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Mechanisms of Accumulation and Biological Consequences of Polynuclear Platinum Compounds

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MECHANISMS OF ACCUMULATION AND BIOLOGICAL CONSEQUENCES OF POLYNUCLEAR PLATINUM COMPOUNDS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry at Virginia Commonwealth University.

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List of Abbreviations

Carboplatin	*cis*-diammine-1,1-cyclobutane dicarboxylateplatinum (II)
Cisplatin, c-DDP	*cis*-diamminedichloroplatinum(II)
IC_{50}	Concentration to inhibit 50% of growth
NER	Nucleotide Excision Repair
MRP	Multi-drug resistance-associated proteins
Oxaliplatin, Ox	*trans*-L-diamininocyclohexaneoxalatoplatinum(II)
PBS	Phosphate buffered saline
CSA	Cyclosporin A
Ly	LY294002
Des.	Desipramine
5-FU	5-Fluorouracil
HMG	High mobility group protein
GSH	Glutathione
SOD1	Superoxide dismutase
hCTR1	Human copper transporter 1
OCT	Organic cation transporter
WND	Wilson's disease
MNK	Menke's disease
ATP7A, 7B	ATPase efflux transporters
Pt	Platinum
TEA	Tetraethylammonium
TEER	Trans epithelial electrical resistance
HEK	Human embryonic kidney cells
EIPA	N-ethyl-N-isopropylamiloride
HSA	Human serum albumin

Abstract

MECHANISMS OF ACCUMULATION AND BIOLOGICAL CONSEQUENCES OF POLYNUCLEAR PLATINUM COMPOUNDS

By PEYMAN KABOLIZADEH

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2007

Major Director: Nicholas P. Farrell
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The novel trinuclear complex, BBR3464 has undergone Phase II clinical trials and been shown to have greater cytotoxicity and cellular uptake than clinical anticancer platinum drugs such as cisplatin, oxaliplatin and carboplatin. The clinical efficacy of cisplatin, oxaliplatin and carboplatin is limited due to acquired resistance and dose limiting side effects. The three major pharmacological factors contributing to the intrinsic cytotoxicity of, and cellular resistance to, platinum drugs are (i) cellular uptake and efflux of platinum; (ii) the frequency and nature of Pt-DNA adducts; and (iii) deactivating

metabolic reactions with sulfur-containing nucleophiles. Since decreased cellular uptake of platinum drugs is a common feature of resistant cells, investigating mechanisms of cellular uptake and efflux is of a great importance in the field of cancer biology. The mechanisms of uptake of Platinum drugs are diverse and complex. Similar to cisplatin, BBR3464 was shown to use copper transporter hCTR1 and ATP7B for influx and efflux respectively. Organic cation transporters (OCT) did not play an important role in BBR3464 cellular uptake, however, desipramine, an OCT inhibitor had synergistic effects on platinum drugs-induced cytotoxicity. This effect is of high clinical relevance since desipramine, an antidepressant, is being used in prostate cancer patients for the treatment of neuropathic pain. The mechanism of this interaction was further addressed.

Due to the high charge of BBR3464, studies have shown that its DNA binding has a non-covalent component. To examine the non covalent component, labile chloride leaving groups were replaced by non labile ammonia groups. Besides having higher cellular accumulation than BBR3464, the non covalent analogue, AH78, had a different mechanism of action in cells and showed promising results *in vivo*. These data confirm the validity of searching for new chemotypes outside the cisplatin structural class to aid in the treatment of recurrent, cisplatin-resistant cancers.

CHAPTER 1: Introduction

1.1. Cancer

Cancer claims the second highest number of deaths in the United States following heart disease. According to the American Cancer Society, the most cancer deaths in the United States are from lung and bronchus carcinomas followed by prostate cancer in males and breast cancer in females. Over the course of a life time, one in every two males and one in every three females will develop some form of cancer.[96] The word cancer is derived from the Latin word *crab* and defined as the uncontrolled, disorderly proliferation of cells which results in malignant tumors. They can invade (spread into neighboring tissue) and metastasize (spread to non-cancerous tissue) and are less differentiated than benign tumors. Cancer cells are marked by anaplasia (poorly differentiated), hyperchromatism, pleomorphism (dark stained nuclei), abnormal mitosis, and increased nuclei-cytoplasmic ratio. It is believed that cancer results from a multi-step process which generally involves genetic abnormalities. Cancer symptoms can vary from a simple cough to hemorrhaging and cachexia. There are multiple treatments for cancer such as radiation therapy, surgery, immunotherapy, and chemotherapy.[1] Chemotherapy is treatment of cancer with anticancer drugs such as cisplatin, oxaliplatin, carboplatin and methotrexate.

1.2. Platinum Drugs

1.2.1. Cisplatin and Its Analogs

Cisplatin (c-DDP) was first synthesized in 1845, and later found to have antitumor activity in 1969 (Figure 1.1).[2] c-DDP is an effective antineoplastic agent that is used for the treatment of cancer, including testicular, head and neck, ovarian, and small cell lung neoplasms with the dose limiting toxicity of nephrotoxicity.[3,4] c-DDP , when combined with vinblastine and bleomycin, increased the cure rate of testicular cancer to 90% [4] and this scientific breakthrough initiated the interest to develop other platinum anticancer drugs. Many cisplatin analogues have entered clinical trials, but only two, carboplatin and oxaliplatin (Figure 1.1) have been approved for clinical use in the United States.

Carboplatin has a similar antitumor spectrum to that of cisplatin but with different dose limiting cytotoxicity, myelosuppression.[6] On the other hand, oxaliplatin is used for treatment of colorectal carcinomas in combination with 5-Fluorouracil (5-FU) with dose limiting cytotoxicity of neuropathy.[97] Another anticancer mononuclear platinum drug is satraplatin (Figure 1.1), which is being evaluated by the FDA for treatment of prostate cancer with dose limiting toxicity of myelosuppression.[98]

1.2.2. Polynuclear Platinum Compounds

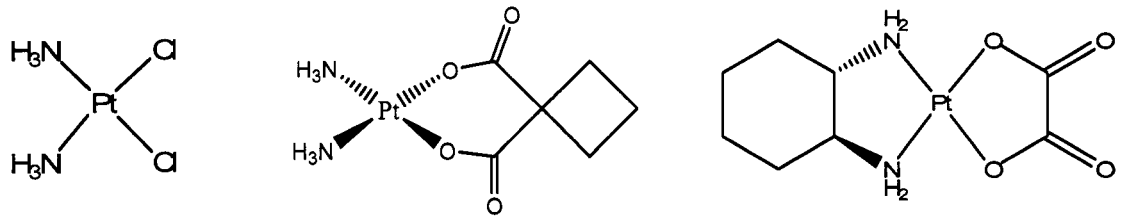
1.2.2a Covalently Binding Polynuclear Platinum Complexes

Although c-DDP and its analogues are effective anticancer drugs, their efficacy is limited due to acquired resistance and dose-limiting side effects.[5] Structurally novel platinum complexes that bind to DNA in a different manner than c-DDP may have distinct

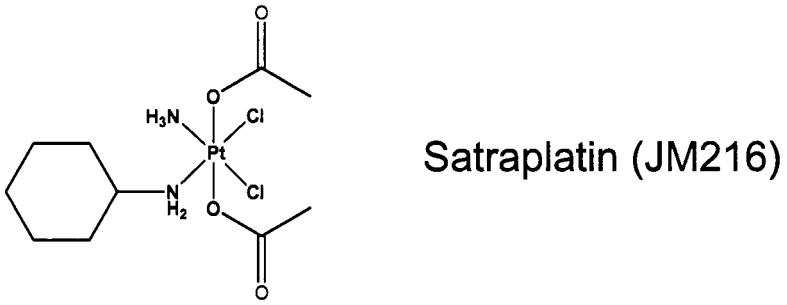
cytotoxicity and side effect profiles. The trinuclear complex, BBR3464, is one example of a class of polynuclear platinum drugs in which the platinum coordination units are linked by alkanediamine chains (Figure 1.1).[6] BBR3464 is significantly more cytotoxic than c-DDP and retains activity against c-DDP-resistant cell lines and tumors *in vitro* as well as *in vivo*. [6-10] BBR3464 has undergone Phase II clinical trials in cisplatin-resistant and refractory cancers. [6-10]

1.2.2b Non-Covalently Binding Polynuclear Platinum Complexes

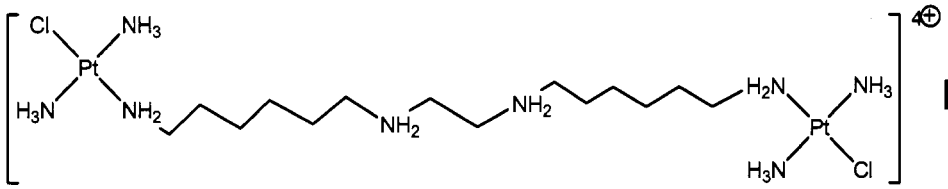
Previous studies have indicated that BBR3464 DNA binding has a non-covalent preassociation component which affects the binding kinetics and the final structure of the Pt-DNA adducts.[11,12] As the structure of the drug/DNA adduct determines the repair mechanisms, protein recognition, and activation of down stream signaling cascades, it is essential to investigate the formation and biological consequences of these DNA adducts. To examine preassociation component of BBR3464, compounds such as AH44 and AH78 (Figure 1.1) were synthesized with inert ammonia or amine groups in place of the labile chloride ligands.[13,14]



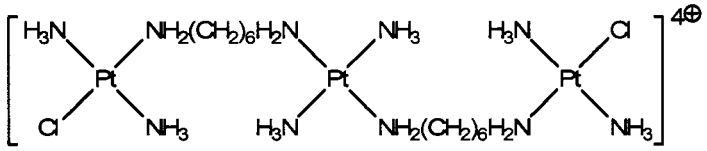
c-DDP Carboplatin Oxaliplatin



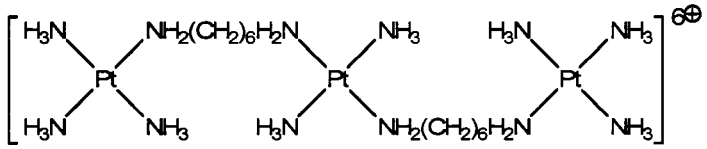
Satraplatin (JM216)



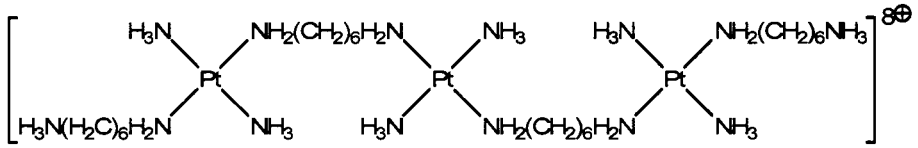
BBR3610



BBR3464



AH44



AH78

Figure 1.1: Structure of Platinum Drugs

1.3. Pharmacological Factors of Cytotoxicity

The three major pharmacological factors contributing to the intrinsic cytotoxicity of, and cellular resistance to, platinum drugs are (i) the frequency and nature of Pt-DNA adducts; (ii) deactivating metabolic reactions with sulfur-containing nucleophiles; and (iii) cellular uptake and efflux of platinum.

1.3.1. Platinum-DNA Adducts

It is believed that c-DDP-induced cytotoxicity is mediated by interactions with DNA and inhibition of DNA synthesis and replication by formation of bifunctional interstrand and intrastrand cross-links.[4] c-DDP mainly forms 1,2 intrastrand adducts at adjacent guanines and to a lesser extent 1,2-intrastrand adducts between one guanine and one adenine, and 1,3 intrastrand and 1,2 interstrand cross-links at guanines (Figure 1.2). [6] There are many cellular proteins that interact with c-DDP adducts such as high mobility group (HMG) proteins which shield the DNA adducts from repair mechanisms, perhaps one factor for its clinical efficacy.[6] On the other hand, polynuclear platinum compounds form a variety of different inter- and intra-strands cross-links. BBR3464 mainly forms 1,4- and 1,6-interstrand and 1,5- intrastrand cross-links at guanines[6] (Figure1.3). Its formed adducts are not recognized by HMG since they do not significantly bend the double helix structure of DNA.[6] Nucleotide excision repair (NER) is another cellular repair process in which destabilizing DNA adducts and damaged nucleotides from DNA are removed. NER is reported to be a major mechanism contributing to c-DDP acquired resistance. [15] Studies have shown that BBR3464-mediated 1,4-interstrand crosslinks cannot be removed by NER, perhaps one reason for its enhanced cytotoxicity. [16]

Unlike BBR3464, c-DDP, and oxaliplatin, non-covalent binding polynuclear platinum drugs such as AH44 and AH78 interact with DNA with high affinity in an electrostatic manner without the potential for covalent binding to DNA. AH78 binds to phosphate oxygen atoms associating with the backbone.[17] It extends along the phosphate backbone (backbone tracking) and spans the minor groove (groove spanning). The high positive charges of AH44 and AH78 not only increased interactions with DNA but also resulted in increased cellular uptake. [18]

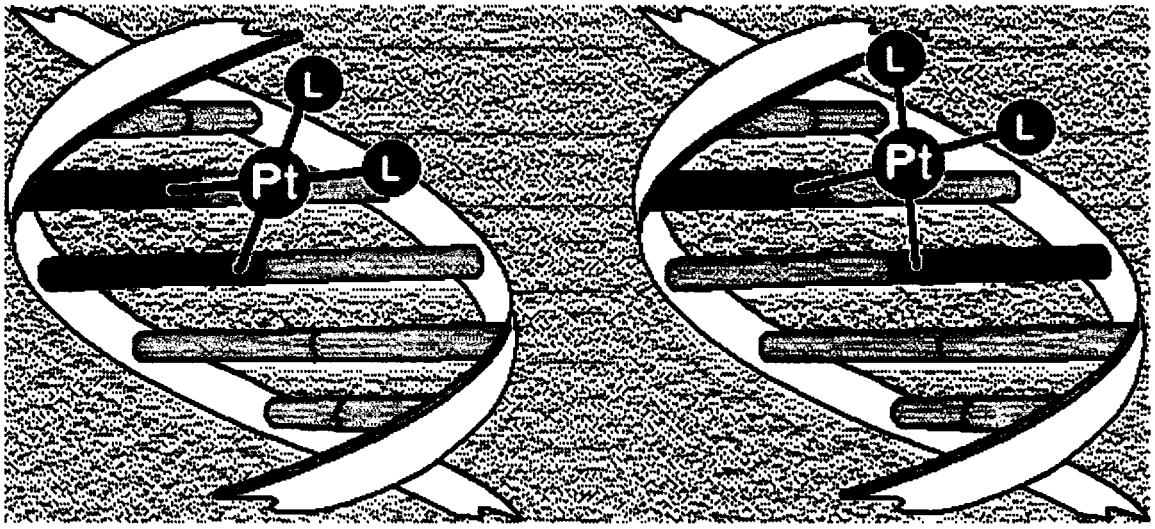
Binding of platinum drugs to DNA results in multiple different signal transduction cascades activations such as mitogen activated protein kinase (MAPK) and p53 pathways. The activation of such pathways, especially p53, play an important role in Pt-induced cytotoxicity in cancer cells. [19]

1.3.2. Metabolic Reactions

Platinum drugs interact with a large range of proteins before binding to DNA. It is estimated that <5% of the administered platinum drugs will reach DNA. One of the important factors in cytotoxicity and pharmacology of Pt drugs is deactivation by sulfur-containing proteins such as glutathione (GSH) and human serum albumin (HSA). [20] These interactions may be responsible for the remainder of the metabolic reactions of Pt-drugs.

1.3.3. Cellular Uptake

Acquired resistance to c-DDP in patients has been a topic of intensive research. The role of cisplatin uptake and efflux is increasingly being seen as a critical determinant in clinical



1,2 Intrastrand **1,2 Interstrand**

Figure 1.2: Cisplatin-DNA adducts

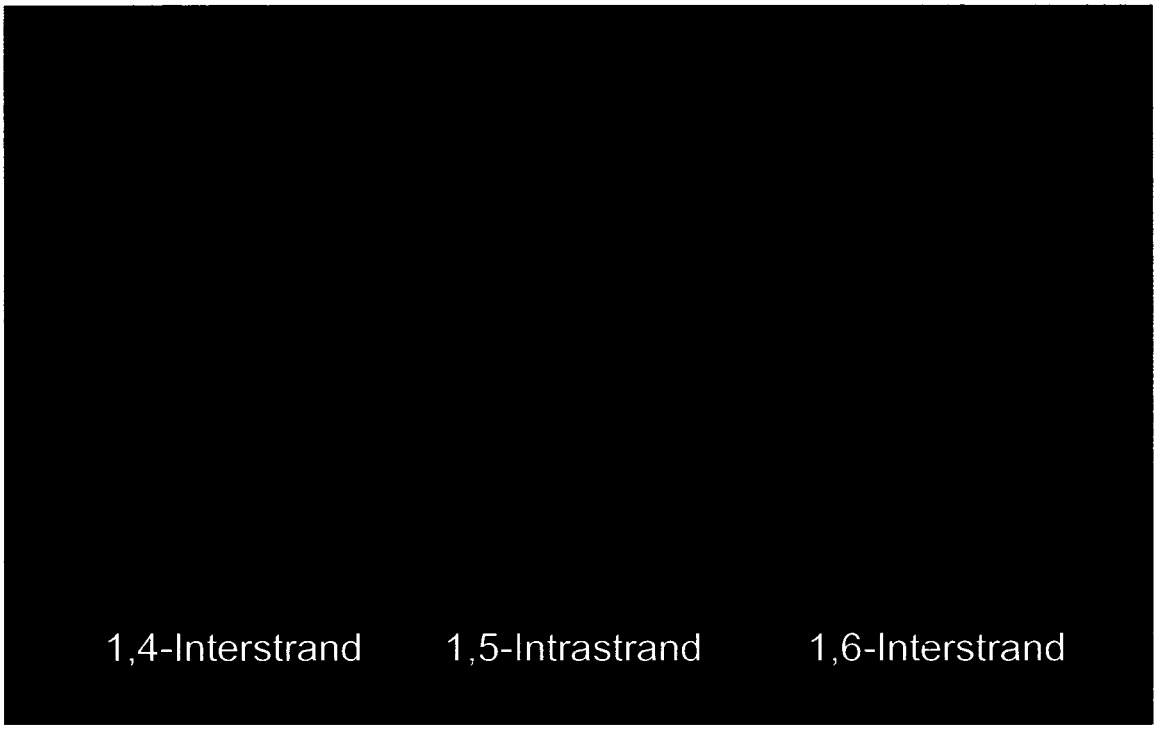


Figure 1.3: Novel BBR3464-DNA adducts[6]

resistance. [21] It was once believed that c-DDP enters cells by a process of passive diffusion; however, multiple studies have shown that the cellular uptake of c-DDP, oxaliplatin and carboplatin is mediated through the use of multiple pathways and transporters. The means of cellular transportation of platinum drugs are diverse and perhaps a factor that can contribute to the efficacy of platinum anticancer drugs. Potential candidates for cellular transportation of Pt-drugs include copper transporters, organic cation transporters, and endocytosis. Polyamine pathways and the chemical status of c-DDP were also indicated to be an important factor in cellular uptake. All of these parameters of cellular uptake will be described in detail in the following sections.

1.4. Copper Homeostasis and Platinum Drugs Uptake

1.4.1. Copper Homeostasis

Copper is an essential metal and functions as a cofactor of different enzymes, such as cytochrome c oxidase and superoxide dismutase, which play an important role in electron transfer and antioxidant defense processes respectively. Copper excess however is toxic to cells due to production of reactive oxygen species as the result of the free copper oxidation. Different groups of proteins, including chaperones and essential enzymes, contain copper binding domains which are rich in cysteine, metionine and histidine residues, therefore maintaining copper homeostasis. Transportation of copper through the human body is a complex process. Copper is absorbed through the intestines and enters the blood by the action of transporter ATP7A.[22] In blood, copper binds to L-histidine and human serum albumin. [22,23]

Copper enters and exits cells *via* different trans-membrane transporters. Human copper transporter 1 (hCTR1) is a 190 amino acid protein with three transmembrane domains involved in the cellular influx of copper.[22] hCTR1 is mainly expressed in the kidney, liver, and heart and to a lesser extent in the intestines, brain and muscles. hCTR1 which resides in the plasma membrane has the methionine and histidine rich N-terminus which plays an important role in copper binding.[22,23] It is important to note that the cellular uptake of copper is temperature, pH, and K^+ ion dependant. Once copper enters the cell, it is transferred from hCTR1 to different chaperone molecules.[22,23] Chaperones play a vital role in transferring copper to essential proteins, as cofactors, and in controlling the toxicity of free copper in cells by keeping it bound. As a result, the free copper inside cell is less than 10^{-18}M.[22,23] Superoxide dismutase (SOD1), which is an antioxidant enzyme, acquires its copper from the chaperone for SOD1 (CCS). COX17 is another chaperone required to deliver copper to cytochrome-c oxidase. [22,23]

Excess copper is toxic to human cells; therefore normal cellular levels of copper are maintained by efflux copper transporters. Antioxidant protein 1 (ATOX1 or HAH1) is a chaperone involved in delivering copper to efflux transporters ATP7A and ATP7B. Copper efflux transporters are P-type ATPase copper transporters which excrete copper from intestinal and liver cells respectively. The ATP7B gene is located on chromosome 13 and is mainly expressed in the liver and to lesser extend in the kidneys, brain and placenta.[22,23] The ATP7B transporter is located in the *trans*-Golgi network but, under high copper concentrations, it is relocated to a more peripheral compartment such as the plasma membrane to function as an exporter. [23]

ATP7A protein is located on the X chromosome and is mainly expressed in the intestinal epithelium. ATP7B and ATP7A proteins contain eight trans-membrane domains, six of which have a high affinity to copper and can play an important role in transporting copper out of cells.[22,23]

Two heriditory defects in copper homeostasis, Menke's (MNK) and Wilson's (WND) diseases, domonstrate the importance of copper in homeostasis and medicine. Mutations in ATP7B cause Wilson's disease, an autosomal recessive genetic disease resulting from copper accumulation in liver, brain, and kidneys due to impaired excretion of copper into bile.[22,23] It is characterized by basal ganglia degeneration, liver cirrhosis, neurological symptoms, and deposition of green or golden pigment in the cornea of the eye.[22,23]

Mutation of the ATP7A gene results in Menkes disease.[22,23] It is an X-linked genetic disorder caused by impaired absorption of copper by the intestinal walls.[22,23] Defective efflux of copper from intestinal cells into the blood results in copper deficiency and severe cerebellar neurodegenration in early childhood. Other symptoms include sparse hair, mental retardation, seizure, hypothermia, feeding difficulty and decreased muscle tone. [22,23]

1.4.2. Cross Resistance and Uptake in Resistant Cell Lines

While there is some cross resistance between c-DDP, oxaliplatin and carboplatin; c-DDP resistant cells have also shown some cross resistance to copper, therefore indicating the connection between copper homeostasis and platinum drug metabolism. [22] Cells that were

selected for resistance to cisplatin showed resistance to copper and vice versa, indicating the bidirectional cross resistance between copper and cisplatin. [24] Ketano *et al.* also demonstrated that cellular resistance to c-DDP in ovarian carcinoma cell lines was accompanied by alteration in pharmacokinetics (efflux and influx) of cisplatin as well as copper.[25] The accumulation of copper and c-DDP was lower in the resistant cell line than the sensitive cell line. [25] In addition, the decreased uptake of cisplatin was shown to be consistent across different resistant cell lines including ovarian carcinoma, osteosarcoma, and cervix squamous cell carcinoma cell lines. [26] Furthermore, intracellular uptake and DNA platination of c-DDP in resistant ovarian and cervical cell lines (A2780cis/HeLACK) was lower than in the parental cell lines (A2780/HeLa), while no difference in DNA repair was observed. [13]

1.4.3. Copper and Its Connection to Platinum Drugs Accumulation

The involvement of copper transporters in platinum drug metabolism has been indirectly examined by investigating the effect of copper ions on the cellular uptake of platinum drugs such as c-DDP and BBR3464.[27] When c-DDP was used at a high concentration (1mM), copper decreased c-DDP uptake in yeast as reported by Ishida *et al.* [14] Kabolizadeh *et al.* observed the same result in ovarian carcinoma cells (A2780) by adding 1mM c-DDP with copper. [27] High concentrations of c-DDP (1mM) may saturate hCTR1, and hence compete with copper for binding. [14] However, this concentration is not clinically relevant, as the approximate blood concentrations of c-DDP in a patient with an administered dosage of 50-120 mg/m^2 body surface area corresponds to 66μM

(20μg/ml).[3,28] When more clinically relevant concentrations of c-DDP and BBR3464 were used (10-20 μM), copper increased the cellular concentration of both platinum drugs in ovarian and colorectal carcinoma cell lines. [27] These data are supported by recent studies showing that c-DDP enhances cellular copper accumulation in MCF-7 breast cancer cells.[29] Collectively, these data would suggest that at physiological concentrations, c-DDP does not directly compete for hCTR1 binding but, rather, may compete with copper for the efflux transporter, resulting in enhanced platinum accumulation. [27,29] Kapp *et al.* did not observe any enhancement of cellular copper uptake by *m*-4f-PtDMSO-DAH , a dinuclear platinum complex, indicating that copper transporters may not be involved in the uptake of this drug.[29] Moreover, Ohashi *et al* observed no change in cellular accumulation of c-DDP (150μM) by treatment of *Saccharomyces cerevisae* cells with low concentration of copper (1 μM). [30] Despite the concentration of copper and c-DDP used, copper has been shown to decrease c-DDP cytotoxicity in different cell lines (ovarian, colorectal, head and neck, breast, mast cell carcinoma, yeast *Saccharomyces cerevisae*). [14,27,30]

The effect of copper on mediated cytotoxicity caused by polynuclear platinum drugs such as BBR3464 was shown to be different than for c-DDP. [27] c-DDP-induced apoptosis was significantly decreased by copper, as observed in yeast. [14] By contrast, BBR3464-induced apoptosis was enhanced by copper in both ovarian and colorectal carcinoma cell lines. Thus, the differential response to copper suggests that c-DDP and BBR3464 have distinct modes of transportation or metabolism leading to apoptosis. [27]

It is believed that metallothionein is involved in c-DDP acquired resistance, and Ohashi *et al.* observed an increased level of mRNA for yeast metallothionin in cells treated

with copper.[30] However, there was no difference in the cytotoxicity of c-DDP seen when the ACE1 gene, a factor that regulates transcription of the yeast gene for metallothionien, was disrupted.[30] Therefore, a decrease in c-DDP-induced cytotoxicity by copper was not due to induction of metallothionein or to the inhibition of uptake as reported by Ohashi *et al.* [30].

Interestingly, Kabolizadeh *et al.* observed that the effect of copper on p53 induction directly correlated with its differential effects on Pt-mediated cell death (c-DDP and BBR3464).[27] Copper decreased c-DDP-mediated p53 induction by more than 50%, while BBR3464-induced p53 expression remained unchanged. Since c-DDP-mediated activation of the effector caspases downstream of p53 was reduced by copper, while BBR3464-induced caspase activation was enhanced, this effect appeared to be functionally significant. Similarly, expression of the caspase substrate PARP was increased by copper in c-DDP treated samples, mirroring the effect on caspase activation. [27]

The importance of p53 to the differential effects observed with copper addition was made clear by use of p53-deficient HCT116 cells, an isogenic companion set to the HCT116 cells. [27] In the absence of p53, both Pt compounds induced cell death, albeit with a slower time course (72h versus 24h). However, the differential effects of copper on c-DDP and BBR3464 activity were completely lost when p53 was deleted. Moreover, caspase activation and PARP levels in p53-deficient HCT116 cells clarify the differential effects of copper. These data support the conclusion that copper enhances the uptake of both c-DDP and BBR3464, but differentially affects their cellular location or metabolism, therefore, altering p53 activation and cell death. This divergent effect of copper is one

indication that while c-DDP and BBR3464 may share transport pathways, their mechanisms of action, including the means by which they activate p53, are distinct. [27] It is clear now that the decrease in c-DDP-induced cytotoxicity was due to the influence of copper on p53 pathway.

1.4.4. hCTR1 (copper influx transporter) and platinum drugs uptake

As mentioned before, hCTR1 is the cellular influx transporter for copper. A 6-Å projection structure was reported in a phospholipids bilayer for hCTR1 using electron crystallography of 2D protein crystals. This projection showed a <40 A° wide symmetrical trimer, revealing a novel architecture close to the channel protein.[95] Ishida *et al.* has shown that the deletion of the yeast CTR1 gene results in an increased cisplatin resistance as well as decreased cellular accumulation and cisplatin adduct formation in yeast cells.[14] Studies have shown that CTR1 internalization and degradation was triggered by high concentrations of copper.[31,32] The same effect was seen using cisplatin supporting the connection between CTR1 and cisplatin transport. [14,33,34] In contrast, Guo *et al.* showed that c-DDP did not alter hCTR1 endocytosis and degradation and in fact, stabilized a hCTR1 multimeric complex. The connection between hCTR1 and c-DDP was further investigated by Ishida *et al.* using mouse cell line lacking CTR1 alleles indicating increased cisplatin resistance and decreased cisplatin accumulation. [14] Lin *et al* also confirmed the importance of yeast CTR1 in cisplatin and its analogs (carboplatin, oxaliplatin), by showing reduced accumulation of Pt- drugs in CTR1-deficient cells. [35]

Holzer *et al.* have shown the importance of hCTR1 in cisplatin cellular transportation using A2780 ovarian carcinoma cell line.[36] Increased expression of hCTR1 in A2780 cells demonstrated increased cellular cisplatin uptake and marginal increase in cisplatin cytotoxicity. [36] It was also shown by the same group, using A2780 cell lines, that cisplatin triggers hCTR1 down regulation in a concentration and time dependent manner.[33] Kabolizadeh *et al.* confirmed this observation by demonstrating that hCTR1 regulates cisplatin and BBR3464 cellular uptake.[27] A2780/hCTR1 cells were shown to be more sensitive than wild type A2780 to c-DDP-induced apoptosis, and entered a G2 cell cycle arrest more readily in response to BBR3464. It is shown that hCTR1 over-expression in A2780 had little effect on c-DDP-induced growth inhibition by MTT assays, while A2780/hCTR1 cells were more sensitive to BBR3464. Results showed clearly that hCTR1 is an important mediator of both mononuclear and polynuclear platinum drugs. The functional importance of hCTR1 appears to be more important in cell death than cell cycle arrest. [27] Holzer *et al.* published the importance of hCTR1 in cisplatin, carboplatin, and oxaliplatin cellular transportation and their induced cytotoxicity using wild-type murine embryonic fibroblasts ($CTR1^{+/+}$) and its $CTR1^{-/-}$ cell line in which both CTR1 alleles were deleted.[37] Oxaliplatin transportation was shown to be dependant on CTR1 at low concentrations, whereas its dependence faded when high concentrations of oxaliplatin were used, indicating that there may be other means of cellular transportation for oxaliplatin. [37] Sook Song *et al.* extended this study and demonstrated the importance of hCTR1 in cisplatin, carboplatin and oxaliplatin cellular uptake using small cell lung cancer cell line (SCLC) and its cisplatin resistant subline (SR2).[38] In comparison to its sensitive cell line,

hCTR1 expression was reduced while the expression of copper efflux transporters was unchanged in SR2 cell line. Interestingly, the SR2 cell line showed resistance toward cisplatin and carboplatin but not oxaliplatin. Over-expression of hCTR1 by transfection increased SCLC and SR2 cellular uptake of copper, cisplatin, carboplatin as well as oxaliplatin. Unlike c-DDP and carboplatin, oxaliplatin-induced cytotoxicity was not enhanced by hCTR1 over-expression in SR2 cells, indicating that SR2 cells have a unique cellular resistance mechanism to oxaliplatin-mediated cytotoxicity.[38]

In contradiction, it was shown by Beretta *et al.* that over expression of hCTR1 in resistant cell lines (A341/Pt) failed to restore cellular uptake and cytotoxicity of cisplatin. In addition, parental cell line (A341) cellular uptake was not affected by increased hCTR1 expression. These results indicate that hCTR1 does not have an important role in cisplatin cellular transportation and induced cytotoxicity. [26]

Satraplatin is also another important Pt-drug showing activity against prostate cancer cells and maintaining its cytotoxicity against c-DDP resistant cell lines. It was shown that hCTR1 is not involved in JM118, the most abundant species of satraplatin found in plasma, cellular uptake and induced cytotoxicity. [39]

As noted above, published studies indicate the importance of hCTR1 in acquired resistance and cellular uptake of several platinum drugs such as c-DDP, carboplatin, oxaliplatin and BBR3464 (Figure 1.4).

1.4.5. ATP7A, ATP7B (copper efflux transporter)

As discussed before, ATP7B and ATP7A are the cellular efflux transporters for copper. It is important to mention that Zisowsky *et al.* showed that ATP7A, and ATP7B expression in resistant cell lines is cell type specific. [13] The role of ATP7B in c-DDP resistant cells was addressed by Komatsu *et al.* by transfection of human epidermoid carcinoma cells with ATP7B cDNA which resulted in decreased c-DDP cellular accumulation. The transfected cells were 8.9 fold more resistant to c-DDP and 2 fold more resistant to copper in comparison to the mock transfected cells. [40] This observation was further addressed by Katano *et al.* by transfecting ovarian and head and neck carcinoma cells with ATP7B expressing vector, which resulted in increased resistance to c-DDP, carboplatin as well as copper. Similarly, Samimi *et al.* transfected Menkes' disease fibroblasts to express ATP7B. While copper level was reduced, c-DDP, oxaliplatin, and carboplatin cellular levels were increased in the transfected cells, however mainly residing in their vesicular compartments. Cells expressing ATP7B were more resistance to copper, c-DDP and carboplatin but not oxaliplatin. This hypersensitivity to oxaliplatin was supported by an increased level of oxaliplatin reaching DNA. These results confirm that ATP7B is important in delocalization of some platinum drugs to subcellular compartments which results in limiting their cytotoxicity. [41]

Katano *et al.* confirmed the interaction of c-DDP with ATP7B using human ovarian carcinoma cells expressing a cyan fluorescent protein (ECFP)-tagged ATP7B.[42] The ATP7B expression increased cellular resistance to copper and c-DDP and decreased the cellular content of both drugs. The study of confocal digital microscopy showed that

copper and c-DDP exposure relocalized ECFP-ATP7B from perinuclear region to more peripheral vesicular structure. A fluorescein-labeled form of c-DDP colocalized with ECFP-ATP7B in the transfected ovarian carcinoma cells. [42]

The importance of ATP7A in Pt drugs was further addressed by Samimi *et al.* by over-expressing ATP7A in ovarian carcinoma cells. [43] An increase in expression of ATP7A induced resistance to c-DDP, carboplatin, and oxaliplatin in ovarian carcinoma cells; however total cellular platinum level was increased which resulted in increased vesicular content of Pt drugs. Likewise, Samimi *et al* has further shown that JM118, the most abundant species in plasma after oral ingestion of satraplatin, interact with ATP7A and ATP7B using Menkes' disease fibroblast over-expressing ATP7A or ATP7B.[39] Cell over expressing ATP7A or ATP7B, were more resistant to JM118 as well as to c-DDP. This over-expression increased whole cell platinum level after exposure to c-DDP but decreased the amount of platinum in DNA. In case of JM118, cellular platinum level was increased due to over-expression of ATP7A or ATP7B, however, the amount platinum in DNA was also increased, indicating difference in the mechanisms of cellular uptake for c-DDP and JM118. [39]

Kabolizadeh *et al.* extended this study to polynuclear platinum drugs cellular uptake. BBR3464- and c-DDP- induced cytotoxicity was not affected by over-expression of ATP7B in ovarian carcinoma cells and their cellular uptake was decreased slightly. [27] Copper efflux transporters have been shown to have an important role in mononuclear drug cytotoxicity and uptake by either decreasing the cellular content of platinum drugs or by sequestering them into vesicular compartments (Figure 1.4).

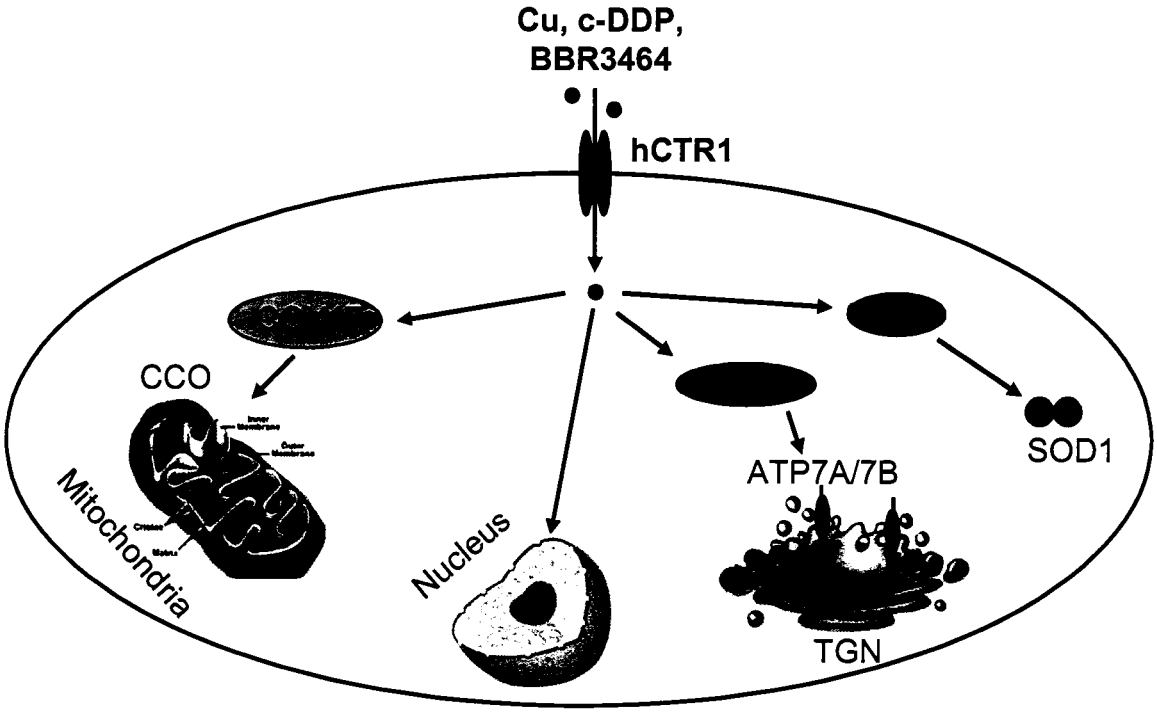


Figure 1.4: Copper Transporters and their cellular function. Copper enters cells *via* hCTR1 and is transferred to chaperones ATOX1, CCS, and COX17. ATOX1, CCS and COX17 transfer copper to ATP7A/ATP7B, Cu-Zn superoxide dismutase (SOD1) and cytochrome c oxidase (CCO) respectively.

1.5. Organic Cation Transporters (OCT)

Another set of transporters which have been recently studied for their effect on platinum drug uptake are the organic cation transporters. These carriers (commonly referred to as OCT1, 2 and 3) are plasma membrane transporters of the SLC22A family and have been characterized functionally and their molecular components identified. [44-46] They mediate movement of a wide variety of organic cations including endogenous compounds such as monoamine neurotransmitters, choline and coenzymes, and also numerous drugs and xenobiotics such as cimetidine and tetraethylammonium (TEA). [45] Some of the cloned organic cation transporters accept one main substrate or structurally similar compounds (oligospecific transporters), while others translocate a variety of structurally diverse cations (polyspecific transporters). [45] In humans, OCT1 is mostly expressed in the liver and to a lesser extent in the intestines. Whereas, OCT2 is mostly expressed in the kidney, OCT3 is expressed in many tissues such as liver, heart, plecenta, and skeletal muscles.[44,47,48]

A common technique to examine the involvement of OCT transporters in cellular uptake of platinum drugs is to use inhibitors such as cimetidine and tetraethylammonium chloride (TEA). TEA, as a competing substrate, significantly decreased the cellular uptake of c-DDP in renal OK cells. [49] Likewise, cimetidine inhibited the decrease in Trans Epithelial Electrical Resistance (TEER) induced by c-DDP in renal MDCK-C7 cells, showing decreased cellular apoptosis. [50] Ciarimboli *et al.* extended this study using human embryonic kidney cortex (HEK) cells transfected with OCT1 or OCT2 and measuring the cellular uptake of ASP (4-[4-(dimethyl-amino)stryil]-methylpyridinium), an organic cation

substrate for OCTs.[51] c-DDP decreased the cellular uptake of ASP in OCT2 transfected cells. Concomitantly, OCT2 transfection increased the cellular uptake of c-DDP and cimetidine decreased c-DDP-induced apoptosis. [51]

Cisplatin and oxaliplatin, but not carboplatin or nedaplatin, have been reported as substrates for human OCTs. The cytotoxicity and cellular uptake of c-DDP was increased in OCT2 transfected human embryonic kidney (HEK) cells and to a lesser extend in OCT1 transfected cells. Those of oxaliplatin were enhanced by OCT2 and OCT3 expression. [52] However, recent studies suggest that OCT1 and OCT2 are important for oxaliplatin uptake but not for c-DDP and carboplatin in transfected canine and human kidney cells. [53] Human OCT 1 and 2 increased the cytotoxicity and cellular uptake of oxaliplatin in transfected cells. In a variety of colon cancer cell lines, the presence of cimetidine decreased cytotoxicity of oxaliplatin, on average 5-11 fold, but had little effect on sensitivity to c-DDP. This differential uptake profile can account for oxaliplatin being active against colorectal tumors, whereas c-DDP is significantly less so. These differences may be attributed in part to enhanced oxaliplatin uptake due to increased expression of OCT1 in colorectal carcinoma cells. The role of OCTs in platinum drug uptake may be both tissue-specific and compound-specific, where the role of carrier ligand is important. [53] Kapp *et al.* further extended this study by examining the involvement of OCTs in cellular uptake of polynuclear platinum compounds.[29] Cimetidine had no effect on transport of c-DDP or a dinuclear platinum compound based on ethylenediamine as a carrier ligand in MCF-7 breast cancer cells. [29] Likewise, TEA did not have any effect on c-DDP cellular uptake but decreased the dinuclear platinum compound cellular uptake by 20%. [29]

The data published provides evidence that organic cation transporters mediate resistance and cellular uptake of c-DDP and oxaliplatin by regulating drug influx. Oxaliplatin is indicated to be a better substrate for organic cation transporters than c-DDP, one reason perhaps why oxaliplatin is more cytotoxic and efficient in colorectal carcinoma cells.

1.6. The Polyamine Pathway and Uptake of Platinum Drugs

1.6.1. Polyamine pathways

Another important pathway that is involved in cellular uptake and cytotoxicity of Pt drugs is the polyamine transport pathway. Among the genes up-regulated by c-DDP and oxaliplatin exposure, there are metabolic pathway genes including those in polyamine pathways such as Spermidine/spermine N1-acetyltransferase (SSAT).[54] Spermidine and spermine are organic cationic polyamines that are essential for cellular proliferation. [55] Deletion of such cellular polyamines resulted in growth inhibition. [56-59] SSAT alters spermidine and spermine by acetylation resulting in their cellular export. [60] It is indicated that polyamines have some implication in cancer since their cellular levels are higher in cancer cells than in normal cells. [61-63] The induction of SSAT mRNA by c-DDP and oxaliplatin was related to the drug-induced growth inhibition in ovarian carcinoma cells, A2780. [54] Likewise, Varma *et al.* showed down-regulation of polyamine biosynthetic pathway genes and up-regulation of a catabolic pathway gene (SSAT) in ovarian carcinoma cells after exposure to c-DDP or oxaliplatin.[64] Roberts *et al.* extended this study by investigating the importance of polyamine pathways and their transporters in

induced cytotoxicity of c-DDP and polynuclear platinum drugs such as BBR3464 and BBR3571.[65] The cytotoxicity of Pt drugs were studied in polyamine uptake deficient murine cells (L1210/MGBG) which are also resistant to the polyamine synthesis inhibitor, methylglyoxal-bis(guanylhydrazone) (MGBG). While the cytotoxicity of c-DDP was not affected, BBR3464- and BBR3571- induced cytotoxicity was significantly increased in L1210/MGBG cells. These results indicate the minor role of polyamine transporters in BBR3464 and BBR3571 cellular uptake and the importance of cellular polyamines in their induced cytotoxicity. [65] The studies published indicate the significant role of polyamine pathways in Pt- drugs-mediated cytotoxicity.

1.7. Endocytosis and Interaction of Platinum Drugs with Membranes

In addition to transporters, cellular uptake of Pt drugs can occur *via* endocytosis. Endocytosis is a process by which cells take up nutrients and materials via engulfing their membrane around them forming a small membrane bound vesicle (endosome). Endocytosis can be divided into three general types: pinocytosis, receptor mediated endocytosis, and phagocytosis. Pinocytosis refers to cells taking up a small amount of extracellular fluid containing dissolved materials. However, in receptor mediated endocytosis, a specific receptor on the cell membrane binds specifically to a molecule (ligand) and then the plasma membrane undergoes endocytosis (vesicle formation). Phagocytosis also refers to the same process in which larger molecules such as bacteria or other molecules are internalized to be degraded by lysosomes. [66-68]

Positively charged molecules are likely to be adsorbed due to their interactions with negatively charged cellular membrane which can result in adsorptive endocytosis. [69] It has been recently shown by Liu *et al.* that the interactions of BBR3464 (Structure, Figure 1.1) with phospholipid membrane models were significantly stronger than c-DDP.[70] Such interactions may represent a possible mechanism of uptake for highly-charged drugs. Kapp *et al.* extended this study by examining the effect of endocytosis inhibitors in a multinuclear platinum compound (*m*-4F-PtDMSO-DAH) cellular uptake.[29] Inhibitors of macropinocytosis such as amiloride, N-ethyl-N-isopropylamiloride (EIPA), wortmannin and cytochalasin D decreased the cellular uptake of *m*-4F-PtDMSO-DAH significantly, indicating the importance of endocytosis. They results provide strong evidence that endocytosis may have a significant role in the cellular uptake of charged polynuclear platinum drugs and their cytotoxicity.

1.8. Importance of Chemical Status in Platinum Drugs Uptake

The chemical status of platinum drugs can alter their cellular uptake profile. Generally, c-DDP is mixed with 154 mM aqueous NaCl in its clinical formulation which also contains a monoaquated species of c-DDP (Figure 1.5). [71] Under physiological condition, a larger concentration of monoaquated species will be formed due to the lower chloride concentration (~105mM) in blood. [71] Pereira-Maia *et al.* has shown that cellular uptake of the aquated forms of c-DDP was significantly higher, with cellular aqua-Pt concentration being 20-30 times higher than the extracellular aqua-Pt form.[72] Furthermore, using different agents modulating membrane potential, it was shown that the aquated form of

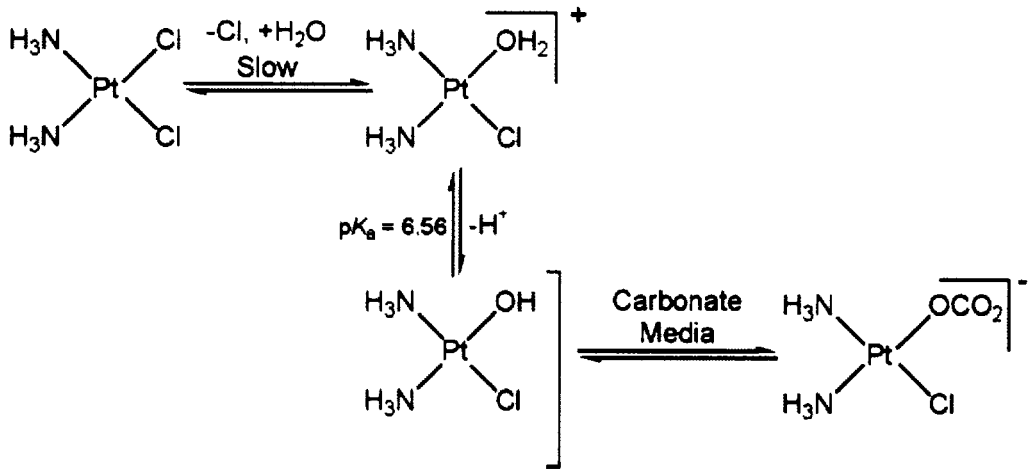


Figure 1.5: Structure of different chemical status of cisplatin

c-DDP accumulates inside the mitochondria indicating the energy and membrane potential-dependence of this process. [73] It was further shown by Palmer *et al.* and Acharya *et al.* that the aquated form of c-DDP will react with carbonate in culture media to form the carbonato complex (Figure 1.5). [74-76] To extend this study, Centerwall *et al.* demonstrated that Jurkat cells rapidly modify the influxed monocarbonato form of c-DDP in culture followed by their efflux. Jurkat cells were unable to reuptake the modified version and this indicates the defense mechanism of Jurkat cells to prevent the carbonato complex to reach the cytosol or DNA by modifying and perhaps inactivating it. This implicates the importance of the carbonato complex and perhaps the importance of chemical status of c-DDP in its induced cytotoxicity and cellular uptake. [77]

1.9. Intracellular Localization and Imaging of Platinum Drugs

Because the mechanism of cellular uptake of Pt drugs is not fully understood, studying the intracellular distribution of Pt compounds is essential in identifying the compartments in which Pt drugs reside. Digital fluorescence microscopy has been used to examine the intracellular distribution using Pt drugs with fluorescent tag. Safaei *et al.* demonstrated fluorescein labeled c-DDP (F-DDP) sub-cellular distribution in ovarian carcinoma cells.[78] F-DDP was localized in vesicles, Golgi and lysosomes and colocalized with vesicle expressing ATP7A. Vesicle trafficking inhibitors such as brefeldin and wortmannin, increased F-DDP in pre-Golgi associated vesicles. Concomitantly, whole cellular uptake of F-DDP was increased by inhibitors such as brefeldin and wortmannin.[78] Safaei *et al.* further compared the lysosomal compartments of c-DDP sensitive ovarian carcinoma cells

with c-DDP resistant cells. The c-DDP resistant cells had 60% less lysosomal compartments in compare to that of sensitive parental cells. Furthermore, c-DDP resistant cellular exosomes contained 2.6 fold more platinum than those from parental cells. The released exosomes also had higher levels of ATP7A and ATP7B transporters, showing the importance of the efflux copper transporter in c-DDP cellular efflux. [79]

Kalayda *et al.* extended this study to examine the polynuclear platinum complexes with fluorescence tags.[80] One hour after treatment, the dinuclear platinum drugs reached the nucleus, but the fluorescence intensity weakened with time, indicating the drugs clearance and delocalization from nucleus to other cellular compartments. The drugs accumulated and localized into the Golgi compartments several hours later and their localization was still observed 24h after internalization. Perhaps the Golgi is the area in which Pt drugs may accumulate before being exported out by exocytosis. [80]

Studies have shown that cellular uptake of polynuclear platinum drugs is enhanced in comparison to c-DDP; perhaps one reason for their higher cytotoxicity. [27,65,81-84] Harris *et al.* further indicated that cellular uptake of highly charged trinuclear platinum compounds was greater than c-DDP and was enhanced further through increasing the charge of the drug by addition of amine moieties.[83] In contrast, addition of a hydrophobic anthraquinone (1C3) moiety to the mononuclear Pt complex sequesters the Pt drug into the lysosomes preventing its accumulation in the nucleus. In conclusion, the Golgi and lysosomes play important roles in cellular resistance toward Pt drugs. In addition, carrier ligands play an important role in Pt drugs cellular uptake and their intracellular distribution.

1.10. Importance of Platinum Drug Uptake in Clinical Outcome

c-DDP, oxaliplatin, and carboplatin are effective clinical anticancer drugs. However, development of resistance is the major cause of treatment failure as well as reducing Platinum drug clinical efficacy. The mechanism of such resistance has not been characterized. However, the most common feature of c-DDP resistant cells is decreased cellular uptake of the drug. Influx copper transporter, hCTR1 and efflux copper transporters such as ATP7A, and ATP7B have been indicated to be involved in cellular transportation of such anticancer drugs. As it was stated previously (sec. 1.3), a strong link between the expression of copper transporters and cellular resistance has been shown. Therefore, it is essential to explain the possible role of copper transporters in patient survival and clinical resistance to Pt drugs.

It has been reported that ATP7B was over-expressed significantly in ovarian carcinoma cells of patients with moderately /poorly differentiated cancers treated with c-DDP.[85,86] Interestingly, patients with higher gene expression of ATP7B before treatment had poorer prognosis than those with low ATP7B gene expression. This indicates a possible role of ATP7B as an independent prognostic factor in patients with ovarian carcinoma. [85,86] Similarly, ATP7B was over-expressed in breast or gastric carcinoma cells in compare to normal adjacent tissue cells, and its expression was higher in patients with poorly differentiated carcinomas than that in well /moderately differentiated carcinomas.[87,88]

Intrinsic or acquired resistance to c-DDP in oral squamous cell, esophageal, and endometrial carcinomas is one of the major causes of treatment failure.[89-91] Patients over-

expressing ATP7B had a significantly inferior response to chemotherapy in comparison to patients with ATP7B-negative tumors resulting in lower survival rate for ATP7B-positive patients.[89-91] Likewise, the expression of ATP7B correlated with decreased responsiveness to c-DDP in patients with esophageal carcinomas. [90]

Samimi *et al.* extended this study to another copper efflux transporter, ATP7A by indicating that its over-expression before treatment resulted in lessened actuarial survival in patients with ovarian carcinoma. [92] In contrast to copper efflux transporters, the influx transporter (hCTR1) did not show any significance role in cancer chemotherapy as its expression was not enhanced in malignant tissues. [93] These results show the important role of efflux copper transporter in c-DDP acquired clinical resistance and in patient survival rate.

1.11. Concluding Remarks and the Doctoral Projects

Platinum drugs play an important role in the treatment of cancer but, due to acquired resistance, their clinical efficacy is limited. Cellular uptake is an important factor involved in clinical resistance to Pt drugs. As it is explained in previous sections (Figure 1.6), the transportation of platinum drugs depends on multiple factors. Platinum drugs influx is mediated through the copper transporter, hCTR1, organic cation transporters and endocytosis. On the other hand, cellular efflux of Pt drugs is mediated through copper transporters ATP7A and ATP7B and organic cation transporters. The chemical status of platinum drugs, especially c-DDP, also plays an important role in their cellular uptake.

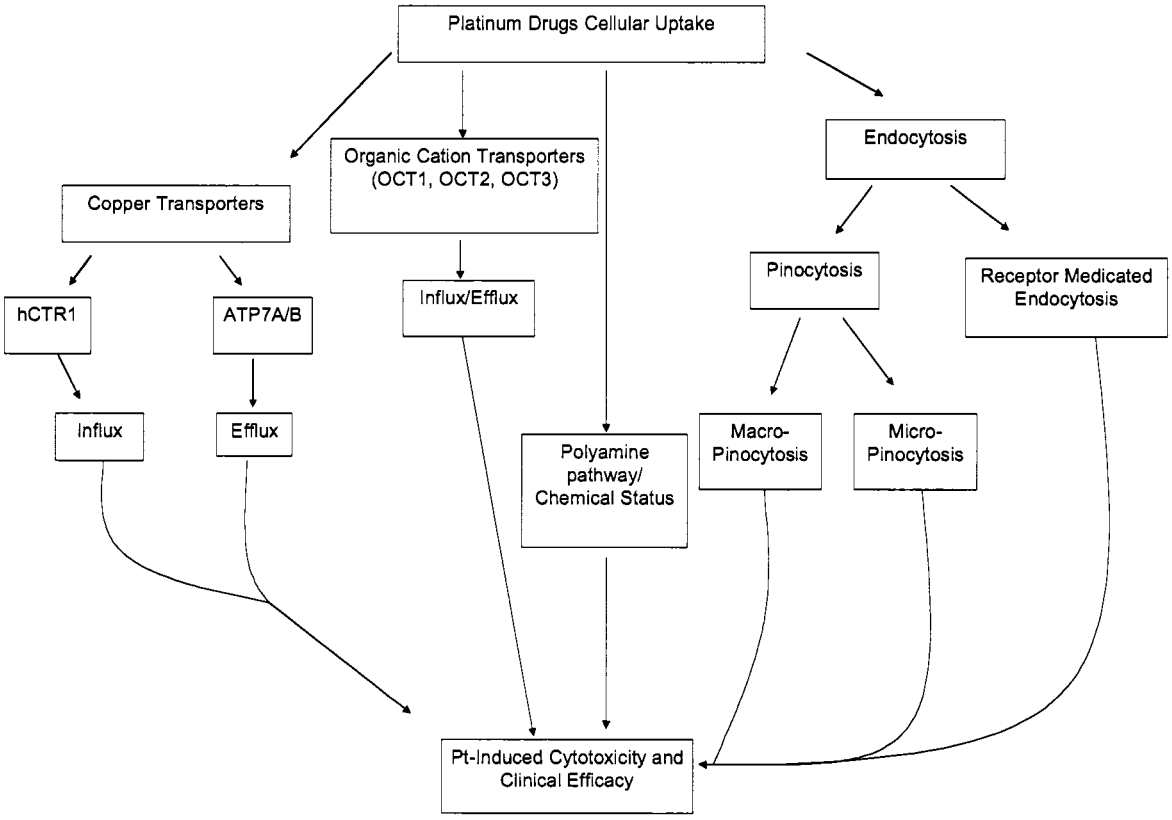
Multiple pathways can contribute to platinum drugs cytotoxicity, uptake and intracellular localizations.

The objective of this thesis was to investigate accumulation mechanisms of polynuclear platinum drugs as well as their mechanism of actions. Chapter 1 of this thesis presents an overview of platinum drugs and their cellular uptake which is in preparation for publication as a review. In chapter II, the importance of copper transporters in BBR3464 cellular uptake and its distinct mechanism of action were examined. The work of chapter 2 was completed and published in the journal, Biochemical Pharmacology. [27]

Chapter 3 addresses the importance of organic cation transporters in BBR3464 cellular uptake and its induced cytotoxicity. The interesting synergistic effect of an antidepressant, desipramine on Pt-induced cytotoxicity was further addressed. The work of chapter 3 was submitted to Cancer Research.

The studies in chapter 4 address the non-covalent binding drugs such as AH78, and their mechanism of cellular uptake and cytotoxicity. The research of the section focuses on the difference between AH78 and other anticancer drugs, BBR3464, c-DDP, and oxaliplatin. These studies are being prepared for publication in Cancer Research.

Studies of chapter 5 addresses the importance of endocytosis and signal transduction pathways in BBR3464 cellular uptake and its mediated cytotoxicity. Studies in Appendix 1 describe the biological consequences of another polynuclear platinum drug BBR3610 in comparison to BBR3464. These studies have been published in the journal, Molecular Pharmacology. [94]



Scheme 1.1: Different routes of platinum drugs cellular uptake

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List of References

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CHAPTER 2: Differences in the Cellular Response and Signaling Pathways of Cisplatin and BBR3464 ([{*trans*-$PtCl(NH_3)_2$}$_2\mu$-(*trans*-$Pt(NH_3)_2(H_2N(CH_2)_6\text{-}NH_2)_2$)]$^{4+}$) Influenced by Copper Homeostasis

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2.1 Abstract

[{*trans*-$PtCl(NH_3)_2$}$_2\mu$-(*trans*-$Pt(NH_3)_2(H_2N(CH_2)_6\text{-}NH_2)_2$)]$^{4+}$ (BBR3464) is a cationic trinuclear platinum drug that is being evaluated in phase II clinical trials for treatment of lung and ovarian cancers. The structure and DNA binding profile of BBR3464 is different from drugs commonly used clinically. It is of great interest to evaluate the difference between the mechanisms of uptake employed by BBR3464 and cisplatin (c-DDP), as altered uptake may explain chemoresistance. Using transfected cell lines, we show that both c-DDP and BBR3464 use the copper transporter hCTR1 to enter cells and to a lesser extent, the ATP7B transporter to exit cells. Copper influenced c-DDP and BBR3464

uptake similarly; it increased the c-DDP and BBR3464 uptake in ovarian (A2780) and colorectal (HCT116) carcinoma cell lines as detected by ICP-OES. However, the effects of copper on c-DDP- and BBR3464-mediated cytotoxicity differed. Copper decreased c-DDP-induced apoptosis, caspase-3/7 activation, p53 induction and PARP cleavage in cancer cell lines. In contrast, copper increased BBR3464-induced apoptosis, and had little effect on caspase activation, PARP cleavage, and p53 induction. It was concluded that BBR3464 employs mechanisms of intracellular action distinct from c-DDP. Although these drugs use the same cellular transporters (hCTR1 and ATP7B) for influx and efflux, downstream effects are different for the two drugs. These experiments illustrate fundamental differences in the mechanisms of action between cisplatin and the novel Pt-based drug BBR3464.

2.2 Introduction

Cisplatin (c-DDP) is an effective antineoplastic agent that is used for treatment of cancer, including testicular, head and neck, ovarian, and small cell lung neoplasms.[1] Its cytotoxicity is mediated mainly through interactions with DNA and inhibition of DNA synthesis and replication by formation of bifunctional interstrand and intrastrand cross links.[2,3] Its efficacy is limited due to acquired resistance and dose-limiting side effects, mainly nephrotoxicity.[4] Structurally novel platinum complexes that bind to DNA differently than c-DDP may have distinct cytotoxicity and side effect profiles. The trinuclear complex, BBR3464, is one example of the polynuclear class of platinum drugs in which the platinum coordination units are linked by alkanediamine chains.[4] BBR3464 is

significantly more cytotoxic than c-DDP and retains activity against c-DDP-resistant cell lines and tumors *in vitro* as well as *in vivo*.[6,7] The drug has undergone Phase II clinical trials in cisplatin-resistant and refractory cancers.

Acquired resistance to c-DDP in patients has been the topic of intensive research. The three major pharmacological factors contributing to the intrinsic cytotoxicity of, and cellular resistance to, platinum drugs are (i) cellular uptake and efflux of platinum; (ii) the frequency and nature of Pt-DNA adducts; and (iii) deactivating metabolic reactions with sulfur-containing nucleophiles. The role of cisplatin uptake and efflux is increasingly being seen as a critical determinant of clinical resistance.[8] Highly charged polynuclear platinum drugs have recently been shown to display higher cellular uptake than c-DDP – a factor which may contribute to their enhanced efficacy.[9] The factors affecting the differential cellular uptake of mononuclear and polynuclear platinum drugs are likely to be multiple. Recently, the interactions of c-DDP and BBR3464 with phospholipid membrane models showed significantly stronger interactions for the trinuclear drug [10] – such interactions may represent a possible mechanism of uptake for highly-charged drugs.

A second possible differential factor is the relationship between Cu homeostasis and platinum drug uptake. Copper is critical for cellular functions such as electron transport, oxygen activation and reactive oxygen detoxification.[11] Free copper is toxic to cells but the free intracellular copper concentration is maintained as low as 10^{-18}M by sequestration and binding to chaperones.[12,13] Copper enters cells by hCTR1 (human copper transport protein 1) and is then delivered to the chaperones.[14,15] Copper exits cells by ATP7A and ATP7B transporters (P-type ATPase cation transporters). Mutation in

ATP7A or ATP7B results in Menkes' or Wilson's disease, respectively.[16,17] hCTR1-mediated copper (and c-DDP) transportation is affected by temperature as well as extracellular proton (pH) and potassium ion concentration.[18]

Since c-DDP-resistant cells often show impaired drug uptake, attention has been directed toward understanding the mechanism of c-DDP cellular uptake and transport.[19-22] Cells resistant to c-DDP show cross-resistance to copper, indicating the possibility of shared transporters.[22,23] In fact, recent studies demonstrated that c-DDP uptake is in part regulated by the hCTR1 and ATP7B copper transporters in human ovarian carcinoma cells.[24,25] Since the efficacy of BBR3464 in c-DDP-resistant cells may be related to enhanced cellular uptake,[9] it is important to understand the role of copper and its transporters in the cellular action of BBR3464. This paper compares the influence of copper and its transport mechanisms on c-DDP and BBR3464 cellular uptake and cytotoxicity, as well as downstream effects on p53 and caspase activation. The study reveals novel and clinically significant differences in c-DDP and BBR3464-mediated cell death and the role of copper in this process.

2.3 Materials and Methods

2.3.1 Compound Synthesis.

Drug compounds were synthesized according to methods reported previously,[26] Fig. 2.1. Copper was obtained in the form of Copper (II) sulfate pentahydrate from Sigma-Aldrich (St. Louis, MO).

2.3.2 Cell System.

Ovarian carcinoma A2780/hCTR1, 2008/pRC/CMV7B (2008/ATP7B), 2008/EV pRC/CMV (2008) cells were the kind gift of S. Howell, University of California at San Diego. These cell lines were cultured with 500 μg/ml G418. The colorectal carcinoma cell lines HCT116, and matched p53-deficient HCT116 cells (HCT116-/-) were the kind gift of Bert Vogelstein (Johns-Hopkins University, Baltimore, MD). HCT116 cells, ovarian carcinoma cells (A2780), head and neck carcinoma cells (HN22), mastocytoma cells (PDMC-1) and breast carcinoma cells (MDA-MB-435) were cultured with RPMI 1640 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) in humidified air with 5% CO2. HN22 and MDA-MB-435 were obtained from D. Lebman and S. Deb respectively (VCU, Richmond, VA).

2.3.3 Growth Inhibition Assay (Detection of cell proliferation).

Cells were cultured at 10000 cells per well in a 96-well microplate. They were incubated with different concentration of c-DDP and BBR3464 for 72h. Drug-containing medium was aspirated and cells were washed with PBS twice before the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, sigma chemicals) to each well. Cells were incubated with 100μl of MTT solution (2mg/1ml PBS) for 3h. MTT solution was aspirated and 100μl of DMSO (Sigma chemicals) was added to each well for determination of viable cell number through measuring the absorbance at 490nm.

2.3.4 Drug Cytotoxicity Assay.

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 and the sub-diploid DNA content was measured by PI DNA Staining, as described below. c-DDP and BBR3464 concentrations were adjusted to achieve approximately 25% apoptosis, allowing us to measure enhancement or inhibition.

2.3.5 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.[27] Samples were then analyzed for subdiploid DNA content 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.

2.3.6 Caspase Activation Assays.

Staining for active caspases was performed using caspase kits (Immunochemistry Technologies, LLC, Bloomington, MN), as specified by the manufacturer. Cells were incubated with a cleavable substrate that binds to the active caspases-3 and -7. Substrate

cleavage results in increased fluorescence intensity, which is interpreted as caspase-positive cells. The percentage of caspase-positive cells was measured by flow cytometry.

2.3.7 Platinum Accumulation Assays.

Cells were plated at 2.0×10^6 cells/ml. BBR3464 or c-DDP was added in different concentrations alone or 60 minutes after the addition of copper (II) sulfate. After 8 or 16h cells were harvested 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 simultaneous inductively coupled plasma optical emission spectroscopy (ICP-OES) at 265 nm (Varian Inc., Palo Alto, CA). Standards and blank were prepared the same as the samples.

2.3.8 Western Blotting.

Whole-cell lysates were blotted with goat polycolonal antibody against hCTR1 (1:500) (Santa Cruz Biotechnology, INC.), rabbit polycolonal antibody against ATP7b (1:1000) (NOVUS Biologicals), mouse monoclonal antibody against p53 (Cell Signaling Technology), monoclonal antibody against poly-ADP ribose polymerase (PARP) (Trevigen, Inc.) or mouse monoclonal antibody against β-actin (Sigma-Aldrich, St. Louis, MO) and resolved with secondary antibody conjugated with horseradish peroxidase. Blots were then treated with a chemiluminescent substrate (Pierce, Rockford, IL, USA) and

exposed to film. Band intensity was measured by densitometry using an Eagle Eye II system (Stratagene, La Jolla, CA).

2.3.9 Statistical Analysis:

Results are the mean and standard error. Statistical analysis was performed using *t*-test for two data points using SysStat9 software (SPSS, Chicago, IL, USA). $p < 0.05$ was considered to be significant.

2.4 Results

2.4.1 Importance of Copper and Copper Transporters in c-DDP and BBR3464 Uptake and Cytotoxicity.

2.4.1a Influence of hCTR1

hCTR1 has been shown to mediate c-DDP cellular uptake.[24] The influence of hCTR1 in c-DDP versus BBR3464 cellular metabolism was determined by comparing uptake levels in the parental ovarian carcinoma cell line A2780 with A2780 cells over-expressing hCTR1 (A2780/hCTR1). As shown in Fig. 2.1A, hCTR1 is over-expressed 3.5-fold in A2780/hCTR1 cells. Cells were cultured for 16 h in the presence of c-DDP or BBR3464, and platinum uptake was determined by ICP-OES. Platinum levels in c-DDP-treated cells were 1.8 times greater in A2780/hCTR1 cells than in A2780 cells, while BBR3464 uptake was increased 2.6-fold in A2780/hCTR1. These data indicated a role for hCTR1 in transport of both compounds (Fig. 2.1B,C).

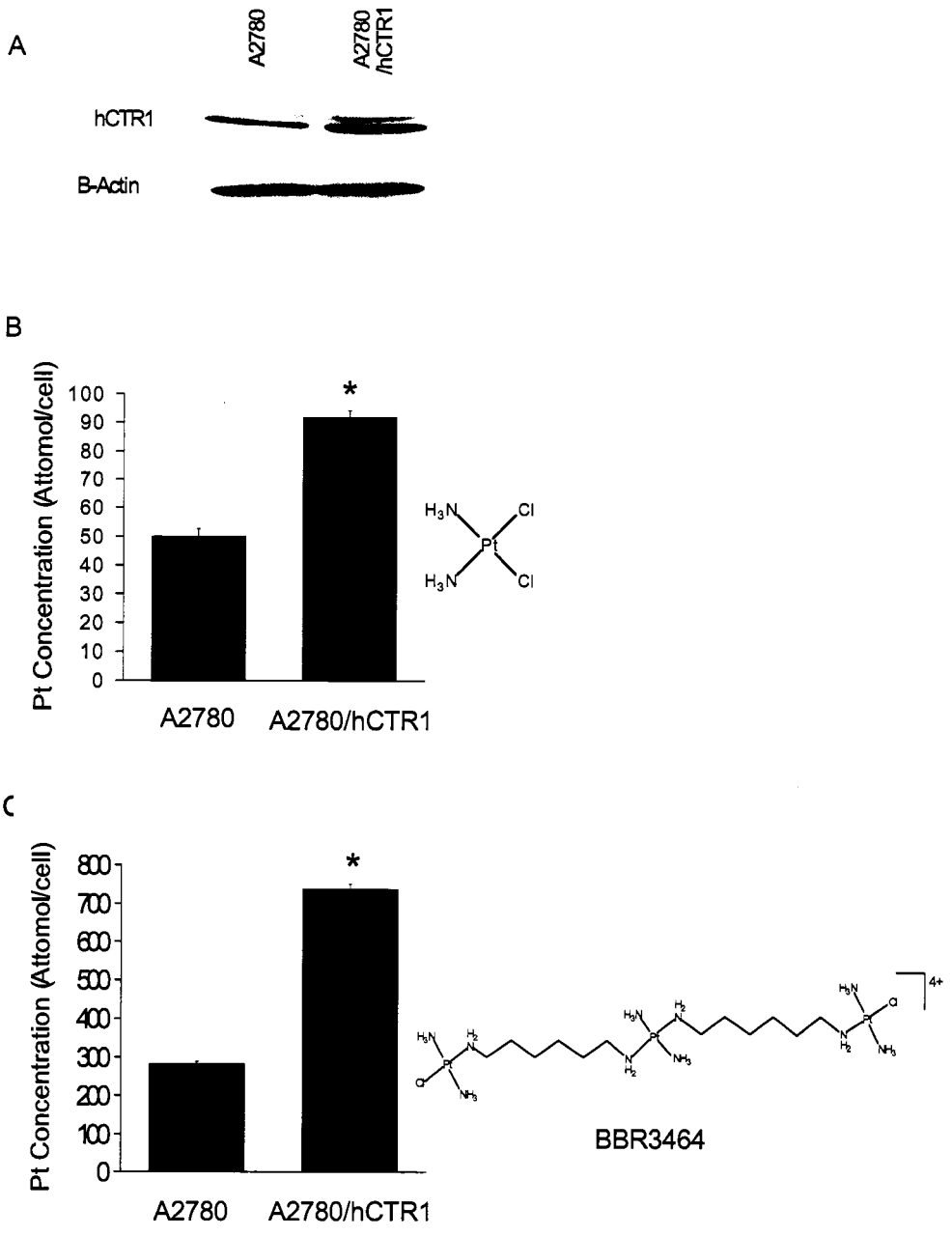

Figure 2.1. The importance of hCTR1 in platinum drug uptake in ovarian carcinoma cells. A. The expression of hCTR1 in A2780 and A2780/hCTR1 was detected by western blot. The membrane was stripped and re-probed for β-actin to show loading. B. c-DDP uptake was measured by ICP in 20 million cells treated with 10 μM c-DDP for 16h before harvesting. C. BBR3464 uptake in A2780 and A2780/hCTR1 cells treated with 10μM BBR3464. Each bar indicates the average (+/- SEM) of three independent experiments. *, $p<0.05$. The structure of c-DDP and BBR3464 are illustrated in B and C.

To determine if the increase in cellular uptake observed in hCTR1-transfected cells was functionally significant, we measured c-DDP- and BBR3464-mediated apoptosis in the A2780 and A2780/hCTR1 carcinoma cells. We also measured cell cycle arrest after treatment with BBR3464, as this effect was more pronounced than apoptosis.[28] DNA fragmentation and G_2 cell cycle arrest were measured by PI-DNA staining. A2780/hCTR1 cells were more sensitive to c-DDP- and BBR3464-induced apoptosis (c-DDP IC_{50} = 3μM, BBR3464 IC_{25}=<0.5μM) than parental A2780 cells (c-DDP IC_{50}=6μM, BBR3464 IC_{25}=10μM), (Fig. 2.2). Additionally, there was 2-3 fold more G_2 arrest in A2780/hCTR1 cells treated with low concentrations of BBR3464 (0.5μM) than in parental A2780 cells (Fig. 2.2B). The BBR3464 cytotoxicity data shows a more sudden increase in apoptosis reaching platue at low doses. The Data in figure 2.2B,C confirms that G2 cell cycle arrest plays a more important role in BBR3464-induced cytotoxicity than apoptosis since percent of cells in G2 cell cycle arrest was increased as more concentration of BBR3464 was used. Moreover, perhaps there is a possibility of hCTR1 saturation at lower doses of BBR3464. Note that growth inhibition concentrations for BBR3464 in A2780 cells have been previously shown to be lower than those for c-DDP.[29] These results support a role for hCTR1 in the efficacy of both c-DDP and BBR3464.

2.4.1b Influence of ATP7B.

The copper transporter ATP7B has been shown to be important for reducing cytosolic levels of c-DDP by promoting cellular efflux.[25] The role of ATP7B in BBR3464

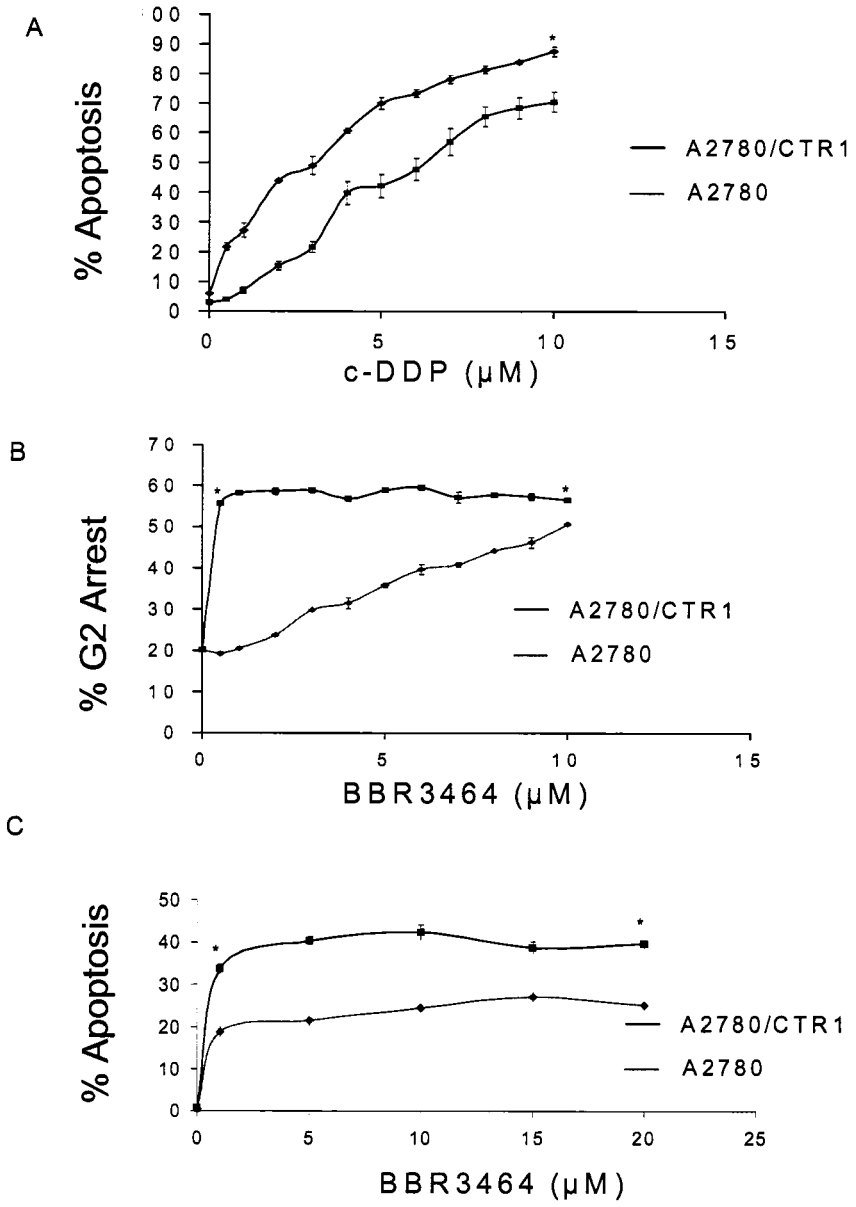

Figure 2.2 The effects of hCTR1 expression on c-DDP- and BBR3464-induced apoptosis and cell cycle arrest. In A and B A2780 and A2780/hCTR1 cells were cultured in the indicated concentrations of c-DDP and BBR3464 for 72h. DNA content was measured by PI-DNA staining to determine apoptosis (sub G_0/G_1) and G2 arrest by flow cytometry, as described in Materials and Methods. C. A2780 and A2780/hCTR1 cells were cultured in the presence of BBR3464 for 96h and sub-diploid DNA content was determined as an indicator of apoptosis. Each point is the average (+/- SEM) of three independent experiments. In each figure, A2780 and A2780/hCTR1 responses were found to be significantly different by Student's t-test; *, $P<0.05$.

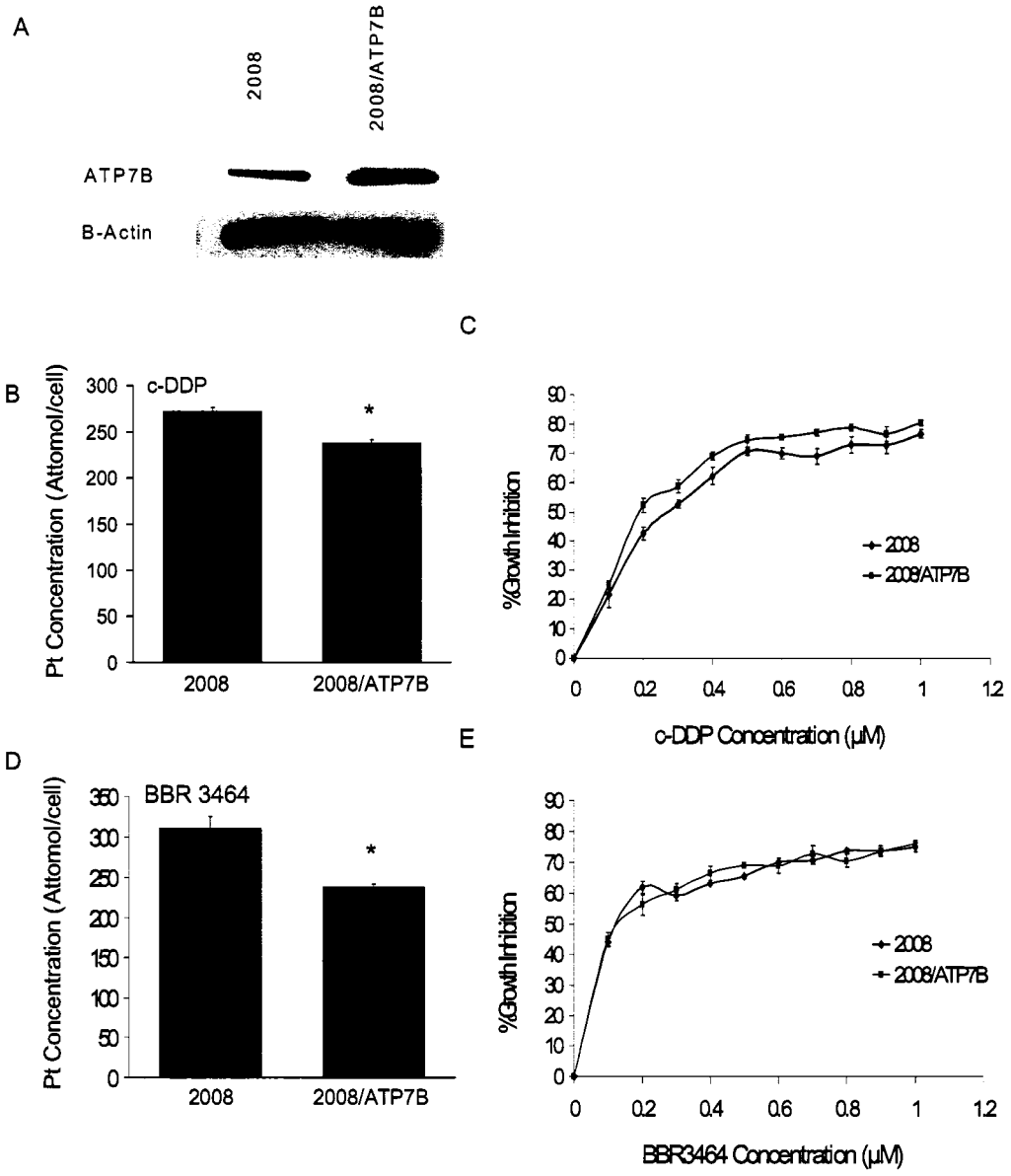

Figure 2.3 Effect of ATP7B transporter on platinum drug retention and Pt-mediated growth inhibition. A. The expression of ATP7B in 2008 ovarian cells was detected by western blot. The membrane was stripped and re-probed for β-actin. B, D. Cellular platinum levels after treatment with 20μM c-DDP or 10μM BBR3464 for 24h were determined by ICP. Each bar indicates the average (+/- SEM) of three independent experiments. In C and E, 2008/pRC/CMV7B ("2008/ATP7B") and 2008/EV pRC/CMV ("2008") cells were treated with the indicated concentrations of c-DDP and BBR3464. Percent growth inhibition was determined by comparing live cell numbers in treated and untreated cultures after 72h, as measured by PI-DNA staining and timed counting via flow cytometry. Each point is the average (+/- SEM) of three independent experiments. *, $p<0.05$ as determined by t test.

metabolism was examined by comparing cellular platinum levels in the parental ovarian carcinoma cell line (2008) to 2008/ATP7B, transfected to over-express ATP7B. As shown in Fig. 2.3A, ATP7B expression is 2-fold greater in 2008/ATP7B cell than in parental cells. This over-expression conveyed a small but reproducible decrease in cellular platinum levels when cells were treated with c-DDP or BBR3464 (Fig. 2.3B,D).

The sensitivity of the 2008 cell lines was examined by measuring growth inhibition, calculated as the decrease in live cell numbers measured *via* PI-DNA staining. Despite the reduction in cellular platinum levels, ATP7B over-expression conveyed no difference in sensitivity to treatment with either c-DDP or BBR3464 (Fig. 2.3C,E).

2.4.1c Effects of Copper on c-DDP and BBR3464 Uptake and Cytotoxicity in Different Cell Lines.

Besides generation of paired cell lines with over-expression of the copper transporters, the influence of copper on platinum drug uptake illustrates how the cellular effects of platinum drugs are modulated. c-DDP and BBR3464 uptake was measured in carcinoma cell lines treated with or without copper prior to the addition of platinum drugs (Fig. 2.4). Copper (as Cu^{2+}) enhanced the uptake of both c-DDP and BBR3464 in the parental ovarian carcinoma cell line (A2780) (Fig. 2.4A, C). In extension to other cell lines, the colorectal carcinoma cell line HCT116 also demonstrated enhanced Pt uptake in the presence of copper, with the effects being more pronounced for c-DDP than for BBR3464 (Fig. 2.4B, D).

We next determined if the enhanced Pt uptake induced by the presence of copper had a commensurate effect on apoptosis. No significant cell death occurred at specified

time points of 16h and 8h, where uptake of c-DDP and BBR3464 were measured, respectively. At later time points (24h), significant differences appeared between the two drugs. These differences were apparent at the concentrations used for uptake measurements but are seen more clearly at higher platinum drug concentrations. Surprisingly, and despite the enhanced uptake, c-DDP-induced apoptosis was inhibited by the presence of copper (Fig. 2.5A,B). At the highest copper concentration studied, c-DDP-induced apoptosis was decreased by 61% and 81% in A2780 and HCT116 cells respectively, relative to c-DDP-alone (Fig. 2.5A,B). In contrast to the inhibitory effects with c-DDP, BBR3464-induced apoptosis was increased by prior copper treatment, paralleling the enhanced uptake. The effects of BBR3464 and copper appeared to be additive rather than antagonistic. At the highest copper concentration studied, BBR3464-induced apoptosis was increased by 74% and 40% in A2780 and HCT116 respectively, relative to BBR3464-alone (Fig. 2.5C,D). This divergent effect of copper on c-DDP- and BBR3464-mediated apoptosis was consistent across a variety of cell lines, including PDMC1 (mastocytoma), HN22 (head and neck), and MDA-MB-435 (breast) tumor cells (Fig 2.6).

2.4.2 Effect of Copper on c-DDP- and BBR3464-mediated p53 Activation.

Given its divergent effects on apoptosis, the biological mechanisms responsible for the different effects of copper on c-DDP- and BBR3464-induced cell death were examined. Many apoptotic signaling pathways converge at the transcription factor p53. p53 elicits cell death in part by inducing mitochondrial damage that activates the death effector

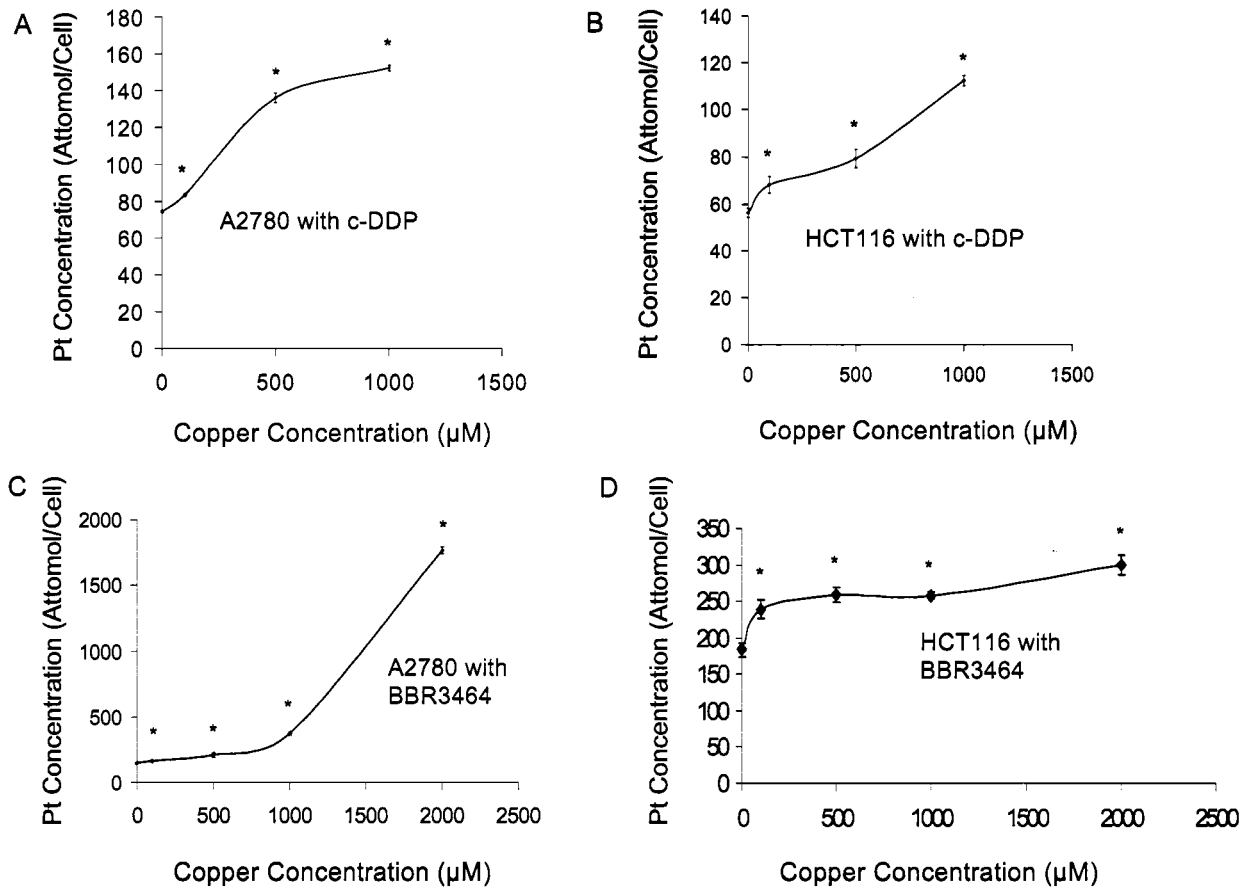

Figure 2.4 Effect of copper on c-DDP and BBR3464 uptake in A2780 and HCT116 carcinoma cell lines. In A and B, A2780 or HCT116 cells were cultured with 10 or 20μM c-DDP, respectively, for 16h in the absence or presence of copper. In C and D, A2780 or HCT116 cells were treated with 10 or 20μM BBR3464, respectively, for 8h. Drugs were added to the media after 1h of treatment with copper. At least 95% of cells were viable at these time points as measured by PI-DNA staining. Each point represents the average (+/- SEM) of three independent experiments. *, $p<0.05$ when comparing cells treated with and without copper, by Student's t-test.

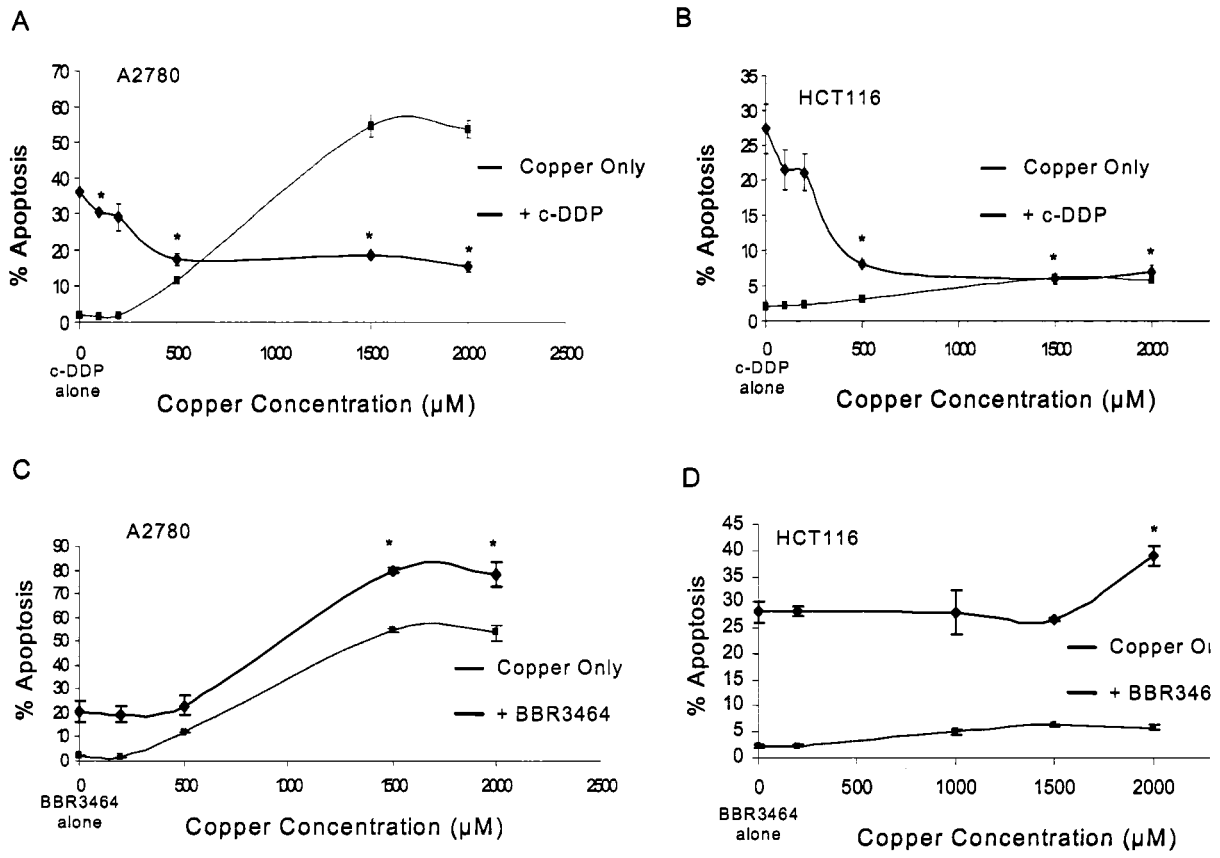

Figure 2.5 Effect of copper on c-DDP- and BBR3464-induced apoptosis in A2780 and HCT116 carcinoma cell lines. Sub-diploid cell content was detected by PI-DNA staining. In A and B, A2780 or HCT116 cells were cultured with 20 or 40μM c-DDP, respectively, for 24h in the absence or presence of copper. In C and D, A2780 or HCT116 cells were treated with 40 or 50μM BBR3464, respectively, for 24h. Drugs were added to the media after 1h of treatment with copper. Each point represents the average (+/- SEM) of three independent experiments. *, $p<0.05$ when comparing cells treated with and without copper, by Student's t-test.

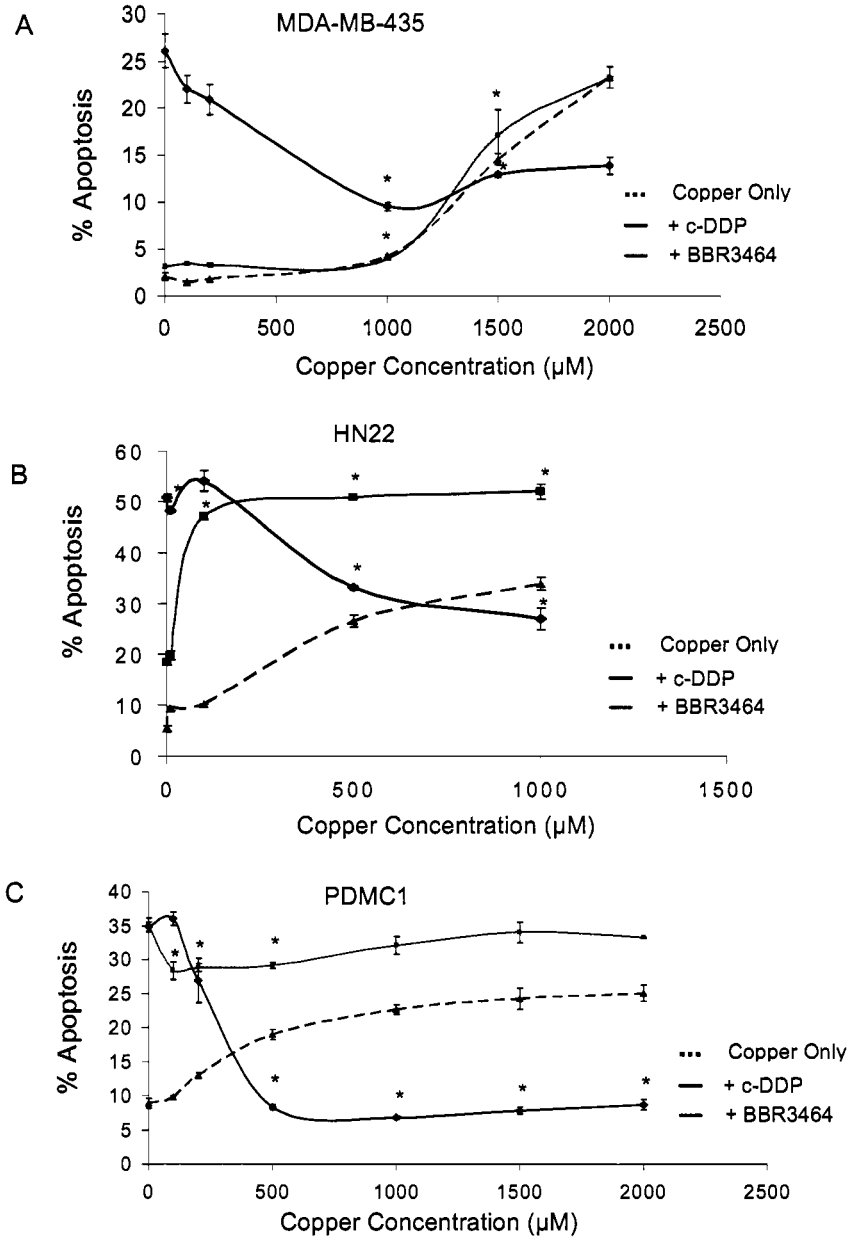

Figure 2.6 Effect of copper on c-DDP- and BBR3464-induced apoptosis in HN22 (head and neck carcinoma), MDA-MB-435 (breast carcinoma), and PDMC1 (mastocytoma) cell lines. Subdiploid cell content was detected by PI-DNA staining. In A, MDA-MB-435 cells were cultured with 30μM c-DDP and 40μM BBR3464 in the presence or absence of copper for 24h. In B, HN22 cells were cultured with 30μM c-DDP and 40μM BBR3464 in the absence or presence of copper for 24h. In C, PDMC1 cells were cultured with 4μM c-DDP and 0.2μM BBR3464 in the absence and presence of copper for 24h. Each point is the average (+/- SEM) of three independent experiments. *, $p<0.05$ when comparing cells treated with and without copper, by Student's t-test.

caspase enzymes.[30] Since both c-DDP and BBR3464 have been argued to elicit apoptosis in certain cell lineages *via* a p53-dependent pathway,[9,31] we measured p53 activation by western blot analysis. As shown in Figure 2.7A, c-DDP treatment induced robust p53 expression in HCT116 cells. This induction was significantly reduced by the addition of copper. Overall, copper decreased p53 protein levels by more than 50%, when comparing copper to copper plus c-DDP treated samples in 3 separate experiments. In contrast, BBR3464-induced p53 expression was unaltered by prior copper treatment (Fig 2.7A).

If the effects of copper on p53 expression are functionally significant, they should be consistent with the activation of the downstream death-inducing caspase enzymes, which can be triggered by p53. We measured the effect of copper on Pt-induced activation of the effector caspases -3 and -7 and cleavage of the caspase substrate PARP, using the same drug concentrations employed in the apoptosis assays. The effects of copper on caspase activation and total PARP levels mirrored its divergent effects on apoptosis and p53 induction. Caspase-3 activation was decreased 79% in HCT116 carcinoma cells treated with copper prior to c-DDP addition (Fig. 2.7B). On the other hand, BBR3464-induced caspase-3 activation was increased 37% by the addition of copper (Fig. 2.7B). Similarly, uncleaved PARP was increased in HCT116 cells treated with copper prior to c-DDP addition, mimicking the effect of copper on p53 and caspase activation (Fig. 2.7C). These results argue that the distinct effects of copper on c-DDP and BBR3464 are functionally relevant, altering the ability of these Pt compounds to induce downstream signals that elicit cell death.

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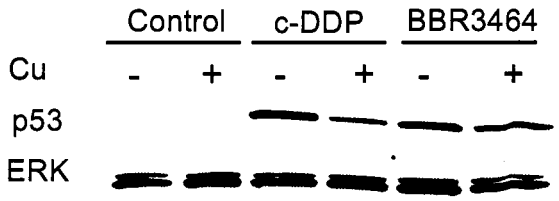

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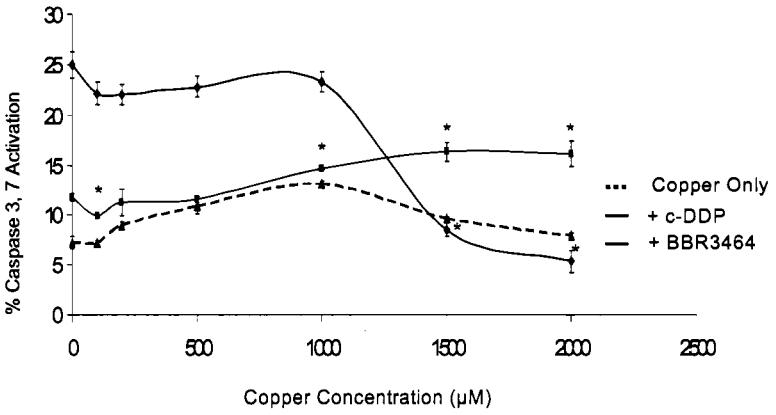

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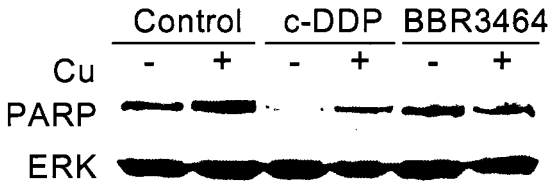


Figure 2.7: For figure legend, refer to next page.

Figure 2.7 Effect of copper on downstream signaling pathways activated by c-DDP and BBR3464. A. Effect of copper on c-DDP- and BBR3464-induced p53 upregulation. HCT116 cells were cultured with 40μM c-DDP or 50μM BBR3464 in the absence and presence of different concentrations of copper for 24h. The expression of p53 was detected by western blotting. The same membrane was stripped and re-probed for ERK to show protein loading. The assay was consistently repeated three times. B. Effect of copper on c-DDP- and BBR3464-induced caspase activation. HCT116 cells were cultured with 40μM c-DDP or 50μM BBR3464 in the absence or presence of copper for 24h. Cells were stained for active caspase-3/7 activation as described in Methods and Materials. Data shown is the percent of the population displaying active caspase-3/7. Each point represents the average (+/-SEM) of three independent experiments. Caspase activation at high concentrations of copper was found to be significantly different than activation in the absence of copper by Student's t-test; *, $p<0.05$. C. The effect of copper on uncleaved PARP expression in c-DDP- and BBR3464-treated samples. HCT116 were cultured with 40μM c-DDP or 50μM BBR3464 in the absence or presence of copper for 24h. The expression of uncleaved PARP was detected by western blotting. The same membrane was stripped and re-probed for ERK to show protein loading.

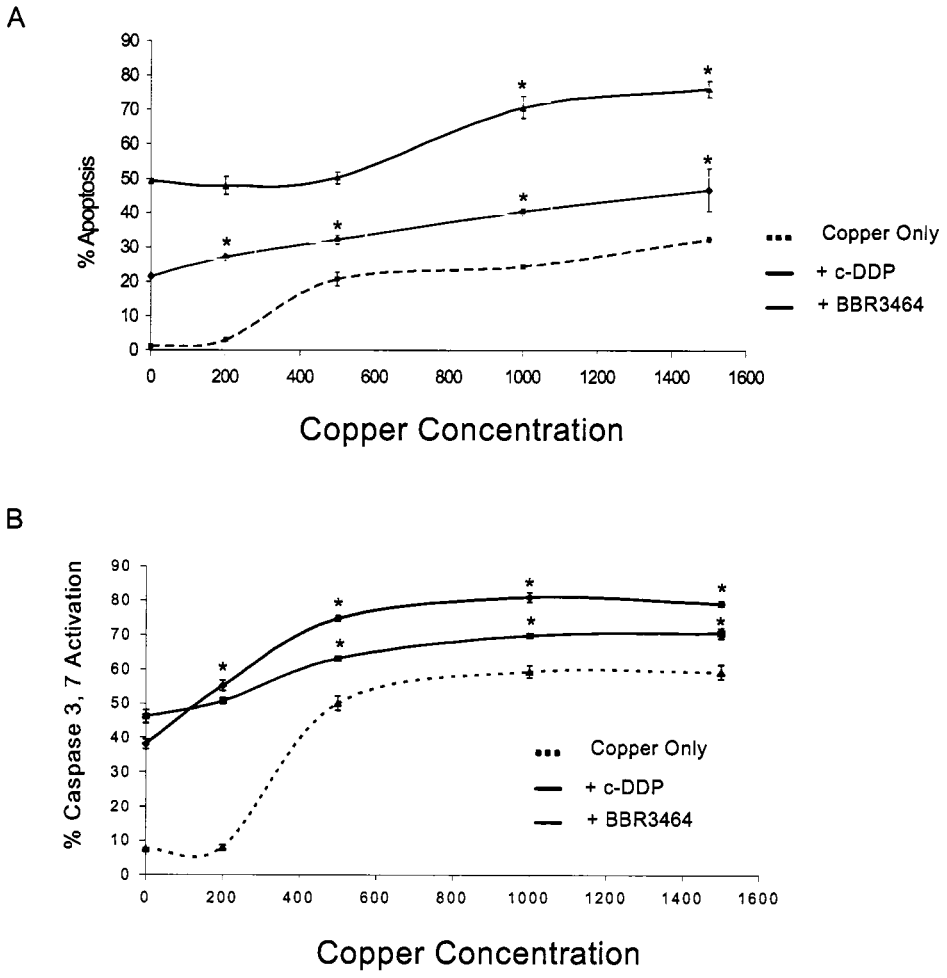


Figure 2.8: For figure legend, refer to next page.

Figure 2.8 Effect of copper on c-DDP and BBR3464 cytotoxicity in HCT116 -/- cells. A. The effect of copper on c-DDP- and BBR3464-induced apoptosis in HCT116 -/- carcinoma cell lines. Sub-diploid cell content was detected by PI-DNA staining. HCT116 -/- cells were cultured with 40μM c-DDP or 50μM BR3464 for 72h in the absence or presence of copper. Drugs were added to the media after 1h of treatment with copper. Each point represents the average (+/- SEM) of three independent experiments. Apoptosis at high concentrations of copper was found to be significantly different than apoptosis in the absence of copper by Student's t-test; *, $p<0.05$. B. The effect of copper on c-DDP- and BBR3464-induced caspase activation. HCT116 -/- cells were cultured with 40μM c-DDP or 50μM BBR3464 in the absence or presence of copper for 72h. Cells were stained for active caspase-3/7 activation as described in Methods and Materials. Data shown are the percent of the population displaying active caspase-3/7. Each point represents the average (+/- SEM) of three independent experiments. Caspase activation at high concentration of copper was found to be significantly different than activation in the absence of copper by Student's t-test; *, $p<0.05$. C. Effect of copper on uncleaved PARP expression. HCT116 were cultured with 40μM c-DDP or 50μM BBR3464 in the absence or presence of copper for 72h. The expression of uncleaved PARP was detected by western blotting. The same membrane was stripped and re-probed for ERK to show protein loading.

To confirm that p53 was the functional link to the differential effects of copper, we cultured a p53-deficient (p53 -/-) isogenic clone of the HCT116 cell line with c-DDP or BBR3464 in the presence or absence of copper. As shown in Figure 2.8A, the addition of copper to these cultures uniformly increased apoptosis for both Pt compounds. Hence the copper-mediated blockade of p53 induction we noted with c-DDP treatment not only coincides with a reduction in apoptosis, but is apparently required for this effect. The effect of copper on Pt-induced apoptosis was consistent with activation of the caspase enzymes in HCT116 p53-/- cells (Fig. 2.8B). Caspase activation was increased for both Pt compounds by copper. Moreover, the effect of copper on PARP cleavage was examined as well. Uncleaved PARP levels were decreased by addition of copper when cells were treated with either c-DDP or BBR3464 (Fig. 2.8C). These data demonstrate that p53 expression is required for the differing effects of copper on c-DDP and BBR3464-mediated cell death, and support the premise that p53 activation is central to Pt-mediated cell death, including how this process is altered by copper.

2.5 Discussion:

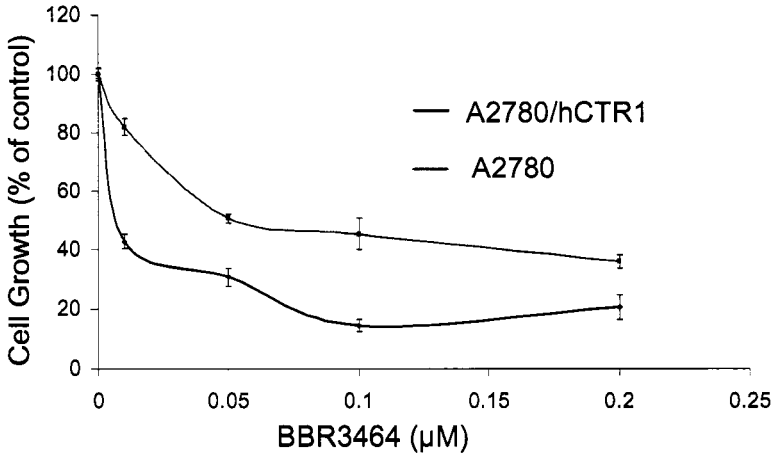
This study shows some similarities and also potentially significant differences in the effect of copper and its transport mechanisms on cellular effects of c-DDP and BBR3464. Over-expression of the copper transporter hCTR1 enhances BBR3464 uptake, similar to the earlier reports for c-DDP.[24] This is the first demonstration that this novel polynuclear platinum compound, structurally discrete from c-DDP, employs similar influx pathways to the parent compound. More importantly, increased uptake correlated with enhanced

efficacy for both c-DDP and BBR3464. A2780/hCTR1 cells were more sensitive than wild type A2780 to c-DDP-induced apoptosis, and entered a G2 cell cycle arrest more readily in response to BBR3464. It is interesting to note that hCTR1 over-expression has previously been shown to have little effect on c-DDP-mediated growth inhibition,[24] a result we confirmed in our MTT assays (Fig. 2.9). hCTR1 over-expression had little effect on c-DDP-induced growth inhibition in MTT assays. On the other hand, A2780/hCTR1 cells were more sensitive to BBR3464, as shown by MTT assay (Fig. 2.9). Our results show clearly that hCTR1 is an important mediator of both mononuclear and polynuclear Pt drugs. The functional importance of hCTR1 appears to be more important to cell death than cell cycle arrest.

In contrast to the important role of hCTR1, over-expressing the copper efflux transporter ATP7B had little effect on c-DDP and BBR3464 uptake and cytotoxicity (Fig. 2.4, 2.5). ATP7B transfection also did not influence cell cycle arrest induced by continuous exposure to c-DDP or BBR3464. It is noteworthy that ATP7B over-expression reportedly reduced growth inhibition caused by a 1-hour exposure to c-DDP.[25] Again, these two results cannot be directly compared because of the different conditions and end-points between the two assays. Our data support the hypothesis that while hCTR1 plays a significant role in c-DDP and BBR3464 uptake and cytotoxicity, the effects of ATP7B may be less critical when cells are exposed to platinum drugs throughout the assay period.

The involvement of copper transporters in platinum drug metabolism was indirectly examined by investigating the effect of copper ion on c-DDP and BBR3464 uptake. Copper decreased c-DDP uptake in yeast when c-DDP was used at a high concentration

A



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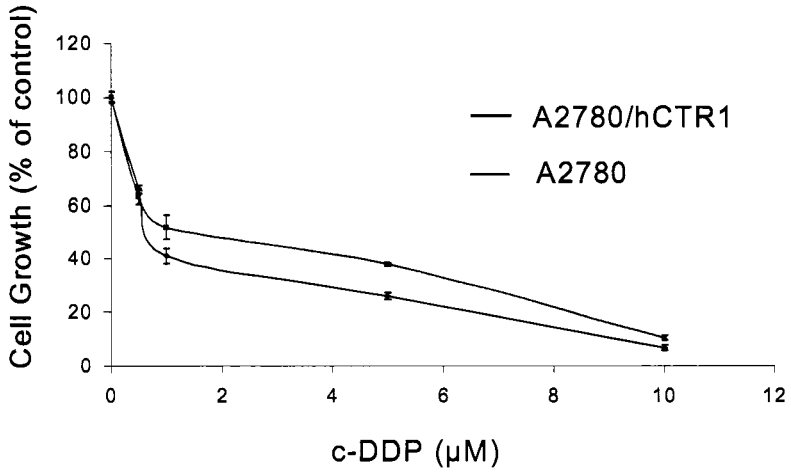

Figure 2.9 The effects of hCTR1 expression on c-DDP- and BBR3464-induced cytotoxicity by MTT assay. In A and B A2780 and A2780/hCTR1 cells were cultured in the indicated concentrations of c-DDP and BBR3464 for 72h. Cell growth was measured by MTT assay as described in Materials and Methods. Each point is the average (+/- SEM) of three independent experiments.

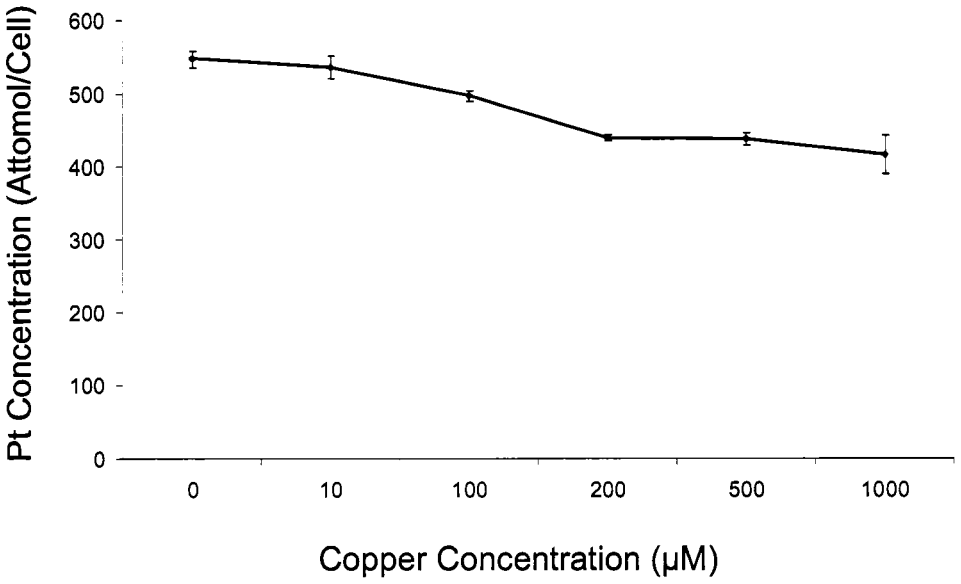

Figure 2.10 Effect of copper on c-DDP uptake in A2780 carcinoma cell line. A2780 cells were cultured with 1mM c-DDP for 3h in the absence or presence of copper. Each point represents the average (+/- SEM) of three independent experiments.

(1mM).[32] We observed the same result in ovarian carcinoma cells (A2780) by adding 1mM c-DDP with copper (Figure 2.10). High concentrations of c-DDP (1mM) may saturate hCTR1, and hence compete with copper for binding.[32] However, this concentration is not clinically relevant, as the approximate blood concentrations of c-DDP in a patient with an administered dosage of 50-120 mg/m^2 body surface area corresponds to 66μM (20μg/ml).[1,33] When more clinically relevant concentrations of c-DDP and BBR3464 were used (10-20 μM), copper increased the cellular concentration of both platinum drugs in ovarian and colorectal carcinoma cell lines (Fig. 2.7). These data are supported by a recent paper showing that c-DDP enhances cellular copper accumulation in MCF-7 breast cancer cells.[34] Collectively, these data would suggest that at physiological concentrations, c-DDP does not directly compete for hCTR1 binding, but rather may compete with copper for the efflux transporter, resulting in enhanced platinum accumulation.

Despite the fact that copper uniformly enhanced the uptake of both Pt drugs, it had different effects on c-DDP- and BBR3464-mediated apoptosis. c-DDP-induced cell death was significantly decreased, as observed previously in yeast.[32] By contrast, BBR3464-induced apoptosis was enhanced in both ovarian and colorectal carcinoma cell lines (Fig. 2.8). Thus, the differential response to copper suggests that c-DDP and BBR3464 have distinct modes of transport or metabolism leading to apoptosis.

Within the realm of cell cycle arrest and apoptosis, perhaps no cell signaling pathway is more relevant than p53 activation. Both Pt compounds increased p53 expression in HCT116. Interestingly, the effect of copper on p53 induction directly

correlated with its differential effects on Pt-mediated cell death. Copper decreased c-DDP-mediated p53 induction by more than 50%, while BBR3464-induced p53 expression was unchanged. This effect appeared to be functionally significant, since c-DDP-mediated activation of the effector caspases downstream of p53 was inhibited by copper, while BBR3464-induced caspase activation was enhanced. Similarly, expression of the caspase substrate PARP was increased by copper in c-DDP treated samples, mirroring the effect on caspase activation. These effects on PARP levels are noteworthy. It was recently demonstrated that PARP interacts with c-DDP-DNA adducts in human cells, and that PARP inhibition can enhance cellular sensitivity to c-DDP.[35]

The importance of p53 to the differential effects observed with copper addition was made clear by use of p53-deficient HCT116 cells, an isogenic companion set to the HCT116 cells. In the absence of p53, both Pt compounds induced death, albeit with a slower time course (72hr versus 24hr). However, the differential effects of copper on c-DDP and BBR3464 activity were completely lost when p53 was deleted. Moreover, caspase activation and PARP levels in p53-deficient HCT116 cells clarify the differential effects of copper. These data support the conclusion that copper enhances the uptake of both c-DDP and BBR3464, but differentially affects their cellular location or metabolism, altering p53 activation and cell death. This divergent effect of copper is one indication that while c-DDP and BBR3464 may share transport pathways, their mechanisms of action, including the means by which they activate p53, are distinct. Our current efforts are focused on revealing these fundamental differences between clinically relevant mononuclear and polynuclear Pt drugs.

In summary, the results demonstrate that hCTR1 may be a common means of entry for Pt-based drugs, including charged polynuclear compounds such as BBR3464. Structurally different platinum drugs with distinct modes of DNA-binding can share this transport mechanism and converge on signal transduction pathways including p53. However, these compounds also employ divergent pathways to induce cell death, as revealed by the differential response to copper. Understanding these pathways will reveal targets that can be exploited for treating drug-resistant tumors. These data confirm the validity of searching for new chemotypes outside the cisplatin structural class to aid in the treatment of recurrent, cisplatin-resistant cancers.

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List of References

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Chapter 3: Platinum Based Drug Cytotoxicity is Enhanced by the Antidepressant Desipramine

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Submitted to the Cancer Research

3.1. Abstract:

Desipramine is a tricyclic antidepressant that is also used for treating neuropathic pain, particularly in prostate cancer patients. Due to its importance and usage as an antidepressant and its role in blocking organic cation transporters, the effects of desipramine on platinum drug uptake and cytotoxicity were investigated. The difference between commonly used platinum drugs cisplatin (c-DDP), oxaliplatin and the cationic trinuclear platinum drug, BBR3464 [{*trans*-$PtCl(NH_3)_2$}$_2\mu$-(*trans*-$Pt(NH_3)_2(H_2N(CH_2)_6NH_2)_2$)]$^{4+}$, which has undergone evaluation in Phase II for activity in resistant lung and ovarian cancers, was also studied. In colorectal carcinoma cell lines, desipramine induced synergic effects on BBR3464-, c-DDP- and oxaliplatin- induced cytotoxicity. Desipramine enhanced cellular accumulation of c-DDP, but had no effect on the accumulation of BBR3464 or oxaliplatin, suggesting that enhanced uptake was not a consistent means by which desipramine altered platinum drug-mediated cytotoxicity. The

synergic effect of desipramine on Pt drug-induced cytotoxicity resulted in increased activation of p53, mitochondrial damage, caspase activation and PARP cleavage. The synergic effect of desipramine was still observed using p53 deficient (p53-/-) HCT116 cells. The results show that desipramine, a safe and effective antidepressant already in use for cancer treatment, greatly augments the cytotoxicity of platinum-based chemotherapeutics. These effects correlated with enhanced activation of the p53-mitochondrial death pathway, but a p53-independent mechanism is also apparent. The study argues that desipramine may be a means of enhancing chemo-responsiveness, and warrants further investigation.

3.2. Introduction:

Clinical cancer treatment currently involves the use of a combination regimen involving one or more drugs. These drugs are comprised of cytotoxics, antimetabolites and, most recently, targeted therapies. Understanding how specific drug-target interactions affect downstream cellular signaling pathways is critical to development of rational combination chemotherapy approaches. Concomitantly, patients receive a variety of medications to offset the physical and psychological effects of anti-cancer drugs. The advances in knowledge on the signaling pathways employed by anticancer drugs also allow study of the effects and consequences of adjuvant drug combinations. In this contribution, we report on an unexpected synergistic effect between the antidepressant desipramine and platinum-based cytotoxic anticancer drugs. Determining the effects of antidepressants like desipramine on platinum drug-mediated cytotoxicity may be a

productive approach to cancer therapy, especially since up to 58% of cancer patients also suffer from depression [1,2] and desipramine is already used for decreasing neuropathic pain caused by prostate cancer chemotherapy. [3,4]

These findings arose from our interest in studying mechanisms of cellular uptake pertinent to the clinical role of platinum drugs. Cisplatin (c-DDP) and oxaliplatin (for structures see Table 3.1) are effective agents for treatment of cancer, including testicular, head and neck, ovarian, small cell lung, and colorectal neoplasms [5]. Their cytotoxicity is mediated mainly through interactions with DNA by formation of bifunctional interstrand and intrastrand cross links.[6,7] Their clinical efficacies are limited due to acquired resistance and dose-limiting side effects.[8,9] The three major pharmacological factors contributing to the intrinsic cytotoxicity of, and cellular resistance to, platinum drugs are (i) cellular uptake and efflux of platinum, (ii) the frequency and nature of Pt-DNA adducts and (iii) deactivating metabolic reactions with sulfur-containing nucleophiles.[10] Modulation of one or other of these factors may likewise result in greater potency or a broader spectrum of treatable tumors. Structurally distinct platinum complexes that bind to DNA differently than c-DDP may thus have complementary cytotoxicity and side effect profiles of clinical relevance. The trinuclear complex, BBR3464, is significantly more cytotoxic than c-DDP or oxaliplatin and retains activity against c-DDP-resistant tumor cells *in vitro* as well as *in vivo*.[11-15] The drug has undergone Phase II clinical trials in cisplatin-resistant and refractory cancers. [16,17]

Defects in c-DDP accumulation are the single most commonly reported feature of cells selected for resistance.[18,19] Multiple pathways contribute to this resistance. Cationic

polynuclear platinum drugs have recently been shown to display higher cellular uptake than c-DDP – a factor which may contribute to their enhanced efficacy in comparison to mononuclear drugs.[20,21] The mechanisms by which small platinum-containing molecules enter cells are diverse – one reason perhaps why they are such useful anticancer agents. The use of cellular copper transporters has been documented as one mechanism of active uptake of platinum drugs.[22,23] BBR3464 and c-DDP use the same cellular transporters (hCTR1 and ATP7B) for influx and efflux; however, downstream effects in the presence of added copper ion are different for the two drugs.[10]

A second set of transporters more recently studied for their effect on platinum drug uptake are the organic cation transporters. These carriers (commonly referred to as OCT1, 2 and 3) are plasma membrane transporters of the SLC22A family and have been characterized functionally and their molecular components identified.[24-26] Substrates include a wide variety of organic cations including endogenous compounds such as monoamine neurotransmitters, choline, and coenzymes, but also numerous drugs and xenobiotics such as cimetidine and tetraethylammonium (TEA).[25] Some of the cloned organic cation transporters accept one main substrate or structurally similar compounds (oligospecific transporters), while others translocate a variety of structurally diverse cations (polyspecific transporters).[25] A common approach to study involvement of transporters in drug uptake is the use of specific inhibitors and/or competing substrates. Cimetidine as an inhibitor or tetraethylammonium chloride (TEA) as a competing substrate significantly decreased c-DDP renal cell accumulation.[27,28] The specific identity of the transporter is, however, unclear.[29] The role of OCT in platinum drug uptake may be both

tissue-specific and compound-specific, where the role of carrier ligand is important. Cisplatin and oxaliplatin, but not carboplatin or nedaplatin, have been reported as substrates for human OCTs.[29] Likewise, recent studies suggest that OCT1 and OCT2 are important for oxaliplatin uptake but not for c-DDP in transfected canine and human kidney cells.[30] In a variety of colon cancer cell lines, the presence of cimetidine decreased cytotoxicity of oxaliplatin, on average 5-11 fold, but had little effect on sensitivity to c-DDP.[30] Oxaliplatin is active against colorectal tumors, whereas c-DDP is significantly less so, and these differences may be due in part to enhanced oxaliplatin uptake.[30] Cimetidine likewise had no effect on the transport of c-DDP or a dinuclear platinum compound based on ethylenediamine as a carrier ligand in MCF-7 breast cancer cells.[31]

Due to its importance and usage as an antidepressant and its role in blocking organic cation transporters, [25] we investigated the effects of desipramine on platinum drug uptake and cytotoxicity. The antidepressant activity of desipramine arises from inhibiting the reuptake of and, therefore, raising the level of, norepinephrine and serotonin in the synaptic cleft.[32] In this paper, we show that desipramine has synergistic effects on platinum drug-induced cytotoxicity *in vitro* and investigate the mechanism by which this occurs. We chose colon cancer cell lines because of the clinical relevance for oxaliplatin. Secondly, a survey of BBR3464 cytotoxicity across the NCI tumor panel showed enhanced sensitivity of colon cancers to the trinuclear drug.[14] Achieving synergistic effects with an anti-depressant in combination with chemotherapeutic platinum drugs provides the advantage of treating two medical conditions at the same time. In addition, since up to 58% of cancer patients also suffer from depression[1,2], a combination of chemotherapy with

antidepressants would provide a new era for drug design and therapy. Combinational therapy is of great interest, since inhibiting or activating specific signal transduction cascades and cellular functions could augment the efficacy of chemotherapy, especially in resistant and refractory cancers.

3.3 Materials and Methods

3.3.1 Compound Synthesis.

BBR3464 and c-DDP compounds were synthesized as previously described.[33] Oxaliplatin and desipramine were obtained from Sigma-Aldrich (St. Louis, MO).

3.3.2 Cell Systems.

The colorectal carcinoma cell lines HCT116, and matched p53-deficient HCT116 cells (HCT116-/-) were the kind gift of Bert Vogelstein (Johns Hopkins University, Baltimore, MD). HCT116 cells were cultured with RPMI 1640 with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mmol/L sodium pyruvate (cRPMI, all from Biofluids, Rockville, MD) in humidified air with 5% CO_2. The colorectal carcinoma cell lines DLD1 and HT29 were grown in cDMEM with 10% fetal bovine serum.

3.3.3 Culture Conditions.

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 cells (adherent and

non-adherent cells) were collected. BBR3464, c-DDP, and oxaliplatin concentrations were adjusted to achieve approximately 30%-40% apoptosis after 72 h of treatment, allowing measurement of enhancement or inhibition.

3.3.4. Propidium Iodide DNA Staining and Analysis of Apoptosis.

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 [34]. Samples were then analyzed for subdiploid DNA content 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.

3.3.5. Assessment of Mitochondrial Membrane Potential

Alteration in mitochondrial membrane potential was assessed by staining with 3,3' – dihexyloxacarbocyanine iodide [$Di(OC_6)_3$; Molecular Probes, Eugene, OR]. $Di(OC_6)_3$ was added to 200 μL of cells at 40 nmol/L final concentration. Cultures were incubated for 30 minutes at 37°C in a CO_2 incubator. The cells were then washed twice with PBS and resuspended in 200 μL PBS for flow cytometric analysis using a forward and side scatter gate sufficiently open to include apoptotic/dying cells.

3.3.6. Assessment of Caspase Activation.

Staining for active caspases was performed with caspase kits (Immunochemistry Technologies, LLC, Bloomington, MN), as specified by the manufacturer. Cells were incubated with a cleavable substrate that binds to the active caspases-3 and -7. Substrate cleavage results in increased fluorescence intensity, interpreted as caspase-positive cells. The percentage of caspase-positive cells was measured by flow cytometry.

3.3.7. Platinum Accumulation Assays.

Cells were plated at 2.0×10^6 cells/mL. Platinum drug was added in different concentrations alone or 60 minutes after the addition of desipramine. After 8 or 16 h, cells were harvested and washed twice with PBS. The cell pellets were then dissolved in hot 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).[20] Platinum analysis was performed on a Vista-MPX simultaneous inductively coupled plasma optical emission spectroscopy at 265 nm (Varian Inc., Palo Alto, CA). Standards and blank were prepared the same as the samples.

3.3.8. Measurement of Platinum Accumulation in DNA.

Cells were plated at 2.0×10^6 cells/ml. Platinum drug was added in different concentrations alone or 60 minutes after the addition of desipramine. After 10h cells were

harvested and washed twice with PBS. DNA was then extracted from the cell pellets using the high salt method [35,36]. Briefly, the cell pellets were treated with nuclei lysis buffer, proteinase K, 10% SDS; and 6M NaCl was added to lysate the pellets. DNA was then precipitated using isopropanol and 3M sodium acetate. DNA was then rinsed using 70% ethanol. The purity of the DNA was measured at absorbance of 260nm. The DNA was then harvested for platinum analysis as explained above.

3.3.9. Western Blotting.

Whole-cell lysates were blotted with goat polycolonal antibody against mouse p53 (BD bioscience), monoclonal antibody against poly-ADP ribose polymerase (PARP) (Trevigen, Inc.), mouse monoclonal antibody against ERK (Cell Signaling Technology), and, subsequently resolved with secondary antibody conjugated with horseradish peroxidase. Blots were then treated with a chemiluminescent substrate (Pierce, Rockford, IL, USA) and exposed to film. Band intensity was measured by densitometry with an Eagle Eye II system (Stratagene, La Jolla, CA).

3.3.10. Colony formation assay. Cells were cultured (250-1500 cells per well of a 6 well plate) and 12 h after plating, cells were treated with drugs for 48 h. Afterward, the drug containing media was carefully removed, the cells were washed once, and fresh media lacking drugs was added. Colony formation assays were cultured for an additional 10-14 days after which the media was removed, cells fixed with methanol, stained with crystal violet and counted manually.

3.3.11. Detection of Growth Inhibition by MTT Assay

Cells were cultured at 10000 cells per well in a 96-well microplate. They were incubated with different concentration of c-DDP and BBR3464 for 72h. Drug-containing medium was aspirated and cells were washed with PBS twice before the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, sigma chemicals) to each well. Cells were incubated with 100μl of MTT solution (2mg/1ml PBS) for 3h. MTT solution was aspirated and 100μl of DMSO (Sigma chemicals) was added to each well for determination of viable cell number through measuring the absorbance at 490nm.

3.3.12. Statistical Analysis.

Statistical analysis was performed using *t*-test for two data points using SysStat9 software (SPSS, Chicago, IL, USA). $p < 0.05$ was considered to be significant. Combination index was calculated with the Calcusyn software from Biosoft (Cambridge, United kingdom) for synergism. The proportion of dead cells was entered into the program, and the degree of synergism determined by combinational index (CI) value according to the Chou and Talaly algorithms. A CI value of <1 shows synergism, whereas CI >1 shows antagonism. When the value remains close to CI=1, additivity is indicated. Results are the mean and standard error.

3.4. Results:

3.4.1. Synergic Effects of Desipramine on Platinum Drug-induced Cytotoxicity.

Desipramine is a tricyclic antidepressant, which is also used for treating neuropathic pain caused by prostate cancer chemotherapy.[3,4,32] To examine the effect of desipramine as a putative OCT inhibitor, the relationship between its effects on cell sensitivity and cellular uptake of platinum drugs was studied. Since the desipramine IC_{50} values for inhibition of OCT 1, 2, 3 are 5.4, 16, and 14μM respectively, constant concentration of 40μmol/L was used in figure 3.1. The influence of desipramine on BBR3464, c-DDP, and oxaliplatin-induced cytotoxicity was determined by comparing the level of apoptosis in colorectal carcinoma HCT116 cells. Cells were cultured at different time points in the presence or absence of desipramine and platinum drugs, and DNA fragmentation was measured by PI-DNA staining. Desipramine alone had no cytotoxic effect on HCT116 survival but augmented BBR3464-induced apoptosis in HCT116 cells, Figure 3.1. The augmentation of BBR3464-induced apoptosis by desipramine was time-dependent, essentially reaching a plateau after 72 h. Apoptosis induced by either desipramine or BBR3464 alone was 2% and 36% respectively, but the combination of BBR3464 and desipramine showed an increase in apoptosis to approximately 80% at that time point. Treatment of cells with c-DDP and oxaliplatin gave similar results. In the case of c-DDP, the enhancement reached a maximum at 48 hours after treatment, where desipramine increased the c-DDP-induced apoptosis from 27% to 79% (Figure 3.1B). For oxaliplatin, a smaller but significant

increase in apoptosis from 49% to 76% was observed after 72 h of treatment in the presence of the antidepressant (Figure 3.1C).

To determine dose-dependence, cells were treated with platinum drugs at different concentrations of desipramine for 72 h, and % apoptosis was measured by PI-DNA staining. The highest percent apoptosis was observed at a desipramine concentration of 20 μmol/L in combination with BBR3464 or c-DDP (Figure 3.2). In the case of oxaliplatin, however, the percent apoptosis peaked with 40 μmol/L desipramine (Figure 3.2).

The combined apoptosis data obtained were subjected to statistical analysis (using calcusyn software, see Materials and Methods), and the combination index (C.I.) calculated for evidence of synergy (Table 3.1). A C.I. < 1 is indicative of synergy and below 0.3 is strong synergy. When C.I. > 1, antagonism is indicated and a C.I. of approximately 1 is considered indicative of an additive rather than synergic response to the combination. The data showed strong synergy for all Pt compounds with desipramine, with the strongest synergy being observed for BBR3464. Note that all platinum drug concentrations were first adjusted to achieve approximately 30%-40% apoptosis after 72 h of treatment, allowing measurement of enhancement or inhibition. Significantly low C.I. values were obtained independent of the dose of either agent used. The C.I. increased somewhat with c-DDP concentration but always within the mathematical index of synergy. Interestingly, at constant c-DDP and BBR3464 concentrations the C.I. was dependent upon the desipramine concentration-dependent between 5 and 20 μmol/L, plateauing thereafter for both drugs, with a higher synergy indicated for BBR3464. In contrast the behavior of oxaliplatin was somewhat different. The lower doses of desipramine yielded additive rather

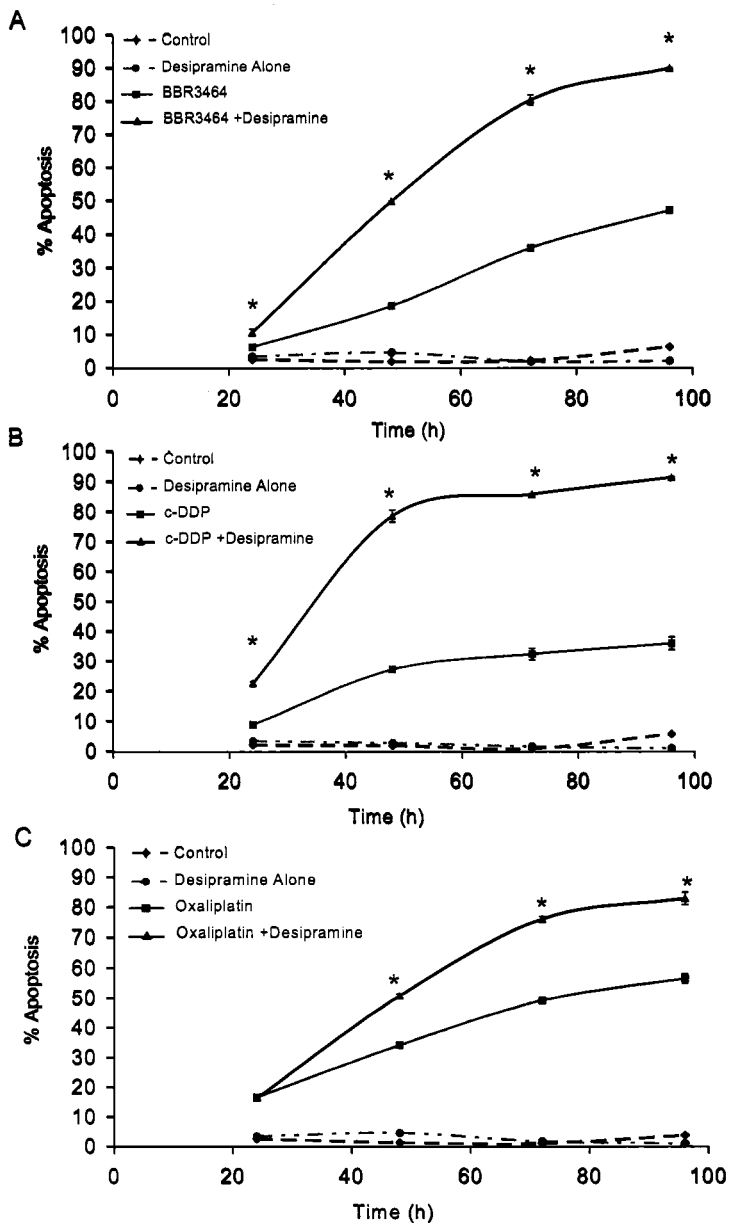


Figure 3.1. The synergic effect of desipramine on Pt-drug-induced apoptosis over time in HCT116 colorectal carcinoma cells. Sub-diploid cell content was detected by PI-DNA staining. In A, B, C, HCT116 cells were cultured with 50μmol/L BBR3464, 10μmol/L c-DDP, or 30μmol/L oxaliplatin respectively for the indicated time points in the absence and presence of 40μmol/L desipramine. Pt-drugs concentrations were adjusted to achieve approximately 20-30% apoptosis after 48h, allowing us to measure enhancement or inhibition. Pt drugs were added to the media after 1h of treatment with desipramine. Each point represents the average (+/- SEM) of three independent experiments. *, $p<0.05$ when comparing cells treated with and without desipramine, by Student's t-test. All points after 48h have $p < 0.05$ for Pt drug with desipramine vs Pt drug alone.

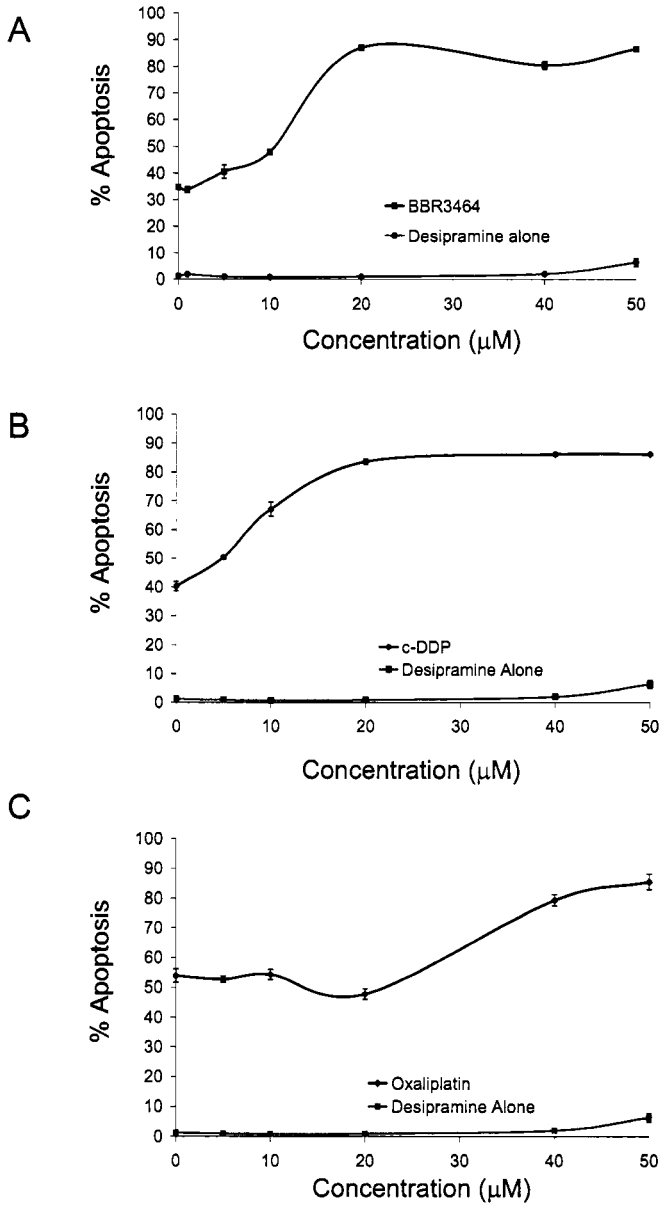


Figure 3.2. The dose response effect of desipramine on Pt-drug-mediated cytotoxicity. DNA content was measured by PI-DNA staining to determine apoptosis (sub G_0/G_1) by flow cytometry, as described in Materials and Methods. In A, B, C, HCT116 cells were cultured with 50μmol/L BBR3464, 10μmol/L c-DDP, or 30μmol/L Oxaliplatin respectively in the presence and absence of desipramine for 72h. Drugs were added to the media after 1h of treatment with desipramine. Each point represents the average (+/- SEM) of three independent experiments. *, $p<0.05$ when comparing cells treated with and without desipramine, by Student's t-test.

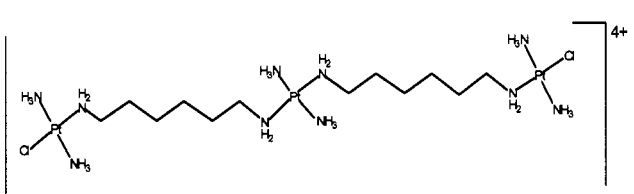
Table1.Analysis of combinational therapy of platinum drugs and desipramine *in vitro*

P.I. DNA Staining (72h) HCT116 WT	BBR3464 † (μM)	Des.* (μM)	% Apop.**	C.I.§	c-DDP †† (μM)	Des.* (μM)	% Apop.**	C.I.§	Ox.‡ (μM)	Des.* (μM)	% Apop.**	C.I.§
A	15	40	58	0.031	5	40	83	0.174	15	40	70	0.13
	25	40	62	0.034	10	40	86	0.303	30	40	79	0.103
	50	40	81	0.008	15	40	88	0.422	45	40	87	0.051
B	50	5	41	0.46	10	5	50	0.922	30	5	52	1.035
	50	10	48	0.233	10	10	67	0.602	30	10	54	0.917
	50	20	87	0.003	10	20	84	0.343	30	20	48	1.508
	50	40	81	0.008	10	40	86	0.303	30	40	79	0.103
	50	50	87	0.003	10	50	86	0.303	30	50	86	0.046
HCT116 p53KO												
A	15	40	33	0.384	5	40	38	0.065	15	40	29	0.453
	25	40	45	0.058	10	40	40	0.115	30	40	50	0.32
	50	40	61	0.006	15	40	51	0.059	45	40	60	0.304

Note: Cytotoxicity was determined after 72h of exposure to Pt drugs using P.I. DNA staining assay as described in Materials and Methods. Combination Index was calculated by Calcusyn program and a value less than 1 indicated synergy. The table shows data from three experiments per data point. A, Constant desipramine concentration was used with different Pt-drug concentrations. B, Constant Pt-drug concentration was used with different desipramine concentrations.

* Desipramine, ** Apoptosis, § Combination Index, † BBR3464 (as NO_3 salt) structure in I, †† c-DDP structure in II,

‡ oxaliplatin structure in III



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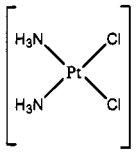

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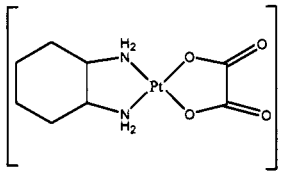

III

than synergistic effects, but above 40 μmol/L (the concentration which gave and optimal increase in apoptosis, Figure 3.2), the C.I. dropped dramatically and was essentially independent of oxaliplatin concentration.

The synergism was also reproduced with BBR3464-treated DLD1 and HT29 colorectal carcinoma cell lines, as measured by the colony formation assay and PI-DNA staining, respectively (Figures 3.3 and 3.4). In the colony formation assay, administered doses ranged from 1-3 nmol/L for BBR3464 combined with 10-30 μmol/L of desipramine. C.I. values were in the 0.8 to 0.9 range, essentially independent of concentrations used (Table 3.2). The C.I. value is thus formally synergistic but without the dramatically low values obtained in apoptosis assays. Nevertheless, the influence of desipramine on platinum-induced cytotoxicity is consistent when other colorectal cell lines and other parameters of cell survival/inhibition are used. Moreover, as shown by Table 3.3, the effect of desipramine on BBR3464-, c-DDP-, and oxaliplatin-induced cellular growth inhibition showed additivity with some synergistic and antagonistic effects as measured by MTT assay. Hence, the functional importance of desipramine appears to be more important to apoptosis than growth inhibition.

3.4.2. Effect of Desipramine on Platinum Drug Cellular Accumulation

The role of uptake and efflux is increasingly being seen as a critical determinant of clinical resistance and cytotoxicity [10,18]. Therefore, the influence of desipramine on platinum drug cellular accumulation was measured (*via* ICP-OES) in cells treated with platinum drug +/- desipramine. Desipramine did not influence BBR3464 or oxaliplatin cellular accumulation

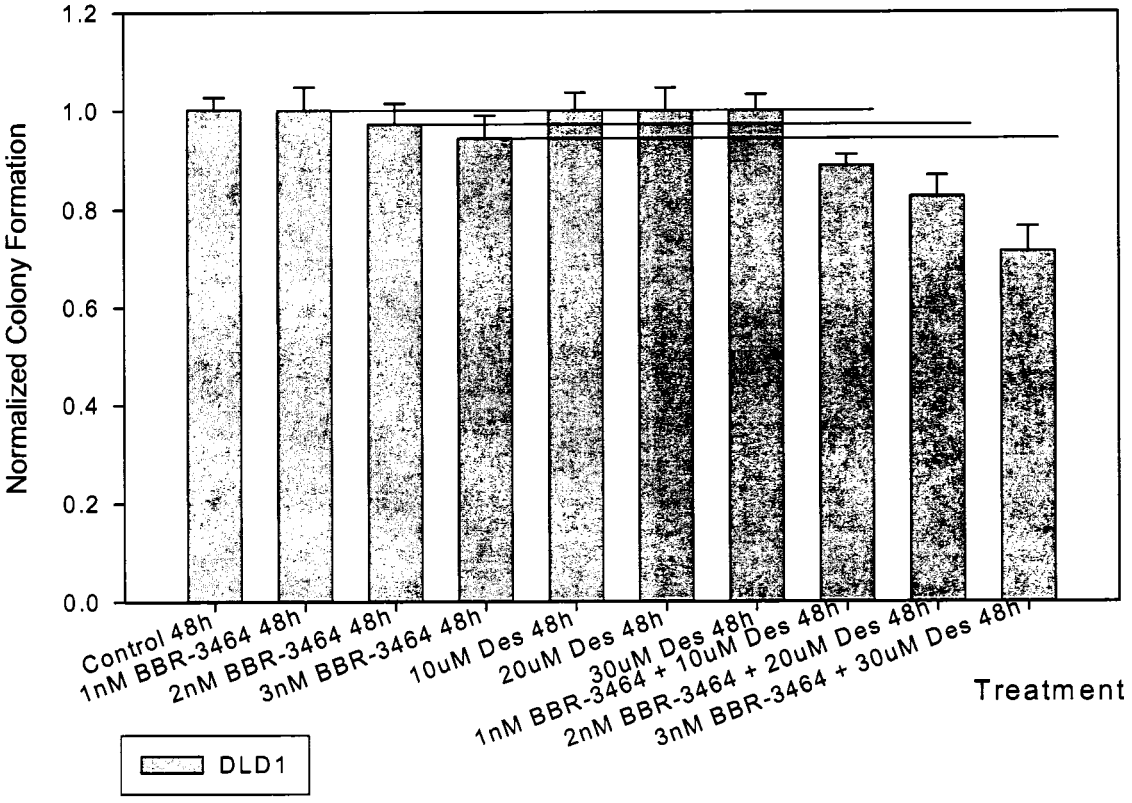

Figure 3.3. The effect of Desipramine on BBR3464-induced apoptosis in DLD1 colorectal carcinoma cells using colony formation assay. DLD1 cells were cultured with indicated concentration BBR3464 for 48h in the absence and presence of different concentrations desipramine. Media containing the drugs was removed afterward, and cells were cultured for 10-14 days to permit > 50 cell colonies to form. The media was removed; the cells were fixed with methanol and stained with crystal blue. Colonies of >50 cells were counted, and the survival for each condition was calculated. Each point represents the average (+/- SEM) of six independent experiments.

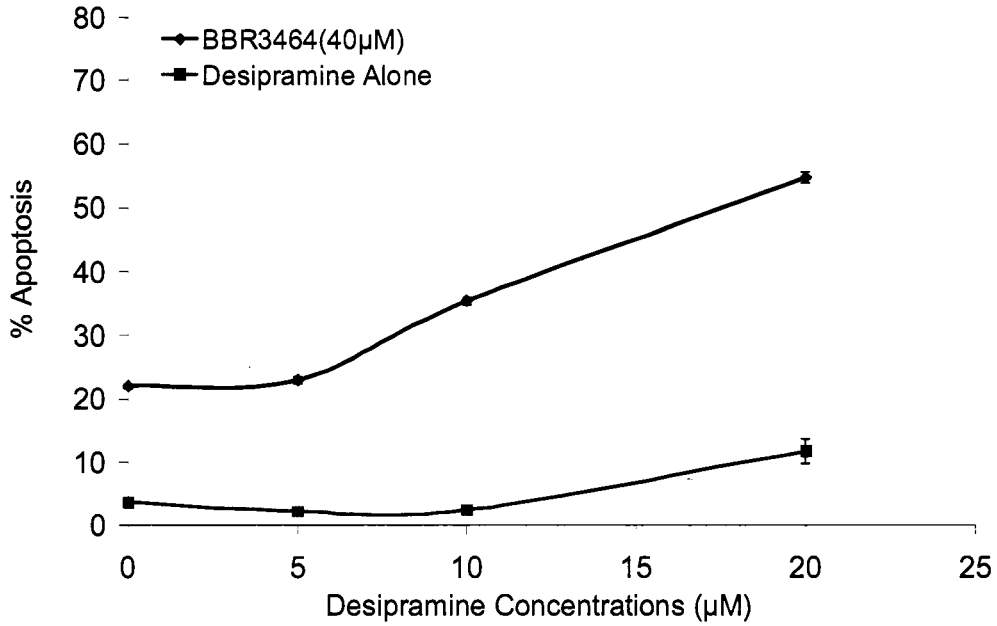

Figure 3.4. The effect of Desipramine on BBR3464-induced apoptosis in HT29 colorectal carcinoma cells. DNA content was measured by PI-DNA staining to determine apoptosis (sub G_0/G_1) by flow cytometry, as described in Materials and Methods. HT29 cells were cultured with 40μmol/L BBR3464 in the presence and absence of desipramine for 72h. Drugs were added to the media after 1h of treatment with desipramine. Each point represents the average (+/- SEM) of three independent experiments.

Table 3.2. Analysis of combinational therapy of BBR3464 and desipramine using colony formation assay

Colony Formation assay (48h) DLD1 Cell Line	BBR3464 (nmol/L)	Desipramine (μmol/L)	Combinational Index
A	1	10	0.813
	2	20	0.864
	3	30	0.802

Note: Cytotoxicity was determined after 48h of exposure to BBR3464 using colony formation assay as described in Materials and Methods. Combination Index was calculated by Calcusyn program and a value less than 1 indicated synergy. The table shows data from three experiments per data point.

Table 3.3: Analysis of Combination therapy of Pt drugs and desipramine using MTT assay

HCT116 (72h)	Desipramine Concentration			
% survival	0	5	10	20
Control	100	100.93	88.24	69.16
BBR3464 (1nM)	99.16	66.33	69.18	48.68
BBR3464 (10nM)	95.93	93.3	81.1	70
BBR3464 (20nM)	92.7	88.4	79.36	71.5
c-DDP (100nM)	101.6	99.07	90.46	74.72
c-DDP (200nM)	88.99	88.77	80.82	62.26
c-DDP (500nM)	74.78	70.76	65.66	43.2
Oxaliplatin (50nM)	106	100	102	93.23
Oxaliplatin (100nM)	92.7	88.95	80.73	60.76
Oxaliplatin (200nM)	95.32	97.12	80.5	63.8
Oxaliplatin (500nM)	62.19	57.5	52	46.76

Note: Cytotoxicity was determined after 72h of exposure to Pt drugs using MTT assay as described in Materials and Methods.

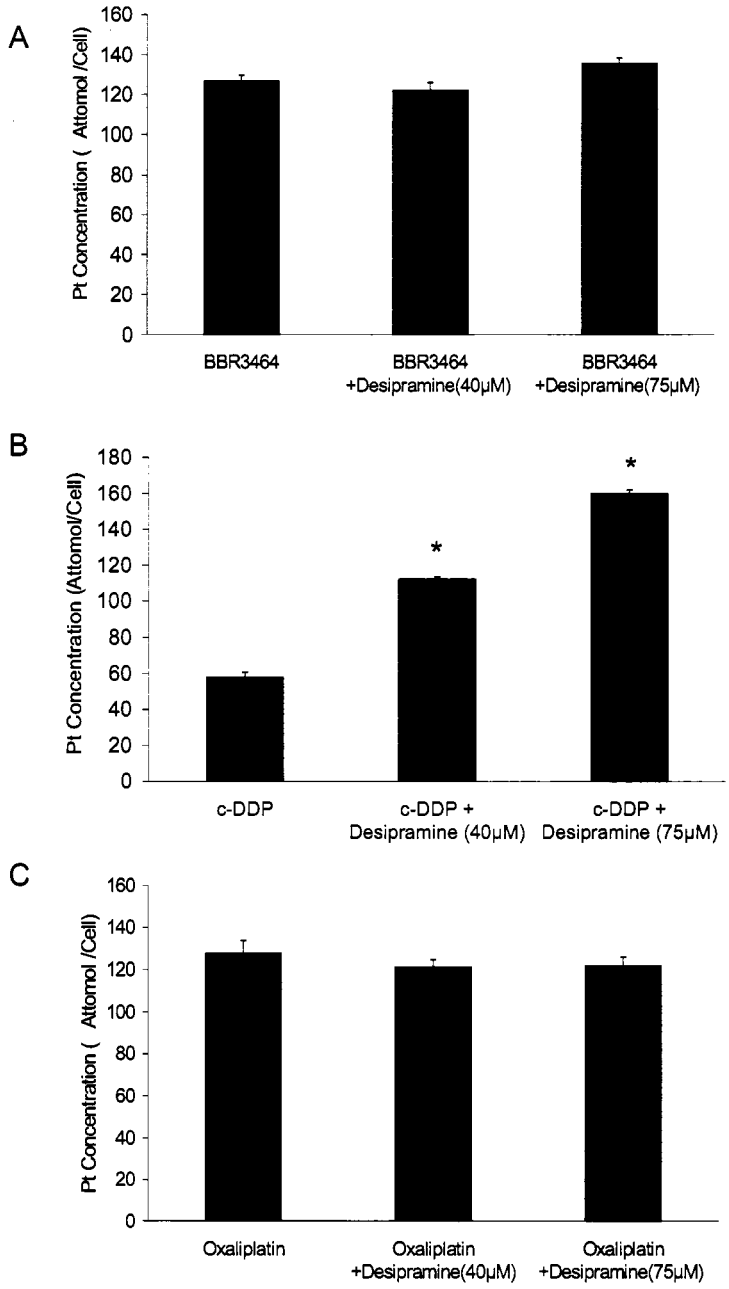

Figure 3.5. The effect of desipramine on Pt-compounds cellular uptake. In A, B, C, HCT116 cells were treated with 20μmol/L BBR3464 for 8h, or c-DDP, Oxaliplatin for 16h, and cellular platinum content was measured by ICP as described in Method and Materials. Each point represents the average (+/- SEM) of three independent experiments. *, $p<0.05$ as determined by t test.

(Fig. 3.5 A, C). In contrast, the c-DDP cellular accumulation was increased more than 2 fold - from 57.9 attomol/cell to 160 attomol/cell, Figure 3.5B. These results, firstly, confirm the high cellular accumulation of the 4+ positively charged compound relative to the neutral c-DDP, especially at earlier time points [10,20]. Secondly, the higher accumulation of oxaliplatin relative to c-DDP in human colon cancer cells at equimolar administered doses was confirmed [30]. The results further imply that modulation of transporter function by desipramine does not play a role in mediating platinum complex uptake in these cells. In contrast, the OCT inhibitor cimetidine decreased the cellular uptake of oxaliplatin and c-DDP, but not BBR3464, in HCT116 cells (Figure 3.7). These results are consistent with previous results of c-DDP- and oxaliplatin-treated HCT116 cells, where the IC_{50} of both drugs was increased in the presence of the cimetidine, although Pt accumulation was not directly measured [30].

Since cellular uptake or DNA binding can alter drug efficacy, the effect of desipramine on cellular DNA binding of BBR3464 was also measured. At doses of 55 μmol/L and 75 μmol/L for BBR3464 and desipramine, only a modest increase of Pt-DNA adducts of approximately 40% was measured (Figure 3.6). These data suggest the synergistic effects of desipramine may be multifunctional, and contributions from altered drug uptake and/or DNA binding may vary, depending on the specific compound studied.

3.4.3. Effect of Desipramine on BBR3464, c-DDP and Oxaliplatin-mediated p53 Activation

The modest effects of desipramine on Pt uptake and/or DNA adduct formation are unlikely to explain its synergistic effects on cell death. Hence we investigated potential biological mechanisms that could be augmented by desipramine. Many apoptotic signaling pathways converge at the transcription factor p53. p53 causes cell death in part by inducing mitochondrial damage that activates the death effector caspase enzymes [37]. Since all platinum drugs have been argued to elicit apoptosis in certain cell lineages *via* a p53-dependent pathway [19,20,38], the induction of p53 was measured by western blot analysis, Figure 3.8. At the relevant concentrations used, augmentation of apoptosis was 44%, 53%, and 27%, for BBR3464, c-DDP and oxaliplatin, respectively. Desipramine increased BBR3464-, and c-DDP-mediated p53 induction more than 71% and 829% respectively, while Oxaliplatin-induced p53 expression was only slightly enhanced (8%), Figure 3.8. Thus, the effects of desipramine on p53 induction resembled its synergistic impact on apoptosis.

To confirm that p53 was the functional link to these synergistic effects, a p53-deficient (p53-/-) isogenic clone of the HCT116 cell line was cultured with BBR3464, oxaliplatin, or c-DDP in the presence or absence of desipramine, Figure 3.9. Interestingly, apoptosis was measurable for all three drugs but reduced in comparison to cells expressing wild type p53 (See Figure 3.1). Despite the fact that levels of apoptosis did not reach those achieved with wild type HCT116 cells, C.I. values were significantly < 1. Hence, p53 has an important role in apoptotic effects of Pt-based cytotoxicity but in addition, there are

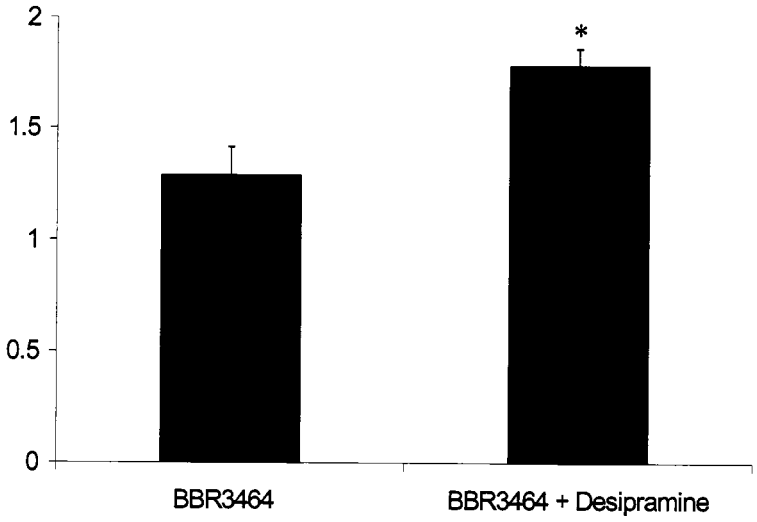

Figure 3.6. The effect of desipramine on BBR3464-mediated DNA adduct formation in HCT116 cells. HCT116 cells were treated with 55 μmol/L BBR3464 for 10h in the absence and presence of 75 μmol/L and cellular DNA adduct formation of BBR3464 was measured by ICP as described in method and materials. Higher concentration of BBR3464 was used in order to detect the platinum level bound to DNA by ICP-OES. Each point represents the average (+/- SEM) of three independent experiments. *, $p<0.05$ as determined by t test.

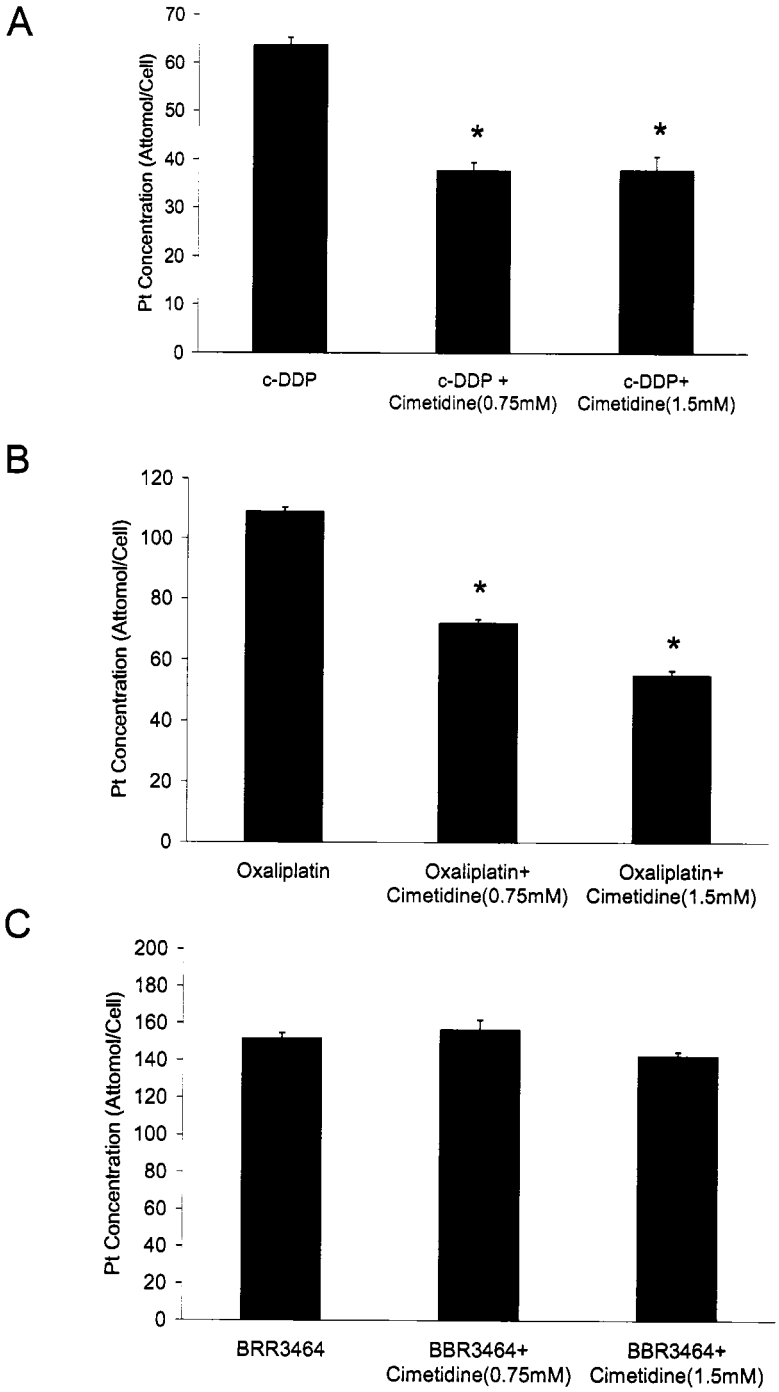

Figure 3.7. The effect of cimetidine on Pt-compounds cellular uptake. In A, B, C, HCT116 cells were treated with 20μmol/L BBR3464 for 8h, and c-DDP, Oxaliplatin for 16h, and cellular platinum content was measured by ICP-OES as described in method and materials. Each point represents the average (+/- SEM) of three independent experiments. *, $p<0.05$ as determined by t test.

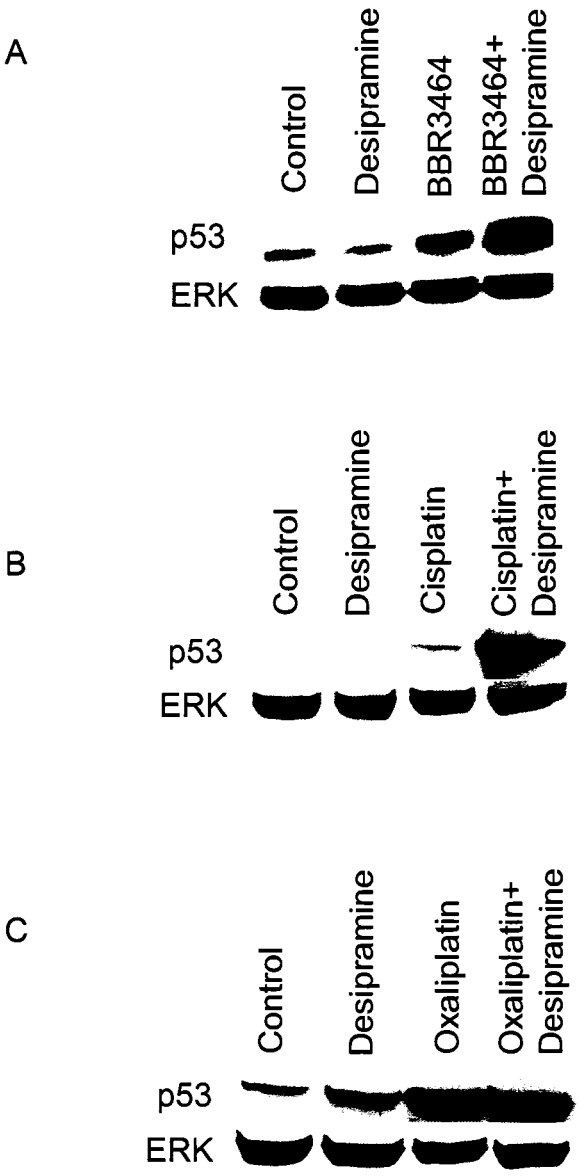

Figure 3.8. Effect of desipramine on downstream signaling pathways activated by BBR3464, c-DDP, and oxaliplatin. A, B, C. Effect of desipramine on BBR3464-, c-DDP- and oxaliplatin-induced p53 upregulation. HCT116 cells were cultured with 50μmol/L BBR3464, 10μmol/L c-DDP or 30μmol/L oxaliplatin in the absence and presence 40μmol/L desipramine for 72h, 48h, or 24h respectively. The expression of p53 was detected by western blotting. The same membrane was stripped and re-probed for ERK to show protein loading. The assay was consistently repeated three times.

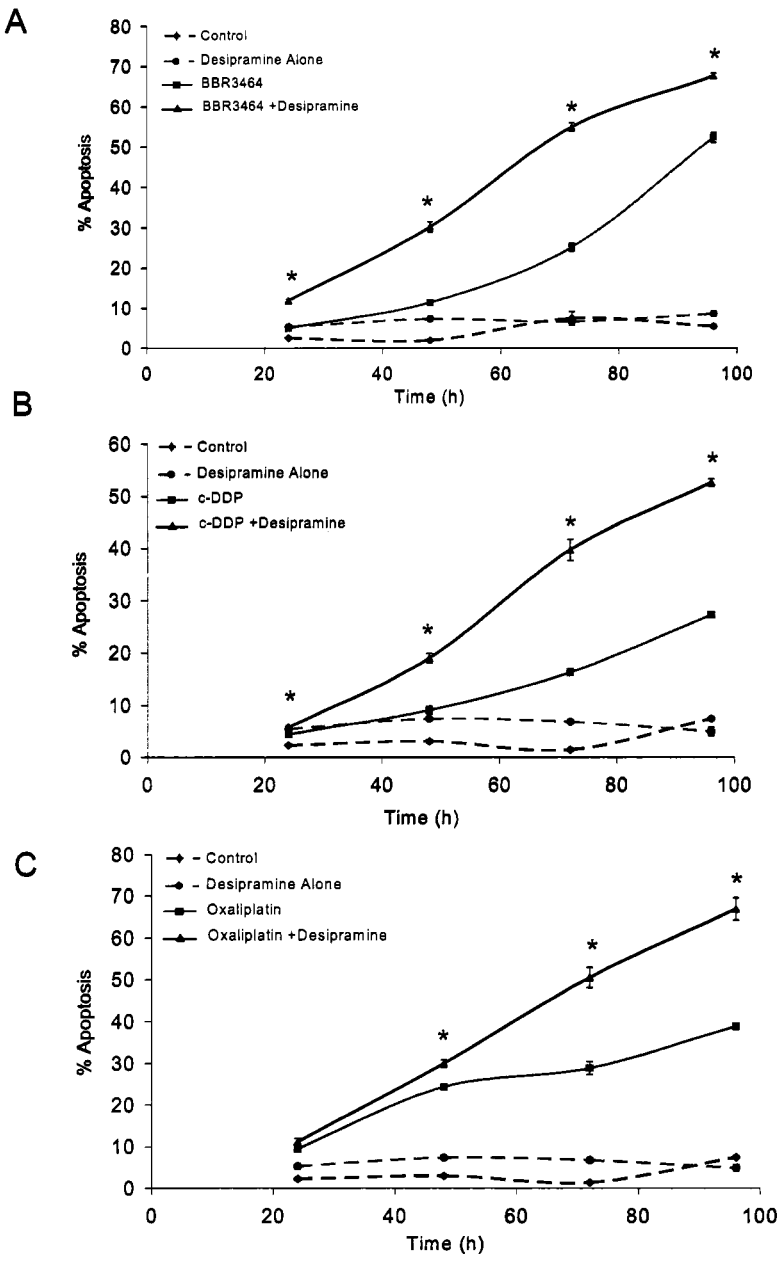

Figure 3.9. Effect of desipramine on BBR3464, c-DDP and oxaliplatin cytotoxicity in HCT116 -/- cells. Sub-diploid cell content was detected by PI-DNA staining. In A, B, C, HCT116 -/- cells were cultured with 50μmol/L BBR3464, 10μmol/L c-DDP or 30μmol/L Oxaliplatin for 72h in the absence or presence of 40μmol/L of desipramine. Drugs were added to the media after 1h of treatment with desipramine. Each point represents the average (+/- SEM) of three independent experiments. *, $p<0.05$ when comparing cells treated with and without desipramine, by Student's t-test.

p53-independent signaling pathways involved in cellular apoptosis induced by desipramine in combination with Pt drugs.

3.4.4. Importance of The p53 Dependent Pathway in Induced Apoptosis

Given that higher levels of apoptosis induction are seen in the cells expressing wild type p53, the relevance of p53 function to the observed synergy was examined. If the effects of desipramine on p53 expression are functionally significant, they should be consistent with increased mitochondrial damage, activation of the downstream death-inducing caspase enzymes and increased cleavage of the caspase substrate PARP, all of which can be triggered by p53 [37]. The same drug concentrations employed in the apoptosis assays of Figure 3.1 were used in these experiments. The effects of desipramine on mitochondrial damage, caspase activation and total PARP levels mirrored the effects on apoptosis and p53 induction, Figure 3.10. The integrity of the mitochondrion was examined by measuring desipramine/platinum drug-mediated loss of mitochondrial membrane potential ($\Delta\psi m$), which leads to apoptosis. Desipramine/platinum drug treatment significantly decreased $\Delta\psi m$, increasing the % of cells showing reduced $\Delta\psi m$ by 121%, 103%, and 78% in HCT116 carcinoma cells treated with BBR3464, c-DDP, and oxaliplatin alone, respectively (Figure 3.10A). In a similar manner, caspase 3 activation was increased 44%, 205%, and 47% in HCT116 carcinoma cells treated with desipramine prior to BBR3464, c-DDP, oxaliplatin addition, respectively (Fig. 3.10B). Finally, the expression of uncleaved PARP was decreased in HCT116 carcinoma cells treated under the same experimental conditions, Figure 3.10C. These effects mimic the effect of desipramine on p53 induction,

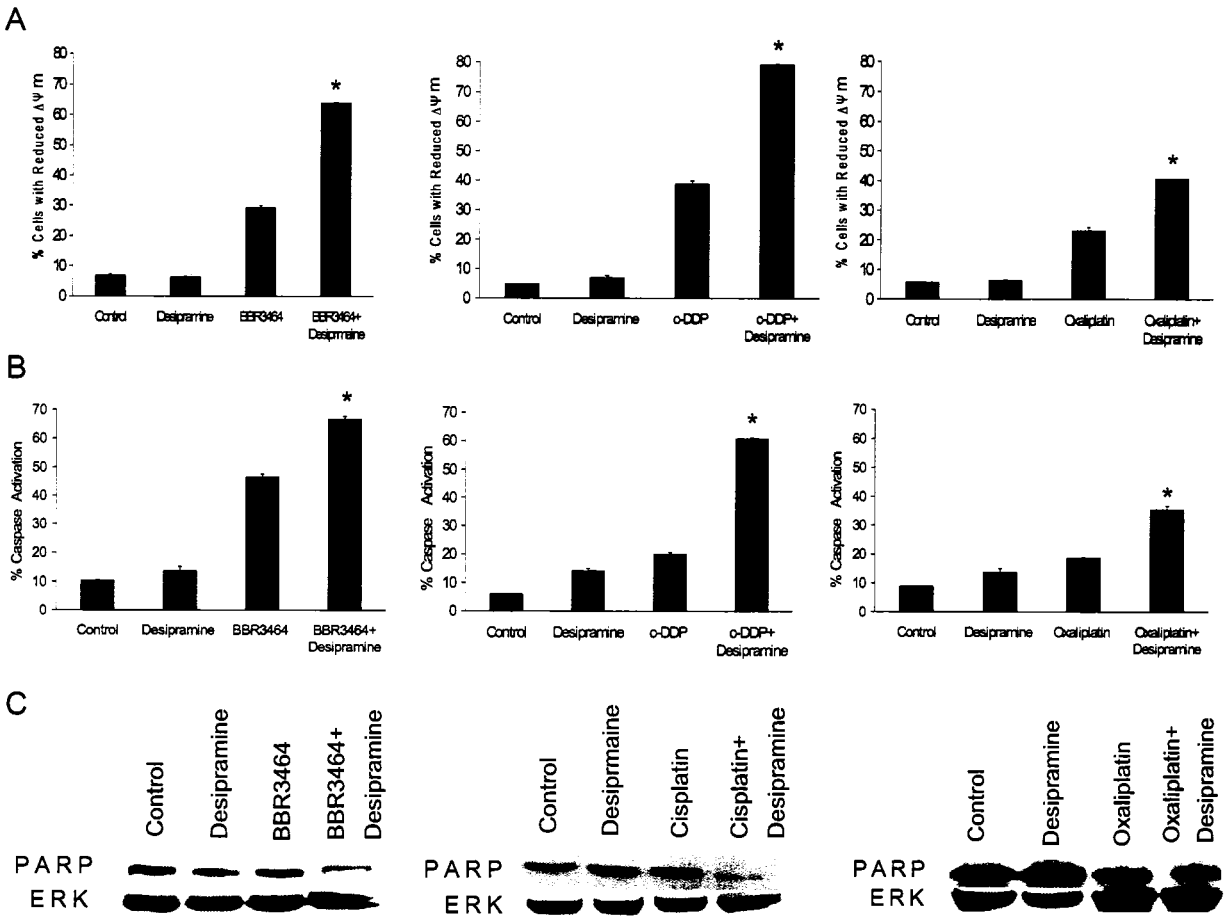

Figure 3.10. Effect of desipramine on downstream signaling pathways of p53 activated by BBR3464, c-DDP and oxaliplatin. A. Effect of desipramine on BBR3464, c-DDP, and oxaliplatin-induced mitochondrial damage. HCT116 cells were cultured with 50μmol/L BBR3464, 10μmol/L c-DDP, or 30μmol/L oxaliplatin in the absence or presence of desipramine for 72h. Reduction in mitochondrial membrane potential was assessed by staining with $[Di(OC_6)_3$ as described in Materials and Methods. Mitochondrial damage by c-DDP and BBR3464 was significantly affected by desipramine. B. Effect of desipramine on Pt-drug-induced caspase activation. HCT116 cells were cultured as in (A). Cells were stained for active caspase-3/7 as described in Materials and Methods. Data shown are the percent of the population displaying active caspase-3/7. Each point represents the average (+/-SEM) of three independent experiments. C. The effect of desipramine on uncleaved PARP expression in BBR3464-, c-DDP- and Oxaliplatin-treated samples. HCT116 were cultured as in (A). The expression of uncleaved PARP was detected by western blotting. The same membrane was stripped and re-probed for ERK to show protein loading. Each experiment was consistently repeated three times. *, $p<0.05$ when comparing cells treated with and without desipramine, by Student's t-test.

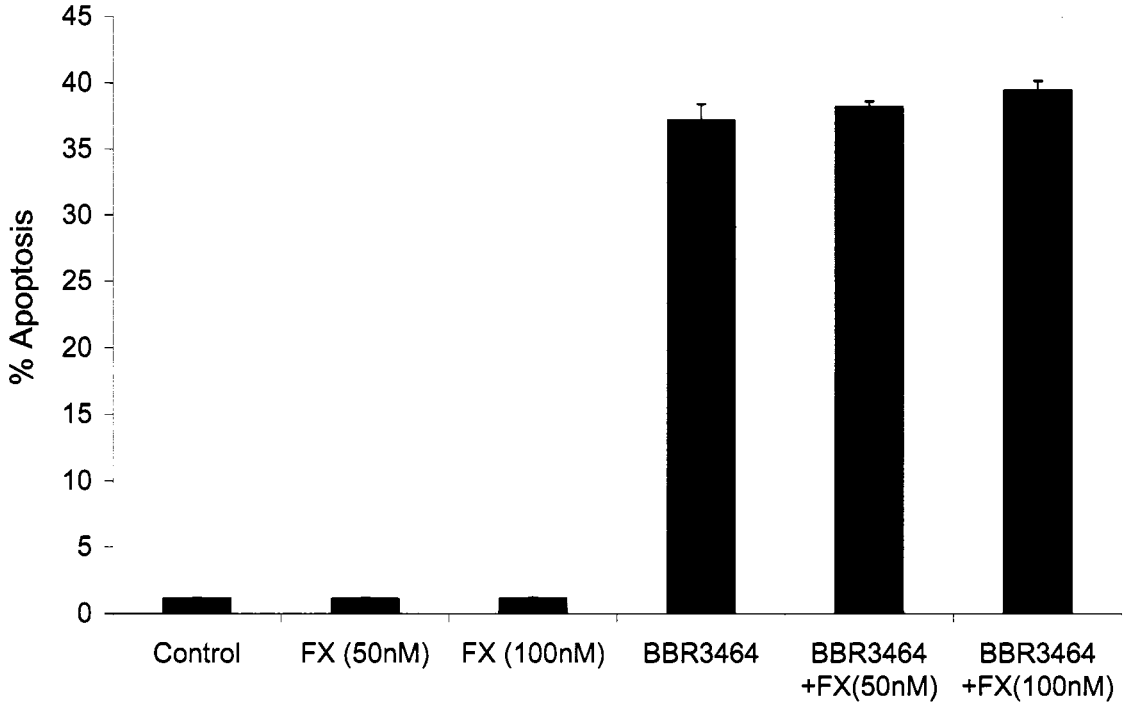

Figure 3.11. Effect of fluoxetine (FX) on BBR3464 cytotoxicity in HCT116 cells. Sub-diploid cell content was detected by PI-DNA staining. HCT116 cells were cultured with 50μmol/L BBR3464 for 72h in the absence or presence of fluoxetine. Drugs were added to the media after 1h of treatment with fluoxetine. Each point represents the average (+/- SEM) of three independent experiments

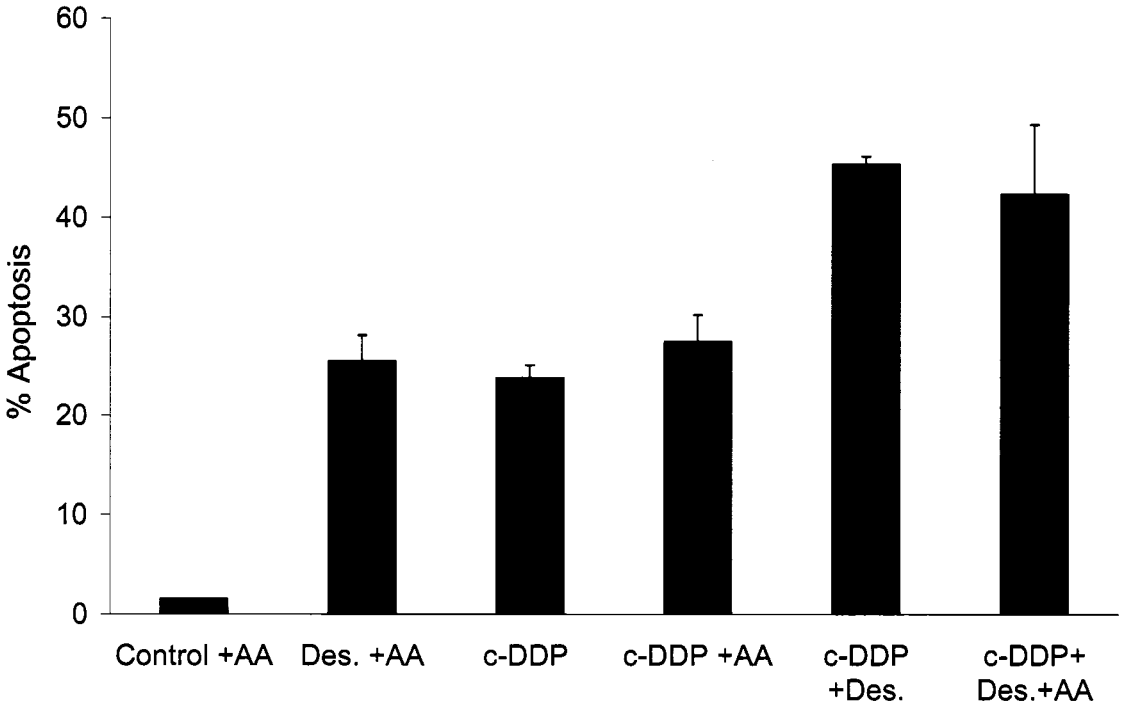

Figure 3.12. Effect of ascorbic acid (AA) on c-DDP cytotoxicity in HCT116 cells. Sub-diploid cell content was detected by PI-DNA staining. HCT116 cells were cultured with 40μmol/L c-DDP for 24h in the absence or presence of 75 μmol/L and 250 μmol/L of desipramine (Des.) ascorbic acid (AA). Drugs were added to the media after 1h of treatment with ascorbic acid. Each point represents the average (+/- SEM) of three independent experiments.

mitochondrial damage and caspase activation (Fig. 3.10C). Moreover, serotonin and oxygen radicals did not have a role in desipramine-induced synergism, as judged by the fact that fluoxetine (a selective serotonin reuptake inhibitor) or ascorbic acid (an oxygen radical scavenger) did not alter BBR3464- and c-DDP-induced apoptosis in HCT116 cells respectively (Figure 3.11, 3.12). These results argue that enhanced activation of the p53 pathway has an important role in the synergistic effects of desipramine on platinum drug-induced cytotoxicity.

3.5. Discussion

The antidepressant desipramine augmented BBR3464-, c-DDP-, and oxaliplatin-mediated cytotoxicity in a highly synergistic manner. The optimal dose of desipramine was well within the range of clinically relevant doses achieved in patients, where the free desipramine concentration in the serum reaches approximately 9.5 μM [39]. Desipramine enhanced c-DDP cellular uptake, but had no effect on the cellular accumulation of BBR3464 or oxaliplatin, suggesting that enhanced accumulation was not a consistent means by which desipramine altered platinum drug-mediated cytotoxicity. Cellular accumulation of c-DDP was increased 2.7 fold by desipramine, which may explain the more rapid kinetics of apoptosis seen (48 h for c-DDP vs. 72 h for BBR3464 and Oxaliplatin) (Figure 3.1). The accumulation results contrast with that found for cimetidine where the cellular accumulation of c-DDP was reduced (Figure 3.7). These results are consistent with underappreciated subtlety - that different inhibitors may affect specific transporters or different transport pathways to varying extents [25,40]. This point is

emphasized in the present case by the fact that OCT1 but not OCT2 expression was found in a panel of colon cancer cell lines [30] and, as a corollary, inhibitors targeted toward specific transporters may display widely varying effects dependent on the specific expression levels in any tumor type. Another factor to consider in the present case is that other transporters are influenced by desipramine [25,32], [41-43] or perhaps desipramine inhibits the efflux transporters. Interestingly, oxaliplatin uptake in OCT-transfected kidney cells [30] and colon cancer cells is diminished in the presence of cimetidine (Figure 3.7). The fact that desipramine has no effect on cellular uptake of oxaliplatin stands in contrast therefore – again, subtle differences in substrate (inhibitor) affinity may be the reason and suggests further methods to manipulate cytotoxic responses. Interestingly, the addition of cimetidine decreased apoptosis in the presence of c-DDP or oxaliplatin but had no effect on induction by BBR3464 (Figure 3.13), in agreement with previous observations on the mononuclear drugs using an MTT assay [30].

The effects of desipramine (and also cimetidine) on BBR3464 are similar to those found for a dinuclear compound [31] and suggest that OCT transport is not an important mechanism of uptake for these compounds. Overall, the combined results suggest further that organic cation transporters do not play a role in desipramine-induced synergistic effects.

Within the realm of cell cycle arrest and apoptosis, perhaps no cell signaling pathway is more relevant than p53 activation. All three Pt compounds induced p53 expression in HCT116 cells. Interestingly, the effect of desipramine on p53 induction directly correlated with its synergic effects on Pt-mediated cell death. As shown in

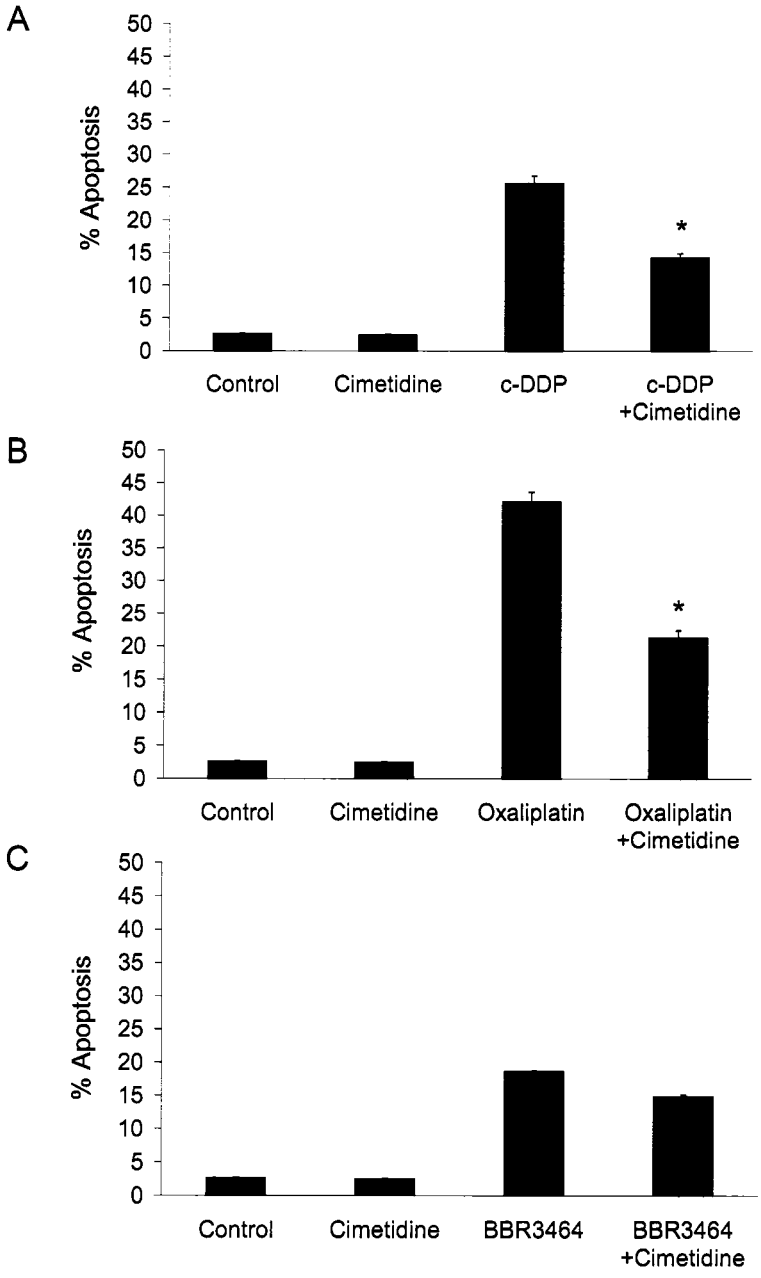

Figure 3.13. Effect of cimetidine on BBR3464, c-DDP and Oxaliplatin cytotoxicity in HCT116 cells. Sub-diploid cell content was detected by PI-DNA staining. A, B, C. HCT116 cells were cultured with 50μmol/L BBR3464, 10μmol/L c-DDP or 30μmol/L Oxaliplatin for 72h in the absence or presence of 0.75 mmol/L of cimetidine. Drugs were added to the media after 1h of treatment with cimetidine. Each point represents the verage (+/- SEM) of three independent experiments. *, $p<0.05$ when comparing cells treated with and without cimetidine, by Student's t-test.

Figure 3.3, all platinum drugs used (BBR3464, c-DDP, and Oxaliplatin) induced robust p53 expression in HCT116 cells. Desipramine further enhanced this upregulation, especially when combined with BBR3464 and c-DDP (Fig. 3.8A, B). The importance of p53 to the synergistic effect of desipramine was determined using p53-deficient HCT116 cells, an isogenic companion set to the HCT116 cells. In the absence of p53, the synergic effect of desipramine was still observed even if apoptosis was lower overall (Figure 3.9). While HCT116 showed ~80% apoptosis at 72h, p53-/- HCT116 cells exhibited ~50% apoptosis at this time point. It is possible that multiple and parallel signaling pathways are invoked by the synergic response, suggesting that this effect is also general and applicable to a broad range of tumors with varying p53 gene status.

In the case of cells bearing wild type p53, enhanced p53 expression appeared to be functionally important based on measures of mitochondrial damage, caspase activation and PARP cleavage. In particular, the effects on PARP levels are noteworthy as it plays an important role in DNA repair as well as DNA replication [19]. It was recently demonstrated that PARP interacts with c-DDP-DNA adducts in human cells, and that PARP inhibition can enhance cellular sensitivity to c-DDP [44]. Combination treatment of desipramine and platinum drugs showed increased PARP cleavage, which could partly explain the increased sensitivity of colon carcinoma cells to cytotoxicity to platinum drugs.

Conclusion. An unexpected result from these studies on the relevance of OCT transporters to platinum drug accumulation is the synergistic effect on cytotoxicity shown by the putative OCT inhibitor desipramine. The tricyclic organic, a safe and effective antidepressant already in use for cancer patients, greatly augments the cytotoxicity of

platinum-based chemotherapeutics. These effects correlated with enhanced activation of the p53- mitochondrial death pathway, but a p53-independent mechanism is also apparent. The study is of high clinical relevance and argues that desipramine may be a means of enhancing chemo-responsiveness to platinum-based anticancer agents, and warrants further investigation for its clinical utility. The study also confirms the potential for rational design of chemotherapeutic regimens based on mechanistic approaches of signaling complementarily.

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List of References:

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Chapter 4: Biological Activity of AH78, a Noncovalent Binding Platinum Drug

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Virginia Commonwealth University

In preparation for Cancer Research

Synthesis by Ralph Kipping, Presented Biological Data by Peyman Kabolizadeh

4.1. Abstract

The structurally novel platinum complex, BBR3464, is a cationic trinuclear compound and has undergone Phase II clinical trial in cisplatin (c-DDP)-resistant and refractory cancers. It is significantly more cytotoxic than c-DDP or oxaliplatin and retains activity against c-DDP-resistant tumor cells *in vitro* as well as *in vivo*. Its mechanism of cytotoxicity was assumed to result from covalent binding to DNA. Previous studies have indicated that the DNA binding of BBR3464 has a preassociation noncovalent component that affects the binding kinetics and the final structure of the formed Pt-DNA adducts. To examine the preassociation component, a compound indicated as AH78 was synthesized with inert ammonia or amine groups instead of the labile chloride ligand, such that the DNA binding can only be obtained through non-covalent interactions. The biological activity of AH78 was investigated and compared to the parent drug, BBR3464. Similar to c-DDP and

BBR3464, it was shown through the use of transfected cell lines, that AH78 used the copper transporter hCTR1 to enter cells and to a lesser extent, the ATP7B transporter to exit cells. AH78 induced p53 in HCT116 cells; however, its induced cytotoxicity was not affected by p53 deletion. AH78 differed from BBR3464, by inducing G1 rather than G2 cell cycle arrest in HCT116 cells. Unlike c-DDP, oxaliplatin, and BBR3464; AH78 was able to circumvent deactivation by glutathione since its cyctoxicity was not affected by inhibition of glutathione synthesis. The antitumor activity of AH78 was also examined *in* vivo showing higher activity against 2008 ovarian carcinoma cells than c-DDP at the doses administered. Our study illustrates fundamental differences in the mechanisms of action between noncovalent binding drugs like AH78 and structurally related covalent binding drugs such as c-DDP, oxaliplatin, and BBR3464.

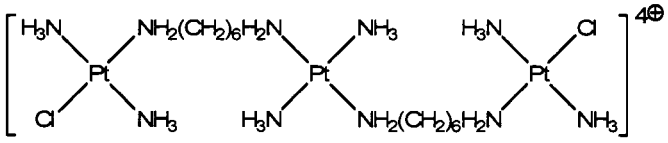
4.2. Introduction:

Cisplatin (c-DDP) and oxaliplatin (Fig. 4.1) are effective antineoplastic agents used for the treatment of cancer, including testicular, head and neck, ovarian, small cell lung, and colorectal neoplasms.[1] Their cytotoxicity is mediated mainly through interactions with DNA and inhibition of DNA synthesis and replication by formation of bifunctional interstrand and intrastrand cross links.[2,3] Their clinical efficacies are limited due to acquired resistance and dose-limiting side effects.[4]

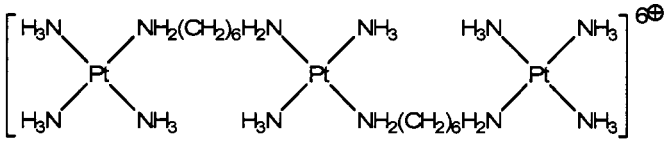
Structurally novel platinum complexes that bind to DNA in a different manner than c-DDP may have complementary cytotoxicity and side effect profiles of clinical relevance. The trinuclear complex, BBR3464 (Fig. 4.1), is significantly more cytotoxic than c-DDP

c-DDP

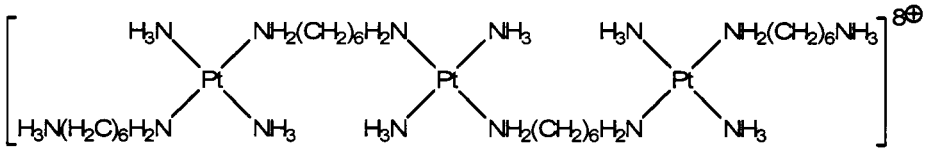
Oxaliplatin



BBR3464



AH44



AH78

Figure 4.1. Structures of c-DDP, Oxaliplatin, BBR3464, AH44 and AH788.

or oxaliplatin and retains activity against c-DDP-resistant tumor cells *in vitro* as well as *in vivo*.[5-9] The drug has undergone Phase II clinical trials in cisplatin-resistant and refractory cancers [10,11]. Its mechanism of cytotoxicity was assumed to result from covalent binding to DNA. Since BBR3464 has a high positive charge, its DNA binding has a preassociation noncovalent component that affects the binding kinetics and final structure of the formed PT-DNA adducts.[12,13] Because the drug/DNA adduct structure determines the repair mechanisms, protein recognition, and activation of downstream signaling cascades, it is important to investigate the formation and biological consequences of DNA adducts. To examine the preassociation component, compounds indicated as AH44, and AH78 (Fig. 4.1) were synthesized with substitution of inert ammonia or amine group instead of the labile chloride ligand respectively (Fig. 4.1).[14,15] Unlike BBR3464, c-DDP, and Oxaliplatin, AH44 and AH78 interact with DNA with high affinity, in an electrostatic manner, without the potential for covalent binding.[15, 16] AH78 binds to phosphate oxygen atoms associated with the DNA backbone. It extends along the phosphate backbone (backbone tracking) and spans the minor groove (groove spanning).[16] The positive charges of AH44 and AH78 not only increased their interactions with DNA but also resulted in increased cellular uptake. [15]

Due to acquired resistance to clinical Pt drugs, their mechanism of action has been studied extensively. The three major pharmacological factors contributing to the intrinsic cytotoxicity of, and cellular resistance to, platinum drugs are (i) cellular uptake and efflux of platinum, (ii) the frequency and nature of Pt-DNA adducts and (iii) deactivating metabolic reactions with sulfur-containing nucleophiles. [17] The role of cisplatin uptake and

efflux is increasingly being seen as a critical determinant of clinical resistance.[18] Defects in c-DDP accumulation are the single most commonly reported feature of cells selected for resistance.[19] Multiple pathways contribute to this resistance. Highly charged polynuclear platinum drugs have recently been shown to display higher cellular uptake than c-DDP – a factor which may contribute to their enhanced efficacy in comparison to mononuclear drugs. [15,20] The mechanisms by which small platinum-containing molecules enter cells are diverse – which is perhaps one reason why they are such useful anticancer agents. The use of cellular copper transporters has been documented as one mechanism of active uptake of platinum drugs.[21,22] BBR3464 and c-DDP use the same cellular transporters (hCTR1 and ATP7B) for influx and efflux; however, downstream effects are different for the two drugs, hence BBR3464 employs mechanisms of intracellular action distinct from c-DDP.[17]

Another important factor in cytotoxicity and pharmacology of Pt drugs is deactivation by sulfur-containing proteins such as glutathione (GSH).[23] The normal intracellular concentration of GSH ranges from 5 to 10 mM [24], therefore the binding of platinum-containing drugs to GSH at its sulfur position is highly probable. Studies have shown that cells with elevated glutathione (>10mM) are more resistant to cisplatin [25] indicating its clinical importance. There is a possibility that noncovalent binding drugs such as AH44 and AH78 may circumvent deactivation by glutathione. Therefore, it is of interest to examine platinum drugs such as AH78 or AH44 with no chloride to act as a leaving group and their interactions with and deactivation by glutathione.

In this paper, the apoptotic, cellular effects, and uptake mechanisms of non-covalent binding drugs were investigated using carcinoma cell lines. The antitumor

activity of AH78 against the 2008 ovarian tumor xenograft model was also studied. This study reveals novel and clinically significant differences in cell death and antitumor activity induced by non-covalent binding drugs (AH78) and covalently binding Pt drugs (c-DDP, BBR3464, and Oxaliplatin).

4.3. Materials and Methods

4.3.1. Compound Synthesis.

BBR3464, c-DDP, and AH78 (Fig. 4.1) compounds were synthesized according to methods reported previously.[14,15] Oxaliplatin (Fig. 4.1) was obtained from Sigma-Aldrich (St. Louis, MO). Buthionine sulphoximine (BSO) was obtained from Sigma-Aldrich (St. Louis, MO).

4.3.2. Cell System.

The colorectal carcinoma cell lines HCT116, and matched p53-deficient HCT116 cells (HCT116-/-) were the kind gift of Bert Vogelstein (Johns-Hopkins University, Baltimore, MD). HCT116 cells were cultured with RPMI 1640 in 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) in humidified air with 5% CO2. Ovarian carcinoma A2780/hCTR1, 2008/pRC/CMV7B (2008/ATP7B), 2008/EV pRC/CMV (2008) cells were the kind gift of S. Howell, University of California at San Diego. These cell lines were cultured with 500 μg/ml G418.

4.3.3. Culture Conditions.

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 drugs concentrations were adjusted to achieve approximately 15%-20% apoptosis, allowing us to measure enhancement or inhibition.

4.3.4. Propidium Iodide DNA Staining and analysis of apoptosis.

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 [26]. Samples were then analyzed for subdiploid DNA content 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.

4.3.5. Platinum Accumulation Assays.

Cells were plated at 2.0×10^6 cells/mL. c-DDP, Oxaliplatin, BBR3464, or AH78 was added in different concentrations alone or 60 minutes after the addition of BSO. After the indicated times, cells were harvested 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 simultaneous inductively coupled plasma optical emission spectroscopy at 265 nm (Varian Inc., Palo Alto, CA). Standards and blank were prepared the same as the samples.

4.3.6. Western Blotting.

Whole-cell lysates were blotted with mouse monoclonal antibody against p53 (BD bioscience), or mouse monoclonal antibody against ERK (Cell Signaling Technology), and resolved with secondary antibody conjugated with horseradish peroxidase. Blots were then treated with a chemiluminescent substrate (Pierce, Rockford, IL, USA) and exposed to film. Band intensity was measured by densitometry using an Eagle Eye II system (Stratagene, La Jolla, CA).

4.3.7. Statistical Analysis.

Results are the mean and standard error. Statistical analysis was performed using *t*-test for two data points using SysStat9 software (SPSS, Chicago, IL, USA). $p < 0.05$ was considered to be significant.

4.3.8. In Vivo Experiments.

4.3.8a Preparation of samples.

The experimental compounds were stored at 4°C. Solutions were made in saline immediately before administration. Cisplatin was obtained from the Peter Mac Pharmacy (East Melbourne, VIC) and diluted in saline immediately prior to administration.

4.3.8b 2008 xenograft model.

Female Balb/c nude mice (Age 5 weeks) were inoculated s.c. on the flank with 3.5 x 10^6 2008 cells in PBS:Matrigel (1:1). Animals were randomized into 4 groups of 9 animals once mean tumor volume of ~140 mm^3 (defined as day 0) was achieved. The animals were treated on Days 0, 4, 8 with either (i) 25 mg/kg AH78 by ip injection or (ii) saline (0.1 ml/10 g body weight) or, on Days 0, 7, 14 with (iii) 4 mg/kg cisplatin by ip injection. Tumors were measured twice weekly using electronic calipers. Tumor volume was calculated as length/2 x $width^2$. Animals were harvested once the tumors reached 4 times their Day 0 volume. On Day 45, any animals remaining were harvested.

Drug efficacy was assessed using two analyses. The tumor growth delay index (GDI) was calculated as (T/C) where T is the median time in days for tumors in drug treated mice to reach 3 or 4 times their starting volume and C is the time in days for tumors in control mice (saline) to reach the same volume. Percent tumor growth inhibition (TGI) was calculated as 100 - (median relative tumour volume of treated group/ median relative tumor volume of control group x 100) on a specified day. Absolute Growth Delay (AGD) was calculated as median time in days to reach 3 or 4 times starting tumor volume.

Differences between the groups were tested for significance using a one-way ANOVA followed by Tukey's test.

4.4. Results:

4.4.1. Importance of Copper Transporters in AH78 cellular uptake and cytotoxicity.

4.4.1a Influence of hCTR1

hCTR1 has been shown to mediate c-DDP and BBR3464 cellular uptake [17,27]. The role of hCTR1 in AH78 cellular transportation was determined by comparing uptake level in the parental ovarian carcinoma cell line A2780 with A2780 cells over-expressing hCTR1 (A2780/hCTR1). Cells were cultured for 3h in the presence of AH78 and platinum uptake was determined by ICP-OES. As shown in figure 4.2A, platinum levels in AH78-treated cells were 1.6 times greater in A2780/hCTR1 cells than in A2780 cells. This data shows a role of hCTR1 in transport of AH78.

To determine if the increase in cellular uptake observed in hCTR1-transfected ovarian carcinoma cells was functionally significant, AH78-mediated growth inhibition was measured in the A2780 and A2780/hCTR1 carcinoma cells. Growth inhibition was calculated as the decrease in live cell numbers measured *via* PI-DNA staining. A2780/hCTR1 cells were more sensitive to AH78–induced cytotoxicity (AH78 $IC_{50}\approx1.2\mu M$) at low concentration (up to 4μM) than parental A2780 cells ($IC_{50}\approx2.2\mu M$) (Fig. 4.2B). However, the sensitivity of hCTR1 transfected cells was diminished at AH78 concentration higher than 4μM. These results support a role for hCTR1 in AH78-mediated cytotoxicity at low concentration.

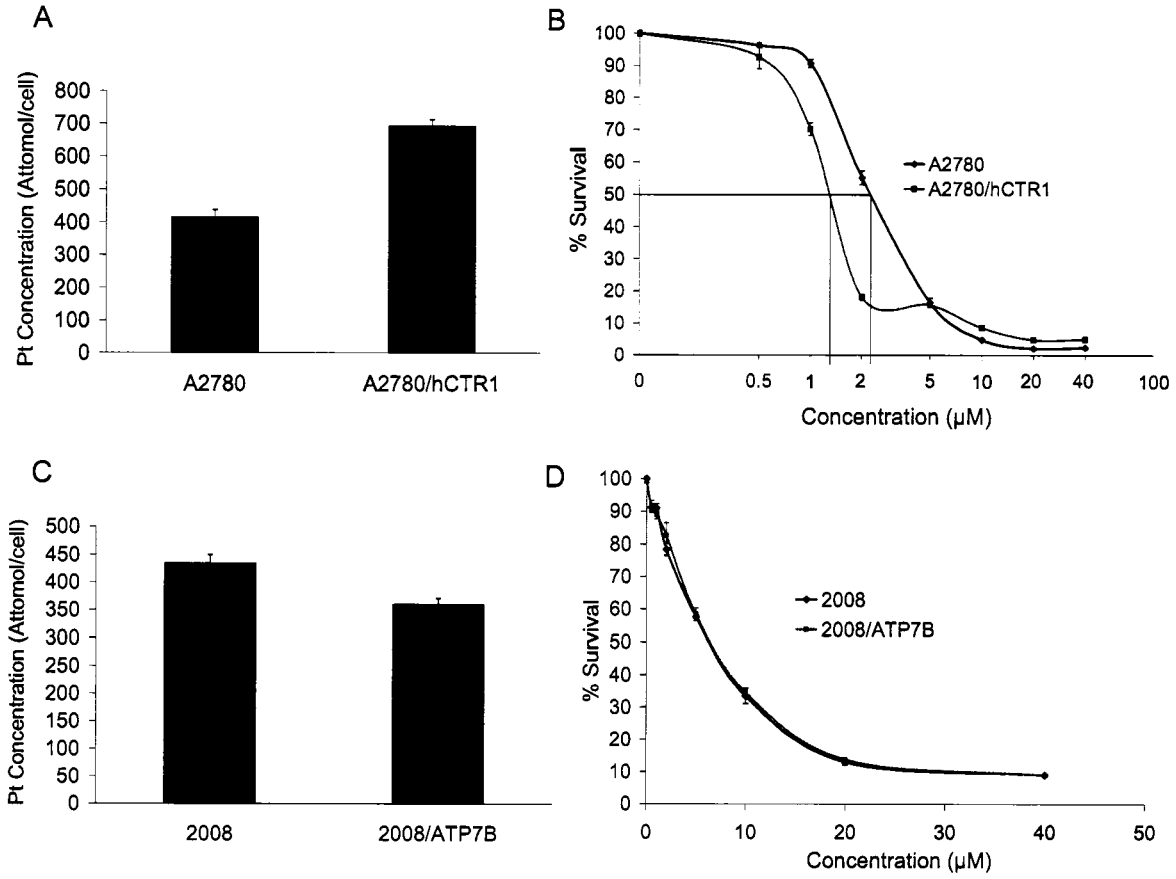


Figure 4.2. The importance of Copper Transporters in platinum drug uptake in ovarian carcinoma cells. A. AH78 uptake was measured by ICP in 10 million cells treated with 20 μM c-DDP for 3h before harvesting. B. The effects of hCTR1 Expression on AH78-mediated growth inhibition. A2780 and A2780/hCTR1 cells were cultured in the indicated concentrations of AH78 for 72h. Percent growth inhibition was determined by comparing live cell numbers in treated and untreated cultures after 72h, as measured by PI-DNA staining and timed counting via flow cytometery as described in Materials and Methods. Each point is the average (+/- SEM) of three independent experiments. C. Effect of ATP7B transporter on platinum drug retention. Cellular platinum levels after treatment with 20μM AH78 for 3h were determined by ICP. Each bar indicates the average (+/- SEM) of three independent experiments. In D, 2008/pRC/CMV7B ("2008/ATP7B") and 2008/EV pRC/CMV ("2008") cells were treated with the indicated concentrations of AH78. Percent growth inhibition was determined by comparing live cell numbers in treated and untreated cultures after 72h, as measured by PI-DNA staining and timed counting via flow cytometry. Each point is the average (+/- SEM) of three independent experiments. *, $p<0.05$ as determined by t test.

4.4.1b Influence of ATP7B.

The copper transporter ATP7B has been shown to be important for reducing cytosolic levels of c-DDP and BBR3464 by promoting cellular efflux [17,28]. The role of ATP7B in AH78 cellular uptake was examined by comparing cellular platinum levels in the parental ovarian carcinoma cell line (2008) to 2008/ATP7B cells, transfected to over-express ATP7B. This over-expression conveyed a small but reproducible decrease in cellular platinum levels when cells were treated with AH78 (Fig. 4.2C).

The sensitivity of the 2008 cell lines was examined by measuring growth inhibition *via* PI-DNA staining. Despite the reduction in cellular platinum levels, ATP7B over-expression conveyed no difference in sensitivity to treatment with AH78 (Fig. 4.2D).

4.4.2. Importance of p53 in AH78-mediated cytotoxicity.

Many apoptotic signaling pathways converge at the transcription factor p53. p53 elicits cell death in part by inducing mitochondrial damage that activates the death effector caspase enzymes [29]. Both c-DDP, Oxaliplatin and BBR3464 have been argued to elicit apoptosis in certain cell lineages *via* a p53-dependent pathway [19,20]. Given its similar cellular transportation mechanism to BBR3464 and c-DDP [17], the importance of p53 function in AH78-mediated cytotoxicity was investigated in the colorectal carcinoma HCT116 cells.

As shown in figure 4.3A, AH78 increased p53 protein expression more than 180% at the highest concentration of AH78 used. To determine the importance of p53 function in AH78-mediated cytotoxicity in comparison to other Pt drugs, the wild type HCT 116

colorectal carcinoma cells and its p53-deficient (p53ko) isogenic clone were cultured with AH78, c-DDP, oxaliplatin, and BBR3464, and growth inhibition was measured by PI-DNA staining. There was no significant difference observed in AH78-mediated cytotoxicity in the two isogenic colorectal cell lines (AH78 $IC_{50}\approx2.5\mu M$), indicating a non-essential role for p53 (Fig. 4.4). By comparison, HCT116 p53 deficient cells were 2-3 times less sensitive to c-DDP, Oxaliplatin, BBR3464–induced cytotoxicity (c-DDP $IC_{50}=1.5\mu M$, Oxaliplatin $IC_{50}\approx2.25\mu M$, BBR3464 $IC_{50}\approx50nM$) than wild type HCT116 cells (c-DDP $IC_{50}\approx0.75\mu M$, Oxaliplatin $IC_{50}\approx0.65\mu M$, BBR3464 $IC_{50}\approx28nM$).

Additionally, cell cycle arrest was analyzed after treatment with AH78 to determine its potential difference with BBR3464, which elicits a prominent G2 arrest [30]. Unlike BBR3464, AH78 induced a G1 cell cycle arrest (Fig. 4.5) in both wild type and p53-deficient colorectal carcinoma cell lines. As shown in figure 4.5A,B, the percent G1 arrest was increased to 72 % or 53% in HCT116 wild type and p53 deficient cells respectively after treatment with AH78. Concomitantly, G2 arrest was reduced to 21% or 36% in HCT116 wild type and p53 deficient cells respectively by AH78. This result was completely reversed when cell were treated with BBR3464, showing more G2 than G1 cell cycle arrest (Fig. 4.5C,D).

4.4.3. Importance of glutathione in AH78-mediated cytotoxicity.

Platinum complexes interact with molecules other than DNA in cells, especially sulfur-containing moieties. One of the main cellular biomolecules is glutathione (GSH), which has an important role in determination of cellular sensitivity to cytotoxic drugs. Therefore,

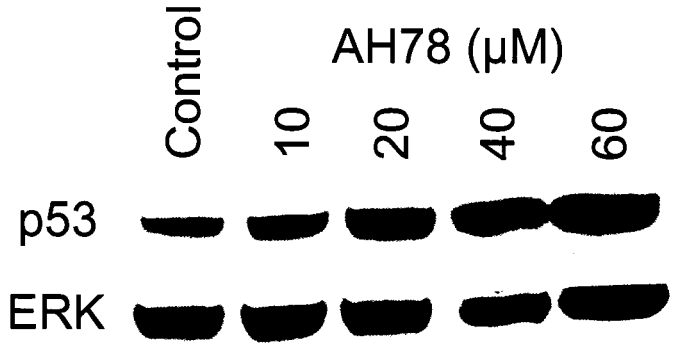


Figure 4.3. Effect of AH78 on p53 upregulation. HCT116 cells were cultured with indicated concentrations of AH78 for 24h. The expression of p53 was detected by western blotting. The same membrane was stripped and re-probed for ERK to show protein loading. The assay was consistently repeated three times.

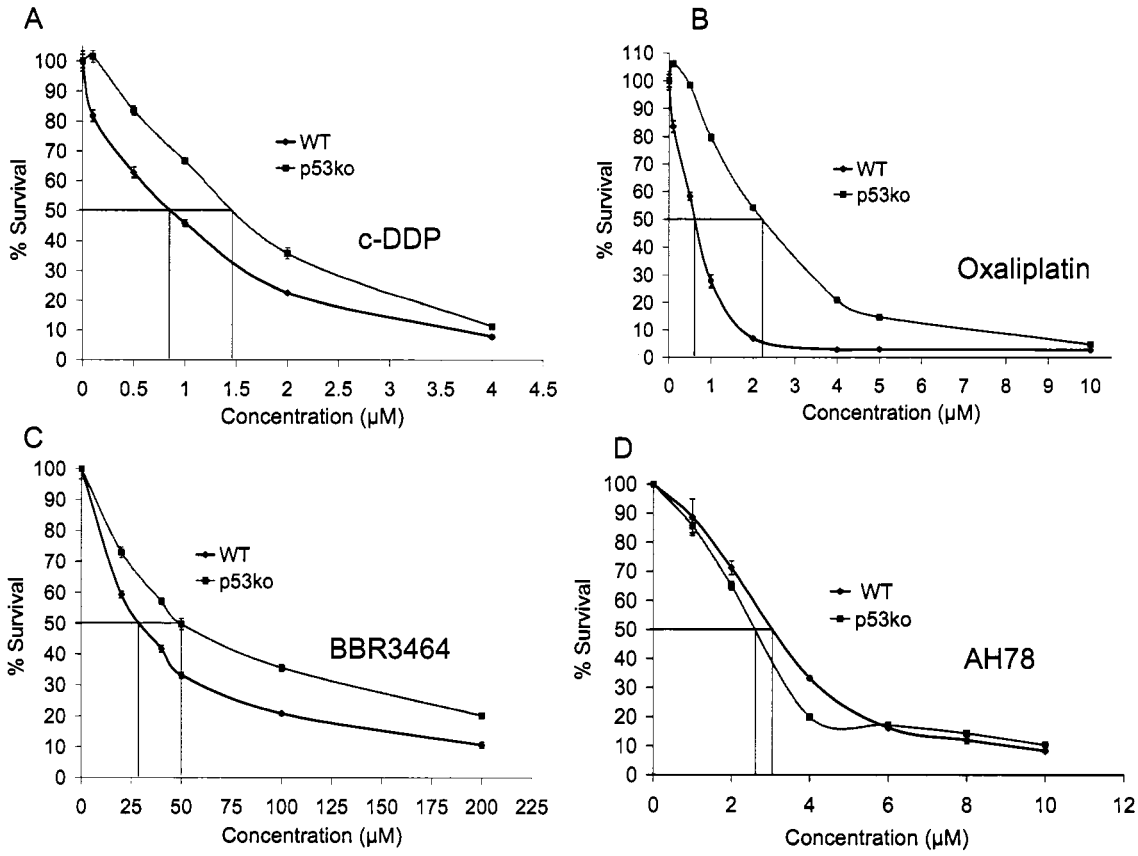


Figure 4.4. Importance of p53 role in Pt Drug- mediated cytotoxicity. A-D. The effects of p53 expression on c-DDP, Oxaliplatin, BBR3464, and AH78-mediated growth inhibition was measured using isogenic colorectal carcinoma cells lines (HCT116-wt, and HCT116/p53ko). HCT116 wt and HCT116/p53ko cells were cultured in the indicated concentrations of platinum drugs for 72h. Percent growth inhibition was determined by comparing live cell numbers in treated and untreated cultures after 72h, as measured by PI-DNA staining and timed counting via flow cytometery as described in Materials and Methods. Each point is the average (+/- SEM) of three independent experiments.

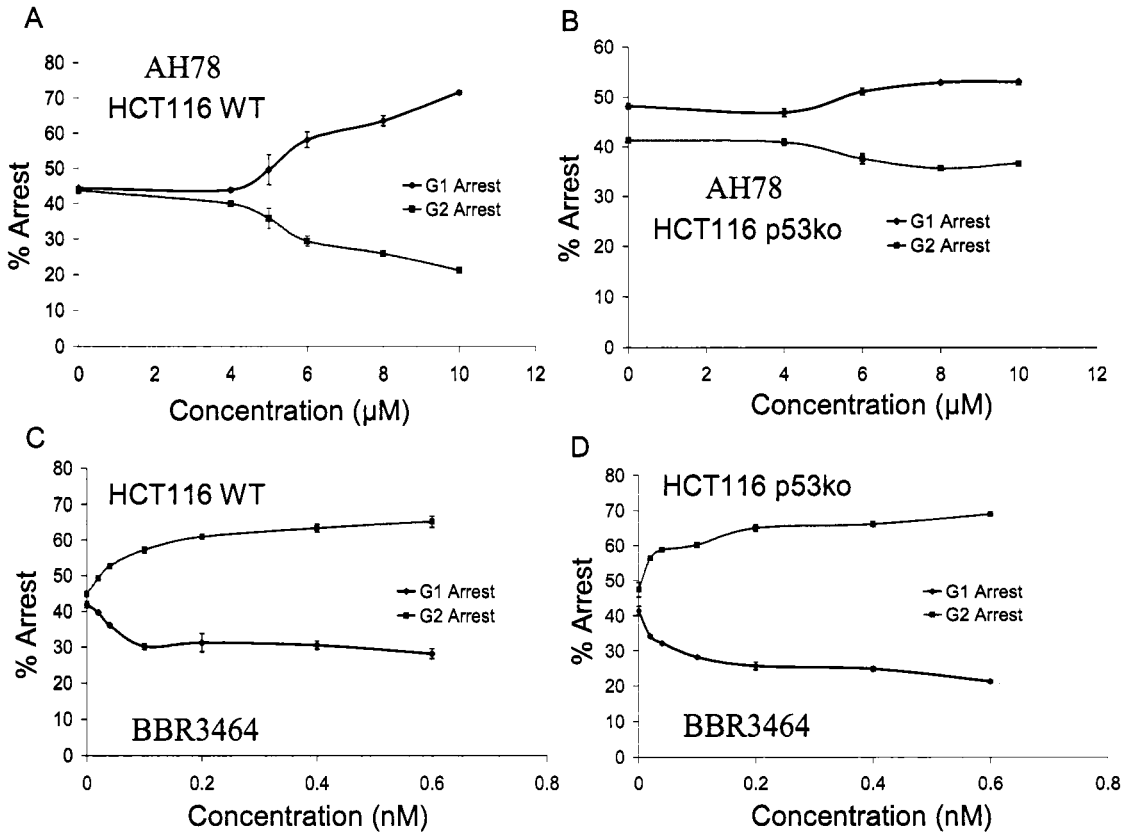


Figure 4.5. Effect of AH78 on cell cycle arrest measured by PI DNA staining. In A and B HCT116-wt and HCT116/p53ko cells were cultured in the indicated concentrations of AH78 for 72h. DNA content was measured to determine G1 and G2 arrest by flow cytometry. In C and D, HCT116-wt and HCT116/p53ko Carcinoma cells were cultured in the presence of BBR3464 for 72h and DNA content was measured to determine G1 and G2 arrest by flow cytometry. Each point is the average (+/- SEM) of three independent experiments.

it is important to assess the role of glutathione in the cellular response to the noncovalent binding platinum drugs such as AH78 or AH44. The cellular glutathione level was decreased using buthionine sulphoximine (BSO), which inhibits gamma glutamylcysteine synthetase, a rate limiting enzyme in glutathione synthesis. As the cellular glutathione level was reduced, c-DDP, oxaliplatin, and BBR3464-induced apoptosis was augmented, demonstrating their interactions with glutathione. As it is shown in Figure 4.6, after 48 hours 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%. The results showed consistency in testing c-DDP and Oxaliplatin. BSO increased c-DDP-induced apoptosis from 14% to 45%, and augmented oxaliplatin-induced cytotoxicity from 16% to 36% (Figure 4.6). In striking contrast to these covalent Pt drugs, AH78 or AH44-induced cytotoxicity was completely unaltered by inhibiting glutathione synthesis (Fig. 4.6). Hence, the lack of leaving groups appeared to alter sensitivity to GSH.

Since multi-drug resistance associated protein (MRP)-mediated efflux has been shown to be dependant on glutathione,[31] the effects of BSO on platinum drugs cellular uptake was measured. Cellular platinum level was measured *via* ICP-OES in cells treated with platinum drug +/- BSO to determine if the increase observed in cytotoxicitiy is the result of enhanced cellular uptake of platinum drugs. As shown in figure 4.7, BSO did not influence c-DDP, Oxaliplatin, BBR3464, and AH78 cellular uptake (Fig. 4.7A,B,C,D). Hence, cellular uptake does not play a role in the augmentation of Pt-induced cytotoxicity by BSO.

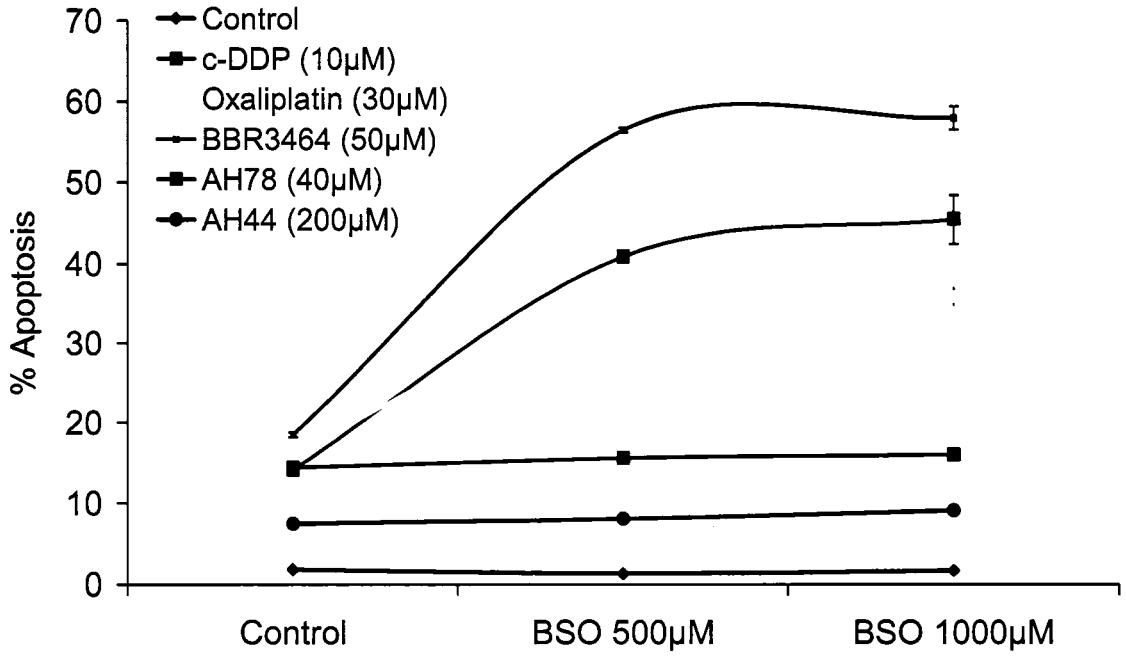


Figure 4.6. Effect of BSO on platinum drug-induced cytotoxicity in HCT116-wt cells. Sub-diploid cell content was detected by PI-DNA staining. HCT116-wt cells were cultured with 50μM BBR3464, 10μM c-DDP, 30μM Oxaliplatin, 200μM AH44 or 40μM AH78 for 72h in the absence or presence BSO. Drugs were added to the media after 1h of treatment with BSO. Each point represents the average (+/- SEM) of three independent experiments. *, $p<0.05$ when comparing cells treated with and without desipramine, by Student's t-test.

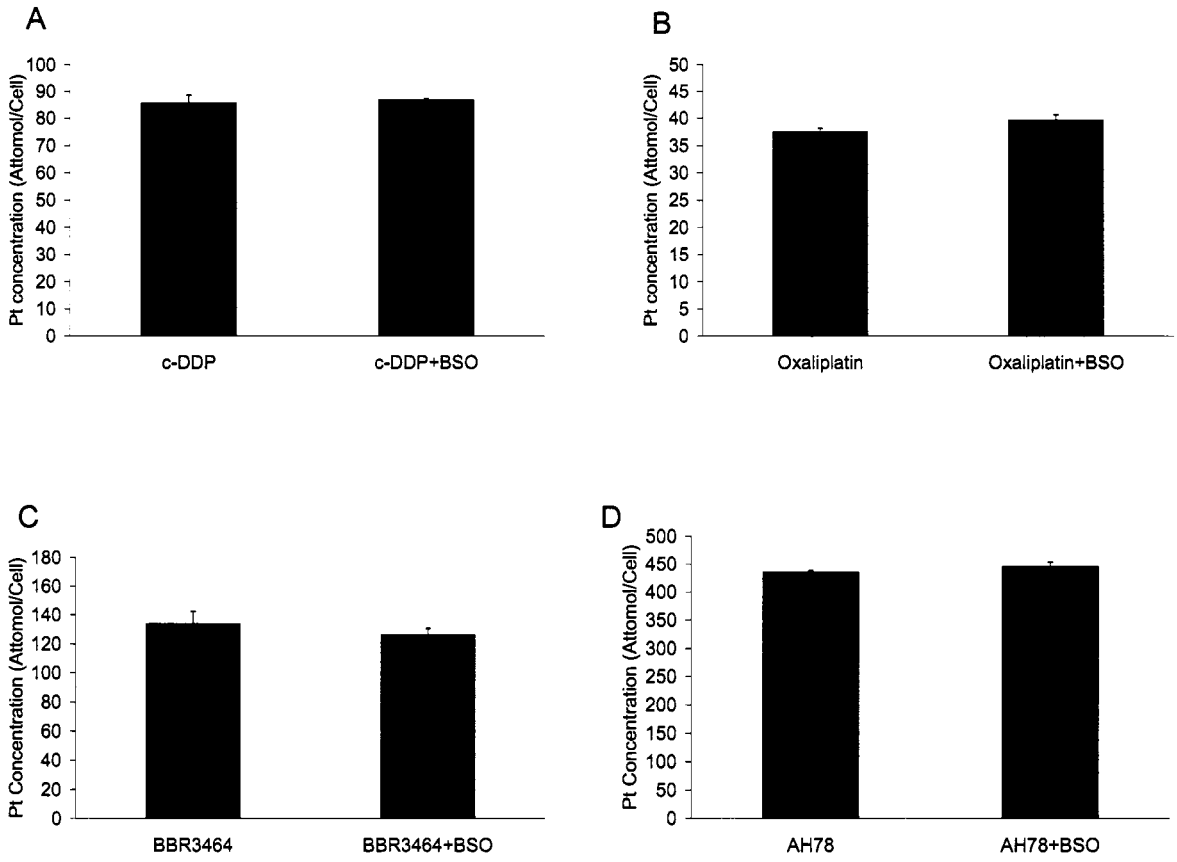


Figure 4.7. Effect of BSO on platinum drugs cellular uptake in HCT116-wt carcinoma cell lines. In A, B, HCT116 cells were cultured with 20μM c-DDP, or Oxaliplatin for 16h, in the absence or presence of 500μM BSO. In C and D, HCT116-wt cells were treated with 20μM BBR3464, or AH78 for 8h or 3h respectively. Drugs were added to the media after 1h of treatment with BSO. Each point represents the average (+/- SEM) of three independent experiments.

4.4.4. Antitumor activity of AH78 against the 2008 ovarian tumor xenograft model.

The antitumor activity of AH78 in colorectal (HCT116) and Ovarian carcinoma (A2780, and 2008) cells *in vitro* showed promising results, therefore, the effect of AH78 was investigated in human tumor xenografts using the human 2008 ovarian carcinoma model. The antitumor effects of AH78 when administered at its maximum tolerated dose were evaluated in comparison to cisplatin (Fig. 4.8A, Table 4.1). Tumor volume was measured twice weekly following the start of treatment. Tumor growth in cisplatin-treated groups was similar to that of saline control. In contrast, tumor growth was significantly delayed by AH78 treatment in comparison to the saline control and c-DDP-treated group, especially on day 10 (Table 4.1). Moreover, as shown by growth delay index, a significant difference was seen in the time for AH78 treated tumor to reach 3 times their day zero volume as compared to the saline control and c-DDP group (Table 4.1). Hence, AH78 demonstrated antitumor activity against 2008 ovarian carcinoma model more effectively than cisplatin at doses administered.

4.5. Discussion:

This study shows some similarities and also significant differences in cellular effects of non-covalently binding Pt drugs such as AH78 and covalently binding drugs like c-DDP, oxaliplatin and BBR3464. Over-expression of the copper transporter hCTR1 increased AH78 uptake, similar to the earlier reports for c-DDP and BBR3464.[17,27] This is the first demonstration that this novel polynuclear platinum compound, structurally similar

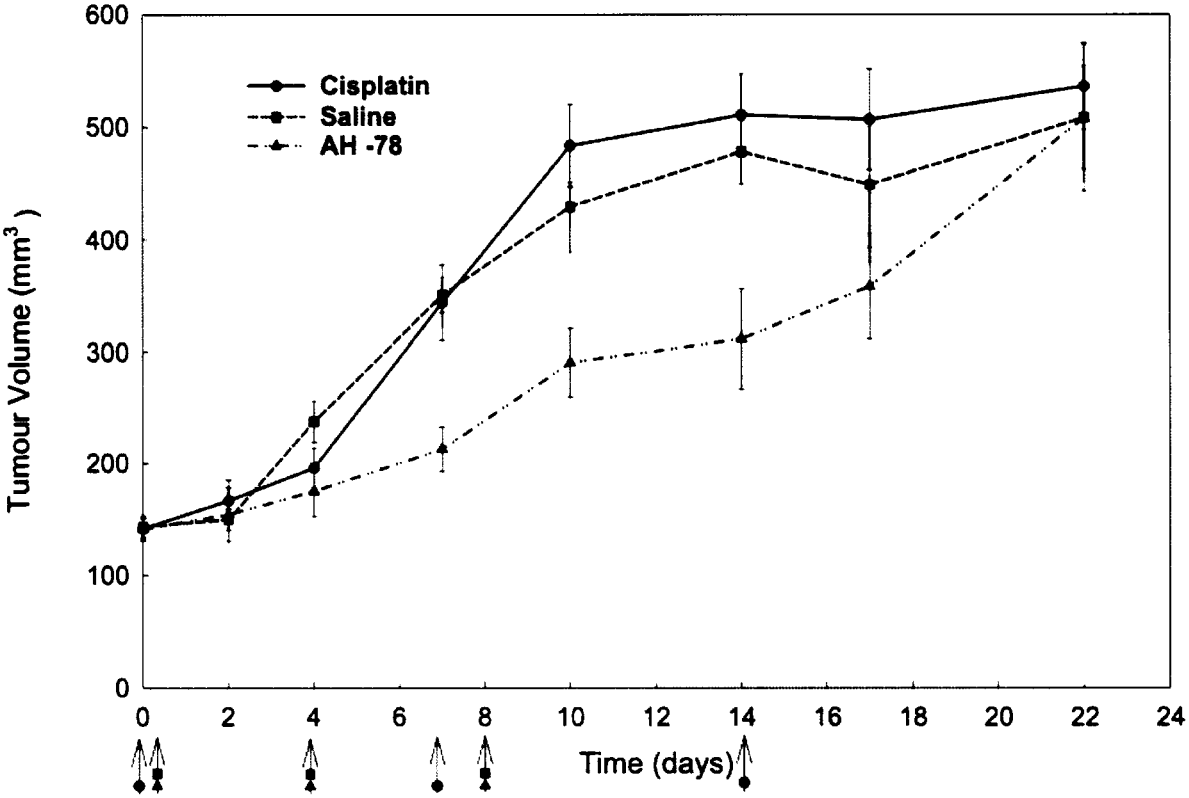


Figure 4.8. Antitumor activity of AH78 against the 2008 ovarian tumor xenograft model. A. Effect of cisplatin (4 mg/kg, ip), AH78 (25 mg/kg, ip) on 2008 tumor growth. The animals were treated on Days 0, 4, 8 with either (i) 25 mg/kg AH78 by ip injection or (ii) saline (0.1 ml/10 g body weight) or, on Days 0, 7, 14 with (iv) 4 mg/kg cisplatin by ip injection as it was explained in materials and methods section. The control group was treated with saline at 0.1 ml/10 g body weight. Each group consisted of 8-9 mice. The data represents the mean of tumor volume (mm^3) of each group (+/- SEM).

Table 4.1. Efficacy of AH78 and cisplatin against the 2008 ovarian carcinoma

Drug	Dose (mg/kg)	TGI (%)[a]	GDI[b]	GDI[c]	AGD[f] (Days to 300%)
Saline	4	-	1	1.1	8.5
Cisplatin	25	32[d]	2.5[e]	1.2	8.8
AH78	55	-	1.1	1.3	21.25

Note: [a] , was determined on Day 10 (tumor is x % smaller than the control tumor on this day). [b/c] ,Tumor took x times as many days to reach 3b or 4c times its starting size. [d]$P < 0.05$. Statistical significance of differences in tumor volumes between groups on Day 10 was tested using the One Way Analysis of Variance and Tukey's test. [e]$P < 0.05$. Statistical significance of differences in tumour growth delay between the groups was tested using the One Way Analysis of Variance and Tukey's test. [f], Absolute growth delay calculated as median time in days to reach 3 time starting tumor volume.

to BBR3464 but having no chloride leaving group, employs similar influx pathways to the parent compound. More importantly, increased uptake correlated with enhanced efficacy for AH78, similar to results seen for both c-DDP and BBR3464 as reported previously [17]. A2780/hCTR1 cells were more sensitive than wild type A2780 to AH78-induced cytotoxicity at low concentrations of AH78 (Fig. 4.2). However, this difference was diminished at higher concentrations, implicating the involvement of other transporters in AH78 cellular uptake. Hence, at high concentrations, it is possible that hCTR1 is saturated and there are other means of cellular transportation involved in AH78 uptake. In contrast to the important role of hCTR1, over-expressing the copper efflux transporter ATP7B had little effect on AH78, c-DDP or BBR3464 uptake and cytotoxicity (Fig. 4.2). Our data support the hypothesis that while hCTR1 plays an important role in AH78 cellular uptake and cytotoxicity, the effects of ATP7B may be less critical when cells are exposed to platinum drugs throughout the assay period.

Although AH78 is a non covalent binding drug, it can still induce cellular stress and apoptosis. Within the realm of cell cycle arrest and apoptosis, perhaps no cell signaling pathway is more relevant than p53 activation. AH78 induced p53 expression in HCT116 cells (Fig. 4.3) similar to c-DDP and BBR3464 [17]. The importance of p53 in AH78-induced cytotoxicity was made clear by use of p53-deficient HCT116 cells, an isogenic companion set to the HCT116 cells. Unlike c-DDP, Oxaliplatin, and BBR3464, AH78-induced cytotoxicity was not affected by p53 deletion (Fig. 4.4). Furthermore, AH78 induced a distinctly different cell cycle arrest (G1 cell cycle arrest) than its parental drug, BBR3464 (G2 cell cycle arrest) (Figure 4.5). These data demonstrate that altering Pt

compounds from covalent to electrostatic interactions with DNA has both quantitative and qualitative effects on the cellular response, completely changing the type of cell cycle arrest.

Another important factor in platinum drug-induced cytotoxicity is their deactivation and binding to sulfur-containing biomolecules such as glutathione. It is possible that noncovalently binding drugs like AH78 circumvent deactivation by glutathione since decreasing the level of cellular glutathione did not alter their cytotoxicity. Hence, their induced cytotoxicities were not limited by binding to glutathione.

In addition to its distinct mechanism of action, the antitumor activity of AH78 *in* vivo showed promising results as tumor growth was significantly delayed by AH78 treatment compared to the saline control and cisplatin-treated groups (Fig. 4.8B). Moreover, a significant difference was seen in the time for AH78 treated tumor to reach 3 times their day zero volume, as compared to the saline control and cisplatin group (Fig. 4.8A, B). Hence, AH78 may be a clinically effective drug, and represents a new class of electrostatic Pt based compounds.

In summary, the results demonstrate that hCTR1 may be a common means of entry for Pt-based drugs, including charged non covalent polynuclear compounds such as AH78. Structurally different platinum drugs with distinct modes of DNA-binding can share this transport mechanism but also employ different pathways to induce cell death, as revealed by the differential cell cycle arrest by AH78. Understanding these pathways will reveal targets that can be exploited for treating drug-resistant tumors. In addition, not having the potential to bind covalently to glutathione can decrease the pharmacokinetic problems

associated with sulfur deactivation. Furthermore, AH78 showed promising results *in vivo* indicating its efficacy in more clinically related models. These data confirm the validity of searching for new chemotypes outside the cisplatin structural class to aid in the treatment of recurrent, cisplatin-resistant cancers.

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List of References

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Chapter 5: Investigating the Role of Different Signal Transduction Pathways in BBR3464-Mediated Cytotoxicity and Cellular Uptake

5.1. Introduction:

Cisplatin, oxaliplatin and carboplatin comprise a group of antineoplastic drugs used for treatment of a variety of cancers including testicular, head and neck, ovarian, small cell lung and colorectal neoplasms.[1] They induce cytotoxicity through their covalent interactions with DNA and inhibition of DNA synthesis and replication by formation of bifunctional interstrand and intrastrand cross links.[2,3] The clinical efficacy of cisplatin is limited due to acquired resistance and dose-limiting side effects.[4,5] However, it has been indicated that structurally novel platinum compounds which bind to DNA differently than mononuclear platinum drugs, such as cisplatin (c-DDP), oxaliplatin and carboplatin may have distinct cytotoxicity, clinical efficacy, and side effect profiles. The trinuclear platinum complex, BBR3464, is a multinuclear platinum drug in which three platinum coordination units are linked by alkanediamine chains [6]. BBR3464 is significantly more cytotoxic than c-DDP and retains activity against c-DDP-resistant cell lines and tumors *in vivo* as well as *in vitro*.[6-10] This is the first multinuclear platinum drug that has undergone phase II clinical trials.

Acquired resistance to c-DDP in patients has been the topic of intensive research.[4,5] The three major pharmacological factors contributing to the intrinsic cytotoxicity of, and cellular resistance to, platinum drugs are (i) cellular uptake and efflux of platinum, (ii) the frequency and nature of Pt-DNA adducts and (iii) deactivating metabolic reactions with sulfur-containing nucleophiles.[11] A decrease in c-DDP cellular accumulation is the most commonly reported feature of resistance in cancer cells [12,13] which can be the result of multiple cellular pathways. Cationic polynuclear platinum drugs have recently been shown to display higher cellular uptake than c-DDP, which may contribute to their enhanced cytotoxicity in comparison to mononuclear platinum drugs.[14,15]

The mechanisms by which small platinum-containing molecules enter cells are diverse. Cellular copper transporters have been indicated to be involved in cellular uptake of platinum drugs.[16,17] BBR3464 and c-DDP use the same cellular transporters (hCTR1 and ATP7B) for influx and efflux; however, downstream effects in the presence of added copper ion are different for the two drugs.[11].

A second set of transporters that are studied for their effect on platinum drug uptake are the organic cation transporters (OCT). Cisplatin and oxaliplatin, but not carboplatin or nedaplatin, have been reported to act as substrates for human OCTs.[18] Likewise, recent studies indicate that OCT1 and OCT2 are important for oxaliplatin cellular uptake, but not for c-DDP in transfected canine and human kidney cells.[19]

In addition to transporters, cellular uptake of Pt drugs can occur *via* endocytosis. This process has also been implicated in cellular uptake of dinuclear platinum compounds.[20] Moreover, it has been recently shown that the interactions of BBR3464 with

phospholipid membrane models were significantly stronger than c-DDP.[21] Endocytosis is therefore considered as one of the mechanisms that may be involved in the uptake of BBR3464, a positively charged polynuclear platinum compound.

Combinational therapy is currently used for clinical cancer treatment, since inhibiting or activating specific signal transduction cascades and cellular functions could augment the efficacy of chemotherapy, especially in resistant and refractory cancers. Understanding how combinational therapies affect downstream cellular signaling cascades is important in developing better and more efficient chemotherapy agents. Different adjuvant therapy may modulate one or other of these pharmacologic cytotoxicity factors resulting in greater potency or a broader spectrum of treatable tumors.

In this study, we investigated different inhibitors of specific signal transduction pathways and their effects on platinum drugs-induced cytotoxicity and cellular uptake. The Importance of different signal transduction pathways in Pt-mediated cytotoxicity was demonstrated. Moreover, due to the importance of PI-3kinase in endocytosis, the importance of this process in BBR3464 cellular uptake was addressed.

5.2. Materials and Methods

5.2.1. Compound Synthesis.

BBR3464 and c-DDP compounds were synthesized as previously described.[22] Oxaliplatin, rapamycin, amiloride and eipa were obtained from Sigma-Aldrich (St. Louis, MO). Ly294002, U0126, SB203580, and roscovitine, Cytochalasin D were obtained from Calbiochem (San Diego, CA).

5.2.2. Cell Systems.

The colorectal carcinoma cell line HCT116 was the kind gift of Bert Vogelstein (Johns-Hopkins University, Baltimore, MD). HCT116 and the ovarian carcinoma cell line cells, A2780 were cultured with RPMI 1640 with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mmol/L sodium pyruvate (cRPMI, all from Biofluids, Rockville, MD) in humidified air with 5% CO_2.

5.2.3. Culture Conditions.

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 cells (adherent and non-adherent cells) were collected. BBR3464, c-DDP, and oxaliplatin concentrations were adjusted to achieve approximately 20%-30% apoptosis after 72h of treatment, allowing measurement of enhancement or inhibition.

5.2.4. Propidium Iodide DNA Staining and analysis of apoptosis.

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 [23]. Samples were then analyzed for subdiploid DNA content 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.

5.2.5. Platinum Accumulation Assays.

Cells were plated at 2.0×10^6 cells/ml. Platinum drug was added in different concentrations alone or 60 minutes after the addition of desipramine. After 8 or 16h, cells were harvested and washed twice with PBS. The cell pellets were then dissolved in hot 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 simultaneous inductively coupled plasma optical emission spectrometer at 265 nm (Varian Inc., Palo Alto, CA). Standards and blank were prepared using the same sample preparation protocol.

5.2.6. Statistical Analysis.

Statistical analysis was performed using *t*-test for two data points using SysStat9 software (SPSS, Chicago, IL, USA). $p < 0.05$ was considered to be significant. Results are the mean and standard error.

5.3. Results:

5.3.1. The Importance of Phosphatidylinositole 3-kinase (PI3-kinase) in Pt Drug-Mediated Cytotoxicity

Phosphatidylinositole 3-kinase (PI3-kinase) plays an important role in cellular function and survival. PI3-kinase induces AKT (serine/threonine protein kinase) activation which results in cellular proliferation, implicating its importance in tumorigenesis. Increased activation of AKT results in activation of multiple pro-proliferation/survival signal transduction proteins including MDM2 and mTOR. In addition, inhibition of other apoptotic/ anti-proliferative proteins including BAD and p21 occurs. Activation and increased expression of AKT results in increased cellular survival and decreased apoptotic processes.[24,25]

It is of a great interest to investigate whether inhibiting PI3-kinase will increase the efficacy of platinum drugs. LY294002 is a commonly used agent to inhibit the PI-3 kinase pathway. The effect of PI3-kinase inhibitor on cytotoxicity was examined. The influence of LY294002 on BBR3464, c-DDP, and oxaliplatin-induced cytotoxicity was determined by comparing the level of apoptosis in colorectal carcinoma HCT116 cells. Cells were cultured in the presence or absence of LY294002 and platinum drugs. Resulting DNA fragmentation was measured by PI-DNA staining. As shown in figure 5.1A, C, inhibition of PI-3 kinase increased the cytotoxicity of c-DDP and BBR3464 after 24h treatment at the highest concentration of LY294002 (50μM) used. However, Oxaliplatin-induced apoptosis was not changed by LY294002 (Figure 5.1B). c-DDP-induced apoptosis was increased from 23% to 45% by LY294002. Treatment of cells with BBR3464 gave similar results

(Figure 5.1). Furthermore, the augmentation of BBR3464-induced apoptosis by LY294002 was time-dependent, essentially reaching a maximum augmentation after 48h. Apoptosis induced by either LY294002 (50μM) or BBR3464 alone was 8% and 16% respectively, but the combination of BBR3464 and LY294002 showed an increase in apoptosis to approximately 43% at that time point (Figure 5.2).

5.3.2. Effect of Immunosuppressants on Pt. Drugs-Mediated Cytotoxicity.

5.3.2.1a Rapamycin

Rapamycin is a new immunosuppressant that is being used for kidney transplantation. It inhibits the cellular response to IL-2, therefore, stopping the activation and proliferation of cells (B and T cells in the case of the immune system). It binds to the FK-binding protein 12 (FKBP12) forming a complex (rapamycin/FKBP12) which inhibits the mTOR (mammalian target of rapamycin) pathway which results in inhibition of proliferation [26]. Rapamycin is also a candidate in phase II clinical trials for different types of cancers. Inhibiting the cellular proliferation by rapamycin could increase the cytotoxicity of platinum drugs. The effect of rapamycin on BBR3464, c-DDP, and oxaliplatin-induced cytotoxicity was determined by comparing the level of apoptosis in colorectal carcinoma HCT116 cells. Cells were cultured at different time points in the presence or absence of rapamycin and platinum drugs, and DNA fragmentation was measured by PI-DNA staining. As shown in figure 5.3, it had minor effects on Pt-drug-mediated cytotoxicity. It is therefore concluded that mTOR pathway is not important in platinum drug-mediated cytotoxicity.

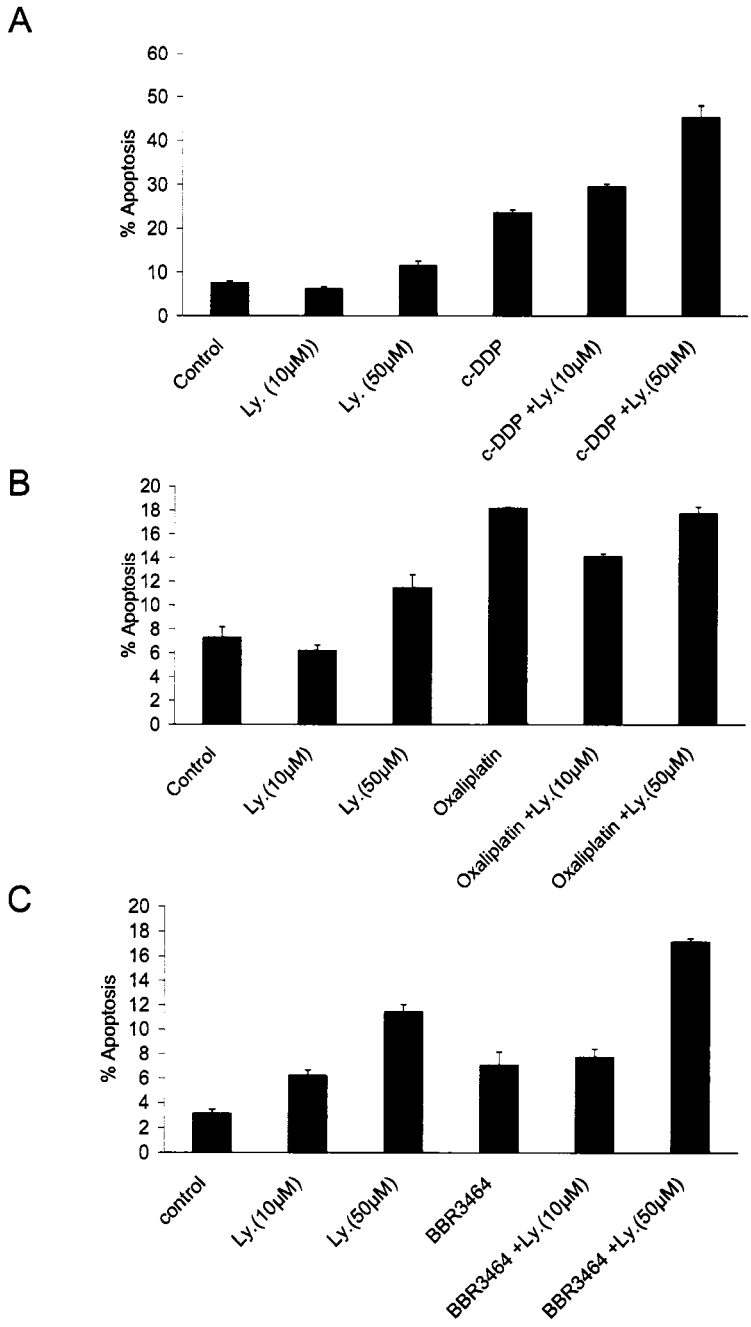

Figure 5.1. The effect of LY294002 (Ly) on Pt-drug-mediated cytotoxicity. DNA content was measured by PI-DNA staining to determine apoptosis (sub G_0/G_1) by flow cytometry, as described in Materials and Methods. In A, B, C, HCT116 cells were cultured with 40μM c-DDP, 30μM oxaliplatin, or 50μM BBR3464, respectively in the presence and absence of LY294002 for 24h. Drugs were added to the media after 1h of treatment with LY294002. Each point represents the average (+/- SEM) of three independent experiments. *, $p<0.05$ when comparing cells treated with and without LY294002, by Student's t-test.

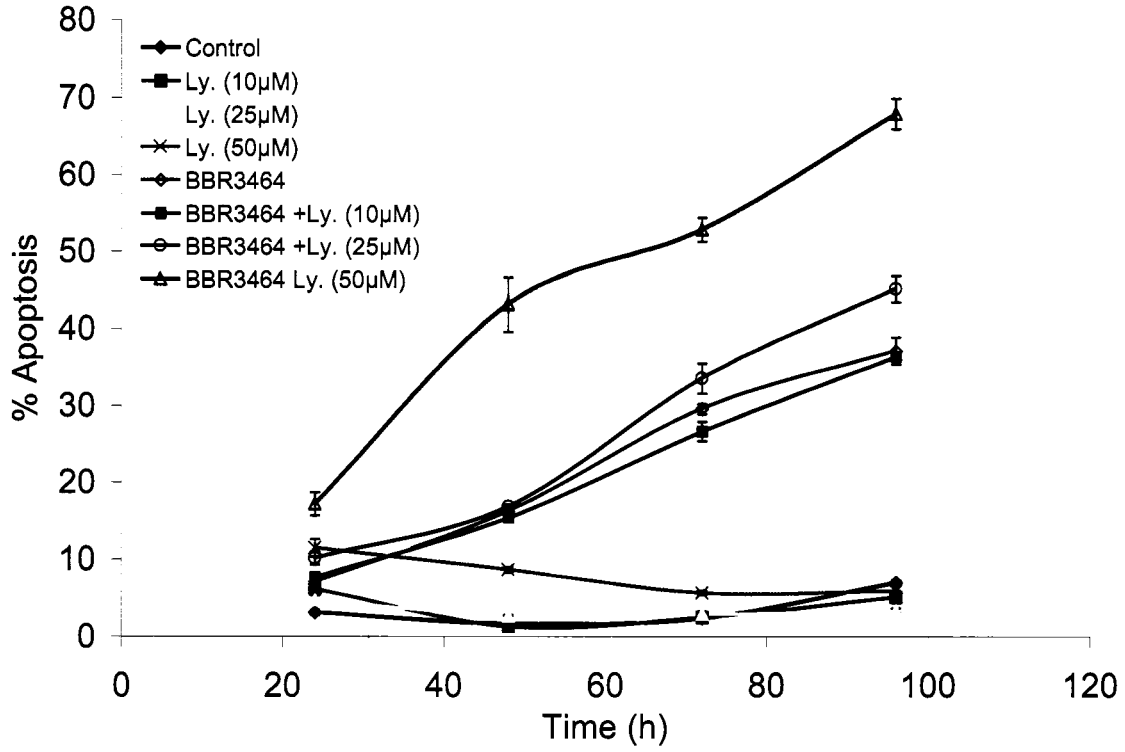

Figure 5.2. The effect of LY294002 on BBR3464-induced apoptosis over time in HCT116 colorectal carcinoma cells. Sub-diploid cell content was detected by PI-DNA staining. HCT116 cells were cultured with 50μM BBR3464 for the indicated time points in the absence and presence of 10 or 50μM LY294002. Pt. drug concentration was adjusted to achieve approximately 20-30% apoptosis after 48h, allowing us to measure enhancement or inhibition. BBR3464 were added to the media after 1h of treatment with LY294002. Each point represents the average (+/- SEM) of three independent experiments. *, $p<0.05$ when comparing cells treated with and without LY294002, by Student's t-test. All points after 48h have $p<0.05$ for Pt drug with LY294002 vs Pt drug alone.

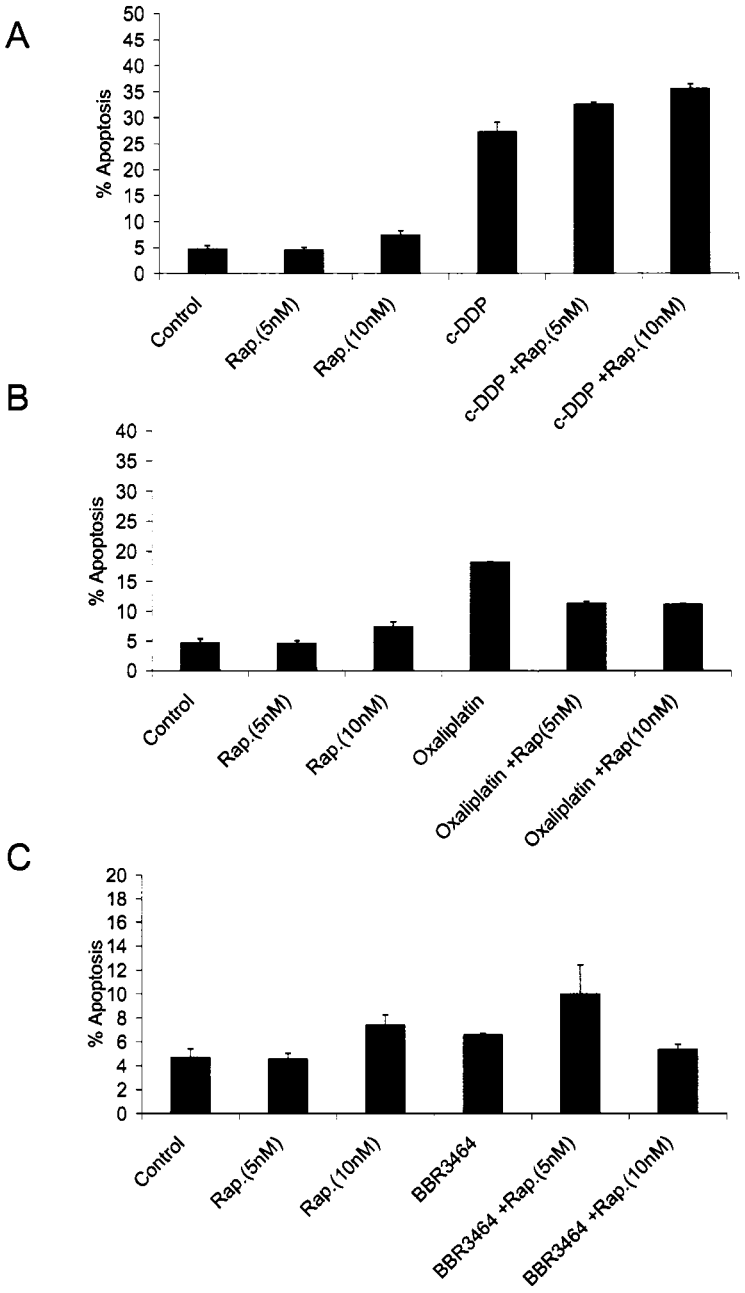

Figure 5.3. The effect of rapamycin (Rap) on Pt-drug-mediated cytotoxicity. DNA content was measured by PI-DNA staining to determine apoptosis (sub G_0/G_1) by flow cytometry, as described in Materials and Methods. In A, B, C, HCT116 cells were cultured with 40μM c-DDP, 30μM oxaliplatin, or 50μM BBR3464, respectively in the presence and absence of rapamycin for 24h. Drugs were added to the media after 1h of treatment with rapamycin. Each point represents the average (+/- SEM) of three independent experiments. *, $p<0.05$ when comparing cells treated with and without rapamycin, by Student's t-test.

5.3.2b Cyclosporine A

Cyclosporine A (CSA) is another immunosuppressant which binds to cyclophilin in the cytosol, especially in T cells. The cyclosporine/cyclophilin complex inhibits calcineurin, a protein which activates IL-2 transcription. Therefore, it reduces proliferation of cancer cells by inhibiting the production of IL-2 in cells.[27] Inhibiting proliferation by CSA can increase platinum-induced apoptosis.

The influence of CSA on BBR3464, c-DDP, and oxaliplatin-induced cytotoxicity was determined by comparing the level of apoptosis in colorectal carcinoma HCT116 cells. Cells were cultured at different time points in the presence or absence of CSA and platinum drugs, and DNA fragmentation was measured by PI-DNA staining. As shown in figure 5.4A,B,C, cyclosporine A increased the cytotoxicity of c-DDP, and BBR3464, but had no effects on Oxaliplatin-mediated cytotoxicity after 24h treatment at the highest concentration of CSA used. c-DDP-induced apoptosis was increased from 43% to 58% by CSA . Treatment of cell with BBR3464 gave similar results (Figure 5.4C). Furthermore, the augmentation of BBR3464-induced apoptosis by CSA is time-dependent, essentially reaching a maximum augmentation after 48h. Apoptosis induced by either CSA (12μM) or BBR3464 alone was 2% and 24% respectively, but the combination of BBR3464 and Ly294002 showed an increase in apoptosis to approximately 57% at that time point (Figure 5.5).

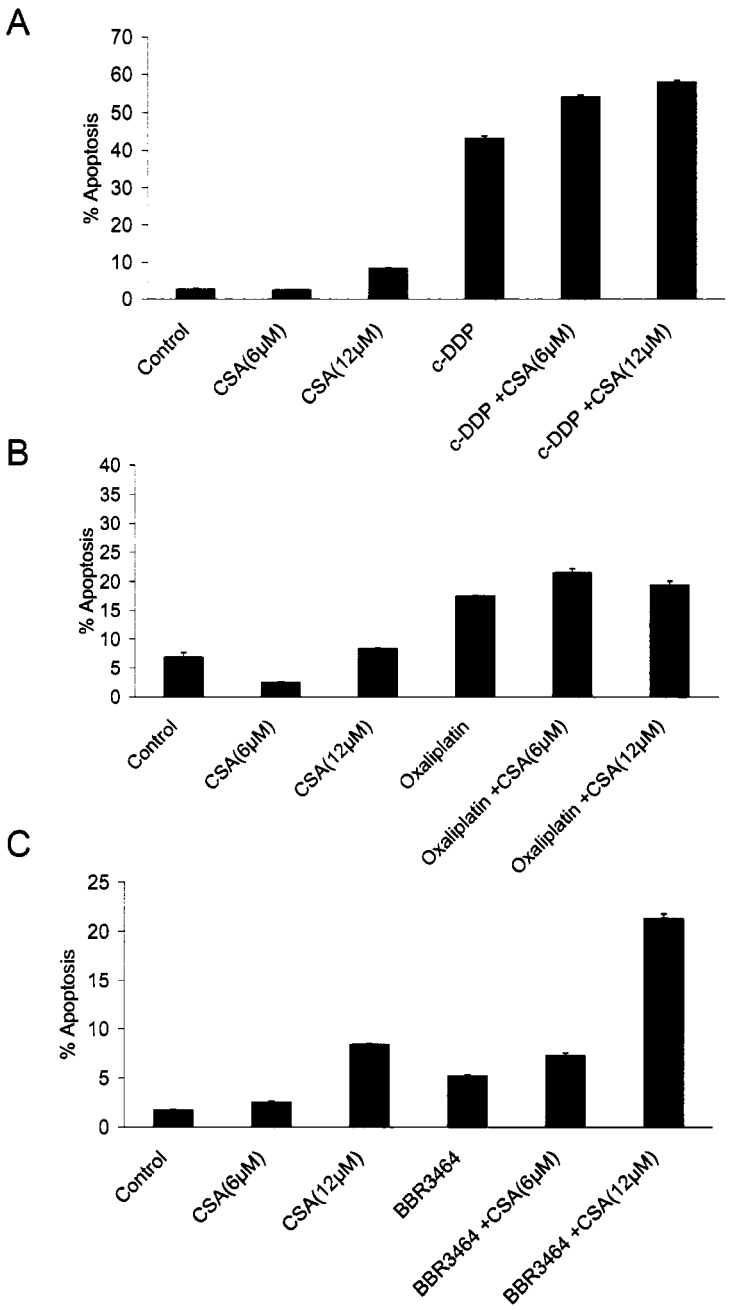

Figure 5.4. The effect of cyclosporine A (CSA) on Pt-drug-mediated cytotoxicity. DNA content was measured by PI-DNA staining to determine apoptosis (sub G_0/G_1) by flow cytometry, as described in Materials and Methods. In A, B, C, HCT116 cells were cultured with 40μM c-DDP, 30μM oxaliplatin, or 50μM BBR3464, respectively in the presence and absence of CSA for 24h. Drugs were added to the media after 1h of treatment with CSA. Each point represents the average (+/- SEM) of three independent experiments. *, $p<0.05$ when comparing cells treated with and without CSA, by Student's t-test.

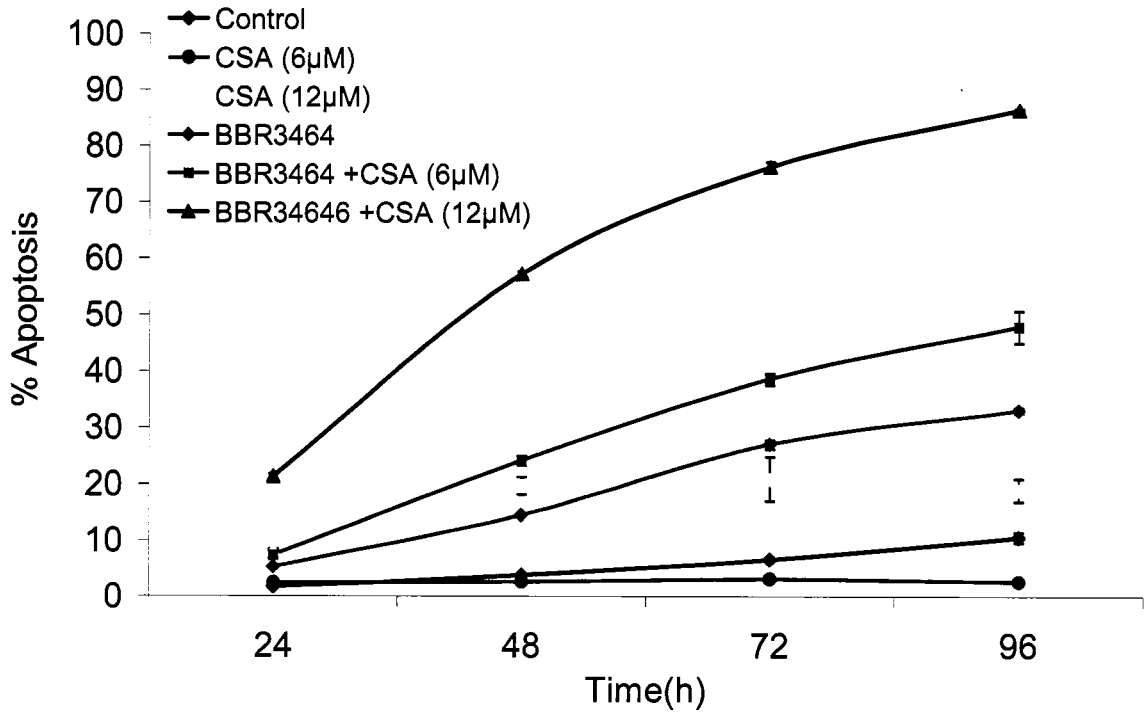

Figure 5.5. The effect of cyclosporineA (CSA) on Pt-drug-induced apoptosis over time in HCT116 colorectal carcinoma cells. Sub-diploid cell content was detected by PI-DNA staining. HCT116 cells were cultured with 50μM BBR3464 for the indicated time points in the absence and presence of 6 or 12μM CSA. Pt. drug concentration was adjusted to achieve approximately 20-30% apoptosis after 48h, allowing us to measure enhancement or inhibition. BBR3464 were added to the media after 1h of treatment with CSA. Each point represents the average (+/- SEM) of three independent experiments. *, $p<0.05$ when comparing cells treated with and without CSA, by Student's t-test. All points after 48h have $p<0.05$ for Pt drug with CSA vs Pt drug alone.

5.3.3. The Importance of Other Signal Transduction Pathways in Platinum Drug-Induced Cytotoxicity

Other signal transduction cascades of interest were JNK, MEK, p38 and cyclin dependant kinase (CDK) pathways, which were inhibited by JNK inhibitor, U0126, SB203580, and roscovitine respectively. The importance and role of these signaling proteins in BBR3464 and c-DDP- induced cytotoxicity was examined by comparing the level of apoptosis in colorectal carcinoma HCT116 cells. Cells were cultured in the presence or absence of specific inhibitors and platinum drugs, and DNA fragmentation was measured by PI-DNA staining. As shown in figure 5.6, JNK inhibitor, U0126 and SB203580 did not influence c-DDP and BBR3464-mediated cytotoxicity, showing their minor role in platinum drug-mediated cytotoxicity.

5.3.4. Effect of Endocytotis on Pt-drug Cellular Uptake

The role of uptake is increasingly being seen as a critical determinant of clinical resistance and cytotoxicity [11,12]. Phosphatidylinosititol 3-kinase has also been implicated to be an important protein in endocytosis. It phosphorylates the 3' position of the inositol ring in Pins (Phosphotidyl inositol), which in turn induces ARF/GTP by activating ARF GTPases. ARF/GTP influences binding of cytosolic coat proteins like clathrin.[28-32] Therefore, the influence of LY294002 on platinum drug cellular uptake was measured (*via* ICP-OES) in cells treated with platinum drug +/- LY294002. As shown in figure 5.7, the uptake of BBR3464 was decreased by 30% when endocytosis was inhibited by LY294002 in A2780

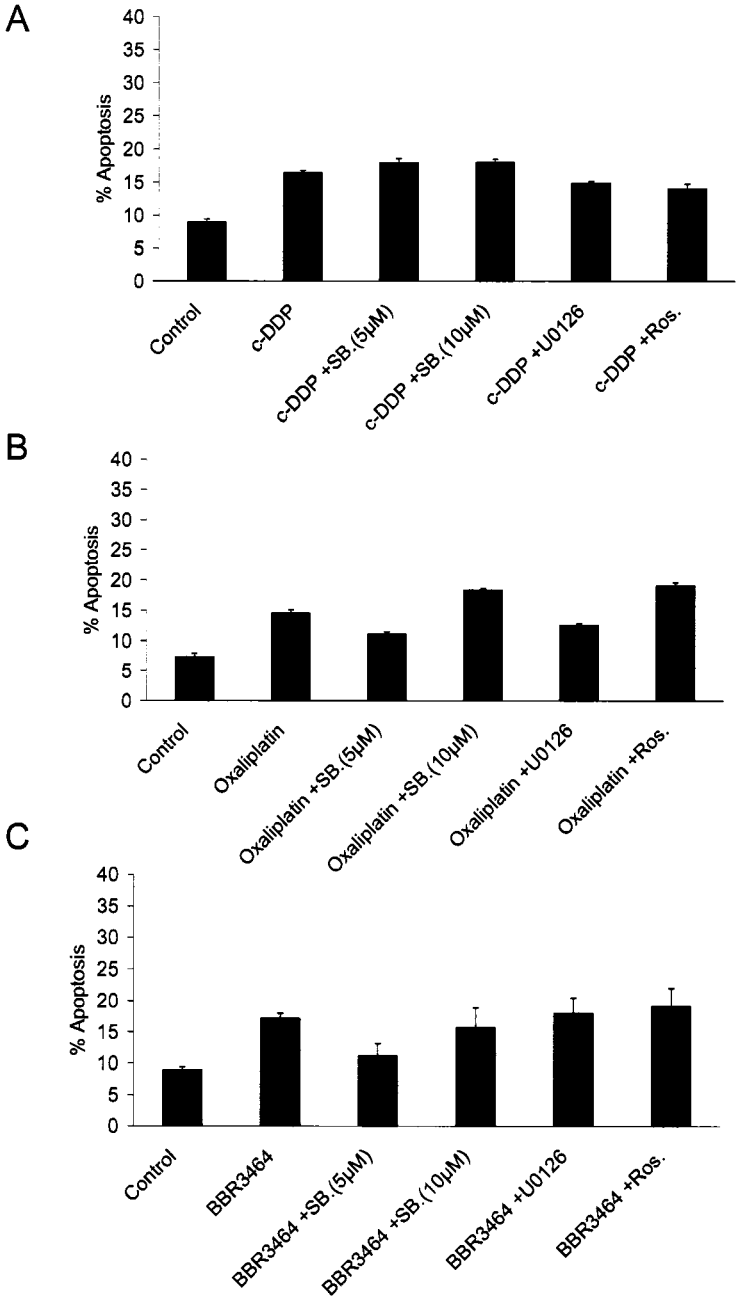

Figure 5.6. The effect of SB203580 (SB) on Pt-drug-mediated cytotoxicity. DNA content was measured by PI-DNA staining to determine apoptosis (sub G_0/G_1) by flow cytometry, as described in Materials and Methods. In A, B, C, HCT116 cells were cultured with 40μM c-DDP, 30μM oxaliplatin, or 50μM BBR3464, respectively in the presence and absence of SB203580 for 24h. Drugs were added to the media after 1h of treatment with SB203580. Each point represents the average (+/- SEM) of three independent experiments. *, $p<0.05$ when comparing cells treated with and without SB203580, by Student's t-test.

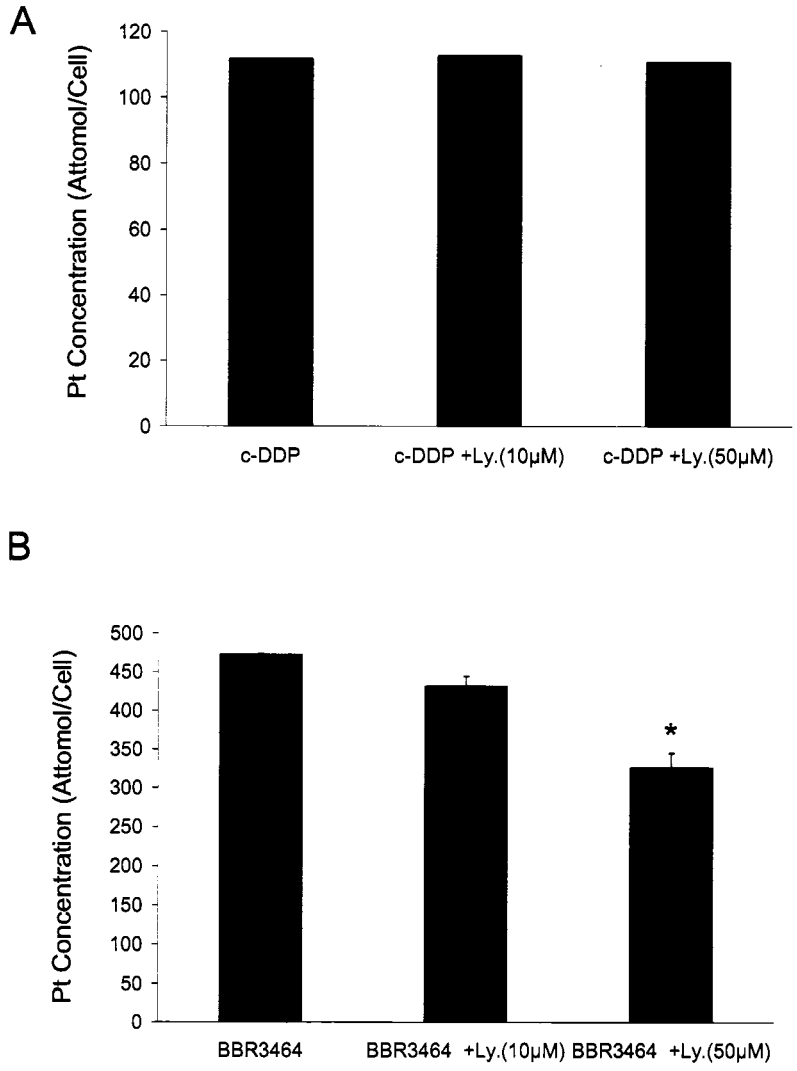

Figure 5.7. The effect of LY294402 (Ly) on Pt-compounds cellular uptake. In A, B, HCT116 cells were treated with 20μM c-DDP or 10μM BBR3464 for 24h, and cellular platinum content was measured by ICP-OES as described in method and materials. Each point represents the average (+/- SEM) of three independent experiments. *, $p<0.05$ as determined by t test.

ovarian carcinoma calls. In contrast, inhibiting PI-3kinase did not change the cellular uptake of c-DDP (figure 5.7).

To further investigate the role of endocytosis in BBR3464 cellular uptake, amiloride, EIPA (ethylisopropyl amiloride) and Cytochalasin D were used to inhibit endocytosis by interacting with the Na^+ -H^+ exchanger in endocytic vesicles or by blocking F-actin microfilament rearrangement. Cytochalasin D decreased BBR3464 cellular uptake by 28% at its highest concentration (20μM). These results show that F-actin polymerization near the plasma membrane is important in facilitating endosome internalization process and speeds up the movement of vesicles (endosomes) into the cytosol away from the plasma membrane.[33]

Moreover, amiloride and EIPA decreased BBR3464 cellular uptake by 21% and 35%, respectively (figure 5.8). These results indicate that acidification of endocytic compartment which depends on a sodium gradient (Na^+ -H^+ exchanger) [22,28,34], is important in BBR3464 cellular uptake.

5.4. Discussion:

This study indicates the important pathways in different Pt drugs-induced cytotoxicity. PI-3kinase pathway was shown to be important in c-DDP and BBR3464-mediated apoptosis, as inhibiting PI-3kinase augmented their cytotoxicity (Figure 5.1). In addition, Oxaliplatin-induced apoptosis was not affected by Ly294002 indicating that a mononuclear platinum drug with different ligands induce different signal transduction pathways. Similarly,

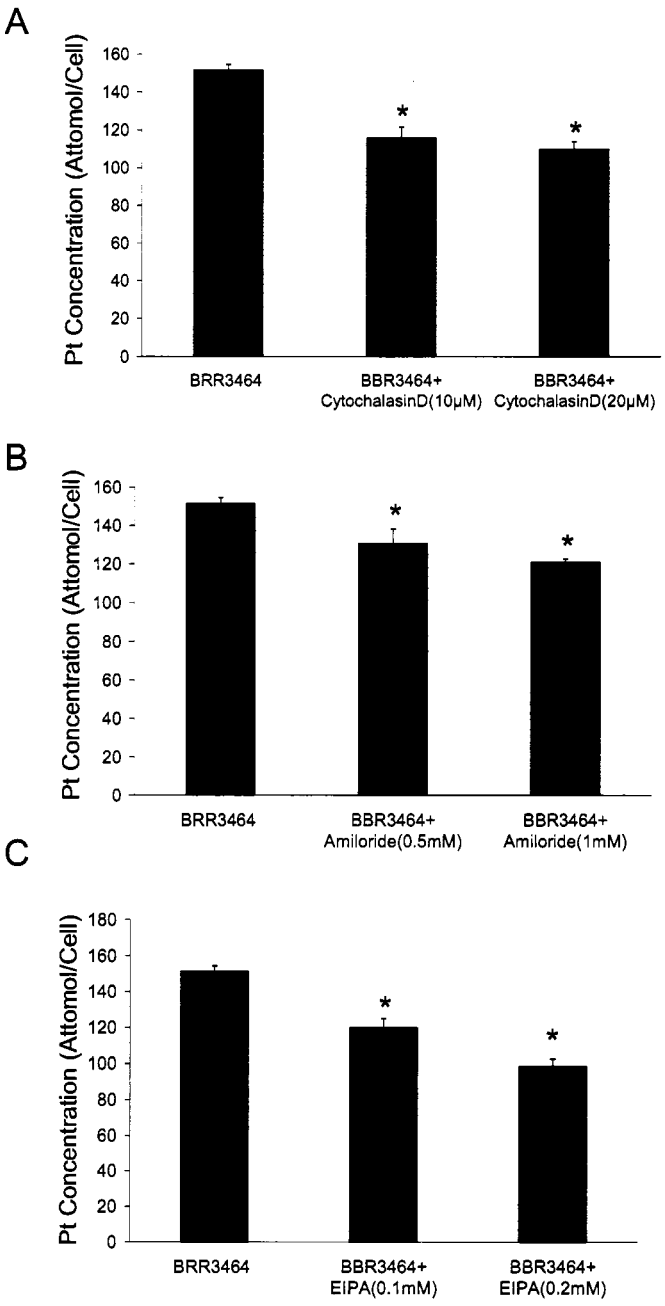

Figure 5.8. The effect of endocytosis inhibitors on Pt-compounds cellular uptake. In A, B,C, HCT116 cells were treated with 20μM BBR3464 for 24h, and cellular platinum content was measured by ICP-OES as described in method and materials. Each point represents the average (+/- SEM) of three independent experiments. *, $p<0.05$ as determined by t test.

cyclosporine A augmented c-DDP and BR3464-mediated cytotoxicity, not Oxaliplatin (figure 5.5). Hence, calcineurin/NFAT pathway plays an important role in BBR3464 and c-DDP mediated cytotoxicity.

Since it was recently shown that the interactions of BBR3464 with phospholipid membrane models were significantly stronger than c-DDP [21], endocytosis is a possible mean by which BBR3464 can be transported. Using different endocytosis inhibitors such as PI-3kinase inhibitor, amiloride, EIPA, and cytochalasin D, the cellular uptake of BBR3464 was decreased indicating importance of endocytosis in BBR3464 cellular uptake. Since this decrease in cellular uptake can not explain the total cellular uptake of BBR3464 and it is been shown that other transporters like hCTR1, and ATP7B are involved in BBR3464 cellular uptake [11], it is reasonable to suggest that there are multiple means by which BBR3464 is transported. Note that the PI-3kinase inhibitor (LY294002) decreased the cellular uptake of BBR3464 by inhibiting cellular endocytosis process, but enhanced the drug induced cytotoxicity. There is enhancement of BBR3464-induced cytotoxicity even with less cellular level of platinum drugs indicating the critical survival role of PI-3kinase pathway in BBR3464-induced cytotoxicity. To further complete this study, the effect of CSA and LY294002 on cellular accumulation of platinum drugs, and p53, caspase activation is warranted. Moreover, the role of endocytosis and the energy dependence of BBR3464 cellular uptake will be investigated further.

The study is of high clinical relevance and argues that inhibitors such as Ly294002 and cyclosporine A may be a mean of enhancing chemo-responsiveness to platinum-based anticancer agents, and warrants further investigation for its clinical utility. However, in

choosing different inhibitors, their effects on cellular uptake of platinum drugs should be addressed, as decreasing cellular uptake can reduce their efficacy. The study also confirms the potential for rational design of chemotherapeutic regimens based on mechanistic approaches of signaling complementarity.

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Chapter 6: Conclusions

c-DDP, oxaliplatin, and carboplatin are effective clinical anticancer drugs. However, the clinical efficacies of these drugs are limited due to acquired resistance and dose limiting side effects such as nephrotoxicity.[1-4] The trinuclear platinum compound, BBR3464, is a novel anticancer drug which has higher cellular uptake than clinical platinum drugs such as c-DDP, oxaliplatin and carboplatin -one possible reason for its greater cytotoxicity and efficacy. The three major pharmacological factors contributing to the intrinsic cytotoxicity of, and cellular resistance to, platinum drugs are (i) cellular uptake and efflux of platinum; (ii) the frequency and nature of Pt-DNA adducts; and (iii) deactivating metabolic reactions with sulfur-containing nucleophiles. Acquired resistance is a major factor contributing to cancer treatment failure.[5] Since decreased cellular uptake is the most common feature reported for resistant cells, the role of cellular uptake and efflux is being seen as an important factor in clinical resistance to Pt drugs. [6] Developing anticancer drugs with different mechanisms of uptake will help to circumvent the acquired resistance against c-DDP. This study improved the understanding of cellular uptake of polynuclear platinum drugs in comparison to c-DDP.

The uptake mechanisms of small platinum-containing molecules are diverse and complex – one reason perhaps why they are such useful antitumor agents. The use of cellular copper transporters has been documented as one mechanism of active uptake of

platinum drugs.[7,8] The same influx transporter, hCTR1 and efflux transporter, ATP7B showed to play an important role in BBR3464 and c-DDP cellular accumulation; however, their mechanisms of action are different in the presence of added copper ion.[9] Collectively, the data from chapter 2 would suggest that at physiological concentrations, c-DDP or BBR3464 does not directly compete for hCTR1 binding, but rather may compete with copper for the efflux transporter, resulting in enhanced cellular platinum accumulation. Structurally different platinum drugs with distinct modes of DNA-binding can share this transport mechanism and converge on signal transduction pathways including p53. However, these compounds also employ divergent pathways to induce cell death, as revealed by the differential response to copper (chapter 2).

A second set of transporters more recently studied for their effects on platinum drug uptake, are the organic cation transporters. OCTs have been shown to have an important role in oxlaliplatin and c-DDP cellular accumulation.[10,11] Unlike cisplatin and oxaliplatin, it was demonstrated that OCTs have minor roles in BBR3464 cellular accumulation. However, desipramine, the antidepressant and OCT inhibitor, showed a synergistic effect on c-DDP, oxaliplatin, and BBR3464-mediated cytotoxicity. The optimal dose of desipramine used was well within the range of clinically relevant doses achieved in patients, where the free desipramine concentration in the serum reaches approximately 9.5 μM.[18] The study of desipramine in combinational therapy with Pt drugs is currently under going *in vivo* testing which ultimately can demonstrate the efficacy of such treatment. Since desipramine is also being used in prostate cancer for treatment of neuropathic pain, this study can be expanded to satraplatin, a potential candidate for

prostate cancer chemotherapy. In summary, the tricyclic organic, a safe and effective antidepressant already in use for cancer patients, greatly augments the cytotoxicity of platinum-based chemotherapeutics. These effects correlated with enhanced activation of the p53- mitochondrial death pathway, but a p53-independent mechanism is also apparent. The study presented in chapter 3 is of high clinical relevance and argues that desipramine may be a means of enhancing chemo-responsiveness to platinum-based anticancer agents, and warrants further investigation for its clinical utility. The study also confirms the potential for rational design of chemotherapeutic regimens based on mechanistic approaches of signaling complementarily.

In addition to transporters, cellular uptake of Pt drugs can occur *via* endocytosis. It is another process that has also been implicated to be involved in cellular accumulation of dinuclear platinum complexes.[12] Since it was recently shown that the interactions of BBR3464 with phospholipid membrane models were significantly stronger than c-DDP, endocytosis is a possible mean by which BBR3464 can be transported.[17] Using different endocytosis inhibitors such as PI-3kinase inhibitor, amiloride, EIPA, and cytochalasin D, the cellular uptake of BBR3464 was decreased indicating importance of endocytosis in BBR3464 cellular uptake. On the other hand, LY294002 didn't change the cellular accumulation of c-DDP indicating the minor role of endocytosis in cisplatin cellular transport. Note that the PI-3kinase inhibitor (LY294002) decreased the cellular uptake of BBR3464 by inhibiting cellular endocytosis process, but enhanced the drug induced cytotoxicity. There is enhancement of BBR3464-induced cytotoxicity even with less

cellular level of platinum drugs indicating the critical survival role of PI-3kinase pathway in BBR3464-induced cytotoxicity (chapter 5).

Since BBR3464 has a high positive charge, its DNA binding has a preassociation noncovalent component that affects the binding kinetics and final structure of Pt-DNA adducts.[13,14] To examine the preassociation component, compounds indicated as AH78 were synthesized by replacing the labile chloride ligand with inert ammonia or amine groups.[15,16] Similar to c-DDP and BBR3464, AH78 has been shown to use the same copper transporters for influx and efflux, however, its mechanism of action was distinct. AH78 induced G1 cell cycle arrest and its induced cytotoxicity showed to be p53 and glutathione independent. As presented in chapter 4, AH78 showed promising results *in vivo* indicating its potential for use as a new anti-tumor compound.

In conclusion: Although previously not well characterized, cellular transport studies of BBR3464 have been shown, through this work, to proceeds via multiple routes. Since the role of transporters in platinum drugs uptake may be both tissue and compound-specific, platinum drugs may display widely varying effects dependent on the specific expression levels of transporters in any tumor type. Studying the mechanism of cellular accumulation of Pt drugs will aid in understanding the association between compound's structure and its mechanism of cellular uptake, ultimately resulting in developing better and more efficient antitumor drugs against recurrent and cisplatin –resistant cancers. Understanding cellular transportation of BBR3464 will aid in the development of new chemotypes outside the cisplatin structural class. In addition, non-covalent compounds were shown to potentially be a new class of platinum drugs with promising clinical

efficacy. Finally, the synergistic effect of desipramine on Pt- mediated cytotoxicity is of high clinical relevance since clinical cancer treatment currently involves the use of combination regimen involving one or more drugs.

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Appendix 1: Low dose BBR3464 toxicity in colon cancer cells in p53-independent and enhanced by inhibition of ERBB1-PI3K signaling

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Molecular Pharmacology, 2007 (Epub ahead of print)

Cellular uptake and DNA binding studies by Peyman Kabolizadeh

Abstract.

We have examined the mechanisms by which the multinuclear platinum chemotherapeutic agent, BBR3610 kills human colon cancer cells. BBR3610 more efficiently killed HCT116, DLD1, SW480 and HT29 cells than BBR3464, cisplatin or oxaliplatin. The amount of platinum uptake per cell and its incorporation into DNA were identical for BBR3464 and BBR3610. BBR3610 lethality (IC75) was unaltered comparing HCT116 wild type and p53 -/- cells; was reduced in p21 -/- cells; and enhanced in K-RAS D13 null cells. Small molecule or molecular inhibition of ERBB1 or PI3K enhanced BBR3610 toxicity in HCT116, DLD1 and SW480 cells. Small molecule or molecular inhibition of caspase 8 function abolished the toxicity of BBR3610 and of BBR3610 + ERBB1 inhibitor treatments whereas inhibition of caspase 9 suppressed the ability of ERBB1 inhibitors to

enhance BBR3610 lethality. Treatment with BBR3610 reduced AKT activity; expression of dominant negative AKT enhanced, and expression of constitutively active AKT suppressed, respectively, the toxicity of BBR3610 and of BBR3610 + ERBB1 inhibitor treatments. Treatment with BBR3610 reduced expression of c-FLIP-s and MCL-1, levels that were maintained in cells expressing constitutively active AKT. Over-expression of c-FLIP-s or loss of BID function suppressed BBR3610 toxicity whereas over-expression of XIAP or BCL-XL suppressed the potentiation of cell killing by ERBB1 inhibitors. Collectively our data argue that BBR3610 promotes cell killing via a caspase 8 –dependent mechanism which can be enhanced by ERBB1 / PI3K inhibitors that promote additional BBR3610-dependent cell killing via activation of BAX and caspase 9.

Introduction:

Cisplatin is known to be one of the most active anti-tumor drugs. In addition to cisplatin a variety of mononuclear platinum compounds have been developed including carboplatin and oxaliplatin [1]. However, it has also been established that polynuclear platinum compounds in which two or three platinum coordination units are linked through alkanediamine or polyamine chains are an important and structurally distinct new class of potential therapeutic agents with greater activity than the first generation mononuclear platinum compounds [2-5]. The first multi-platinum compound to undergo human clinical trials, the trinuclear BBR3464, demonstrated objective responses in Phase I trials and Phase II ovarian cancer and non-small cell lung cancer [5-7]. The toxic effects of BBR3464 were noted to be p53 independent and appear to be mechanistically different from those of

cisplatin with respect to their DNA interactions [8]. The "central" platinum unit in BBR3464 contributes to DNA binding only through hydrogen-bonding and charge effects which also contribute [5]. The "2nd generation" agent BBR3610 contains two platinum coordination units and was designed to replace the central platinum unit of BBR3464 by an amine but with the same overall length between the Pt-Cl bonds responsible for DNA platination [5]. The dinuclear polyamine-linked compounds in general display activity profiles similar to BBR3464 and cytotoxicity is dependent on the nature of the polyamine [9]. BBR3610 treatment of glioma cells caused more killing in vitro on a Molar basis than BBR3464. Cell killing correlated with a G2/M cell cycle arrest which was in part dependent upon enhanced ERK1/2 signaling [10].

The apoptotic cell death threshold of cells is modulated by the activities of multiple signal transduction pathways and the expression, usually controlled by the signaling pathways, of multiple pro- and anti-apoptotic proteins [11-13]. Activation of the epidermal growth factor receptor (EGFR, also called ERBB1) stimulates signaling through both the PI3K-AKT and RAF-ERK1/2 pathways which have been linked to increased expression of many cyto-protective proteins including BCL-2 family members and inhibitor of apoptosis proteins. In colon cancer cells, activation of ERBB1 and expression of mutated active K-RAS proteins has been shown to protect this cell type from a wide variety toxic stresses, including platinum therapeutic agents [14,15]. In general, PI3K-AKT signaling in tumor cells has been argued to be a greater protective signal than signaling by RAF-ERK1/2, and in contrast to a general perceived role of RAF-ERK1/2 signaling being protective, it should be noted that several studies exist using small molecule inhibitors of MEK1/2 suggest that

platinum agent – induced ERK1/2 signaling plays a key role in promoting drug toxicity, including BBR3610 [16,17].

The mechanisms by which cells process the DNA damage of mononuclear drugs cisplatin and oxaliplatin leading to tumor cell death have been extensively investigated [18,19]. In a cell type dependent fashion, cisplatin and oxaliplatin have been shown to promote activation of caspase 8 (extrinsic apoptosis pathway) or cause mitochondrial dysfunction and activation of caspase 9 (intrinsic apoptosis pathway) [20-22]. In a variety of colon cancer cell lines, oxaliplatin has been shown to promote predominantly activation of the extrinsic, or to a lesser extent intrinsic, apoptosis pathways [14,23-26]. Cisplatin toxicity can be enhanced by inhibition of cell cycle checkpoints, signaling pathways, and in combination with other therapeutic modalities such as ionizing radiation. The present studies were performed to understand in greater detail the molecular mechanisms by which the novel dinuclear platinum containing agent BBR3610 (as the chloride salt) caused colon carcinoma tumor cell death; whether inhibition of ERBB1 function enhanced the lethality of the platinum drug in a synergistic fashion, and the molecular mechanisms by which these events occurred.

Materials and Methods.

Materials.

Phospho-/total-ERK1/2, Phospho-/total-JNK1/2, Phospho-/total-p38 MAPK, Anti-S473 AKT and total AKT, caspase 8, c-FLIP, XIAP, MCL-1, BCL-XL antibodies were purchased from Cell Signaling Technologies (Worcester, MA). All the secondary

antibodies (anti-rabbit-HRP, anti-mouse-HRP, and anti-goat-HRP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The PI3K inhibitor (LY294002), JNK inhibitor peptide (JNK IP) and ERBB1 inhibitor (AG1478) were supplied by Calbiochem (San Diego, CA) as powder, dissolved in sterile DMSO, and stored frozen under light-protected conditions at –80oC. Enhanced chemiluminescence (ECL) kits were purchased from Amersham Enhanced Chemi-Luminescence (ECL) system (Bucks, England) and NEN Life Science Products (NEN Life Science Products, Boston, MA). Trypsin-EDTA, DMEM were purchased from GIBCOBRL (GIBCOBRL Life Technologies, Grand Island, NY). BAX/BAK -/-, BIM -/- and BID -/- fibroblasts were kindly provided by Dr. S. Korsmeyer (Harvard University, Boston, MA). Transformed PKR like endoplasmic reticulum (PERK) -/- cells were a kind gift from the Ron Laboratory, Skirball Institute, NYU School of Medicine. The BCL-Xl and activated MEK1 EE adenoviruses were kindly provided by Dr. J. Moltken (University of Cincinnati). Other reagents were of the highest quality commercially available [27-29].

Methods.

Culture of human colon cancer cells and drug treatments for short term viability assays.

Cells were plated (5×10^4 per cm^2) and 24h after plating either infected with recombinant adenovirus (see section below for details) or treated with either a Pt containing agent (0-3.0 μM, as indicated), or AG1478 / LY294002 / PD184352 (0-10.0 μM), for up to 96h, as indicated.

Cellular Platinum Accumulation Assays.

Cells were plated at 2.0×10^6 cells/ml. BBR3464 or BBR3610 was added in 20μM. After 8h, cells were harvested 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 simultaneous inductively coupled plasma optical emission spectroscopy at 265 nm (Varian Inc., Palo Alto, CA). Standards and blank were prepared the same as the samples.

Assessment of Platinum Accumulation in DNA.

Cells were plated at 2.0×10^6 cells/ml. BBR3464 or BBR3610 was added at 55μM. After 10h, cells were harvested and washed twice with PBS. DNA was then extracted from the cell pellets using high salt method (Montagna et al, 2002; Miller et al, 1988). Briefly, the cell pellets were treated with nuclei lysis buffer, proteinase K, 10% SDS and 6M NaCl. DNA was then precipitated using isopropanol and 3M sodium acetate. DNA was rinsed using 70% ethanol and the purity of the DNA was measured at absorbance of 260nm. The DNA was then harvested for platinum analysis according to the United States Environmental Protection Agency procedure 3050b as explained above.

Culture of human colon cancer cells and drug treatments for colony formation assays.

Cells were plated (250-1,500 cells per well of a 6 well plate) and 12h after plating treated with either Pt agent (0-3.0 μM, as indicated), or AG1478/LY294002/PD184352 (0-10.0 μM), for 48h, as indicated. After 48h, the drug containing media was carefully removed, the cells washed once, and fresh media lacking drugs added. Colony formation assays were cultured for an additional 10-14 days after which the media was removed, cells fixed with methanol, stained with crystal violet and counted manually.

SDS-PAGE and Western blot analysis.

Cells were treated with either Pt agent or AG1478/LY294002/PD184352 as indicated. Cells were isolated For SDS PAGE and immunoblotting, cells were lysed in either a non-denaturing lysis buffer, and prepared for immunoprecipitation or in whole-cell lysis buffer (0.5 M Tris-HCl, pH 6.8, 2%SDS, 10% glycerol, 1% β-mercaptoethanol, 0.02% bromophenol blue), and the samples were boiled for 30 min. After immunoprecipitation, samples were boiled in whole cell lysis buffer. The boiled samples were loaded onto 10-14% SDS-PAGE and electrophoresis was run overnight. Proteins were electrophoretically transferred onto 0.22 μm nitrocellulose, and immunoblotted with various primary antibodies against different proteins. All immunoblots were visualized by ECL. For presentation, immunoblots were digitally scanned at 600 dpi using Adobe PhotoShop 7.0, and their color removed and Figures generated in MicroSoft PowerPoint. Densitometric analysis for E.C.L. immunoblots were performed using a Fluorochem 8800 Image System

and the respective software (Alpha Innotech Corporation, San Leandro, CA) and band densities were normalized to that of a total protein loading control.

Recombinant adenoviral vectors; generation and infection in vitro.

We generated and purchased previously noted recombinant adenoviruses to express constitutively activated and dominant negative AKT and MEK1 proteins, dominant negative ERBB1 (COOH-terminal 533 amino acid deletion; CD533), dominant negative caspase 9, CRM A (caspase 8 inhibitor), XIAP, c-FLIP-s and BCL-XL (Vector Biolabs, Philadelphia, PA). Colon cancer cells were infected, 24h after plating, with these adenoviruses at an approximate m.o.i. of 50 for 4h with gentle rocking after which time the media was replaced. Cells were further incubated for 24 hours to ensure adequate expression of transduced gene products prior to drug exposures. For transformed mouse embryonic fibroblasts, cells were infected at 150 moi due to lower CAR levels on these cells.

Detection of cell death by Trypan Blue assays.

Cells were harvested by trypsinization with Trypsin/EDTA for ~10 min at 37 ^{o}C. As some apoptotic cells detached from the culture substratum into the medium, these cells were also collected by centrifugation of the medium at 1,500 rpm for 5 min. The pooled cell pellets were resuspended and mixed with trypan blue dye. Trypan blue stain, in which blue dye incorporating cells were scored as being dead was performed by counting of cells using a light microscope and a hemacytometer. Five hundred cells from random fields were

counted and the number of dead cells was counted and expressed as a percentage of the total number of cells counted.

Transfection of DLD1 cells with small interfering RNA molecules.

RNA interference or gene silencing for down-regulating the expression of FADD and CD95 (FAS receptor) was performed using validated target sequences designed by Ambion, Inc. (Austin, Texas). For transfection, 10 nM of the annealed SiRNA targeting FADD or CD95, the positive control doubled stranded SiRNA targeting GAPDH or the negative control (a "scrambled" sequence with no significant homology to any known gene sequences from mouse, rat or human cell lines) were used. The siRNA molecules were transfected into cells according to the manufacturer's instructions. Cells were cultured for 48h after transfection prior to any additional experimentation.

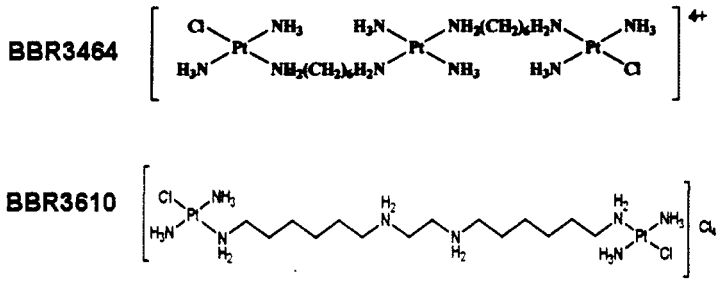
Data analysis.

Comparison of the effects of various treatments was performed using one way analysis of variance and a two tailed Student's *t*-test. Differences with a *p*-value of < 0.05 were considered statistically significant. Experiments shown are the means of multiple individual points (± SEM). Characterization of synergistic and antagonistic interactions in cells exposed to a range of BBR3610 and AG1478 concentrations administered at a fixed ratio was done using median dose effect analysis in conjunction with a commercially available software program (CalcuSyn, Biosoft, Ferguson, MO).

Results.

Initial studies examined the toxicity of the novel dinuclear platinum agent BBR3610 in colon cancer cells in comparison to established platinum chemotherapeutic agents and to a multinuclear platinum chemotherapeutic that has undergone Phase II trials, BBR3464 (Figure 1A). BBR3610 was a significantly more toxic agent in colony formation assays than BBR3464, cisplatin or oxaliplatin (Figures 1B and 1C). The expression of both p53 and p21 has been linked to the toxicity profiles of cells treated with Pt containing therapeutic agents [30,31]. At approximately the IC75 for BBR3610, loss of p53 function in HCT116 cells did not significantly alter drug toxicity whereas loss of p21 function modestly suppressed cell killing (Figure 1B). Although BBR3610 was a more toxic agent than BBR3464, we did not observe significant differences in the uptake of either drug or comparing the incorporation of Pt from either drug into DNA (Figure 1D). One established mechanism of chemotherapeutic and radio-resistance in colon cancer is due to the high incidence of expression of mutated (active) K-RAS proteins. Deletion of the single K-RAS D13 allele in HCT116 cells markedly enhanced the toxic effects of BBR3610 compared to wild type cells; an effect that was not readily apparent for oxalipatin (Figure 1E, panel on left); expression of H-RAS V12 in HCT116 cells at low BBR3610 doses enhanced BBR3610 toxicity beyond that in wild type cells, the opposite to the enhanced survival effects of H-RAS V12 expressing cells after irradiation (Figure 1E, panel on right).

A



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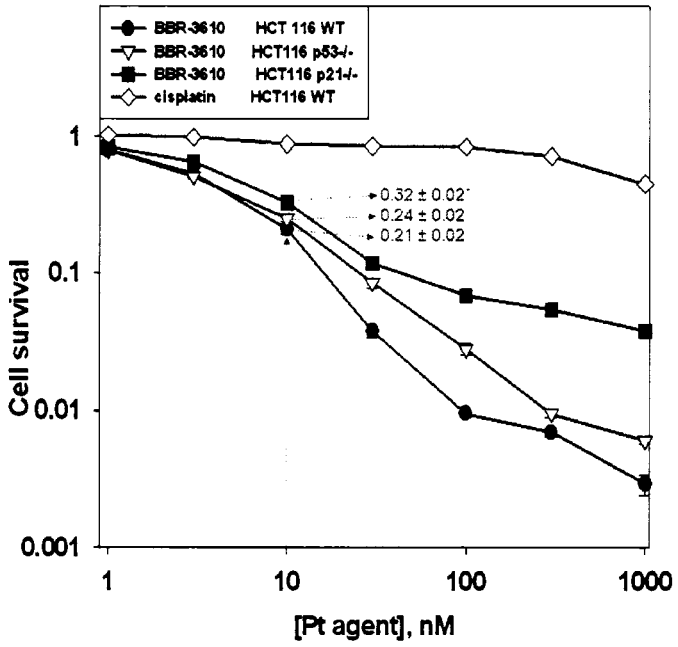

Figure 1. BBR3610 is a more toxic agent than BBR3464, oxaliplatin and cisplatin in colon cancer cells. Panel A. The chemical structures of BBR3464 and BBR3610. **Panel B.** HCT116 cells (wild type, WT; deleted for p53, p53 -/-; deleted for deleted for p21, p21 -/-) growing in log phase were trypsinized and single cells re-plated in 60 mm dishes (250-2,500 cells / dish). Twelve hours after plating cells were treated with BBR3610 or cisplatin as indicated. Media containing the drug was removed 48h after treatment, the plates carefully washed with drug free media and then fresh drug free media added to the cells. Cells were cultured for 10-14 days to permit > 50 cell colonies to form. The media was removed, the cells fixed with methanol and stained with crystal blue. Colonies of > 50 cells were counted, the survival for each condition calculated. The data is from a representative experiment (n = 2) of six plates per data point (± SEM, n = 6).

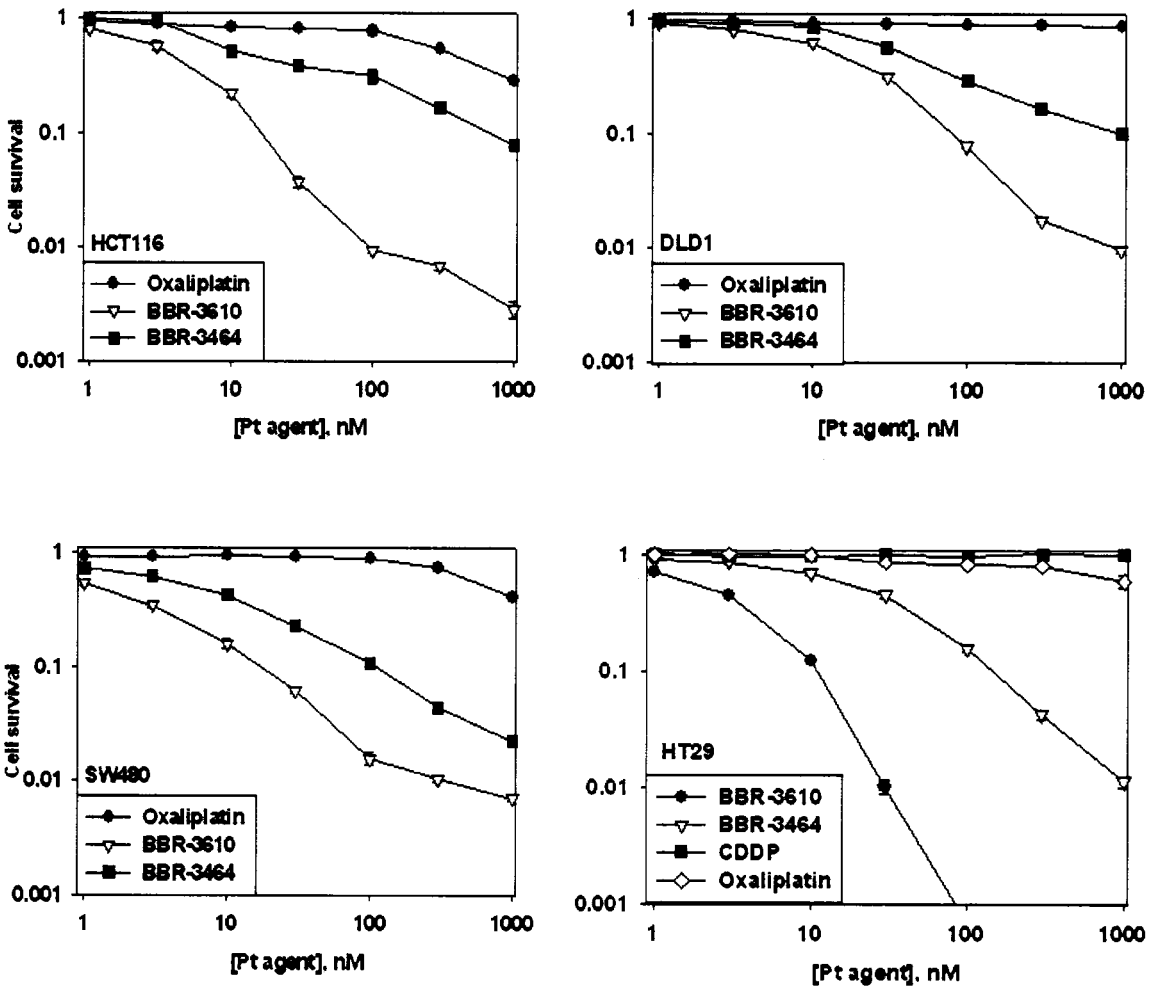

Figure 1, Panel C. HCT116, DLD1, SW480 and HT29 cells growing in log phase were trypsinized and single cells re-plated in 60 mm dishes (250-2,500 cells / dish). Twelve hours after plating cells were treated with BBR3610, cisplatin (CDDP) or oxaliplatin as indicated. Media containing the drug was removed 48h after treatment, the plates carefully washed with drug free media and then fresh drug free media added to the cells. Cells were cultured for 10-14 days to permit > 50 cell colonies to form. The media was removed, the cells fixed with methanol and stained with crystal blue. Colonies of > 50 cells were counted, the survival for each condition calculated. The data is from a representative experiment (n = 2) of six plates per data point (± SEM, n = 6).

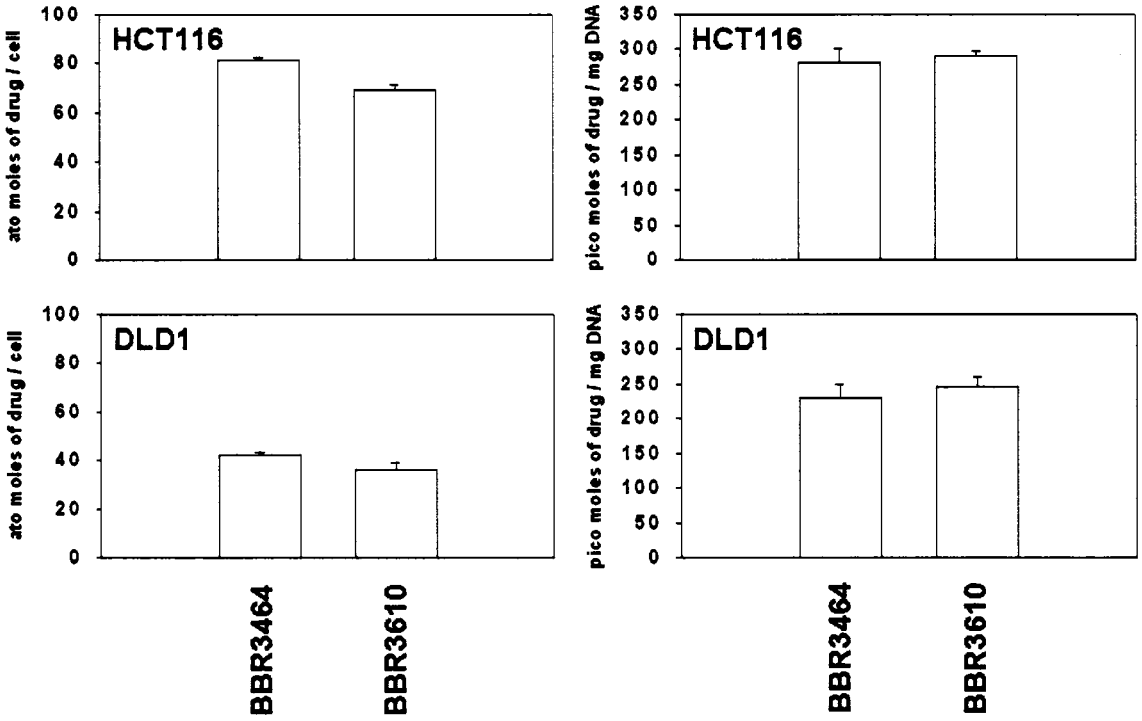

Figure 1, Panel D. The uptake and DNA adduct formation of BBR3464 and BBR3610 was measured as described in the Methods section. Uptake data are expressed as the number of free unbound ato moles of platinum agent within each cell. DNA adduct formation data are expressed as the number of pico moles of Pt drug incorporated per milligram of DNA. Data are in triplicate ± SEM.

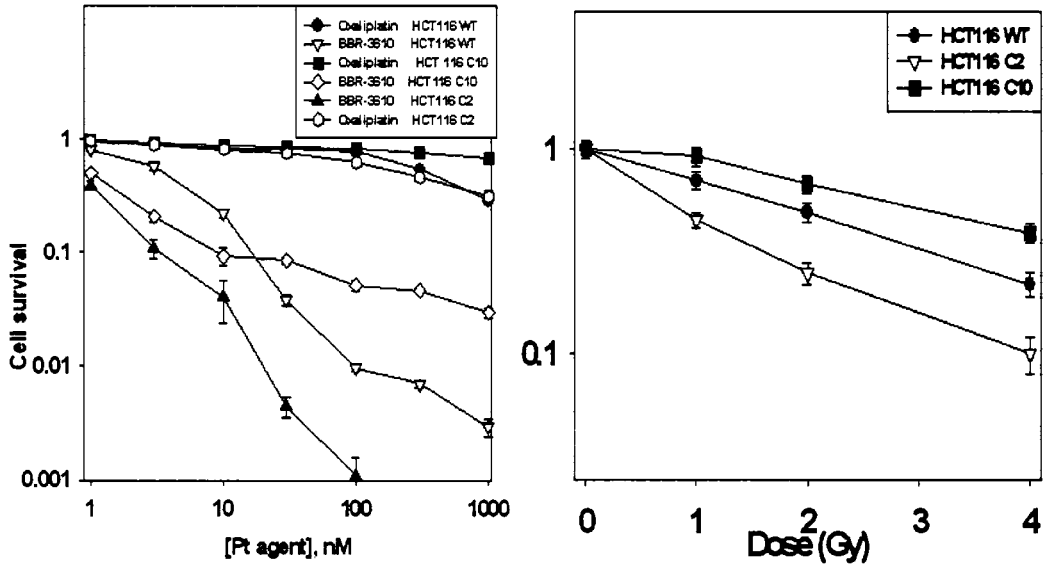

Figure 1, Panel E. HCT116 cells (wild type, WT; deleted for the single allele of mutated active K-RAS D13, C2; C2 cells stably expressing low levels of H-RAS V12) were plated as described above for colony formation assays and either treated with BBR3610 or oxaliplatin or irradiated (Yacoub et al, 2006a). Colony formation was determined 10-14 days after drug / radiation exposure and the survival of cells plotted. Data are from 3-6 data points per treatment condition ± SEM (n = 2-3).

In addition to mutated RAS proteins, colon cancer cells are known to over-express ERBB receptors, and for ERBB receptors to play an important role in colon cancer cell survival. Treatment of HCT116, DLD1 and SW480 cells with low marginally toxic concentrations of an ERBB1 inhibitor, AG1478, enhanced BBR3610 lethality, which was p53-independent (Figure 2A). Based on median dose effect analyses using colony formation assays, with a combination index of less than one, the interaction between BBR3610 and AG1478 was judged to be synergistic in DLD1 and HCT116 cells (Table 1 data not shown). In all lines tested inhibition of PI3K, but not MEK1/2, enhanced the lethality of BBR3610 (Figure 2B). As the data presented in Figure 1 and Table 1 take several weeks to perform, we next determined whether BBR3610 and AG1478 interacted to rapidly promote cell killing within 48-96h of treatment as judged in trypan blue viability assays. AG1478 promoted BBR3610 lethality in DLD1 and in HCT116 tumor cells 48h and 96h after drug exposure (Figure 2C, data not shown). In DLD1 cells expression of a truncated dominant negative ERBB1 protein (ERBB1-CD533) enhanced BBR3610 toxicity to a similar extent as that induced by the small molecule ERBB1 inhibitor AG1478 (Figure 2D).

Chemotherapeutic agents such as Ara C or ionizing radiation promote tumor cell killing by causing activation of the JNK1/2 and/or p38 MAPK pathways. Low doses of BBR3610, in the presence or absence of ERBB1 inhibitor, weakly enhanced signaling through the p38 MAPK and JNK1/2 pathways in DLD1 cells (Figure 3A, lower section). In contrast, BBR3610 significantly reduced the levels of PAKT and transiently increased P-ERK1/2 levels at 24h (Figure 3A, upper section). Inhibition of ERBB1 modestly further

suppressed AKT activity 24-48h after exposure. Expression of constitutively active AKT protected DLD1 cells from BBR3610 and BBR3610 + AG1478 toxicity, whereas expression of dominant negative AKT enhanced the toxicity of BBR3610, AG1478 and of BBR3610 + AG1478 (Figure 3B).

Unlike the relatively specific MEK1/2 inhibitor PD184352, expression of dominant negative MEK1 enhanced the toxicity of BBR3610; of AG1478 and of BBR3610 + AG1478 (Figure 3C). The toxicity of BBR3610 + AG1478 remained significantly greater than the additive individual toxicity of BBR3610 or AG1478 which was in contrast to our observations expressing dominant negative AKT where the greater than additive interaction between BB3610 and AG1478 was lost to the greater enhancement of cell killing (Figures 3B and 3C). Expression of constitutively active MEK1 did not alter BBR3610 toxicity and weakly suppressed the toxicity of BBR3610 + AG1478. Although neither p38 MAPK nor JNK1/2 were strongly activated by any drug treatment, inhibition of either p38 MAPK or JNK1/2 suppressed the ability of ERBB1 inhibition to enhance BBR3610 toxicity (Figure 3D).

To determine by a molecular approach whether the promotion of BBR3610 toxicity caused by suppression of ERBB1 function was AKT-dependent, we co-expressed dominant negative ERBB1 and constitutively active AKT and treated cells with BBR3610 and/or AG1478. Expression of constitutively active AKT suppressed BBR3610 toxicity in the presence or absence of dominant negative ERBB1 (Figure 3E). We also examined the impact of these genetic manipulations on anti-apoptotic protein expression. Inhibition of ERBB1 did not alter basal c-FLIP expression but enhanced the suppression of c-FLIP

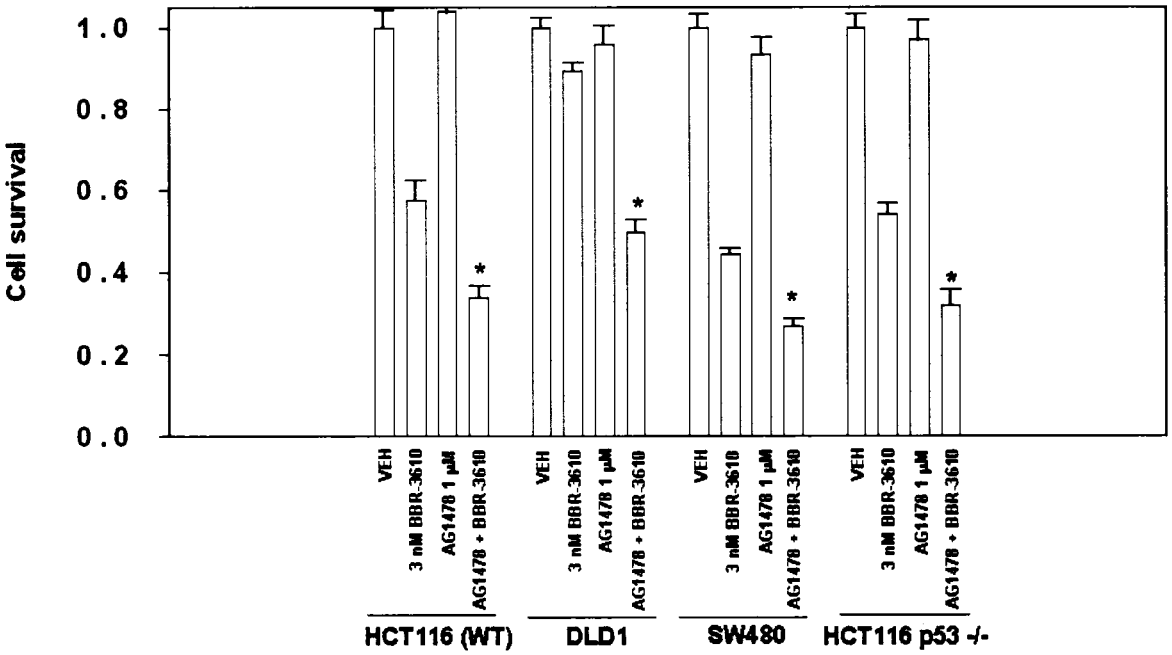

Figure 2. The lethality of BBR3610 is p53-independent and is enhanced by inhibition of ERBB1 or PI3 kinase signaling. Panel A. HCT116 (wild type, WT; deleted for expression of p53, p53 -/-), DLD1 and SW480 cells growing in log phase were trypsinized and single cells re-plated in 60 mm dishes (250-2,500 cells / dish). Twelve hours after plating cells were treated with BBR3610 and AG1478 as indicated. Media containing the drugs was removed 48h after treatment, the plates carefully washed with drug free media and then fresh drug free media added to the cells. Cells were cultured for 10-14 days to permit > 50 cell colonies to form. The media was removed, the cells fixed with methanol and stained with crystal blue. Colonies of > 50 cells were counted, the survival for each condition calculated. The data is from a representative experiment (n = 2) of six plates per data point (± SEM, n = 6).

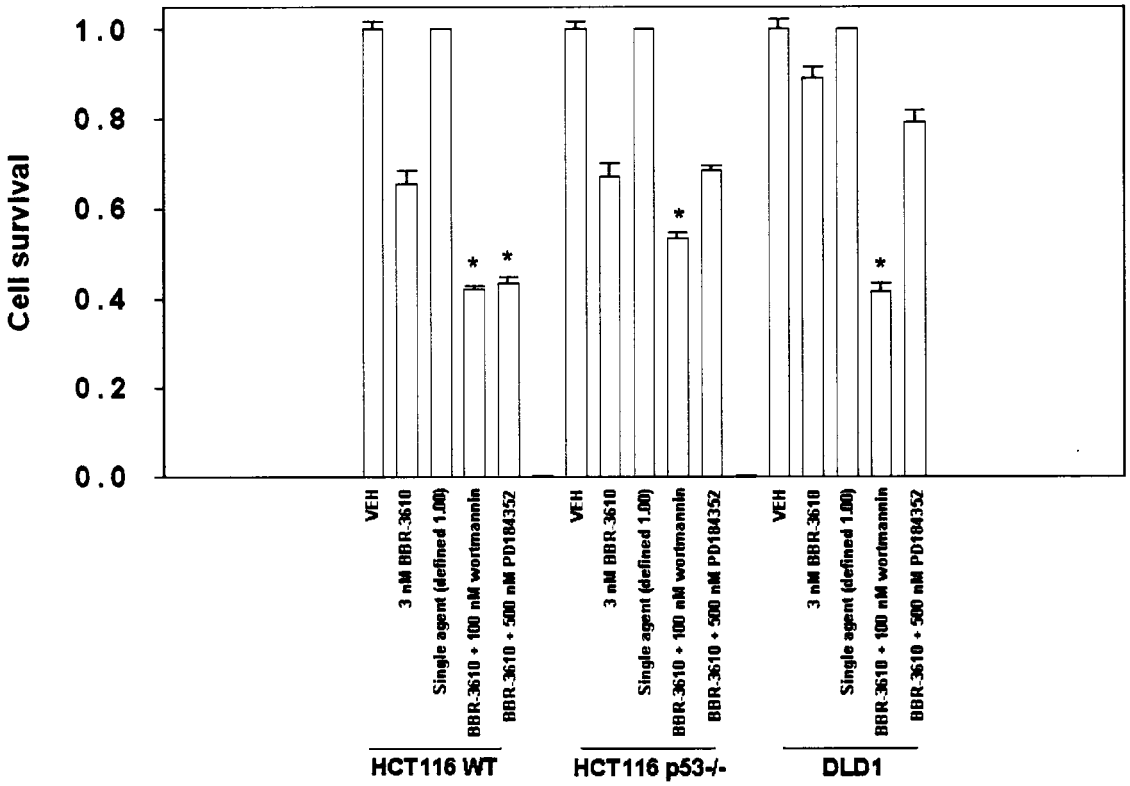

Figure 2, Panel B. HCT116 (wild type, WT; deleted for expression of p53, p53 -/-), and DLD1 cells growing in log phase were trypsinized and single cells re-plated in 60 mm dishes (250-2,500 cells / dish). Twelve hours after plating cells were treated with BBR3610, PD184352 or wortmannin as indicated. Media containing the drugs was removed 48h after treatment, the plates carefully washed with drug free media and then fresh drug free media added to the cells. Cells were cultured for 10-14 days to permit > 50 cell colonies to form. The media was removed, the cells fixed with methanol and stained with crystal blue. Colonies of > 50 cells were counted, the survival for each condition calculated. The data for single agent PD184352 or wortmannin treatment is normalized and defined as 1.00 is the Figure. The data is from a representative experiment (n = 2) of six plates per data point (± SEM, n = 6).

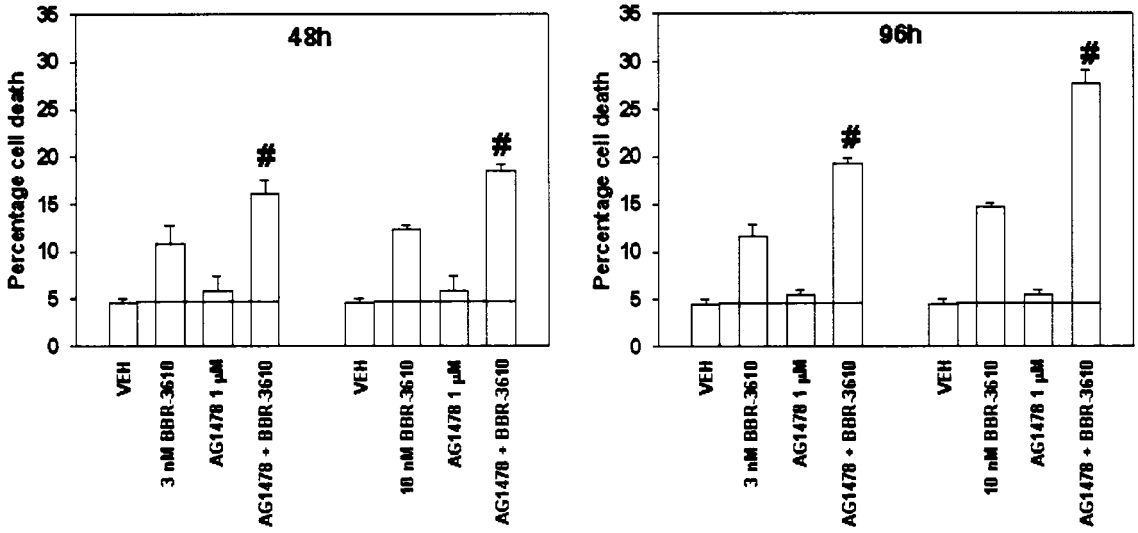

Figure 2, Panel C. DLD1 cells were plated in triplicate and 24h after plating treated with the indicated concentrations of BBR3610 and AG1478. Cells were isolated 48h and 96h after drug exposure and trypan blue cell viability assays performed. Data are from a representative experiment (n = 2) of triplicate samples ± SEM. (# $p < 0.05$ greater than treatment with BBR3610 alone).

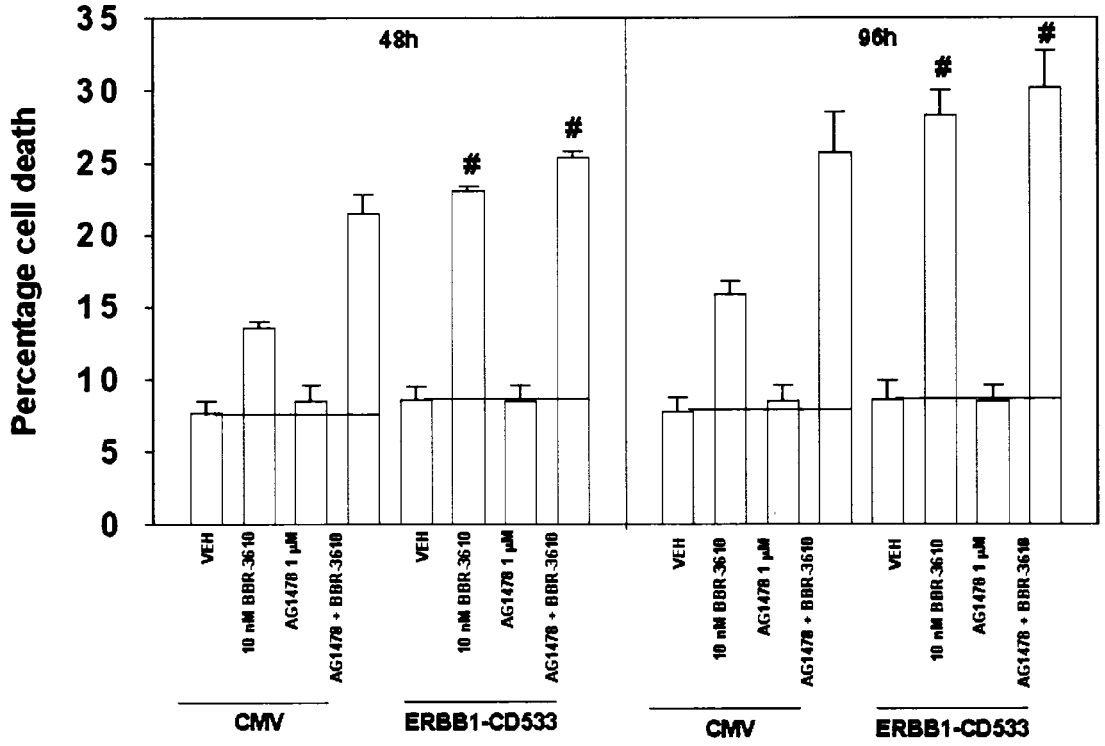

Figure 2, Panel D. DLD1 cells were plated in triplicate and 12h after plating infected with either a control empty vector virus (CMV) or with a virus to express dominant negative ERBB1-CD533. Twenty four hours after infection cells were treated with the indicated concentrations of BBR3610 and AG1478. Cells were isolated 48h and 96h after drug exposure and trypan blue cell viability assays performed. Data are from a representative experiment (n = 2) of triplicate samples ± SEM. (# $p < 0.05$ greater than treatment with BBR3610 alone).

BBR-3610 (nM)	AG1478 (μM)	Fa	CI
1.5	0.5	0.23	0.32
3	1	0.31	0.52
4.5	1.5	0.40	0.66

Table 1. BBR3610 and the ERBB1 inhibitor AG1478 interact in a synergistic fashion to kill colon cancer cells in vitro. DLD1 cells growing in log phase were trypsinized and single cells re-plated in 60 mm dishes (250-2,500 cells / dish). Twelve hours after plating cells were treated with BBR3610 and AG1478 at a fixed ratio. Media containing the drugs was removed 48h after treatment, the plates carefully washed with drug free media and then fresh drug free media added to the cells. Cells were cultured for 10-14 days to permit > 50 cell colonies to form. The media was removed, the cells fixed with methanol and stained with crystal blue. Colonies of > 50 cells were counted, the survival for each condition calculated and values entered into the Calcusyn program. The table shows data from a representative experiment of six plates per data point (n = 2). A combination index (CI) values of less than 1.00 indicates synergy (Fa; fraction afftected

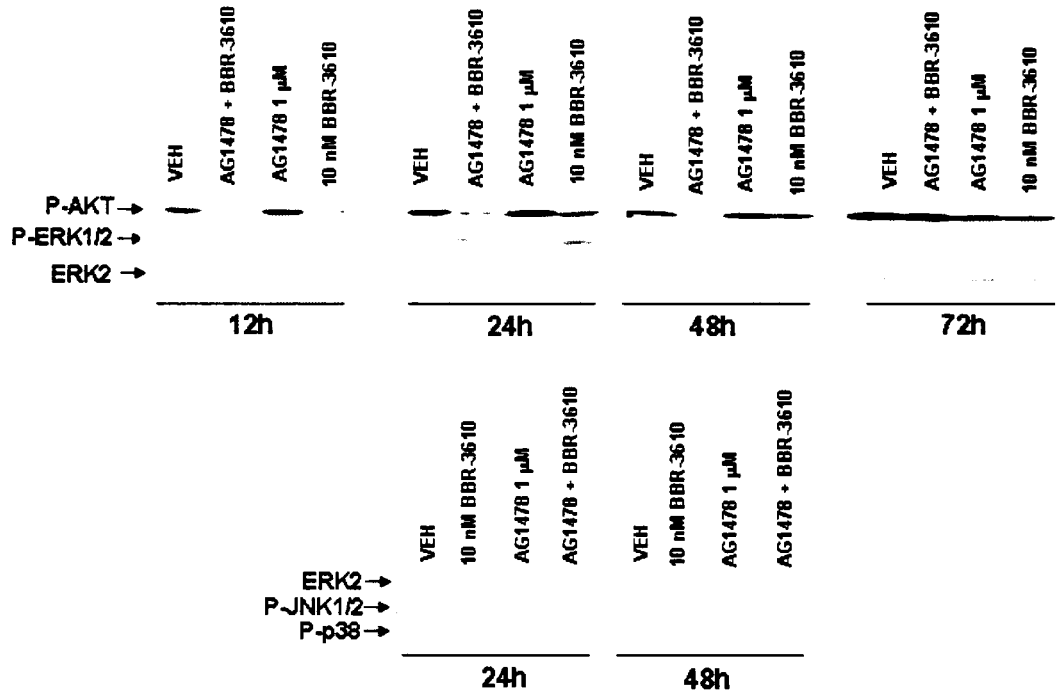

Figure 3. Activation of AKT suppresses BBR3610 and BBR3610+ERBB1 inhibition lethality whereas activation of MEK1 partially suppresses BBR3610+ERBB1 inhibition lethality: activation of AKT maintains c-FLIP and MCL-1 expression.

Panel A. DLD1 cells were plated in 60 mm dishes (10^6 cells) and 24h after plating treated with BBR3610 and/or AG1478, as indicated. Cells were isolated at the indicated time points and subjected to SDS PAGE and immunoblotting then performed to determine the phosphorylation (activity) status of ERK1/2, AKT (S473), p38 MAPK and JNK1/2. Data are from a representative experiment (n = 2).

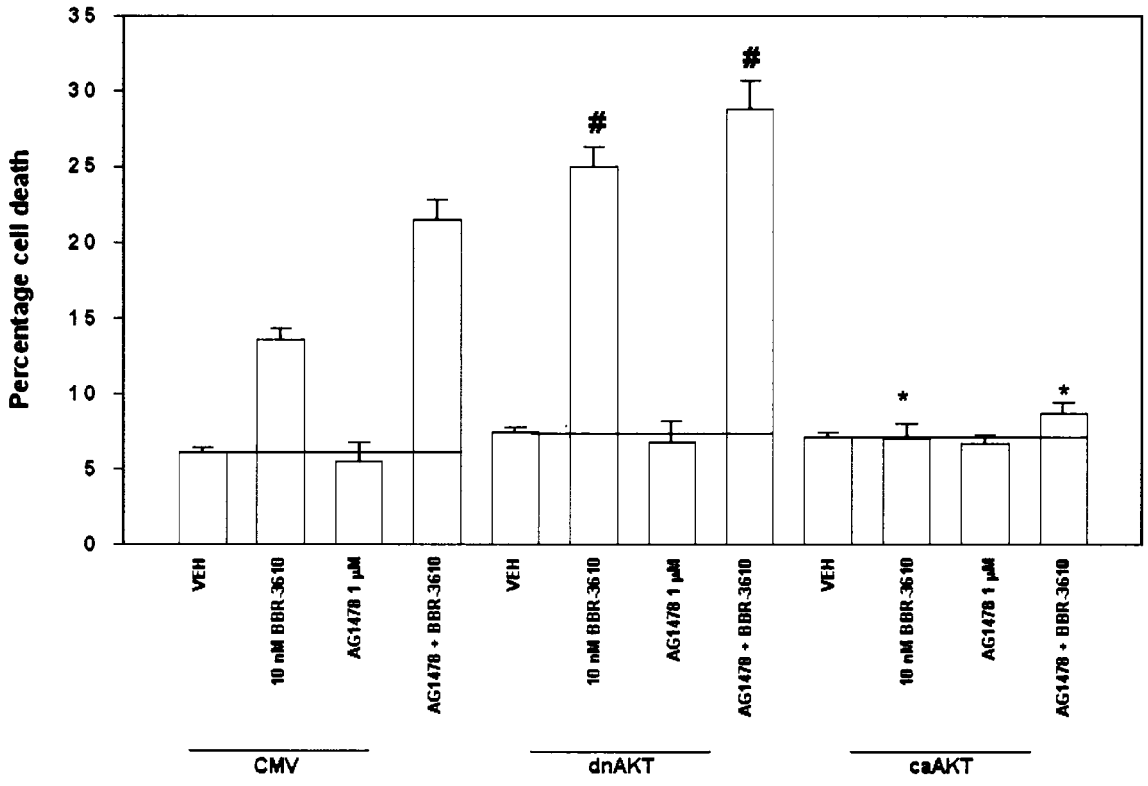

Figure 3, Panel B. DLD1 cells were plated in triplicate and 12h after plating infected with either a control empty vector virus (CMV) or with a virus to express dominant negative AKT or constitutively active AKT. Twenty four hours after infection cells were treated with the indicated concentrations of BBR3610 and AG1478. Cells were isolated 96h after drug exposure and trypan blue cell viability assays performed. Data are from a representative experiment (n = 2) of triplicate samples ± SEM. (* $p < 0.05$ less than treatment with BBR3610 alone).

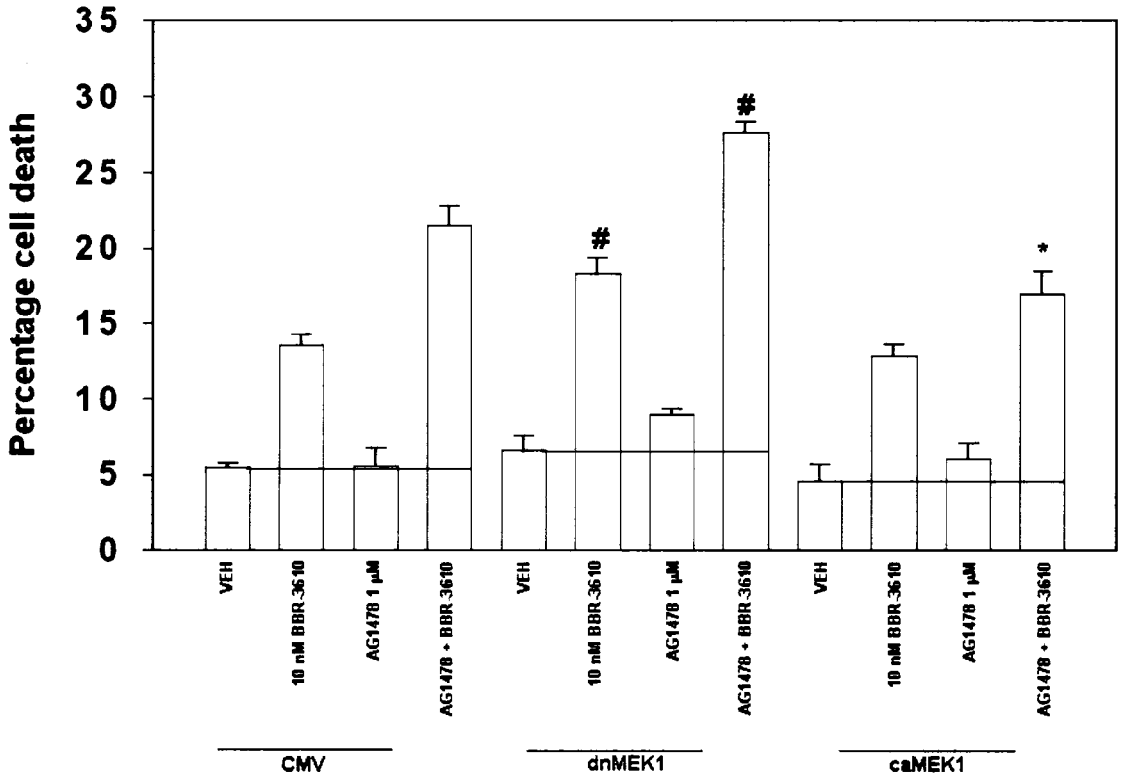

Figure 3, Panel C. DLD1 cells were plated in triplicate and 12h after plating infected with either a control empty vector virus (CMV) or with a virus to express dominant negative MEK1 or constitutively active MEK1. Twenty four hours after infection cells were treated with the indicated concentrations of BBR3610 and AG1478. Cells were isolated 96h after drug exposure and trypan blue cell viability assays performed. Data are from a representative experiment (n = 2) of triplicate samples ± SEM. (# $p < 0.05$ greater than treatment with BBR3610 alone; * $p < 0.05$ less than treatment with BBR3610 alone).

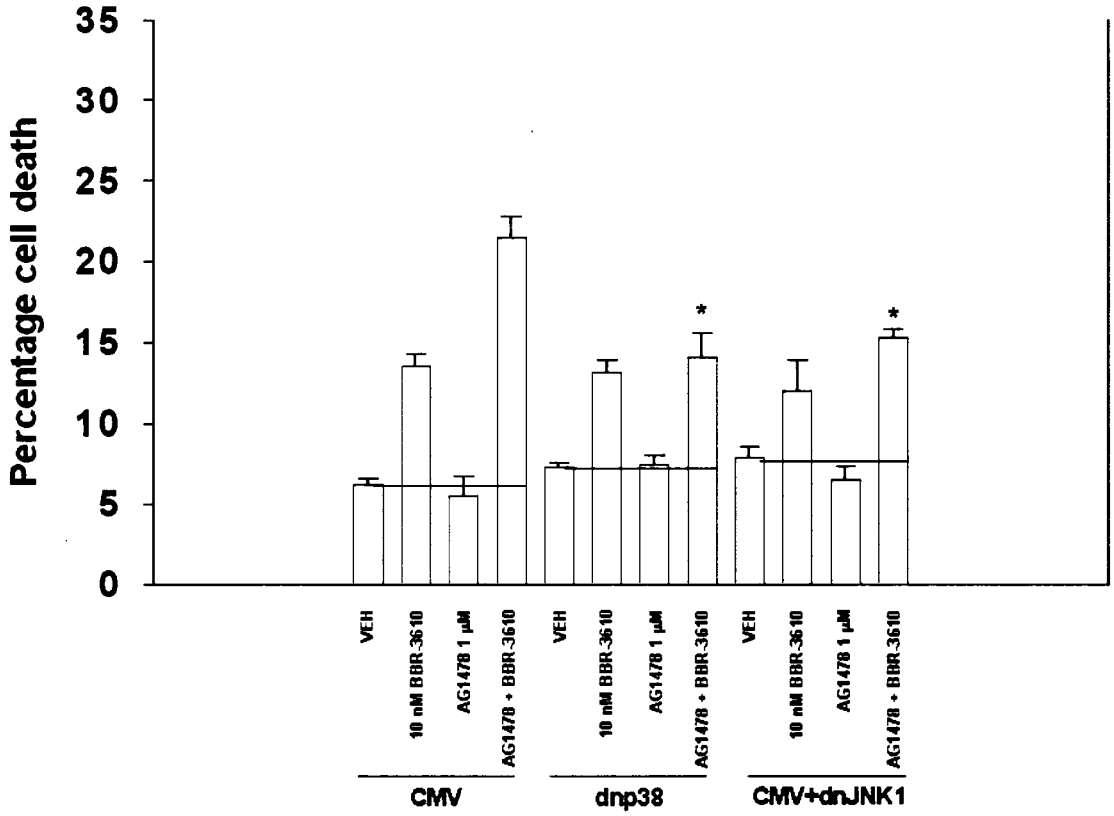

Figure 3, Panel D. DLD1 cells were plated in triplicate and 12h after plating infected with either a control empty vector virus (CMV) (3 x 8 plates) or with a virus to express dominant negative p38 MAPK (3 x 4 plates). Twenty four hours after infection one portion of CMV infected cells (3 x 4 plates) were treated with the JNK inhibitory peptide (JNK-IP); the other plates were treated with vehicle control (DMSO). Thirty minutes after JNK-IP treatment, cells were treated with the indicated concentrations of BBR3610 and AG1478. Cells were isolated 96h after drug exposure and trypan blue cell viability assays performed. Data are from a representative experiment (n = 2) of triplicate samples ± SEM. (* $p < 0.05$ less than treatment with BBR3610 alone).

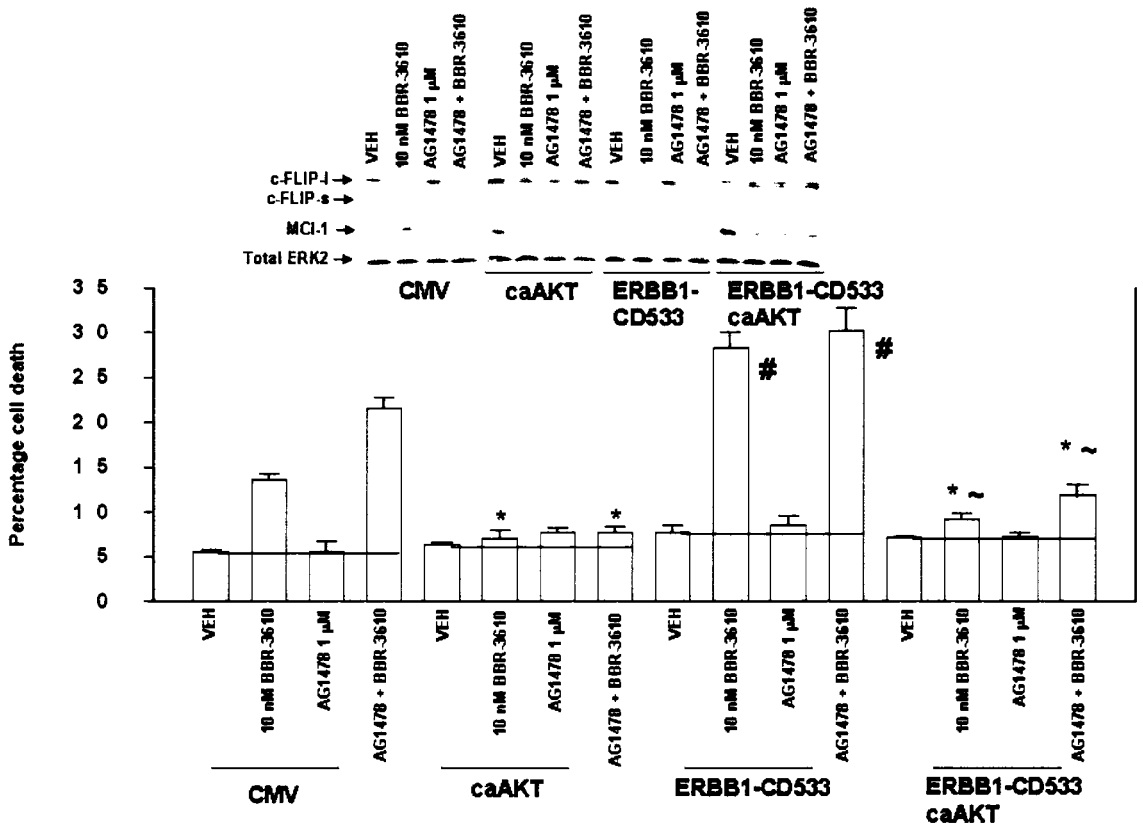

Figure 3, Panel E. DLD1 cells were plated in triplicate and 12h after plating infected with either a control empty vector virus (CMV), with a virus to express dominant negative ERBB1-CD533 or constitutively active AKT, as indicated in the Figure. Twenty four hours after infection cells were treated with the indicated concentrations of BBR3610 and AG1478. Cells were isolated 96h after drug exposure and trypan blue cell viability assays performed. Data are from a representative experiment (n = 2) of triplicate samples ± SEM. (# $p < 0.05$ greater than treatment with BBR3610 alone; * $p < 0.05$ less than treatment with BBR3610 alone; ~ $p < 0.05$ less than cells expressing ERBB1-CD533 that do not express activated AKT). Inset Panel. Cells were infected and treated with drugs in an identical manner to those in the graphical panel. Cells were isolated 48h after drug exposure and subjected to SDS PAGE followed by immunoblotting to determine the expression of c-FLIP proteins, MCL-1 and the total expression of ERK2 (n = 2).

levels caused by BBR3610 treatment. Inhibition of ERBB1 suppressed MCL-1 expression. Activation of AKT enhanced basal levels of c-FLIP and MCL-1; AKT activation maintained c-FLIP and MCL-1 levels in cells expressing dominant negative ERBB1 and when treated with BBR3610 (Figure 3E, inset panel). Collectively, our data argues that BBR3610 suppresses AKT activity which is causal in cell death and that inhibition of ERBB1 – MEK1/2 signaling, in the presence of already suppressed AKT signaling, further elevates BBR3610 toxicity.

We next examined the molecular pathways by which BBR3610 killed colon cancer cells in vitro. Incubation of DLD1 cells with a pan-caspase inhibitor (zVAD) or a caspase 8 inhibitor (IETD) reduced BBR3610 toxicity whereas a caspase 9 inhibitor (LEHD) did not alter BBR3610 lethality but abolished the ability of AG1478 to enhance cell killing (Figure 4A). Similar data were obtained when the caspase 8 inhibitor CRM A and dominant negative caspase 9 were expressed in DLD1 cells (Figure 4B). XIAP is an inhibitor of caspase 9; c-FLIP-s is an inhibitor of caspase 8; and BCL-XL is a protein that in a similar manner to MCL-1 maintains mitochondrial function downstream of caspase 8 – BID signaling as well as from BAX/BAK/BIM –induced cytochrome c release. Treatment of cells with BBR3610 increased cleavage of pro-caspase 8 24h and 48h after exposure (data not shown). Over-expression of c-FLIP-s abolished BBR3610 toxicity (Figure 4C). Expression of XIAP did not alter BBR3610 lethality, but abolished the enhancement of cell killing by ERBB1 inhibition; over-expression of BCL-XL partially suppressed BBR3610 lethality and abolished the enhancement of cell killing by ERBB1 inhibition (Figure 4C).

Additional studies were then performed in transformed mouse embryonic fibroblasts (MEFs) lacking key pro-apoptotic genes which cause mitochondrial dysfunction. In transformed MEFs, BB3610 lethality was suppressed to a greater extent by loss of BID function than by loss of BAX/BAK or BIM expression (Figure 4D). The ability of ERBB1 inhibition to enhance BBR3610 lethality was suppressed in cells lacking BAX/BAK function. In addition to caspase 8, BID cleavage and subsequent mitochondrial dysfunction can also be catalyzed by cathepsin B, and loss of cathepsin B expression in cathepsin B -/- MEFs suppressed BBR3610 toxicity (data not shown). Pro-caspase 8 is activated by death receptors via FADD but has also been noted to auto-catalyze its own activation independently of FADD when expression of caspase 8 inhibitors such as c-FLIP-s is reduced. Knock down of FADD and of the FAS death receptor (CD95) suppressed BBR3610 toxicity in DLD1 cells (Figure 5). Collectively these findings demonstrate that BBR3610 initiates cell killing via a death receptor-dependent activation of pro-caspase 8 that is facilitated by reduced expression of c-FLIP proteins and reduced AKT signaling; inhibition of ERBB1 promotes BBR3610 lethality by facilitating BID-and BAX/BAK/BIM –dependent mitochondrial dysfunction and activation of the caspase 9 pathway.

Discussion

Platinum containing cancer therapeutic drugs are used in the treatment of many malignancies including testicular, ovarian, lung and colon cancer. However the development of resistance to mononuclear platinum agents is common and the synthesis of

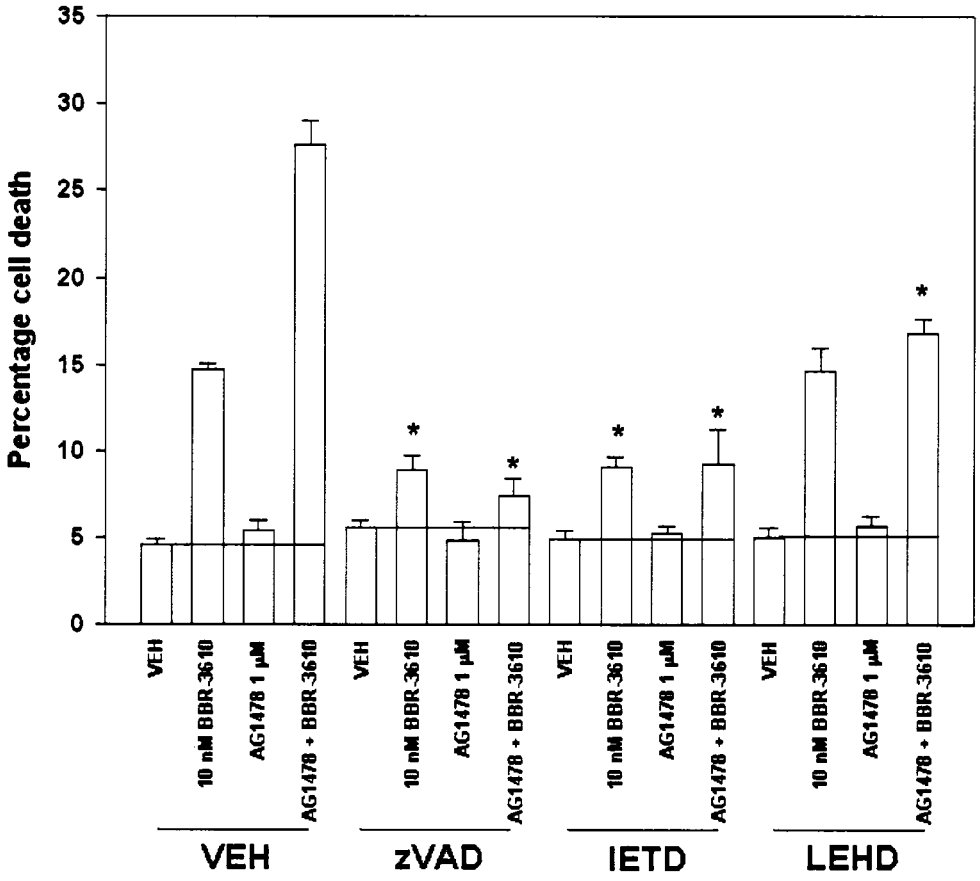

Figure 4. BBR3610 promotes colon cancer killing by activation of caspase 8; the potentiation of BBR3610 lethality by ERBB1 inhibition is caspase 9 -dependent. Panel A. DLD1 cells, 24h after plating, were treated with BBR3610 and AG1478, as indicated. In parallel, cells were co-treated with vehicle (VEH, DMSO), a pan-caspase inhibitor (zVAD, 50 µM), a caspase 8 inhibitor (IETD, 50 µM) or a caspase 9 inhibitor (LEHD, 50 µM). The caspase inhibitors were re-supplemented to the media every 24h. Cells were isolated by trypsinization 96h after the addition of BBR3610 and AG1478 and trypan blue assays performed. Data are from a representative experiment (n = 2) of triplicate samples ± SEM. (* $p < 0.05$ less than corresponding treatment in VEH treated cells).

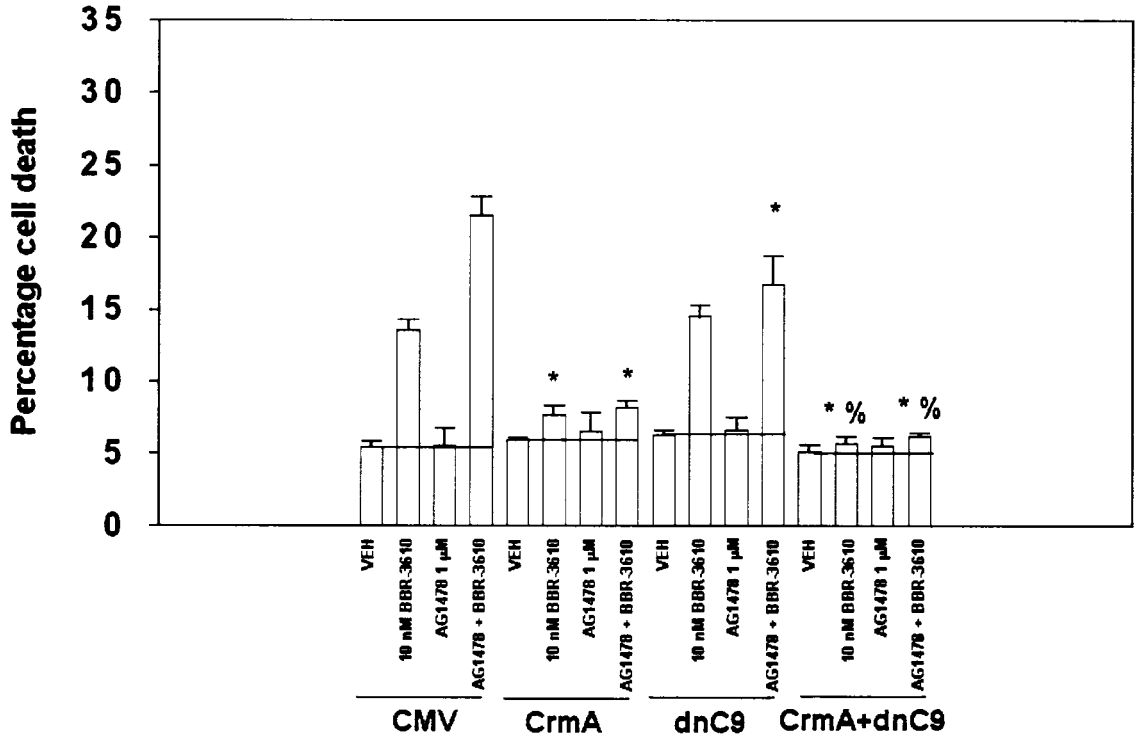

Figure 4, Panel B. DLD1 cells were plated in triplicate and 12h after plating infected with either a control empty vector virus (CMV), with a virus to express dominant negative caspase 9 or a viral inhibitor of caspase 8, CRM A, as indicated in the Figure. Twenty four hours after infection cells were treated with the indicated concentrations of BBR3610 and AG1478. Cells were isolated 96h after drug exposure and trypan blue cell viability assays performed. Data are from a representative experiment (n = 2) of triplicate samples ± SEM. (* $p < 0.05$ less than treatment with BBR3610 alone; % $p < 0.05$ less than corresponding value in cells expressing only CRM A).

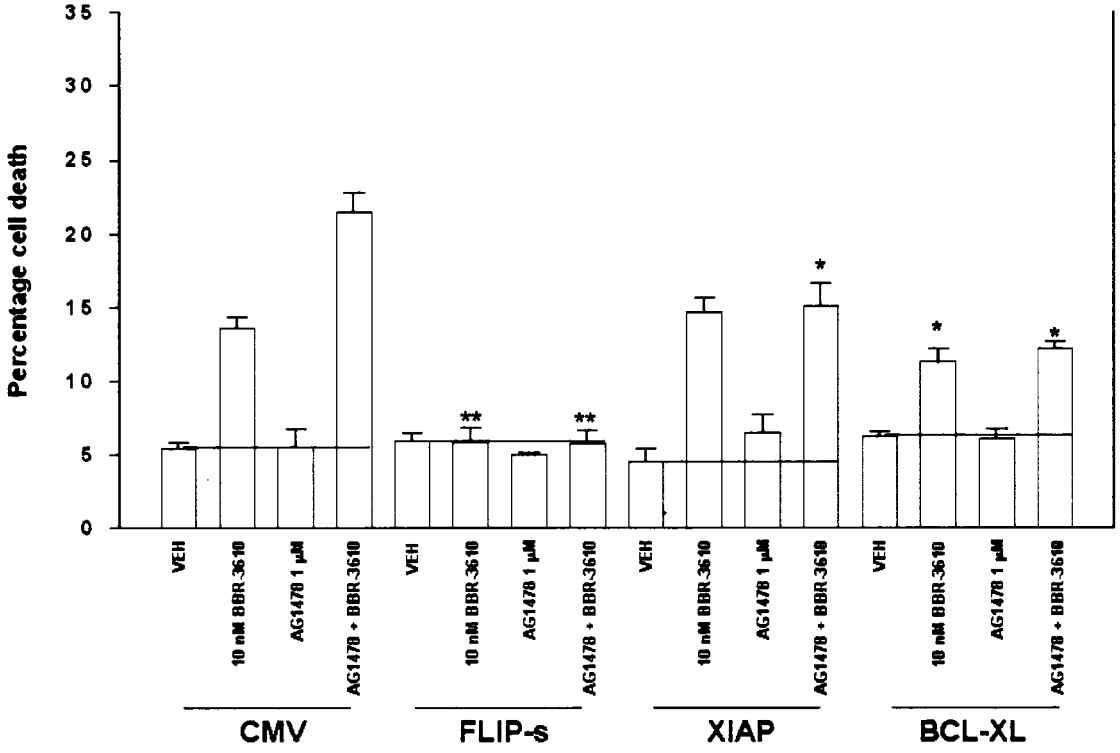

Figure 4, Panel C. DLD1 cells were plated in triplicate and 12h after plating infected with either a control empty vector virus (CMV), with a virus to express the mitochondrial protective protein BCLXL, with a virus to express the caspase 9 inhibitor XIAP or with a virus to express the caspase 8 inhibitor c-FLIP-s, as indicated in the Figure. Twenty four hours after infection cells were treated with the indicated concentrations of BBR3610 and AG1478. Cells were isolated 96h after drug exposure and trypan blue cell viability assays performed. Data are from a representative experiment (n = 2) of triplicate samples ± SEM. (* $p < 0.05$ less than corresponding treatment in CMV infected cells; ** $p < 0.05$ less than corresponding values in all other groups of virally infected cells).

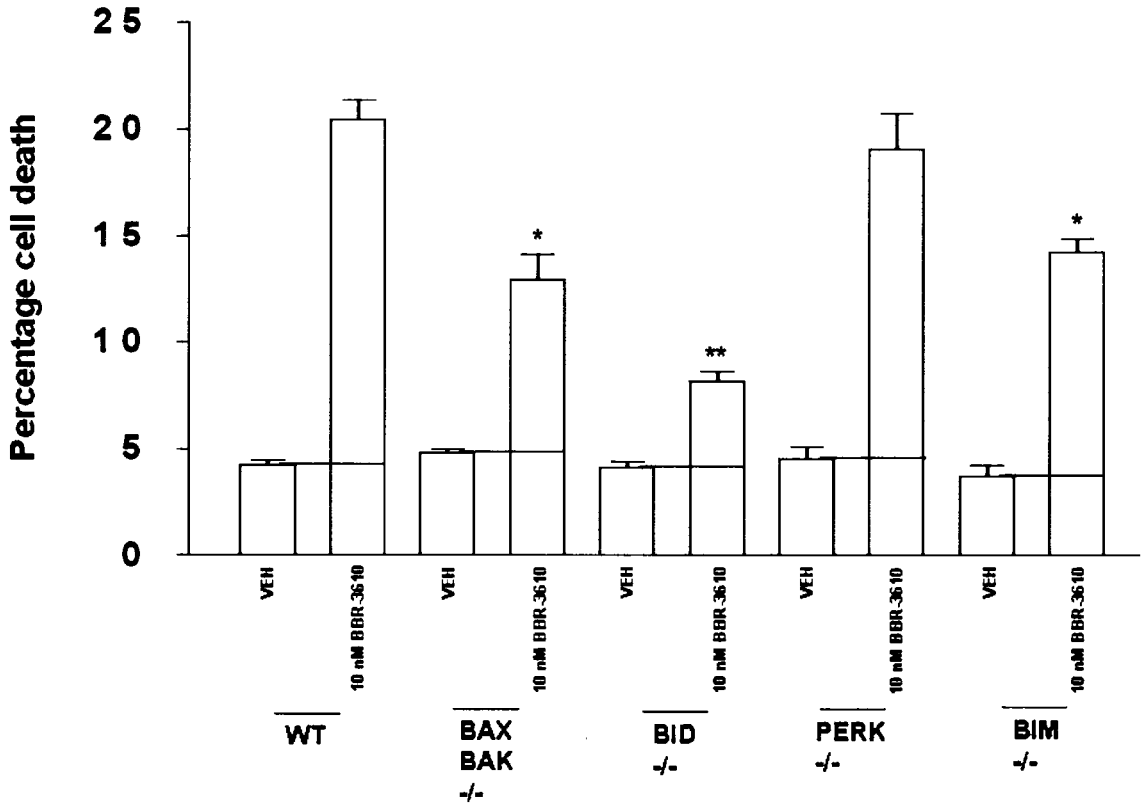

Figure 4, Panel D. SV40 transformed mouse embryonic fibroblasts (wild type, WT; lacking BAX and BAK, BAX/BAK -/-; lacking BIB, BID -/-; lacking PKR like endoplasmic reticulum kinase (PERK), PERK -/-; lacking BIM -/-), 24h after plating, were treated with BBR3610. Cells were isolated 96h after drug exposure and trypan blue cell viability assays performed. Data are from a representative experiment (n = 2) of triplicate samples ± SEM. (* $p < 0.05$ less than the value in WT cells; ** $p < 0.05$ less than the value in all other fibroblast cell types).

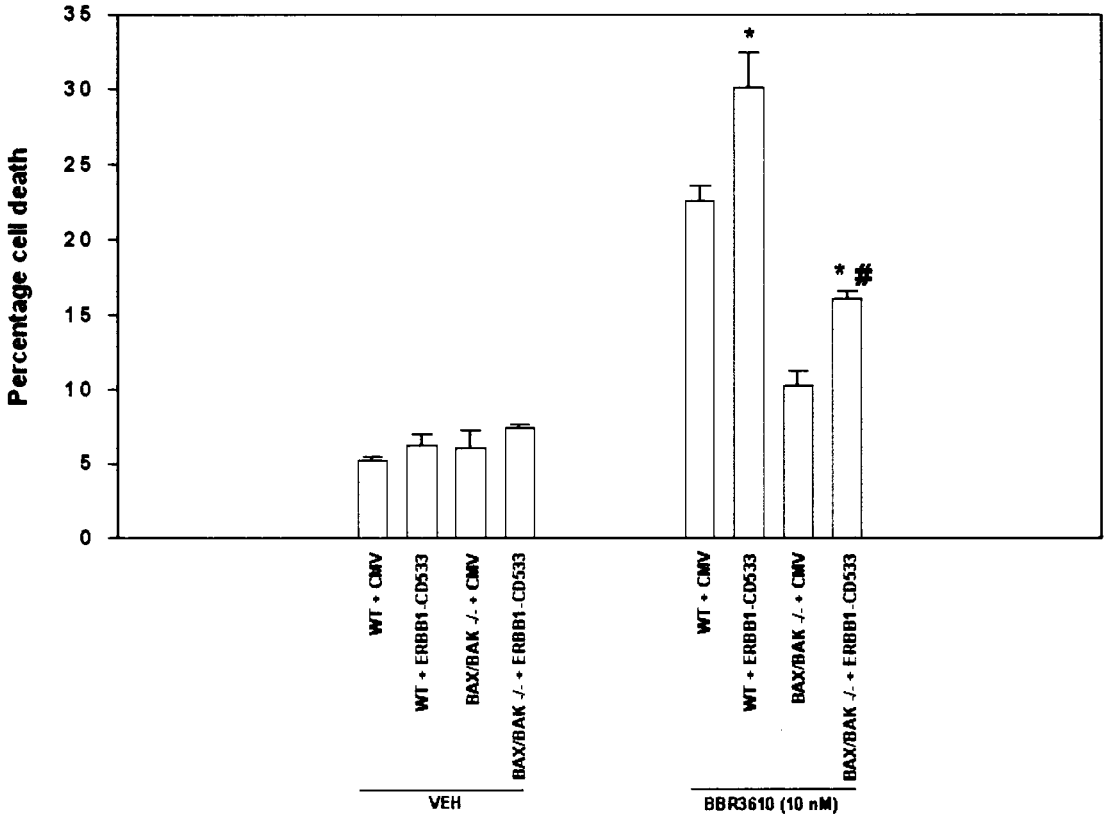

Figure 4, Panel E. Wild type and BAX/BAK -/- fibroblasts, 12h after plating were infected with either a control empty vector virus (CMV), or with a virus to express dominant negative ERBB1-CD533, as indicated in the Figure. Twenty four hours after infection cells were treated with the indicated concentrations of BBR3610 and AG1478. Cells were isolated 96h after drug exposure and trypan blue cell viability assays performed. Data are from a representative experiment (n = 2) of triplicate samples ± SEM.

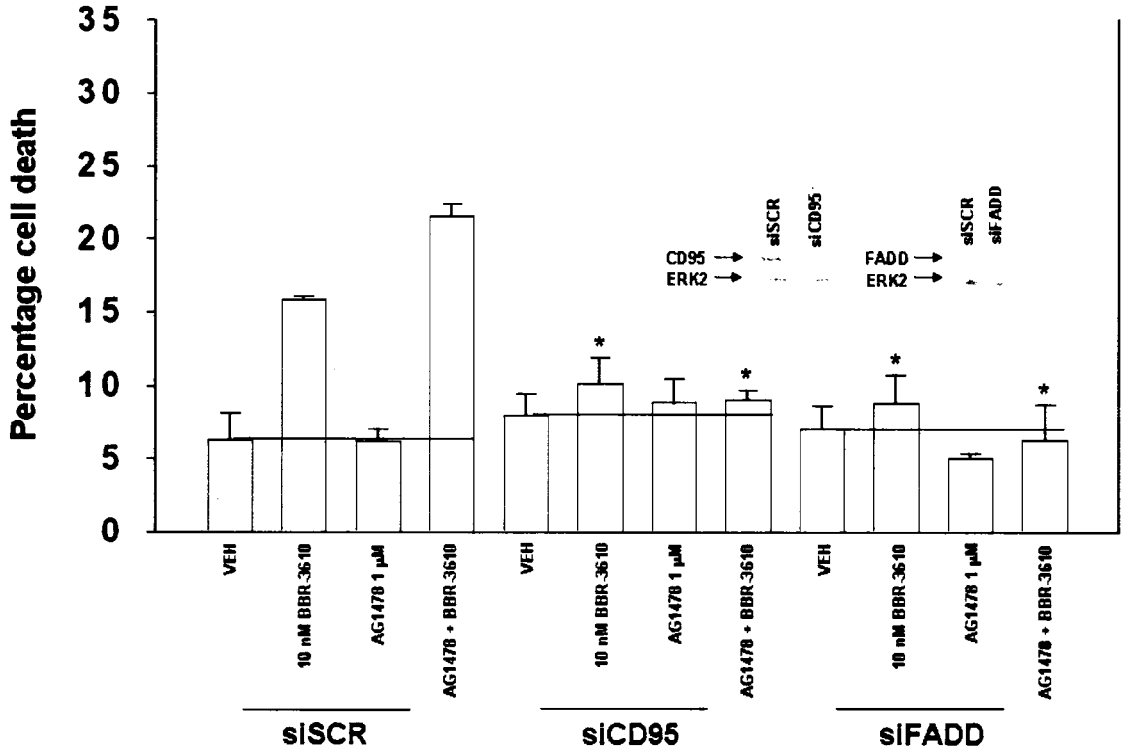

Figure 5. Knock down of CD95 or of FADD suppresses the lethality of BBR3610. DLD1 cells, 12h after plating were transfected with siRNA molecules to knock down the expression of FADD or of CD95 (FAS receptor), or with a scrambled siRNA (SCR) as described in the Methods section. Forty eight hours after transfection, cells were treated with BBR3610 and/or AG1478. Forty eight hours after drug treatment, cells were isolated and cell viability determined using trypan blue exclusion assays. Data are from a representative experiment (n = 2) of triplicate samples ± SEM. (* $p < 0.05$ less than the value in SCR transfected cells). Inset Panel: Forty eight hours after transfection, cells from each condition prior to drug treated were isolated and subjected to SDS PAGE followed by immunoblotting to determine the expression of CD95, FADD and ERK2.

new platinum containing therapeutic drugs that exhibit a greater lethality, and activity in tumors historically resistant to the established drugs such as cisplatin, is an important developmental goal for better tumor control and patient outcomes. In vitro the dinuclear platinum agent BBR3610 is a more toxic agent in multiple colon cancer cell lines compared to the trinuclear platinum agent BBR3464; a differential effect in cell killing that was not initially explained by either drug uptake or drug incorporation into DNA. Furthermore, we have reported previously that BBR3464 and BBR3610 are at least equipotent as anti-tumor agents in xenograft mouse studies, and more potent than cisplatin [32]. Future studies will be required to determine at the molecular level why BBR3463 and BBR3610 have differential in vitro toxicities. In the present studies we have defined how BBR3610 acted to kill colon cancer cells. BBR3610 suppressed AKT activity, that was further enhanced by inhibition of ERBB1, and which correlated with reduced expression of c-FLIP proteins and of BCL-XL. Expression of activated AKT maintained the expression of c-FLIP proteins and BCL-XL in cells treated with BBR3610 with or without ERBB1 inhibition. Expression of activated AKT suppressed BBR3610 lethality and the ability of ERBB1 inhibition to promote BBR3610 toxicity whereas expression of dominant negative AKT enhanced BBR3610 lethality to an extent that was not further enhanced by inhibition of ERBB1. BBR3610 lethality was dependent upon activation of the death receptor - extrinsic / caspase 8 apoptosis pathway as judged by the ability of CRM A, IETD and knock down of FADD and CD95 expression to significantly suppress all drug-induced lethality effects. BBR3610 lethality as a single agent was independent of p53 in HCT116 cells, in contrast to the findings of others using oxaliplatin [23]. The ability of ERBB1

inhibitors to promote BBR3610 lethality was reliant upon mitochondrial dysfunction / BAX-BAK / caspase 9 activation, and was also p53 independent, as judged by the suppression of this effect by expression of dominant negative caspase 9 or XIAP. Note that all cell killing effects were dependent upon prior Pt-agent induced activation of caspase 8, as judged by over-expression of the specific caspase 8 inhibitor c-FLIP-s abolishing drug-induced increases in cell death.

Previous studies using mononuclear platinum agents and also BBR3464, have correlated Pt-agent –induced ERK1/2 activation to a pro-apoptotic response, with many of these studies using the MEK1/2/5 inhibitors PD98059 and U0126 [10]. Using the more specific MEK1/2 inhibitor PD184352 and molecular approaches, we now show that small molecule inhibition of the RAF-ERK1/2 pathway at the time of Pt-agent exposure enhanced BBR3610 lethality in HCT116 cells in a p53-dependent fashion. Molecular inhibition of the RAF-ERK1/2 pathway modestly enhanced both BBR3610 and BBR3610 and ERBB1 inhibitor lethality, however unlike expression of dominant negative AKT, expression of dominant negative MEK1 did not abolish the ability of the ERBB1 inhibitor AG1478 to promote BBR3610 lethality. Collectively, our data argue that treatment of colon cancer cells with low doses of BBR3610 promotes inactivation of AKT that is further enhanced in these cells by inhibition of ERBB receptor function, which lead to suppressed expression of multiple anti-apoptotic proteins permitting activation of the intrinsic and extrinsic apoptosis pathways.

The molecular mechanisms by which BBR3610 stimulates both death receptor signaling as well as inhibition of AKT activity remain to be determined. One potential

mechanism by which BBR3610 could cause cell killing, which would correlate with death receptors and with dephosphorylation of AKT, could be increased levels of the pro-apoptotic lipid ceramide. Ceramide has been linked to stimulating both ligand independent clustering of death receptors and to activation of PP2A isoforms [21,33]. Cisplatin has been shown to increase ceramide levels in tumor cells and further studies will be required to determine whether BBR3610 significantly alters ceramide levels in colon cancer cells. As knock-down of CD95 expression abolished BBR3610-induced cell killing our findings indicate that BBR3610-induced changes in CD95 function are likely to be a primary effector in the chain of pro-apoptotic events that occur via two pathways: CD95 – FADD – caspase 8 – caspase 3 and caspase 8 -BID – mitochondrial dysfunction – caspase 9 – caspase 3.

Prior studies using oxaliplatin have argued that treatment of colon cancer cells with Tumor necrosis factor-related ligand (TRAIL) or knock down of FLIP protein expression can enhance the toxicity of the Pt agent [14,25]. Of particular note, whereas over-expression of c-FLIP-s protected HCT116 cells from BBR3610 toxicity, over-expression of c-FLIP-l but not c-FLIP-s protected cells from oxaliplatin lethality [14]. Griffiths et al. argued in colon cancer cells that Src-induced expression of BCL-XL and –suppression of CD95 function blunted oxaliplatin lethality [26]. Our data argued that BBR3610 as a single agent can suppress MCL-1 levels as well as those of FLIP proteins. Collectively, our findings together with those of other groups argue that BBR3610 lethality has the potential to be enhanced not only by inhibitors of ERBB1-PI3K signaling but also by inhibitors of Src

kinases e.g. BMS354825 (dasatinib) or additional mitochondrial protective proteins such as BCL-2 and BCL-XL e.g. ABT-737 [34,35].

Inhibition of ERBB1 enhanced BBR3610 toxicity that was caspase 9-dependent, an effect that was also reliant upon the initial BBR3610 –dependent activation of caspase 8. Neither BBR3610 nor inhibition of ERBB1 enhanced JNK1/2 or p38 MAPK activity in total cell lysates but inhibition of either pathway suppressed the ability of ERBB1 inhibition to promote mitochondrial dysfunction and cell killing. A variety of studies have argued that mitochondrial toxic agents can promote activation of BAX and BAK via JNK1/2 and p38 MAPK signaling and loss of BAX/BAK function suppressed BBR3610 toxicity and the ability of ERBB1 inhibition to promote BBR3610 toxicity [29]. Additional studies beyond the scope of this manuscript will be required to determine whether selected pools of JNK1/2 and p38 MAPK become activated following BBR3610 and AG1478 drug treatment, and play a role in activating BAX and BAK. It will be of interest in future studies to determine whether clinically relevant proprietary inhibitors of ERBB receptor signaling such as cetuximab and Lapatinib can also promote BBR3610 lethality in a similar manner to AG1478 and ERBB1CD533.

Understanding the mechanisms by which a particular agent causes cell killing permits the subsequent rational combination of that agent with other drugs that activate complementary death –inducing processes. With the advent of targeted drugs to the clinic, understanding how they can best be combined with clinically useful cytotoxics is an important developmental goal. The present studies have demonstrated that BBR3610 kills colon tumor cells by activating the extrinsic and, to a lesser extent, intrinsic pathways, and

that inhibition of ERBB1 facilitates killing by permitting additional mitochondrial dysfunction to take place. Based on our data, it could be hypothesized that BBR3610 toxicity could also be enhanced by: (a) therapeutic agents which cause further activation of the extrinsic pathway e.g. TRAIL; (b) therapeutic agents which cause further DNA damage and mitochondrial dysfunction e.g. ionizing radiation; (c) therapeutic agents which suppress expression of multiple cytoprotective proteins e.g. flavopiridol. Further studies will be required to determine whether TRAIL, CDK9 inhibitors or ionizing radiation can further enhance the lethal actions of (BBR3610 + ERBB1 inhibitor) treatment in human colon cancer cells.

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List of References

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VITA

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