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MOL (18762)

Title Page

Biological Consequences of Trinuclear Platinum Complexes: Comparison of BBR 3464 to its Non-covalent Congeners

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Running Title Page

Comparison of BBR 3464 to its Non-covalent Congeners

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Abbreviations: c-DDP, cisplatin
BMMC, Bone Marrow Mast Cell
ICP-OES, Inductively coupled plasma optical emission spectroscopy

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Abstract

BBR3464 is a 4+ cationic trinuclear platinum drug, [{*trans*-$PtCl(NH_3)_2$}$_2$μ-(*trans*-$Pt(NH_3)_2(H_2N(CH_2)_6NH_2)_2$)]$^{4+}$, which has undergone Phase II clinical trials in ovarian and lung cancer. 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 non-covalent contribution to DNA binding. To examine this effect further, the biological activity of two BBR 3464 analogues that bind DNA only through non-covalent interactions was investigated in this study and their cellular effects compared to those caused by the "parent" drug. The compounds were [{*trans*-$PtL(NH_3)_2$}$_2$μ-(*trans*-$Pt(NH_3)_2(H_2N(CH_2)_6NH_2)_2$)]$^{n+}$ (L = NH_3, n = 6, **I** and L = $H_2N(CH_2)_6NH_3$, n = 8, **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 for 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) cyotoxicity for **II**. Cellular accumulation was enhanced in mastocytomas over primary mast cells, suggesting a mechanism for enhancement of tumor cell selectivity.

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Introduction

The phase II chemotherapeutic drug BBR 3464 is a tri-nuclear platinum compound whose presumed mechanism of action arises from covalent binding to DNA. (Farrell, 2004). Due to the high positive charge, its DNA binding has been shown to have a significant non-covalent (preassociation) component that influences the binding kinetics as well as the structure of the final adducts (Hegmans *et al.*, 2004),(Qu *et al.*, 2003). Since 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. In order to investigate the preassociation with DNA, a series of compounds that contain an inert ammonia or amine group instead of the chloride were synthesized, **I** and **II** (Figure 1) (Qu *et al.*, 2004), (Harris *et al.*, 2005b). These compounds cannot covalently bind guanine residues as traditional Pt 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 hexamine (Qu *et al.*, 2004). **I** and **II** displace ethidium bromide from DNA and increase the binding affinity for Hoechst dye in an A/T rich 12 mer (Harris *et al.*, 2005a). These non-covalent compounds have also been shown to associate with the A/T regions of the DNA minor groove (Harris *et al.*, 2005b).

Interestingly, charge dispersion along the molecule as in **II** not only enhances the strength of the DNA interaction but results in enhanced cellular uptake in an ovarian tumor cell line (A2780) contributing, in part, to an enhanced cytotoxicity of **II** in comparison to **I.** The cytotoxicity of compound **II** in A2780 cells is in the 1-10

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micromolar range, lower than that of BBR 3464, but similar to cisplatin (c-DDP) (Harris *et al*., 2005b). Deactivation by sulfur-containing proteins, such as glutathione, is a critical factor in the pharmacology of covalently binding drugs such as c-DDP and BBR 3464 (Oehlsen *et al*., 2003). It is possible that non-covalent 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.*, 2005b). Further, 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 BBR3464 and **I** and **II,** that have distinct profiles of DNA and protein binding.

In this study we investigated the apoptotic and cellular effects of the non-covalent compounds **I** and **II** in comparison to BBR 3464 and c-DDP, using a mouse mast cell system. Study of these analogues of BBR 3464 will increase our understanding of the role that non-covalent forces play in the action of BBR 3464, assisting in the design of next generation chemotherapeutics. The *in vitro* system employing 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.

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Materials and Methods

Compound Synthesis:

Drug compounds were synthesized using previously reported methods (Harris *et al.*, 2005b), (Qu *et al.*, 2004).

Cell System:

Bone marrow mast cells were extracted from the femurs and tibias of C57BL/6x129 mice ("wild-type"; from Taconic Farms, Germantown, NY, USA), p53 or Bax deficient mice (Jackson Labs, Bar Harbor, ME, USA) according to previously published methods. Cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 mM sodium pyruvate (all from Biofluids, Rockville, MD, USA) 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 cells 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 x 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 (PI) DNA Staining:

Samples were fixed in an ethanol and FBS solution, washed with PBS and then stained with a solution of propidium iodide and RNase A, as described previously.(Yeatman *et al.*, 2000) Samples were then analyzed for sub-diploid DNA on a Becton Dickinson

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FACScan flow cytometer. Importantly, 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 are 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) 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, LLC (San Diego, CA, USA). Solid was dissolved to make a 10 mM stock solution in DMSO, 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 hours for caspase 3 activation and DNA damage by PI DNA staining.

Pt compound Uptake:

Mast cell tumor lines (either P815 or PDMC-1) were plated at 5.0 x 10^5 cells/mL. Compounds **I**, **II**, BBR 3464, or c-DDP were added to give a concentration of 10 μM drug in culture. After 2 or 6 hours, 5.0 x 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 EPA procedure 3050b (all volumes reduced by 1/10) and diluted with MilliQ water. Pt analysis was

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preformed on a Varian Vista-MPX CCD Simultaneous ICP-OES at 265 nm. Standards and blank were prepared the same as the sample.

Results

To determine the cytotoxicity of trinuclear Pt compounds that bind DNA via non-covalent interactions, apoptosis was measured. Primary mast cells were cultured for 72 hours in the presence or absence of **I**, **II**, BBR 3464, or c-DDP, and DNA 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 Figure 2A. While the IC_{50} values of the non-covalent compounds were over one hundred times higher than BBR 3464, they were similar to that of cisplatin with IC_{50} in the micromolar range. When the cytotoxicity was assayed over time at doses that were approximately the IC_{90}, **II** showed very rapid death-inducing kinetics despite its 8+ charge (Figure 2B).

Platinum uptake was then measured in primary BMMC cultures (Figure 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 higher than the other compounds. The uptake is consistent with the more rapid killing by **II** of target cells (Figure 2B). These data are consistent with uptake in A2780 ovarian tumor cells where **II** also has a much higher uptake and **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*., 2005b). Cisplatin data is not shown because under these conditions the amount of cisplatin taken into cells was below the limit of quantitation for the instrument. For the two non-covalent 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

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cisplatin analogues dictated that Pt compounds had to be neutral to be taken up by cells (Jamieson and Lippard, 1999; Andrews, 2000; Essigmann and Kartalou, 2001).

Pt-DNA adducts induce a cellular signaling response regulating survival and apoptosis. Given the difference in DNA binding profile and cellular uptake between covalent and non-covalent 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 (Figure 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 pro-apoptotic transcription factor, and Bax, which is also a pro-apoptotic 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 (Zamble and Lippard, 1999). This is consistent with the cytotoxicity of c-DDP across the NCI tumor panel, which appears 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 Pt-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 still much less than noted with the other Pt compounds.

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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 if treatment with Pt compounds activated these enzymes, we measured caspase 9 and caspase 3 activity following Pt treatment, using a fluorometric assay (Figure 5). Both caspases were activated with kinetics very similar to the PI-DNA staining results.

To further assess the importance of caspase function in Pt-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 (Figure 6A). The decrease in caspase function directly correlated with decreased Pt-mediated apoptosis, as demonstrated by PI-DNA staining (Figure 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 Pt-mediated cell death.

An important parameter of potential therapeutic index and efficacy of anti-tumor compounds is cytotoxic differentiation between “normal” (in this case primary) and transformed cells. For this reason, the Pt compounds were tested in two mastocytoma cell lines, PDMC and P815. Importantly, all four Pt compounds induced apoptosis in these mastocytoma cell lines (Figures 7A and B). Furthermore, the IC_{50} value for apoptosis induced by **II** in mastocytomas was 10x lower than in the primary mast cells. In contrast, the IC_{50} for c-DDP in both tumor cell lines was essentially identical to the primary cells (Figures 2 and 7). This significant enhancement of cytotoxicity in the tumor cell lines also produces the interesting result that, while BBR 3464 remained the most potent

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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 Figures 2 and 7) where the "promotion" of the relative cytotoxicity of Compound **II,** especially when compared to c-DDP, is readily apparent.

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

Discussion

While Pt based chemotherapy has largely been based on the theory that covalent interactions are essential to cytotoxicity, the highly charged compound **II** demonstrates cytotoxicity in the micromolar range, similar to cisplatin and analogues. 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 on A2780 cells, where the presence of the dangling amine and the increased charge (8+) in **II** was shown to significantly enhance cytotoxicity over the 6+ analogue **I** (Harris *et al*., 2005b). Further, **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. The relative potencies of **II** and c-DDP are inverted when the data for primary and tumor mast cells is compared (Figures 2 and 7).

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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 (i) structure and frequency of target (DNA) adducts, (ii) cellular uptake and efflux and (iii) metabolic deactivation by sulfur nucleophiles. The enhanced cellular uptake of **II** in tumor over primary mast cells (cf Figures 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 Pt 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 that increasing the charge through 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, non-covalent binding. The kinetics of covalent binding of the multinuclear Pt compounds to DNA is,

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however, relatively slow, whereas the non-covalent interaction is manifested much more rapidly once the compounds enter cells. Related to this, the kinetics of cellular uptake of compound **II** are quite rapid compared with both c-DDP and the other multinuclear compounds – this effect may produce a sufficiently critical frequency of target (DNA) interactions manifested as observed by the rapid onset of apoptosis.

The mechanism of Pt-mediated cell death appears 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 Pt compounds 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 use of a mitochondrial pathway in Pt-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 insentive to c-DDP, but quite sensitive to BBR 3464, and studies in an astrocytoma line which demonstrated that p53 was not upregulated after

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treatment with BBR 3464, in contrast to c-DDP (Orlandi *et al.*, 2001;Servidei *et al.*, 2001). The p53 dependency 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 non-covalent interactions in the mechanism of BBR3464 cytotoxicity, and suggests compounds such as **II** as a possible new class of anti-tumor agents in their own right. Many of the Pt compounds previously synthesized for anti-tumor 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 Pt compound in the clinic today, has improved pharmacokinectic parameters compared to cisplatin, but not a different spectrum of activity (Wong and Giandomenico, 1999). This is likely due to similar mechanisms of activity. Carboplatin forms the same DNA lesions as cisplatin and appears to activate the same downstream cascades. The multinuclear non-covalent compounds have the potential for activity different from those of any Pt compounds currently used, as they appear to bind DNA in the minor groove rather than forming 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 as well as in vivo analyses to test this theory.

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Footnotes

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Legends for Figures

Figure 1. Structures of trinuclear Pt complexes

Figure 2. Cytotoxicity of platinum drugs in BMMC, shown in (A) as a function of dose at 72 hours. In (B) the concentration of IC_{90} at 72 hours (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.

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

Figure 4. The effect of p53 or Bax deficiency on Pt-induced apoptosis. WT, p53 KO, or Bax KO BMMC were cultured for 72 hours in IL-3+SCF in the presence of the indicated concentrations of each Pt compound. Drug concentrations are the same as figure 1B. Apoptosis was measured by PI-DNA staining to detect sub-diploid DNA content. Each point represents the average of six samples in three independent experiments with standard errors shown.

Figure 5. Effect of Pt compounds on caspase activation. Cells were incubated with or without Pt compounds for 48 h. At 4, 16, 24, and 48 hours 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 figure 1B. Data represents the average of six samples from three different experiments and standard errors are shown.

Figure 6. Pt-induced apoptosis requires caspase activation. Cells were cultured for 48 hours in media lacking growth-supporting cytokines (media alone), or in media with

cytokines and the indicated Pt compound or vehicle. Black bars represent data from cultures not receiving the caspase inhibitor, while gray bars show samples given the pan caspase inhibitor Q-VD-OPH (25μM). **II** 50μM, BBR 3464 1 μM, c-DDP 10 μM, and **I** 500 μM. Panel A shows caspase-3 activation, while panel B shows PI-DNA staining results.

Figure 7. Cytotoxity in Mastocytomas. PI-DNA staining was performed on mast cell tumor lines grown in cRPMI for 72 hours with the indicated platinum drugs. Uptake samples were preformed at two and six hours. In both experiments the average of six samples from at least two separate experiments are shown. Panel A shows data from P815 cells and panel B data from PDMC-1 cells.

Figure 8. Uptake in Mastocytomas. Uptake samples were preformed at two and six hours. In both experiments the average of six samples from at least two separate experiments are shown. Panel A shows data from P815 cells and panel B data from PDMC-1 cells.

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Table 1. Apoptosis in mast cells. The concentration (μM) required to kill 50 % of the cells by apoptosis as determined by PI staining is listed. This was determines at 72 hours for the mastocytomas and 96 hours for the primary cells.

Drug	Primary BMMC	P815	PDMC
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

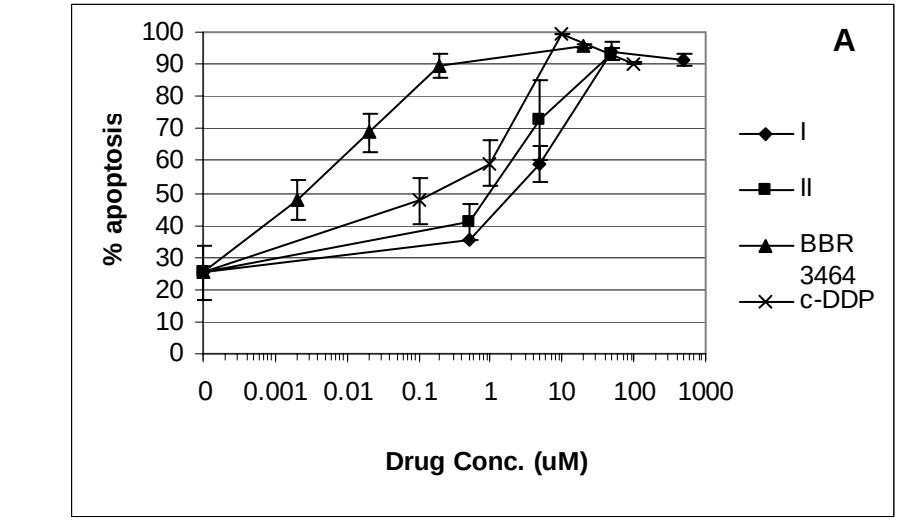
Figure 1.

BBR 3464

I

II

Figure 2.



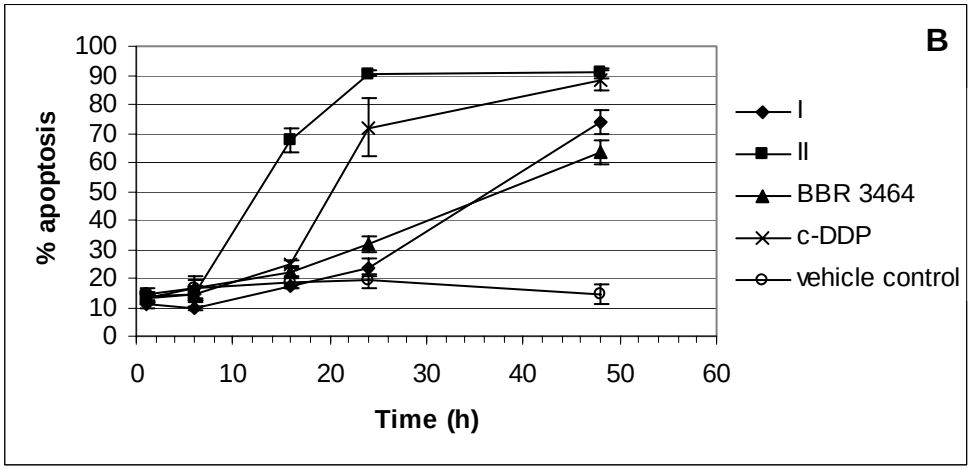


Figure 3.

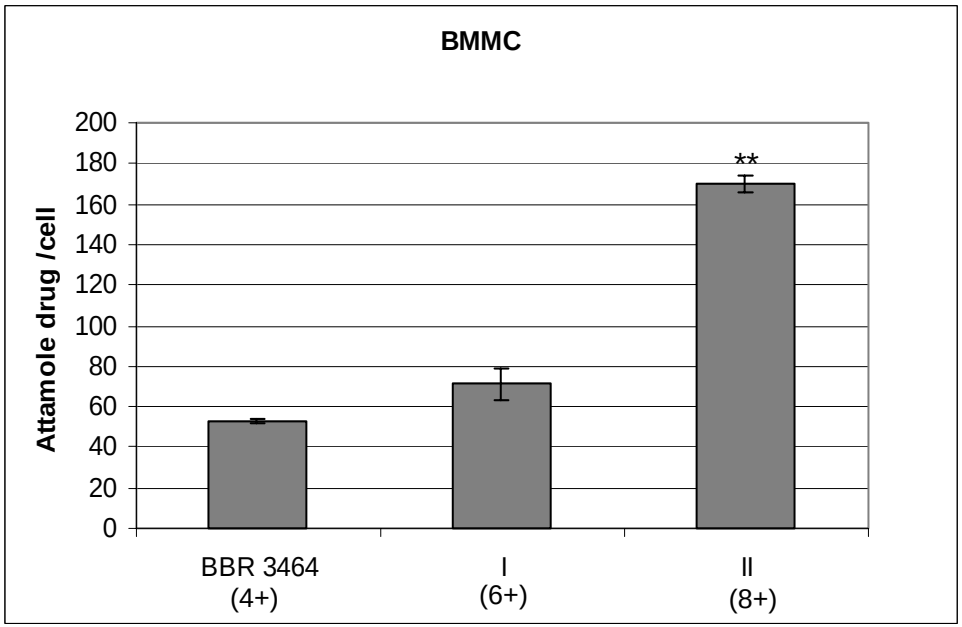


Figure 4.

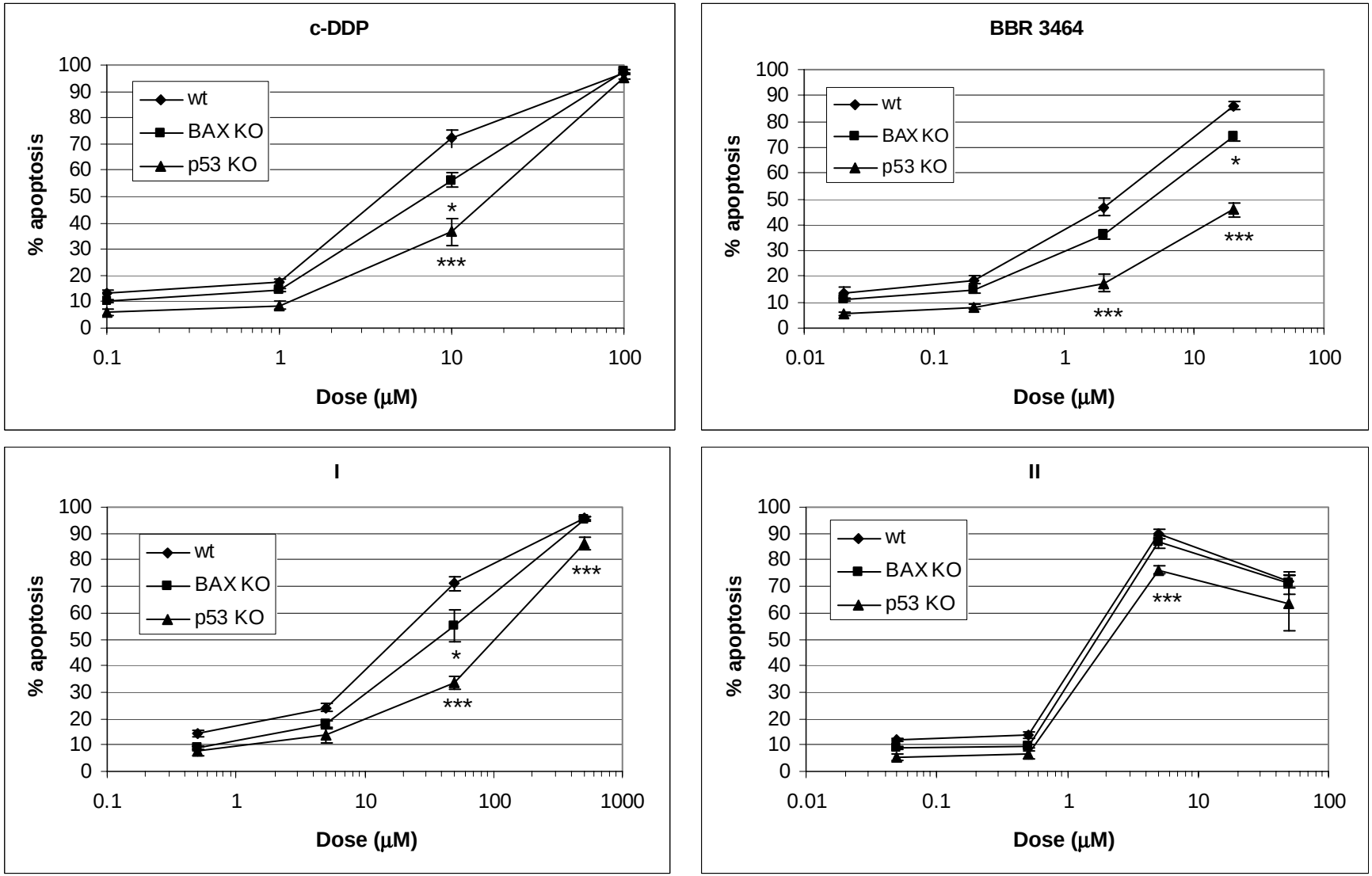


Figure 5.

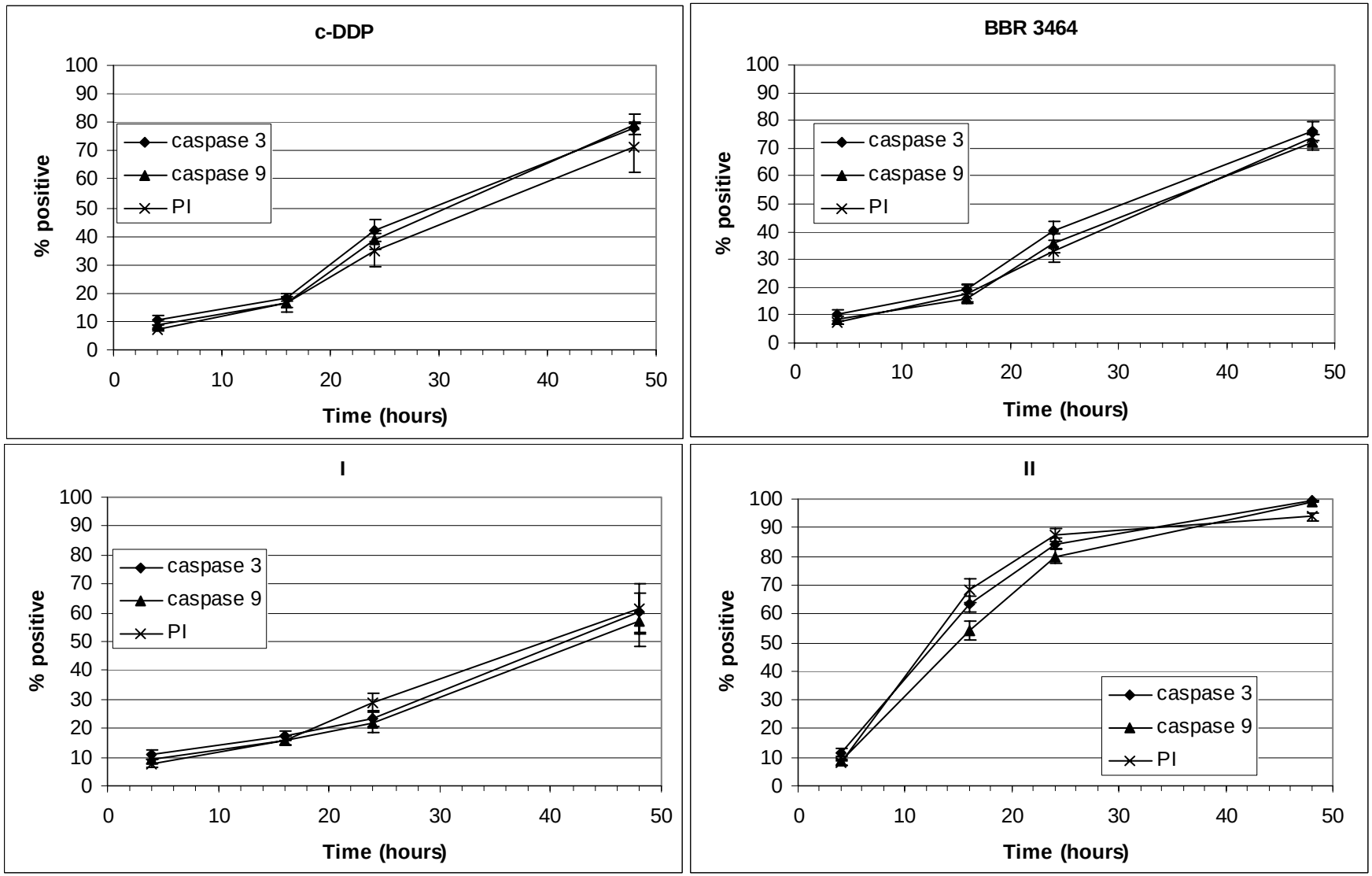
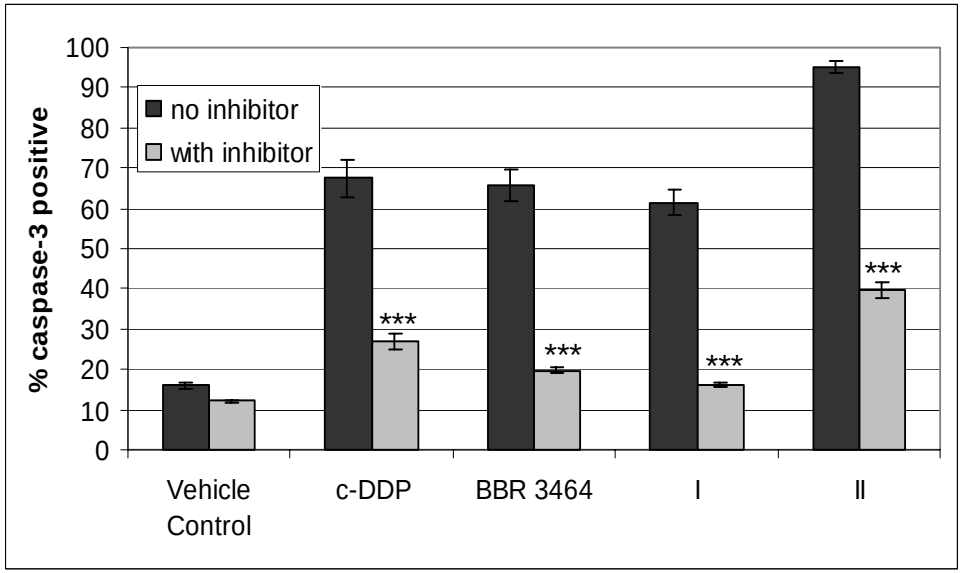


Figure 6.



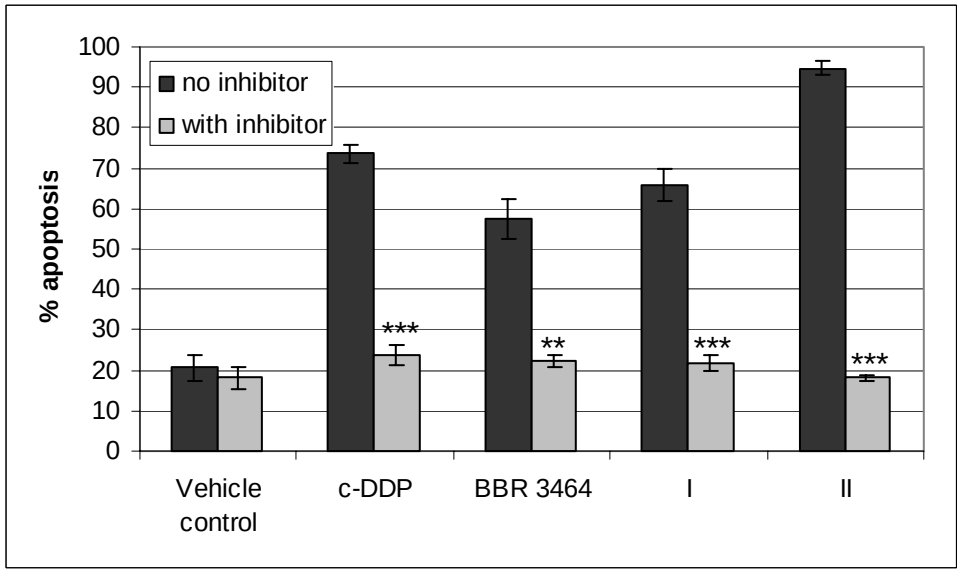
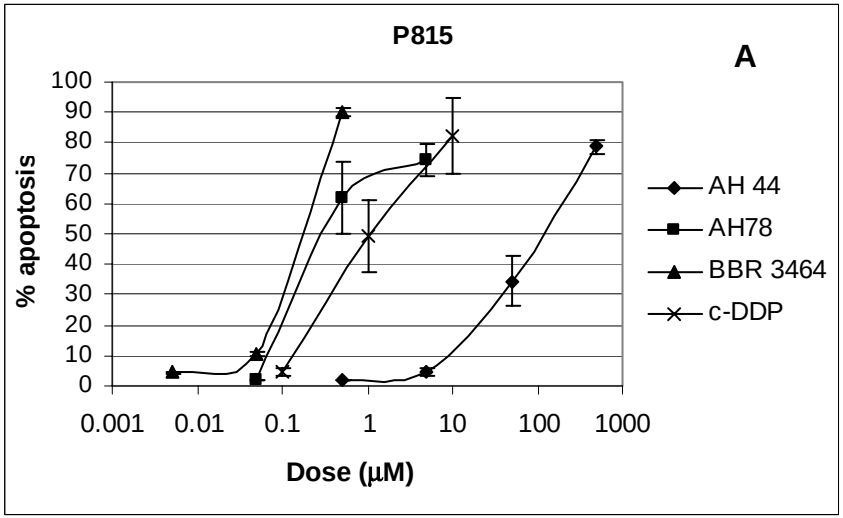


Figure 7.



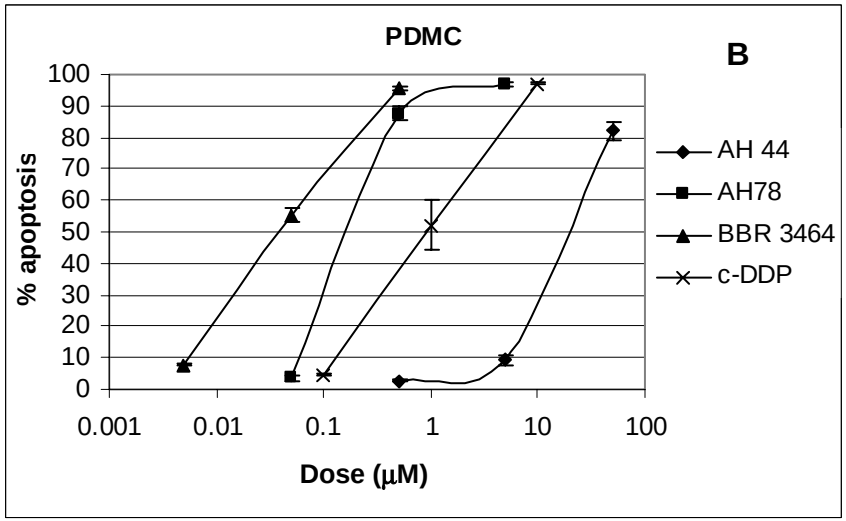


Figure 8.