Arch Pharm Res Vol 29, No 12, 1067-1073, 2006

Archives of Pharmacal Research
http://apr.psk.or.kr

Platinum Transporters and Drug Resistance

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(Received October 26, 2006)

Cisplatin, a platinum coordinated complex, is a widely used antineoplastic agent for the treatment of metastatic tumors of the testis, metastatic ovarian tumors, lung cancer, advanced bladder cancer and many other solid tumors. The cytotoxic action of the drug is often thought to be associated with its ability to bind DNA to form cisplatin-DNA adducts. The development of resistance to cisplatin during treatment is common and constitutes a major obstacle to the cure of sensitive tumors. Although to understand the clinically relevant mechanisms of resistance, many studies have been aimed at clarifying the biochemical/molecular alterations of cisplatin-resistance cells, these studies did not conclusively identify the basis of cellular resistance to cisplatin. In this review, cisplatin resistance was discussed in terms of the relevant transporters, such as copper transporters (CTRs), organic cation transporters (OCTs) and multi-drug resistance related transporters (MDRs). These transporters seem to be contributed to cisplatin resistance through the reduction of drug accumulation in the cell. Better understanding the mechanism of cisplatin resistance associated with transporters will provide the useful informations for overcoming the cisplatin resistance.

Key words: Cisplatin, Resistance, Transporters, CTRs, OCTs, MDRs

INTRODUCTION

Cisplatin has been recognized as an important antitumor agent since its introduction into clinical trials (Prestayko *et al.*, 1979). It has been effective against several types of human malignancies including testicular, ovarian, cervical, bladder, head and neck, and small cell lung cancers. Particularly, about 80% of all patients with metastatic germ cell cancer will be cured after cisplatin-based combination chemotherapy (Kollmannsberger *et al.*, 2006). However, many patients eventually relapse and develop resistance, representing a major limitation of cisplatin-based chemotherapy (Giaccone, 2000).

Two major strategies have been adopted to improve the efficacies of cisplatin-based chemotherapies: One is the development of platinum analogues with better therapeutic indices. Of these, carboplatin exhibits comparable efficacy and more favorable toxicity profiles than the first generation cisplatin (Lokich and Anderson, 1998). Third generation (e.g., Eloxatine®) has been developed with the desire of finding new structures that show broad spectrum of antitumor activity, lack of cross-resistance with cisplatin and carboplatin, and reduced toxicity (Weiss and Christian, 1993; Raymond *et al.*, 2002). These developments have made platinum-based chemotherapy one of the most important treatments for human cancers.

The other strategy is through better understanding the resistance mechanisms of cisplatin so that methods for circumventing drug resistance can be developed. Inside the cell, cisplatin forms intrastrand crosslink adducts on DNA, interferes with DNA synthesis and activates cell death pathways (Siddik, 2002, 2003). Thus, reduction in adduct formation (Fink *et al.*, 1998) and enhanced repair of adducts (Johnson *et al.*, 1997) have been suggested as important mechanisms of resistance. Another important issue relevant to the toxicity and resistance of cisplatin is transport system of cisplatin. In fact, defective uptake of cisplatin has been one of the most consistently identified characteristics of cells selected for cisplatin resistance both *in vivo* and *in vitro* (Anderson and Howell, 1990; Gately and Howell, 1993). Moreover, many reports point to reduced drug accumulation as a significant mechanism of cisplatin resistance (Ishida *et al.*, 2002; Katano *et al.*, 2002). The intracellular concentration of cisplatin is a balance of uptake process into the cell and disposition process from the cell. Therefore, copper transporters are known to play an important role in the homeostasis of

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cisplatin, and also thought to be key transporters in cisplatin resistance.

CISPLATIN TRANSPORTERS

Copper transporters

Homeostasis of the essential metal copper is maintained by complicated mechanisms and involved in many kinds of proteins such as copper transporter 1 (Ctr1), metallochaperones, and P-type ATPase transporters (e.g., ATP7A and ATP7B). These proteins have a conserved characteristic that contains unique cysteine, methionine or histidine-rich domains referred to metal binding sequence (MBS; GMXCXXCC). MBS binds to Cu(I) in a protective pocket and hand it to the next protein through an intimate protein-protein interaction such that copper is virtually never free in the cell (Safaei and Howell, 2005).

Ctr1 transporter

Copper entry into cells is mainly controlled by copper transporter 1 (Ctr1) and to a lesser extent, by divalent metal transporter (DMT1) and divalent cation transporter (DCT1) (Puig and Thiele, 2002). The essential role of these Cu transporters have been established by the observations that ctr1-knock out animals are embryonic lethal (Lee *et al.*, 2001; Kuo *et al.*, 2001).

Deletion of the yeast Ctr1 (yCtr1) and mouse Ctr1 (mCtr1) gene resulted in increased cisplatin resistance and reduced accumulation of several platinum analogs including cisplatin, carboplatin, oxaliplatin, and ZD0473 (Ishida *et al.*, 2002; Lin *et al.*, 2002), suggesting the importance of Ctr1 in cisplatin resistance. Moreover, cells selected from resistance to cisplatin are cross-resistant to Cu and vice versa (Katano *et al.*, 2002). Song *et al.* (2004) reported that decreased mRNA expression of human Ctr1 (hCtr1) and decreased uptake rate as well as increased IC_{50} values of cisplatin, carboplatin, and oxaliplatin in cisplatin resistant small cell lung cancer cell line (SR2) compared to cisplatin sensitive cell line (SCLC). The SR2 cell line was sensitized by the overexpression of hCtr1 transporter, suggesting the involvement of hCtr1 transporter in cisplatin resistance. However, modulation of cisplatin resistance by hCtr1 expression was not observed in other cell types (Beretta *et al.*, 2004; Song *et al.*, 2004; Helleman *et al.*, 2006). For example, mRNA level of hCtr1 in A2780-Pt cisplatin resistant ovarian carcinoma cells was not increased compared to parent cells (Helleman *et al.*, 2006). As compared with A431/Pt cisplatin resistant cervix squamous cell carcinoma, A431/Pt transfectants overexpressing hCTR1 (3.4-fold) exhibited increased uptake of copper, thereby supporting the expression of a functional transporter. But no changes in cisplatin uptake and cellular sensitivity to drug were observed (Beretta *et al.*, 2004). Taken together, It seem that the role of hCtr1 in the cisplatin resistance is controversial depends on cell types. This controversy can be solved through understanding the underlying mechanism of how copper and platinum drugs regulate the expression and function of hCtr1 transporter.

A recent study demonstrated that cisplatin down-regulates very rapidly both endogenous and exogenously expressed hCtr1 in ovarian carcinomas, which reduces the uptake of Cu and occurs at clinically relevant cisplatin concentrations (Holzer *et al.*, 2004). Another recent study demonstrated that, at very high concentrations, cisplatin crosslinked the N-terminal domain of hCtr1, and that this effect was dependent on the methionines in the M1 and M2 motifs of the hCtr1 (Guo *et al.*, 2004a), suggesting that cisplatin itself regulate Ctr1 transporter and, as a result, modulate cisplatin resistance. In this point of view, studies on function and regulation of Ctr1 have gained substantial attention in the recent years. The Ctr is a family of evolutionarily conserved membrane protein from yeast to human (Puig and Thiele, 2002). All Ctr members contain three putative transmembrane domains, with extracellular N-terminal domain and intracellular C-terminal domain. Methionine rich motifs in N-terminal regions in all the Ctr1 and other methionine residues (e.g., Met^{150} and Met^{154} in hCtr1) located in the transmembrane domain are important in transport function (Puig *et al.*, 2002; Aller *et al.*, 2004; Guo *et al.*, 2004b; Eisses and Kaplan, 2005). It has been proposed that oligomerization of hCtr1 monomer, probably involving the N-terminal residues (Klomp *et al.*, 2003; Petris *et al.*, 2003), is important for the formation of independent translocation pore for the transport of copper (Lee *et al.*, 2002; Klomp *et al.*, 2003; Petris *et al.*, 2003; Aller and Unger, 2006). Nonetheless, the molecular details how hCtr1-mediated copper movement, including extracellular metal capture, passage through the membrane lipid bilayer, and transfer it to the intracellular copper chaperones, remain largely unknown.

Multiple layers of regulation are involved in the biosynthesis of Ctr1 family members. Transcriptional up-regulation during copper starvation and down-regulation during copper proficiency has been observed for both yCtr1 and yCtr3 genes (Dancis *et al.*, 1994; Pena *et al.*, 2000). Moreover, posttranslational regulation has also been reported in response to copper concentration. Ooi *et al.* (1996) reported that membrane spanning yCtr1 protein is degraded when cells are exposed to high concentrations of copper and the degradation process is independent of endocytotic pathway, although there was a fraction of internalized yCtr1. Unlike yCtr1, yCtr3 is regulated neither by protein degradation nor by endocytosis in response to copper concentrations (Puig *et al.*, 2002).

Available information regarding regulation mechanism

of hCtr1 in response to extracellular copper concentrations is controversial. First, posttranslational mechanism has been suggested to play a major role in the regulation of hCtr1 by copper concentrations. Using cultured cell system overexpressing epitope-tagged recombinant hCtr1, it was reported that copper exposure was associated with a rapid (within 10 min) internalization of hCtr1 from plasma membrane (Petris *et al.*, 2003; Guo *et al.*, 2004b). The copper-dependently internalized hCtr1 is then degraded for the maintenance of hCtr1 homeostasis. However, using baculoviral expression of hCtr1 in insect (sf9) cells, Eisses and Kaplan (2002) observed that initial rates of copper uptake remained linear for at least 1 hr in sf9 cells in the continued presence of copper. Subsequent studies (Eisses *et al.*, 2005) showed no evidence of a copper-dependent regulation of internalization of hCtr1 in the same system. Second, in contrast to yCtr1, the steady-state levels of mammalian Ctr1 mRNA were not changed in response to cellular Cu availability, suggesting that transcriptional and/or post-transcriptional regulation are not involved (Lee *et al.*, 2000).

Metallochaperone

Ctr1 hands the copper to one of three different chaperones, each of which serves to deliver copper to a specific target in the cell. Cytochrome c oxidase 17 (COX17) delivers copper to cytochrome *c* oxidase in the mitochondria; copper chaperone for Cu, Zn superoxide dismutase (CCS) delivers copper to superoxide dismutase (SOD) in cytosol, and human atox-1 homologue (HAH1 or antioxidant protein-1 (ATOX1)) delivers copper to a P-type ATPase transporter (ATP7A or ATP7B) in trans-golgi compartment (Amaravadi *et al.*, 1997; Culotta *et al.*, 1997; Klomp *et al.*, 1997).

A series of deletion strains carrying defects in gene which mediate intracellular copper trafficking and utilization such as atox1, ccc2 (copper efflux transporter; homologues to ATP7B), fet3 (homologues to ceruloplasmin), lys7 (a yeast copper chaperone for SOD; homologues to CCS), sod1, cox17, and sco1 (cytochrome c oxidatse family) were examined, but none of these mutants resulted in significant enhancement of cisplatin resistance (Pufahl *et al.*, 1997; Valentine and Gralla, 1997; Culotta *et al.*, 1999; Ishida *et al.*, 2002). Moreover, HAH1 expression is not changed in cisplatin resistant ovary cancer cells compared to sensitive cells, whereas the expression of ATP7A and ATP7B was increased (Katano *et al.*, 2002). Taken together, copper chaperones apparently do not play a major role in cisplatin resistance even though they play an important role in maintaining copper homeostasis.

P-type ATPase

Two P-type ATPase transporters, ATP7A and ATP7B, shuttle between trans-golgi and plasma membrane to mediate efflux of intracellular copper and mutations in these transporters have relation to the human genetic diseases of Cu metabolism, Menkes and Wilson's diseases, respectively. Impaired cellular efflux of Cu is the main cause of excessive Cu accumulation in the liver, brain, and kidney of patients with Wilson's disease and in the brain of those with Menkes disease.

There are reports showing that overexpression of ATP7B conferred cisplatin resistance associated with decreased accumulation of cisplatin and carboplatin (Komatsu *et al.*, 2000; Katano *et al.*, 2003), suggesting that cisplatin efflux transporters may at least in part also carry platinum drugs and modulate platinum resistance.

Transfection of ATP7B expression vector into head and neck and ovarian carcinoma cells increased resistance in these cells to copper, cisplatin, and carboplatin (Katano *et al.*, 2003). Interestingly, ATP7B transfected cells were found to have lower basal levels of Cu than empty vector transfected cells (Katano *et al.*, 2003). Increased expression of ATP7B protein in ovarian carcinoma cells not only reduced the whole cell and DNA content of platinum in cisplatin and carboplatin treated cells but also increased the rates of the primary and secondary phases of efflux for these platinum drugs (Katano *et al.*, 2003). Moreover, immunohistochemical and mRNA analyses have demonstrated that, in many cell types, higher expression of ATP7B correlates with unfavorable response to platinum drug treatment. The data published so far provide strong evidence that ATP7B mediates resistance to the platinum based drugs by regulating drug efflux.

Study of the cellular pharmacology of copper and cisplatin into ATP7A-deficient human Menkes fibroblast cell line demonstrated that lack of ATP7A function was associated with increased accumulation of both copper and cisplatin and hypersensitivity to both substrates (Samimi *et al.*, 2003). A small increase in ATP7A expression produced resistance to all three of the clinically available platinum drugs (Howell, 2004), whereas large increase in ATP7A expression did not reduce accumulation of the platinum drugs. Moreover, platinum drugs did not trigger ATP7A relocalization. Thus, although ATP7A is also an important determinant of the efflux of platinum drugs, substantially less information is available on the ability to modulate drug sensitivity and cellular pharmacology of platinum drugs.

Organic cation transporter

Although cisplatin is an effective anticancer agent, severe nephrotoxicity limits its clinical application. It was reported that an increase in the serum creatinine concentration was observed in 41% of patients treated with high dose cisplatin (de Jongh *et al.*, 2003). The major site of cisplatin induced renal injury is the proximal tubule

(Dobyan *et al.*, 1980). In addition, cisplatin induced tubular toxicity, followed by an increase in the serum creatinine level (Ichimura *et al.*, 2004). Moreover, the tubular toxicity caused a decrease in the glomerular filtration rate (GFR), resulting in acute renal failure (Thadhani *et al.*, 1996). Recently, Ludwig *et al.* (2004) reported that cisplatin induced cytotoxicity was specifically observed from the basolateral site, and the toxicity was ameliorated in the presence of cimetidine, suggesting the involvement of basolateral drug transporters in the cisplatin uptake (Okuda *et al.*, 1999). Organic cation transporter 2 (OCT2) is the most abundant organic cation transporter expressed in the basolateral side of the kidney among other organic cation transporter family and mediated the accumulation of various cationic drugs such as tetraethyl ammonium, MPP^+, procainamide, metformin, and cisplatin into proximal tubular epithelial cells from blood circulation (Urakami *et al.*, 1998). The accumulation of platinum was greater in OCT2 overexpressed HEK293 cells treated with cisplatin than that in control vector transfected cells. Moreover, cimetidine and corticosterone, OCT2 inhibitors, inhibited the cytotoxicity and the transport of cisplatin in OCT2 overexpressed HEK293 cells, indicating that renal OCT2 expression is the major determiant of cisplatin-induced tubular toxicity (Yonezawa *et al.*, 2005).

Polynuclear platinum complexes which show a different toxicity profile from that of cisplatin and a significantly different mode of action in terms of DNA binding accumulated independent of cisplatin and copper. The uptake of polynuclear platinum complex was slightly inhibited by TEA and cimetidine, representative OCT inhibitors, and dramatically inhibited by the treatment of amiloride, EIPA, and cytochalasin D, potent inhibitors of endocytosis, suggesting that polynuclear platinum complexes is uptaken by endocytosis and, to a lesser extent, by OCT transporter (Kapp *et al.*, 2006). Because these drugs had different transport system and DNA binding mechanism, polynuclear platinum complexes did not show cross resistance with cisplatin and carboplatin.

Multi-Drug Resistance related transporters

MDR

Multidrug resistance (MDR) of tumors is frequently associated with decreased cellular accumulation of anticancer drugs and elevated expression of MDR transporters such as MDR1, MRP1, and BCRP1. Therefore, it is of importance to investigate the correlation between MDR gene expression and cisplatin resistance. The expression profile of MDR1, MRP1, and BCRP1 mRNA and protein was not correlated with cisplatin resistance or intracellular/intranuclear cisplatin accumulation of non small cell lung cancer cell (NSCLC) and six ovarian cancer cell lines (e.g., MZOV4, EFO27, SKOV3, OAW42, OTN14, MZOV20) (Kanzaki *et al.*, 2002; Nakayama *et al.*, 2002; Schondorf *et al.*, 2003; Ikuta *et al.*, 2005). The relationship between MDR gene expression and cisplatin resistance is needed to be more investgated. Available information was limited regarding the other cancer cell lines. Therefore, the involvement of MDR transporters in the cisplatin resistance needs further investigation.

MRP

Early works demonstrated that exposure of human ovarian cancer cell lines to cisplatin led to development of cell lines that exhibited increasing degrees of drug resistance, which were closely correlated with increase of the levels of cellular GSH (Godwin *et al.*, 1992; Meijer *et al.*, 1992; Schroder *et al.*, 1996; Sasada *et al.*, 2000; Jansen *et al.*, 2002; Mishima *et al.*, 2006). Moreover, GSH depletion by BSO is associated with increased sensitivities to cisplatin (Godwin *et al.*, 1992; Yao *et al.*, 1995; Dedoussis and Andrikopoulos, 2001; Ikeda *et al.*, 2001; Ryoyano *et al.*, 2001). Biosynthesis of GSH is controlled by the rate-limited enzyme, g-glutamylcysteine synthetase which consists of catalytic or heavy subunit (-GCSh) and regulatory or light subunit (-GCSl) (Masters *et al.*, 1996); and in many case when -GCSh contents were measured, elevated levels of -GCSh mRNA were correlated with cisplatin resistance. Moreover, GSH levels in testicular tumors and bladder tumors that are intrinsically sensitive and resistant to cisplatin chemotherapy, respectively, are lower in testicular tumor cell lines than in bladder tumor cell lines (Masters *et al.*, 1996). Multiple mechanisms have been proposed to explain GSH-mediated cisplatin resistance: (i) Cisplatin can be covalently linked to GSH and platinum-GSH conjugate can be eliminated by ATP-dependent efflux pump, MRP/GS-X pump (Ishikawa and Ali-Osmann, 1993; Minamino *et al.*, 1999). (ii) Alternatively, GSH may protect cells by maintaining proper nucleotide pools for DNA repairing system for cisplatin-induced DNA damages (Lai *et al.*, 1989). While these observations, collectively, have been strongly suggested that intracellular GSH levels play an important in regulating cisplatin resistance, the roles of GSH in cisplatin resistance remain to be critically investigated for the following reasons: (i) Unlike redox-active metals, copper, cadmium, and ferrous ions, reaction of Pt to GSH is a very slow process (Valko *et al.*, 2006). Moreover, there is overexpression of MRP1 in cultured cells do not confer resistance to cisplatin (Cole *et al.*, 1994). (ii) Report also shows that correlation between glutathione content and platinum sensitivity is not associated with corresponding change in the accumulation of platinum (Ikeda *et al.*, 2001). (iii) Most importantly, the observations described above are mostly correlative in nature, and no cause-effective relationship has been established as yet.

CONCLUDING

If the expression level of copper transporters (i.e., hCtr1, ATP7A, and ATP7B) is an important determinant of cisplatin resistance, it will be of interest to try to develop active platinum derivatives that have distinct uptake and efflux mechanisms compared to cisplatin. Platinum compounds that can be efficiently accumulated by a copper transporter-independent manner may be useful for overcoming resistance since these compounds may have novel toxicity spectra. In this point of view, polynuclear platinum complex that was accumulated via endocytosis but not copper transporter, for example, will be a good candidate for next generation of platinum compounds.

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