Inorg. Chem. **2005**, *44*, 9598–9600

Synthesis, Characterization, and Cytotoxicity of a Novel Highly Charged Trinuclear Platinum Compound. Enhancement of Cellular Uptake with Charge

Amanda L. Harris,[†] Xiaohong Yang,[†] Alex Hegmans,[†] Lawrence Povirk,[‡] John J. Ryan,[§] Lloyd Kelland,[||] and Nicholas P. Farrell*,[†]

Chemistry Department, Virginia Commonwealth University, 1001 W. Main Street, Richmond, Virginia 23284-2006, Biology Department, Virginia Commonwealth University, 1000 W. Cary Street, Richmond, Virginia 23284-2006, Pharmacology Toxicology Department School of Medicine, Virginia Commonwealth University, Richmond, Virginia 23284-2006, and The Institute of Cancer Research, Sutton, Surrey, U.K.

Received August 15, 2005

Charge delocalization (6+ to 8+) in "noncovalent" linear trinuclear platinum complexes produces compounds with cytotoxicity in some cases equivalent to cisplatin. The cellular uptake of a novel 8+ compound is greater than that of neutral cisplatin as well as other multinuclear Pt compounds.

The phase II clinical drug BBR 3464 (1,0,1/t,t,t) is a highly cytotoxic and antitumor-active trinuclear platinum compound that appears to exert these effects through its DNA binding.[1] The frequency and structure of Pt-DNA adducts are distinct from those formed by cisplatin and its congeners. The cellular uptake of BBR 3464 is also significantly higher than that of cisplatin in A2780 and SKOV-3 cells.[2] The early structure−activity relationships for platinum antitumor drugs emphasized the need for neutral compounds. Thus, novel structural classes, such as the charged polynuclear series, challenge not only the paradigm of DNA binding but also other critical pharmacological factors such as cellular accumulation.[3]

The DNA binding of compounds such as BBR 3464 is covalent, but a significant "noncovalent" component arises from the presence of the central platinum(tetraamine) unit, which interacts with DNA only through electrostatic and hydrogen-bonding effects. Studies on the kinetics of interstrand cross-link formation by BBR 3464 in the duplex $(ATATGTACATAT)_2$ by $\{^1H,^{15}N\}$ HSQC NMR spectroscopy showed perturbations of the central AT pairs consistent with preassociation in the minor groove.[4] Interstrand cross-links formed by BBR 3464 occur in both 5′ → 5′ and 3′ → 3′ directions.[5] The extent of preassociation affects the structure (direction) of the cross-link as well as the kinetics of formation. The solution structure of the 1,4-interstrand cross-link formed by BBR 3464 in the 8-mer, $d(5'\text{-ATG-TACAT-}3')_2$, also showed contacts between the central platinum unit and AT protons residing in the minor groove.[6]

To examine the effects of electrostatic and hydrogen bonding in the absence of covalent Pt-DNA bond formation, the noncovalent analogue of BBR 3464 (**I**, Figure 1) has been synthesized.[7] The highly charged compound induces B → A and B → Z conformational changes in canonical sequences of DNA. The binding affinity is sufficiently high such that they are not readily displaced from the helix by intercalators such as ethidium bromide, resulting in an essential irreversibility of the conformational change.[7] The interaction with DNA of the minor-groove binding dye Hoechst 33258 is cooperatively enhanced in the presence of the charged compound.[8] In this paper, we show how further extension of the noncovalent concept by use of dangling amines to increase charge and charge dispersion along these linear cations surprisingly results in significantly enhanced cellular accumulation, even for compounds with a formal charge of 8+. A consequence of this accumulation is enhanced cytotoxicity, despite the reversible nature of DNA binding (Figure 1).

* To whom correspondence should be addressed. E-mail: nfarrell@mail1.vcu.edu.
[†] Chemistry Department, Virginia Commonwealth University.
[‡] Pharmacology Toxicology Department School of Medicine, Virginia Commonwealth University.
[§] Biology Department, Virginia Commonwealth University.
[||] The Institute of Cancer Research.

(1) Farrell, N. *Met. Ions Biol. Syst.* **2004**, *42*, 251−296.
(2) Manzotti, C.; Pratesi, G.; Menta, E.; Di Domenico, R.; Fiebig, H. H.; Kelland, L. R.; Farrell, N.; Polizzi, D.; Supino, R.; Pezzoni, G.; Zunino, F. *Clin. Cancer Res.* **2000**, *6*, 2626−2634.
(3) Farrell, N. P. *Semin. Oncol.* **2004**, *31*, 1−9.
(4) Hegmans, A.; Berners-Price, S. J.; Davies, M. S.; Thomas, D. S.; Humphreys, A. S.; Farrell, N. *J. Am. Chem. Soc.* **2004**, *126*, 2166−2180.
(5) Kasparkova, J.; Zehnulova, J.; Farrell, N.; Brabec, V. *J. Biol. Chem.* **2002**, *277*, 48076−48086.
(6) Qu, Y.; Scarsdale, N. J.; Tran, M.; Farrell, N. P. *J. Biol. Inorg. Chem.* **2003**, *8*, 19−28.
(7) Qu, Y.; Harris, A.; Hegmans, A.; Kabolizadeh, P.; Penazova, H.; Farrell, N. *J. Inorg. Biochem.* **2004**, *98*, 1591−1598.
(8) Harris, A.; Qu, Y.; Farrell, N. P. *Inorg. Chem.* **2005**, *44*, 1196−1198.

10.1021/ic051390z CCC: $30.25
Published on Web 11/30/2005

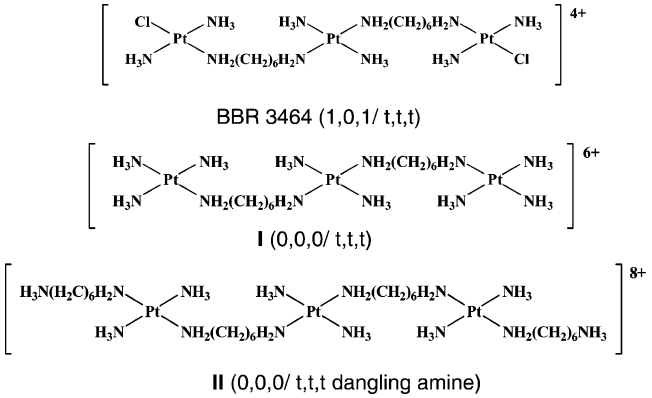
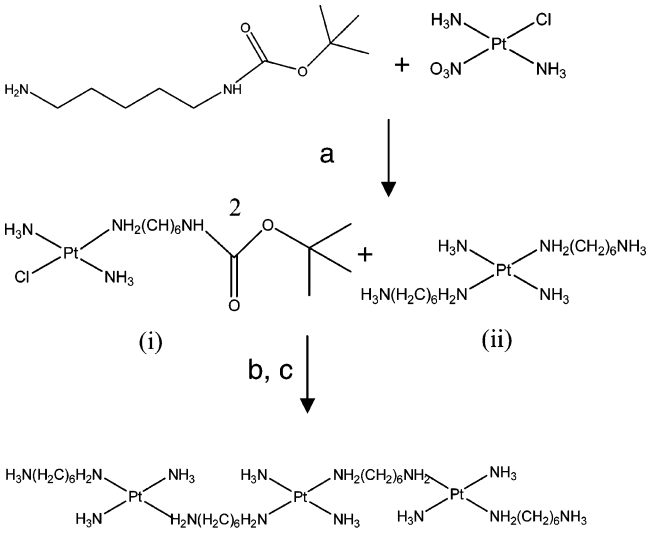


Figure 1. Stuctures of multinuclear Pt compounds.

Scheme 1. Synthetic Pathway Reagents and Conditions[a]



[a] Monoactivated transplatin was produced by stirring transplatin with $AgNO_3$ overnight in DMF at room temperature in the dark. AgCl was removed by filtration. (a) The Boc-protected hexanediamine was then stirred with the monoactivated transplatin for 3 h at −20 °C in DMF and then 1 h at room temperature. (b) A total of 2 equiv of **i** was stirred overnight in DMF with $AgNO_3$. AgCl was removed and **ii** added in DMF with NaOH in MeOH and stirred for 48 h. (c) The deprotection was accomplished by dissolving the compound in MeOH, adding HCl in EtOH, and then stirring for 72 h.

The synthesis of **I**, where the bridging unit is the *trans*-$[Pt(NH_3)_2\{NH_2(CH_2)_6NH_2\}_2]$ unit, and its dinuclear spermine and spermidine-linked congeners have been reported.[7] The synthesis of **II** is illustrated in Scheme 1. The central coordination sphere of a trinuclear compound is generally inequivalent to the terminal ones, and the preparation follows the route

$$2Pt(t) + 1Pt(c) \rightarrow Pt(t)-Pt(c)-Pt(t)$$

Specifically for **II**, hexanediamine selectively protected at one amine end is allowed to react with monoactivated transplatin (step a). The product **i** serves as the terminal precursor and was then allowed to react with the "central" portion, *trans*-$[Pt(NH_3)_2\{H_2N(CH_2)_6NH_3\}_2]^{4+}$ (**ii**),[9] to produce the trinuclear product (step b), which upon deprotection with methanolic HCl affords **II**. The product was then converted to the nitrate salt by reaction with $AgNO_3$ for 2 h. Elemental analysis, ^{1}H NMR, and reversed-phase high-performance liquid chromatography (HPLC) confirmed the product and purity.[10]

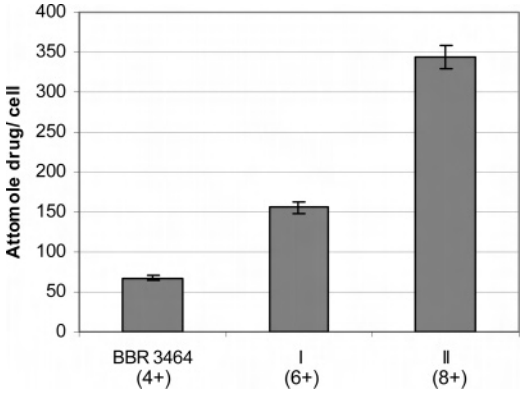


Figure 2. Uptake into A2780 human ovarian tumor cells. A total of 5 million cells were incubated in 10 μM **I**, **II**, or BBR 3464 for 6 h. Samples were then trypsinized to remove cells from the plate, washed three times with PBS, heated in nitric acid followed by the addition of H_2O_2 and HCl, and analyzed by inductively coupled plasma (ICP) optical emission spectroscopy. Full details are given in the Supporting Information. Compound **II** shows a much greater uptake than BBR 3464 despite its higher charge. Each bar represents the average of six samples collected in two separate experiments, and error bars reflect the standard errors. Cisplatin uptake was below the ICP limit of detection under these conditions.

Table 1. 96-h IC_{50} (μM) Cytotoxicity (Resistance Factors in Parentheses) in Ovarian Carcinoma Cell Lines (Sulforhodamine B Assay)[11,13]

	A2780	A2780 cisR	CH1	CH1 cisR	SKOV-3
I	41.0	23.0 (0.56)	28.0	56.0 (2.0)	>100
II	4.3	1.6 (0.37)	3.7	7.4 (2.0)	2.6
BBR 3464	0.048	0.355 (7.4)	0.016	0.019 (1.2)	0.25
c-DDP	0.76	4.2 (5.5)	0.15	0.425 (2.8)	2.4

The major pharmacological parameters of cytotoxicity in platinum compounds are the frequency and nature of DNA adducts, the extent of metabolic inactivation by sulfur-containing biomolecules such as human serum albumin and glutathione, and the efficiency of cellular platinum uptake. Surprisingly, the noncovalent compounds display even higher cellular uptake and, remarkably, **II** with a higher charge (8+) has about 5 times greater cellular uptake than the "parent" BBR 3464 (4+) (Figure 2). Further, uptake is rapid and is significant even at the relatively early time point of 6 h.

The consequences of such high uptake may be seen by comparing the cytotoxicity of **I** and **II**. The results of cytotoxicity tests across a panel of ovarian tumor cell lines selected for sensitivity, as well as intrinsic and acquired resistance to cisplatin, are shown in Table 1. Incorporation of the dangling amine into the chemotype produces a significant increase in cytotoxicity of **II** over that of the 6+ compound (**I**) for all cell lines tested. The *enhancement* of cytoxicity with the addition of the dangling amine is also noted for the dinuclear spermine- and spermidine-linked analogues, but the effect is most significant for the trinuclear species (data not shown). The cytotoxicity of noncovalent

(9) Da Re, G.; Di Domenico, R.; Spinelli, S.; Farrell, N. U.S. Patent 6,-313,333, 2001.

(10) For **II**:^{1}H NMR δ 3.00 (t), 2.67 (m), 1.65 (m), 1.38 (m) ppm; HPLC purity 97%. Elem Anal. Found (calcd): C, 16.84 (16.91); H, 5.12 (5.32); N, 17.93 (18.08).

compounds is clearly not as high as that of BBR 3464, but the cytotoxicity of **II** is comparable to that of cisplatin in some cell lines. The lack of resistance in the p53 null SKOV-3 cell line suggests that the cytotoxicity of **II** may be p53-independent. The vast majority of mononuclear DNA-binding platinum agents display cytotoxicity in the micromolar range,[11] and thus the low micromolar range attained for **II** is remarkable given the charge and noncovalent nature of the compound.

It is notable that the resistance factors in A2780cisR cells are less than 1 for both **I** and **II**, an unusual result even for structurally novel Pt complexes (cf. BBR 3464).[2,12] Increased glutathione expression is indicated as a major contributing factor to the resistance in this cell line.[13] The trinuclear structure of BBR 3464 and dinuclear analogues is rapidly degraded in the presence of glutathione.[14,15] ^{1}H NMR studies of **I** in the presence of glutathione showed no breakdown of the trinuclear structure even after 24 h, indicating little thiol reactivity in the absence of substitution-labile Pt−Cl bonds.

The general properties of DNA binding as elucidated previously for **I**[7]—conformational changes, cooperative binding of the minor-groove binder—are shared by the new agent, with **II** being effective at concentrations lower than those published for the previously reported compound. It can displace ethidium bromide about 30% more effectively than **I** at equivalent ratios of compound to DNA. Cationic noncovalent platinum compounds have been shown to associate in the minor groove by spectroscopic (NMR) and molecular biology techniques.[16] Interestingly, the single-crystal X-ray diffraction structure of **II** in the presence of the Drew−Dickerson 12-mer d(5′-CGCGAATTCGCG-3′)$_2$ shows interaction of **II** along the phosphate backbone of the DNA.[17] In solution, and in agreement with the observations on BBR 3464, the ^{1}H NMR spectrum of **I** in the presence of the 10-mer d(5′-GGTAATTACC-3′)$_2$ shows nuclear Overhauser effect contacts between the central platinum unit and the central AATT base pairs.[18] These results suggest some association of **II** in the DNA minor groove, also confirmed by an adaptation of a melphalan competition assay.[19,20] Approaches to platinum-based minor-groove binding agents have included conjugation of known minor-groove DNA binding ligands and intercalators into discrete platinum complexes as well as noncovalent dinuclear and trinuclear complexes using the bridging 4,4′-dipyrazolylmethane ligand.[21] In comparison to these latter examples, the IC_{50} values for **II** are noteworthy and indeed, in some cases, also similar to those reported in ovarian cancer cells for putative minor-groove platinum binding agents based on acridine.[22] The mechanism of the remarkable cellular uptake of **II** may help contribute to the understanding of cellular accumulation of platinum complexes in general and its clinical relevance. Alterations in cellular accumulation (intake/efflux) are of clinical relevance in cisplatin resistance.[3] Recently, considerable interest has focused on the relationship of Cu transporters to Pt accumulation. In the present case, polyspecific organic cation transporters[23] are possible candidates for drug binding. Further study of the details of the remarkable cellular accumulation of **II** will contribute to understanding the diversity of heavy-metal ion uptake. Likewise, the electrostatic/hydrogen-bonding motifs may result in both backbone and minor-groove binding of DNA different in consequences from covalently binding agents. The results further underscore the importance of noncovalent interactions in determining the factors responsible for the biological activity of this discrete class of antitumor agents.

Acknowledgment. This work has been supported by an operating grant from The National Institutes of Health (Grant CA-78754). The Massey Cancer Center is gratefully acknowledged for support of X.Y. The authors would also like to thank Atilio Anzellotti, Genevieve Bulluss, and John Bradley Mangrum for the preparation of the cover graphic.

Supporting Information Available: Experimental synthesis and ICP-OES information for the central portion *trans*-[Pt(NH$_3$)$_2$\{H$_2$N(CH$_2$)$_6$NH$_3$\}$_2$]Cl$_2$ and ends *trans*-[PtCl(NH$_3$)$_2$NH$_2$-(CH$_2$)$_6$NHBOC]NO$_3$. This material is available free of charge via the Internet at http://pubs.acs.org.

IC051390Z

(11) Fojo, T.; Farrell, N.; Ortuzar, W.; Tanimura, H.; Weinstein, J.; Myers, T. G. *Crit. Rev. Oncol./Hematol.* **2005**, *53*, 25−34.
(12) Farrell, N. Polynuclear Charged Platinum Compounds as a New Class of Anticancer Agents. Toward a New Paradigm. In *Platinum-Based Drugs in Cancer Therapy*; Kelland, L. R., Farrell, N., Eds.; Humana Press: Totowa, NJ, 2000; pp 321−338.
(13) Hills, C. A.; Kelland, L. R.; Abel, G.; Siracky, J.; Wilson, A. P.; Harrap, K. R. *Br. J. Cancer* **1989**, *59*, 527−534.
(14) Oehlsen, M.; Qu, Y.; Farrell, N. *Inorg. Chem.* **2003**, *42*, 5498−5506.
(15) Jansen, B. A.; Brouwer, J.; Reedijk, J. *J. Inorg. Biochem.* **2002**, *89*, 197−202.
(16) Collins, J. G.; Wheate, N. J. *J. Inorg. Biochem.* **2004**, *98*, 1578−1584 and references cited therein.
(17) Komeda, S.; Sato, T.; Chikuma, M.; Farrell, N. P.; Williams, L. D. Crystal structures of d(CGCGAATTCGCG)$_2$ cocrystallized with multinuclear platinum(II) complexes: Noncovalent interactions between DNA and potential anticancer drugs. Poster Presentation at European Biological Inorganic Chemistry Conference, Garmisch−Partenkirchen, Germany, 2004.
(18) Harris, A.; Qu, Y.; Yang, X.; Povirk, L.; Farrell, N. Unpublished results, 2005.
(19) Harris, A. Dissertation, Virginia Commonwealth University, Richmond, VA, 2005.
(20) Wang, P.; Bauer, G.; Bennet, R.; Povirk, L. *Biochemistry* **1991**, *30*, 11515−11521.
(21) Wheate, N. J.; Webster, L. K.; Brodie, C. R.; Collins, J. G. *Anti-Cancer Drug Des.* **2000**, *15*, 313−322.
(22) Martins, E. T.; Baruah, H.; Kramarczyk, J.; Saluta, G.; Day, C. S.; Kucera, G. L.; Bierbach, U. *J. Med. Chem.* **2001**, *44*, 4492−4496.
(23) Burckhardt, G. *Mol. Pharmacol.* **2005**, *67*, 1391−1392.