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Biological Consequences of Trinuclear Platinum Complexes: Comparison of [{*trans*-PtCl(NH_3)$_2$}$_2\mu$-(*trans*-Pt(NH_3)$_2$($H_2N(CH_2)_6NH_2$)$_2$)]$^{4+}$ (BBR 3464) with Its Noncovalent Congeners

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ABSTRACT

[{*trans*-PtCl(NH_3)$_2$}$_2\mu$-(*trans*-Pt(NH_3)$_2$($H_2N(CH_2)_6NH_2$)$_2$)]$^{4+}$ (BBR 3464) is a 4+ cationic trinuclear platinum drug that has undergone phase II clinical trials in the treatment of ovarian and lung cancers. The chemical structure of BBR 3464 is distinct from that of clinically used agents such as cisplatin and oxaliplatin. As a consequence, the modes of DNA binding and the structures of BBR 3464 DNA adducts are also structurally distinct from those formed by cisplatin and oxaliplatin. Previous chemical and spectroscopic measurements on BBR 3464 had elucidated a significant noncovalent contribution to DNA binding. To examine this effect further, the biological activity of two BBR 3464 analogs that bind DNA only through noncovalent interactions was investigated in this study, and their cellular effects were compared with those caused by the "parent" drug. The compounds were [{*trans*-PtL(NH_3)$_2$}$_2\mu$-(*trans*-Pt(NH_3)$_2$($H_2N(CH_2)_6NH_2$)$_2$)]$^{n+}$, with L = NH_3, n = 6 for compound **I,** and L = $H_2N(CH_2)_6NH_3$, n = 8 for compound **II**. All compounds induce caspase-dependent apoptosis in both primary mast cells and transformed mastocytomas, although with a smaller IC_{50} value in the transformed cells. In cells deficient in either the tumor suppressor proteins p53 or Bax, apoptosis was least affected in the case of **II,** but in all cases the effect of p53 deficiency was greater than that of Bax. Surprisingly, cellular uptake was actually enhanced for the more highly charged compounds, resulting in significant (micromolar) cytotoxicity for **II**. Cellular accumulation was enhanced in mastocytomas over primary mast cells, suggesting a mechanism for enhancement of tumor cell selectivity.

The phase II chemotherapeutic drug BBR 3464 is a trinuclear platinum compound whose presumed mechanism of action arises from covalent binding to DNA (Farrell, 2004). Because of the high positive charge, its DNA binding has been shown to have a significant noncovalent (preassociation) component that influences the binding kinetics as well as the structure of the final adducts (Qu et al., 2003; Hegmans et al., 2004). Because the structure of the DNA adducts determines repair, protein recognition, and other downstream cellular events, an understanding of their formation and biological consequences is essential to further drug development. To investigate the preassociation with DNA, a series of compounds that contain an inert ammonia or amine group instead of the chloride were synthesized, compounds **I** and **II** (Fig. 1) (Qu et al., 2004; Harris et al., 2005a). These compounds cannot covalently bind guanine residues as traditional platinum agents do. They still associate with DNA with high affinity, however, producing B-to-A and B-to-Z transitions in susceptible sequences at concentrations lower than those required by cobalt hexammine (Qu et al., 2004). **I** and **II** displace ethidium bromide from DNA and increase the binding affinity for Hoechst dye in an adenine/thymine-rich 12-mer (Harris et al., 2005b). These noncovalent compounds have also been shown to associate with the adenine/thymine regions of the DNA minor groove (Harris et al., 2005a).

Charge dispersion along the molecule as in **II** not only enhances the strength of the DNA interaction, but also results in enhanced cellular uptake in an ovarian tumor cell line (A2780), contributing, in part, to an enhanced cytotoxicity of **II** compared with **I.** The cytotoxicity of compound **II** in A2780 cells is in the 1 to 10 μM range, lower than that of BBR 3464 but similar to cisplatin (c-DDP) (Harris et al., 2005a). Deactivation by sulfur-containing proteins, such as glutathione, is a critical factor in the pharmacology of covalently

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ABBREVIATIONS: BBR 3464, [{*trans*-PtCl(NH_3)$_2$}$_2\mu$-(*trans*-Pt(NH_3)$_2$($H_2N(CH_2)_6NH_2$)$_2$)]$^{4+}$; c-DDP, cisplatin; BMMC, bone marrow mast cell; KO, knockout; PBS, phosphate-buffered saline; PI, propidium iodide; IL, interleukin; **I**, [{*trans*-Pt NH_3(NH_3)$_2$}$_2\mu$-(*trans*-Pt(NH_3)$_2$($H_2N(CH_2)_6NH_2$)$_2$)]$^{6+}$; **II**, [{*trans*-Pt$H_2N(CH_2)_6NH_3$(NH_3)$_2$}$_2\mu$-(*trans*-Pt(NH_3)$_2$($H_2N(CH_2)_6NH_2$)$_2$)]$^{8+}$; Q-VD-OPH, *N*-(2-quinolyl)-L-valyl-L-aspartyl-(2,6,-difluorophenoxy) methylketone; SCF, stem cell factor.

binding drugs such as c-DDP and BBR 3464 (Oehlsen et al., 2003). It is possible that noncovalent compounds can circumvent the pharmacokinetic problems associated with sulfur deactivation. These considerations make the cellular pharmacology of **I** and **II** interesting for study in their own right (Harris et al., 2005a). Furthermore, the sum of the pharmacological factors affecting cytotoxicity of platinum agents, structure and frequency of target (DNA) adducts; cellular uptake and efflux; and metabolic deactivation by sulfur nucleophiles also combine to affect signaling pathways, leading to apoptosis (Siddik, 2003). It was therefore of interest to examine the similarities and/or differences in cellular effects of compounds closely related in structure, such as BBR 3464 and **I** and **II,** which have distinct profiles of DNA and protein binding.

In this study, we investigated the apoptotic and cellular effects of the noncovalent compounds **I** and **II** in comparison with BBR 3464 and c-DDP using a mouse mast cell system. Study of these analogs of BBR 3464 will increase our understanding of the role that noncovalent forces play in the action of BBR 3464, assisting in the design of next-generation chemotherapeutics. The in vitro system using primary mast cells was chosen for its ability to mimic the factor-dependent and polyclonal nature of the in vivo environment. Comparison between primary and transformed mast cell populations is also reported. The results indicate that cellular signaling pathways to apoptosis are affected by compound structure.

Materials and Methods

Compound Synthesis. Drug compounds were synthesized using methods reported previously (Qu et al., 2004; Harris et al., 2005a).

Cell System. Bone marrow mast cells were extracted from the femurs and tibias of C57BL/6 × 129 mice (wild type; obtained from Taconic Farms, Germantown, NY) and p53- or Bax-deficient mice (The Jackson Laboratory, Bar Harbor, ME) according to methods published previously. Cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate (all from Biofluids, Rockville, MD) and either 20% WEHI 3B cell-conditioned media or IL-3 (5 ng/ml) and SCF (50 ng/ml). Cells were allowed to mature 30 days before use in experiments. Mast cell phenotype was confirmed by the expression of FcεRI and Kit by flow cytometry.

Cytotoxicity Assays. Cells were plated in 96-well plates at 3.0×10^5 cells/ml. Drug or a vehicle (H_2O) control was added to each well. IL-3 or IL-3 plus SCF was added as specified.

Propidium Iodide DNA Staining. 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 (Yeatman et al., 2000). Samples were then analyzed for subdiploid DNA on a Becton Dickinson FACScan flow cytometer (BD Biosciences, San Jose, CA). 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, we were able to detect intact versus fragmented DNA, revealing discrete stages of the cell cycle and the percentage of the population undergoing apoptosis.

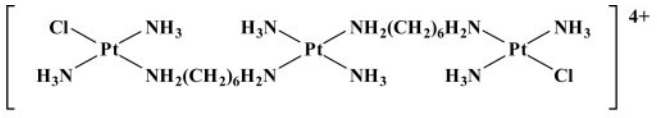
Caspase Activation Assays. Caspase staining for active caspases was performed using caspase kits (Immunochemistry Technologies, LLC, Bloomington, MN), as specified by the manufacturer.

Caspase Inhibition Study. Pan-caspase inhibitor *N*-(2-quinolyl)-L-valyl-L-aspartyl-(2,6,-difluorophenoxy) methylketone (Q-VD-OPH) was obtained from Axxora Life Sciences, Inc. (San Diego, CA). Solid was dissolved to make a 10 mM stock solution in dimethyl sulfoxide, which was then diluted with PBS for use in culture. The final concentration of Q-VD-OPH in culture was 25 μM. Samples were analyzed at 24 and 48 h for caspase 3 activation and DNA damage by PI DNA staining.

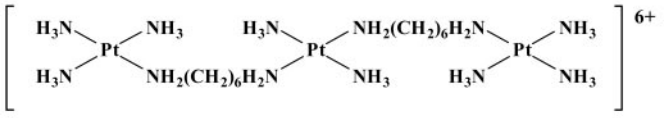
Platinum Compound Uptake. Mast cell tumor lines (either P815 or PDMC-1) were plated at 5.0×10^5 cells/ml. Compounds **I** and **II**, BBR 3464, or c-DDP was added to give a concentration of 10 μM drug in culture. After 2 or 6 h, 5.0×10^6 cells were harvested from each condition and washed twice with PBS. The cell pellets were then heated in nitric acid followed by the addition of hydrogen peroxide and hydrochloric acid, according to the United States Environmental Protection Agency procedure 3050b (all volumes reduced by 1/10) and diluted with Milli-Q water (Millipore Corporation, Billerica, MA). Platinum analysis was performed on a Vista-MPX charge-coupled device simultaneous inductively coupled plasma optical emission spectroscopy at 265 nm (Varian Inc., Palo Alto, CA). Standards and blank were prepared the same as the sample.

Results

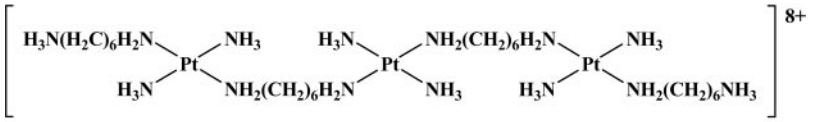
To determine the cytotoxicity of trinuclear platinum compounds that bind DNA via noncovalent interactions, apoptosis was measured. Primary mast cells were cultured for 72 h in the presence or absence of **I**, **II**, BBR 3464, or c-DDP, and DNA



BBR 3464



I



II

Fig. 1. Structures of trinuclear platinum complexes.

fragmentation was measured by PI DNA staining of fixed cells treated with RNase A. **I** and **II** were shown to be cytotoxic in BMMC cultures, as shown in Fig. 2A. Whereas the IC_{50} values of the noncovalent compounds were more than 100 times greater than that of BBR 3464, they were similar to that of cisplatin, with IC_{50} values in the micromolar range. When the cytotoxicity was assayed over time at doses that were approximately the IC_{90} value, **II** showed very rapid death-inducing kinetics, despite its 8+ charge (Fig. 2B).

Platinum uptake was then measured in primary BMMC cultures (Fig. 3). BBR 3464 and **I** showed very similar uptake, whereas **II**, despite the 8+ charge, was taken up by cells at levels more than 2-fold greater than the other compounds. The uptake is consistent with the more rapid killing by **II** of target cells (Fig. 2B). These data are consistent with uptake in A2780 ovarian tumor cells in which **II** also has a much higher uptake than **I** or BBR 3464, although the overall intracellular levels of these compounds are much lower in primary cells than in A2780 cells (Harris et al., 2005a). Cisplatin data are not shown, because under these conditions, the amount of cisplatin taken into cells was lower than the limit of quantitation for the instrument. For the two noncovalent compounds, increasing charge is in fact correlated with increased uptake. This is particularly interesting, because the structure-activity relationship used for many years to develop cisplatin analogs dictated that platinum compounds had to be neutral to be taken up by cells (Jamieson and Lippard, 1999; Andrews, 2000; Kartalou and Essigmann, 2001).

Platinum-DNA adducts induce a cellular signaling response regulating survival and apoptosis. Given the difference in DNA binding profile and cellular uptake between covalent and noncovalent compounds, it was important to examine whether these features are reflected in differences in the downstream events induced. Cytotoxicity was therefore measured in gene-targeted primary BMMC in an attempt to determine the mechanism by which apoptosis occurred (Fig. 4). Of the many proteins that are implicated in the apoptotic pathway of c-DDP, two that are frequently associated with malignancy are p53, a proapoptotic transcription factor, and Bax, which is also a proapoptotic protein that destabilizes the mitochondrial membrane (Siddik, 2003). p53 is also an interesting candidate, because in addition to playing a role in c-DDP-mediated apoptosis, it is mutated in approximately 50% of human tumors (O' Connor et al., 1997). This is consistent with the cytotoxicity of c-DDP across the NCI tumor panel, which seems to be p53-dependent (O'Connor et al., 1997). p53- or Bax-deficient (KO) cells were cultured with or without each platinum compound. The absence of p53 or Bax diminished the apoptosis induced by c-DDP, BBR 3464, and **I**, indicating that these proteins are involved in platinum-induced apoptosis. In all cases, the effect of p53 deficiency was more pronounced than that of Bax deficiency. **II**-mediated apoptosis was least affected by either p53 or Bax mutations. Bax deficiency had no significant effect on **II**-induced death. The effect of p53 deficiency on **II**-induced apoptosis was statistically significant at 5 μM but was still much less than noted with the other platinum compounds.

p53 activation and its up-regulation of Bax expression leads to mitochondrial damage. The mitochondrial pathway to apoptosis results in activation of the caspases-9 and -3 (Danial and Korsmeyer, 2004). To determine whether treatment with platinum compounds activated these enzymes, we measured caspase-9 and caspase-3 activity after platinum treatment using a fluorometric assay (Fig. 5). Both caspases were activated with kinetics very similar to the PI DNA staining results.

To further assess the importance of caspase function in platinum-induced apoptosis, the pan-caspase inhibitor Q-VD-OPH was added to the cultures. Caspase activation was reduced by the presence of the caspase inhibitor, as expected (Fig. 6A). The decrease in caspase function directly

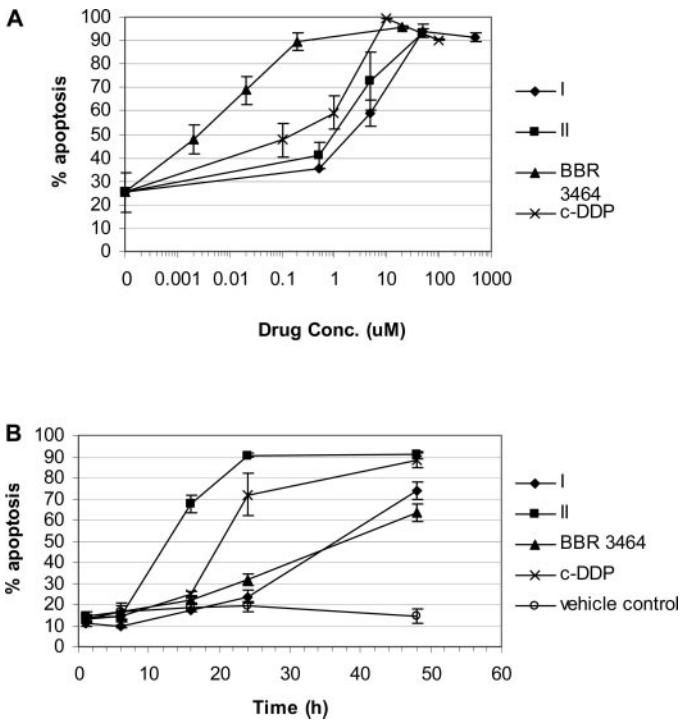


Fig. 2. Cytotoxicity of platinum drugs in BMMC, shown in A as a function of dose at 72 h. In B, the concentration of drug that led to 90% cytotoxicity at 72 h (500 μM **I**, 50 μM **II**, 10 μM c-DDP, and 1 μM BBR 3464) was followed over time. **II** shows remarkably rapid kinetics. Each point represents the average of six samples tested in at least two independent populations. Standard error measurements are shown.

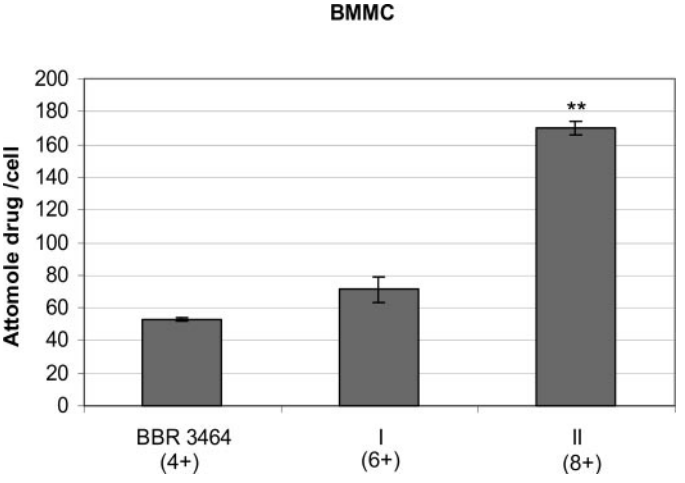


Fig. 3. Platinum uptake in mast cells. Graphs show the uptake of platinum compounds into BMMC after 6 h. Each bar represents the average of three samples, and standard errors are shown.

correlated with decreased platinum-mediated apoptosis, as demonstrated by PI DNA staining (Fig. 6B). These data clearly showed that apoptosis was dependent on caspase function. The results confirm that cell death occurs via apoptosis rather than necrosis (Gonzalez et al., 2001) and supports a role for the mitochondrion and caspases in platinum-mediated cell death.

An important parameter of potential therapeutic index and efficacy of antitumor compounds is cytotoxic differentiation between "normal" (in this case, primary) and transformed cells. For this reason, the platinum compounds were tested in two mastocytoma cell lines, PDMC and P815. It is noteworthy that all four platinum compounds induced apoptosis in these mastocytoma cell lines (Fig. 7, A and B). Furthermore, the IC_{50} value for apoptosis induced by **II** in mastocytomas was 10 times lower than in the primary mast cells. In contrast, the IC_{50} value for c-DDP in both tumor cell lines was essentially identical with the primary cells (Figs. 2 and 7). This significant enhancement of cytotoxicity in the tumor cell lines also produces the interesting result that, whereas BBR 3464 remained the most potent compound, **II** now was more active than c-DDP in apoptosis induction in both the tumor cell lines, as assessed by PI DNA staining. Table 1 summarizes IC_{50} values for apoptosis in both the primary BMMC and tumor P815 and PDMC cells (data from Figs. 2 and 7) in which the "promotion" of the relative cytotoxicity of compound **II,** especially compared with c-DDP, is readily apparent.

To determine the nature of this cytotoxic enhancement, platinum uptake was measured in the two mastocytoma tumor cell lines. In both P815 and PDMC-1 cells, the platinum loading of **II** was three to five times higher (depending on the time and cell line) than that of the other two compounds (Fig. 8). The platinum levels of all three compounds were significantly greater than in the primary mast cells (Fig. 3) at the same administered dose. Once again, under these experimental conditions, the amount of platinum recovered from the cisplatin samples was lower than the limit of quantitation for the inductively coupled plasma atomic emission spectroscopy, so no data are shown.

Discussion

Whereas platinum-based chemotherapy has largely been derived from the theory that covalent interactions are essential to cytotoxicity, the highly charged compound **II** demonstrates cytotoxicity in the micromolar range, similar to cisplatin and analogs. In all cases, BBR 3464 was the most toxic compound, as predicted from published data. The results in mast cells confirm and expand on the preliminary results with A2780 cells, in which the presence of the dangling amine and the increased charge (8+) in **II** were shown to significantly enhance cytotoxicity over the 6+ analog **I** (Harris et al., 2005a). Furthermore, **II** proved to be quite toxic to the tumor cells, with IC_{50} values that were approximately 10-fold lower than those observed with primary mast cells.

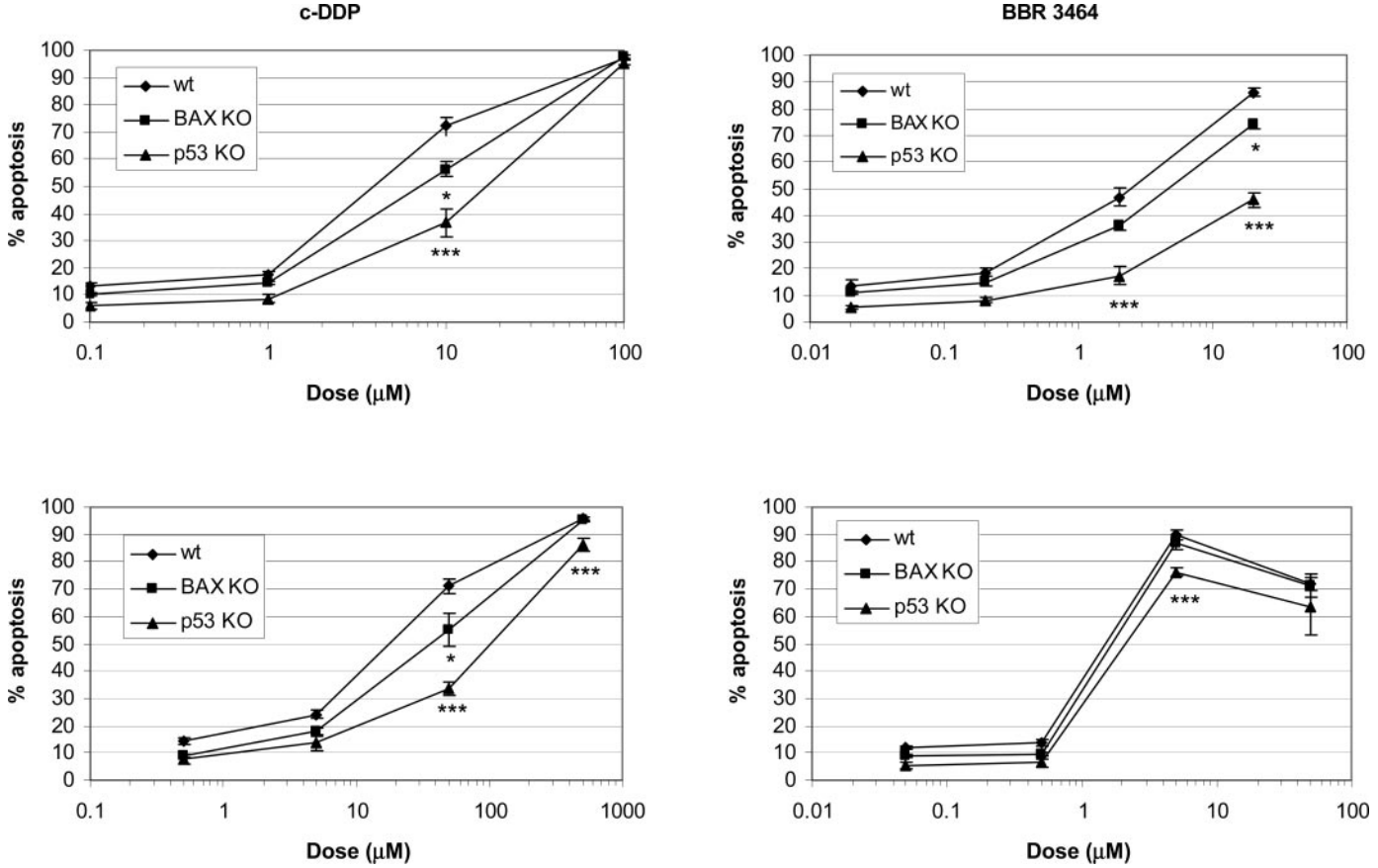


Fig. 4. The effect of p53 or Bax deficiency on platinum-induced apoptosis. Wild type (wt), p53 KO, or Bax KO BMMC were cultured for 72 h in IL-3 + SCF in the presence of the indicated concentrations of each platinum compound. Drug concentrations are the same as in Fig. 1B. Apoptosis was measured by PI DNA staining to detect subdiploid DNA content. Each point represents the average of six samples in three independent experiments with standard errors shown.

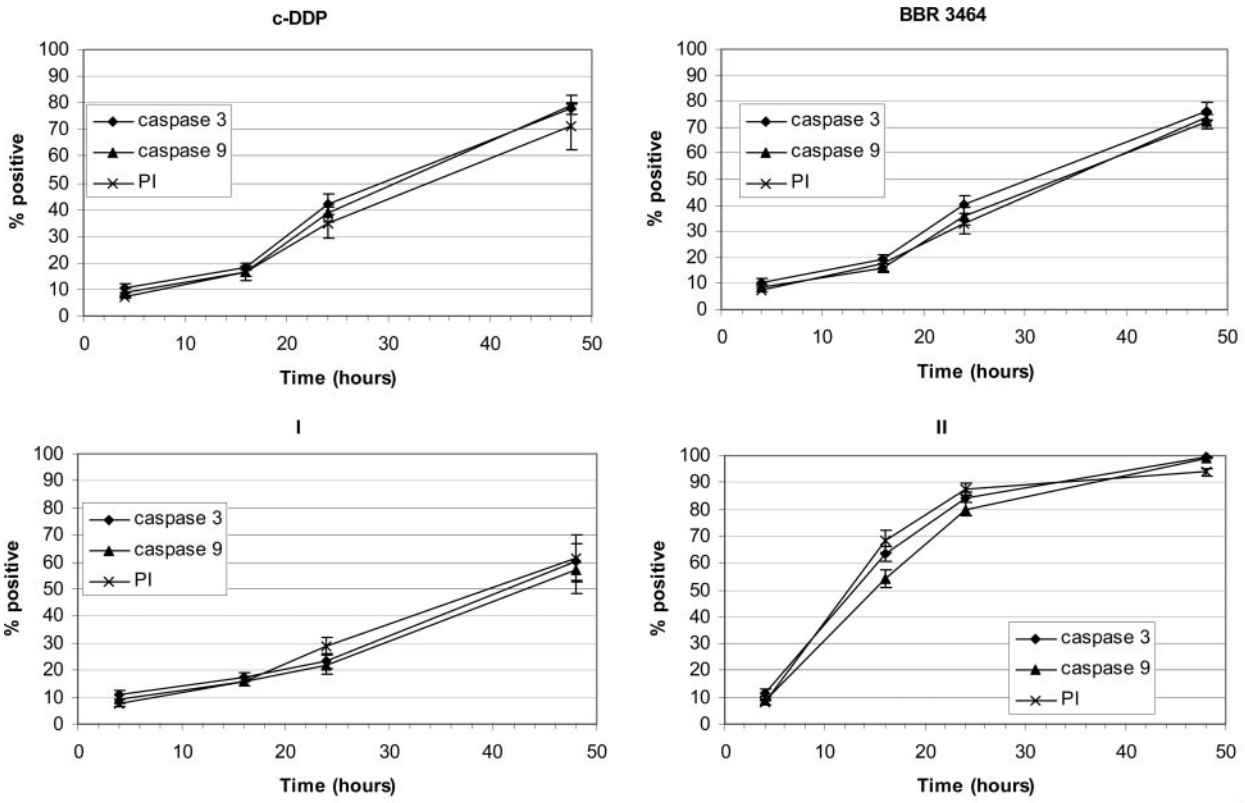


Fig. 5. Effect of platinum compounds on caspase activation. Cells were incubated with or without platinum compounds for 48 h. At 4, 16, 24, and 48 h, samples were stained for DNA cleavage with propidium iodide or for the activation of caspase-3 or -9. Drug concentrations are the same as those shown in Fig. 1B. Data represent the average of six samples from three different experiments, and standard errors are shown.

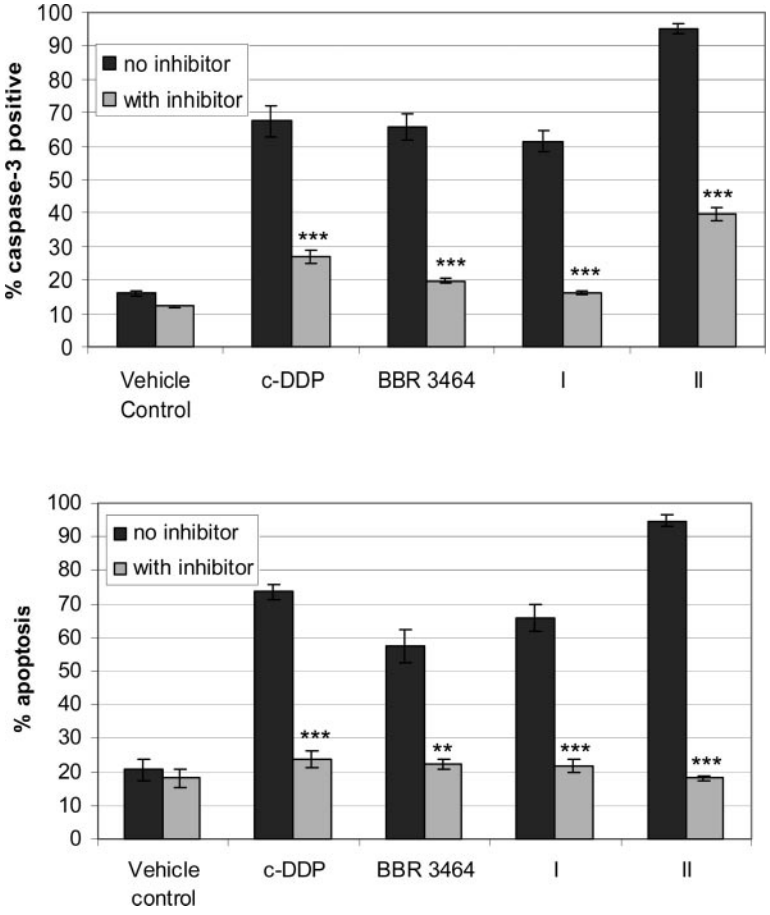


Fig. 6. Platinum-induced apoptosis requires caspase activation. Cells were cultured for 48 h in media lacking growth-supporting cytokines (media alone) or in media with cytokines and the indicated platinum compound or vehicle. ■, data from cultures not receiving the caspase inhibitor; ▨, samples given the pan-caspase inhibitor Q-VD-OPH (25 μM); **II,** 50 μM; BBR 3464, 1 μM; c-DDP, 10 μM; and **I,** 500 μM. A, caspase-3 activation. B, PI DNA staining results.

The relative potencies of **II** and c-DDP are inverted when the data for primary and tumor mast cells are compared (Figs. 2 and 7). To our knowledge, this inversion is the first demonstration of this unique effect for platinum complexes.

The origin of this effect may be multifactorial. The pharmacological factors affecting platinum drug cytotoxicity are 1) structure and frequency of target (DNA) adducts, 2) cellular uptake and efflux, and 3) metabolic deactivation by sulfur nucleophiles. The enhanced cellular uptake of **II** in tumor over primary mast cells (Figs. 3 and 8) is likely to be a significant factor in the higher apoptosis observed. However, there is no clear correlation between uptake and cytotoxicity within the set of tumor cells tested; for example, there is a significant difference in cytotoxicity of **I** in the P815 and PDMC cells despite relatively similar uptake. Nevertheless, the results confirm the remarkable uptake of an 8+ compound. The paradigm for many years in platinum chemistry was that the neutral dichloride form of c-DDP was taken up by cells, and that it was only in the lower chloride ion concentration present inside the cell that this compound was converted to the active aquated form (Jamieson and Lippard, 1999). For this reason, development of second-generation compounds like carboplatin focused on neutral species. BBR 3464 and other multinuclear compounds were a significant challenge to that paradigm. This work shows that not only can these large, highly charged compounds enter cells but also that increasing the charge by adding the dangling amine moiety consistently enhances cellular uptake.

With respect to DNA interactions, it is inherent that covalent binding and the production of long-lived, and essentially chemically irreversible, intra- and interstrand adducts are likely to be more toxic than reversible, relatively weaker noncovalent binding. The kinetics of covalent binding of the multinuclear platinum compounds to DNA are, however, relatively slow, whereas the noncovalent interaction is manifested much more rapidly once the compounds enter cells. Related to this, the kinetic of cellular uptake of compound **II** is quite rapid compared with both c-DDP and the other multinuclear compounds; this effect may produce a sufficiently critical frequency of target (DNA) interactions as observed by the rapid onset of apoptosis.

The mechanism of platinum-mediated cell death seems to be highly complex. For instance, c-DDP has been shown to increase Bax expression, destabilizing the mitochondria by influencing the Bax/Bcl-2 ratio (Siddik, 2003). However, c-DDP has also been shown to induce apoptosis through the Fas/FasL pathway, independently of the mitochondria (Siddik, 2003). In this study, all four platinum compounds

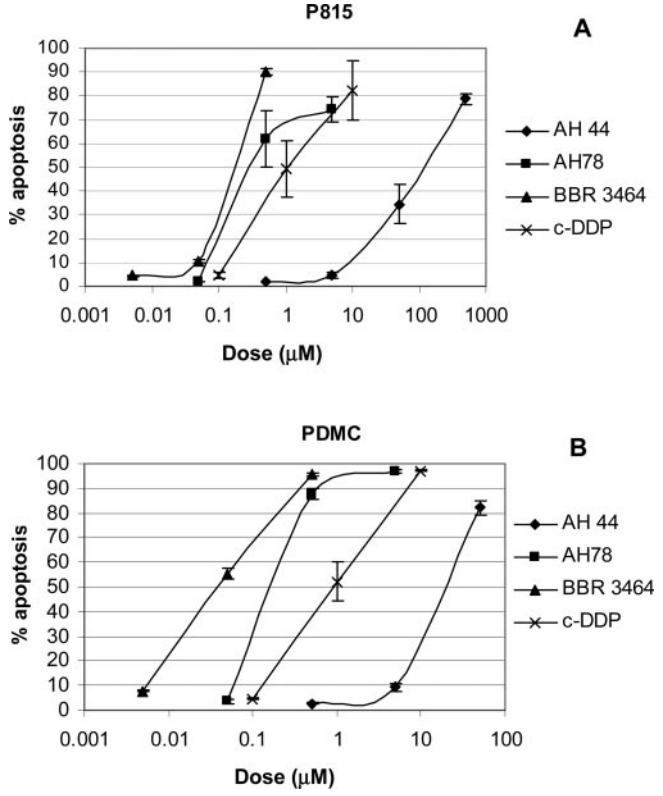


Fig. 7. Cytotoxicity in mastocytomas. PI DNA staining was performed on mast cell tumor lines grown in cRPMI for 72 h with the indicated platinum drugs. Uptake samples were performed at 2 and 6 h. In both experiments, the average of six samples from at least two separate experiments are shown. A, data from P815 cells. B, data from PDMC-1 cells.

TABLE 1

Apoptosis in mast cells

The concentration required to kill 50% of the cells by apoptosis as determined by PI staining is listed. This was determined at 72 h for the mastocytomas and at 96 h for the primary cells.

Drug	Primary BMMC	P815	PDMC
		μM	
BBR 3464	0.004	0.27	0.04
I	3.27	208.46	30.22
II	1.79	0.41	0.30
c-DDP	0.27	0.82	0.96

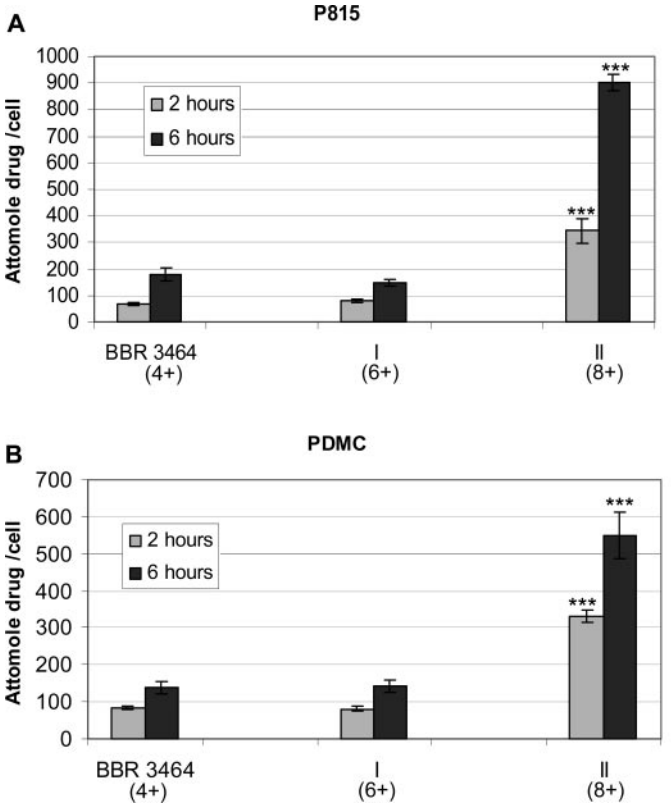


Fig. 8. Uptake in mastocytomas. Uptake samples were performed at 2 and 6 h. In both experiments, the average of six samples from at least two separate experiments are shown. A, data from P815 cells. B, data from PDMC-1 cells.

showed some dependence on p53, with **II** being the least affected. Cell death correlated with activation of caspase-9, which is most commonly cleaved in response to mitochondrial damage downstream of p53 function. These results and the strict dependence on caspase function argue for the use of a mitochondrial pathway in platinum-mediated apoptosis, but one which is not uniquely dependent on p53 status. Lipophilic organic cations have been shown to induce apoptosis through mitochondrial poisoning (Modica-Napolitano and Aprille, 2001); hence, it is possible that apoptosis induced by **II** functions via this mechanism, essentially bypassing the need for p53 to achieve mitochondrial damage and subsequent caspase activation.

Earlier work has suggested that BBR 3464 works in a p53-independent manner (Pratesi et al., 1999; Manzotti et al., 2000). Further evidence included activity in a lung carcinoma with mutant p53 that is insensitive to c-DDP but quite sensitive to BBR 3464 and studies in an astrocytoma line which demonstrated that p53 was not up-regulated after treatment with BBR 3464, in contrast to c-DDP (Orlandi et al., 2001; Servidei et al., 2001). The p53 dependence of this compound, however, may be dose- or lineage-dependent, as has been indicated for c-DDP (Siddik, 2003). The activity of **II** in p53 and Bax KO cells is of interest given the role of p53 as a potent inducer of apoptosis and where its mutation in nearly half of human tumors precludes this function (Ruley, 1996). It is striking that a slight modification in structure can reduce dependence on p53 function.

Finally, this study highlights the potential importance of noncovalent interactions in the mechanism of BBR 3464 cytotoxicity and suggests compounds such as **II** as a possible new class of antitumor agents in their own right. Many of the platinum compounds synthesized previously for antitumor use have shown a spectrum of activity similar to cisplatin as well as cross-resistance in cisplatin-resistant tumors (Farrell, 2004). For example, carboplatin, the most commonly used platinum compound in the clinic today, has improved pharmacokinetic parameters compared with cisplatin but not a different spectrum of activity (Wong and Giandomenico, 1999). This is probably because of similar mechanisms of activity. Carboplatin forms the same DNA lesions as cisplatin and seems to activate the same downstream cascades. The multinuclear noncovalent compounds have the potential for activity different from those of any platinum compounds currently used, because they seem to bind DNA in the minor groove rather than form covalent adducts with guanine in the major groove. Coupled with its relative resistance to p53 or Bax mutation, **II** may have real clinical value if it consistently shows a bias toward killing transformed cells. We have begun an expanded set of in vitro studies and in vivo analyses to test this theory.

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