available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Differences in the cellular response and signaling pathways of cisplatin and BBR3464 ([{*trans*-$PtCl(NH_3)_2$}$_2$μ-(*trans*-$Pt(NH_3)_2(H_2N(CH_2)_6$-$NH_2)_2$)]$^{4+}$) influenced by copper homeostasis

Peyman Kabolizadeh [a], *John Ryan* [b], *Nicholas Farrell* [a,*]

[a] *Department of Chemistry, Virginia Commonwealth University, 1001 W. Main Street, Richmond, VA 23284, United States*
[b] *Department of Biology, Virginia Commonwealth University, Richmond, VA, United States*

ARTICLE INFO

Article history:
Received 8 September 2006
Accepted 12 December 2006

Keywords:
Platinum
Copper
hCTR1
ATP7B
Chemotherapy
Apoptosis

ABSTRACT

[{*trans*-$PtCl(NH_3)_2$}$_2$μ-(*trans*-$Pt(NH_3)_2(H_2N(CH_2)_6$-$NH_2)_2$)]$^{4+}$ (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.

1. 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 [5]. BBR3464 is significantly more cytotoxic than c-DDP and retains

* *Corresponding author*. Tel.: +1 804 828 6320; fax: +1 804 828 8599.
E-mail address: npfarrell@vcu.edu (N. Farrell).
0006-2952/$ – see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.bcp.2006.12.016

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 transportation [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. Materials and methods

2.1. Compound synthesis

Drug compounds were synthesized according to methods reported previously [26], Fig. 1. Copper was obtained in the form of Copper(II) sulfate pentahydrate from Sigma–Aldrich (St. Louis, MO).

2.2. 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% CO_2. HN22 and MDA-MB-435 were obtained from D. Lebman and S. Deb, respectively (VCU, Richmond, VA).

2.3. Growth Inhibition assay (detection of cell proliferation)

Cells were cultured at 10,000 cells per well in a 96-well microplate. They were incubated with different concentration of c-DDP and BBR3464 for 72 h. 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 100 μl of MTT solution (2 mg/1 ml PBS) for 3 h. MTT solution was aspirated and 100 μl of DMSO (Sigma chemicals) was added to each well for determination of viable cell number through measuring the absorbance at 490 nm.

2.4. 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.

2.5. 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.

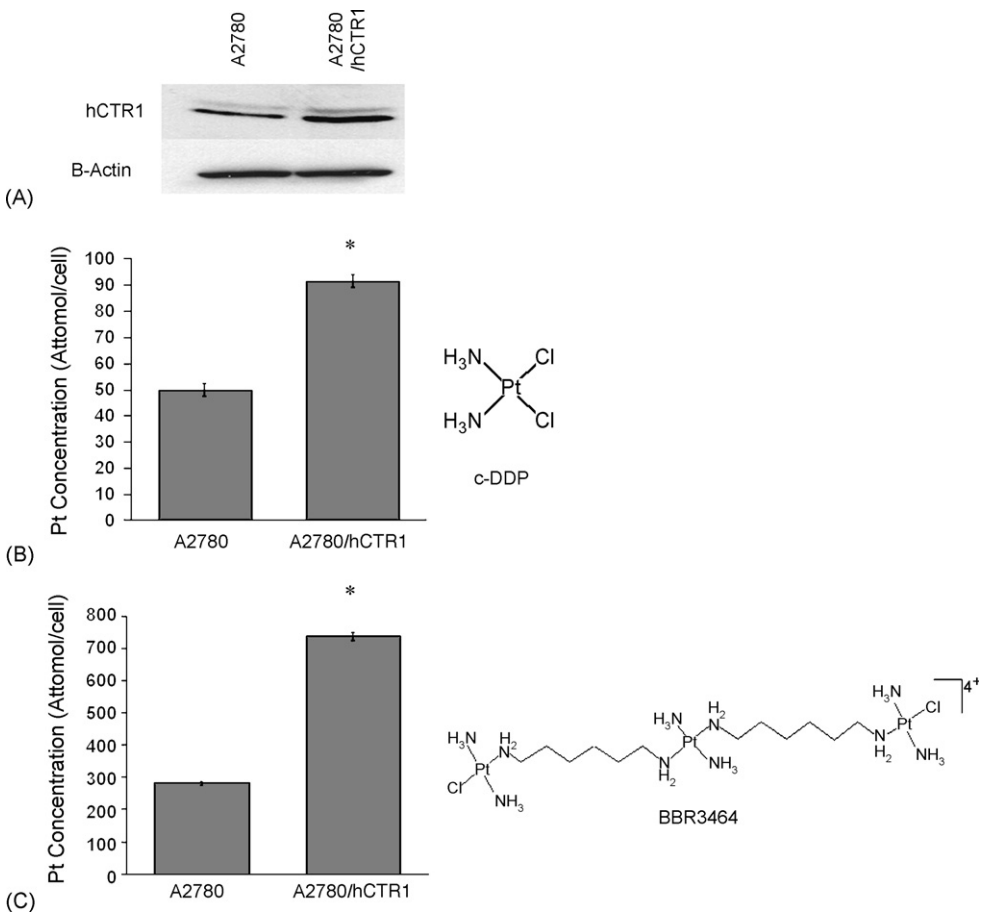

Fig. 1 – The importance of hCTR1 in platinum drug uptake in ovarian carcinoma cells. (A) The expression of hCTR1 in A2780 and A2780/hCTR1 was detected by Western blot. The membrane was stripped and re-probed for β-actin to show loading. (B) c-DDP uptake was measured by ICP in 20 million cells treated with 10 μM c-DDP for 16 h before harvesting. (C) BBR3464 uptake in A2780 and A2780/hCTR1 cells treated with 10 μM BBR3464. Each bar indicates the average (±S.E.M.) of three independent experiments. $^{*}p < 0.05$. The structure of c-DDP and BBR3464 are illustrated in B and C.

2.6. *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.

2.7. *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 min after the addition of copper (II) sulfate. After 8 or 16 h 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.

2.8. *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).

2.9. 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.

3. Results

3.1. Importance of copper and copper transporters in c-DDP and BBR3464 uptake and cytotoxicity

3.1.1. 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. 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. 1B 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 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 $IC_{50} = 3$ μM, BBR3464 $IC_{25} \leq 0.5$ μM) than parental A2780 cells (c-DDP $IC_{50} = 6$ μM, BBR3464 $IC_{25} = 10$ μM) (Fig. 2). Additionally, there was two- to three-fold more G_2 arrest in A2780/hCTR1 cells treated with low concentrations of BBR3464 (0.5 μM) than in parental A2780 cells (Fig. 2B). 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.

3.1.2. 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 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. 3A, ATP7B expression is two-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. 3B and 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. 3C and E).

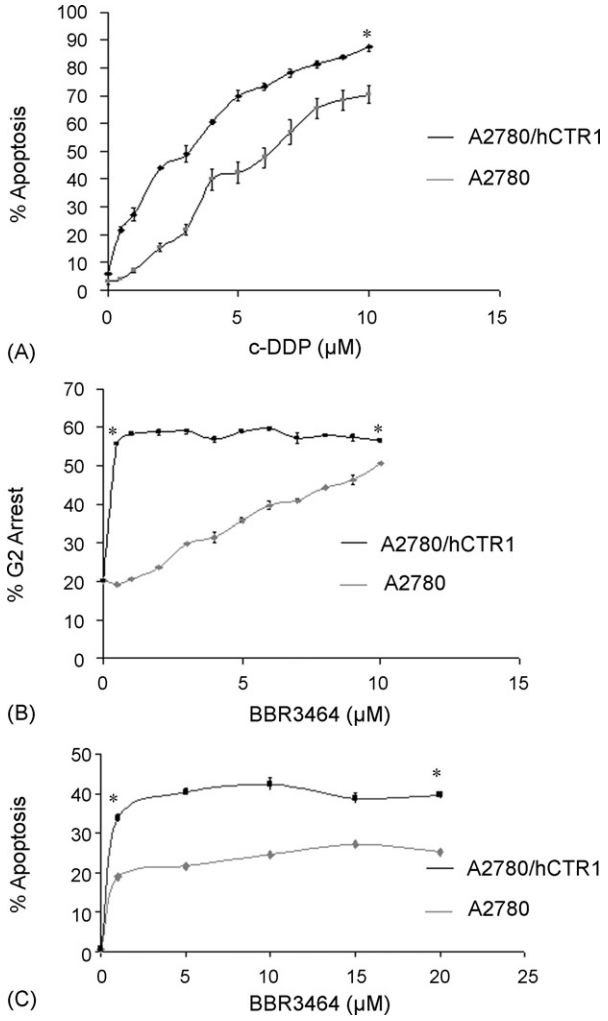

Fig. 2 – The effects of hCTR1 expression on c-DDP- and BBR3464-induced apoptosis and cell cycle arrest. In (A) and (B) A2780 and A2780/hCTR1 cells were cultured in the indicated concentrations of c-DDP and BBR3464 for 72 h. DNA content was measured by PI-DNA staining to determine apoptosis (sub G_0/G_1) and G2 arrest by flow cytometry, as described in Section 2. (C) A2780 and A2780/hCTR1 cells were cultured in the presence of BBR3464 for 96 h and sub-diploid DNA content was determined as an indicator of apoptosis. Each point is the average (±S.E.M.) of three independent experiments. In each figure, A2780 and A2780/hCTR1 responses were found to be significantly different by Student's *t*-test; $^{*}P < 0.05$.

3.1.3. 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

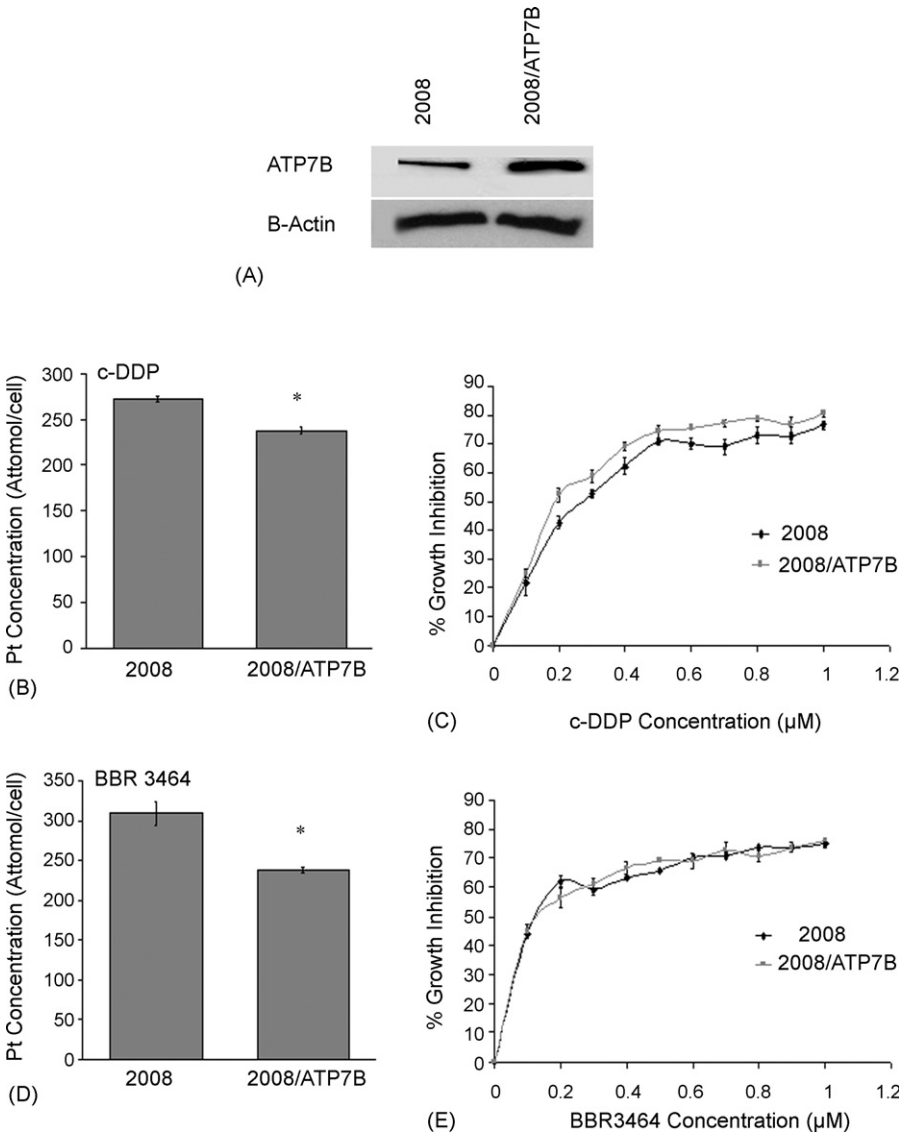

Fig. 3 – Effect of ATP7B transporter on platinum drug retention and Pt-mediated growth inhibition. (A) The expression of ATP7B in 2008 ovarian cells was detected by Western blot. The membrane was stripped and re-probed for β-actin. (B and D) Cellular platinum levels after treatment with 20 μM c-DDP or 10 μM BBR3464 for 24 h were determined by ICP. Each bar indicates the average (±S.E.M.) 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 72 h, as measured by PI-DNA staining and timed counting via flow cytometry. Each point is the average (±S.E.M.) of three independent experiments. *$p < 0.05$ as determined by t-test.

copper prior to the addition of platinum drugs (Fig. 4). Copper (as Cu^{2+}) enhanced the uptake of both c-DDP and BBR3464 in the parental ovarian carcinoma cell line (A2780) (Fig. 4A and 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. 4B and 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 16 and 8 h, where uptake of c-DDP and BBR3464 were measured, respectively. At later time points (24 h), 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. 5A and 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. 5A and 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

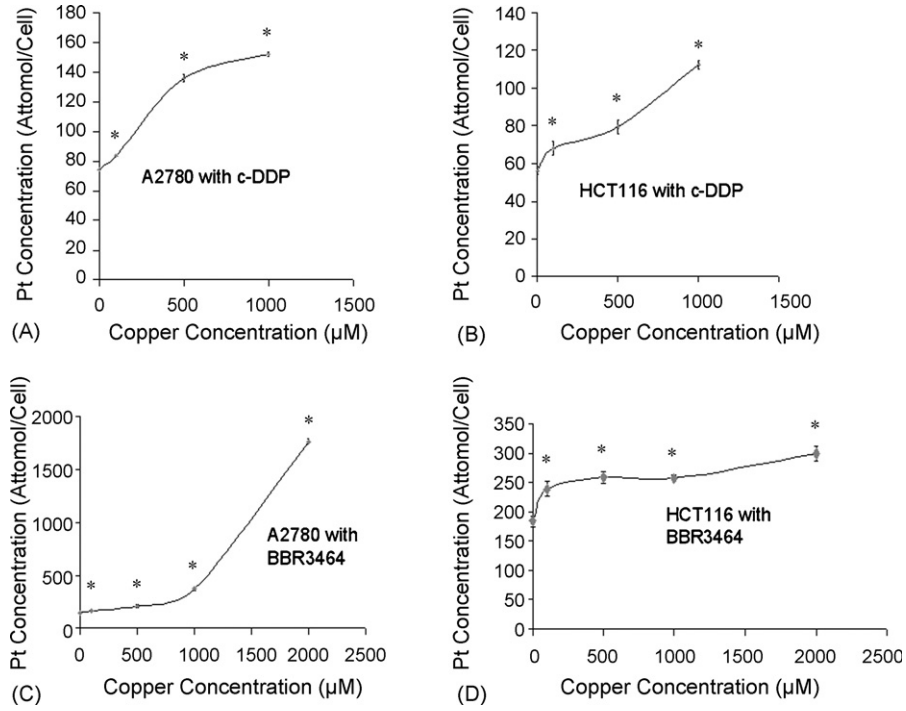

Fig. 4 – Effect of copper on c-DDP and BBR3464 uptake in A2780 and HCT116 carcinoma cell lines. In (A) and (B), A2780 or HCT116 cells were cultured with 10 or 20 μM c-DDP, respectively, for 16 h in the absence or presence of copper. In (C) and (D), A2780 or HCT116 cells were treated with 10 or 20 μM BBR3464, respectively, for 8 h. Drugs were added to the media after 1 h 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 (±S.E.M.) of three independent experiments. $^{*}p < 0.05$ when comparing cells treated with and without copper, by Student's *t*-test.

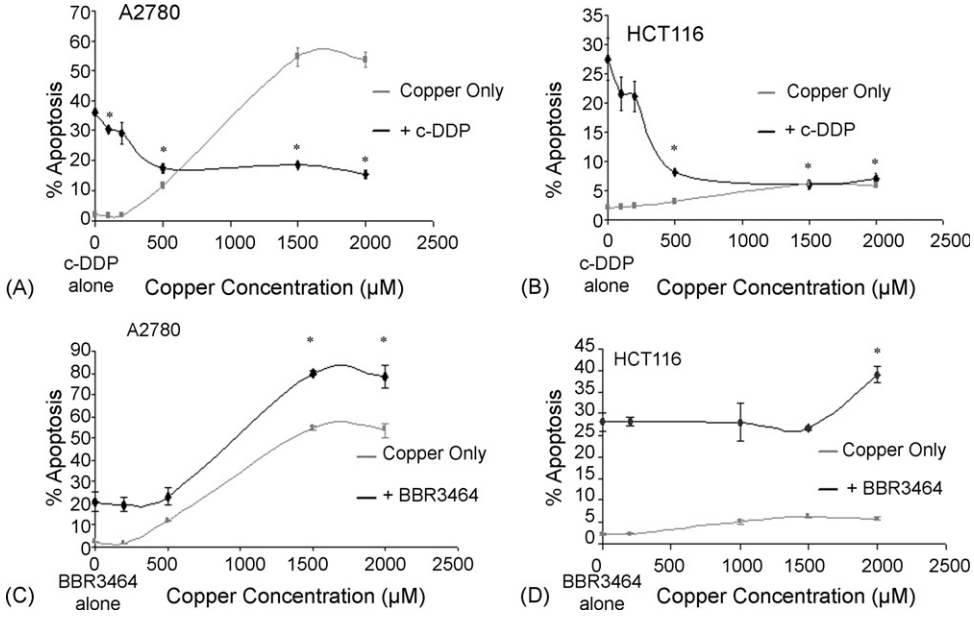

Fig. 5 – Effect of copper on c-DDP- and BBR3464-induced apoptosis in A2780 and HCT116 carcinoma cell lines. Sub-diploid cell content was detected by PI-DNA staining. In (A) and (B), A2780 or HCT116 cells were cultured with 20 or 40 μM c-DDP, respectively, for 24 h in the absence or presence of copper. In (C) and (D), A2780 or HCT116 cells were treated with 40 or 50 μM BBR3464, respectively, for 24 h. Drugs were added to the media after 1 h of treatment with copper. Each point represents the average (±S.E.M.) of three independent experiments. $^{*}p < 0.05$ when comparing cells treated with and without copper, by Student's *t*-test.

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. 5C and 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. S1).

3.2. *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 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 Fig. 6A, 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 three separate experiments. In contrast, BBR3464-induced p53 expression was unaltered by prior copper treatment (Fig. 6A).

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. 6B). On the other hand, BBR3464-induced caspase-3 activation was increased 37% by the addition of copper (Fig. 6B). 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. 6C). 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.

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 Fig. 7A, 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. 7B). Caspase activation was increased for both Pt compounds by copper.

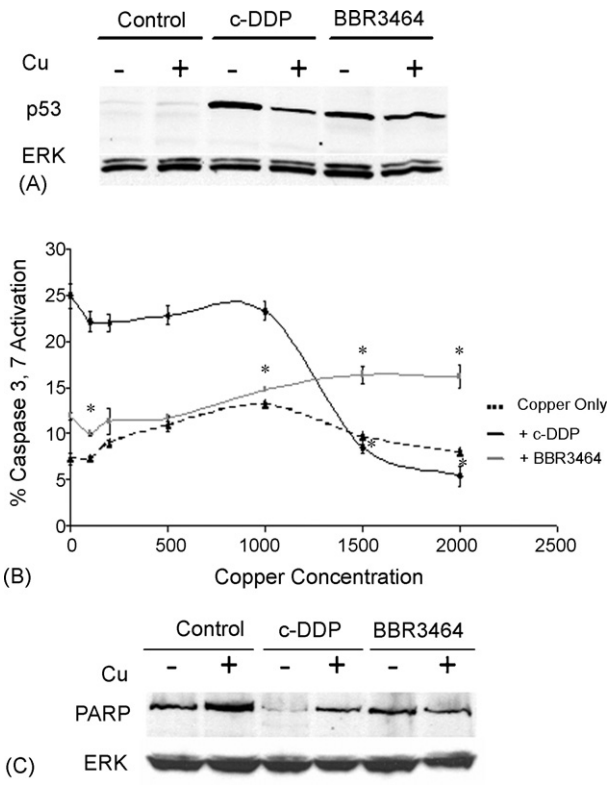

Fig. 6 – Effect of copper on downstream signaling pathways activated by c-DDP and BBR3464. (A) Effect of copper on c-DDP- and BBR3464-induced p53 upregulation. HCT116 cells were cultured with 40 μM c-DDP or 50 μM BBR3464 in the absence and presence of different concentrations of copper for 24 h. The 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 40 μM c-DDP or 50 μM BBR3464 in the absence or presence of copper for 24 h. Cells were stained for active caspase-3/7 activation as described in Section 2. Data shown is the percent of the population displaying active caspase-3/7. Each point represents the average (±S.E.M.) 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 40 μM c-DDP or 50 μM BBR3464 in the absence or presence of copper for 24 h. The expression of uncleaved PARP was detected by Western blotting. The same membrane was stripped and re-probed for ERK to show protein loading.

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. 7C). 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

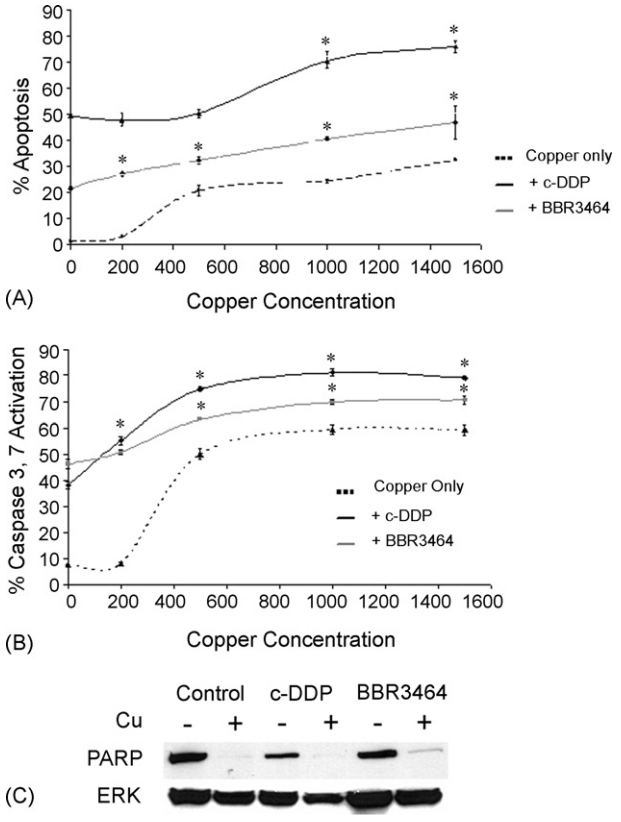

Fig. 7 – Effect of copper on c-DDP and BBR3464 cytotoxicity in HCT116−/− cells. (A) The effect of copper on c-DDP- and BBR3464-induced apoptosis in HCT116−/− carcinoma cell lines. Sub-diploid cell content was detected by PI-DNA staining. HCT116−/− cells were cultured with 40 μM c-DDP or 50 μM BR3464 for 72 h in the absence or presence of copper. Drugs were added to the media after 1 h of treatment with copper. Each point represents the average (±S.E.M.) 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 40 μM c-DDP or 50 μM BBR3464 in the absence or presence of copper for 72 h. Cells were stained for active caspase-3/7 activation as described in Section 2. Data shown are the percent of the population displaying active caspase-3/7. Each point represents the average (±S.E.M.) 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 40 μM c-DDP or 50 μM BBR3464 in the absence or presence of copper for 72 h. The expression of uncleaved PARP was detected by Western blotting. The same membrane was stripped and re-probed for ERK to show protein loading.

p53 activation is central to Pt-mediated cell death, including how this process is altered by copper.

4. 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. S2). 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. S2). 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 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 (Figs. 4 and 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-h 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 (1 mM) [32]. We observed the same result in ovarian carcinoma cells (A2780) by adding 1 mM c-DDP with copper (unpublished data). High concentrations of c-DDP (1 mM) 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 66 μM (20 μg/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. 6). 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. 7). 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 induced 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 HCT166 cells. In the absence of p53, both Pt compounds induced death, albeit with a slower time course (72 h versus 24 h). 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.

Acknowledgments

We thank Dr. Stephen B. Howell for providing the A2780/hCTR1, 2008/EV pRC/CMV, and 2008/pRC/CMV7B cell lines. We thank Dr. S. Deb for providing breast carcinoma cell line (MDAMB-435). We thank Dr. D. Lebman for providing head and neck carcinoma cell line (HN22). This work was supported by a grant from the National Institutes of Health (R01-CA78754).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2006.12.016.

REFERENCES

[1] Loehrer PJ, Einhorn LH. Drugs five years later. Cisplatin. Ann Intern Med 1984;100(5):704–13.
[2] Jamieson ER, Lippard SJ. Structure, recognition, and processing of cisplatin-DNA adducts. Chem Rev 1999;99(9):2467–98.
[3] Wong E, Giandomenico CM. Current status of platinum-based antitumor drugs. Chem Rev 1999;99(9):2451–66.
[4] Safirstein R, Winston J, Goldstein M, Moel D, Dikman S, Guttenplan J. Cisplatin nephrotoxicity. Am J Kidney Dis 1986;8(5):356–67.
[5] Farrell N. Polynuclear platinum drugs. Met Ions Biol Syst 2004;42:251–96.
[6] Pratesi G, Perego P, Polizzi D, Righetti SC, Supino R, Caserini C, et al. A novel charged trinuclear platinum complex effective against cisplatin-resistant tumours: hypersensitivity of p53-mutant human tumour xenografts. Br J Cancer 1999;80(12):1912–9.
[7] Riccardi A, Meco D, Ferlini C, Servidei T, Carelli G, Segni G, et al. In vitro and in vivo antitumor activity of the novel trinuclear platinum complex BBR 3464 in neuroblastoma. Cancer Chemother Pharmacol 2001;47(6):498–504.
[8] Farrell NP. Preclinical perspectives on the use of platinum compounds in cancer chemotherapy. Semin Oncol 2004;31(6 Suppl 14):1–9.
[9] Harris AL, Ryan JJ, Farrell N. Biological consequences of trinuclear platinum complexes: comparison of [[*trans*-$PtCl(NH_3)_2]_2\mu$-(*trans*-$Pt(NH_3)_2(H_2N(CH_2)_6$-$NH_2)_2$)]$^{4+}$ (BBR 3464) with its noncovalent congeners. Mol Pharmacol 2006;69(2):666–72.
[10] Liu Q, Qu Y, Van Antwerpen R, Farrell N. Mechanism of the membrane interaction of polynuclear platinum anticancer agents. Implications for cellular uptake. Biochemistry 2006;45(13):4248–56.
[11] Linder MC, Hazegh-Azam M. Copper biochemistry and molecular biology. Am J Clin Nutr 1996;63(5):797S–811S.
[12] Rae TD, Schmidt PJ, Pufahl RA, Culotta VC, O'Halloran TV. Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. Science 1999;284(5415):805–8.
[13] Strausak D, Howie MK, Firth SD, Schlicksupp A, Pipkorn R, Multhaup G, et al. Kinetic analysis of the interaction of the copper chaperone Atox1 with the metal binding sites of the Menkes protein. J Biol Chem 2003;278(23):20821–7.

[14] Zhou B, Gitschier J. hCTR1: a human gene for copper uptake identified by complementation in yeast. Proc Natl Acad Sci USA 1997;94(14):7481–6.
[15] Moller LB, Petersen C, Lund C, Horn N. Characterization of the hCTR1 gene: genomic organization, functional expression, and identification of a highly homologous processed gene. Gene 2000;257(1):13–22.
[16] Tanzi RE, Petrukhin K, Chernov I, Pellequer JL, Wasco W, Ross B, et al. The Wilson disease gene is a copper transporting ATPase with homology to the Menkes disease gene. Nat Genet 1993;5(4):344–50.
[17] Vulpe C, Levinson B, Whitney S, Packman S, Gitschier J. Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase. Nat Genet 1993;3(1):7–13.
[18] Lee J, Pena MM, Nose Y, Thiele DJ. Biochemical characterization of the human copper transporter Ctr1. J Biol Chem 2002;277(6):4380–7.
[19] Safaei R, Howell SB. Copper transporters regulate the cellular pharmacology and sensitivity to Pt drugs. Crit Rev Oncol Hematol 2005;53(1):13–23.
[20] Andrews PA, Jones JA, Varki NM, Howell SB. Rapid emergence of acquired cis-diamminedichloroplatinum(II) resistance in an in vivo model of human ovarian carcinoma. Cancer Commun 1990;2(2):93–100.
[21] Gately DP, Howell SB. Cellular accumulation of the anticancer agent cisplatin: a review. Br J Cancer 1993;67(6):1171–6.
[22] Katano K, Kondo A, Safaei R, Holzer A, Samimi G, Mishima M, et al. Acquisition of resistance to cisplatin is accompanied by changes in the cellular pharmacology of copper. Cancer Res 2002;62(22):6559–65.
[23] Safaei R, Katano K, Samimi G, Naerdemann W, Stevenson JL, Rochdi M, et al. Cross-resistance to cisplatin in cells with acquired resistance to copper. Cancer Chemother Pharmacol 2004;53(3):239–46.
[24] Holzer AK, Samimi G, Katano K, Naerdemann W, Lin X, Safaei R, et al. The copper influx transporter human copper transport protein 1 regulates the uptake of cisplatin in human ovarian carcinoma cells. Mol Pharmacol 2004;66(4):817–23.
[25] Katano K, Safaei R, Samimi G, Holzer A, Rochdi M, Howell SB. The copper export pump ATP7B modulates the cellular pharmacology of carboplatin in ovarian carcinoma cells. Mol Pharmacol 2003;64(2):466–73.
[26] Qu Y, Harris A, Hegmans A, Petz A, Kabolizadeh P, Penazova H, et al. Synthesis and DNA conformational changes of non-covalent polynuclear platinum complexes. J Inorg Biochem 2004;98(10):1591–8.
[27] Yeatman 2nd CF, Jacobs-Helber SM, Mirmonsef P, Gillespie SR, Bouton LA, Collins HA, et al. Combined stimulation with the T helper cell type 2 cytokines interleukin (IL)-4 and IL-10 induces mouse mast cell apoptosis. J Exp Med 2000;192(8):1093–103.
[28] Billecke C, Finniss S, Tahash L, Miller C, Mikkelsen T, Farrell NP, et al. Polynuclear platinum anticancer drugs are more potent than cisplatin and induce cell cycle arrest in glioma. Neuro-oncology 2006;8(3):215–26.
[29] Perego P, Gatti L, Righetti SC, Beretta GL, Carenini N, Corna E, et al. Development of resistance to a trinuclear platinum complex in ovarian carcinoma cells. Int J Cancer 2003;105(5):617–24.
[30] Haupt S, Berger M, Goldberg Z, Haupt Y. Apoptosis—the p53 network. J Cell Sci 2003;116(Pt 20):4077–85.
[31] Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene 2003;22(47):7265–79.
[32] Ishida S, Lee J, Thiele DJ, Herskowitz I. Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals. Proc Natl Acad Sci USA 2002;99(22):14298–302.
[33] Jordan P, Carmo-Fonseca M. Cisplatin inhibits synthesis of ribosomal RNA in vivo. Nucl Acids Res 1998;26(12):2831–6.
[34] Kapp T, Muller S, Gust R. Dinuclear alkylamine platinum(II) complexes of [1,2-Bis(4-fluorophenyl)ethylenediamine]platinum(II): influence of endocytosis and copper and organic cation transport systems on cellular uptake. Chem Med Chem 2006;1(5):560–4.
[35] Zhang CX, Chang PV, Lippard SJ. Identification of nuclear proteins that interact with platinum-modified DNA by photoaffinity labeling. J Am Chem Soc 2004;126(21):6536–7.