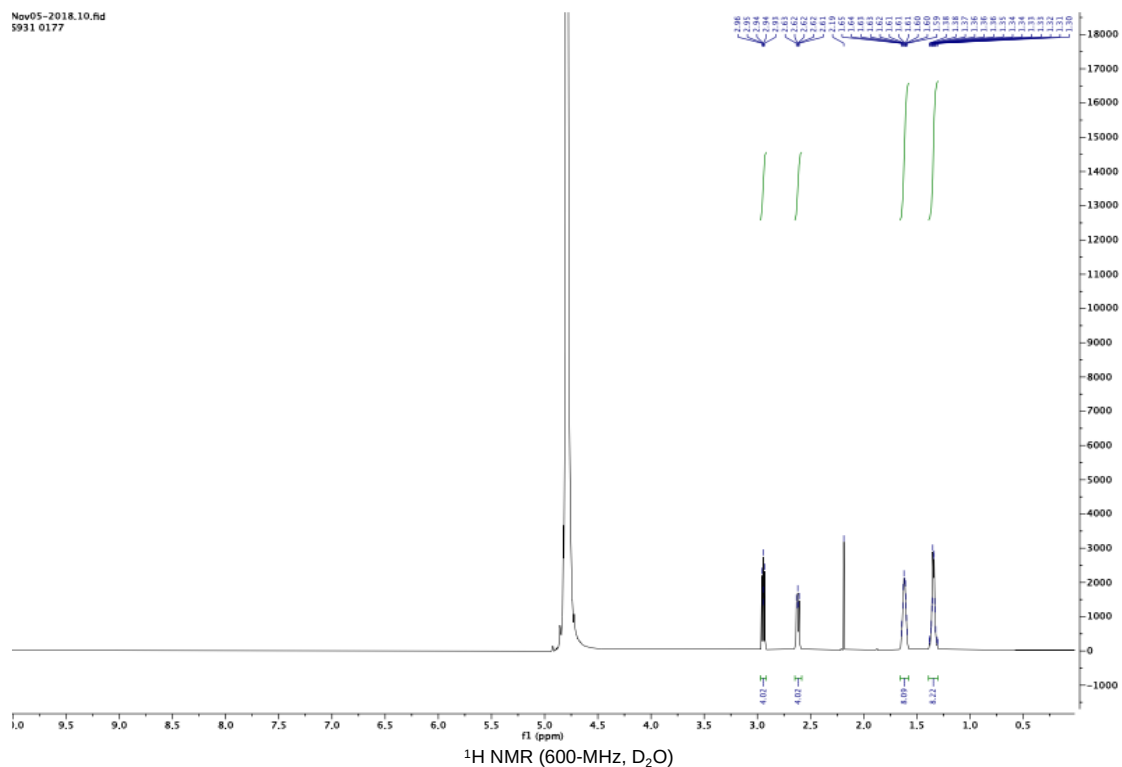
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^1H NMR (600-MHz, CDCl_3)

Figure A- 1. ^1H NMR spectrum of 1-amino-6-azido hexane in CDCl_3 .

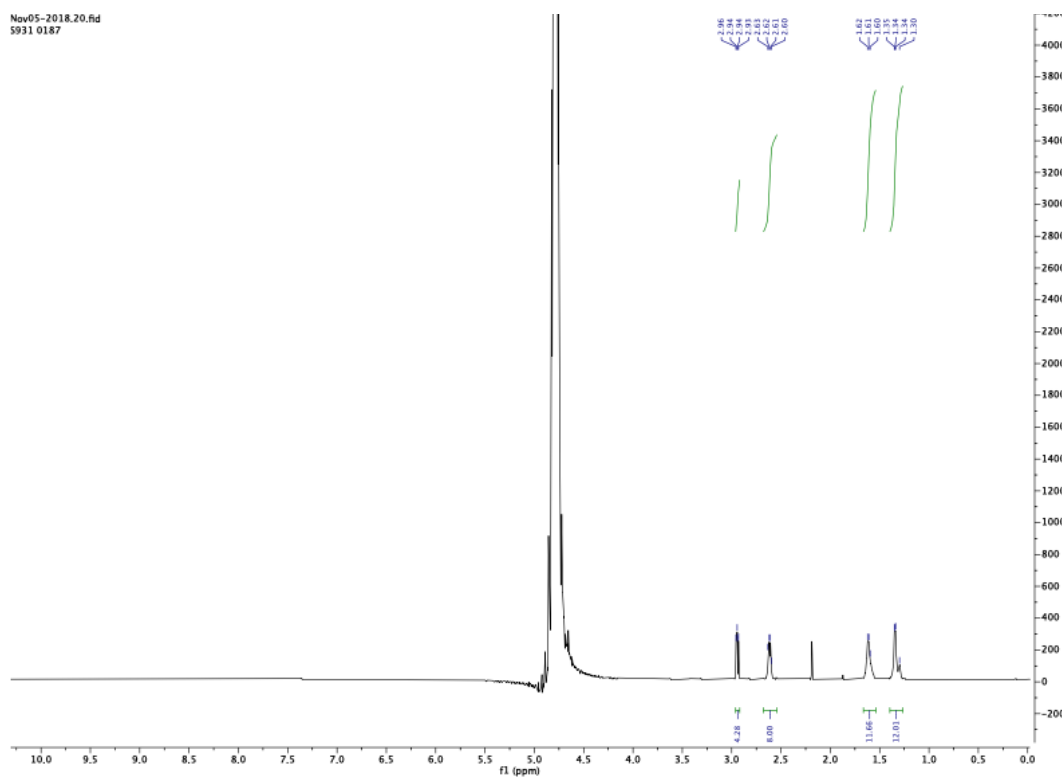
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^1H NMR (600-MHz, D_2O)

Figure A- 2. ^1H NMR spectrum of MonoplatinNC in D_2O .

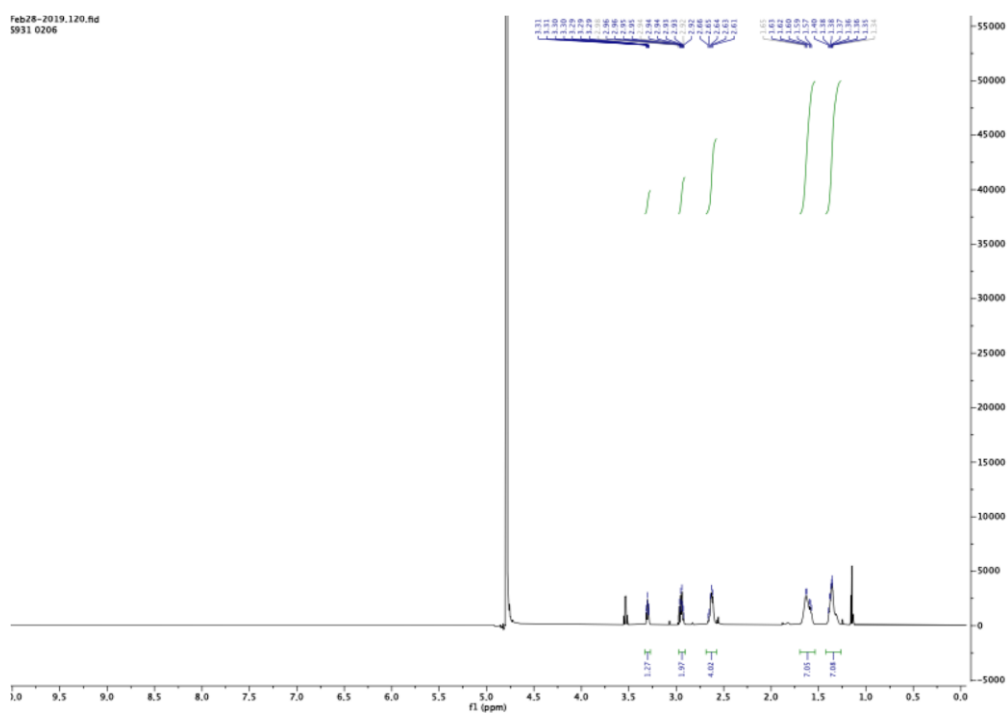
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^1H NMR (600-MHz, D_2O)

Figure A- 3. ^1H NMR spectrum of DiplatinNC in D_2O .

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^1H NMR (600-MHz, D_2O)

Figure A- 4. ^1H NMR spectrum of N_3 -MonoplatinNC in D_2O .

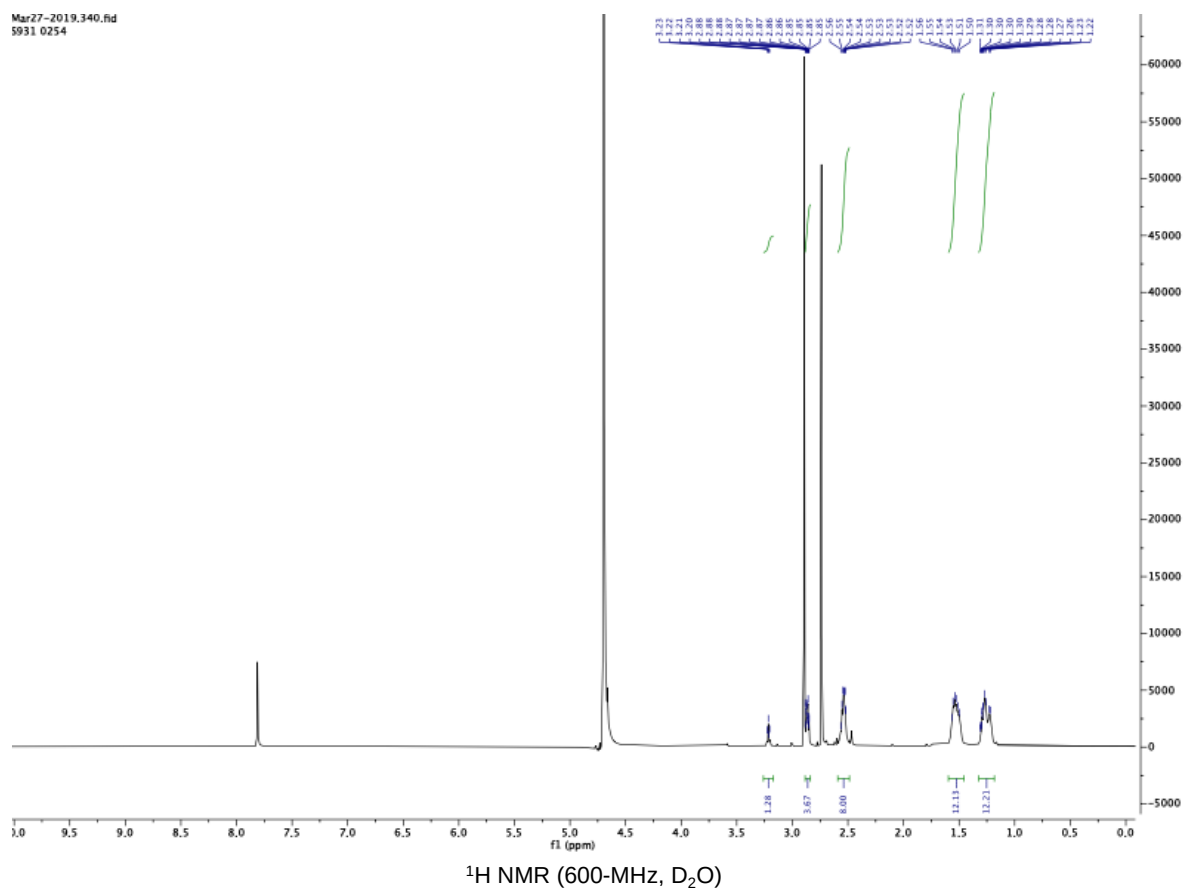
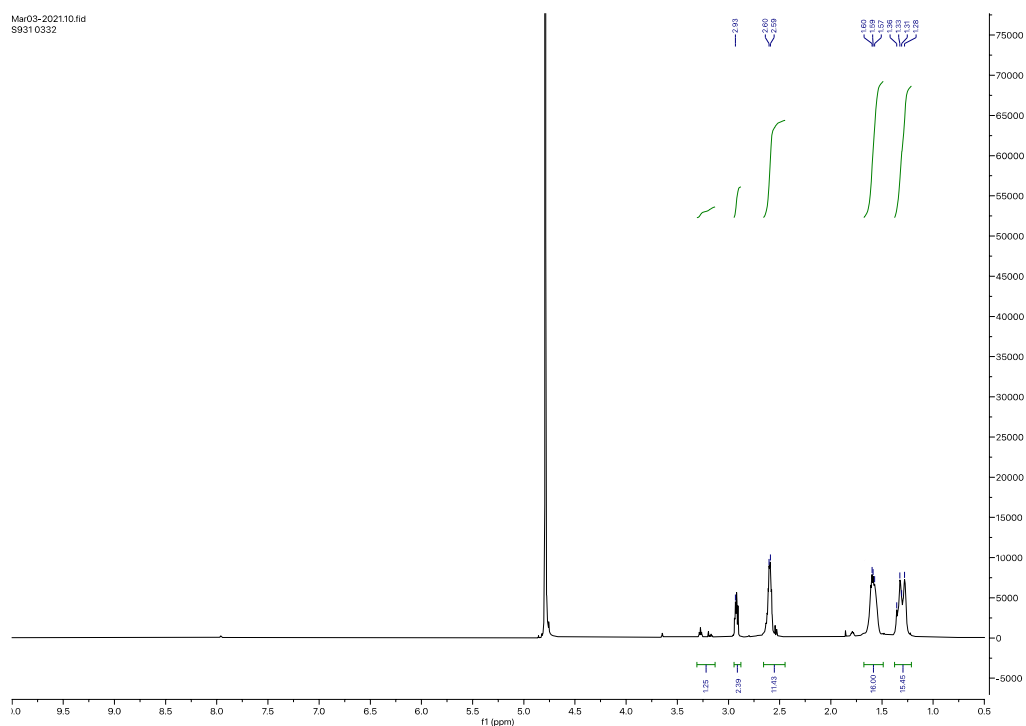


Figure A- 5. ^1H NMR spectrum of N_3 -DiplatinNC in D_2O .



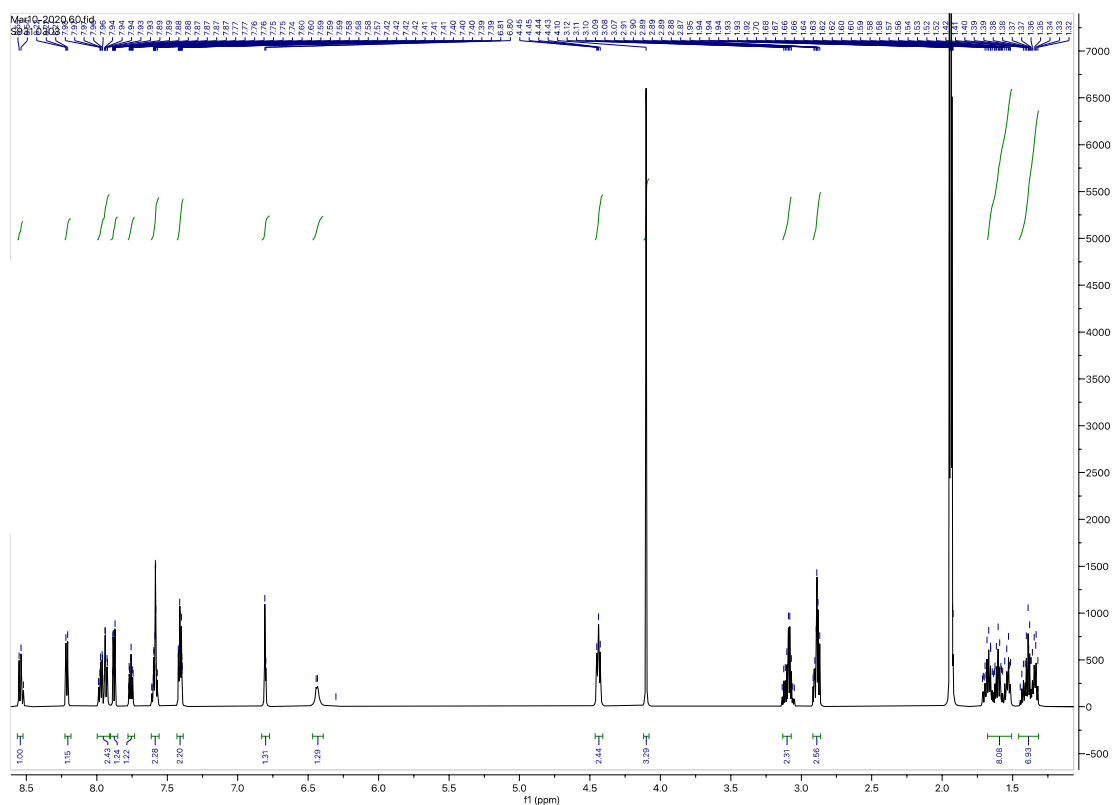


Figure A- 7. ^1H NMR spectrum of thiazole orange ligand in ACN-d_3 .

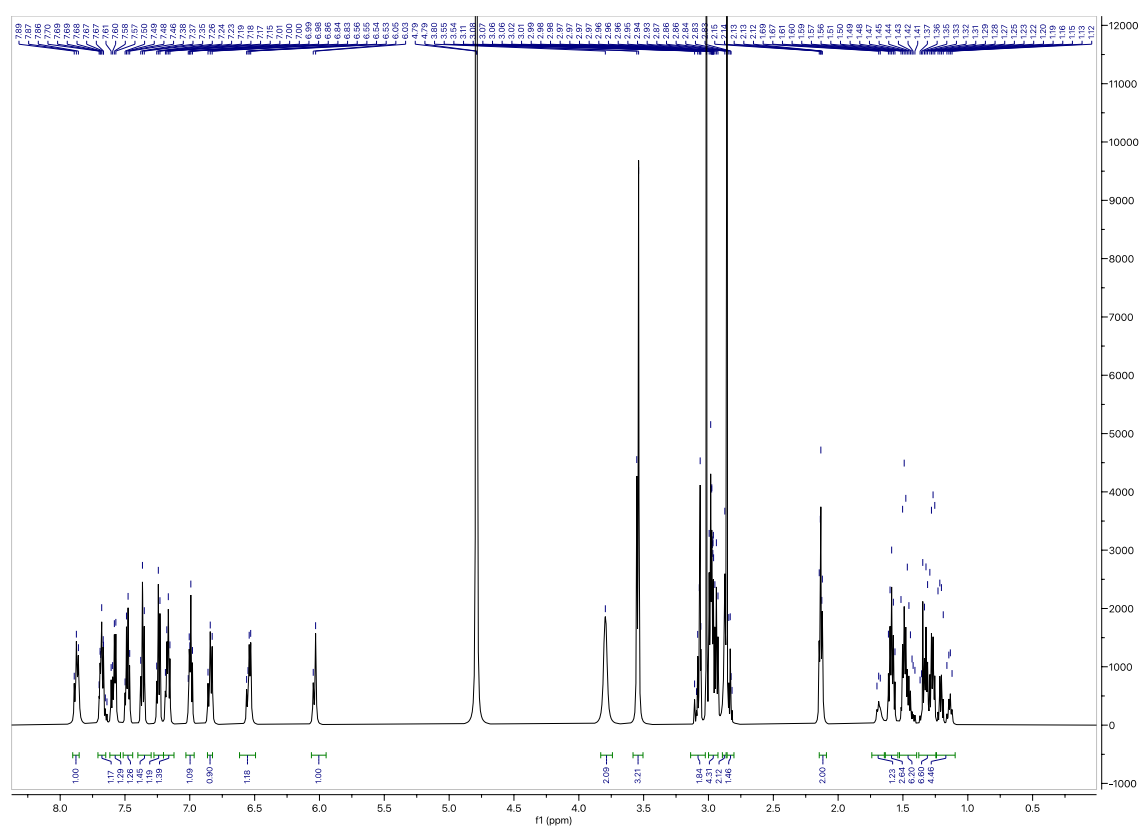


Figure A- 8. ^1H NMR spectrum of $\text{N}_3\text{-TO-MonoplatinNC}$ in D_2O

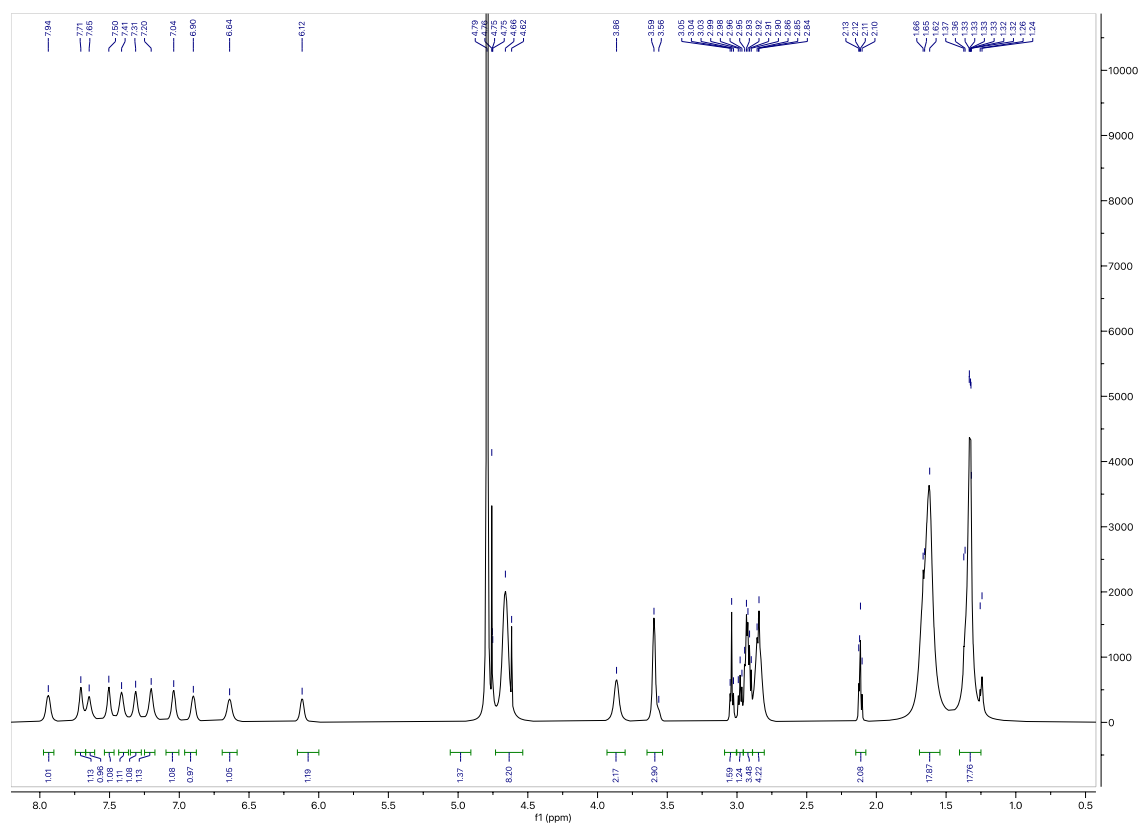


Figure A- 9. ^1H NMR spectrum of $\text{N}_3\text{-TO-DiplatinNC}$ in D_2O .

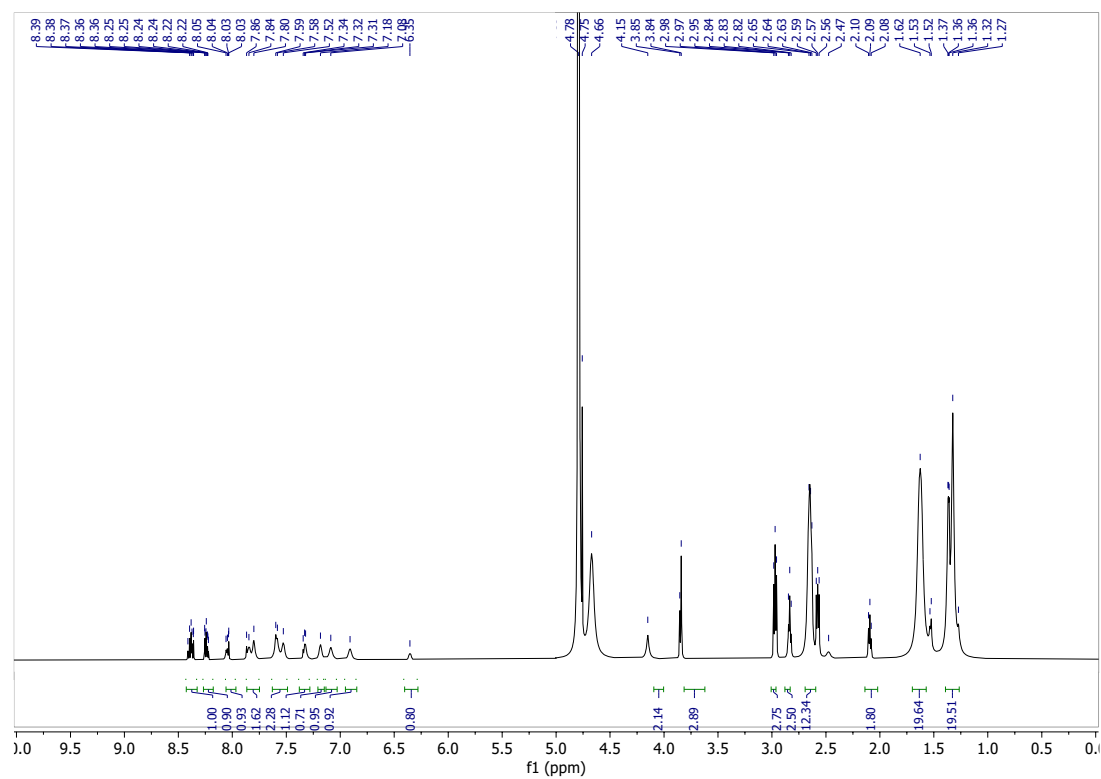


Figure A- 10. ^1H NMR spectrum of $\text{N}_3\text{-TO-TriplatinNC}$ in D_2O

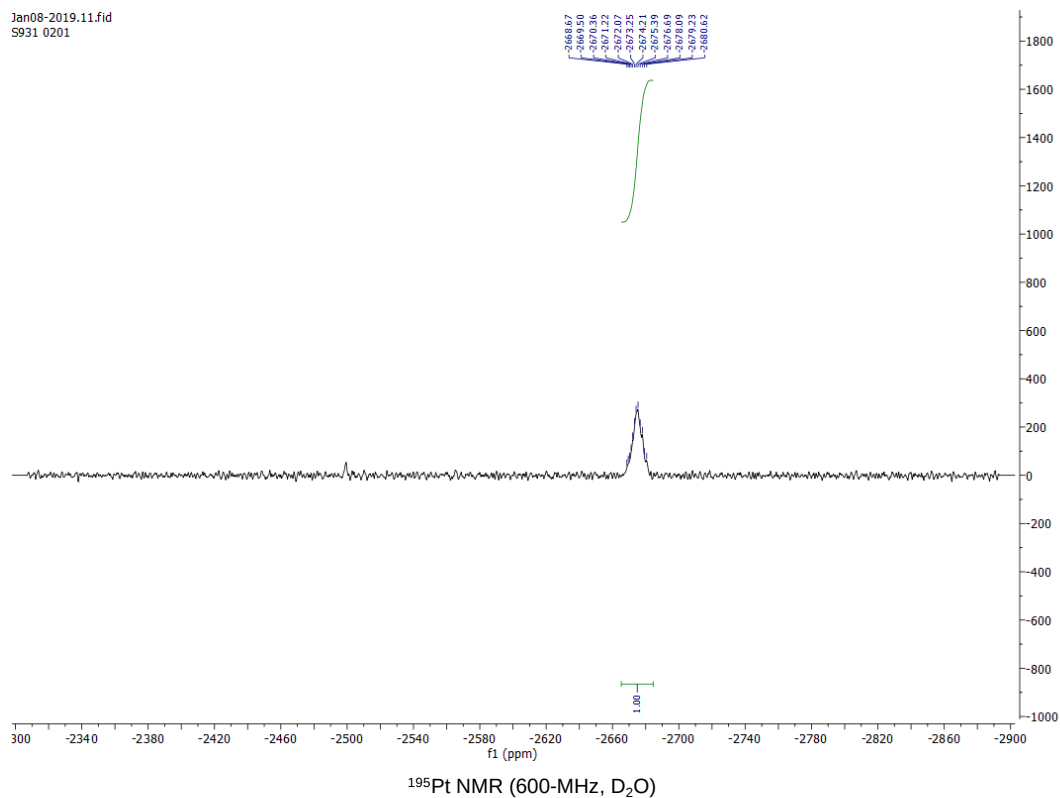


Figure A- 11. ^{195}Pt NMR spectrum of MonoplatinNC in D_2O .

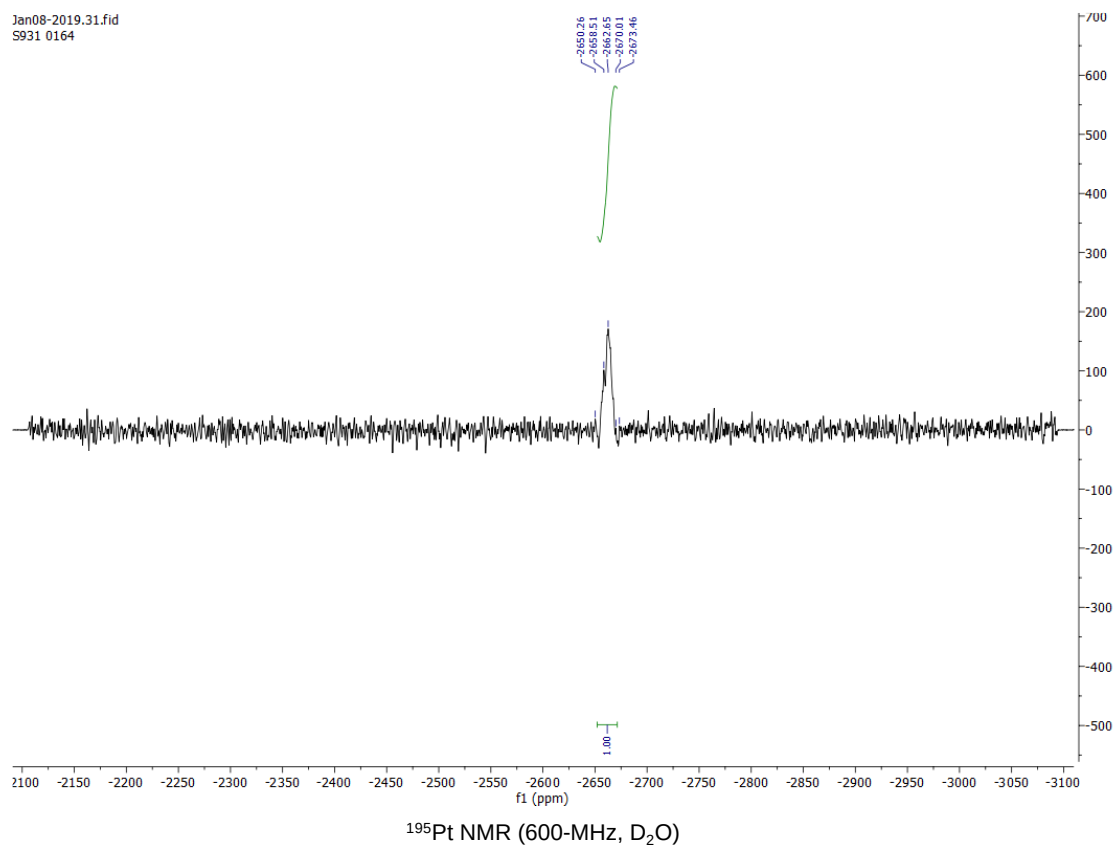
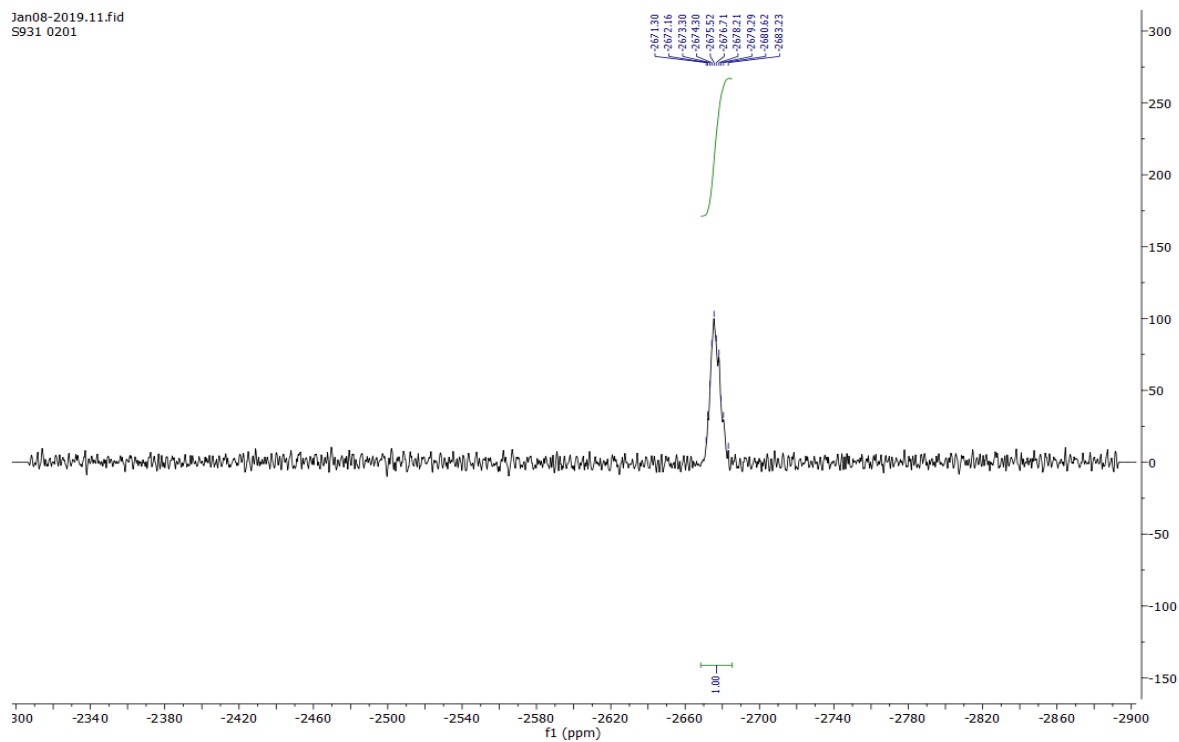


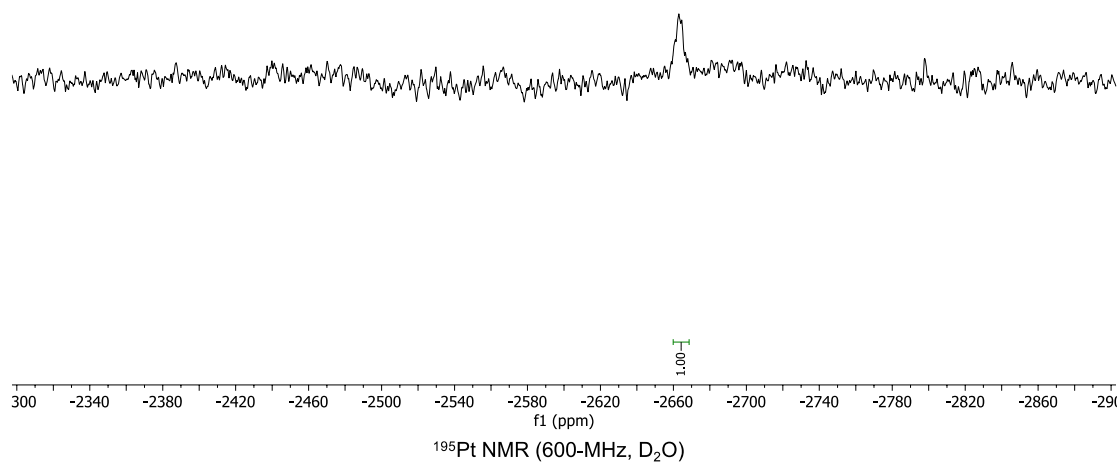
Figure A- 12. ^{195}Pt NMR spectrum of DiplatinNC in D_2O .

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S931 0201



^{195}Pt NMR (600-MHz, D_2O)

Figure A- 13. ^{195}Pt NMR spectrum of N_3 -MonoplatinNC in D_2O .



^{195}Pt NMR (600-MHz, D_2O)

Figure A- 14. ^{195}Pt NMR spectrum of N_3 -DiplatinNC in D_2O .

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5931 0248 TRI

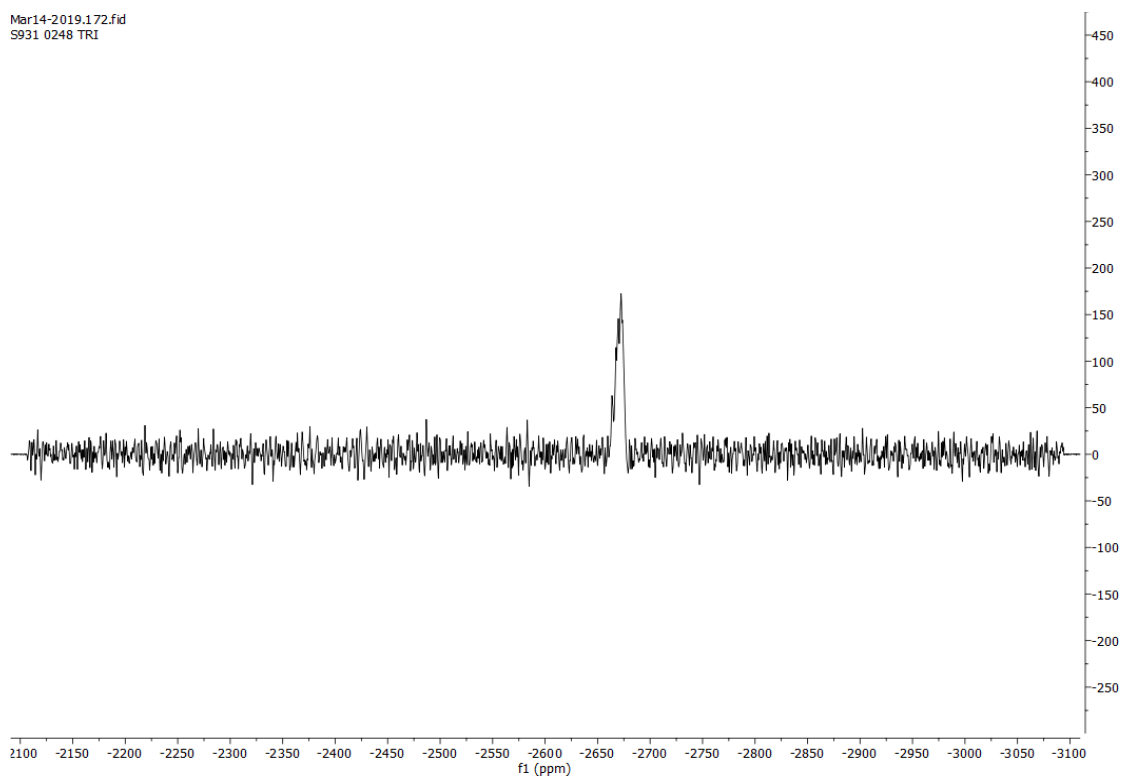


Figure A- 15. ^{195}Pt NMR spectrum of N_3 -TriplatinNC in D_2O .

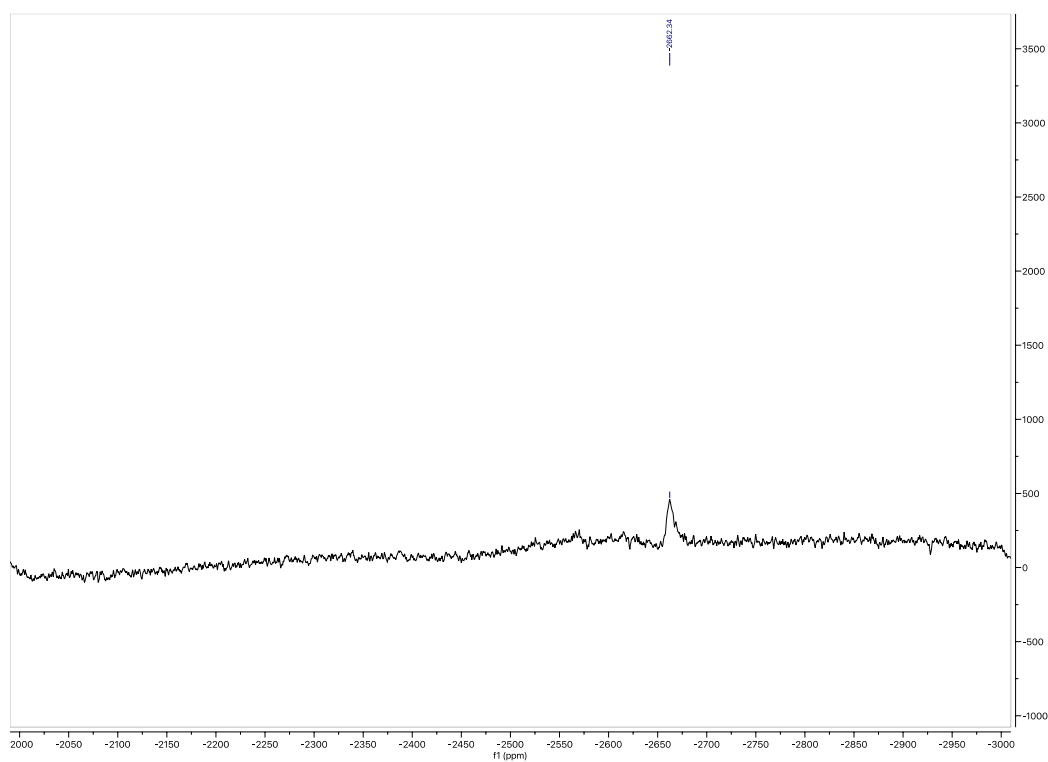


Figure A- 16. ^{195}Pt NMR spectrum of N_3 -TO-MonoplatinNC in D_2O .

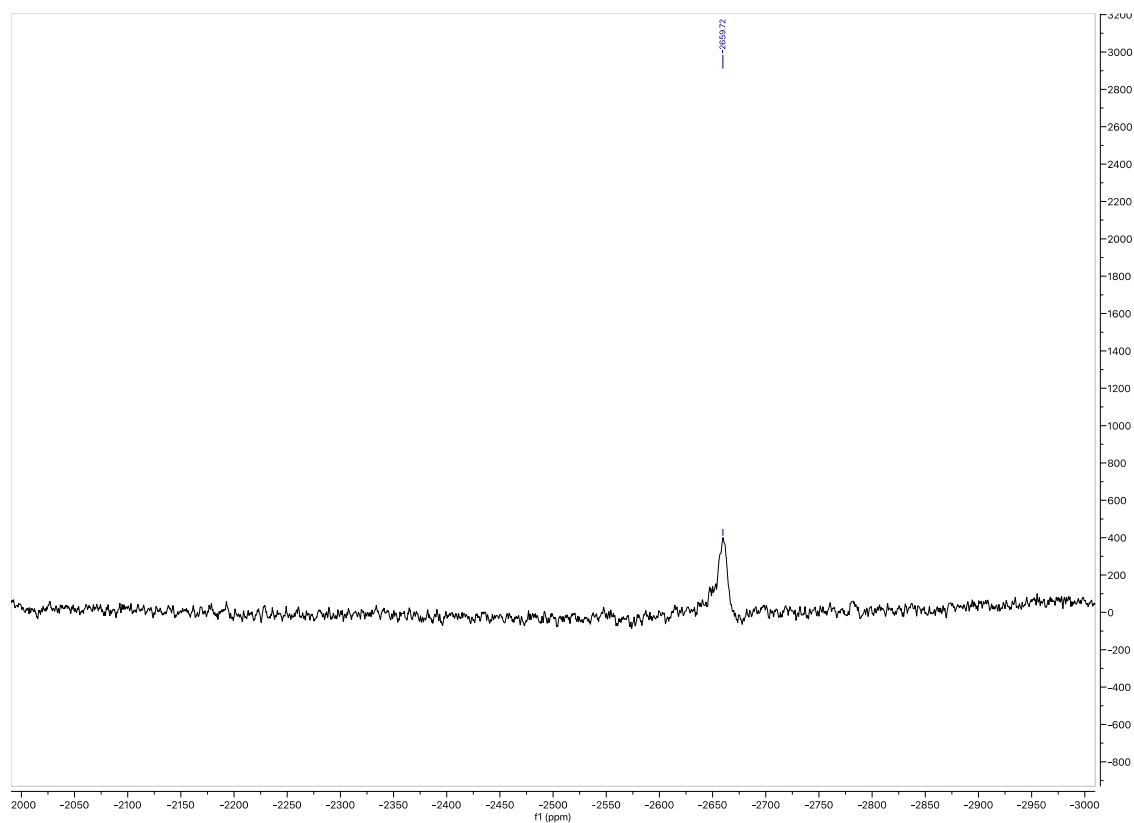


Figure A- 17. ^{195}Pt NMR spectrum of $\text{N}_3\text{-TO-DiplatinNC}$ in D_2O

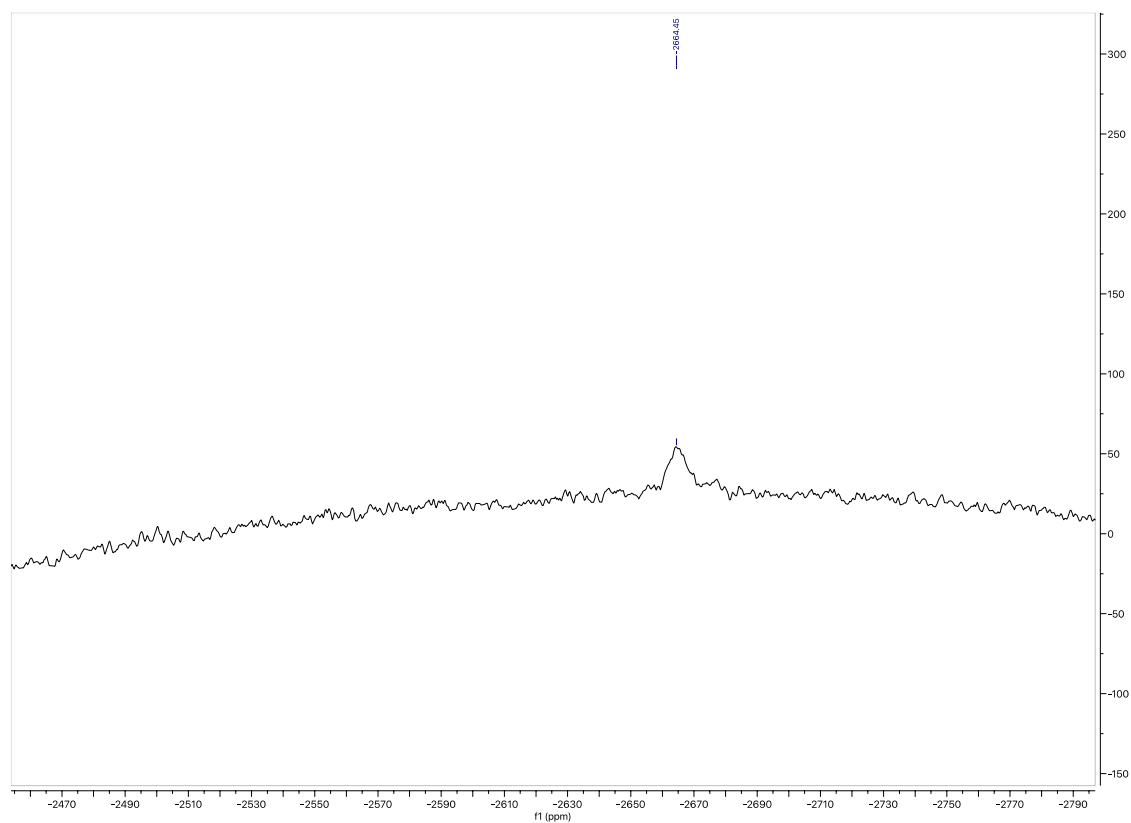


Figure A- 18. ^{195}Pt NMR spectrum of $\text{N}_3\text{-TO-TriplatinNC}$ in D_2O .

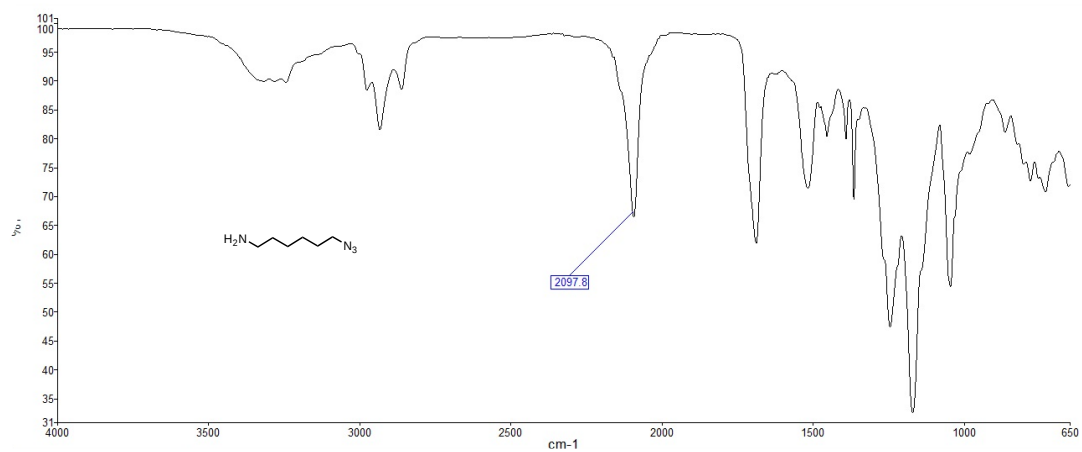


Figure A- 19. FT-IR spectrum of 1-amino-6-azido hexane.

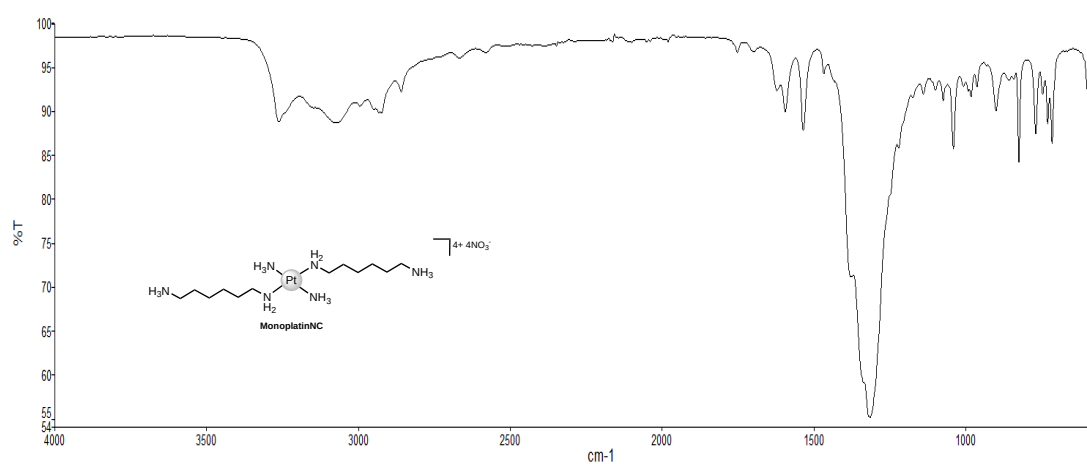


Figure A- 20. FT-IR spectrum of MonoplatinNC.

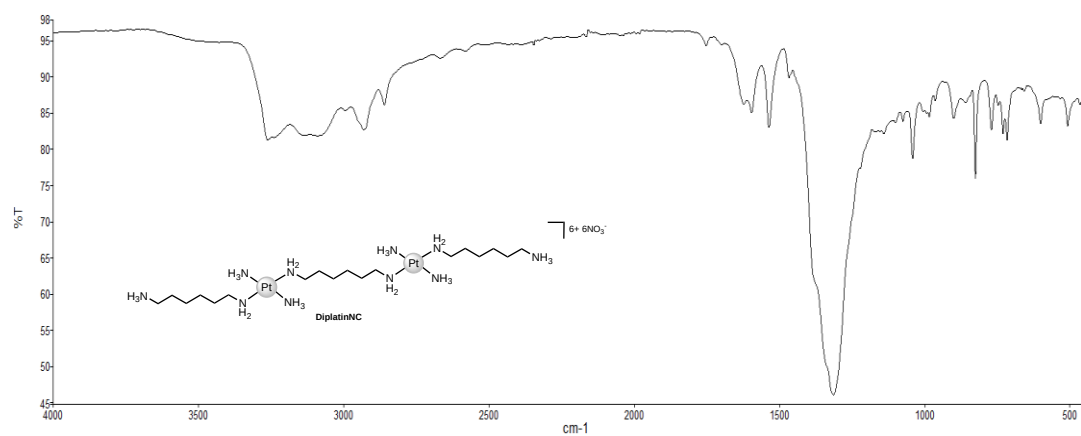


Figure A- 21. FT-IR spectrum of DiplatinNC

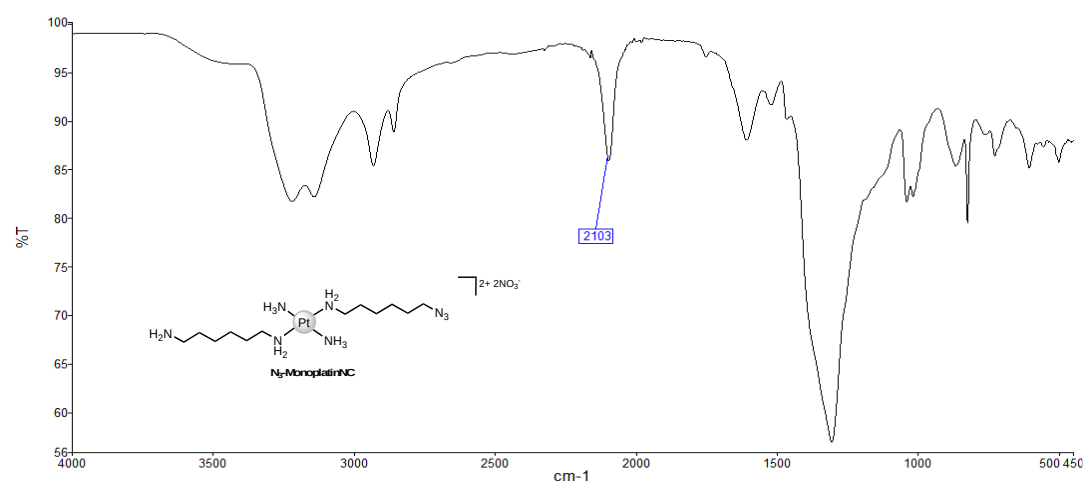


Figure A- 22. FT-IR spectrum of N₃-MonoplatinNC.

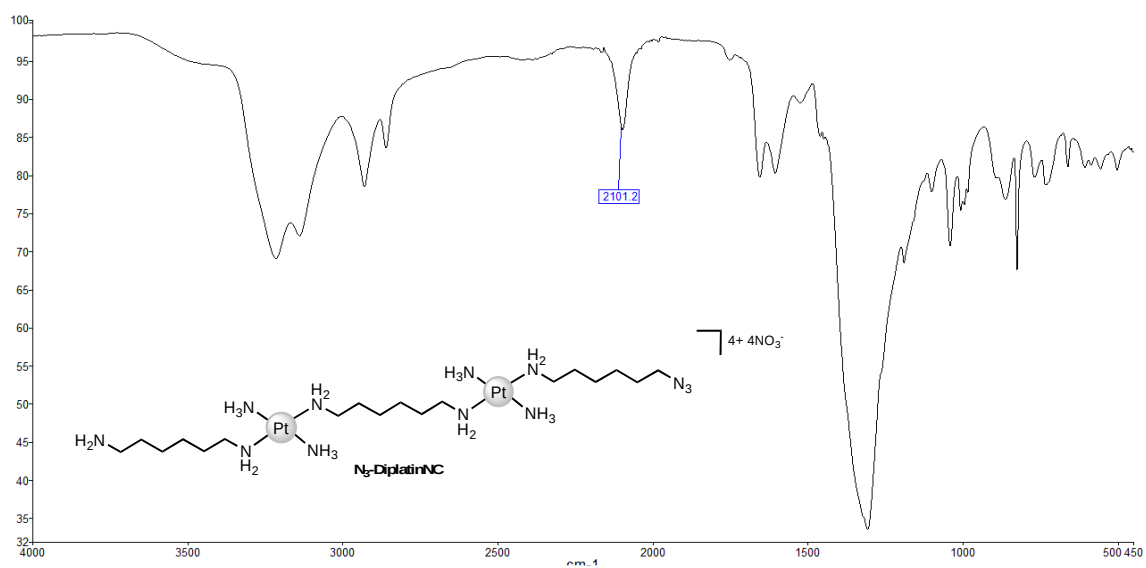


Figure A- 23. FT-IR spectrum of N_3 -DiplatinNC.

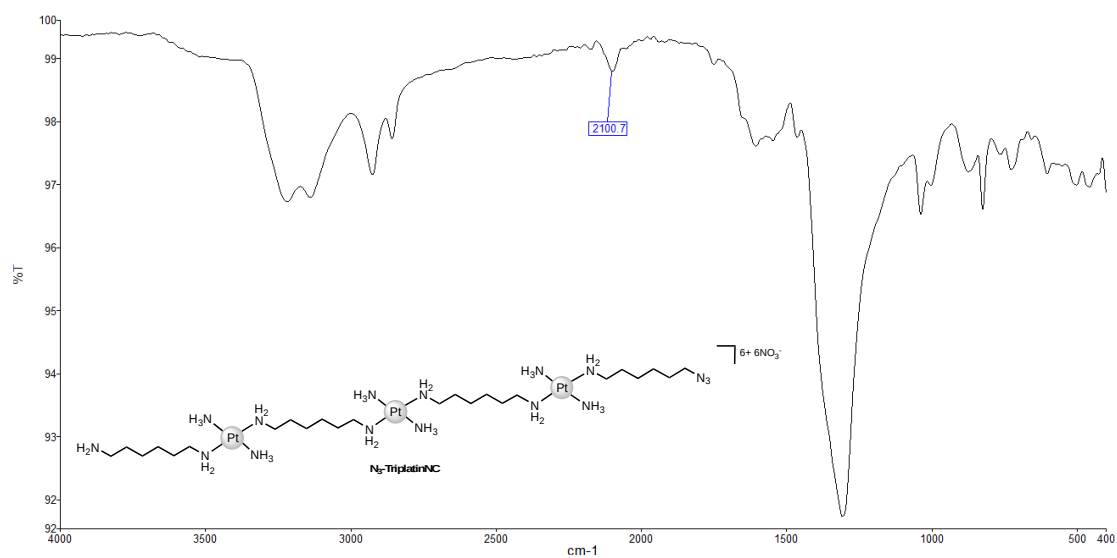


Figure A- 24. FT-IR spectrum of N_3 -TriplatinNC.

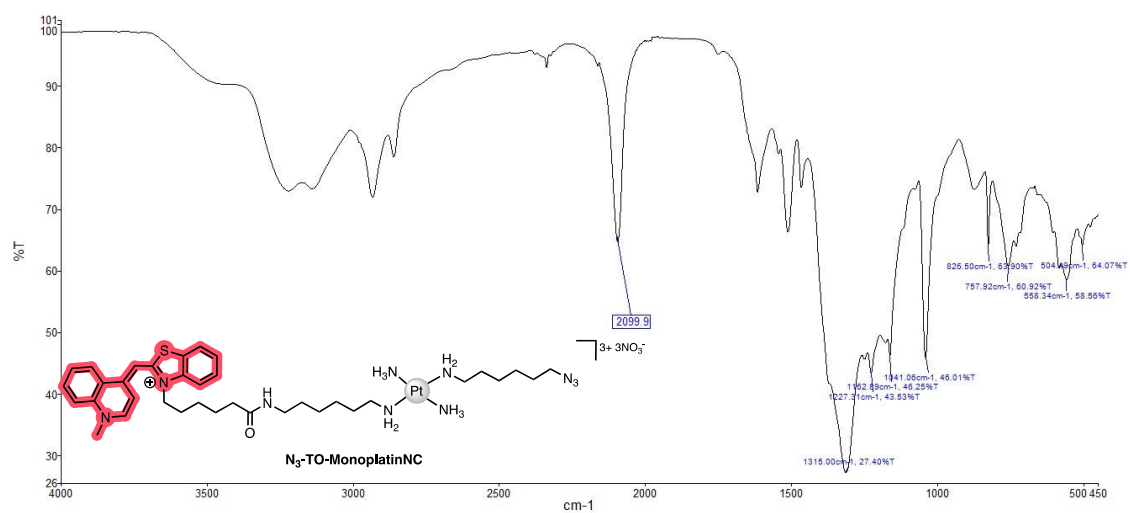


Figure A- 25. FT-IR spectrum of N₃-TO-MonoplatinNC.

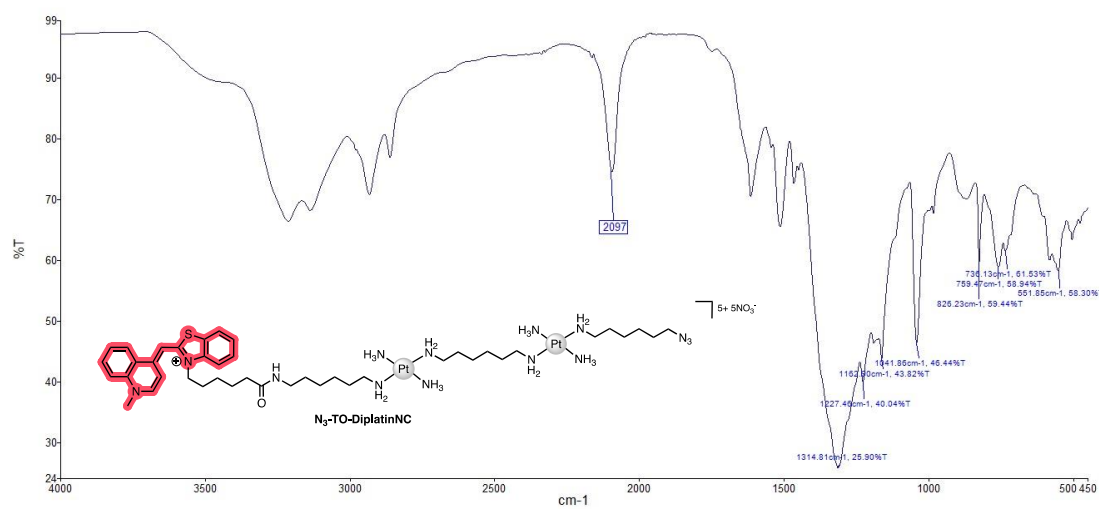


Figure A- 26. FT-IR spectrum of N₃-TO-DiplatinNC

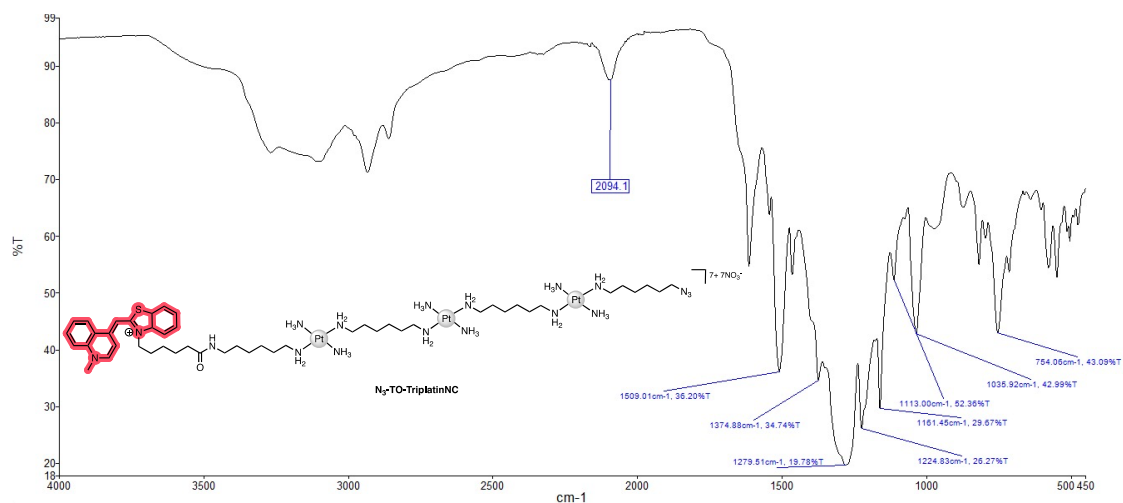


Figure A- 27. FT-IR spectrum of N_3 -TO-TriplatinNC.

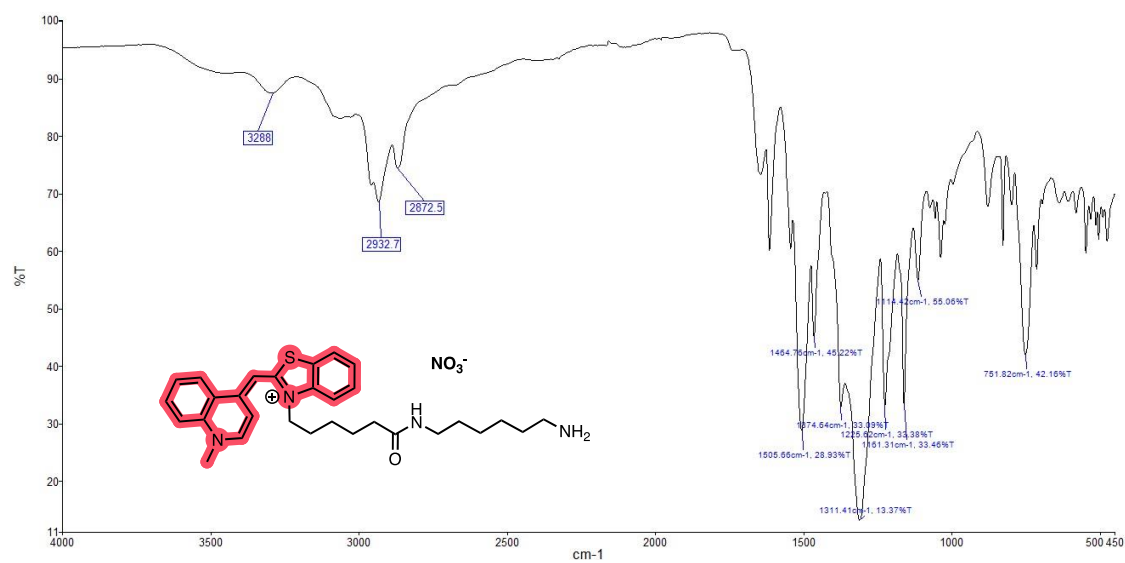


Figure A- 28. FT-IR spectrum of the thiazole orange ligand.

Appendix B

Agarose Gel Electrophoresis

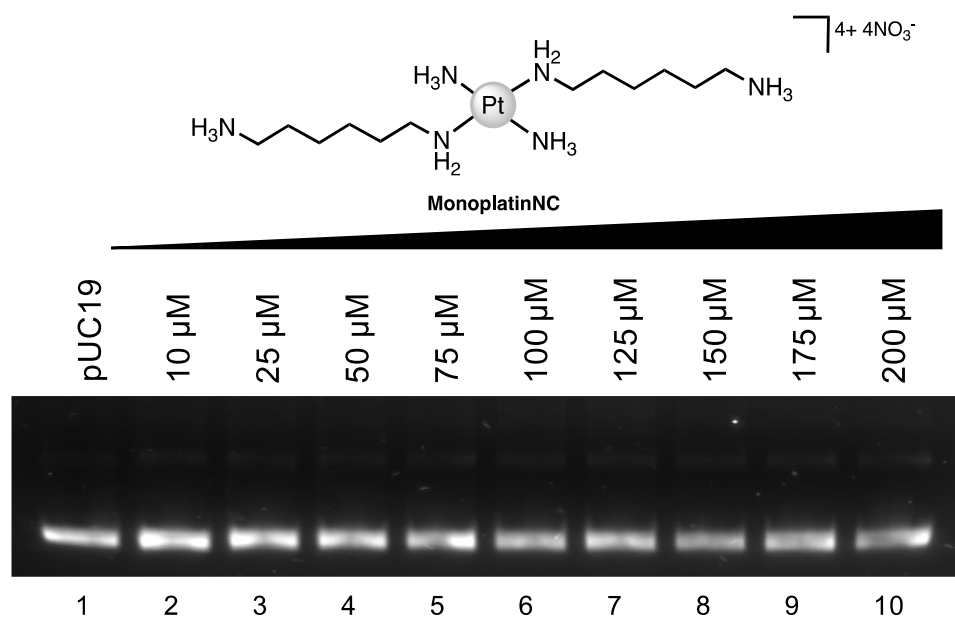


Figure B- 1. Effects of MonoplatinNC on the migration of negatively supercoiled plasmid pUC19 in the native agarose gel.

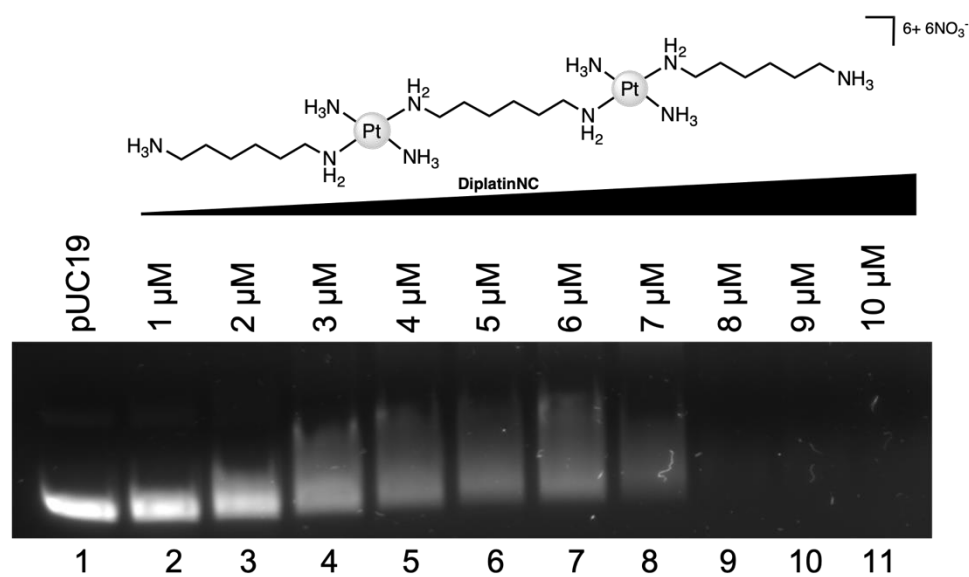


Figure B- 2. Effects of DiplatinNC on the migration of negatively supercoiled plasmid pUC19 in the native agarose gel.

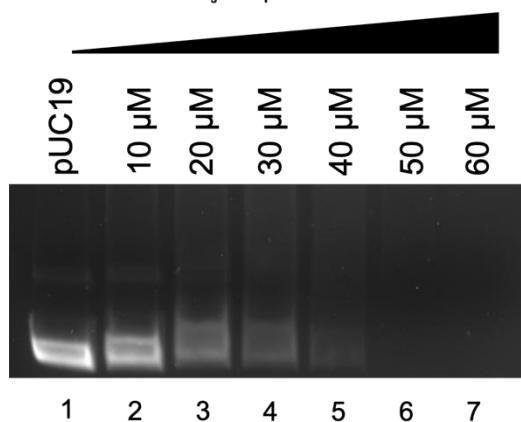
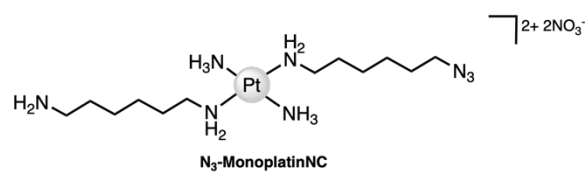


Figure B- 5. Effects of *N*₃-MonoplatinNC on the migration of negatively supercoiled plasmid pUC19 in the native agarose gel.

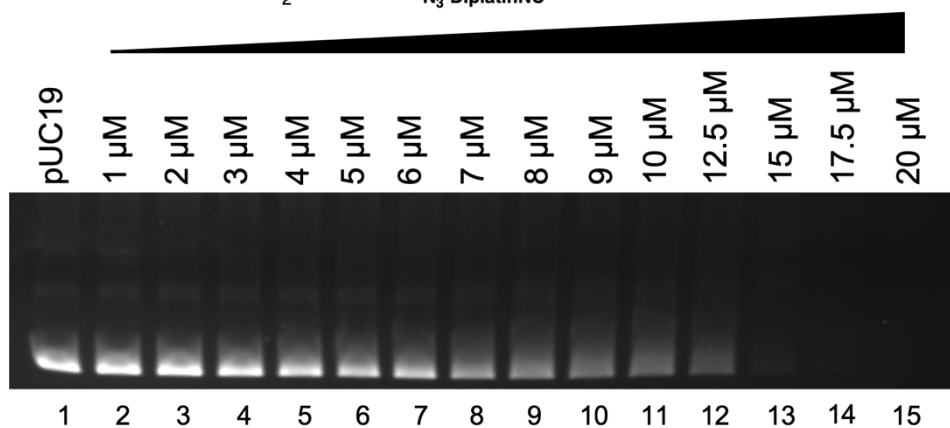
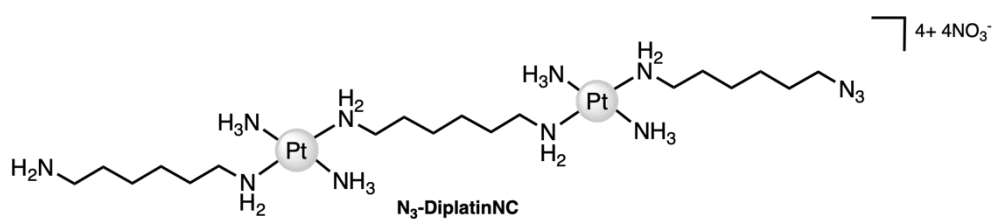


Figure B- 6. Effects of *N*₃-DiplatinNC on the migration of negatively supercoiled plasmid pUC19 in the native agarose gel.

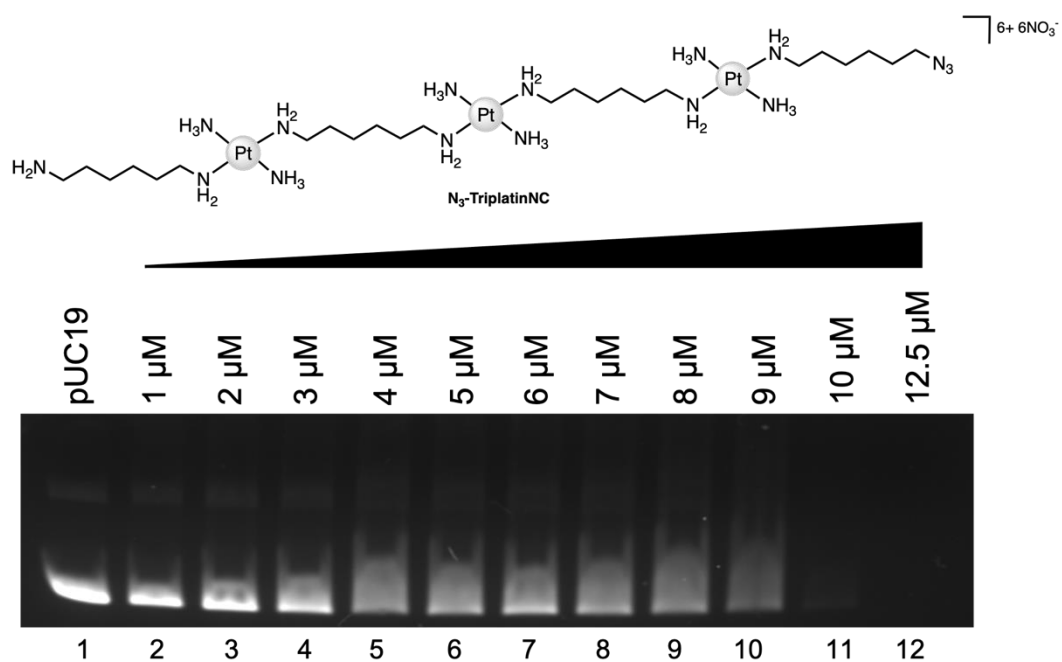


Figure B- 7. Effects of N_3 -TriplatinNC on the migration of negatively supercoiled plasmid pUC19 in the native agarose gel.

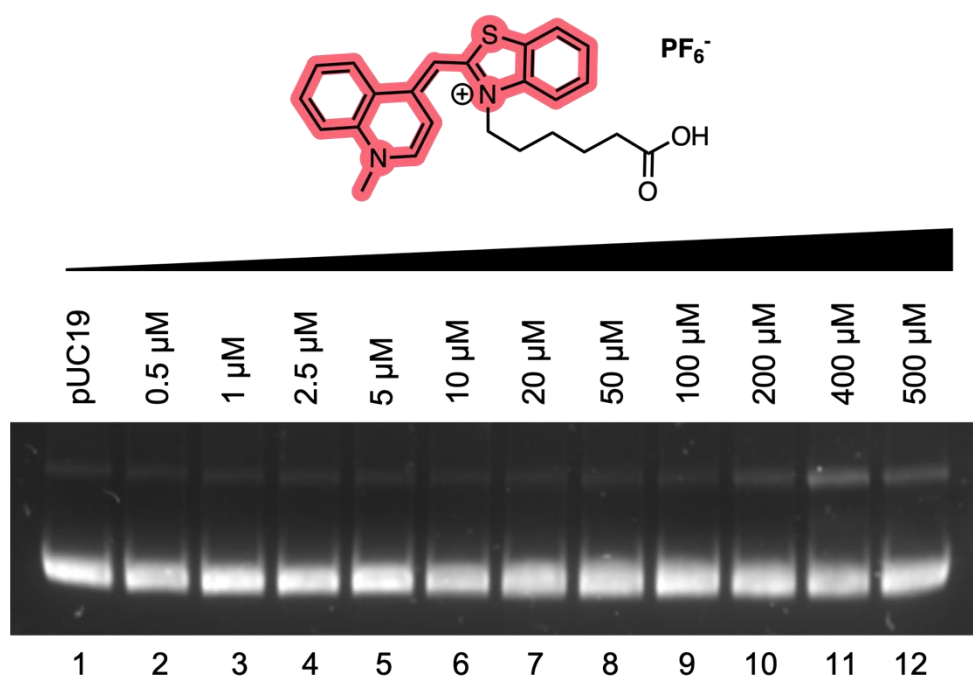


Figure B- 8. Effects of thiazole orange on the migration of negatively supercoiled plasmid pUC19 in the native agarose gel.

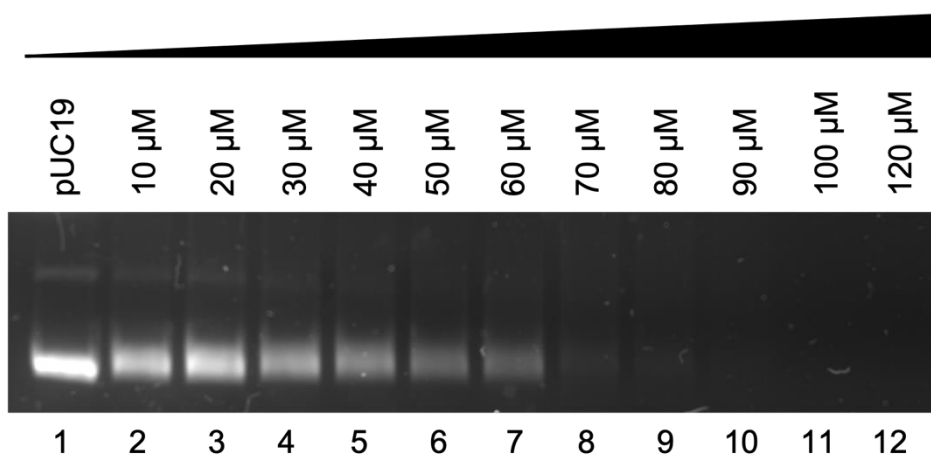
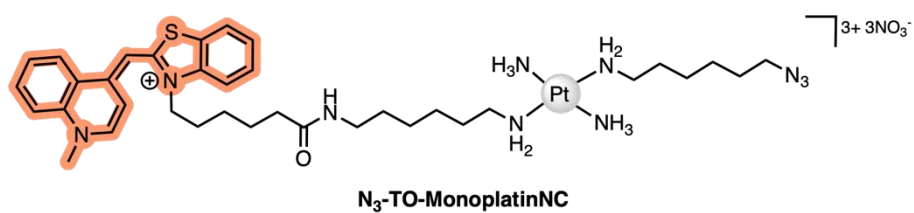


Figure B- 9. Effects of N₃-TO-MonoplatinNC on the migration of negatively supercoiled plasmid pUC19 in the native agarose gel.

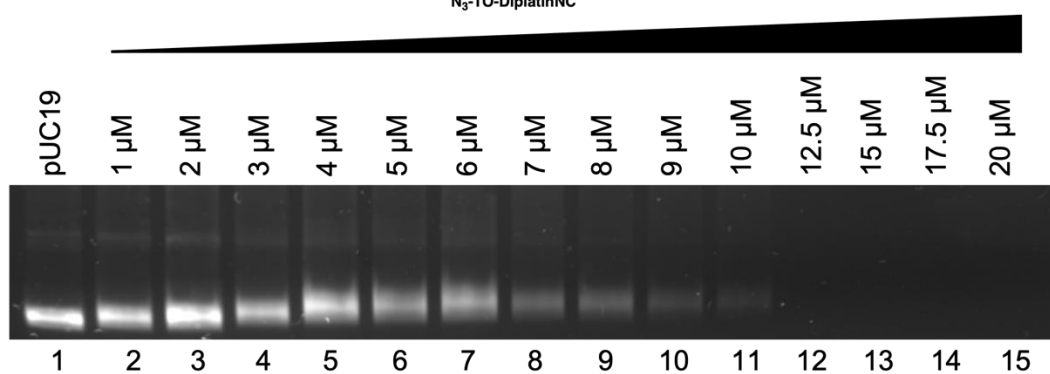
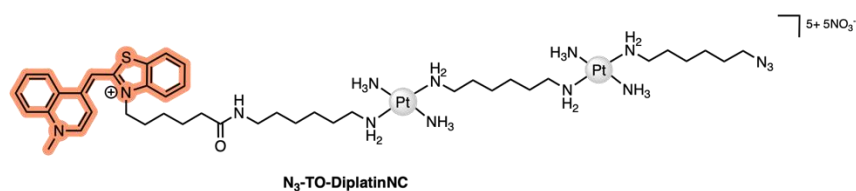


Figure B- 10. Effects of N₃-TO-DiplatinNC on the migration of negatively supercoiled plasmid pUC19 in the native agarose gel

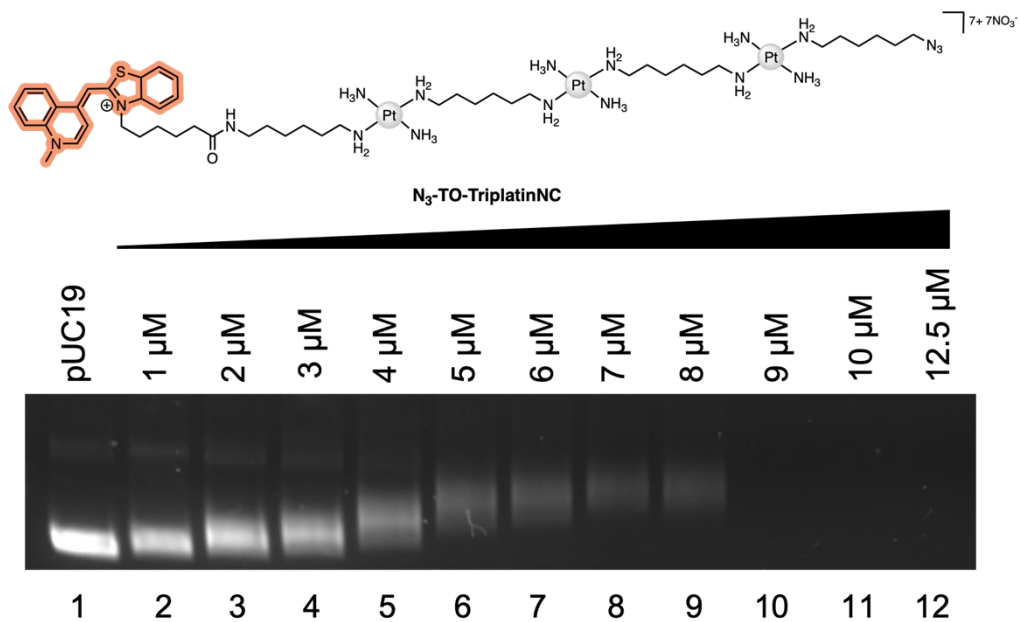


Figure B- 11. Effects of N₃-TO-TriplatinNC on the migration of negatively supercoiled plasmid pUC19 in the native agarose gel.

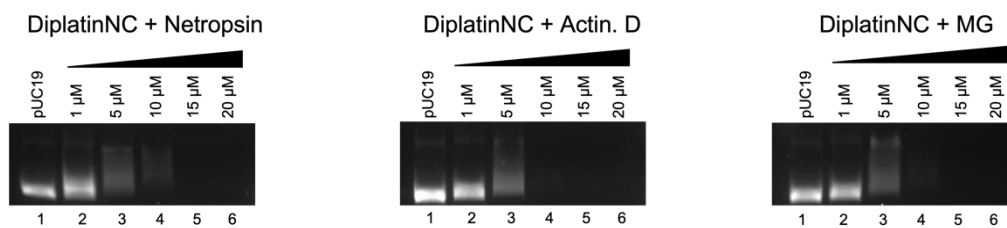


Figure B- 12. Groove blocking studies of DiplatinNC using netropsin, actinomycin D., and methyl green.

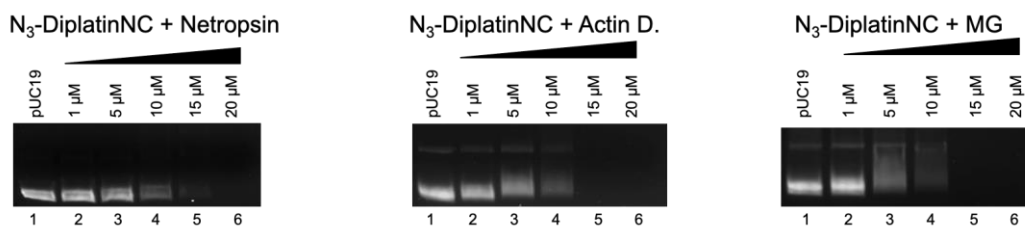


Figure B- 13. Groove blocking studies of N_3 -DiplatinNC using netropsin, actinomycin D., and methyl green.

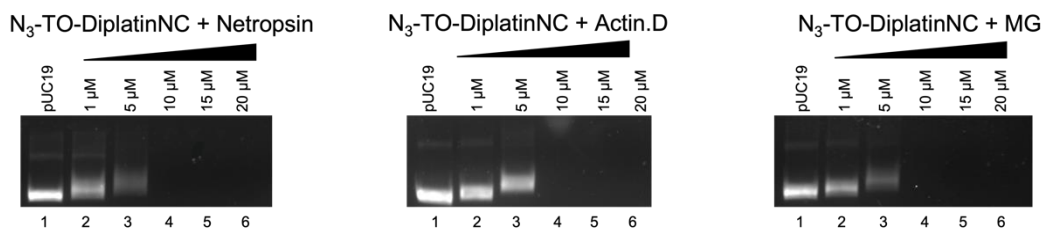


Figure B- 14. Groove blocking studies of N_3 -TO-DiplatinNC using netropsin, actinomycin D., and methyl green.

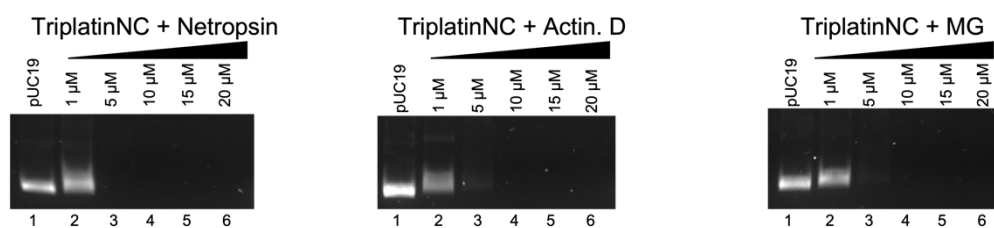


Figure B- 15. Groove blocking studies of TriplatinNC using netropsin, actinomycin D., and methyl green.

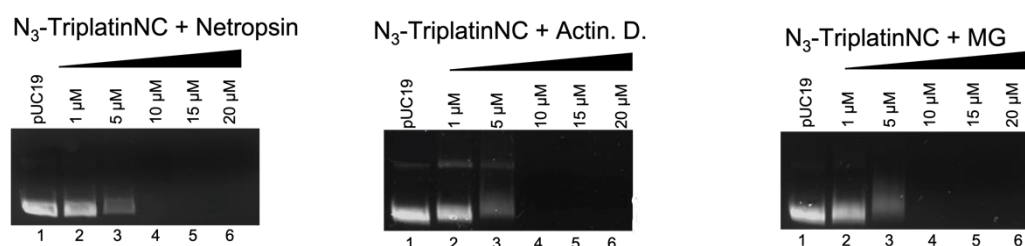


Figure B- 16. Groove blocking studies of N_3 -TriplatinNC using netropsin, actinomycin D., and methyl green.

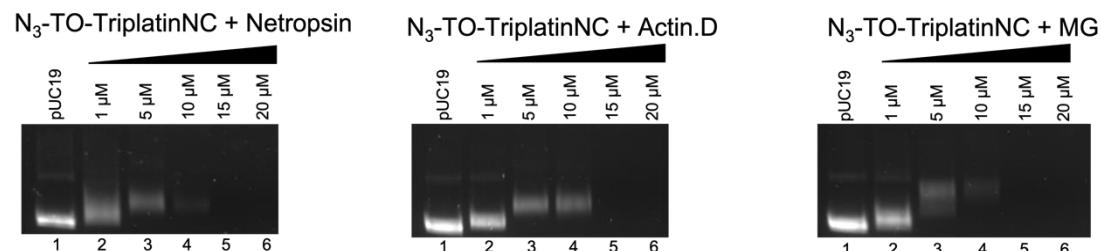


Figure B- 17. Groove blocking studies of N_3 -TO-TriplatinNC using netropsin, actinomycin D., and methyl green.

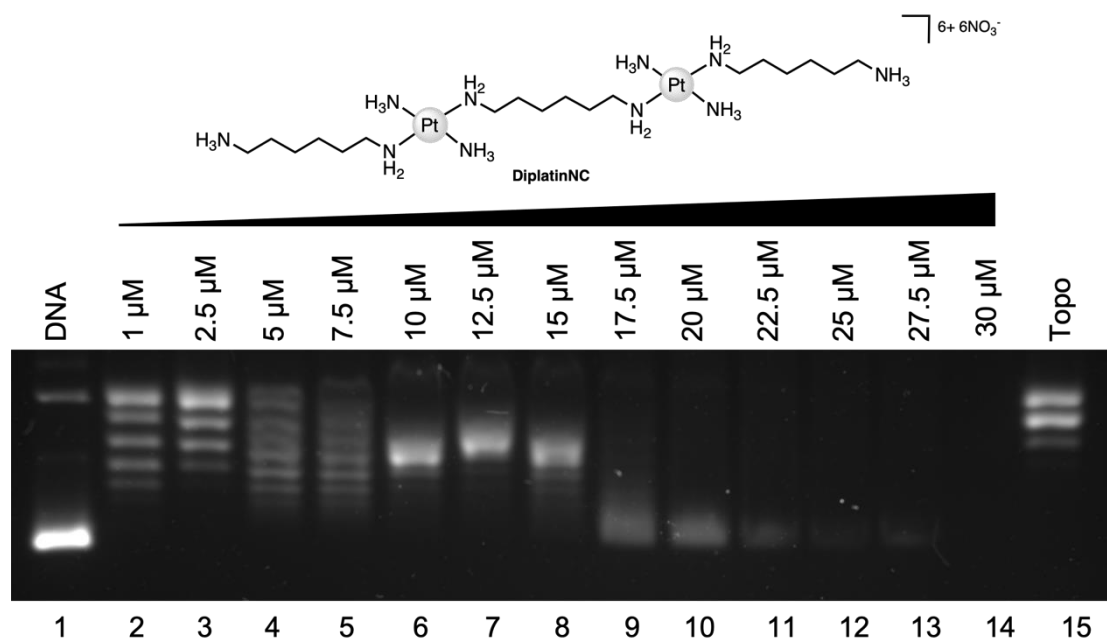


Figure B- 18. Topoisomerase I relaxation inhibition assay with DiplatinNC.

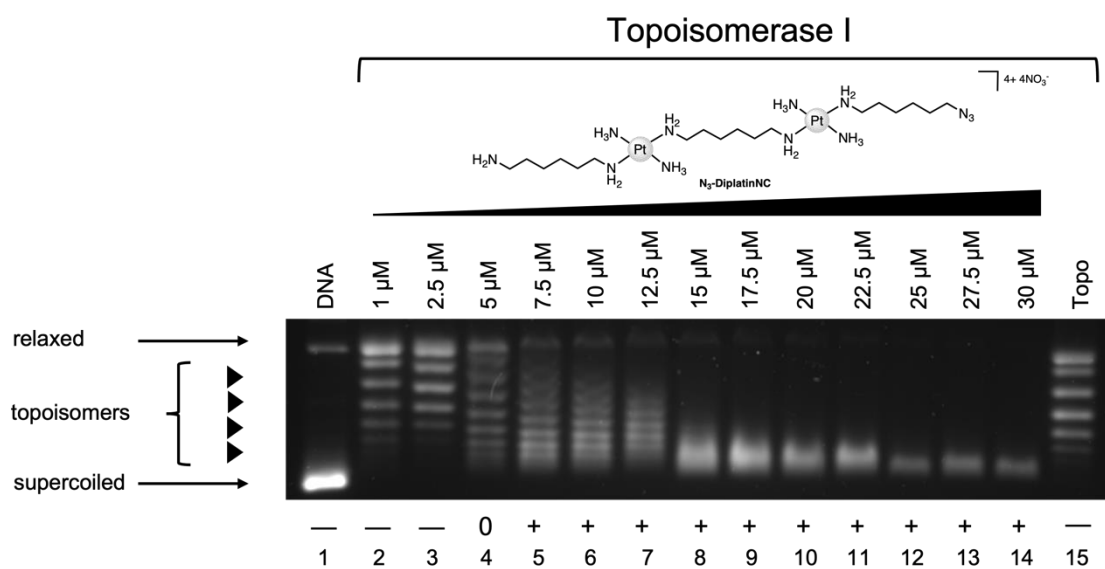


Figure B- 19. Topoisomerase I relaxation inhibition assay with N_3 -DiplatinNC.

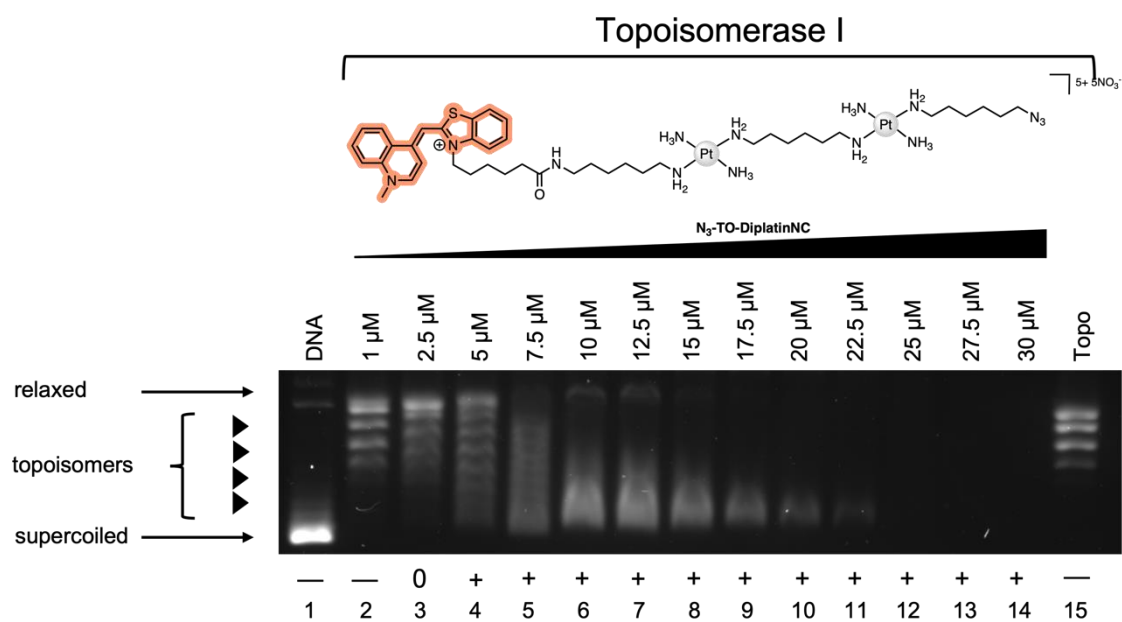


Figure B- 20. Topoisomerase I relaxation inhibition assay with N₃-TO-DiplatinNC.

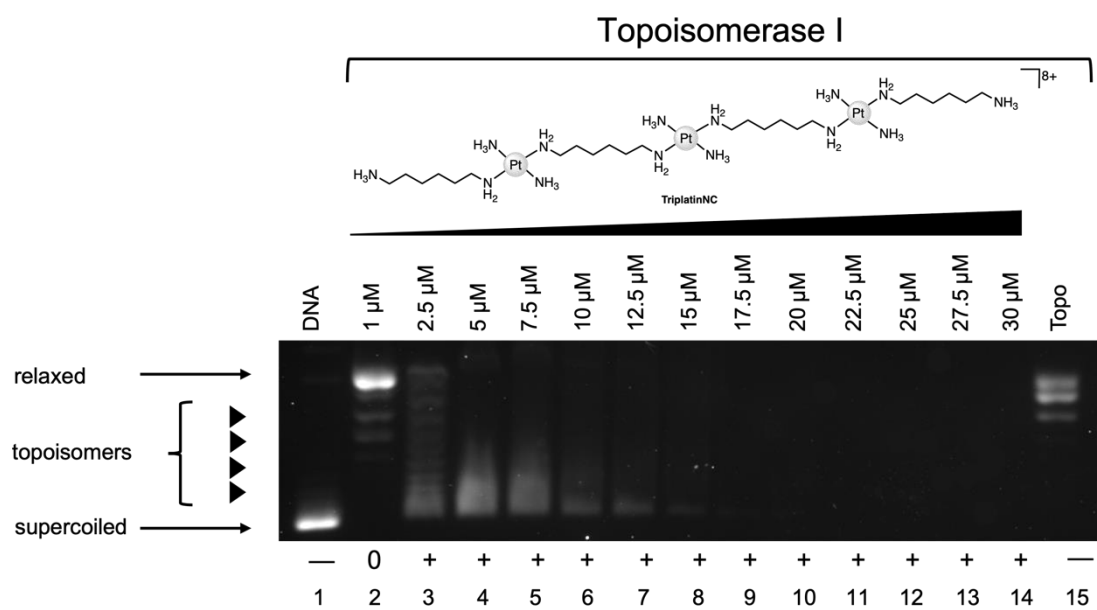


Figure B- 21. Topoisomerase I relaxation inhibition assay with TriplatinNC

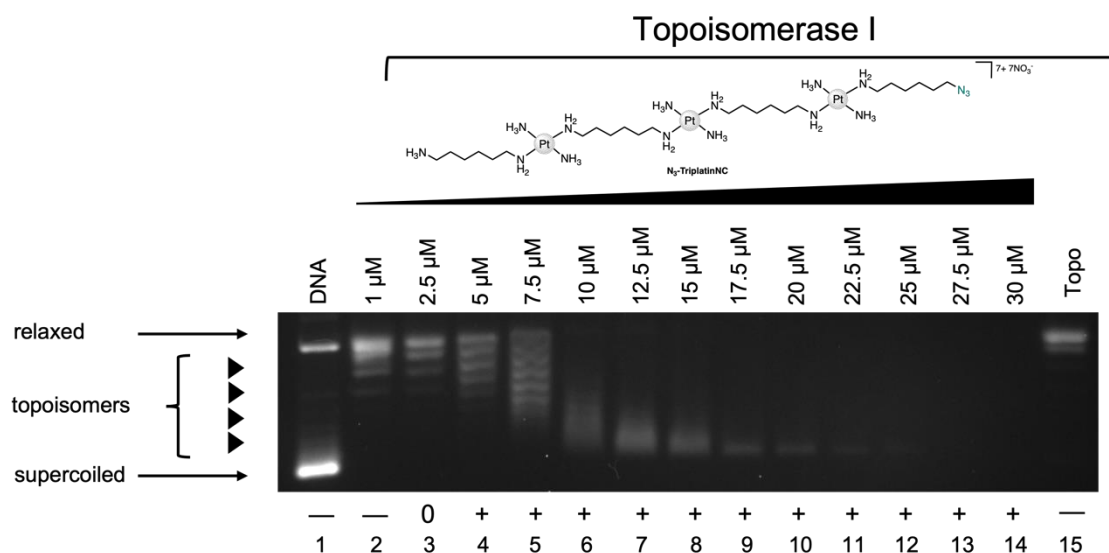


Figure B- 22. Topoisomerase I relaxation inhibition assay with N₃-TriplatinNC.

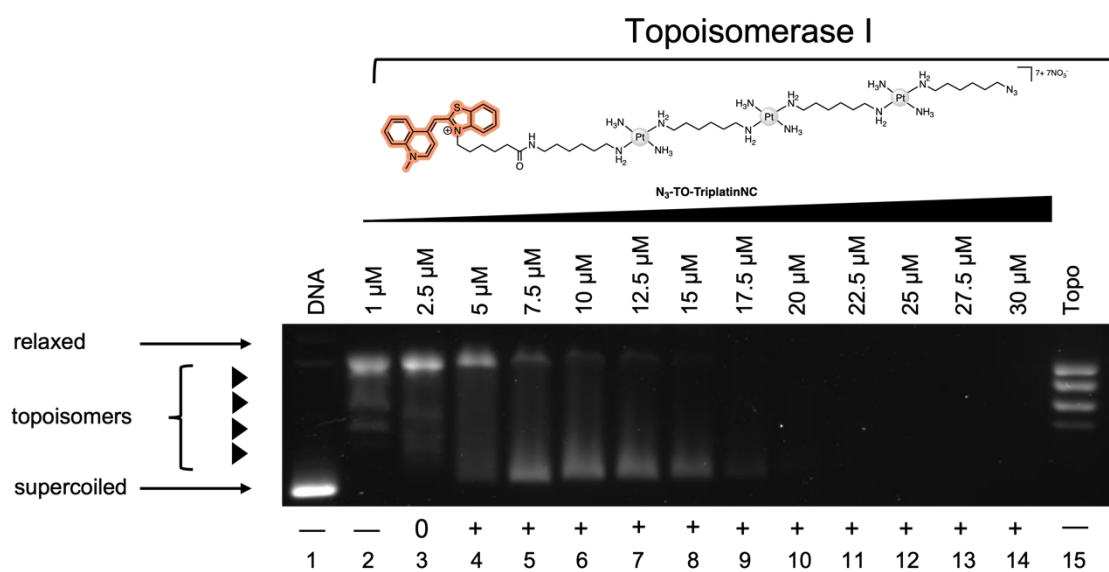


Figure B- 23. Topoisomerase I relaxation inhibition assay with N₃-TO-TriplatinNC.

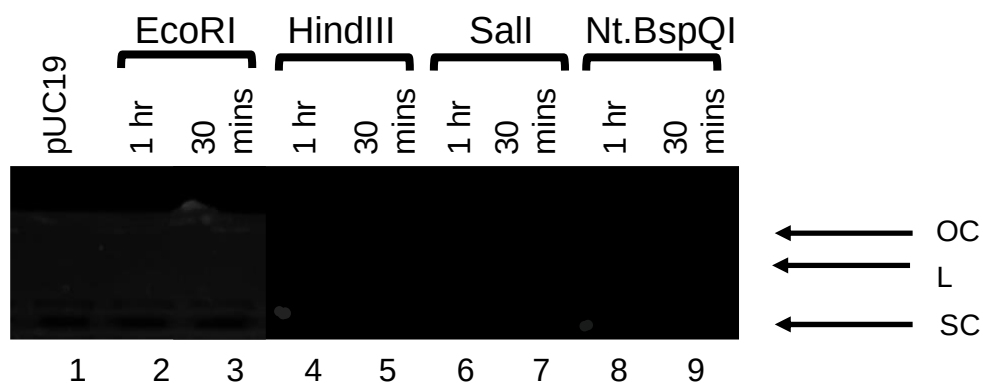


Figure B- 24. Endonuclease activity of *EcoRI*, *HindIII*, *SalI*, and *Nt. BspQI*.

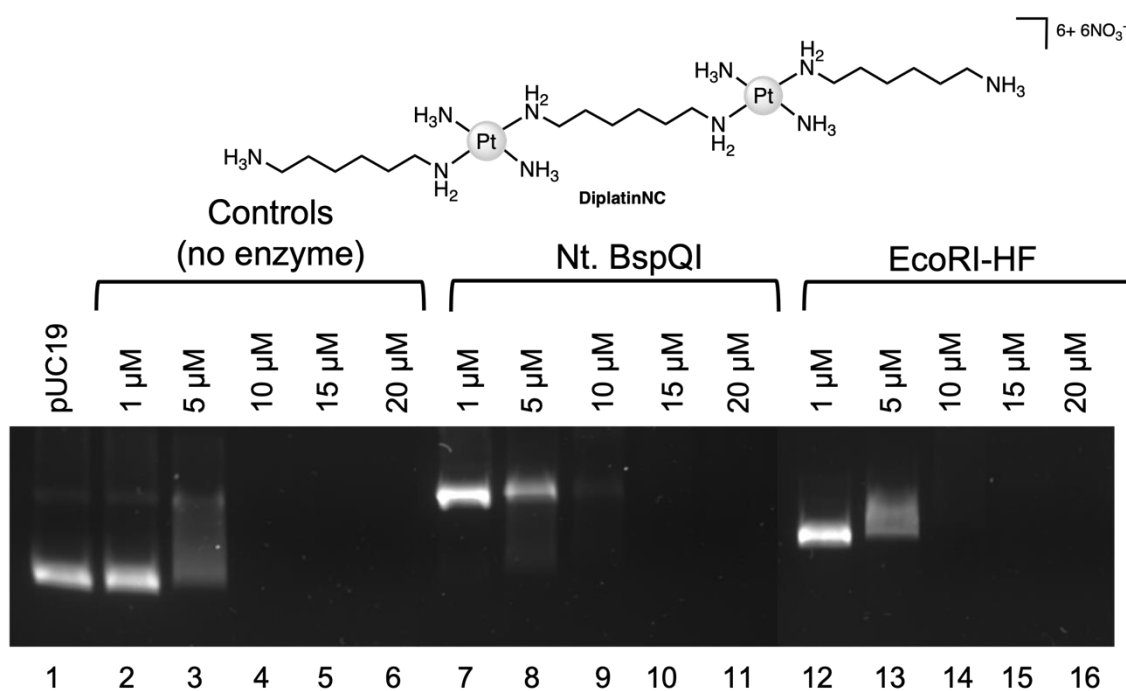


Figure B- 25. Effects of *DiplatinNC* on the endonuclease activity of *EcoRI* and *Nt.BspQI*.



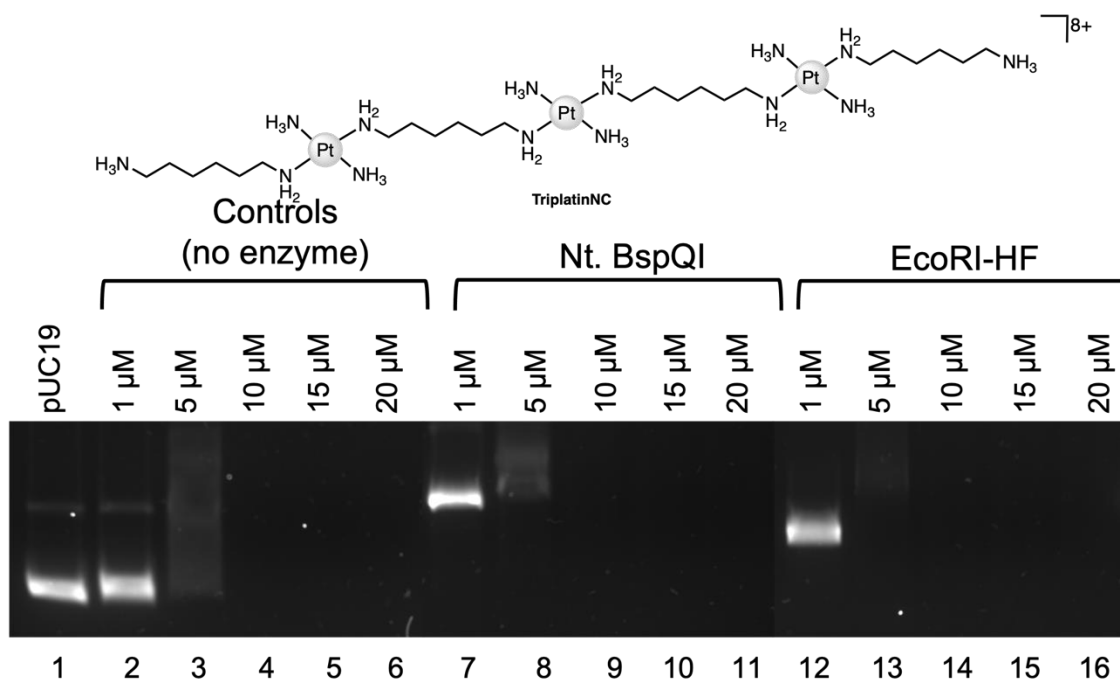


Figure B- 28. Effects of TriplatinNC on the endonuclease activity of EcoRI and Nt.BspQI

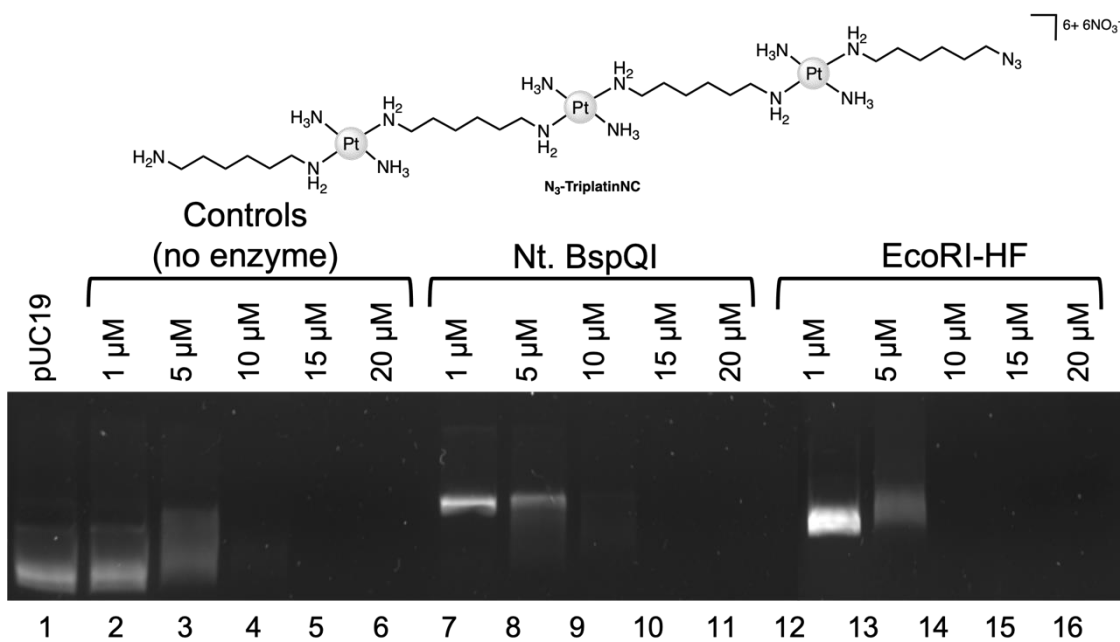


Figure B- 29. Effects of N_3 -TriplatinNC on the endonuclease activity of EcoRI and Nt.BspQI.

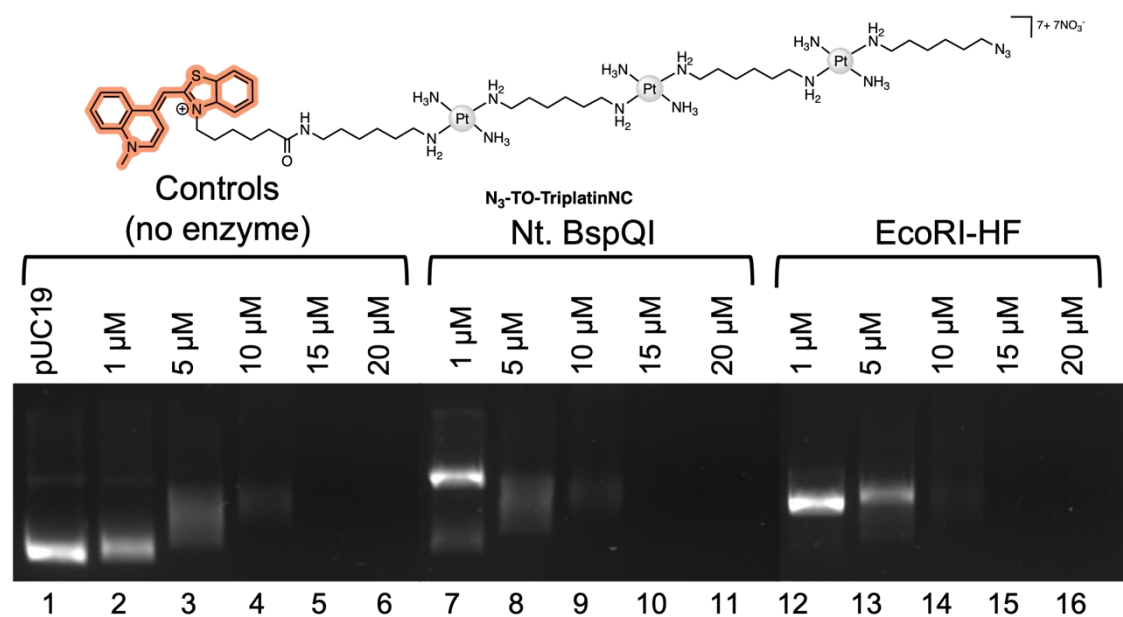


Figure B- 30. Effects of N_3 -TO-TriplatinNC on the endonuclease activity of EcoRI and Nt.BspQI.

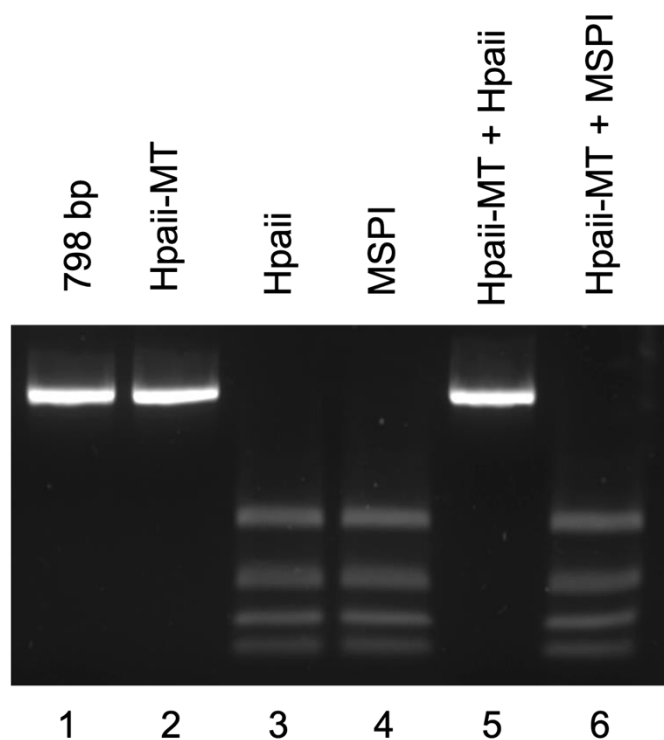


Figure B- 31. Control experiments of the enzymes HpaII and MSPI and the effect cytosine methylation has on the

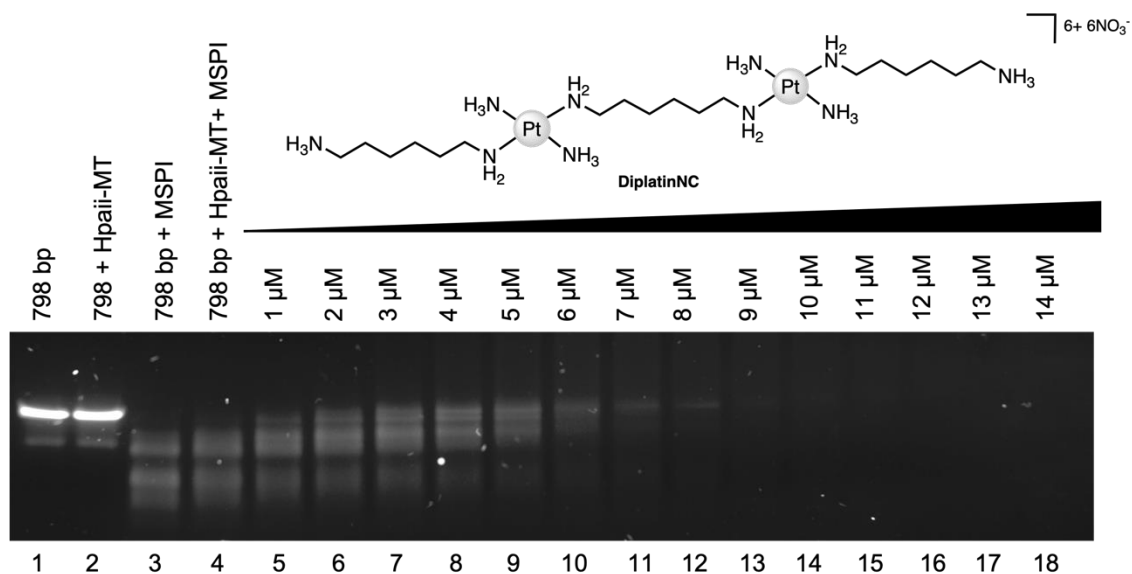


Figure B- 32. Agarose gel electrophoresis of the effects of DiplatinNC on the epigenetic-reading enzyme MSPI.

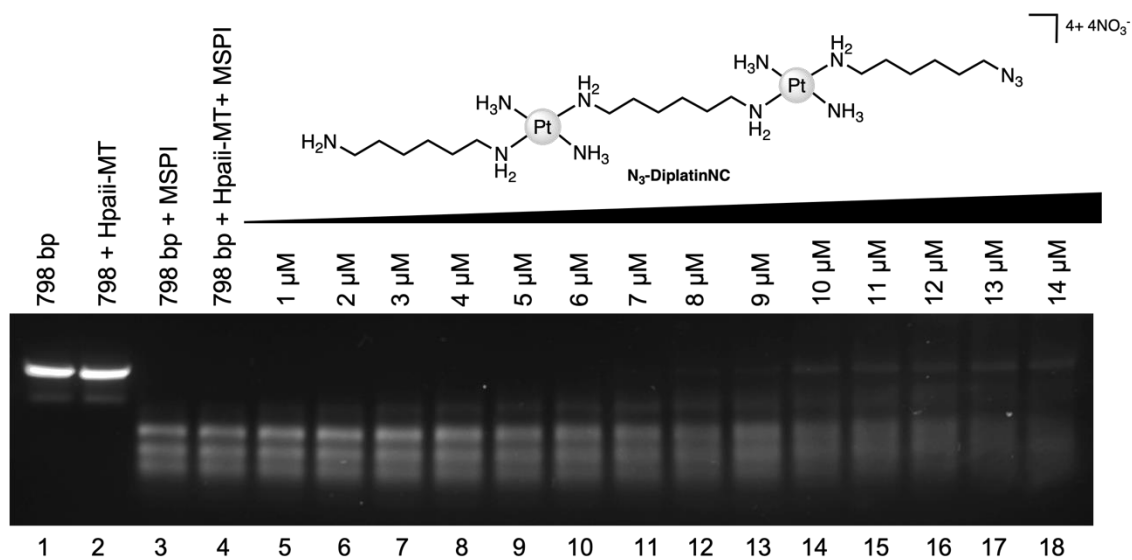


Figure B- 33. Agarose gel electrophoresis of the effects of N_3 -DiplatinNC on the epigenetic-reading enzyme MSPI.

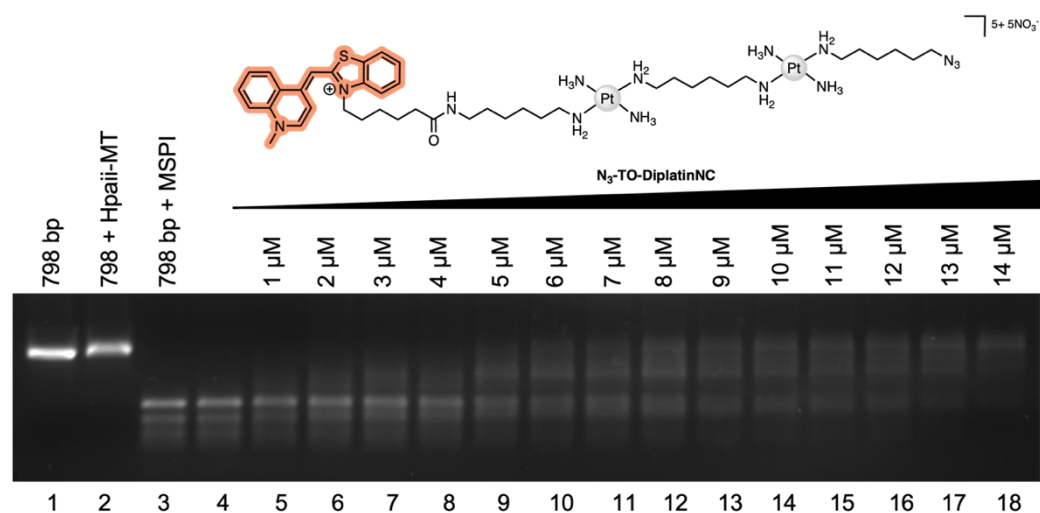


Figure B- 34. Agarose gel electrophoresis of the effects of N_3 -TO-DiplatinNC on the epigenetic-reading enzyme MSPI.

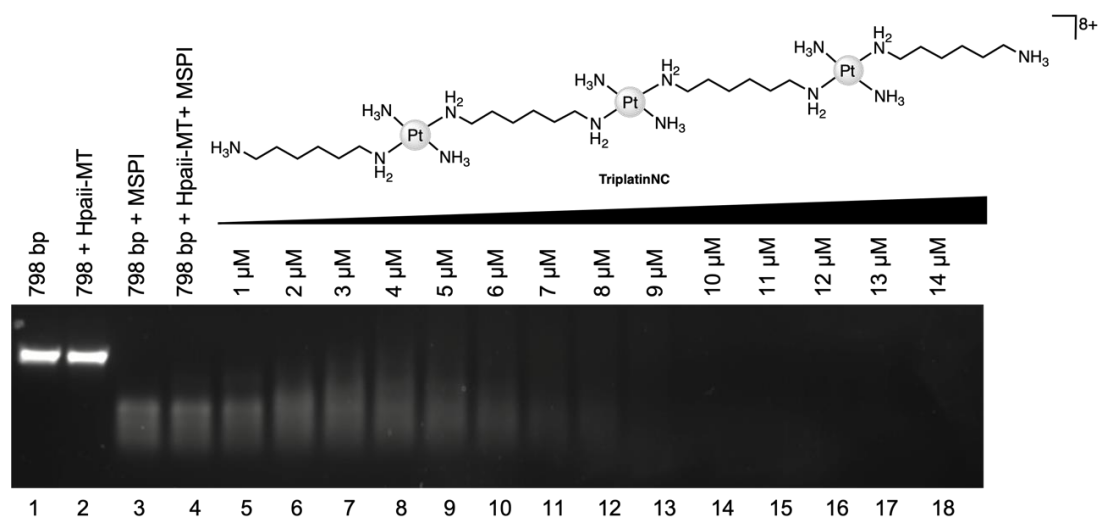


Figure B- 35. Agarose gel electrophoresis of the effects of TriplatinNC on the epigenetic-reading enzyme MSPI.

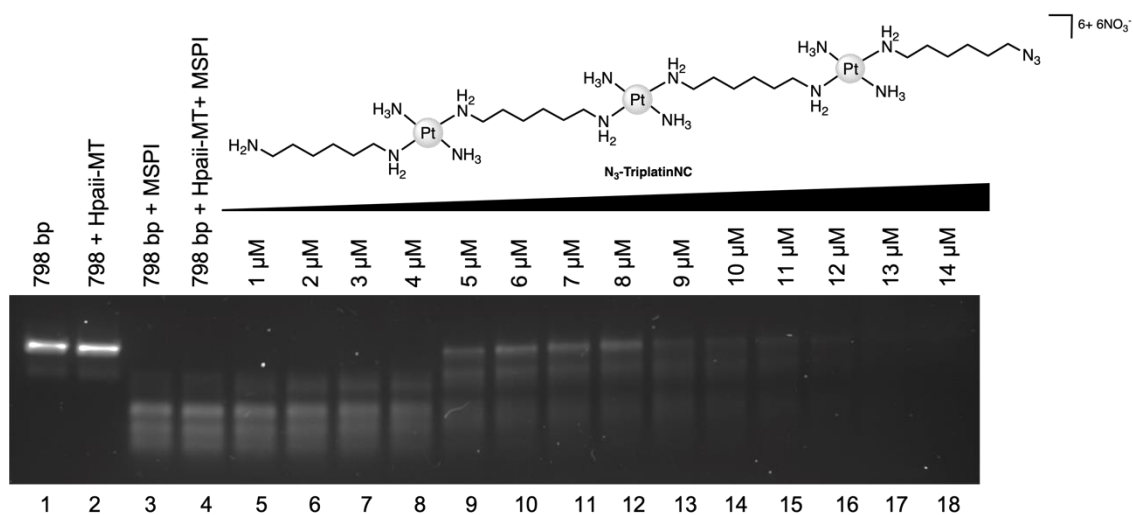


Figure B- 36. Agarose gel electrophoresis of the effects of $\text{N}_3\text{-TriplatinNC}$ on the epigenetic-reading enzyme MSPI.

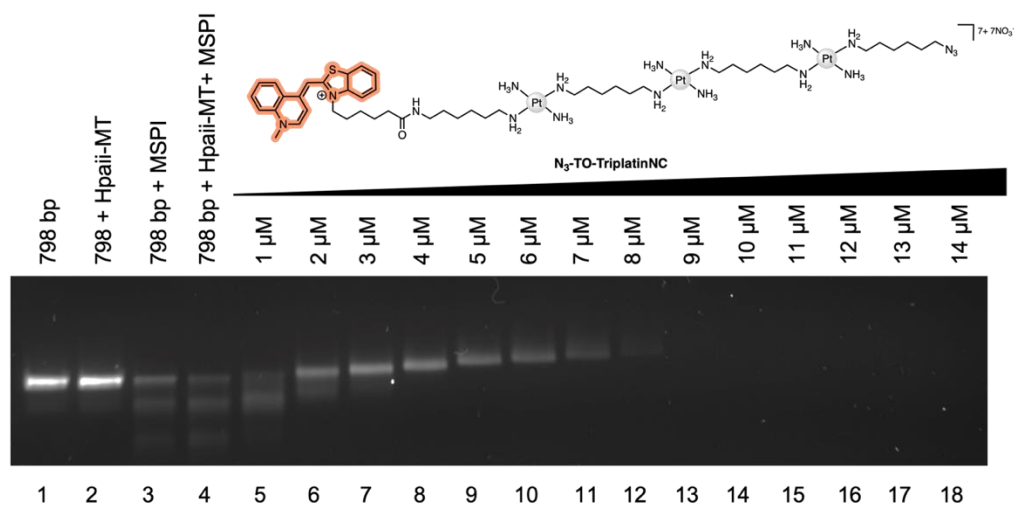


Figure B- 37. Agarose gel electrophoresis of the effects of $\text{N}_3\text{-TO-TriplatinNC}$ on the epigenetic-reading enzyme MSPI.

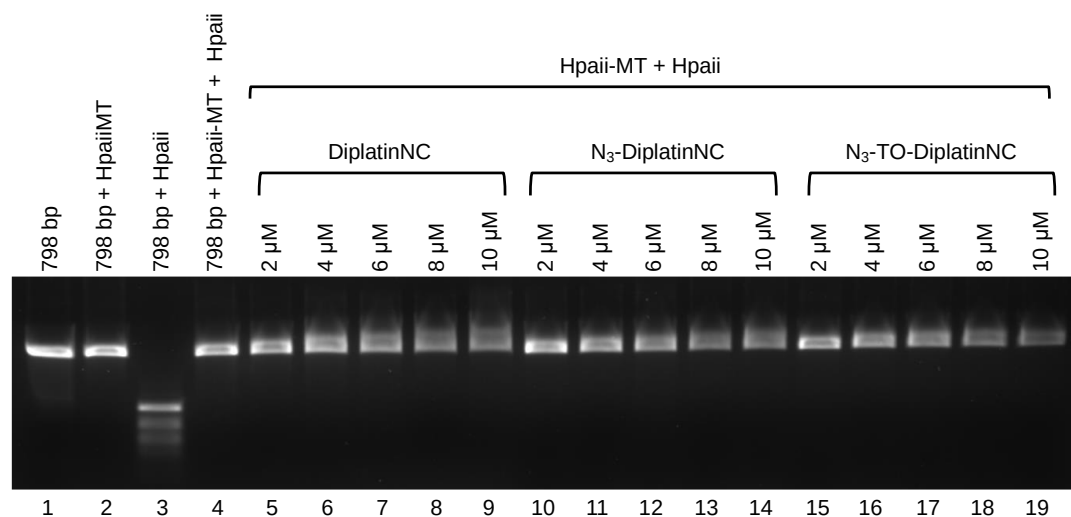


Figure B- 38. Agarose gel electrophoresis studies of the inhibition of the epigenetic writing activity of HpaII-MT by the dinuclear complexes DiplatinNC, N₃-DiplatinNC, and N₃-TO-DiplatinNC.

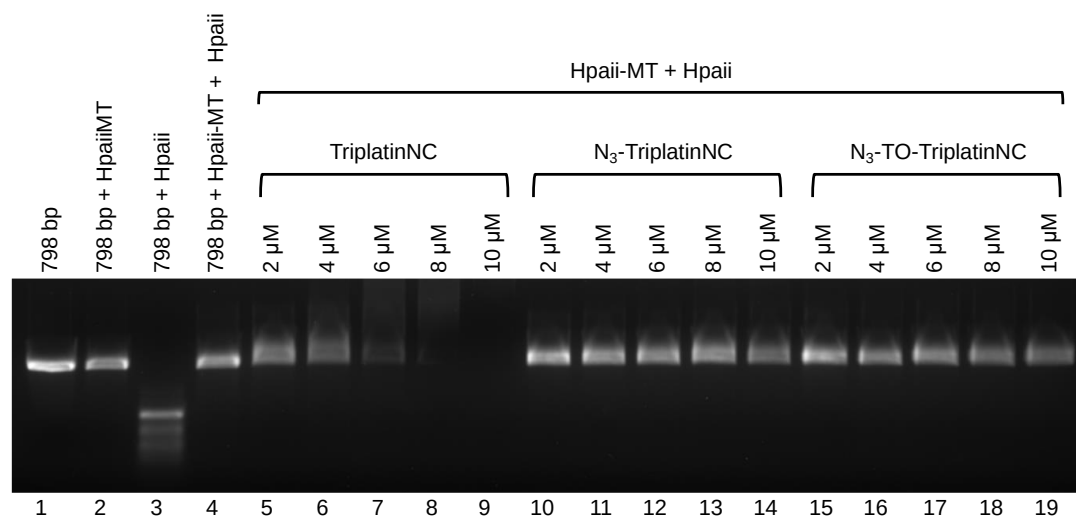


Figure B- 39. Agarose gel electrophoresis studies of the inhibition of the epigenetic writing activity of HpaII-MT by the trinuclear complexes TriplatinNC, N₃-TriplatinNC, and N₃-TO-TriplatinNC.

Appendix C

Biophysical DNA binding studies of the PPCs, N₃-PPCs, and N₃-TO-PPCs

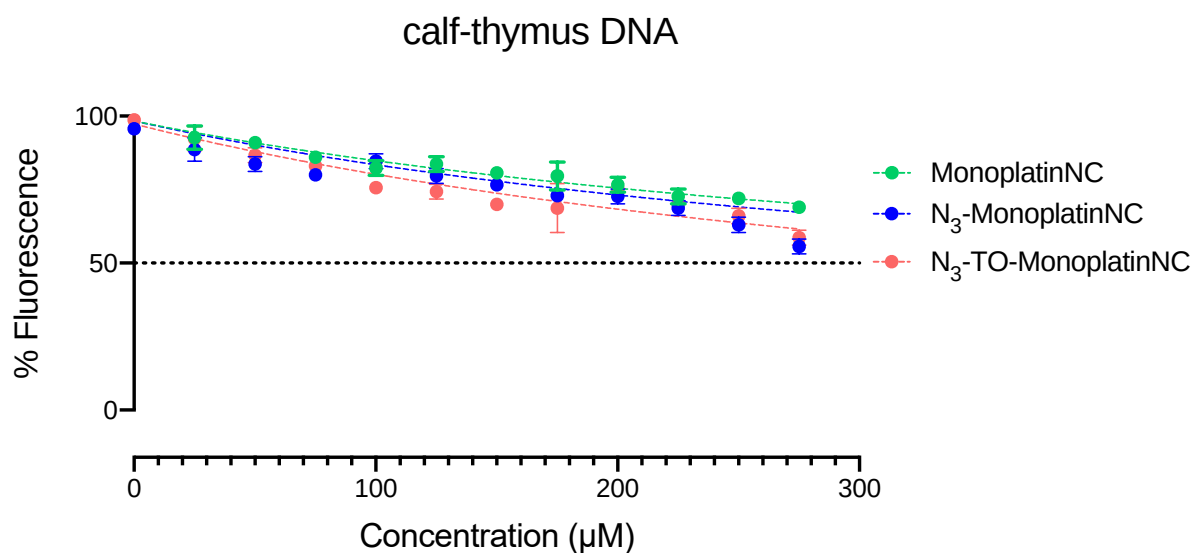


Figure C- 1. Competitive ethidium bromide displacement assay for MonoplatinNC, N_3 -MonoplatinNC, and N_3 -TO-MonoplatinNC on calf-thymus DNA.

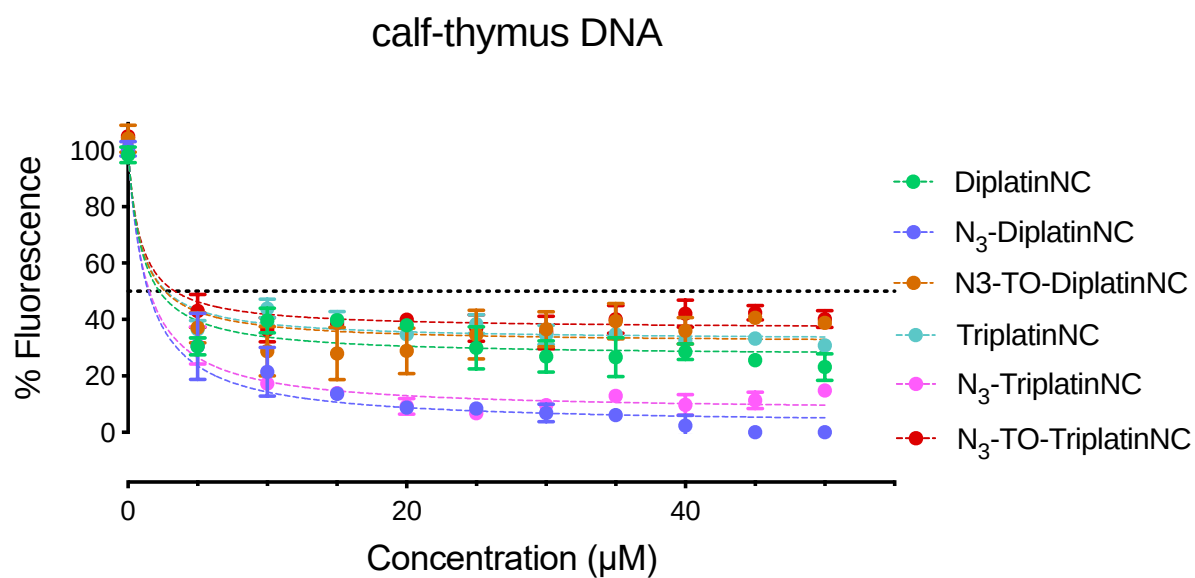


Figure C- 2. Competitive ethidium bromide displacement assay for dinculear and trinuclear PPCS, N_3 -PPCs and N_3 -TO-PPCs on calf-thymus DNA.

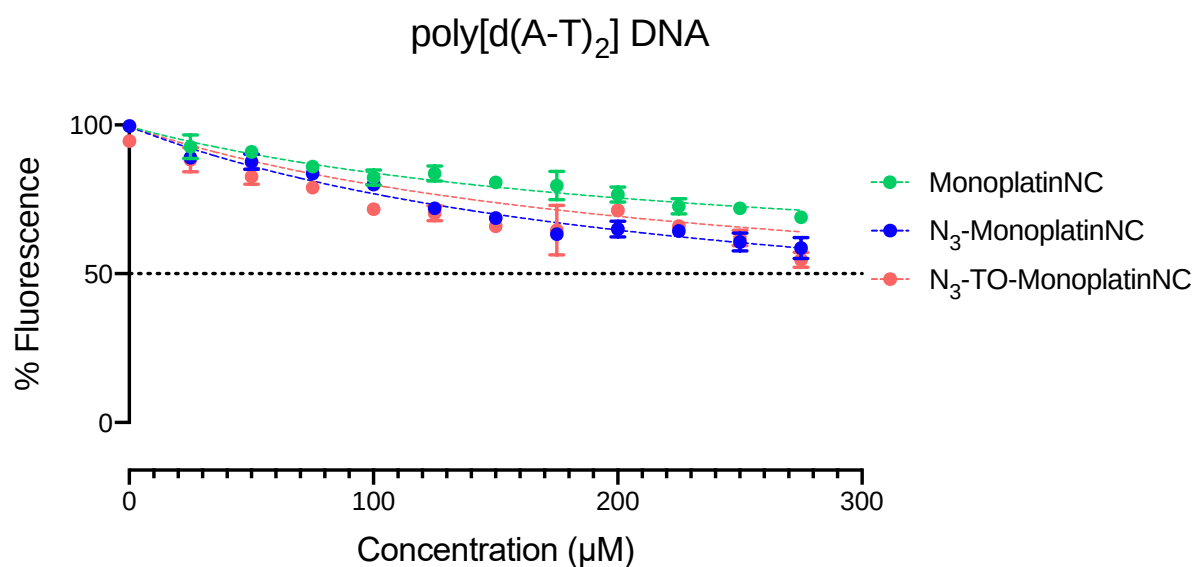


Figure C- 3. Competitive ethidium bromide displacement assay for MonoplatinNC, N₃-MonoplatinNC, and N₃-TO-MonoplatinNC on poly[d(A-T)₂].

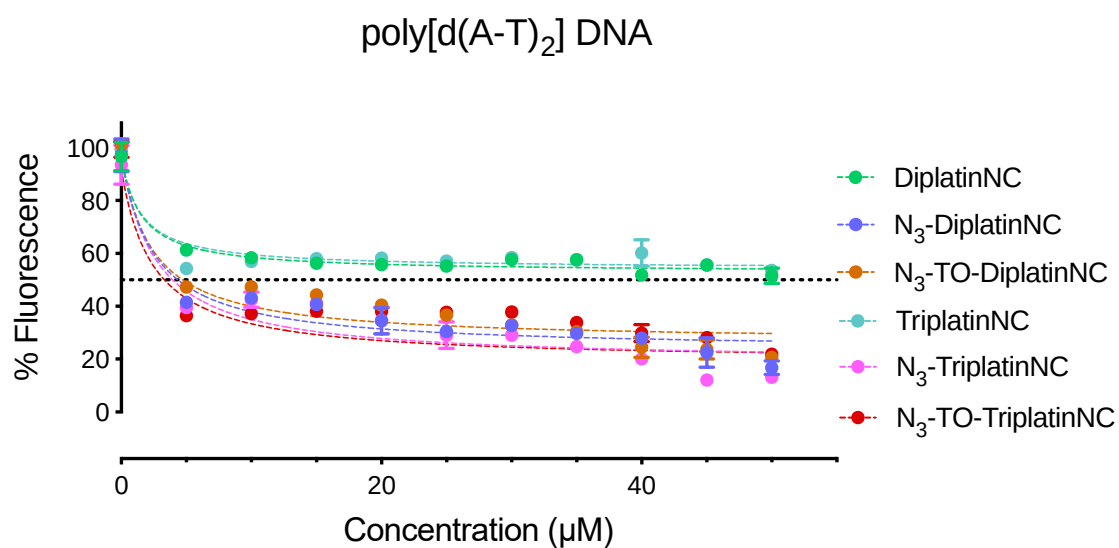


Figure C- 4 Competitive ethidium bromide displacement assay for dinuclear and trinuclear PPCs, N₃-PPCs and N₃-TO-PPCs on poly[d(A-T)₂].

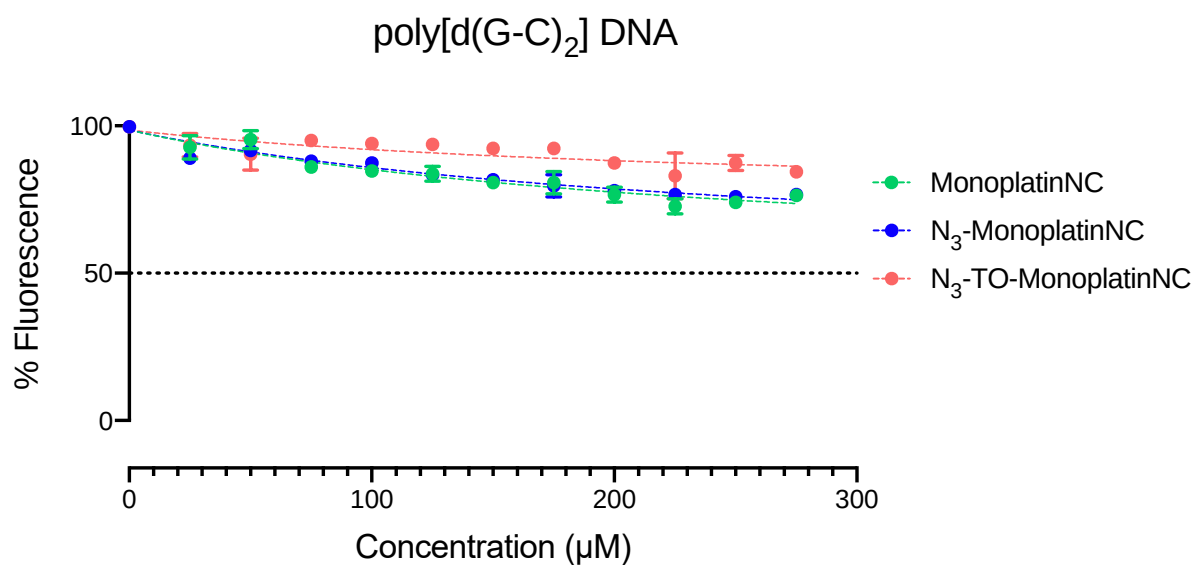


Figure C- 5. Competitive ethidium bromide displacement assay for MonoplatinNC, N₃-MonoplatinNC, and N₃-TO-MonoplatinNC on poly[d(G-C)₂].

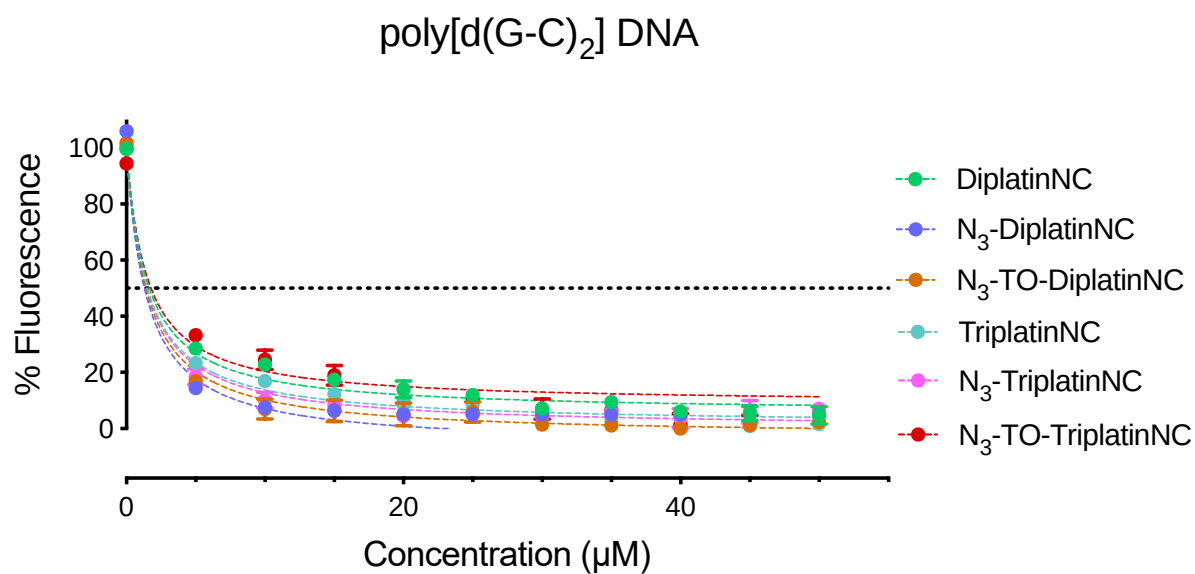


Figure C- 6. Competitive ethidium bromide displacement assay for dinuclear and trinuclear PPCs, N₃-PPCs and N₃-TO-PPCs on poly[d(G-C)₂].

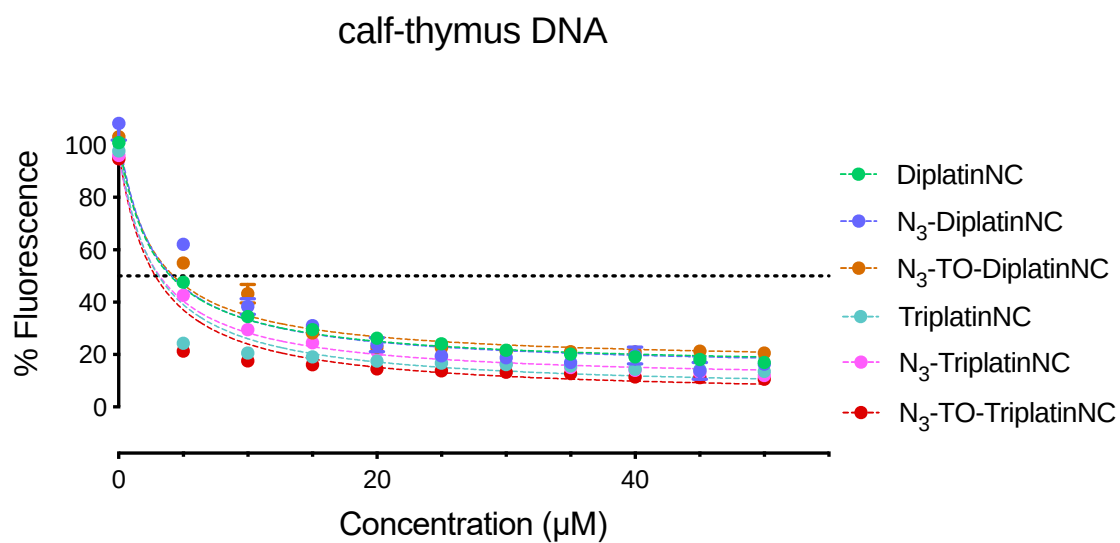


Figure C- 7. Fluorescence quenching studies of the dinuclear and trinuclear PPCs, N₃-PPCs, and N₃-TO-PPCs on calf-thymus DNA.

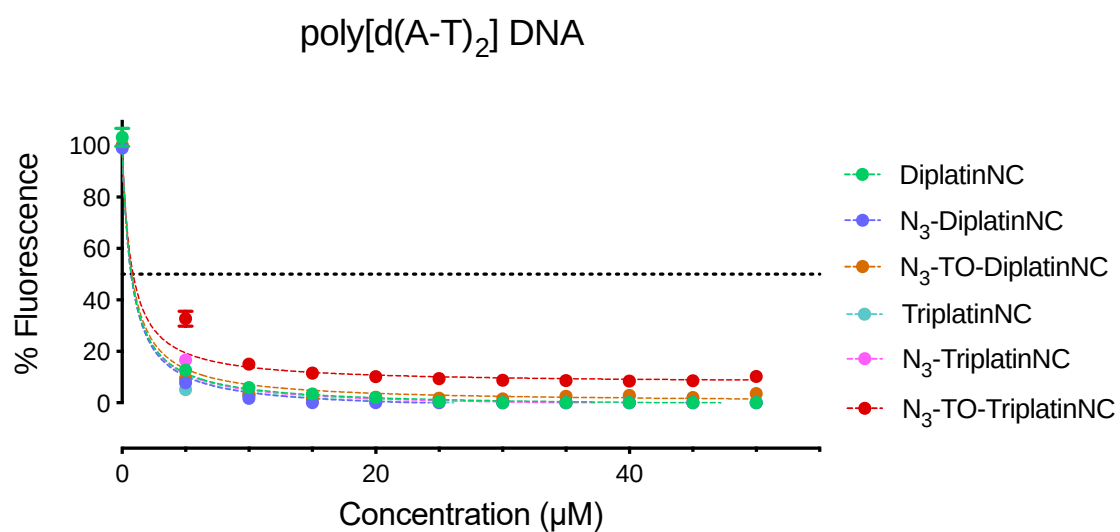


Figure C- 8. Fluorescence quenching studies of the dinuclear and trinuclear PPCs, N₃-PPCs, N₃-TO-PPCs on poly[d(A-T)₂] DNA.

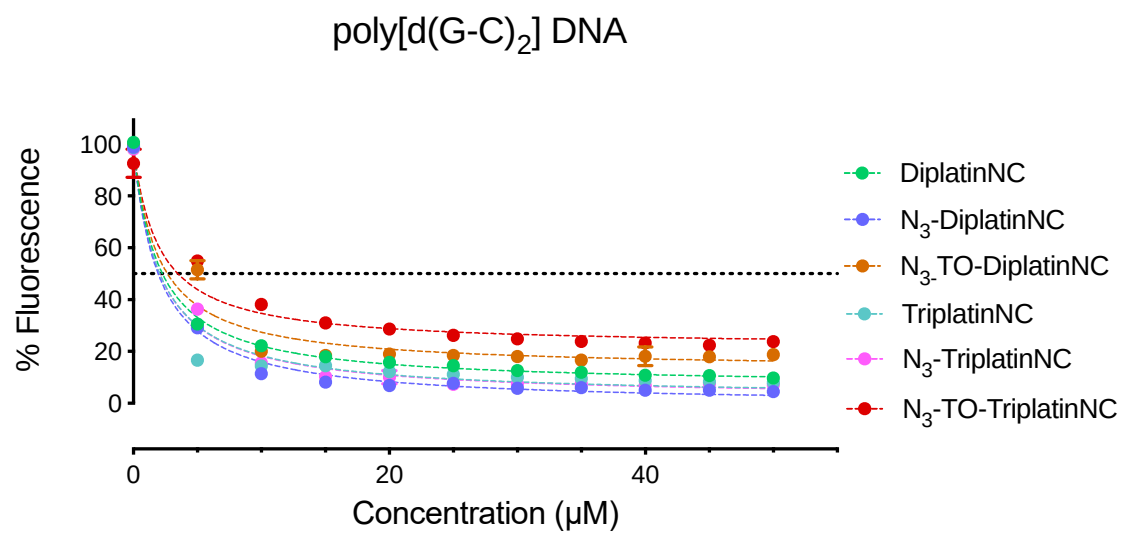


Figure C- 9. Fluorescence quenching studies of the dinuclear and trinuclear PPCs, N₃-PPCs, and N₃-TO-PPCs on poly[d(G-C)₂] DNA.

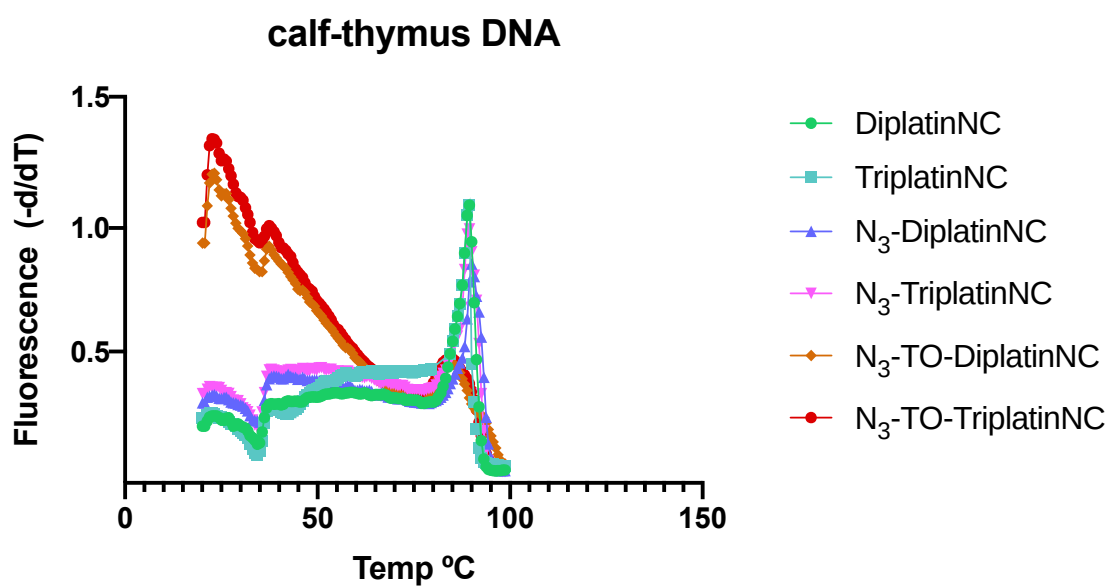


Figure C- 10. Thermal melting studies of the dinuclear and trinuclear PPCs, N₃-PPCs, and N₃-TO-PPCs on calf-thymus DNA.

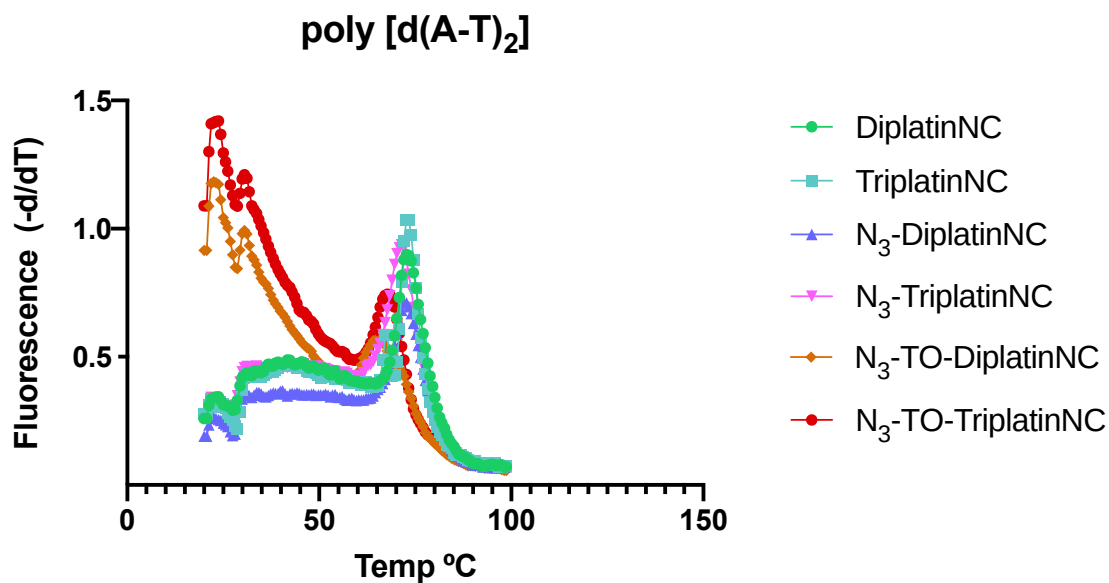


Figure C- 11. Thermal melting studies of the dinuclear and trinuclear PPCs, N₃-PPCs, and N₃-TO-PPCs on poly[d(A-T)₂] DNA.

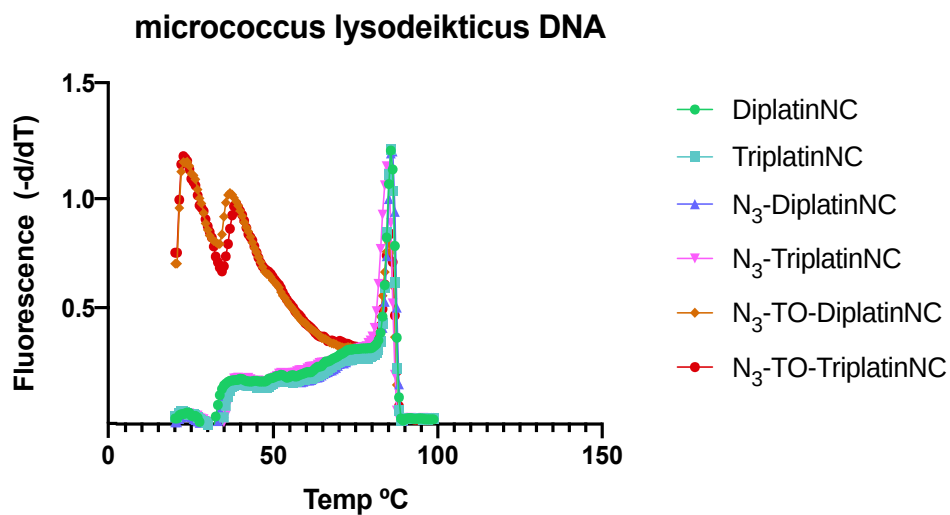


Figure C- 12. Thermal melting studies of the dinuclear and trinuclear PPCs, N_3 -PPCs, and N_3 -TO-PPCs on *micrococcus lysodeikticus* DNA.

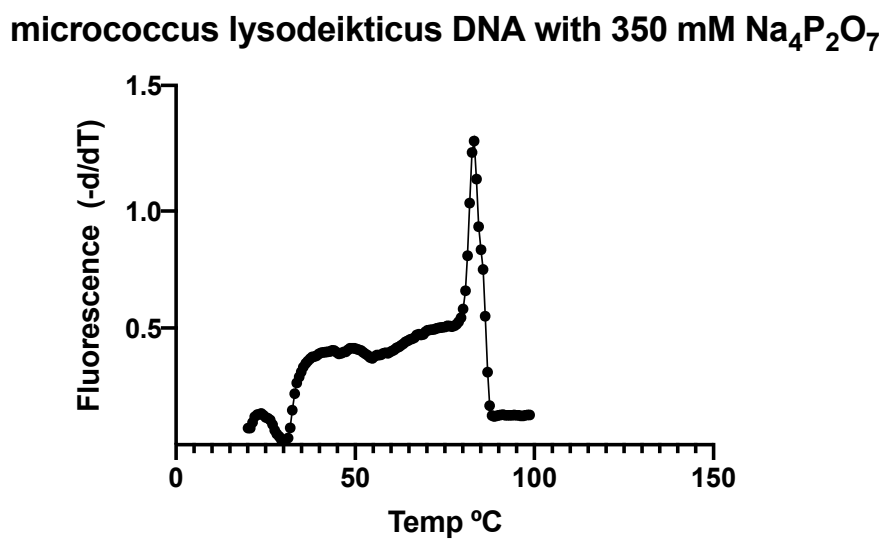


Figure C- 13. Thermal melting studies *micrococcus lysodeikticus* DNA in 350 mM $Na_4P_2O_7$.

Appendix D

In vitro, in vivo, and click labelling studies

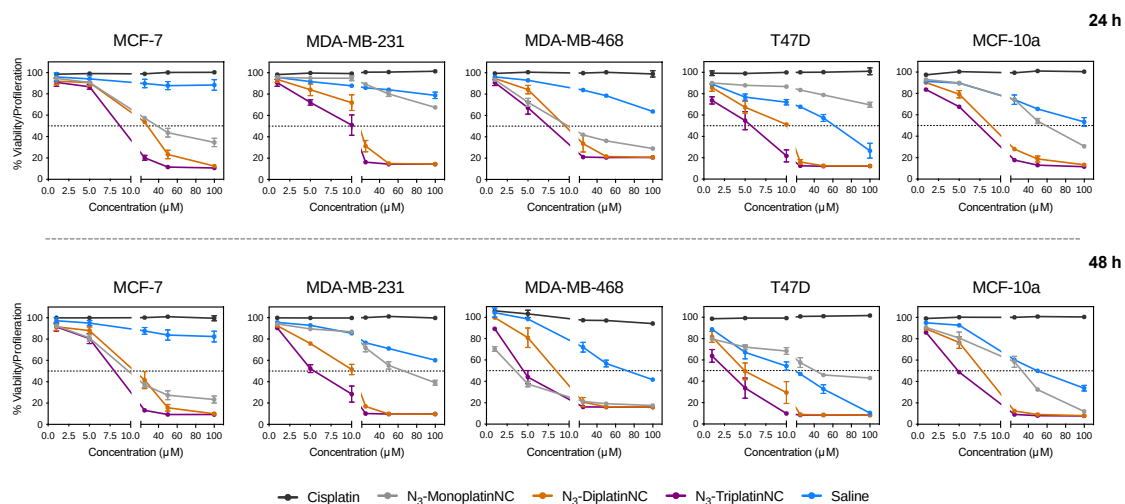


Figure D- 1. In vitro cytotoxicity analysis of N₃-PPCs.

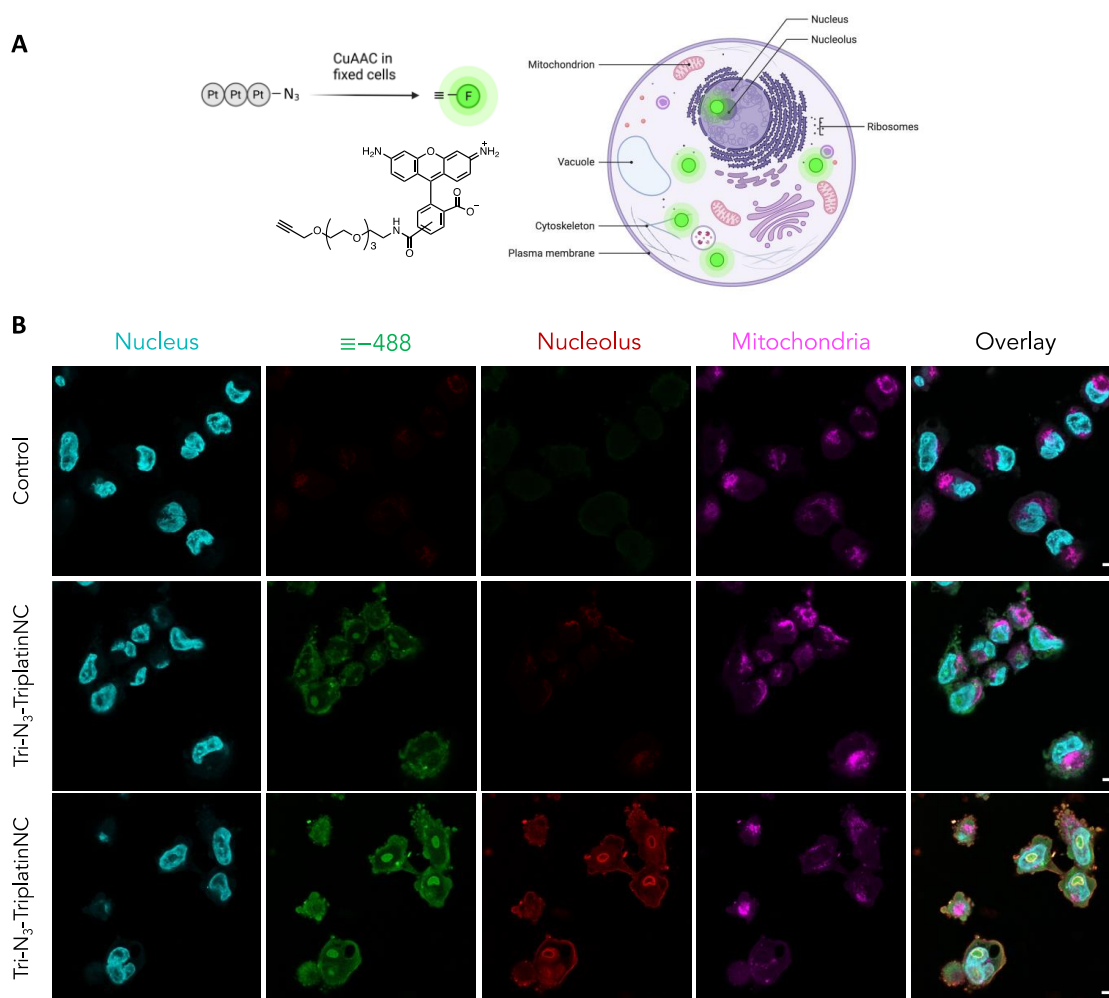


Figure D- 2A. Intracellular fluorescent labelling of N₃-PPC through CuAAC chemistry to examine cellular uptake and localisation; B. Confocal microscope images on 63x oil immersion lens of fixed MDA-MB-231 cells exposed to 10 μ M of N₃-TriplatinNC for 24 h and click to alkyne 488 dye (green). Nuclei were stained with NucBlue Fixed (blue), mitochondria were stained with MitoTracker Deep Red (magenta) and nucleoli were visualised by immunofluorescent staining of NPM (red). Scale bars indicate 10 μ m (overlay, bottom right corner).

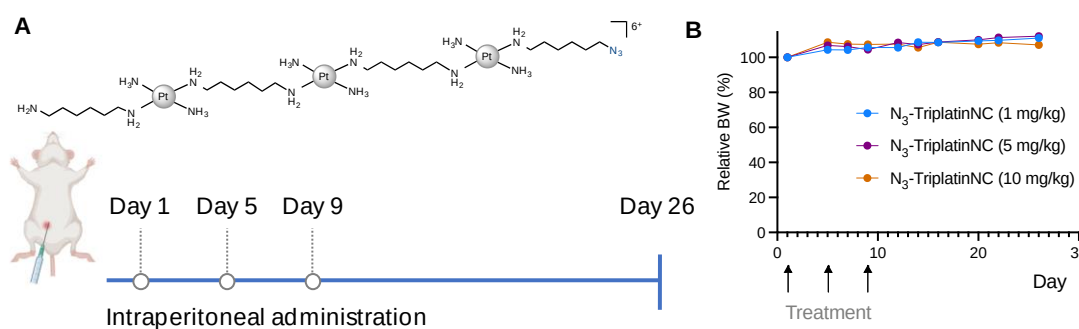


Figure D- 3A Experimental plan of maximum tolerated dose study for N₃-TriplatinNC; B. Relative body weight (BW) expressed as percent.

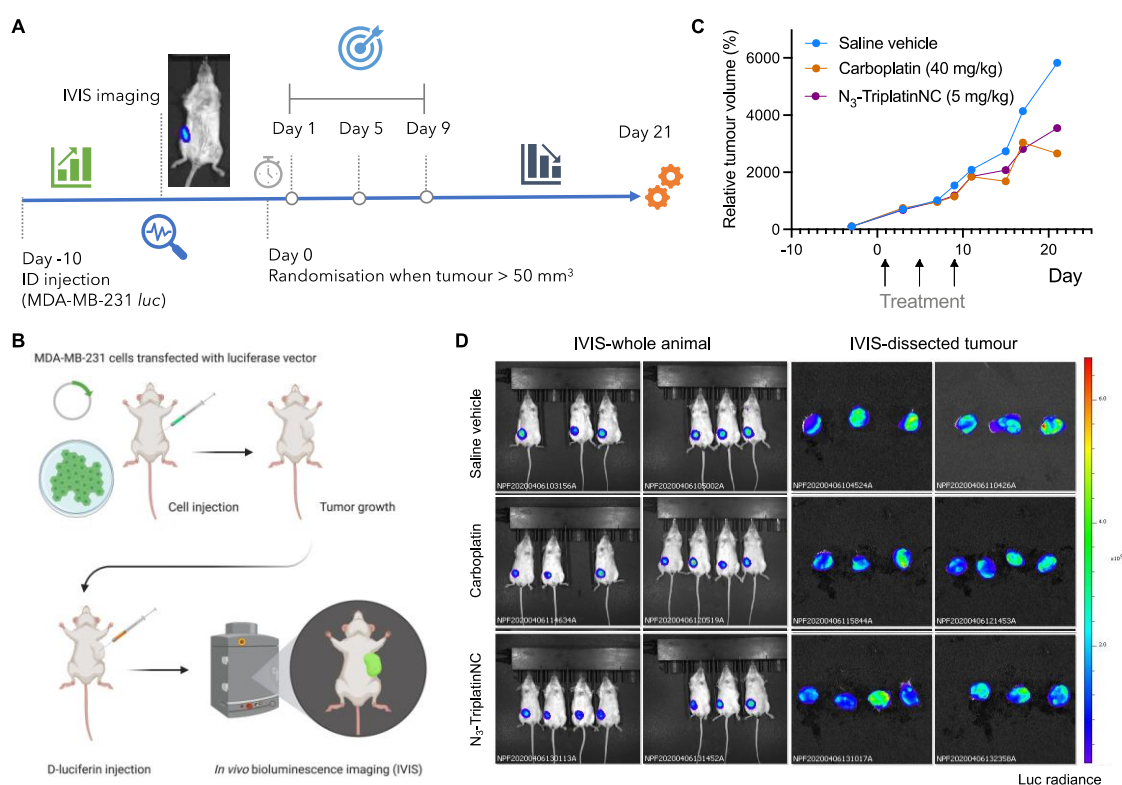


Figure D- 4. Experimental set-up and timeline of xenograft work; B. Transfection, culture, and injection of MDA-MB-231 luciferase (luc) cells for inherent tumour detection; C. Relative tumour volume is represented as a percentage; D. Bioluminescent imaging of tumours in whole animals on day 21 (endpoint) and dissected samples.