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# HEPARAN SULFATE PROTEOGLYCAN-MEDIATED ENTRY PATHWAY FOR CHARGED TRI-PLATINUM COMPOUNDS. DIFFERENTIAL CELLULAR ACCUMULATION MECHANISMS FOR PLATINUM

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## Abstract

We examined the mechanism of accumulation of charged polynuclear platinum complexes (PPCs), based on analogy of polyarginine interactions with the cell surface heparan sulfate proteoglycan (HSPG) family of protein-linked glycosoaminoglycan polysaccharides (GAGs). GAGS such as heparan sulfate (HS) and chondroitin sulfate (CS) mediate the cellular entry of many charged molecules. Fluorescence microscopy and flow cytometry showed that PPCs, but not the neutral cisplatin or oxaliplatin, blocked the cellular entry of TAMRA-$R_9$ (a nonarginine peptide, $R_9$) coupled to the TAMRA fluorescent label 5-(and 6-)carboxytetramethylrhodamine) in Chinese Hamster Ovary (CHO), human colon carcinoma (HCT116), and osteosarcoma (SAOS-2) cells. Furthermore, detection of platinum accumulation in wt CHO, mutant CHO-pgsD-677 (lacking HS), and CHO-pgsA (lacking HS/CS) cells confirms that HSPG-mediated interactions are an important mechanism for PPC internalization, but not so for uncharged cisplatin and oxaliplatin. Endocytosis inhibitor studies show that macropinocytosis, a mechanism of cell entry for heparan sulfate GAGs and arginine-rich peptides, is important in the cellular accumulation of “non-covalent” TriplatinNC, and to a lesser degree, the covalently-binding BBR3464. Clathrin-mediated endocytosis, however, was not involved in either case. Overall the results suggest a new proteoglycan-mediated mechanism for cellular accumulation of PPCs not shared by cisplatin or oxaliplatin. The results have significant implications for rational design of platinum antitumor drugs with distinct biological profiles in comparison to the clinically-used agents as well as expanding the chemotypes for HS proteoglycan-dependent receptors.

### Keywords

Triplatinum compounds; cellular accumulation; cisplatin resistance; heparan sulfate; glycosaminoglycans

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SUPPORTING INFORMATION

Supporting Information available includes control fluorescence experiments of TAMRA-$R_9$; ESI-MS of TriplatinNC-octasaccharide adducts; apoptosis assays and effects of endocytosis inhibitors and cimetidine on cellular accumulation. This information is available free of charge via the Internet at http://pubs.acs.org/.

## INTRODUCTION

Repeating polyarginine sequences are a common recognition motif for protein-DNA/RNA recognition, mediated by the arginine fork involving hydrogen-bonding from the positively-charged guanidine groups of the amino-acid arginine to the phosphate backbone oxygens of the oligonucleotide. (1) Recently, a new, “synthetic”, mode of ligand recognition on DNA analogous to the arginine fork has been crystallographically characterized, Figure 1. (2,3) The X-ray crystal structures of duplex DNA (the Dickerson-Drew dodecamer, DDD) complexed with “non-covalent” polynuclear platinum complexes (PPCs, TriplatinNC and AH44) show hydrogen-bonding to the phosphate oxygens from two mutually *cis*-oriented amine (-$RNH_2$) and ammine ($NH_3$) groups bound to platinum forming a “phosphate clamp”. The backbone tracking and minor groove spanning interactions of the phosphate clamp are discrete from the “classic” minor-groove and intercalator ligand binding modes. The modular nature of this interaction, where three independent platinum coordination units engage in ligand recognition, produces a high-affinity binding to the nucleic acid. PPCs represent a new class of clinically relevant anti-cancer platinum drugs whose chemical and biological properties differ significantly from cisplatin. BBR3464 (Figure 1B) has undergone Phase II clinical trials in cisplatin-resistant and refractory cancers. (Summarized in 4)

Cellular internalization of small molecule drugs and macromolecules is critical to their function. An interesting feature of AH44 and TriplatinNC is that while their DNA-binding is essentially identical in both the solid state and solution (2,3), cellular accumulation differs dramatically between the 6+ and 8+ congeners. (5,6) The cellular accumulation is higher than for cisplatin and actually increases with charge, a perhaps paradoxical situation for Pt. Further, unlike cisplatin, accumulation is higher in transformed mast cells in comparison to the parent cells, suggesting possible tumor selectivity. (5) A common observation in many tumor cells with acquired resistance to cisplatin is reduced platinum accumulation in comparison to the parental cells. (7)

Natural and synthetic polycationic peptides, especially containing the poly(arginine) motif, are efficiently taken up by cells and also facilitate cellular accumulation of a host of molecules. (8) For example, the entry of the exogenous RNA-binding protein of human immunodeficiency virus (HIV-TAT) protein into mammalian cells is critically dependent on the presence of the arginine-rich sequence (TAT48–60 (GRKKRRQRRRPPQC). (9,10) The presence of positive charge and hydrogen-bonding ability are essential features of these cationic protein transduction domains (PTDs), and arginine is preferred over other potential donors such as lysine or histidine with Nona-L-arginine ($R_9$) (Figure 1D) the most efficacious known PTD. (8) The receptor for polyarginine membrane binding is the heparan sulfate proteoglycan (HSPG) family of protein-linked glycosoaminoglycan polysaccharides (GAGs) (11–13). GAGs, including heparan sulfate (HS), heparin, chondroitin sulfate (CS), dermatan sulfate (DS) and keratin sulfate, are composed of a variable number of repeating disaccharide units differing in uronic acid (D-glucuronic acid, D-iduronic acid or galactose), hesoxamine unit (D-galactosamine or D-glucosamine) and number and position of sulfate groups (11–13). With their abundant carboxyl and sulfate groups, GAGs constitute a major source of macromolecular polyanions surrounding almost every cell type, especially mammalian cells.

Binding to cell surface proteoglycans has been identified as the first step in the cellular internalization of PTD peptides (14–17). Further, heparan binding facilitates cell uptake of polyamines such as spermidine and spermine. (11,18). Internalization of a fluorescent arginine probe, TAMRA-$R_9$, decreases in GAG-deficient cell lines, suggesting that binding to heparan sulfate is necessary for PTD internalization (19). Endocytotic pathways are also

unequivocally involved in further phases of PTD cell entry. In this paper we pursue the phosphate clamp-arginine fork analogy to show a new unique pathway of PPC cellular accumulation based on glycososaminoglycan-mediated transduction and subsequent localization, discrete from those used by cisplatin and oxaliplatin. The development of platinum-based compounds that employ different influx/efflux mechanisms is an important area of investigation in cancer chemotherapy capable of contributing to a different biological profile in comparison to clinically-used platinum drugs. The identification of HS proteoglycans as receptors may have further consequences for their celluar structure and function.

## MATERIALS AND METHODS

### Materials

BBR3464, AH44, TriplatinNC and cisplatin were synthesized as previously described (6,20). Oxaliplatin was obtained from Sigma-Aldrich.

### Cell Culture

CHO-K1, CHO-pgsD-677, CHO-pgsA-745, SAOS-2, and HCT116 cells, were obtained from the American Type Culture Collection. CHO cell lines were cultured with Ham's F-12 medium (Cellgro) supplemented with 10 % v/v fetal bovine serum, 100 U/ml penicillin, and 100 μg/mL streptomycin. HCT116 cells were cultured with RPMI 1640 with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate all obtained from Biofluids Inc.. SAOS-2 cells were cultured with McCoy's 5A medium from Cellgro supplemented with 15% fetal bovine serum, 2mmol/L L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mmol/L sodium pyruvate. Cells were grown in a humidified atmosphere at 37 °C and 5% $CO_2$.

### TAMRA-$R_9$ Internalization by Fluorescence Microscopy

CHO cells were seeded in six-well plates ($10^5$ cells/well). 1.0 μM TAMRA-$R_9$ (nona-arginine peptide labeled with 5-(and 6-) carboxytetramethylrhodamine) (Genscript) and Hoescht 33342 (Molecular Probes) were added to each well, and incubated at 37°C for 1 hr. Cells were washed six times with PBS. Cellular localization was visualized using a 1X-70 inverted microscope equipped with a 12-bit black/white F-View CCD camera and processed using Microsuite-B3SV Version 3.2 software (Olympus). Cells were maintained at 37°C using a stage heater (20/20 Technology). Competition of TAMRA-$R_9$ internalization and platinum compounds was performed in the same way described above, with addition of platinum compounds 5 minutes prior to addition of TAMRA-$R_9$.

### TAMRA-$R_9$ Internalization by Flow Cytometry

CHO, SAOS-2 and HCT116 cells were seeded in six-well plates ($10^5$ cells/well). 1.0 μM TAMRA-$R_9$ was added to each well, and incubated at 37°C for 1hr. Cells were washed six times with PBS, harvested using trypsin/EDTA, washed twice with PBS, and then analyzed at 568 nm using a Becton Dickinson FACScan flow cytometer (BD Biosciences). For competition experiments, platinum compounds (10 μM except where stated) were added 5 min prior to addition of TAMRA-$R_9$.

### Intracellular Platinum accumulation

CHO, CHO-pgsD-677, and CHO-pgsA-745 cells were seeded in 100mm dishes ($2 \times 10^6$ cells/plate). After 24hrs, cells were treated with 10uM of the indicated platinum drug. After 1 h, cells were harvested and washed twice with PBS. The cell pellets were dissolved in hot nitric acid followed by the addition of hydrogen peroxide and hydrochloric acid (United

States Environmental Protection Agency procedure 3050b). Platinum analysis was performed on a Vista-MPX simultaneous inductively coupled plasma optical emission spectrometer (ICP-OES) at 265 nm (Varian Inc.). Standards and blanks were prepared the same as the samples.

### MTT Assay

CHO, CHO-pgsD-677, and CHO-pgsA-745 cells were seeded in 96-well plates ($5 \times 10^3$ cells/well) in 100 μL of media. After 24hrs, cells were treated with various concentrations of platinum drugs in sets containing 6 replicates for each concentration. After drug exposure for 24 hours, 1mM MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma), was added to each well and incubated for 4 hours at 37 °C. The MTT reagent was removed, and 100 μL of DMSO was added to each well. Spectrophotometric readings were determined at 492 nm using a microplate reader (Bio-Tek instruments). Percent cell survival was determined as: treated/untreated controls × 100.

### Apoptosis Assay

As described previously (21), samples were fixed in an ethanol and fetal bovine serum solution, washed with PBS, and stained with a solution of propidium iodide (PI) and RNase A. Samples were then analyzed for subdiploid DNA content on a Becton Dickinson FACScan flow cytometer (BD Biosciences). It is noteworthy that this protocol differs significantly from the more common PI-based exclusion, which only differentiates live *versus* dead cells. Through fixation and RNase A treatment, intact *versus* fragmented DNA was detected, revealing discrete stages of the cell cycle and the percentage of the population undergoing apoptosis.

### Cellular Treatment with Inhibitors of Endocytosis

HCT116 cells were seeded in 140mm dishes ($10–15 \times 10^6$ cells/dish) and allowed to attach overnight. In short-term uptake studies, the culture medium was exchanged for isotonic Hepes-NaCl buffer (20 mM Hepes, 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mg/ml glucose, 1 mM sodium pyruvate, pH 7.3) containing or not the inhibitor (nystatin, 50 μg/ml; EIPA, 100 μM; chloropromazine, 30 μM) and, after 30 min pretreatment at 37°C, BBR3664 and TriplatinNC were added at 60 μM and 30 μM, respectively, and incubation was continued for 60 min. To evaluate the effect of hypertonic medium as inhibitor of clathrin-mediated endocytosis, BBR3464 (60 μM) or TriplatinNC (30 μM) were incubated for 90 min in hypertonic Hepes-NaCl-saccharose buffer (20 mM Hepes, 132 mM KCl, 3.5 mM NaCl, 0.43 M sucrose, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mg/ml glucose, 1 mM sodium pyruvate, pH 7.3) and uptake was compared to that in the isotonic Hepes-NaCl buffer. In the long-term uptake studies, the culture medium was exchanged for fresh complete RPMI medium, containing or not the inhibitor (cytochalasin D, 20 μM; EIPA, 50 μM), and after 30 min pretreatment at 37°C, BBR3464 and TriplatinNC were added at 40 μM and 10 μM, respectively, and incubation was continued for 4 h. Following incubation extracellular medium was discarded and cells were washed twice with ice-cold PBS. After harvesting, the cell pellet was stored at −18°C until quantitation of intracellular levels of platinum, as described above. Before the last centrifugation, cells were counted and viability was assessed with trypan blue (cell viability >90%).

# RESULTS

## Effect of Platinum Drugs on TAMRA-$R_9$ Internalization assessed by Fluorescence Microscopy and Flow Cytometry

Cellular uptake of TAMRA-$R_9$ in the presence of platinum drugs (structures in Figure 1) was examined in wt CHO-K1 cells and the heparan sulfate (HS)-deficient and heparan sulfate and chondroitin sulfate(HS/CS)-deficient mutants, CHO-pgsD-677 and CHO-pgsA-745, respectively. (22, 23) Fluorescence microscopy showed that the dye was clearly visible in the wt but almost undetectable in the GAG-deficient cells, confirming previous results, Figure S1. Upon drug incubation 5 minutes prior to that of the probe, TAMRA-$R_9$ cell entry was prevented in a charge-dependent manner, Figure 2. TriplatinNC, with an (8+) charge, completely abrogated TAMRA-$R_9$ fluorescence (Fig. 2D), while the 6+ and 4+ analogs (AH44 and BBR3464) reduced fluorescence to a lesser extent. Neither the neutral cisplatin nor oxaliplatin affected fluorescence, and hence uptake of the polyarginine (Figs 2B and 2C). Competitive inhibition of platinum drugs on cellular uptake of TAMRA- $R_9$ was quantified by flow cytometry (Fig. 3). TriplatinNC dramatically reduced TAMRA-$R_9$ fluorescence in wt CHO cells to approximately 10% in a concentration-dependent manner (Fig. 3A, 3B). The decrease in fluorescence was significantly smaller for AH44 and BBR3464, and negligible for cisplatin and oxaliplatin. Extension to human colon carcinoma, HCT-116, and osteosarcoma, SAOS-2, cell lines showed the same trend for each drug (Fig. 3C, 3D). The quantified data are summarized in Table S1. Importantly, there is significantly increased inhibition in the tumor cell lines for the 4+ (BBR3464) and 6+ (AH44) compared to “normal” CHO (Figure 3 and Table S1) suggesting a possible mechanism of tumor entry selectivity for PPCs, given the overexpression of proteoglycans on certain tumor cells. (12,13) Overall, the results indicate that the PPCs, especially TriplatinNC, competitively inhibit TAMRA-$R_9$ binding to its HSPG substrate. In confirmation, the mass spectrum of TriplatinNC incubated with an octasaccharide (DP8) modeled on heparan sulfate shows clearly the formation of 1:1 adducts between the two species, showing for the first time non-covalent binding of a platinum drug to a heparan sulfate model, Figure S3.

## Platinum accumulation in wt and mutant CHO cells

The cellular accumulation of platinum drugs in all three CHO cells confirmed the dependence on proteoglycan status, Figure 4. Interestingly, the double mutant lacking both HS and CS preferentially affects accumulation for the non-covalent drugs – for TriplatinNC accumulation decreased 25% and 56% in HS-deficient and HS/CS-deficient CHO cells, respectively. These results may suggest a specific role for chondroitin sulfate PGs in receptor activity. The neutral compounds (cisplatin and oxaliplatin) showed no dependence of cellular accumulation on the proteoglycan status, Figure 4. The results confirmed the previously demonstrated enhanced accumulation of trinuclear charged compounds compared to cisplatin, as well as the dependence of overall charge. (4–6)

## Correlation of Platinum Accumulation and Cytotoxicity by MTT and Apoptosis Assays

MTT and apoptosis assays explored the consequences of proteoglycan-dependent cellular accumulation on cytotoxicity, Figure 5 and Table S2. Both mutants are significantly less sensitive to all PPCs whilst retaining equivalent sensitivity to cisplatin. There is not a clear-cut correlation at this point between cytotoxicity and cellular accumulation for the two individual (HS) and (CS/HS) mutants - the CS/HS mutant is somewhat more sensitive than the HS mutant for TriplatinNC and BBR3464 but there is still a distinct difference compared to the wt values (Fig. 5 and Fig. S2). Proteoglycan status similarly altered apoptosis induction for TriplatinNC in CHO cells, where an approximate 10-fold increase in $IC_{50}$ was observed (0.81 μM for wt; 6.99 and 8.72 for HS- and HS/CS-deficient cells, respectively), Figure S4. Again, cisplatin showed no dependence on HSPG status when measuring

apoptosis as an outcome. In analyzing cytotoxicity results, the favorability and frequency of drug-target interactions have to be considered, along with the end-points chosen for cell growth inhibition and/or cell kill, and these are beyond the scope of this initial report, especially considering that we have to take into account both noncovalent and covalent target interactions. Nevertheless, comparing the two noncovalent compounds AH44 and Triplatin NC, the differences in biological activity attributed to cellular accumulation (5,6), rather than DNA binding (2,3), are confirmed here.

## Involvement of Endocytosis Mechanisms in Polynuclear Platinum Accumulation

Endocytosis, an essential cellular process for the internalization of a wide variety of extracellular factors, occurs through functionally distinct mechanisms. (24) Endocytotic pathways are unequivocally involved in PTD cell entry and heparan sulfate-facilitated mechanisms include lipid-raft independent or dependent macropinocytosis, and clatharin-mediated endocytosis, although the contributions of the individual specific processes remain to be elucidated. (19, 25–27) We therefore examined the effect of selected endocytosis inhibitors on BBR3464 and TriplatinNC uptake in HCT116 cells, Table 1 and Figures S5–S7. These two compounds were chosen because of the observed dramatic effects of TriplatinNC while BBR3464 is the “covalent” analog as well as having undergone Phase II clinical trials. (4) The well-established macropinocytosis inhibitors cytochalasin D and EIPA significantly reduced accumulation of both compounds, Table 1 and Figure S5. Nystatin-mediated lipid raft disruption significantly affects TrisplatinNC accumulation (~ 40%) without any effect on BBR3464; further differentiating macropinocytosis mechanisms for the two drugs, (Fig. S6A, 6B). Hypertonic media and chlorpromazine at 30 μM specifically inhibit clathrin-mediated endocytosis. (28) Cells incubated with TriplatinNC and BBR3464 in hypertonic medium showed the same levels of platinum uptake as those incubated in the isotonic buffer, (Fig. S7A, 7B). Chlorpromazine treatment had no effect on the uptake of TriplatinNC but it significantly increased BBR3464 uptake (~ 30%), (Fig. S7C, 7D). The stimulation of BBR3464 uptake is consistent with a previous report where chlorpromazine strongly enhanced accumulation of a dinuclear platinum complex in MCF-7 cells, interpreted as enhanced macropinocytosis. (29) The cumulative data strongly suggest that macropinocytosis, but not clathrin-mediated endocytosis, is involved in the uptake of these cationic trinuclear platinum drugs. Further, lipid raft-mediated macropinocytosis uniquely contributes to TriplatinNC accumulation in HCT116 cells.

## Comparison of Trinuclear and Mononuclear Platinum Drug Cellular Accumulation Mechanisms

How do these results compare with the general understanding of cisplatin and oxaliplatin cellular accumulation? Platinum accumulation pathways are multifactorial, with both passive diffusion and active accumulation by transport proteins (7). One set of plasma membrane transporters implicated in platinum accumulation is the organic cation transporter set (OCT 1, 2, 3), members of the SLC22A family. (7,30) BBR3464 and TriplatinNC accumulation in HCT116 cells was unaffected by the presence of cimetidine, an inhibitor of OCT-mediated cellular accumulation, Figure S8. These results are again consistent with the cimetidine inhibition of a cationic dinuclear platinum compound. (29) In contrast, cimetidine decreased oxaliplatin and cisplatin accumulation in HCT116 cells, consistent with previous reports where the $IC_{50}$ of both drugs was increased in the presence of cimetidine, although Pt accumulation was not directly measured. (31) These results imply that modulation of organic cation transporter function does not play a role in mediating PPC uptake, a feature further differentiating them from the clinically used drugs.

The copper influx (hCTR1) and efflux ATP7B transporters play a substantial role in cisplatin and oxaliplatin accumulation (7,32). Both copper and cisplatin (at clinically

relevant concentrations) rapidly downregulate CTR1 expression in human ovarian cancer cell lines; through CTR1 internalization from the plasma membrane by macropinocytosis, followed by proteasome-based degradation (33). BBR3464 can use hCTR1 to enter cells and to a lesser extent, the ATP7B transporter to exit cells, although downstream effects are different to those of cisplatin. (34).

## DISCUSSION

Polynuclear cationic platinum complexes are a promising class of anticancer drugs with discrete DNA binding modes and the ability to overcome cisplatin resistance. (4,35) The interactions between the amine groups of the triplatinum compounds and the phosphate groups of the DNA backbone are very similar to the interactions of guanidine groups on arginine with phosphates. We have now extended this analogy to isostructural sulfate and have identified for the first time through competitive inhibition, cellular accumulation and cell death assays new receptors for charged platinum drugs - HS proteoglycans. The affinity of TAMRA- $R_9$ to heparin has been measured as $K_d$ = 109 nM, similar to typical receptor-ligand interactions. (19) TriplatinNC must have similar affinity and, as a small molecule analog of the polyarginine motif, is thus likely to engage in many similar biological processes. The results enhance the rich diversity of accumulation pathways of platinum compounds and the discrete accumulation mechanism further differentiates the potential of PPCs over the mononuclear drugs. (7,31,32,36)

The identification of HS proteoglycans as membrane receptors opens a new area for GAG-platinum chemistry. The subtle distinctions between heparan and chondroitin sulfates may impart further selectivity for these platinum complex-biomolecule interactions. Glycans play crucial roles in the pathophysiology of tumour progression – for example NG2, a transmembrane chondroitin sulfate is differently expressed in neoplasms with higher expression in high compared to low-grade gliomas. (37,38) Previous work had suggested some tumor selectivity for PPCs based on cellular accumulation and cytotoxicity between normal and transformed mast cells. (5) PPCs are also exceptionally potent against gliomas and neuroblastomas (39,40) and it will be instructive to examine these results further in the light of the results here. Proteoglycans are therapeutic targets in their own right (38) – conformational changes upon platinum complex binding, observed for biologically relevant metal ion binding, may affect function (41). Finally, proteoglycan conjugation has been used to attempt tissue delivery and enhance selectivity and activity of cisplatin. (42,43) The “natural” affinity of PPCs for GAGs may impart similar desirable “inherent” pharmacological properties.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## References

1. Calnan BJ, Tidor B, Biancalana S, Hudson D, Frankel AD. Arginine-mediated RNA recognition: the arginine fork. Science. 1991; 252:1167–1171.

2. Komeda S, Moulaei T, Woods KK, Chikuma M, Farrell N, et al. Phosphate Clamps: A Novel Mode of DNA Interaction by a Polynuclear Platinum(II) Complex. J Amer Chem Soc. 2006; 128:16092–16103. [PubMed: 17165762]
3. Komeda S, Moulaei T, Chikuma M, Odani A, Kipping R, et al. The Phosphate Clamp: A Small and Independent Motif for Nucleic Acid Backbone Recognition. Nuc Acids Res. 2011; 39:325–336.
4. Farrell N. Polynuclear Platinum Drugs. Metal Ions in Biol Sys. 2004; 41:252–296.
5. Harris AL, Ryan JJ, Farrell N. Biological consequences of trinuclear platinum complexes: comparison of [*trans*-PtCl($NH_3$)$_2$]$_2$μ-(*trans*-Pt($NH_3$)$_2$($H_2N(CH_2)_6NH_2$)$_2$)]4+ (BBR3464) with its noncovalent congeners. Mol Pharmacol. 2006; 69:666–672. [PubMed: 16275707]
6. Harris AL, Yang X, Hegmans A, Povirk L, Ryan JJ, et al. Synthesis and Characterization of the DNA Binding and Cytotoxicity of a Novel Trinuclear Highly Charged Compound. Inorg Chem. 2005; 44:9598–9600. [PubMed: 16363817]
7. Hall MD, Okabe M, Shen DW, Liang XJ, Gottesman MM. The role of cellular accumulation in determining sensitivity to platinum-based chemotherapy. Annu Rev Pharmacol Toxicol. 2008; 48:495–535. [PubMed: 17937596]
8. Fuchs SM, Raines RT. Internalization of Cationic Peptides: the road less (or more ?) travelled. Cell Mol Life Sci. 2006; 63:1819–1822. [PubMed: 16909213]
9. Frankel AD, Pabo CO. Cellular uptake of the tat protein from human immunodeficiency virus. Cell. 1988; 55:1189–1193. [PubMed: 2849510]
10. Vives E, Brodin P, Lebleu B. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. J Biol Chem. 1997; 272:16010–16017. [PubMed: 9188504]
11. Belting M. Heparan sulfate proteoglycan as a plasma membrane carrier. Trends Biochem Sci. 2003; 28:145–151. [PubMed: 12633994]
12. KjeUin L, Lindahl U. Proteoglycans: Structures and Interactions. Annu Rev Biochem. 1991; 60:443–475. [PubMed: 1883201]
13. Lindhard RJ, Toida T. Role of glycosaminoglycans in cellular communication. Acc Chem Res. 2004; 37:431–438. [PubMed: 15260505]
14. Rothbard JB, Jessop TC, Wender PA. Adaptive translocation: the role of hydrogen bonding and membrane potential in the uptake of guanidinium-rich transporters into cells. Adv Drug Deliv Rev. 2005; 57:495–504. [PubMed: 15722160]
15. Tyagi M, Rusnati M, Presta M, Giacca M. Internalization of HIV-1 tat requires cell surface heparan sulfate proteoglycans. J Biol Chem. 2001; 276:3254–3261. [PubMed: 11024024]
16. Ziegler A, Seelig J. Interaction of the protein transduction domain of HIV-1 TAT with heparan sulfate: binding mechanism and thermodynamic parameters. Biophys J. 2004; 86:254–263. [PubMed: 14695267]
17. Fuchs SM, Raines RT. Polyarginine as a multifunctional fusion tag. Protein Sci. 2005; 14:1538–1544. [PubMed: 15930002]
18. Belting M, Persson S, Fransson LA. Proteoglycan involvement in polyamine uptake. Biochem J. 1999; 338:317–323. [PubMed: 10024506]
19. Fuchs SM, Raines RT. Pathway for polyarginine entry into mammalian cell. Biochem. 2004; 43:2438–2444. [PubMed: 14992581]
20. Qu Y, Harris A, Hegmans A, Kabolizadeh P, Penazova H, et al. Synthesis and DNA conformational changes interactions of non-covalent polynuclear platinum complexes. J Inorg Biochem. 2004; 98:1591–1598. [PubMed: 15458821]
21. Yeatman CF 2nd, Jacobs-Helber SM, Mirmonsef P, Gillespie SR, Bouton LA, et al. Combined stimulation with the T helper cell type 2 cytokines interleukin (IL)-4 and IL-10 induces mouse mast cell apoptosis. J Exp Med. 2000; 192:1093–1103. [PubMed: 11034599]
22. Lidholt K, Weinke JL, Kiser CS, Lugemwa FN, Bame KJ, et al. A single mutation affects both N-acetylglucosaminyltrans-ferase and glucuronosyltransferase activities in a Chinese hamster ovary cell mutant defective in heparan sulfate biosynthesis. Proc Natl Acad Sci USA. 1992; 89:2267–2271. [PubMed: 1532254]
23. Esko JD, Stewart TE, Taylor WH. Animal cell mutants defective in glycosaminoglycan biosynthesis. Proc Natl Acad Sci USA. 1985; 82:3197–3201. [PubMed: 3858816]

24. Doherty GJ, McMahan HT. Mechanisms of Endocytosis. Ann Rev Biochem. 2009; 78:857–902. [PubMed: 19317650]
25. Fotin-Mleczek M, Fischer R, Brock R. Endocytosis and cationic cell-penetrating peptides – a merger of concepts and methods. Curr Pharm Des. 2005; 11:3613–3628. [PubMed: 16305498]
26. Wadia JS, Stan RV, Dowdy SF. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. Nat Med. 2004; 10:310–315. [PubMed: 14770178]
27. Richard JP, Melikov K, Brooks H, Prevot P, Lebleu B, et al. Cellular uptake of unconjugated TAT peptide involves clathrin-dependent endocytosis and heparin sulfate receptors. J Biol Chem. 2005; 280:15300–15306. [PubMed: 15687490]
28. Heuser JE, Anderson RGW. Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. J Cell Biol. 1989; 108:389–400. [PubMed: 2563728]
29. Kapp T, Muller S, Gust R. Dinuclear alkylamine platinum(II) complexes of [1,2-bis(4-fluoropheny)ethylenediamine]-platinum(II): influence of endocytosis and copper and organic cation transport systems on cellular uptake. Chem Med Chem. 2006; 1:560–564. [PubMed: 16892392]
30. Koepsell H, Lips K, Volk C. Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. Pharm Res. 2007; 24:1227–1251. [PubMed: 17473959]
31. Zhang S, Lovejoy KS, Shima JE, Lagpacan LL, Shu Y, et al. Organic Cation Transporters Are Determinants of Oxaliplatin Cytotoxicity. Cancer Res. 2006; 66:8847–8857. [PubMed: 16951202]
32. Howell SB, Safaei R, Larson CA, Sailor MJ. Cooper Transporters and the Cellular Pharmacology of the Platinum-containing Cancer Drugs. Mol Pharmacol. 2010; 77:887–894. [PubMed: 20159940]
33. Holzer AK, Howell SB. The internalization and degradation of human copper transporter 1 following cisplatin exposure. Cancer Res. 2006; 66:10944–10952. [PubMed: 17108132]
34. Kabolizadeh P, Ryan J, Farrell N. Differences in the cellular response and signaling pathways of cisplatin and BBR3464 ([{*trans*-PtCl$(NH_3)_2$}$_2\mu$-(*trans*-Pt$(NH_3)_2(H_2N(CH_2)_6$-$NH_2)_2$)]4+) influenced by copper homeostasis. Biochem Pharmacol. 2007; 73:1270–1279. [PubMed: 17234160]
35. Mangrum JB, Farrell NP. Excursions in Polynuclear Platinum-DNA Binding. Chem Comm. 2010; 46:6640–6650. [PubMed: 20694266]
36. Lovejoy KS, Todd RC, Zhang S, McCormick MS, D'Aquino JA, et al. *cis*-Diammine(pyridine)chloroplatinum(II), a monofunctional platinum(II) antitumor agent: Uptake, structure, function, and prospects. Proc Natl Acad Sci USA. 2008; 105:8902–8907. [PubMed: 18579768]
37. Chekenya M, Rooprai HK, Davies D, Levine JM, Butt AM, Pilkington GJ. The NG2 chondroitin sulfate proteoglycan: role in malignant progression of human brain tumours. Int J Dev Neurosci. 1999; 17:421–435. [PubMed: 10571405]
38. Fuster MM, Esko JD. The sweet and sour of cancer: Glycans as novel therapeutic targets. Nature Reviews. 2005; 5:526–542.
39. Billecke C, Finniss S, Tahash L, Miller C, Mikkelsen T, Farrell N, Bögler O. Sensitivity of glioma cells in vitro and in vivo to platinum anticancer drugs. Polynuclear agents are more potent than cisplatin and induce cell cycle arrest. Neurooncology. 2006; 8:215–226.
40. Riccardi A, Meco D, Ferlini C, Servidei T, Carelli G, Segni G, Manzotti C, Riccardi R. In vitro and in vivo antitumor activity of the novel trinuclear platinum complex BBR3464 in neuroblastoma. Cancer Chemother Pharmacol. 2001; 47:498–504. [PubMed: 11459202]
41. Youjin S, Schenaur MR, Leary JA. Biologically relevant metal-cation binding induces conformational changes in heparin oligosaccharides as measured by ion mobility mass spectrometry. Int J Mass Spectrom. 2011; 303:191–198. [PubMed: 21731426]
42. Xie Y, Aillon KL, Cai S, Christian JM, Davies NM, Berkland CJ, Forrest ML. Pulmonary delivery of cisplatin-hyaluronan conjugates via endotracheal instillation for the treatment of lung cancer. Int J Pharm. 2010; 392:156–163. [PubMed: 20363303]

43. Zhang JS, Imai T, Suenaga A, Otagiri M. Molecular-weight-dependent pharmacokinetics and cytotoxic properties of cisplatin complexes prepared with chondroitin sulfate A and C. Int J Pharm. 2002; 240:23–31. [PubMed: 12062498]

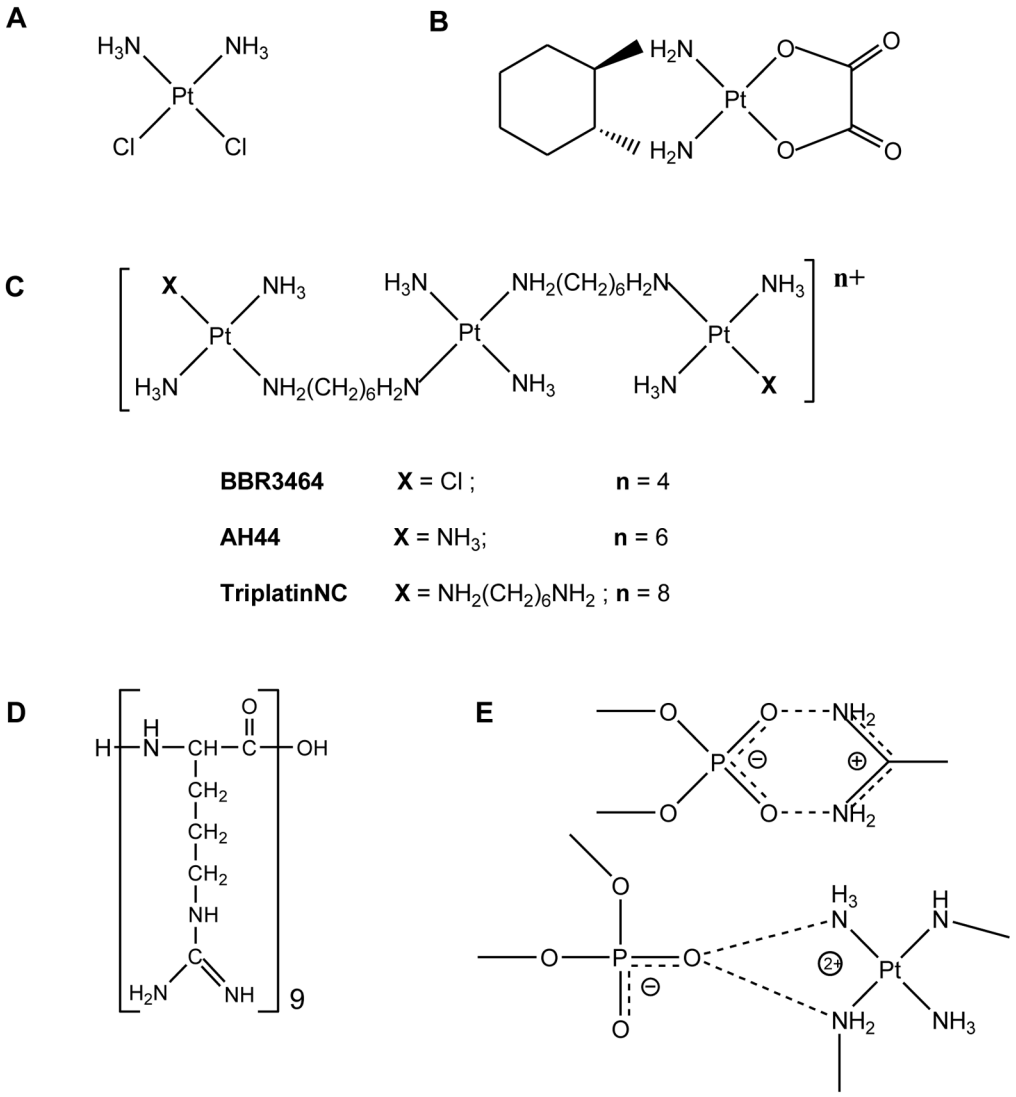


**Figure 1.**
Structures of A) Cisplatin; B) Oxaliplatin; C) Polynuclear Platinum Compounds (PPCs); D) Nonarginine-R9 and E) the analogy between the arginine fork and phosphate clamp.

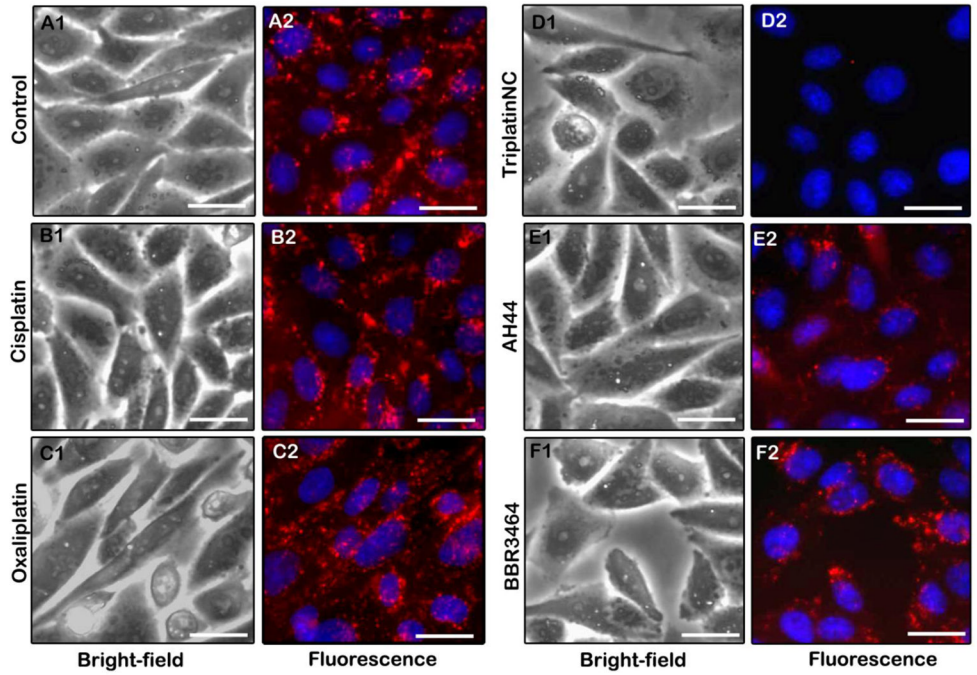


**Figure 2.**

Competition of TAMRA-R9 internalization and Platinum compound in wt CHO cells. Compounds (10 μM) were added 5 min prior to the addition of TAMRA-R9 (1.0 μM) and analyzed by fluorescence microscopy. Cells were counterstained with Hoescht 33342. Panels: (A) Control; (B) Cisplatin; (C) Oxaliplatin; (D) TriplatinNC; (E) AH44; (F) BBR3464. Bar = 10 μm.

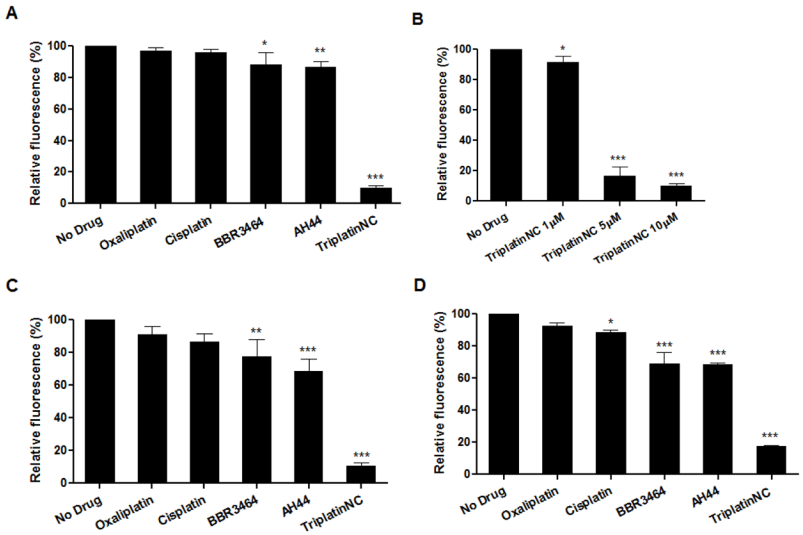


**Figure 3.**

Platinum compound (10 μM except where stated) competition with TAMRA-R9 uptake in wt CHO cells (A). Concentration Dependence of TriplatinNC on TAMRA-R9 uptake (B). Effect of compounds on TAMRA-R9 uptake in colon cancer HCT116 (C), and osteosarcoma SAOS-2 cells (D). Statistics analysis used was One Way ANOVA and Bonferoni post-test (* $p < 0.5$; ** $p < 0.1$; *** $p < 0.01$). Each point is the average (+/− SD) of three independent experiments.

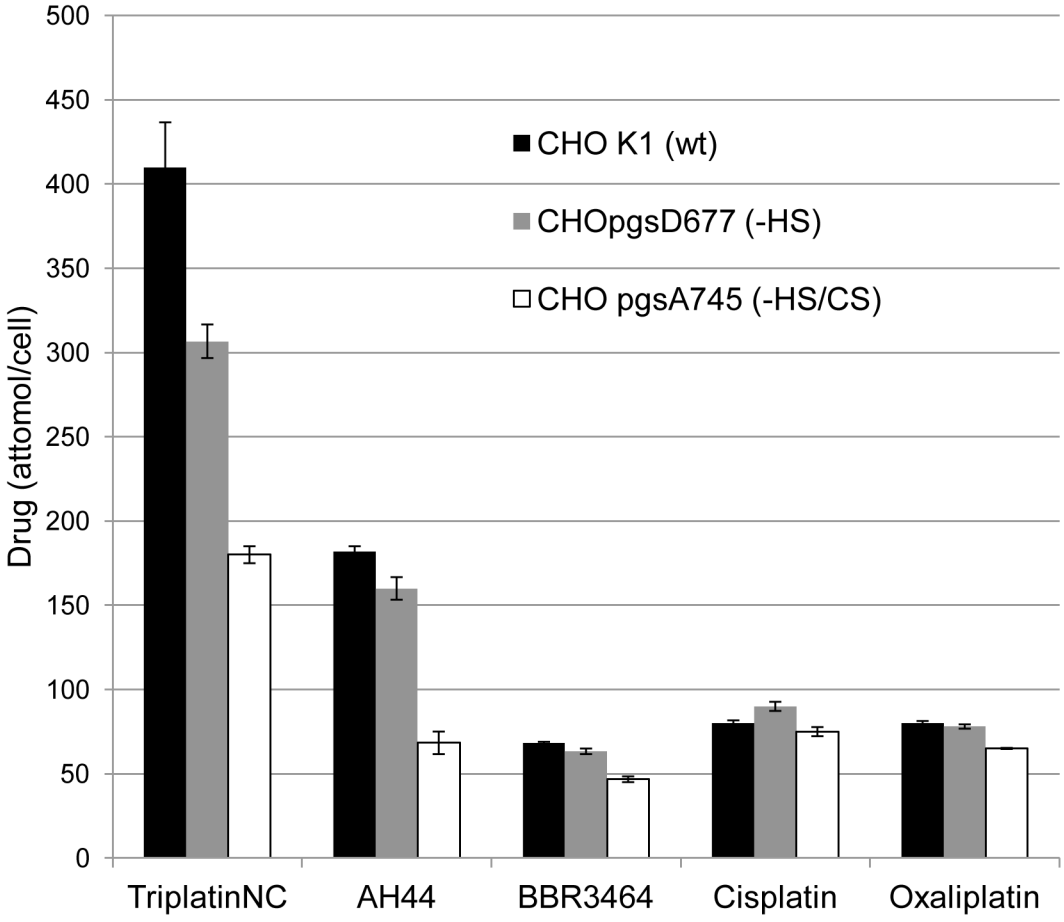


**Figure 4.**

Cellular accumulation of platinum compounds in wt (CHO K1); mutant CHO-pgsD-677 (lack HS) and CHO-pgsA-745 cells (lack HS/CS). Statistics analysis used was One Way ANOVA and Bonferoni post-test, all bars not signalized are significantly different ($p < 0.01$). Each point is the average (+/− SD) of three independent experiments. Values for trinuclear compounds adjusted to account for the presence of 3Pt.

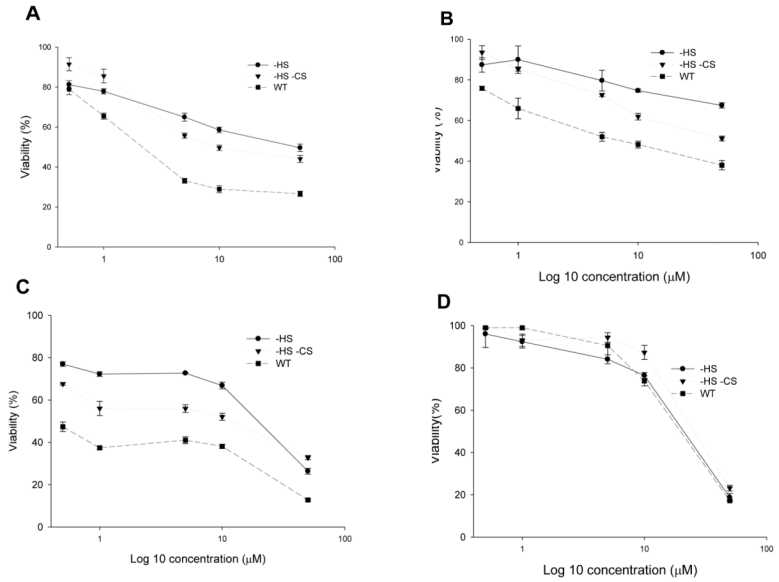


**Figure 5.**

Cytotoxicity of platinum compounds in wt CHO-K1; CHO-pgsD-677 (lack HS); and CHO-pgsA-745, (lack HS/CS) cells by MTT assay. A) TriplatinNC; B) AH44; C) BBR3464; D) Cisplatin. Each point is the average (+/− SD) of three independent experiments.

**TABLE 1**

Inhibition efficiency (%) of different treatments on the accumulation of PPCs in HCT116 cells.[a]

| Drug | Lipid raft endocytosis inhibitor (nystatin) | Macropinocytosis inhibitors | | Clathrin-mediated endocytosis inhibitors | |
|---|---|---|---|---|---|
| | | Cytochalasin D | EIPA | Hypertonic medium | Chlorpromazine |
| BBR3464 | ns | −13 * | −13 ** | ns | +33 * |
| TriplatinNC | −41 ** | −32 ** | −30 ** | ns | ns |

[a]; See Materials and Methods. Data are shown as mean (n=3). For SD values, see supplemental Figures S5–S8.

* $p = 0.05$,

** $p<0.01$, Student t test. ns: non-significant