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This tutorial review summarizes chemical, biophysical and cellular biological properties of formally substitution-inert "non-covalent" polynuclear platinum complexes (PPCs). We demonstrate how modulation of the pharmacological factors affecting platinum compound cytotoxicity such as cellular accumulation, reactivity toward extracellular and intracellular sulfur-ligand nucleophiles and consequences of DNA binding is achieved to afford a profile of biological activity distinct from that of covalently-binding agents. The DNA binding of substitution-inert complexes is achieved by molecular recognition through minor groove spanning and backbone tracking of the phosphate clamp. In this situation, the square-planar tetra-am(m)ine Pt(II) coordination units hydrogen bond to phosphate oxygen OP atoms to form bidentate N-O-N motifs. The modular nature of the polynuclear compounds results in high-affinity binding to DNA and very efficient nuclear condensation. These combined effects distinguish the phosphate clamp as a third mode of ligand-DNA binding, discrete from intercalation and minor-groove binding. The cellular consequences mirror those of the biophysical studies and a significant portion of nuclear DNA is compacted, a unique effect different from mitosis, senescence or apoptosis. Substitution-inert PPCs display cytotoxicity similar to cisplatin in a wide range of cell lines, and sensitivity is indifferent to p53 status. Cellular accumulation is mediated through binding to heparan sulfate proteoglycans (HSPG) allowing for possibilities of tumor selectivity as well as disruption of HSPG function, opening new targets for platinum antitumor agents. The combined properties show that covalently-binding chemotypes are not the unique arbiters of cytotoxicity and antitumor activity and meaningful antitumor profiles can be achieved even in the absence of Pt-DNA bond formation. These dual properties make the substitution-inert compounds a unique class of *inherently* dual-action anti-cancer agents.

Introduction

Platinum-based anti-cancer drugs are the most widely prescribed cytotoxics and are used as components of almost half of all cancer treatments. The 1978 Federal Drug Administration (FDA) approval of cisplatin, Figure 1, for use in testicular cancer is considered the real start of the anticancer platinum era. In the intervening period the development of the field has been typical of drug development – the search for safer, better tolerated analogs and rational attempts to expand the efficacy profile of the clinically used drugs, especially to combat the onset of clinical resistance. Currently, we count three FDA-approved agents (cisplatin, carboplatin and oxaliplatin) and other more restricted agents – notably nedaplatin (Japan), lobaplatin (China) and heptaplatin (Korea) as comprising the anti-cancer platinum armamentarium, out of approximately 30 analogs which underwent human clinical trials.[1,2,3] All of these agents share the basic mononuclear *cis*-$[PtX_2(amine)_2]$ chemotype (X = leaving group, amine = neutral or carrier group), predicated on early structure-activity relationships and the acceptance of DNA as the molecular target of platinum drugs. The toxicity of platinum drugs is typical of cytotoxics – The Chemotherapy Handbook Springhouse (ISBN 0-87434-618-5) lists similar dose-limiting side effects for taxol and vinblastine, to use two examples.[4] Early on, much was made of the platinating agent-alkylating agent analogy, as both may be considered electrophilic agents forming covalent bonds with the purine and pyrimidine bases of the nucleic acid. Indeed the time span of platinum drug development and FDA approval, from cisplatin in 1978 to oxaliplatin in 2002, mirrors to some extent that of the alkylating agents with a 40-year span between approval of the first alkylating agent mechlorethamine in 1949 and the metabolically activated version of ifosfamide in 1988.

The most recent period of drug development has not been kind to platinum with the failure of the most studied candidates in the mid-1990's to late 2000's – notably Picoplatin, Satraplatin and Triplatin (BBR3464) – to advance to full clinical use. This

period also coincided with (and was somewhat preceded by) the emphasis on "targeted" drug discovery in the hope of more specific drug action. Advances in understanding of cancer biology and signaling pathways leading to apoptosis (the ultimate goal of an anti-cancer agent) does give the chemist significant inspiration for "smart" drug design to ally with the substitution kinetics and pharmacokinetics of platinum compounds, where reactions take place usually on the scale of hours or days. This review summarizes chemical and biological studies on polynuclear platinum complexes (PPCs) – a discrete chemotype distinct in structure and mechanism from the mononuclear agents, Figure 1. Especially, emphasis will be on recent advances showing formally substitution-inert PPCs as a distinct sub-class with a unique profile of biological activity in their own right. The drug profile of cisplatin and analogs has been well documented and will not be repeated here.[1,2,3]

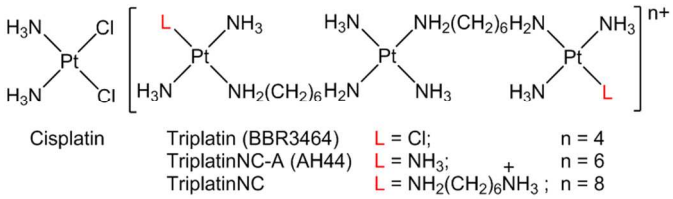


Figure 1. Structures of principal cationic polynuclear platinum complexes (PPCs) to be discussed. AH44 (TriplatinNC-A) and TriplatinNC (AH78) and are substitution-inert complexes derived by displacement of Pt-Cl in parent BBR3464.

The study of PPCs had its origin in the hypothesis that "Complexes capable of molecular interactions not accessible to monomeric complexes or acting by different mechanisms might also display a broader spectrum of clinical activity".[5] Their development represents an approach to systematically altering the cellular response induced by cisplatin by changing the nature and structure of the DNA lesion induced. Discrete downstream effects of protein recognition could be manipulated to eventually afford a therapeutic advantage over other clinical agents. To achieve this goal it was necessary to challenge the accepted structure-activity relationships, design new chemotypes and delineate their biological action. Proof of the success of this approach was given by the advance of Triplatin (See Figure 1, BBR3464, a tetrapositive, trinuclear bifunctional DNA-binding agent whose adducts are structurally different to those of the mononuclear drugs) to Phase II human clinical trials, the only non-cisplatin analog to be introduced to humans.[1,6] With this advance the paradigm of cisplatin-based antitumor agents was altered. Triplatin remains the only example of a non-cisplatin structure to enter human trials (and likely to remain so for some time yet) and remains proof of principle for the utility and promise of new structural chemotypes in platinum anti-cancer drug development. The nature of the PPC chemotype means it is a discrete and modular DNA binding device with high potential as a drug-design scaffold. It should be noted that the generic dinuclear platinum formula is given by $[\{PtCl_m(NH_3)_{3-m}\}_2\mu\text{-}(H_2N(CH_2)_nNH_2)]^{(2-m)+}$ where m = 1 or 2 and thus a great variety of structures is possible, including those containing two linked cis-platin like moieties.[6] Additionally the simple diamine linker can be modified to incorporate bridging μ-spermidine and μ-spermine linkers as well as the central $\mu\text{-}\{Pt(NH_3)_2(\mu\text{-}H_2N(CH_2)_nNH_{2\text{-}})_2\}$ linker leading to Triplatin. To make systematic study manageable from this wide array, the complexes containing $[PtClN_3]$ coordination spheres were prioritized as being most different from the mononuclear *cis*-$[PtX_2(amine)_2]$.

Biological and Clinical Activity of Triplatin (BBR3464). Triplatin clinical results have been documented but bear brief repetition here.[1,6] It is an exceptionally potent cytotoxic agent with IC_{50} values at least 20-fold lower than cisplatin across a very broad range of human tumors sensitive, resistant and refractory to cisplatin. The effective *in vivo* doses - 0.3 to 0.6 mg/kg compared to standard cisplatin doses of 3-6 mg/kg - parallel the greater cytotoxicity. Impressive efficacy included complete tumor regressions in lung tumors. Triplatin showed a superior activity against p53-mutant tumors as compared to those carrying the wild-type gene. In the NCI "60-cell line" panel breast, renal and colon cancers were, on the whole, deemed more susceptible to the trinuclear drug than cisplatin, mirroring to some extent the later clinical findings.[1,6] In many cell lines Triplatin cellular accumulation and DNA-bound platinum are much higher than those observed for cisplatin.[6] Overall, these pre-clinical data confirmed the potential for a genuinely different profile compared to the mononuclear drugs. The most notable features of the clinical trials were:

i. Phase I studies fixed a dose of 0.9 –1.1 mg/m^2 as maximum tolerated dose (MTD) and extrapolated well from the preclinical studies. Dose limiting toxicity is diarrhea (treatable with loperamide) with no evidence of nephrotoxicity, neurotoxicity or severe emesis.

ii. Partial responses in Phase I were seen in metastatic pancreatic cancer (for four months confirmed from computerized tomography scans), metastatic melanoma and bronchiolo-alveolar carcinoma. A Phase I combination with 5-fluorouracil (5-FU) gave a confirmed response in breast cancer.

iii. In Phase II clinical trials 5/28 partial responses were seen with patients with cisplatin-relapsed ovarian cancer. 4/5 of these patients were p53wt. The response in some cases was durable (in two of the partial responses duration was > 2.5 years, a unique result and one which would not be replicated by carboplatin alone, for example). 1 partial response was observed in cisplatin-refractory ovarian cancer although notably the patient was p53 mutant.

iv. Pharmacokinetic analysis did show drug decomposition in blood with bridge cleavage resulting in production of inactive mononuclear and dinuclear metabolites.[7] Decomposition in human plasma was three times quicker than for murine plasma with both reversible and non-reversible (bond-forming) components. The products of drug decomposition can be reproduced upon reaction with sulfur nucleophiles, especially glutathione (GSH).[8]

The pharmacokinetic issues (albeit not dissimilar to cisplatin), a relatively narrow Therapeutic Index, coupled to complications

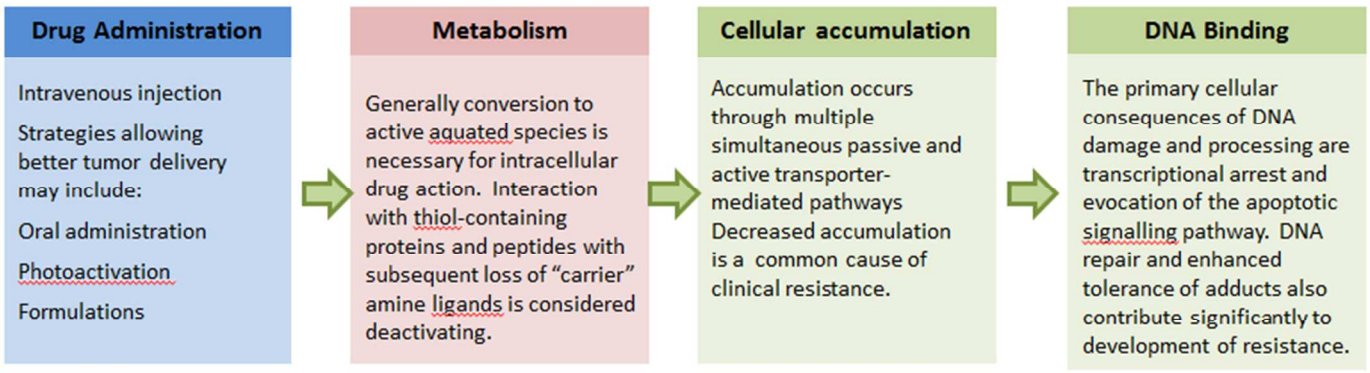


Figure 2. Determinants of platinum drug cytotoxicity and antitumor activity suggesting potential avenues for rational design of new chemotypes.

from drug company takeovers and realignment, meant that Triplatin did not advance beyond Phase II clinical trials – an unfortunate but common fate in anti-cancer chemotherapy.[9] Given the current nature of the drug development process, deviations and delays caused by small company realignments should not be underestimated.

1. Mechanisms of Action of Platinum Anti-cancer Agents. Three principal factors – cellular accumulation, the frequency and structure of DNA adducts, and the extent of metabolizing interactions - control the cytotoxicity and antitumor activity of platinum anticancer drugs, Figure 2. All of these factors are clearly susceptible to chemical manipulation. Coupled with the chemical and pharmacological aspects, an understanding of how the nature of the Pt-DNA adducts affect signaling pathways may lead to molecular rationales for tissue specificity and/or combination chemotherapy with targeted drugs.[1,10,11] Cellular sensitivity to cisplatin correlates with the presence of the tumor suppressor protein wtp53.[12] The efficacy and eventual utility of any anti-cancer agent is a balance between target inhibition (in this case DNA) and metabolic interactions. A common estimate is that significantly < 5% of administered platinum is bound to DNA. Deactivation by sulfur nucleophiles in plasma proteins, especially Human Serum Albumin (HSA), and intracellular glutathione (GSH), is considered a principal source of non-DNA biochemical interactions of Pt drugs.[13,14] In the following sections we will contrast the properties of mononuclear and multinuclear complexes with respect to the fundamental predictors of platinum complex antitumor activity.

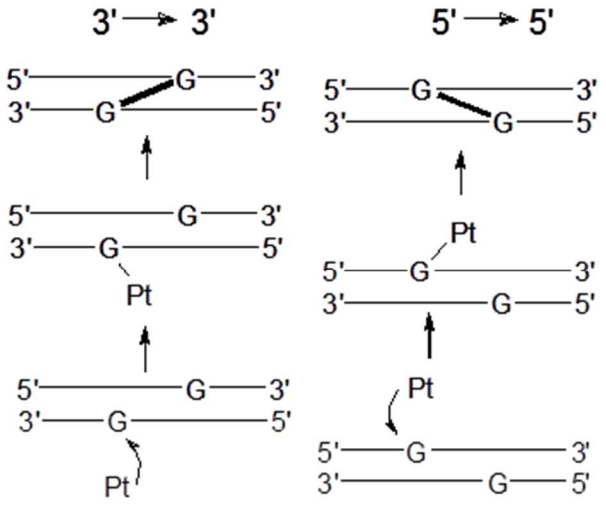


Figure 3. Schematic of directional isomers in long-range (Pt,Pt) IXLs.

DNA Binding of Triplatin. The acceptance of DNA as cellular target for cisplatin and structural analogs has led to detailed understanding of the modes of binding (*via* aquation, monofunctional, then bifunctional binding), structural consequences, protein recognition and effects on signalling pathways.[2,3,10] The mononuclear cisplatin forms predominantly bifunctional 1,2-intrastrand crosslinks between adjacent guanines (GG). Secondary adducts are 1,2-intrastrand crosslinks between a G and an A (adenine); 1,3-intrastrand crosslinks and 1,2-interstrand crosslinks between neighboring guanines in adjacent (GC) base pairs. The structural features of the 1,2-intrastrand GG adduct are helical unwinding and a directed bend into the major groove.[10,15] The primary cellular consequence of DNA damage and processing is considered transcriptional arrest through stalling of RNA polymerase.[11]

In contrast, Triplatin interactions with DNA – long-range (Pt,Pt) inter and intra-strand crosslinks where the platination sites may be separated by up to 4 intervening base pairs - are distinct from the mononuclear-based agents and, indeed, unlike those of any DNA-damaging agent in clinical use.[16] A unique aspect of this research has been the first comprehensive description of non-interconvertible *directional isomers* of long-range interstrand crosslinks (IXLs). Crosslinks occur not only in the ''normal'' 5' -> 5' direction, since DNA is read from the 5'-side, but also in the ''opposite'' antiparallel 3' -> 3' direction, Figure 3. Complementary molecular biology and spectroscopic approaches showed that both crosslinks occur as a pair of conformers and that all four conformers affect DNA in a distinctly different way. Using fully-^{15}N labelled Triplatin and the 12-mer 5′-d(ATAT**G**TACATAT)$_2$ duplex (5' > 5' between **G** and the G of complementary C) 2D {^{1}H,^{15}N} HSQC NMR confirmed formation of two distinct, non-interconvertible conformers.[17] In contrast, studies with 5′–d{TATACTA**G**TATA}$_2$ (now a 3' -> 3' IXL) showed that the reaction did not yield a single distinct 1,4-GG IXL, and numerous cross-linked adducts formed. Molecular dynamics

simulations showed a distorted structure with fraying of the end base pairs and considerable widening of the minor groove. Overall the global conformational changes of both 5' -> 5' and 3' -> 3' long-range crosslinks are distinguished by flexible bending and "Z-DNA-like" structure around the platinated sites.[16] The adducts are not substrates for high mobility group (HMG) protein recognition which binds avidly to the cisplatin 1,2-GG intrastrand adduct, confirming that modification of DNA structure can lead to differential protein recognition. This fact separates the mononuclear and polynuclear chemotypes at the level of DNA-protein function. The overall results support the view that the multiple DNA cross-links formed by BBR3464 may all contribute substantially to its cytotoxicity, so that the overall cytotoxicity could be the sum of the contributions of different interstrand and intrastrand adducts.

2. Substitution-inert Polynuclear Platinum Complexes. Antitumor Activity through "Non-Covalent" Binding. The high charge on Triplatin and similar PPCs with monofunctional [$PtClN_3$] coordination spheres results in pre-association through hydrogen-bonding and electrostatic interactions with many biomolecules including DNA,[17,18] Human Serum Albumin[19] and phospholipids.[20] The observation of a reversible component in human plasma pharmacokinetics can also be reasonably attributed to "non-covalent" interactions. The relevance of pre-association in the pharmacokinetics, and ultimately target binding and biological activity, may be examined by study of substitution-inert compounds where the Pt-Cl bonds are displaced by NH_3 or "dangling" amine $-H_2N(CH_2)_nNH_3^+$. This direction has led to new and exciting avenues for drug design. This review will focus on the two compounds described in Figure 1 – TriplatinNC containing the dangling amine, as the paradigm for substitution-inert compounds, and its analog TriplatinNC-A with the simple NH_3 group in place of the Cl^- ligand. The properties will be contrasted with cisplatin and the covalently-binding BBR464, from which they are derived.

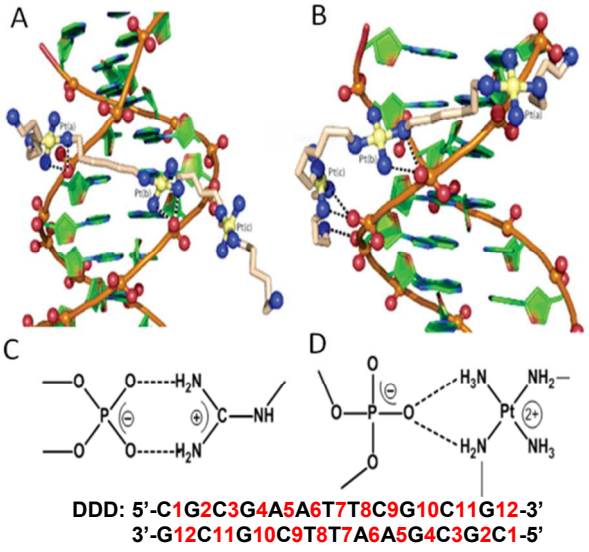


Figure 4. Structure of the TriplatinNC complexed to DDD (NBD 2DYW) showing (A.) groove spanning (B.) backbone tracking [21,22] Comparison of arginine fork (C.) and phosphate clamp (D.) Adapted from ref. 20

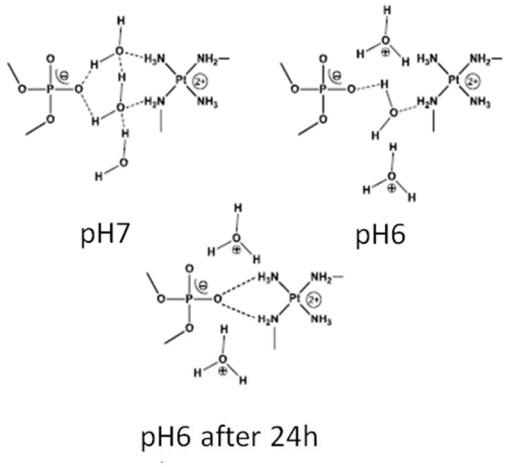
DNA Binding and Affinity of Substiution-inert PPCs. ***The Phosphate Clamp as Novel DNA Binding Motif.*** The X-ray crystal structure of the Dickerson-Drew Dodecamer, (DDD, $[d(CGCGAATTCGCG)]_2$) with TriplatinNC showed a third mode of ligand-DNA recognition distinct from the conventional modes of intercalation and groove binding (NDB entry 2DYW).[21] Hydrogen bonding with phosphate oxygens results in either backbone tracking or groove spanning through formation of "phosphate clamps" where the square-planar tetra-am(m)ine Pt(II) coordination units all form bidentate N-O-N complexes with phosphate oxygen OP atoms, Figure 4. The generality of the "phosphate clamp"-DNA binding motif was confirmed by a second crystal and molecular structure with TriplatinNC-A.[22] In both cases, the conformation in the DDD-Phosphate Clamp complexes differs significantly from that of the native structure (NDB entry bdl084). The axial bend and the axial path length shortening ratio are significantly greater than those of control (DDD-TriplatinNC-A: 28°/2.0%; DDD-TriplatinNC:27°/2.4%; DDD: 12.9°/0.66%); helical parameters are perturbed and the minor groove width profile is modestly impacted. Note that the bending is similar to that achieved by the bifunctional 1,2-intrastrand adducts of cisplatin. Circular Dichroism spectroscopy indicate very similar conformations for both compounds in solution. The TriplatinNC-DNA interactions are similar in some ways to those of the guanidino group of arginine which shows an analogous, but attenuated, OP clamping ability in which two OP atoms form a clamp-like structure, the Arginine Fork, Figure 4.[21,22] Preassociation of Triplatin on DNA prior to covalent bond formation plays an important role in the binding kinetics as well as the structure of the final DNA adducts, and is likely mediated through the phosphate clamp interactions.[17,18]

Do the solution binding properties reflect the crystallographically determined modes of groove spanning and backbone tracking ? The presence of the phosphate clamp motif in solution was confirmed by 2D ^1H NMR studies on the DDD duplex where significant A-T contacts, mainly on nucleotides A6, T7 and T8 were observed implying a selective bridging from C9G10 in the 3' direction to C9G10 of the opposite strand (See Figure 4 for duplex sequence). {^1H, ^{15}N} HSQC NMR Spectroscopy using the fully ^{15}N-labelled compound TriplatinNC showed at pH6 significant chemical shift and $^1J(^{195}Pt\text{-}^{15}N)$ coupling constant differences from free complex and DDD-TriplatinNC at pH 7 indicative of formation of the phosphate clamp.[23] The signals associated with the formation of the phosphate clamp result in downfield shifts of approximately 1.5 ppm in the proton dimension and 20 ppm in the nitrogen dimension for both cross-peaks indicating a dramatic change in the chemical environment of the amino/ammine hydrogens rather than the platinum, Table 1. Typical coupling constants are ~300 Hz for $^{195}Pt\text{-}N_4$ complexes or for $Pt\text{-}^{15}NH_3$ *trans* to another NH_3 (Z), for example $[Pt(^{15}NH_3)_2(Z_2)]^{n+}$ complexes. The phosphate clamp signals exhibit satellite peaks with a lower coupling frequency of $^1J(^{195}Pt\text{-}^{15}N)$ 240-270 Hz for both $^{15}NH_3$ and $^{15}NH_2$ ligands. Hydrogen-bonding to highly electronegative phosphate oxygen will reduce electron density on the nitrogen

	Pt-NH_3(pH 7.0)		Pt-NH_2CH_2-(pH 7.0)		Pt-NH_3(pH 6.0 PC)		Pt-NH_2CH_2-(pH 6.0 PC)	
	$\delta(^1H/^{15}N)$	$^1J(^{195}Pt\text{-}^{15}N)$, Hz	$\delta(^1H/^{15}N)$	$\delta(^1H/^{15}N)$	$\delta(^1H/^{15}N)$	$^1J(^{195}Pt\text{-}^{15}N)$, Hz	$\delta(^1H/^{15}N)$	$^1J(^{195}Pt\text{-}^{15}N)$, Hz
TpNC	4.19/-63.5	293	4.72/-44.2	4.72/-44.6	N/A	N/A	N/A	N/A
TpNC-DDD	4.30/-63.9	303	4.84/-44.3	4.84/-44.3 4.85/-44.6	5.82/-43.3 5.94/-43.0	270 259	6.25/-24.2 6.39/-24.0	249 241

Table 1 Chemical shifts (^1H / ^{15}N in ppm) and coupling constants ($^1J^{(195}Pt\text{-}^{15}N$ in Hz) of free TriplatinNC (TpNC) and bound to the Dickerson-Drew-Dodecamer (DDD) at varying pH. The PC designation indicates shifts and coupling constants arising from the formation of the phosphate clamp.

am(m)ine and the "delocalization" of the NH bond may be considered deshielding for both ^1H and ^{15}N. At neutral pH the association of TriplatinNC to the DNA backbone is most probably water mediated, as has been found for many minor-groove binding agents to DDD.[24] Upon lowering the pH to 6 the hydrogen-bond network and with it the interface between the backbone and the drug is partly disrupted; hence the association of TriplatinNC is no longer mediated by water but can interact directly with the negatively charged oxygen:



DNA Binding Affinity. The apparent binding constants on Calf Thymus (CT) DNA for TriplatinNC of K_{app} ~5 x10^7 M^{-1} are significantly higher compared to the K_{app} values "classical" minor-groove binders netropsin and pentamidine of 2.55 x 10^6 and 8.77 x 10^5 $M(bp)^{-1}$, respectively.[25] The affinity is also higher than for ethidium bromide where the intrinsic binding constant (K_b) was identified as 8.8 x 10^6 $M(bp)^{-1}$ through direct spectrophotometry. The binding affinities do not change significantly with structural variation – i.e. altering chain length within the TriplatinNC structure or indeed, for non-terminally functionalised trinuclear TriplatinNC-A and the polyamine-bridged dinuclear compounds $[\{Pt(NH_3)_3\}_2\text{-}\mu\text{-spermidine}]^{5+}$ and $[\{Pt(NH_3)_3\}_2\text{-}\mu\text{-spermine}]^{6+}$ (K_{app}~3 x10^7 M^{-1}).[26,27]

Base-specific nucleic acid interactions. The "classical" minor groove binders such as netropsin and Hoechst 32258 by definition show distinct binding preferences for A-T tracts. In the case of the substitution-inert PPCs the two canonical modes of binding elucidated by crystallography and NMR spectroscopy are reflected in base-specific interactions. There is a clear correlation between A·T content and the stabilising effect of TriplatinNC. The difference in melting temperature increases with the amount of A·T base pairs from ΔT_M = +7.0 °C for CT DNA to +22.6 °C for *Clostridium perfringens* DNA (73% A·T), and then finally to > +28 °C for pure A·T polynucleotides. The ΔT_M of TriplatinNC-modified homopolymeric and alternating copolymeric sequences are almost identical showing that stabilisation arises from the discrete binding of TriplatinNC, and is not influenced by the initial melting temperature of the untreated polynucleotide. The two distinctive limiting interaction modes may be distinguished by their formational dependence on tertiary DNA helical topology and G·C content.[25] Comparison of biophysical studies and NMR data for the TriplatinNC adduct of the G-C rich sequence $\{5'\text{-AACGCGCGCGAA-}3'\}_2$ compared to that of the DDD suggests that binding occurs predominantly in a backbone tracking manner rather than minor-groove spanning. DNA condensation (see below) is driven by minor-groove recognition.

Condensation Effects. UV/Visible spectroscopy, total intensity light scattering, gel retardation effects and atomic force microscopy all confirmed that TriplatinNC is very effective in condensing nucleic acids. The efficacy of various condensing agents in inducing DNA or RNA condensation can be quantified by determining the EC_{50} value, the concentration of a condensing agent at the midpoint of the condensation. The EC_{50} value of TriplatinNC (0.15 ± 0.1 μM) is ~27-fold lower than that of spermine obtained at the same conditions.[26] By atomic force microscopy it was shown that low concentrations (~3.125 μM), TriplatinNC induced the formation in linearized plasmid pSP73 (linearized by *Nde* I restriction endonuclease, which cuts only once within the plasmid) of massive multimolecular aggregates (>3 μm in diameter) containing structures reminiscent of flowers. With increasing concentration, the morphologies of DNA condensates become more compact leading to the formation of DNA particles rather than flat and single-layered compact DNA patterns.

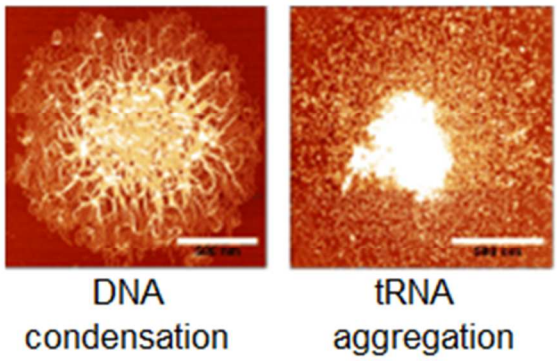


Figure 5. Atomic Force Microscopy shows TriplatinNC induces highly efficient nucleic acid condensation through the phosphate clamp. [26,27] Adapted from ref. 26.

RNA Condensation. Whereas Calf Thymus DNA with an average length of several kilobase pairs can be compacted by either monomolecular condensation with distinguishable morphologies or multimolecular aggregation with irregular morphology, tRNA molecules, typically 60-95 nucleotides are too short in length to be individually condensed and are compacted only by multimolecular aggregation. TriplatinNC is also very effective in tRNA aggregation with an EC_{50} value of 0.24 ± 0.01 μM, slightly higher than that obtained for the condensation of CT DNA. Spermine did not induce tRNA aggregation even at 50 μM concentration. These results were in some contrast to fluorescence quenching experiments carried out similar to the standard CTDNA studies, where little binding was observed. The results are not inconsistent if TriplatinNC molecules bind tRNA at non-fluorescent EtBr binding sites that have negligible influence on thermal denaturation. Reflecting the high affinity of phosphate clamp-oligonucleotide binding, samples containing plasmid pSP73 DNA or tRNA condensed by TriplatinNC treated with high concentrations of NaCl did not fully restore the DNA to its relaxed form even up to 2 M NaCl.[26]

DNA-Protein Interactions. It is axiomatic that strong ligand (metal complex)-DNA binding may affect protein recognition and processing. As distinct from a covalent adduct, where the protein binding sites are unlikely to displace the strong Pt-purine(pyrimidine) bond, non-covalent ligands may be physically displaced from their DNA recognition sites if the protein affinity is significantly higher than that of the ligand-DNA interaction. As seen, the modular nature of the polynuclear platinum complex does result in both high DNA affinity, allied to very efficient condensation. This combination is sufficient to block protein recognition, with consequent biological effects. Three highly relevant examples are endonuclease inhibition; prevention of Topoisomerase I-mediated relaxation of DNA and inhibition of TBP (**T**ATA **B**inding **P**rotein) to its cognate DNA sequence.[25,26,28]

Substitution-inert PPCs inhibit superhelical pUC19 plasmid DNA (pDNA) migration as shown by agarose gel electrophoresis with complete condensation also occurring at low concentrations. This behavior is in contrast to the covalently-binding agents cisplatin and Triplatin (BBR3464) where the Pt-DNA bond formation results in concentration-dependent unwinding of the Form I superhelix. Linearisation of the plasmid by the *Aat*II restriction enzyme followed by Triplatin NC binding and then exposure to the endonucleases *Bam*HI, *Eco*RI or *Sal*I, (all of which have only one recognition sequence on pUC19) showed efficient inhibition of plasmid cleavage. A novel on-chip DNA microfluidic method was used to distinguish between inhibition caused by simple condensation rather than as a consequence of ligand (TriplatinNC) binding. Concentration dependent inhibition was identified for all endonucleases and thus, in the presence of Triplatin complex, the pUC19 vector is protected from site-selective endonuclease excision.

Topoisomerases participate in important events related to DNA metabolism including replication, transcription and recombination and play a vital role in the control of the topological state of DNA. They are important therapeutic targets and topoisomerase inhibitors are promising anticancer agents.[29] The topoisomerase I-mediated relaxation of supercoiled DNA was inhibited by TriplatinNC at an approximately 250-fold lower concentration than that of spermine.

A very relevant example of the strength of the phosphate clamp *versus* the arginine fork is given by the effective inhibition by TriplatinNC of the TATA-box binding protein (TBP) recognition of a 24mer duplex containing the T-A-T-A-A-A-A-G DNA consensus sequence, Figure 6. TATA-binding protein is a critical transcription factor for all three eukaryotic RNA polymerases. TBP inhibition initiates upon 0.63 μM of drug exposure with higher concentrations (>1.25 μM) completely inhibiting protein binding. The A-T rich sequence is expected to have high affinity for minor groove binders and it is notable that, given the comparison of the phosphate clamp with "classical" minor-groove binders netropsin is significantly less efficient at TBP binding inhibition.[28] A principal motif of TBP-DNA recognition is that of the arginine fork, Figure 6, as protein DNA-binding in the minor groove bends the helix toward the major groove.[30] Thus, the phosphate clamp can directly compete with the arginine fork of the protein for DNA sites. Hypotheses on how platinum drugs inhibit transcription have been divided into three categories: (i.) hijacking of transcription factors, (ii.) physical inhibition of the enzyme and (iii.) inhibition at the level of chromatin reorganization.[11] The results in this case would correspond therefore to physical blocking of the enzyme since there is no Pt-DNA bond formation and represent the first example of a substitution-inert Pt complex inhibiting the important TBP-DNA recognition.

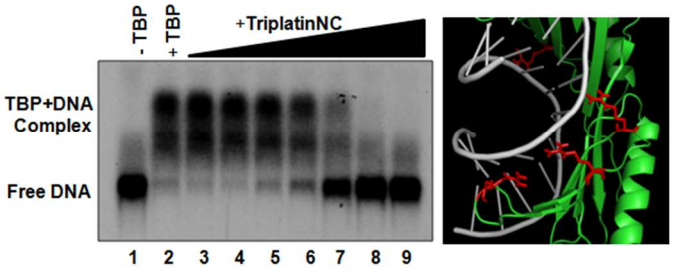


Figure 6. TriplatinNC-DNA binding is sufficiently strong to inhibit TBP-DNA interaction and is competitive with multiple arginine recognition sites. Briefly, dsDNA (24mer {5'-GAAGGGGGGCTCTAAAAGGGGGTG-3'}$_2$ containing the AdML TATA box) was incubated for 15 min. at 30°C with varying drug concentrations (Lanes 3-9; 2nM ^{32}P-labeled DNA incubated with 0.08, 0.16, 0.31, 0.63, 1.25, 2.5, and 5uM drug, respectively). Where indicated, 100ng rTBP was added and reactions were further incubated for 30 min. Lane 1 is the 'free DNA' control and Lane 2 is the 'TBP/DNA complex' positive control [24,28]. On right, structure of TBP-DNA showing multiple arginine fork-DNA recognition [30].

Summary of Chemical and Biophysical Studies. The combined properties of high affinity and very efficient nuclear condensation effects distinguish the phosphate clamp DNA binding mode of substitution-inert PPCs (with their high positive charge) from typical minor-groove binders, even though both share high affinity for A-T-rich sequences. The chemotype is further distinct from both the polyamine class of spermine and spermidine. With respect to the latter, the rigidity afforded by the Pt square planar coordination enhances the ability to form strong H-bonding motifs. Thus, the phosphate is justifiably seen as a third discrete mode of ligand-DNA binding. clamp-DNA TriplatinNC-DNA binding leads to (i) cooperative binding of the minor-groove-binding Hoechst 33258, (ii) fluorescence quenching of intercalated ethidium bromide, (iii) prevention of minor-groove alkylation and (iv) inhibition of protein-recognition and function for critical DNA processing by topoisomerases and the TATA Binding protein.[6,16] The general mechanisms of biogical activity of substitution-inert PPCs may be associated with these properties and their unique ability to condense/aggregate nucleic acids with consequent inhibitory effect on crucial enzymatic activities.

It is appropriate at this juncture to note that the great variety of coordination motifs can lend itself to other novel "non-covalent" binding motifs with great potential for diverse biological effects, away from the more "direct" covalent binding which has been the norm. The supramolecular cylindrical helicates pioneered by Hannon are yet another example of a discrete mode of ligand (substitution-inert metal complex)-DNA binding.[31] The focus in this review, as stated, is on substitution-inert compounds.

3. Biological (Cellular) Effects. Do these physicochemical and biochemical findings translate to cellular effects ? DNA-modifying agents such as the minor groove binders and intercalators have proven a rich source of anti-cancer drugs and medicines.[24,28] It is axiomatic then that the discrete phosphate clamp motif should exert effects in cells. Compounds as exemplified by TriplatinNC with strong affinity for DNA indeed show distinct and significant biological properties in their own right, as the prototype for the substitution-inert PPC chemotype. Conceptualizing the structure as a small-molecule polyarginine mimic has been exceptionally useful in elucidating the cellular profile of this distinct class. Two major cellular properties distinct from cisplatin are nucleolar localization and nucleic acid condensation.

Nucleolar targeting. The nucleolus for drug intervention is increasing in importance and rRNA synthesis is a shared target of many clinically important anticancer agents including the platinum-based drugs, oxaliplatin and cisplatin, which inhibit the transcriptional rate of the long 47S rRNA precursor transcript, and the antimetabolite, 5-Fluorouracil (5-FU), which disrupts processing of the precursor into shorter, mature 28S, 18S, and 5.8S rRNA transcripts.[32] Oxaliplatin, cisplatin, and 5-FU interactions are not limited to the nucleolus but are nonselective, genotoxic agents that incorporate into the total limiting genotoxic events. Positive charge is a major factor in localization and retention of molecules to the nucleolus. Mutagenesis studies of nucleolar proteins, such as nucleolin, fibrillarin, and the viral HIV TAT, show that clusters of positively charged amino acids, arginine and lysine, serve as pool of nucleic acid. An important challenge for small molecule therapeutics is to specifically target rRNA synthesis, and thereby nucleolar localization signals.[33]

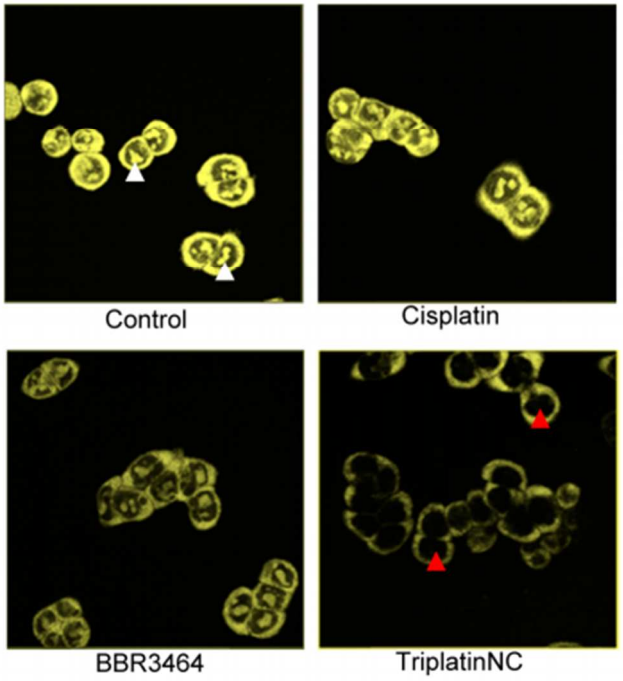


Figure 7. TriplatinNC is a nucleolus-targeting agent. It competes with the nonaarginine TAMRA-R9 for localization to the nucleolus (white, red tabs). Cells were incubated with or without 10μM drug for 10 min. followed by addition of 1μM Tamra-R9. After 15 minutes, cells were fixed, mounted, and visualized by confocal microscopy [28]. Adapted from 28.

Nucleolar localization of TriplatinNC was confirmed by competitive inhibition studies with the fluorescent polyarginine probe TAMRA-R_9, (which is composed of a nonaarginine peptide R_9 coupled to the fluorescent TAMRA 5-(and 6-) carboxytetramethylrhodamine label. Treatment of HCT116 colon cancer cells with 10μM TriplatinNC (4 x IC_{50}), 10μM BBR3464, or 10μM cisplatin for 10 minutes followed by addition of the fluorescent dye allows the pattern of intracellular localization of TAMRA-R_9 in HCT116 cells and the effects of each compound on the dye's localization to be monitored. Tamra-R9 readily enters cells after Cisplatin treatment but significantly less Tarma-R9 enters the cells after treatment with BBR3464 and, especially, TriplatinNC as evidenced by diminution of the intrinsic fluorescence signal of the labelled peptide. TriplatinNC, but not BBR3464 (or cisplatin), competes with the polyarginine for localization to the nucleolus.[28]

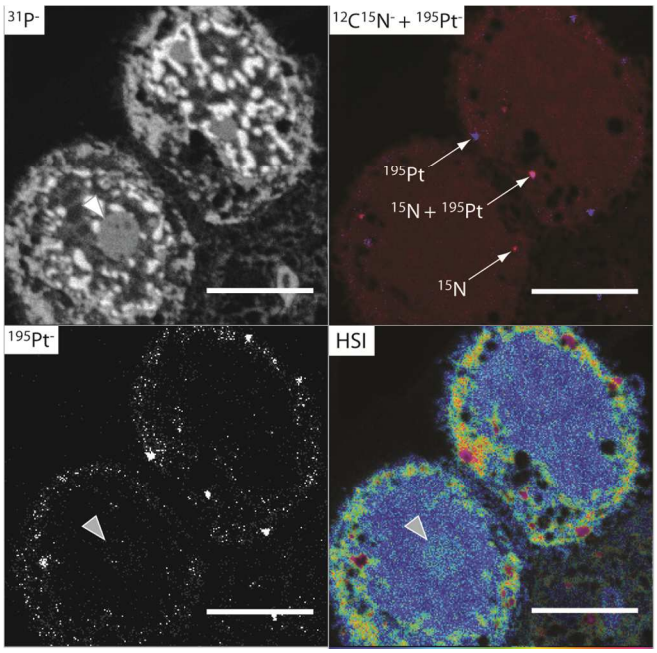


Figure 8. A novel use of dual-element imaging by NanoSIMS confirms nucleolar targeting. Secondary ion maps acquired of MCF7 cells treated with 20μM ^{15}N-TriplatinNC for 2h. The ^{195}Pt-secondary ion map and the hue-saturation intensity (HSI) representation of the $^{12}C^{15}N^-/^{12}C^{14}N^-$ ratio map clearly show localization of both ^{195}Pt and ^{15}N within the nucleolus (grey arrow); scale bars = 5 μm. [35] Adapted from Ref. 35.

The nucleolar targeting was further confirmed by spectrometric techniques. Significant advances have been made in visualising cellular distribution of metal-based therapeutics through the application of highly sensitive surface analysis techniques, such as secondary ion mass spectrometry (SIMS), to cellular imaging.[34] Nano-scale secondary ion mass spectrometry combines exquisite spatial resolution (50 nm) and the simultaneous detection of heavy and light elements. A novel application of our ^{15}N-labeleld compounds (normally used for many applications of {^{1}H,^{15}N} HSQC NMR Spectroscopy) for NanoSIMS confirmed the nucleolus as target of TriplatinNC with a cellular distribution distinctly different compared to cisplatin, Figure 8.[35] Analysis of the ^{15}N enrichment determined for whole cells and individual subcellular compartments support the observation of ^{15}N enrichment in the nucleolus of the cells, with a small enrichment within the nucleus. The enrichment of ^{15}N in the nucleoli of the cells treated with TriplatinNC, in comparison to the surrounding nuclear regions suggests that a nucleolar targeting mechanism is responsible.

Downstream Effects of Nucleolar Localization. Inhibition of Transcription. The immediate downstream effects of nucleolar localization include a decrease in rRNA transcription, G1 arrest, and eventually apoptosis. In principle, because the nucleolus is not membrane-enclosed, any soluble molecule can diffuse in and out of the nucleolar compartment. Therefore, it is generally accepted that retention of a molecule within the nucleolus must occur as a direct interaction with its components, the two most obvious examples for platinum compounds being ribosomal RNA and DNA. The abundance of newly formed 47S precursors, 32S intermediate cleavage products, and mature 28S and 18S rRNA may be sufficiently labeled for visualization by autoradiography.[32] In HCT116 cells, the production rate of 47S rRNA precursor transcripts was dramatically reduced in a dose-dependent manner as an early event after drug treatment. The rate of 47S rRNA processing does not appear to be affected as the abundance of 32S, 28S, and 18S rRNA products decreases proportionally to that of the precursor. These results confirm the *in vitro* results of inhibition of transcriptional activity of the circular form of pBR322 DNA in the presence of TriplatinNC.

The signaling pathway leading to cell cycle arrest after exposure to antitumor agents has been studied in detail.[36] HCT116 cells treated with 20μM cisplatin (IC_{90}) were shown to induce accumulation in S-phase at 24hrs, and finally in G_2 at 48hrs, Figure 9. Treatment of HCT116 cells with 20μM TriplatinNC, on the other hand, induced an arrest in G_1 at 24hrs

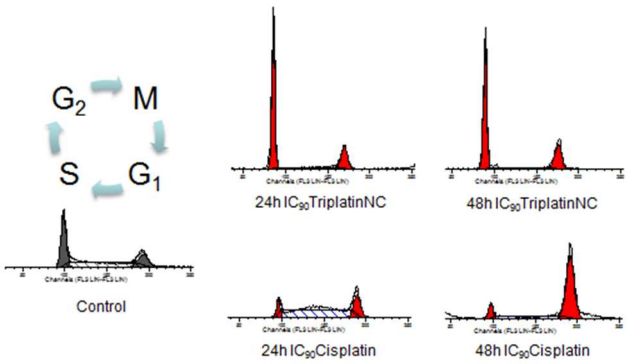


Figure 9. Cell cycle analysis of HCT116 cells treated with 20μM TriplatinNC or Cisplatin for 24 and 48 h. [28]

continuing to 48hrs.

It was surprising, therefore, to observe by confocal microscopy that a significant percentage (28.3 ± 4.1%) of cells treated with TriplatinNC for 24hrs contained DNA that appeared compacted or condensed, Figure 10.[28] The DNA compaction events induced by TriplatinNC are separate from the DNA condensation events of mitosis, senescence, and apoptosis. During mitosis, the DNA condenses and the nuclear membrane recedes, and the cytoplasmic pools of TriplatinNC are likely exposed to DNA that are otherwise inaccessible. TriplatinNC may prevent the decondensation of DNA at this point, leaving the cell suspended between cytokinesis and G_1. This unique effect mirrored the efficient condensation of tRNA and DNA in cell-free systems.[26,27] This cellular effect is unique to TriplatinNC treatment and has not been previously described for other platinum compounds, or in general.

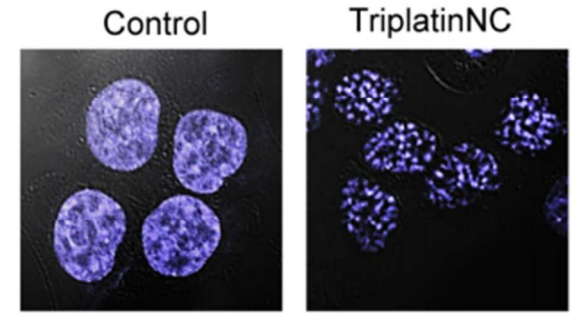


Figure 10. Nuclear condensation in HCT116 cells treated with 20μM TriplatinNC for 24 h were stained with DAPI and visualized by confocal microscopy.[28] Adapted from Ref. 28.

Table 2 Cytotoxicity Comparison (IC_{50}, μM) [28,47]

Cell Lines	TriplatinNC	Cisplatin
HCT116 p53wt	2.5 ± 0.6	3.5 ± 0.8
HCT116 p53-/-	2.8 ± 0.5	5.2 ± 1.0
A2780	4.1 ± 0.9	3.0 ± 0.8
MDA-MB-435	3.05 ± 0.7	5.4 ± 1.2
Mast Cell Lines	**TriplatinNC**	**Cisplatin**
BMMC(primary)	1.79	0.27
P815(transformed)	0.41	0.82
PDMC(transformed)	0.3	0.96

Cytotoxicity of TriplatinNC. Across a broad range of tumor cell lines TriplatinNC shows micromolar cytotoxicity equivalent to that of cisplatin. Nearly all cancers harbor genetic defects that directly, or indirectly, inhibit proapoptotic or tumor suppressor functions of p53.[36] The absence of p53 does not affect the ability of TriplatinNC to effectively induce cell cycle arrest and cell death whereas the ability of cisplatin to inhibit cell growth was significantly limited in the absence of p53, Table 2. The advantage of DNA compaction may increase the effectiveness of platinum compounds in apoptosis-defective cell lines.

A further distinct property of the substitution-inert TriplatinNC is that unlike cisplatin or BBR3464 the cytotoxicity is independent of the GSH status of the cell.[37] Upon treatment with buthionine sulfoximine (BSO), to reduce cellular glutathione levels, cisplatin and BBR3464-induced apoptosis was augmented whereas TriplatinNC induced cytotoxicity was unaltered. Treatment of A2780 ovarian carcinoma cells with HSA-bound cisplatin (cisplatin/HSA) and cisplatin preincubated with whole serum showed dramatic decreases in cytotoxicity, cellular accumulation, and DNA adduct formation compared to treatment with cisplatin alone. Similar effects are seen with BBR3464. In contrast, TriplatinNC, the HSA bound derivative (TriplatinNC/HSA), and TriplatinNC pretreated with whole serum retained identical cytotoxic profiles and equal levels of cellular accumulation at all time points.[37]

4. New Mechanisms of Cellular Accumulation and the Concept of Metalloglycomics. Polynuclear platinum compoundss, especially those containing a central charged/H-bonding capacity, are actually accumulated to a significantly greater extent than neutral compounds and cellular accumulation is even charge-dependent and possibly tumor-selective.[1,6,47] While the solid-state and solution DNA-binding chemistry profile of AH44 and TriplatinNC is essentially identical, cellular accumulation differs dramatically and increases with charge between the 6+ and 8+ congeners of Figure 1. In both cases the cellular accumulation is higher than for the neutral cisplatin, as is always Triplatin itself. This is a

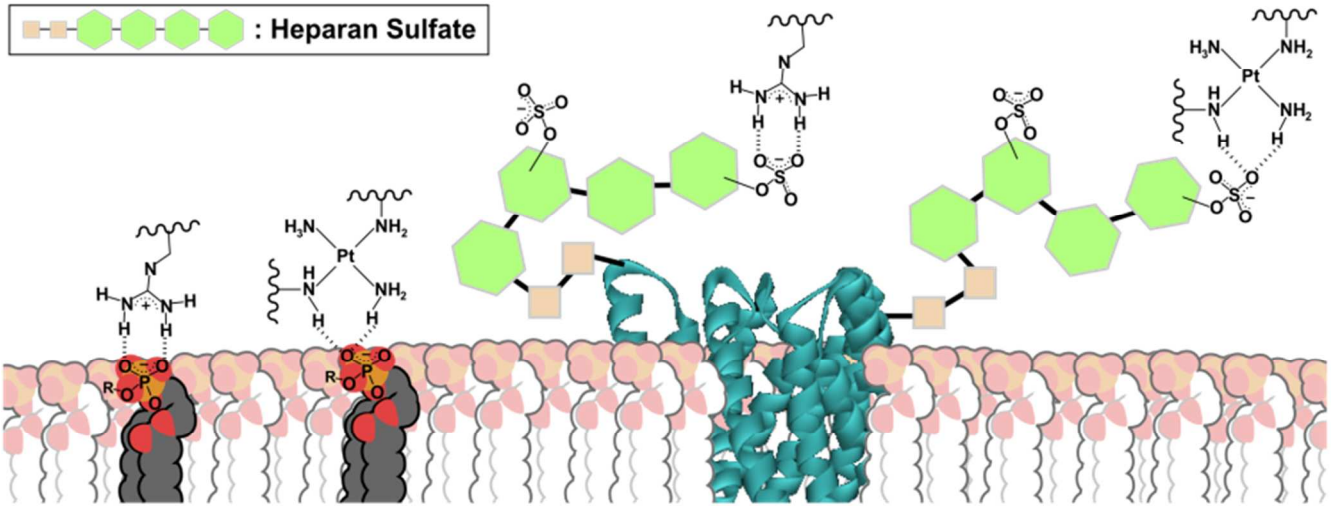


Figure 11. PPCs as small molecule polyarginine mimics – the phosphate and "sulfate" clamp applied to accumulation studies.

Transporter/Receptor	Platinum Compounds							References
	Cisplatin	Carboplatin	Oxaliplatin	Satraplatin	Picoplatin	Triplatin	TriplatinNC	
Copper Transporter	+	+	±	-	+	+	±	38,43,44
Organic Cation Transporters	+	-	+	+	+	-	-	38,44
Heparan Sulfate Proteoglycans	-		-			+	+	41

Table 3. Transporter and Receptor-mediated Accumulation Mechanisms of Platinum Drugs.

paradoxical situation for Pt, not envisaged in the early structure-activity relationships. A common observation in many tumor cells with acquired resistance to cisplatin is reduced platinum accumulation in comparison to the parental cells.[38] Thus, the higher accumulation may explain in part the increased activity of all highly charged polynuclear platinum complexes. A novel extension of the phosphate clamp-arginine fork analogy was suggested to explain this cellular accumulation. The arginine-rich polycationic peptides are taken up efficiently by cells as well as being recognized as protein translocation domains facilitating cellular accumulation of a host of molecules.[39] The molecular target for polyarginine binding is the Heparan Sulfate Proteoglycan (HSPG) family of polysaccharides.[39,40] We reasoned that the phosphate clamp-arginine fork analogy should extend to isostructural sulfate – the analogies using membrane biomolecules are shown in Figure 4. The concept of a possible "sulphate-clamp" for molecular recognition on the cell membrane led to the confirmation that HSPGs are indeed receptors for cellular accumulation of the highly-charged PPCs.[41]

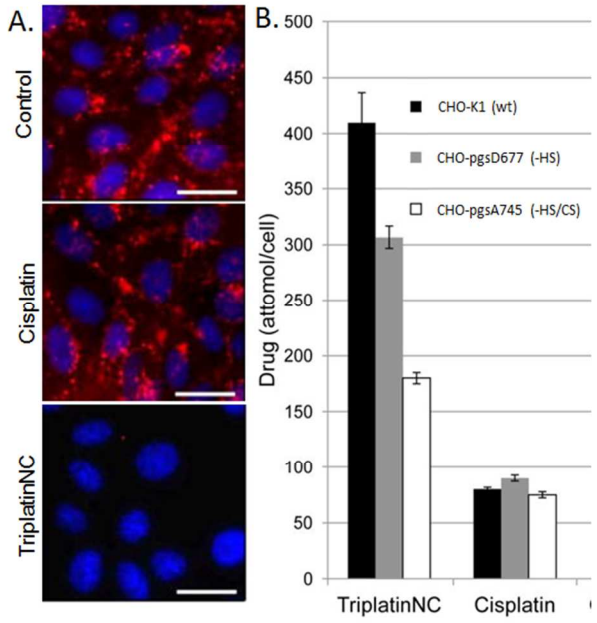


Figure 12. A. TriplatinNC inhibits polyarginine uptake into CHO cells. Competitve inhibition of TAMRA-R_9 (1μM) uptake by Pt compounds (10μM) in wtCHO cells. B. Effect on Pt accumulation in wtCHO and GAG-deficient mutants. Cytotoxicity is related to accumulation. Note dramatic increase of TriplatinNC *vs*. cisplatin. of PPC relative to cisplatin.[41] Adapted from Ref. 41.

Fluorescence microscopy and flow cytometry showed that PPCs, but not the neutral cisplatin or oxaliplatin, blocked the cellular entry of TAMRA-R_9 in Chinese Hamster Ovary (CHO) cells, Figure 12. Accumulation of TriplatinNC in mutant CHO-pgsD-677 (lacking heparan sulfate, HS), and CHO-pgsA (HS/CS, lacking HS and chondroitin sulfate CS) cells decreased relative to wt CHO. Apoptosis and growth inhibition assays paralleled the effect of mutant cells on accumulation. We conclude that polynuclear platinums inhibit the poly(arginine) binding and that HSPG-receptor mediated interactions are an important mechanism for their internalization. Fluorescence inhibition was also significant for TriplatinNC in human colon carcinoma (HCT116) and osteosarcoma (SAOS-2) tumor models.[41] These results confirm that the highly-charged PPCs are competitive inhibitors of the polyarginine-heparan sulfate recognition.

Molecular Mechanism of HSPG-mediated cellular Accumulation. Heparan Sulfate is composed of a complex array of highly sulfated oligosaccharides. Confirming the potential for high-affinity binding to oligosaccharides, ESI-MS spectra of a model DP8 octasaccharide in the presence of the 8+ (TriplatinNC) ions showed clear evidence of 1:1 adducts and stabilization toward sulphate loss with a difference of up to 7 sulphate groups protected *versus* free oligosaccharide, Figure 13.[42] The interaction is by its nature non-covalent and is the first demonstration of a platinum compound interaction with a sulphated polysaccharide. The mass spectral results are entirely consistent with strong-PPC-oligosaccharide binding with increased stability of the sulfate group to dissociation which (a) verifies the complexation with sulphate moieties in preference to elsewhere on the glycosidic backbone and (b) may have biological consequences in its own right by reducing the "effective" sulphate concentration on the membrane surface.

Consequences for HSPG Binding. There are several highly significant implications to these findings that can be exploited in drug discovery. Firstly, HSPG-mediated cellular internalization is a completely new mechanism of cellular accumulation for platinum drugs, being discrete for PPCs, thus further differentiating their biological profile from the mononuclear drugs, Table 3. Clinically, the initial cycles of chemotherapy with platinum drugs produce some evidence of response but resistance emerges during continued therapy. Cellular accumulation of platinum drugs is intricate and

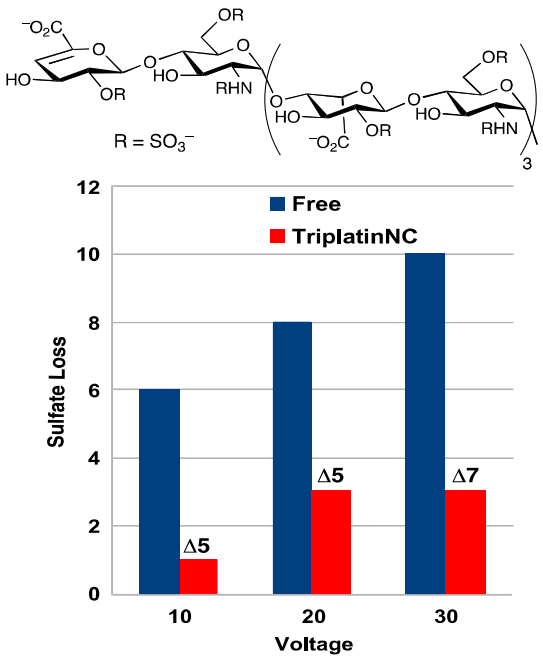


Figure 13. TriplatinNC binds strongly to the octasaccharide (dp8) and protects against elimination of the fragile sulphate groups. Δn is the difference in sulphate loss between free and adducted dp8. [42] Adapted from Ref. 42.

proceeds through complementary passive and active mechanisms. Indeed this may be an important feature in the utility of small diffusible inorganic compounds as drugs. Across a wide range of cell lines, however, decreased accumulation, reflecting defects in uptake and/or efflux, is the most commonly observed. In laboratory cell lines, acquired resistance is quite stable and the phenotype is co-dominant in somatic cells. Multiple pathways contribute to this resistance.[43] The cellular transport and cytotoxicity of cisplatin, carboplatin and oxaliplatin are all modulated both by the hCTR1 copper influx transporter and the ATP7B efflux transporters.[44] Organic cation transporters are also determinants of both cisplatin and oxaliplatin cytotoxicity, but not PPCs. As stated, the HSPG-mediated cellular accumulation is a property shared by all charged PPCs of Figure 1, including the clinically-relevant Triplatin. Thus, in the latter case, cellular accumulation allied to the distinct DNA binding further differentiates the drug from the neutral mononuclear-based agents.

Tumor Selectivity. Proteoglycans are a major source of macromolecular polyanions surrounding almost every cell type, especially mammalian cells and are expressed 2-3 times more in many tumor cells lines.[45,46] Thus, HSPG-mediated cellular accumulation presents a distinct mechanism for tumor selectivity of cytotoxic platinum. It is instructive to reexamine early cytotoxicity and anti-tumor data in the light of these more recent findings – can we find correlations with this new receptor-mediated mechanism ? Strict comparisons are hard to find but it is noteworthy that transformed P-815 mast cells were more selective for PPC uptake, and thus more sensitive, than their bone marrow progenitor-derived primary mast cells (BMMCs), See Table 2.[47] The activity of TriplatinNC compared to cisplatin is enhanced in the transformed cells. P-815s mastocytoma cells produce significant amounts of chondroitin-4-sulfate rather than the normal heparin, suggesting the possibility of a role for glycans in this "promotion" of the cytotoxicity.[48] The MDA-MB-435 breast cancer tumor line also has naturally high levels of endogenous heparanase and again is more sensitive to the TriplatinNC.[28] Gliomas are also especially sensitive to PPCs and also contain high levels of proteoglycans.[1,46]

Interference of HSPG function. Thirdly, the consequences of strong glycan binding extend to interference of *function*. Glycans regulate many important events in tumor progression (including proliferation, invasion, angiogenesis and metastasis) and proteoglycans and their associated enzymes are significant emerging drug targets of high biological relevance.[45,46] They are present on both the cell surface as well as in the extracellular matrix and the basement membrane, bind to a wide variety of proteins and exercise important normal physiological functions such as cell-cell and cell-extracellular matrix adhesion and are receptors for adhesion molecules and growth factors. Heparan Sulphate proteoglycans (HSPGs) are degraded by mammalian and bacterial enzymes. In the case of the mammalian endoglycosidase heparanase, degradation releases angiogenic and growth factors leading to tumor cell migration, growth and angiogenesis. Heparanase is over-expressed in tumors and there is significant correlation between metastatic potential and heparanase activity.[49] The bacterial lyase heparinase is important as a carbon source and degradation of heparin and heparan sulfate leads to biologically active oligosaccharides with significant clinical and pharmaceutical implications.

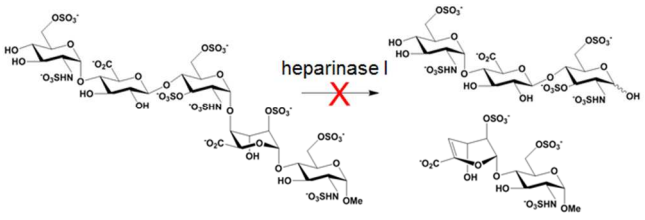


Figure 14. PPCs inhibit oligosaccharide cleavage by heparinase I.[42]

Design of mimetics for competitive enzyme inhibition involves the complex synthesis of small (tetra/penta) oligosaccharides. A relevant example is the paradigmatic pentasaccharide Fondaparinux, the fully synthetic methyl glycoside of the antithrombin III (ATIII)-activating pentasaccharide sequence of heparin.[50] Incubation of Fondaparinux with TriplatinNC prior to enzyme exposure and cleavage confirmed that inhibition of heparinase cleavage was effective in a charge and concentration-dependent manner for the non-covalent

compounds.[42] The clinical agent Triplatin also inhibited cleavage, attributed to a contribution from covalent binding by Pt-Cl substitution (only possible for BBR3464). Cisplatin does not inhibit cleavage, consistent with the fact that it is not a substrate for HSPG-mediated internalization.[41] The ability to inhibit oligosaccharide degradation with PPC "metalloshields" presents an exciting alternative approach to glycan targeting and enzyme inhibition, distinct from the complex design and synthetic chemistry of oligosaccharide mimics. of angiogenesis. These concepts may be extended to inhibition of the heparanase/growth factor interaction. The definitive 'end-point" of inhibition of heparanase and growth factor binding to heparan sulphate is the inhibition of angiogenesis. This new direction, stemming from an initial desire to understand the non-covalent interactions of a clinically relevant drug (Triplatin) suggests a systematic approach to designing anti-metastatic rather than cytotoxic platinum.

Conclusions. This review summarizes the chemical, biophysical and cellular profile of a prototypical substitution-inert polynuclear platinum complex. The advent to clinical trials of the covalently-binding parent compound Triplatin (BBR3464) altered the paradigm for design of clinically useful platinum agents. There is now a wide variety of mononuclear structures differing from cisplatin, including monofunctional Pt-intercalator conjugates and *trans*-platinum compounds, under study for their biological effects.[1,3] Nevertheless, development and design of all these chemotypes has still been predicated on the necessity for formation of a Pt-DNA bond. While it is always possible that a very small percentage of TriplatinNC hydrolyses to produce more substitution-labile Pt-aquo species, the array and rate of biological reactions suggests that the cellular events are a consequence of interactions of the intact TriplatinNC molecule. The extension from covalent biomolecule interactions, generally considered as belonging to the field of chemistry, to "non-covalent" ones, somewhat characteristic of the biodisciplines, has extended the possibilities for tumor selectivity and targeting and is a further shift in the paradigm of structure-activity relationships for platinum anti-cancer drugs.

If we examine the main points of Figure 2 we see that three principal factors – cellular accumulation, the frequency and structure of DNA adducts, and the extent of metabolizing interactions – are considered to control the cytotoxicity and antitumor activity of platinum anticancer drugs. Eliminating Pt-DNA bond formation as an end point opens up many interesting avenues for drug design and development. Comparing the data from TriplatinNC to cisplatin we see the systematic modulation of these factors – (i) the substitution-inert TriplatinNC is resistant to degradation by sulfur nucleophiles, unlike cisplatin, thus providing a pool of circulating drug; (ii) secondly, cellular accumulation is significantly higher than the neutral species and is mediated by specific membrane-based mechanisms not available to the neutral species and finally, (iii) high-affinity phosphate clamp binding is at least as effective as the covalent Pt-DNA bond formation of cisplatin in altering DNA structure and function. These features combine to produce a compound with comparable cytotoxicity to cisplatin over a very wide range of tumor cell lines - a remarkable result for an 8+ compound. Further, the compound has demonstrated *in vivo* activity, thus again emphasizing modulation to produce a drug with a distinct profile.[28]

Beyond the mere evaluation of cytotoxicity, however, interesting and clinically relevant patterns emerge. Of these, nucleolar targeting and nuclear compaction present pathways to more specific genotoxic agents. The nucleic acid compaction events noted in biophysical studies are mirrored in cells – presenting a unique biological (cellular) consequence for a platinum agent, or indeed any chemotherapeutic drug.

The concept of the polyarginine analogy, first noted for DNA structure, has been especially useful in discovering a new mechanism of cellular accumulation through HSPG-mediation. Understanding the molecular details of this accumulation has identified heparan sulfate proteoglycans and their associated enzymes as valid molecular targets for intervention by platinum agents. The potential for tumor selectivity is allied to the potential for systematic development of anti-angiogenic and anti-metastatic, rather than cytotoxic, agents. Metalloglycomics and the associated concept of metalloshielding has rich and multiple applications in a new area of endeavor in the field of bioinorganic chemistry distinct from protein and DNA/RNA interactions.

The polynuclear platinum complexes may now be seen as *inherently* dual-function agents combining high-affinity DNA binding with potential tumor-selective glycan interactions. The unique modular nature and the structural dictates of the square-planar platinum coordination sphere ensure their distinctiveness from the polyamines and minor-groove binders in the manifestation of their biological properties. In this context, the idea that the DNA damage may be considered a secondary insult rather than the primary one associated with covalent Pt-DNA formation is provocative. It is the case that Triplatin (BBR3464) itself shares some of the biological properties discovered by examining "pure" substitution-inert compounds in the absence of bond-forming reactions with biomolecules. Science and perhaps especially drug development is not a linear process – new facts and properties constantly emerge which allows us to place well-established results in newer contexts. A reasonable question for us is whether any of the clinical findings of Triplatin(BBR3464) have their origins in these newer findings. The lessons learned can be used in development of "smart" second-generation drugs for disease treatment and betterment of human health.

Acknowledgements

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5. Notes and references.

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