molecular pharmaceutics

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Effects of Noncovalent Platinum Drug−Protein Interactions on Drug Efficacy: Use of Fluorescent Conjugates as Probes for Drug Metabolism

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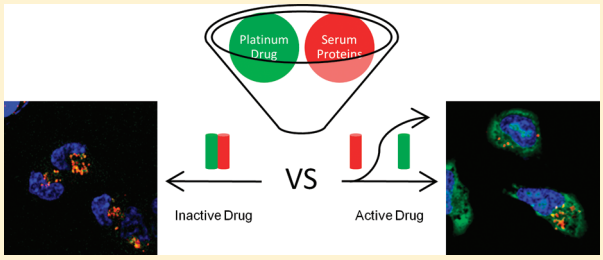
Supporting Information

ABSTRACT: The overall efficacy of platinum based drugs is limited by metabolic deactivation through covalent drug−protein binding. In this study the factors affecting cytotoxicity in the presence of glutathione, human serum albumin (HSA) and whole serum binding with cisplatin, BBR3464, and TriplatinNC, a "noncovalent" derivative of BBR3464, were investigated. 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. Confocal microscopy of both TriplatinNC-NBD, a fluorescent derivative of TriplatinNC, and TriplatinNC-NBD/HSA showed nuclear/nucleolar localization patterns, distinctly different from the lysosomal localization pattern seen with HSA. Cisplatin-NBD, a fluorescent derivative of cisplatin, was shown to accumulate in the nucleus and throughout the cytoplasm while the localization of cisplatin-NBD/HSA was limited to lysosomal regions of the cytoplasm. The results suggest that TriplatinNC can avoid high levels of metabolic deactivation currently seen with clinical platinum chemotherapeutics, and therefore retain a unique cytotoxic profile after cellular administration.



KEYWORDS: cisplatin, BBR3464, TriplatinNC, platinum, human serum albumin, glutathione, fluorescence microscopy

INTRODUCTION

Platinum drugs are among the most commonly used chemotherapeutics for the treatment of testicular, head and neck, ovarian, small cell lung, and colorectal carcinomas.[1−3] The cytotoxicity of cisplatin is accepted to result mainly from the formation of bifunctional DNA cross-links resulting in the inhibition of DNA synthesis and replication.[4] The DNA-reactive platinum species are considered to be monoaquo and diaquo complexes—in general $[Pt(amine)_2(H_2O)_2]^{2+}$ or $[Pt(amine)_2(Cl)(H_2O)]^{+}$—produced upon hydrolysis of the administered drugs, and the chemistry of these species has been well examined.[5] While this concept has been useful in rationalizing some structure−activity relationships, there is increasing understanding that even anions with nominally weak affinity for platinum—CO_3^{2-};[6,7] SO_4^{2-};[8] PO_4^{3-} and even $RCOO^-$[9]—become physiologically relevant ligands because of their high concentrations in plasma and cells. Similarly, cellular accumulation of platinum drugs is multifactorial with evidence for both passive and active transporter-mediated processes.[10]

The three major pharmacological factors affecting the clinical success of any type of platinum-based cancer therapy are cellular uptake, the frequency and the type of DNA adducts formed and metabolic deactivation. Metabolic deactivation is also mediated through interactions of the small molecules with biomolecules, specifically sulfur-containing amino acids in proteins, and these interactions may contribute to the toxic side effects associated with platinum based treatments.[11,12] One of the most plausible extracellular non-DNA targets for platinum compounds is blood serum. Serum is the liquid portion of blood, approximately 55% of the total volume, excluding red and white blood cells, containing all plasma proteins at a concentration of 70 g/L.[13] These proteins include fibrinogen, globulins, and human serum albumin (HSA). Albumin, the most abundant serum protein at 30−50 g/L and the most likely protein candidate for drug metabolic interactions, is a 585 amino acid, 66 kDa, single chain protein involved in transportation of numerous drugs and ligands.[14,15] It is mainly accepted that, once coordinated to

Received: February 3, 2011
Accepted: May 6, 2011
Revised: April 8, 2011
Published: May 06, 2011

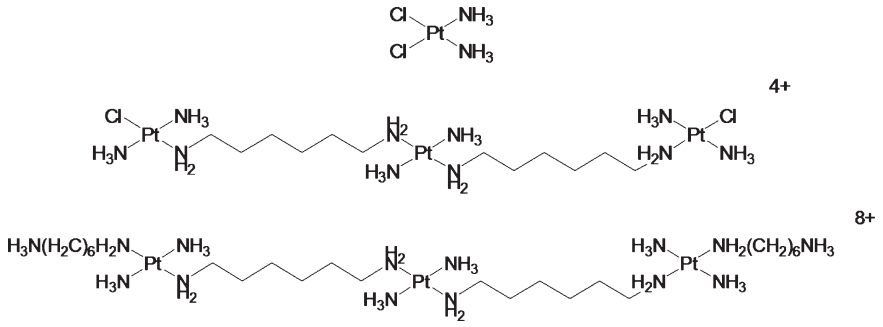


Figure 1. Chemical structures of cisplatin (top), BBR3464 (middle) and TriplatinNC (bottom).

plasma proteins, platinum compounds are effectively "deactivated" and eliminated from the body; thus never reaching their desired site of action.[16,17]

Another important sulfur-containing protein found intracellularly is the tripeptide, glutathione (GSH).[18] The normal intracellular concentration of GSH ranges from 5 to 10 mM,[19] therefore the binding of platinum-containing drugs to GSH is highly probable. Cells with elevated glutathione (>10 mM) are more resistant to cisplatin,[20] indicating its clinical importance. Due to these metabolic consequences resulting in low cellular Pt−DNA binding, it is necessary to fully investigate the interactions of sulfur-containing biomolecules with current clinical and preclinical platinum chemotherapeutics.

Polynuclear platinum compounds are a structurally discrete class of drugs whose chemical and biological properties differ significantly from those of cisplatin.[21−23] BBR3464, the only non-cisplatin-like molecule to enter human clinical trials, has promising activity in cisplatin-resistant, cisplatin-sensitive and p53 mutant tumors.[24−26] BBR3464 is also 40- to 80-fold more potent on a molar basis than cisplatin.[27] Promising preclinical and phase I and II clinical studies of BBR3464 have been offset to some extent by rapid degradation of BBR3464 into inactive metabolites due to enhanced interactions with plasma proteins.[28,29] The production of these inactive metabolites may be replicated by the chemical reactions of BBR3464 with reduced GSH.[18] These pharmacokinetic factors are thought to contribute to reduced activity in gastric and lung cell carcinomas.[30,31] For the development of subsequent generations of platinum derivatives, it is necessary to design platinum compounds which may have the ability to avoid or minimize these metabolic deactivations.

Most platinum chemotherapeutics bind to DNA in a covalent manner by formation of a Pt−DNA adduct. Replacement of the chloride leaving groups of BBR3464 with substitutionally "inert" ammine ligands or "dangling" ammines, $H_2N(CH_2)_6NH_3^+$, gives "noncovalent" polynuclear platinum compounds, most notably TriplatinNC, Figure 1. Although chemically nonreactive, TriplatinNC binds to DNA through the formation of a "phosphate clamp", a ligand mode of binding distinct from the "classical" minor-groove binders and intercalators.[32] TriplatinNC has significantly higher cellular accumulation than either BBR3464 or cisplatin, and in some cases cytotoxicity equivalent to or greater than that of cisplatin.[33,34]

Biophysical studies on the interaction of TriplatinNC with albumin have shown the presence of an initial, electrostatic, association with the protein.[35] Previous studies have shown that the interactions of cationic lipids with albumin give approximate binding ratios of 1−2:1 (lipid:albumin).[36] Due to its high charge, TriplatinNC would be expected to associate to albumin and other serum proteins *in vivo* but, in the absence of a substitution-labile leaving group, would not be expected to undergo the same types of deactivation reactions as seen with the covalent platinum analogues.[12,17,35] This protein association, in the absence of deactivation, could provide a promising and novel pharmacokinetic profile not seen in current clinical platinum regiments. This paper addresses the effects of serum protein and glutathione deactivation on platinum drug trafficking and the consequences for compound efficacy, levels of cellular accumulation, DNA adduct formation, and intracellular distribution. The effects of protein association on intracellular localization were examined by confocal microscopy using a new, versatile fluorophore for platinum drugs based on 7-nitro-2,1,3-benzoxadiazole (NBD).

EXPERIMENTAL SECTION

Materials. BBR3464, cisplatin, and TriplatinNC were synthesized as reported previously.[37−39] The synthesis and characterization of the fluorophore derivatized TriplatinNC-NBD and cisplatin-NBD is described in the Supporting Information. Human plasma was purchased from Innovative Research Inc. Albumin from human serum, minimum 99%, FITC-albumin, and buthionine sulfoximine (BSO) were obtained from Sigma-Aldrich (St. Louis, MO). LysoTracker Red DND-99 was obtained from Invitrogen.

Cell System. HCT116 and A2780 cells were cultured in RPMI 1640 with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin in humidified air with 5% CO_2.

Human Plasma Binding. Solutions of each drug were prepared in PBS and added to human plasma such that the final concentration of complex was 0.9 mM (1:1 v/v). The reactions were incubated at 37 °C for 0, 0.5, 2, and 24 h. Unreacted and protein-bound drug was removed by centrifugation through a Millipore Microcon YM-10 (10,000 kDa) membrane filter. Both ultrafiltrate and protein samples were stored at −20 °C immediately following centrifugation. Analysis of platinum content for both the protein-bound and unreacted samples was performed on a Varian ICP-OES. Protein concentration was determined spectrophotometrically.

Formation of Drug−Albumin Complex. Platinum compounds (cisplatin, cisplatin-NBD, BBR3464, and TriplatinNC) were incubated with HSA for 72 h at 10:1 (drug:protein). Non-protein-bound drug was removed by centrifugation using the method described above. The protein was collected, redissolved in PBS and immediately frozen at −20 °C. Pt concentration was determined using ICP-OES by removing a small aliquot of the Pt−protein solution before freezing.

BSO Treatment, Propidium Iodide DNA Staining, and Analysis of Apoptosis. HCT116 cells were cultured in 6-well plates at an initial density of 7.0×10^4 cells/mL. Different concentrations of drugs were added to each well as indicated. Total cell contents (apoptotic and viable cells) were collected. In experiments using BSO, Pt drug concentrations were adjusted to achieve approximately 15−20% apoptosis, allowing measurement of enhancement or inhibition. 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, as described previously.[40] Samples were then analyzed for subdiploid DNA content on a Becton Dickinson FACScan flow cytometer (BD Biosciences, San Jose, CA).

Cellular Growth Inhibition. The MTT assay was used to determine growth inhibition potency of platinum drugs and the corresponding protein-bound derivatives.[41] A2780 cells were seeded in 96-well plates at 6,500 cells/well in 200 μL of RPMI 1640 medium and allowed to attach overnight. Medium was removed, and 200 μL of each compound was added after serial dilution in quadruplicate wells and exposed to cells for 72 h. For time dependent medium incubation studies 500 μM of each Pt compound was incubated in RPMI 1640 medium supplemented with 10% FBS (1:1 v/v) for 0, 24, 48, and 72 h before cellular treatment. Samples were diluted to treatment concentrations [30−0.25 μM] with fresh medium after appropriate time points, and cells were treated accordingly. Quantification of cell growth in treated and control wells was then assessed by MTT assay. IC_{50} values were determined graphically.

Cellular Uptake of Drug and Drug−HSA Complexes. A2780 cells were seeded at 2×10^6 cells in 10 cm dishes and allowed to attach overnight. Cells were then treated with IC_{90} concentrations of each drug and drug−HSA complex,15 μM for TriplatinNC, 4 μM for cisplatin, and 2.5 μM for BBR3464, in 15 mL of medium for 0, 3, 6, 12, and 24 h. After appropriate incubation times, cells were removed from the dish, counted, and resuspended in 250 μL of ddH_2O. Samples were digested, and analysis of Pt content was performed on a Varian ICP-MS.

Quantification of DNA−Drug Adducts. A2780 cells were seeded at 2×10^6 cells in 10 cm dishes and allowed to attach overnight. Cells were then treated with IC_{90} concentrations of each drug and drug−HSA complex, 4 μM for cisplatin, and 2.5 μM for BBR3464, in 15 mL of medium for 24 h. For time dependent medium incubation studies 500 μM of each Pt compound was incubated in RPMI 1640 medium supplemented with 10% FBS (1:1 v/v) for 0, 24, 48, and 72 h before cellular treatment. After incubation, cells were removed from the dish and suspended in 200 μL of ddH_2O. DNA was extracted using a DNeasy blood and tissue kit (Qiagen). Extent of DNA platination was measured on a Varian ICP-MS.

Confocal Laser Scanning Microscopy of A2780 Cells. A2780 cells were grown on 8-well chamber slides (Lab-TekII Chamber Slide) for 2−3 days until cells reached near-confluency. The cells were treated with cisplatin-NBD (15 μM) and cisplatin-NBD/HSA (1 mg/mL) for 24 h. LysoTracker Red (75nM) was added to the slide for the final 30 min of drug treatment time. For TriplatinNC-NBD (15 μM) and TriplatinNC-NBD/HSA (1 mg/mL), and FITC-albumin (1 mg/mL), A2780 cells were treated for 6 h and LysoTracker Red (75 nM) was added to the slide for the final 30 min of drug treatment time. After treatment, slides were washed 3× with ice-cold PBS and fixed with 3% paraformaldehyde. Paraformaldehyde was removed, and cells were washed again with 3× PBS and allowed to dry. Slides were then mounted with Vectashield mounting medium containing DAPI. Fluorescence was observed by confocal laser scanning microscopy (Zeiss LSM 510).

RESULTS AND DISCUSSION

Importance of Glutathione in TriplatinNC-Mediated Cytotoxicity. One of the main intracellular biomolecules is glutathione (GSH), which has an important role in determination of cellular sensitivity to cytotoxic drugs. The cellular glutathione level in HCT116 cells was decreased using buthionine sulfoximine (BSO), which inhibits gamma glutamylcysteine synthetase, a rate-limiting enzyme in glutathione synthesis. As the cellular glutathione level was reduced, cisplatin- and BBR3464-induced apoptosis was augmented, demonstrating their interactions with glutathione. After 48 h of treatment, BSO alone and BBR3464-induced apoptosis were 1% and 18% (as in control sample), but the combination of BBR3464 and BSO increased apoptosis to 58% (Figure 2A). The results showed consistency in testing with cisplatin. BSO increased cisplatin-induced apoptosis from 14% to 45% (Figure 2A). In striking contrast to these covalent Pt drugs, TriplatinNC-induced cytotoxicity was completely unaltered by inhibiting glutathione synthesis (Figure 2A).

Since some efflux mechanisms have been shown to be dependent on glutathione,[42] the effects of BSO on the cellular accumulation of platinum drugs was measured. Cellular platinum levels were measured *via* ICP-OES in cells treated with platinum drugs ± BSO to determine if the increase observed in cytotoxicity

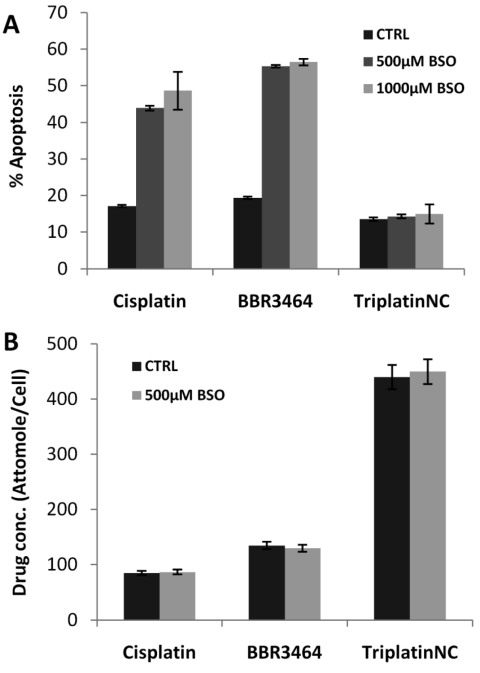


Figure 2. (A) Effect of BSO on platinum drug-induced cytotoxicity in HCT116 cells. Subdiploid cell content was detected by PI-DNA staining. HCT116 cells were cultured with 10 μM cisplatin, 50 μM BBR3464, or 40 μM TriplatinNC for 48 h in the absence or presence BSO. Drugs were added to the medium after 1 h of treatment with BSO. (B) Effect of BSO on platinum drug cellular uptake in HCT116 carcinoma cell lines. HCT116 cells were cultured with 20 μM cisplatin for 16 h, 20 μM BBR3464 for 8 h, or 20 μM TriplatinNC for 3 h in the absence or presence of 500 μM BSO. Drugs were added to the medium after 1 h of treatment with BSO.

is the result of enhanced cellular uptake of platinum drugs. As shown in Figure 2B, BSO did not influence cisplatin, BBR3464, or TriplatinNC cellular accumulation. Hence, cellular accumulation does not play a role in the augmentation of Pt-induced cytotoxicity by BSO.

Effects of Protein Binding on Cisplatin, BBR3464, and TriplatinNC. The pharmacokinetic profiles in phase I clinical trials of BBR3464 and cisplatin have been shown to be vastly different, with protein binding in BBR3464 being much higher than cisplatin at early time points.[27] To evaluate the effect of protein binding on drug deactivation, cisplatin, BBR3464 and TriplatinNC were incubated with human plasma or human serum albumin for selected time points. Analysis of platinum content by ICP-OES shows an increase in protein binding over time for both cisplatin and BBR3464 after incubation with whole serum. This time dependent increase in protein binding represents a displacement of the platinum−chloride bond and the formation of the covalent platinum−protein adduct (Figure S1 in the Supporting Information). In contrast, with the noncovalent compound, TriplatinNC, a 1:1 drug−protein complex is immediately formed, representing a rapid association of the positively charged drug, presumably with negative regions of serum proteins.(Figure S1 in the Supporting Information). Due to the chemical nature of TriplatinNC, the initial noncovalent binding is not followed by covalent drug−protein adduct formation.

Role of Protein Binding on Cytotoxicity of Cisplatin, BBR3464, and TriplatinNC. In theory, once a covalent platinum−protein adduct is formed, the drug "loses" its cytotoxic effect and is rendered inactive.[43] To further understand the differences in binding profile between covalent and "noncovalent" platinum compounds with HSA, cytotoxicity assays were performed on the protein-bound derivatives of cisplatin, BBR3464, and TriplatinNC (cisplatin/HSA, BBR3464/HSA, and TriplatinNC/HSA) and compared to the free drug IC_{50} values in A2780 ovarian carcinoma cell lines. As seen in Figure 3A, A2780 cells treated with cisplatin/HSA show a large decrease in drug efficacy as compared with treatment of cisplatin alone. The same trend can be seen with BBR3464/HSA and BBR3464 (Figure 3B). In stark contrast, A2780 cells showed no change in growth inhibition when treated with the complex TriplatinNC/HSA ($IC_{50} \approx 3\ \mu M$) as compared to treatment of the drug alone, TriplatinNC ($IC_{50} \approx 3\ \mu M$) (Figure 3C).

Incubation of cisplatin, BBR34364 and TriplatinNC with whole serum provides a physiological approach to studying the effects of protein binding on drug efficacy. Cisplatin and BBR3464 both show a time-dependent decrease in cytotoxicity upon incubation followed by cellular exposure after pretreatment with serum (Figure 3D,E). For cisplatin and BBR3464, the observed IC_{50} values decreased from 0.6 μM to >30 μM, and 0.2 μM to >30 μM, respectively, over a 72 h period. Cellular treatment with TriplatinNC after 72 h medium incubation showed no marked decrease in IC_{50} value, remaining approximately 3 μM at all time points (Figure 3F). The data is tabulated in Figure S2 in the Supporting Information.

Effects of Protein Binding on Cellular Drug Accumulation. Since the rate of cellular uptake and total accumulation correlates to the cytotoxic effect of platinum drugs, cellular accumulation of each platinum compound and its protein-bound derivative was examined at IC_{90} concentrations, Figure 4. Treatment of A2780 cells with cisplatin produced an increase in cellular platinum levels in a time dependent manner, reaching approximately 30 attomoles (amol) of drug/cell after 24 h. In contrast, cells treated with cisplatin/HSA showed a drug accumulation of 5 amol of drug/cell or less at all time points (Figure 4A). Cellular treatment with BBR3464 also shows a time dependent increase in drug concentration, reaching approximately 100 amol of drug/cell after 24 h (Figure 4B). In contrast to cisplatin/HSA, the protein-bound derivative of BBR3464 shows a sporadic increase in platinum concentration over time. Although BBR3464 is covalently bound to albumin,[35] the chemical nature of this binding may cause the trinuclear drug to degrade into dinuclear and mononuclear noncovalent, noncytotoxic metabolites as seen

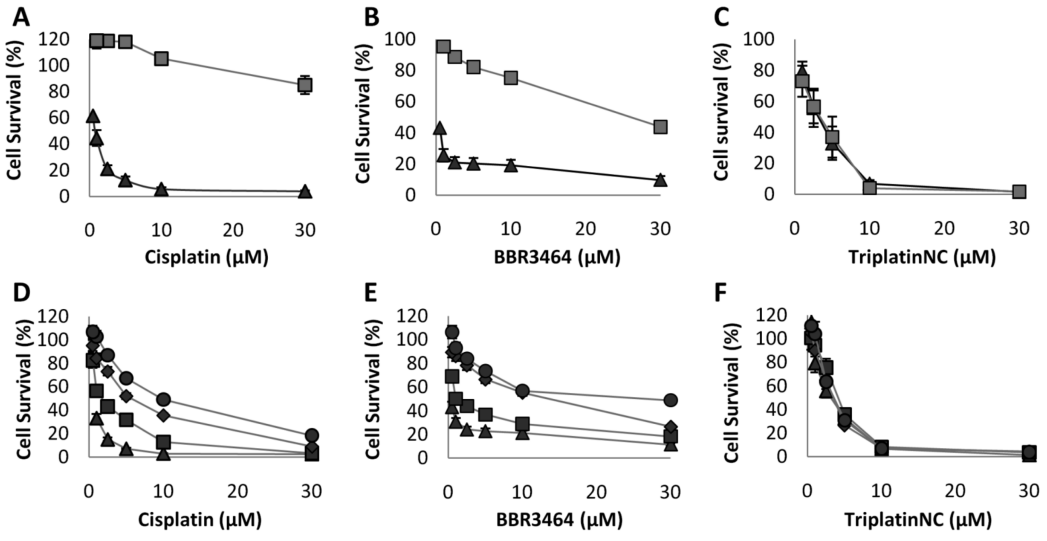


Figure 3. Influence of protein binding on platinum drug efficacy in A2780 ovarian carcinoma cells. (A) Cisplatin cytotoxicity (▲) in A2780 cells compared with the treatment of cisplatin/HSA (■). (B) BBR3464 cytotoxicity (▲) compared with the treatment of BBR3464/HSA (■). (C) TriplatinNC cytotoxicity (▲) compared with the treatment of TriplatinNC/HSA (■). (D−F) Platinum compounds were incubated with serum supplemented medium for 0−72 h. Cellular grown inhibition was then measured after treatment. Efficacy of cisplatin (D), BBR3464 (E), and TriplatinNC (F) following increased serum incubation times (0 (▲), 24 (■), 48 (♦), and 72 (●) h). A2780 cells were cultured in the indicated concentrations of each compound for 72 h. Percent growth inhibition was determined by MTT.

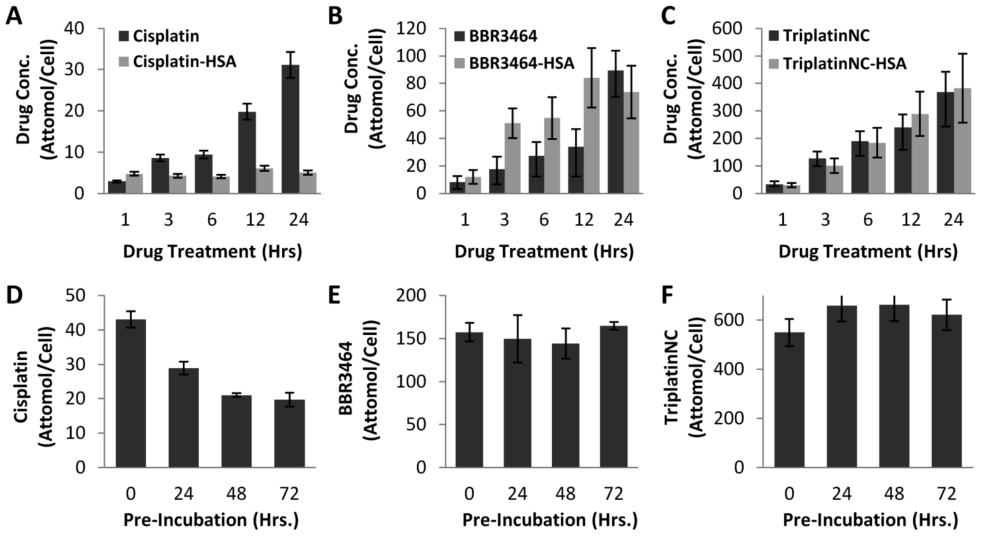


Figure 4. Influence of protein binding on platinum cellular uptake in A2780 ovarian carcinoma cells. A2780 cells were treated with IC_{90} concentrations of cisplatin and cisplatin/HSA (A), BBR3464 and BBR3464/HSA (B), TriplatinNC and TriplatinNC/HSA (C) for 0, 3, 6, 12, and 24 h. For serum incubation studies each platinum drug was preincubated with serum for (0−72 h). A2780 cells were then treated with IC_{90} concentrations of each compound ((D) cisplatin, (E) BBR3464, (F) TriplatinNC) for 24 h. Analysis of platinum content was performed on a Varian ICP-MS.

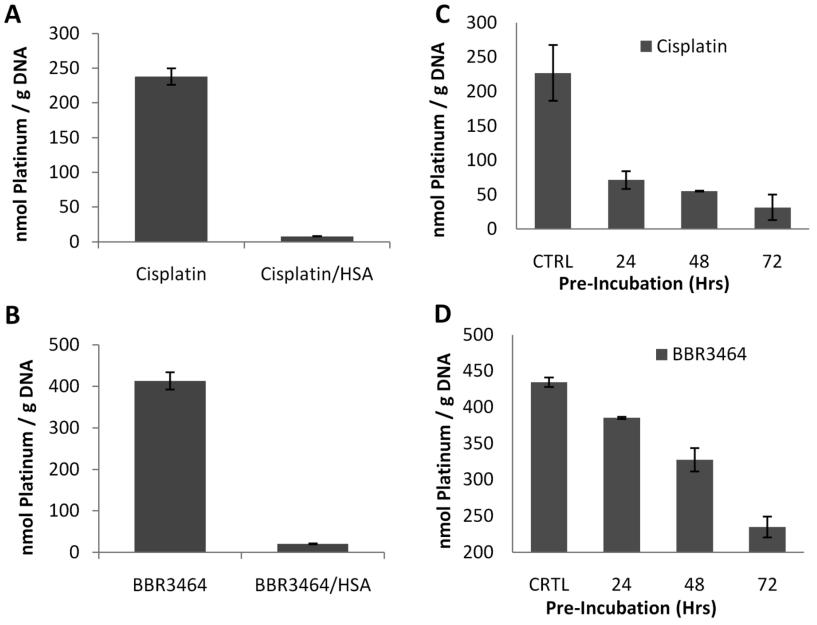


Figure 5. Influence of protein binding on DNA platination in A2780 ovarian carcinoma cells. A2780 cells were treated with IC_{90} concentrations of cisplatin and cisplatin/HSA (A), BBR3464 and BBR3464/HSA (B) for 24 h. For serum incubation studies each platinum drug was preincubated with serum for (0−72 h). A2780 cells were then treated with IC_{90} concentrations of each compound ((C) cisplatin, (D) BBR3464) for 24 h. Cellular DNA was extracted and analysis of platinum content was performed on a Varian ICP-MS.

previously with the treatment of blood cells with BBR3464.[18,29,44] It is reasonable to assume that these dinuclear and mononuclear metabolites may also enter cells and contribute to the measurement of Pt levels. In contrast, platinum accumulation in cells treated with TriplatinNC and TriplatinNC/HSA was nearly identical at all time points, reaching approximately 400 amol of drug/cell at the 24 h time point (Figure 4C). Note again the enhanced accumulation of charged trinuclear drugs in comparison to cisplatin.

To examine the role of whole serum deactivation on the rate of cellular accumulation levels for cisplatin, BBR3464 and TriplatinNC, intracellular platinum levels were determined after 24 h of drug exposure, following specified serum incubation times. Treatment of cisplatin with serum supplemented medium shows a time-dependent decrease in cellular platinum uptake following drug exposure for 24 h (Figure 4D). Treatment of BBR3464 with serum supplemented medium shows a constant level of cellular platinum uptake following drug exposure for 24 h. This platinum accumulation is interpreted to correspond to a mixture of the covalent and noncovalent metabolites of BBR3464, consistent with the metabolic degradation seen after protein binding (Figure 4E). Upon incubation with serum

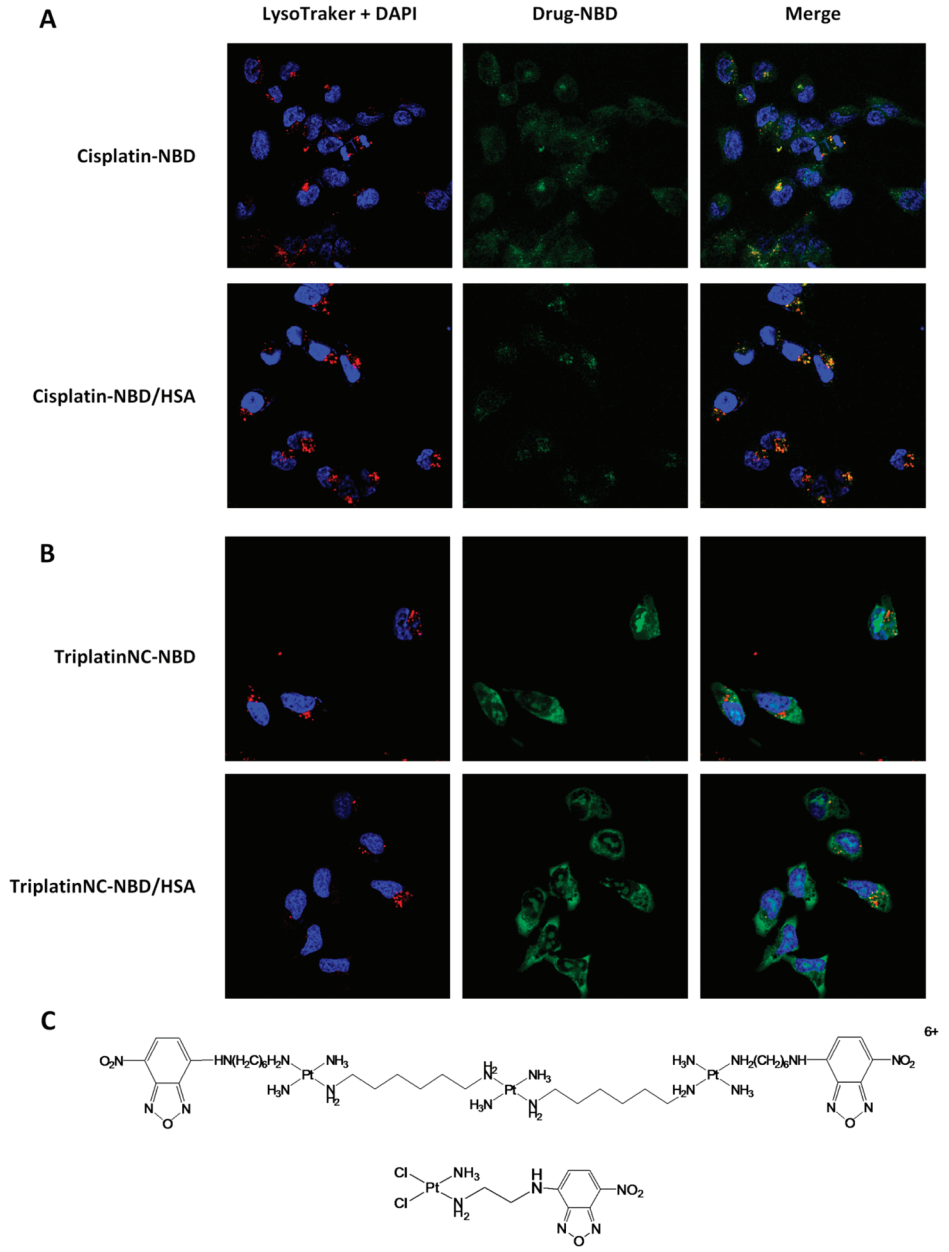


Figure 6. Role of protein binding on the cellular localization and distribution of cisplatin and TriplatinNC. Confocal laser scanning micrographs (A, B) of A2780 cells. (A) (top row) Cisplatin-NBD (15 μM) (green) incubated with A2780 cells for 24 h and LysoTracker Red (75 nM) (red) for final 30 min of drug incubation. (A) (bottom row) Cisplatin-NBD/HSA (1 mg/mL) (green) incubated with A2780 cells for 24 h and LysoTracker Red (75 nM) (red) for final 30 min of drug incubation. Orange represents areas of drug−protein colocalization. (B) (top row) TriplatinNC-NBD (15 μM) (green) incubated with A2780 cells for 6 h and LysoTracker Red (75 nM) (red) for final 30 min of drug incubation. (B) (bottom row) TriplatinNC-NBD/HSA (1 mg/mL) (green) incubated with A2780 cells for 6 h and LysoTracker Red (75nM) (red) for final 30 min of drug incubation. Slides were mounted with Vectashield mounting medium containing DAPI (blue). Fluorescence was observed by confocal laser scanning microscopy (Zeiss LSM 510). (C) Chemical structures of TriplatinNC-NBD (top) and cisplatin-NBD (bottom).

for 24, 48, and 72 h and subsequent cellular treatment, TriplatinNC cellular accumulation levels remains constant (Figure 4F). A constant IC_{50} value over time and a consistent rate of cellular drug accumulation shows the ability of TriplatinNC to "bypass" the typical protein deactivation pathway seen with current platinum chemotherapeutics.

Effects of Protein Binding on Levels of DNA Platination. The cytotoxicity of platinum chemotherapeutics is mainly accepted to be modulated by the frequency and type of platinum−DNA adducts formed during drug treatment. To examine the role of protein binding on the amount of DNA platination, A2780 cells were treated for 24 h with IC_{90} concentrations of cisplatin, cisplatin/HSA, BBR3464, and BBR3464/HSA. DNA platination levels after 24 h of continuous cisplatin treatment reached approximately 225 nmol of Pt/g of DNA, while platinum levels of cisplatin/HSA remained less than 10 nmol of Pt/g of DNA (Figure 5A). The same trend in DNA adduct formation was seen with BBR3464 and BBR3464/HSA, with platinum levels reaching 425 and 20 nmol of Pt/g of DNA respectively (Figure 5B).

dx.doi.org/10.1021/mp2000583 |*Mol. Pharmaceutics* 2011, 8, 940–948

DNA platination levels of cisplatin and BBR3464 were also monitored following serum incubation. After incubation with serum proteins for 24, 48, and 72 h, both cisplatin and BBR3464 DNA platination levels were reduced. Initial levels of DNA platination for cisplatin were approximately 225 nmol of platinum/g of DNA. After 72 h of serum treatment prior to cell treatment, DNA platination levels were reduced to 31 nmol of platinum/g of DNA (Figure 5C). The same trend was seen with serum pretreatment of BBR3464, where platinum levels decrease from 425 nmol of platinum/g of DNA to 230 nmol of platinum/g of DNA (Figure 5D). The decreases in DNA platination seen after protein binding are directly responsible for the reduced activity of both cisplatin and BBR3464.

Due to the noncovalent nature of TriplatinNC-DNA binding,[32] isolation of cellular DNA may disrupt the interaction. To resolve this inherent problem, we have developed a new fluorescent probe for platinum and used fluorescence microscopy as a tool to examine the effects of protein binding on cellular distribution and DNA platination.

Fluorescent Drug Design of TriplatinNC-NBD and Cisplatin-NBD. The use of molecular imaging techniques, such as confocal microscopy, is an important tool in the understanding of platinum drug trafficking and intracellular distribution. To utilize this approach, new fluorescent platinum derivatives were developed using NBD fluorophores. Hambley has summarized recent fluorescent approaches to monitoring platinum trafficking.[45] The advantage of the NBD approach is that, rather than use chelates like ethyelenediamine and cumbersome chemical modifications, the NBD addition is performed on a primary amine and gives analogues very similar to the "parent drugs". Previous attempts at "tagging" platinum compounds have yielded cisplatin derivatives in which FITC and Alexa based fluorophores have been utilized. These derivatives tend to mimic the biological properties of the fluorescent "tag" rather than the platinum moiety itself.[45] In the case of TriplatinNC, we conjugated the NBD fluorophore directly to the primary amine on each end of the complex, and for cisplatin, we replace the existing amine group with NBD-ethane-1,2-diamine. TriplatinNC-NBD shows only a 12% increase in molecular weight, retains a high positive charge (6+), and shows similar cytotoxicity to TriplatinNC (Figure S3A in the Supporting Information). Cisplatin-NBD has a molecular weight increase of 66%, substantially less than seen with FDDP,[45] and also retains a cytotoxic profile in A2780 cells (Figure S3B in the Supporting Information).

Role of Albumin in the Cellular Localization of Cisplatin and TriplatinNC. The mechanisms of cellular uptake and distribution of albumin and other serum proteins have been extensively studied and show that serum proteins localize to lysosomal regions of the cytosol.[46] The use of a noncovalent drug, TriplatinNC, affords a unique handle to examine how macromolecule association affects small platinum molecule biodistribution—in the absence of platinum bond-forming reactions. Confocal microscopy was therefore employed to investigate how internalization and localization of cisplatin and TriplatinNC is affected by albumin. A2780 cells were treated with cisplatin-NBD (15 μM) and cisplatin-NBD/HSA (1 mg/mL) to investigate the role of covalent protein binding on drug localization. LysoTracker Red was used as a lysosomal marker to indicate uptake of HSA (Figure S4 in the Supporting Information). Cisplatin-NBD was shown to localize throughout the cytosol and, to a lesser extent, the nucleus (Figure 6A). This distribution is consistent with the fact that less than 10% of cellular cisplatin is found in the nucleus. Low levels of cisplatin-NBD can also be seen to accumulate in the lysosomal regions of the cell. Due to the weakly basic nature of the NBD fluorophore, it is possible that a percentage of the lysosomal localization seen is a result of lysosomal trapping, a phenomenon commonly seen with basic lipophilic compounds.[47,48] After treatment of cisplatin-NBD/HSA, the cytosolic and nuclear localization was significantly reduced and limited to lysosomal regions of the cytosol. Colocalization of cisplatin-NBD/HSA and LysoTracker Red was seen at a greater extent than with the treatment of cisplatin alone, indicating an increase in drug deactivation after protein binding.

HSA (1 mg/mL) and TriplatinNC-NBD (15 μM) (protein:drug, 1:1) were preincubated in serum-free medium for 2 h to ensure complex formation. Upon treatment with TriplatinNC-NBD/HSA for 6 h, punctate cellular localization of LysoTracker Red was observed in a pattern consistent with lysosomal accumulation while TriplatinNC was shown to localize throughout the cytosol and nucleolus. The uptake of TriplatinNC-NBD alone was shown to be identical to the localization seen upon treatment of the protein-bound complex (Figure 6B). The difference in accumulation patterns between TriplatinNC and LysoTracker Red and, more importantly, the similarity between TriplatinNC-NBD and TriplatinNC-NBD/HSA localization show the ability of TriplatinNC to avoid metabolic deactivation associated with serum binding.

CONCLUSION

This study shows, for the first time, a platinum chemotherapeutic with a substantially enhanced and distinctly different pharmacokinetic profile compared to that of any clinically developed platinum drug. This work has shown the significance of the interaction of TriplatinNC with plasma as well as individual intracellular and extracellular proteins. The noncovalent binding of TriplatinNC to serum proteins allows for an interaction with the protein, but not a deactivation of the platinum compound. The deactivation of cisplatin and BBR3464 upon binding to glutathione, serum or albumin shows the major problems associated with the treatment of cancer using the current platinum chemotherapeutic regiment.

The data also allows one to examine the role of albumin as a drug carrier or drug delivery system for TriplatinNC. Albumin is known to function as an important protein in the maintenance of osmotic pressure and also as a transport protein involved in the distribution of hormones, fatty acids and metal ions.[49,50] Once administered, TriplatinNC would be expected to bind to plasma proteins in a noncovalent manner and then be transported to the cellular surface or become actively transported internally through the endocytosis of albumin.[46] The mechanism of uptake of TriplatinNC and the role of albumin in this process was examined by utilizing novel fluorescently labeled derivatives of both cisplatin and TriplatinNC. Further, the noncovalent drug is a unique probe for studying platinum drug molecule—biomolecule interactions in cells. TriplatinNC not only presents a new mode of DNA binding, distinct from any clinical platinum agent, but also allows for the elimination of covalent Pt—protein adduct formation, thus creating a novel pharmacokinetic and cytotoxicity profile worthy of further development.

ASSOCIATED CONTENT

Supporting Information. Details of the synthesis of cisplatin-NBD and TriplatinNC-NBD. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENT

This work was supported by a grant NIH R01CA78754 to N.P.F. Microscopy was performed at the VCU—Dept. of Neurobiology & Anatomy Microscopy Facility, supported, in part, with funding from NIH-NINDS Center core grant 5P30NS047463.

REFERENCES

(1) O'Dwyer, P.; Stevenson, J.; Johnson, S. Clinical pharmacokinetics and administration of established platinum drugs. *Drugs* **2000**, *59*, 19–27.

(2) Decatris, M. P.; Sundar, S.; O'Byrne, K. J. Platinum-based chemotherapy in metastatic breast cancer: current status. *Cancer Treat. Rev.* **2004**, *30*, 53–81.

(3) Wong, E.; Giandomenico, C. M. Current status of platinum-based antitumor drugs. *Chem. Rev.* **1999**, *99*, 2451–2466.

(4) Jamieson, E. R.; Lippard, S. J. Structure, Recognition, and Processing of Cisplatin-DNA Adducts. *Chem. Rev.* **1999**, *99*, 2467–2498.

(5) Berners-Price, S. J.; Appleton, T. G. *Platinum-Based Drugs in Cancer Therapy*; Humana Press: Totowa, NJ, 2000.

(6) Centerwall, C. R.; Goodisman, J.; Kerwood, D. J.; Dabrowiak, J. C. Cisplatin Carbonato Complexes. Implications for Uptake, Antitumor Properties, and Toxicity. *J. Am. Chem. Soc.* **2005**, *127*, 12768–12769.

(7) Centerwall, C. R.; Tacka, K. A.; Kerwood, D. J.; Goodisman, J.; Toms, B. B.; Dubowy, R. L.; Dabrowiak, J. C. Modification and Uptake of a Cisplatin Carbonato Complex by Jurkat Cells. *Mol. Pharmacol.* **2006**, *70*, 348–355.

(8) Ruhayel, R. A.; Corry, B.; Braun, C.; Thomas, D. S.; Berners-Price, S. J.; Farrell, N. P. Determination of the Kinetic Profile of a Dinuclear Platinum Anticancer Complex in the Presence of Sulfate: Introducing a New Tool for the Expedited Analysis of 2D [1H, 15N] HSQC NMR Spectra. *Inorg. Chem.* **2010**, *49*, 10815–10819.

(9) Zhang, J.; Thomas, D.; Davies, M.; Berners-Price, S.; Farrell, N. Effects of geometric isomerism in dinuclear platinum antitumor complexes on aquation reactions in the presence of perchlorate, acetate and phosphate. *J. Biol. Inorg. Chem.* **2005**, *10*, 652–666.

(10) Hall, M. D.; Okabe, M.; Shen, D.-W.; Liang, X.-J.; Gottesman, M. M. The Role of Cellular Accumulation in Determining Sensitivity to Platinum-Based Chemotherapy. *Annu. Rev. Pharmacol. Toxicol.* **2008**, *48*, 495–535.

(11) Safirstein, R.; Winston, J.; Goldstein, M.; Moel, D.; Dikman, S.; Guttenplan, J. Cisplatin nephrotoxicity. *Am. J. Kidney Dis.* **1986**, *8*, 356–367.

(12) Wang; Guo, Z. The role of sulfur in platinum anticancer chemotherapy. *Anticancer Agents Med. Chem.* **2007**, *7*, 19–34.

(13) Espósito, B. P.; Najjar, R. Interactions of antitumoral platinum-group metallodrugs with albumin. *Coord. Chem. Rev.* **2002**, *232*, 137–149.

(14) Carter, D. C.; Ho, J. X. Structure of Serum Albumin. *Adv. Protein Chem.* **1994**, *45*, 153−176, 176a, 176b, 176c, 176d, 176e, 176f, 176g, 176h, 176i, 176j, 176k, 176l, 177−203.

(15) Peters, T., Jr. Serum Albumin. *Adv. Protein Chem.* **1985**, *37*, 161–245.

(16) Xiaoyong, W.; Zijian, G. The Role of Sulfur in Platinum Anticancer Chemotherapy. *Anti-Cancer Agents Med. Chem.* **2007**, *7*, 19–34.

(17) Timerbaev, A. R.; Hartinger, C. G.; Aleksenko, S. S.; Keppler, B. K. Interactions of Antitumor Metallodrugs with Serum Proteins: Advances in Characterization Using Modern Analytical Methodology. *Chem. Rev.* **2006**, *106*, 2224–2248.

(18) Oehlsen, M. E.; Qu, Y.; Farrell, N. Reaction of polynuclear platinum antitumor compounds with reduced glutathione studied by multinuclear (1H, 1H-15N gradient heteronuclear single-quantum coherence, and 195Pt) NMR spectroscopy. *Inorg. Chem.* **2003**, *42*, 5498–5506.

(19) Kosower, N. S.; Kosower, E. M. The glutathione status of cells. *Int. Rev. Cytol.* **1978**, *54*, 109–160.

(20) Eastman, A.; Richon, V. M. Mechanisms of cellular resistance to platinum coordination complexes. In *Biochemical Mechanisms of Platinum Antitumor Drugs*; McBrien, D. C. H., Slater T. F., Eds.; 1986, IRL Press, Oxford, pp 91−119.

(21) Farrell, N.; Qu, Y.; Hacker, M. P. Cytotoxicity and antitumor activity of bis(platinum) complexes. A novel class of platinum complexes active in cell lines resistant to both cisplatin and 1,2-diaminocyclohexane complexes. *J. Med. Chem.* **1990**, *33*, 2179–2184.

(22) Hegmans, A.; Kasparkova, J.; Vrana, O.; Kelland, L. R.; Brabec, V.; Farrell, N. P. Amide-based prodrugs of spermidine-bridged dinuclear platinum. Synthesis, DNA binding, and biological activity. *J. Med. Chem.* **2008**, *51*, 2254–2260.

(23) Perego, P.; Gatti, L.; Caserini, C.; Supino, R.; Colangelo, D.; Leone, R.; Spinelli, S.; Farrell, N.; Zunino, F. The cellular basis of the efficacy of the trinuclear platinum complex BBR 3464 against cisplatin-resistant cells. *J. Inorg. Biochem.* **1999**, *77*, 59–64.

(24) Manzotti, C.; Pratesi, G.; Menta, E.; Di Domenico, R.; Cavalletti, E.; Fiebig, H. H.; Kelland, L. R.; Farrell, N.; Polizzi, D.; Supino, R.; Pezzoni, G.; Zunino, F. BBR 3464: A Novel Triplatinum Complex, Exhibiting a Preclinical Profile of Antitumor Efficacy Different from Cisplatin. *Clin. Cancer Res.* **2000**, *6*, 2626–2634.

(25) Perego, P.; Caserini, C.; Gatti, L.; Carenini, N.; Romanelli, S.; Supino, R.; Colangelo, D.; Viano, I.; Leone, R.; Spinelli, S.; Pezzoni, G.; Manzotti, C.; Farrell, N.; Zunino, F. A novel trinuclear platinum complex overcomes cisplatin resistance in an osteosarcoma cell system. *Mol. Pharmacol.* **1999**, *55*, 528–534.

(26) Pratesi, G.; Perego, P.; Polizzi, D.; Righetti, S. C.; Supino, R.; Caserini, C.; Manzotti, C.; Giuliani, F. C.; Pezzoni, G.; Tognella, S.; Spinelli, S.; Farrell, N.; Zunino, F. A novel charged trinuclear platinum complex effective against cisplatin-resistant tumours: hypersensitivity of p53-mutant human tumour xenografts. *Br. J. Cancer* **1999**, *80*, 1912–1919.

(27) Sessa, C.; Capri, G.; Gianni, L.; Peccatori, F.; Grasselli, G.; Bauer, J.; Zucchetti, M.; Vigano, L.; Gatti, A.; Minoia, C.; Liati, P.; Van den Bosch, S.; Bernareggi, A.; Camboni, G.; Marsoni, S. Clinical and pharmacological phase I study with accelerated titration design of a daily times five schedule of BBR3464, a novel cationic triplatinum complex. *Ann. Oncol.* **2000**, *11*, 977–983.

(28) Oehlsen, M. E.; Hegmans, A.; Qu, Y.; Farrell, N. Effects of geometric isomerism in dinuclear antitumor platinum complexes on their interactions with N-acetyl-L-methionine. *J. Biol. Inorg. Chem.* **2005**, *10*, 433–442.

(29) Vacchina, V.; Torti, L.; Allievi, C.; Lobinski, R. Sensitive species-specific monitoring of a new triplatinum anti-cancer drug and its potential related compounds in spiked human plasma by cation-exchange HPLC-ICP-MS. *J. Anal. Atom. Spectrom.* **2003**, *18*, 884–890.

(30) Hensing, T. A.; Hanna, N. H.; Gillenwater, H. H.; Gabriella Camboni, M.; Allievi, C.; Socinski, M. A. Phase II study of BBR 3464 as treatment in patients with sensitive or refractory small cell lung cancer. *Anti-Cancer Drugs* **2006**, *17*, 697−704.

(31) Jodrell, D. I.; Evans, T. R. J.; Steward, W.; Cameron, D.; Prendiville, J.; Aschele, C.; Noberasco, C.; Lind, M.; Carmichael, J.; Dobbs, N.; Camboni, G.; Gatti, B.; De Braud, F. Phase II studies of BBR3464, a novel tri-nuclear platinum complex, in patients with gastric or gastro-oesophageal adenocarcinoma. *Eur. J. Cancer* **2004**, *40*, 1872–1877.

(32) Komeda, S.; Moulaei, T.; Woods, K. K.; Chikuma, M.; Farrell, N. P.; Williams, L. D. A third mode of DNA binding: Phosphate clamps by a polynuclear platinum complex. *J. Am. Chem. Soc.* **2006**, *128*, 16092–16103.

(33) Harris, A. L.; Ryan, J. J.; Farrell, N. Biological consequences of trinuclear platinum complexes: comparison of [{trans-PtCl(NH3)(2)}(2)mu-(trans-Pt(NH3)(2)(H2N(CH2)(6)-NH2)(2))](4+) (BBR 3464) with its noncovalent congeners. *Mol. Pharmacol.* **2006**, *69*, 666–672.

(34) Harris, A. L.; Yang, X. H.; Hegmans, A.; Povirk, L.; Ryan, J. J.; Kelland, L.; Farrell, N. P. Synthesis, characterization, and cytotoxicity of a novel highly charged trinuclear platinum compound. Enhancement of cellular uptake with charge. *Inorg. Chem.* **2005**, *44*, 9598–9600.

(35) Montero, E. I.; Benedetti, B. T.; Mangrum, J. B.; Oehlsen, M. J.; Qu, Y.; Farrell, N. P. Pre-association of polynuclear platinum anticancer agents on a protein, human serum albumin. Implications for drug design. *Dalton Trans.* **2007**, 4938–4942.

(36) Charbonneau, D.; Beauregard, M.; Tajmir-Riahi, H.-A. Structural Analysis of Human Serum Albumin Complexes with Cationic Lipids. *J. Phys. Chem. B* **2009**, *113*, 1777–1784.

(37) Qu, Y.; Harris, A.; Hegmans, A.; Petz, A.; Kabolizadeh, P.; Penazova, H.; Farrell, N. Synthesis and DNA conformational changes of non-covalent polynuclear platinum complexes. *J. Inorg. Biochem.* **2004**, *98*, 1591–1598.

(38) Harris, A. L.; Yang, X.; Hegmans, A.; Povirk, L.; Ryan, J. J.; Kelland, L.; Farrell, N. P. Synthesis, Characterization, and Cytotoxicity of a Novel Highly Charged Trinuclear Platinum Compound. Enhancement of Cellular Uptake with Charge. *Inorg. Chem.* **2005**, *44*, 9598–9600.

(39) Kauffman, B., Cowan, D. O. cis- and trans-dichlorodiammine-platinum (II). In *Inorganic Syntheses*; Kleinberg, J., Ed.; 1963; 7, McGraw Hill, New York, pp 239−245.

(40) Yeatman, C. F., 2nd; Jacobs-Helber, S. M.; Mirmonsef, P.; Gillespie, S. R.; Bouton, L. A.; Collins, H. A.; Sawyer, S. T.; Shelburne, C. P.; Ryan, J. J. 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.

(41) Cory, A.; Owen, T.; Barltrop, J.; Cory, J. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Commun.* **1991**, *3*, 207–212.

(42) van Brussel, J. P.; Oomen, M. A.; Vossebeld, P. J.; Wiemer, E. A.; Sonneveld, P.; Mickisch, G. H. Identification of multidrug resistance-associated protein 1 and glutathione as multidrug resistance mechanisms in human prostate cancer cells: chemosensitization with leukotriene D4 antagonists and buthionine sulfoximine. *BJU Int.* **2004**, *93*, 1333–1338.

(43) Moller, C.; Tastesen, H. S.; Gammelgaard, B.; Lambert, I. H.; Sturup, S. Stability, accumulation and cytotoxicity of an albumin-cisplatin adduct. *Metallomics* **2010**, *2*, 811–818.

(44) Oehlsen, M. E.; Hegmans, A.; Yun, Q.; Farrell, N. Effects of geometric isomerism in dinuclear antitumor platinum complexes on their interactions with N-acetyl-L-methionine. *J. Biol. Inorg. Chem.* **2005**, *10*, 433–442.

(45) Klein, A. V.; Hambley, T. W. Platinum Drug Distribution in Cancer Cells and Tumors. *Chem. Rev.* **2009**, *109*, 4911–4920.

(46) Yumoto, R.; Nishikawa, H.; Okamoto, M.; Katayama, H.; Nagai, J.; Takano, M. Clathrin-mediated endocytosis of FITC-albumin in alveolar type II epithelial cell line RLE-6TN. *Am. J. Physiol.* **2006**, *290*, L946–955.

(47) Daniel, W. A.; Wójcikowski, J. Lysosomal trapping as an important mechanism involved in the cellular distribution of perazine and in pharmacokinetic interaction with antidepressants. *Eur. Neuropsychopharmacol.* **1999**, *9*, 483–491.

(48) Hayeshi, R.; Masimirembwa, C.; Mukanganyama, S.; Ungell, A.-L. B. Lysosomal trapping of amodiaquine: impact on transport across intestinal epithelia models. *Biopharm. Drug Dispos.* **2008**, *29*, 324–334.

(49) Curry, S.; Mandelkow, H.; Brick, P.; Franks, N. Crystal structure of human serum albumin complexed with fatty acid reveals an asymmetric distribution of binding sites. *Nat. Struct. Mol. Biol.* **1998**, *5*, 827–835.

(50) Petitpas, I.; Grüne, T.; Bhattacharya, A. A.; Curry, S. Crystal structures of human serum albumin complexed with monounsaturated and polyunsaturated fatty acids. *J. Mol. Biol.* **2001**, *314*, 955–960.