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Targeting drug transport mechanisms for improving platinum-based cancer chemotherapy

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Abstract

Introduction—Platinum (Pt)-based antitumor agents remain important chemotherapeutic agents for treating many human malignancies. Elevated expression of the human high-affinity copper transporter 1 (hCtr1), resulting in enhanced Pt drug transport into cells, has been shown to be associated with improved treatment efficacy. Thus, targeting hCtr1 upregulation is an attractive strategy for improving the treatment efficacy of Pt-based cancer chemotherapy.

Area covered—Regulation of hCtr1 expression by cellular copper homeostasis is discussed. Association of elevated hCtr1 expression with intrinsic sensitivity of ovarian cancer to Pt drugs is presented. Mechanism of copper-lowering agents in enhancing hCtr1-mediated *cis*-diamminedichloroplatinum (II) (cisplatin, cDDP) transport is reviewed. Applications of copper chelation strategy in overcoming cDDP resistance through enhanced hCtr1 expression are evaluated.

Expert opinion—While both transcriptional and posttranslational mechanisms of hCtr1 regulation by cellular copper bioavailability have been proposed, detailed molecular insights into hCtr1 regulation by copper homeostasis remain needed. Recent clinical study using a copper-lowering agent in enhancing hCtr1-mediated drug transport has achieved incremental improvement in overcoming Pt drug resistance. Further improvements in identifying predictive measures in the subpopulation of patients that can benefit from the treatment are needed.

Keywords

cisplatin; copper homeostasis; high-affinity copper transporter 1; hCtr1; Sp1; copper chelators; platinum drug cancer chemotherapy

Financial and competing interest's disclosure

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1. Introduction

The platinum (Pt)-based antitumor drugs, including cDDP, carboplatin, and oxaliplatin, have been the mainstay for treating a broad spectrum of human malignancies over the last four decades [1, 2]. cDDP has particular effectiveness in treating metastatic testicular cancers, where cure rates of greater than 90% can be achieved [3]. Pt drugs are also effective in treating ovarian cancers with response rates of about 70% [4]. Other tumor types that have been effective include head and neck, bladder, lung, breast, and malignant mesothelioma [1]. The primary lethality target of Pt drugs is DNA by forming intra-stranded and inter-stranded crosslinking [5]. It has been reported that the extent of DNA damage is directly correlated with cell lethality, which is also directly correlated with cellular Pt drug contents [6, 7]. Indeed, defective drug transport systems resulting in reduced Pt accumulation have long been recognized as an important mechanism of resistance [8–13]. Andrews and Howell [14] pointed out that almost all cDDP-resistant variants are associated with various degrees of reduced Pt levels in comparison with their cDDP-sensitive counterparts, implicating that drug transport is a widespread mechanism of cDDP resistance, underscoring the importance of understanding cDDP transport mechanisms for targeted therapy.

2. Mechanisms of cDDP transport

Early works reported that cDDP enters cells by means of passive diffusion and independently of protein carrier or channel/transporter, and that transport is unsaturable and un-competetable by Pt analogues [7, 8]. Import transporter for cDDP was identified by using yeast genetic approach as the high-affinity copper transporter 1 (Ctr1). Deletion of *yCtr1* resulted in reduced cellular copper (Cu) and cDDP accumulation [15, 16]. This was confirmed in mouse embryonic fibroblasts with deleted m*Ctr1* alleles (mCtr1 (−/−)) which exhibited a similar phenotype [15]. However, Ctr1 apparently does not account for the total Cu and cDDP transport, because *mCtr1*(−/−) cells show only 60 – 70% reductions of Cu [17] and cDDP [18] transport capacities compared with those in *mCtr1*(+/+) counterparts. It has been suggested that divalent metal transporter 1 (Dmt1) may also transport Cu [19, 20], however, this remains controversial [21] and further investigations is needed.

2.1. Roles of copper transport systems in cDDP transport

Despite substantial differences in the biochemical properties of Cu ions and cDDP, the mechanisms of hCtr1-mediated transport of these two substrates are quite similar. Human Ctr1 (hCtr1) is a 190-amino acid protein of approximately 23 kDa with three transmembrane domains. Biochemical and structural analyses have shown that a functional hCtr1 is composed of three monomeric subunits forming a channel-like configuration [22]. The two highly conserved motifs Met-(X)n-Met (where X can any other amino acids except methionine), one located at the N-terminal extracellular domain and the other at the second transmembrane domain, are essential for Cu and cDDP transport [23]. X-ray fine absorption spectroscopy showed that two Cu^{+1} ions bind to the N-terminus of hCtr1, each coordinated by three sulfur atoms [24]. Deletion/mutation manipulations of these regions also result in impaired cDDP transport [23, 25, 26].

Using the N-terminal hCtr1 sequences as model peptides, it was suggested that prior to entering a cell, cDDP (molecular formula: *cis*-$[PtCl_2(NH_3)_2]$) is activated by interacting with the extracellular methionine-clusters of hCtr1, resulting in release of the carrier ligand [27–29] and formation of the $[Pt(Met)Cl(NH_3)_2]^{+1}$ intermediate which, like Cu^+, has one positive charge. Traverses of Cu^{+1} and cDDP through hCtr1 induce conformational changes of trimeric hCtr1 as probed by a protein crosslinking approach [23]. Site-directed mutations of hCtr1 suggest that more than one molecule of Cu^{+1} or Pt are concurrently associated with one hCtr1 trimer during transport [23]. It has been proposed that the activated cDDP may use methionine-based intermolecular sulfur-sulfur exchange along the axis of the trimeric hCtr1 to facilitate its transport [30], much like the mechanisms underlying Cu^{+1} or Ag^{+1} transport by the CusA efflux pump in bacteria [31].

Once Cu^{+1} has traversed through the membrane, it is carried away by various Cu chaperones to various cuproenzymes in different cellular compartments: to superoxide dismutase 1 in the cytosol by CCS, to mitochondrial cytochrome C oxidase by Cox17, and to two *trans*-Golgi P1B-type ATPases (ATP7A and ATP7B) by Atox1 for loading on ceruloplasmin, a secretory protein for Cu elimination. Recent studies demonstrated that both CCS [32] and Atox1 [33] interact with hCtr1, suggesting a direct transfer of Cu^{+1} from hCtr1 to these carrier proteins (Figs. 1B & 1C). Bindings between cDDP and Atox1 [34–36] and between cDDP and Cox17 [37] have been demonstrated. Give the direct contact between these chaperones and hCtr1, cDDP may be similarly carried to its cellular destinations, but this remains to be demonstrated. Inside the cells, cDDP is transformed into $[Pt(NH_3)_2Cl(OH_2)]^+$ and $[Pt(NH_3)_2(OH_2)_2]^{2+}$ because chloride ligand is slowly displaced by water [5]. The aqua ligands in these complexes are readily displaceable, allowing Pt atom to interact with cellular targets, notably the guanine residue of DNA $[Pt(NH_3)_2Cl(Guanine\text{-}DNA)]^+$ [38] to elicit its cellular lethality mechanisms. Clearly, mechanisms of Pt drugs transport and their biotransformation resulting in formation of Pt-DNA adducts are complex and required further investigations.

Humans have a low-affinity Cu transporter, hCtr2 which has 143 amino acid residues that span through three transmembrane domains. In comparison with hCtr1, hCtr2 lacks the majority of the N-terminal ecto-sequence that is important for Cu^{+1} and cDDP transport but contains a M-X_3-M sequence in the second transmembrane domain that is important for Cu transport. hCtr2 is located mainly in intracellular vesicles and has been proposed for Cu storage. mCtr2(−/−) mice display increased Cu accumulation in several organs compared with wild-type animals, demonstrating that mCtr2 plays a role in regulating systemic Cu distribution. At the cellular level, *mCtr2(−/−)* fibroblasts also elevated endosomal Cu accumulation. mCtr2 may interact with mCtr1 and causes formation of truncated Ctr1 lacking its ecto-domain containing Cu- and cDDP- binding motifs, but the mechanism of the proteolytic cleavage is not known [39]. Study also suggests that hCtr2 expression can also be modulated by Cu bioavailability and also plays a role in regulating cDDP sensitivity [40].

ATP7A and ATP7B are associated with Menke's and Wilson's diseases, respectively. Each contains eight transmembrane spannings and is organized into multiple functional domains involved in nucleotide-binding and catalytic ATP phosphorylation, *trans*-Golgi targeting and retention, and vesicle trafficking [41]. The N-termini of these ATPases contain six highly

conserved CXXC motifs, each can bind one Cu^{+1}. At low cellular Cu concentrations, Cu binding to these ATP7A and ATP7B induces transient catalytical phosphorylation thereby providing Cu^{+1} to cuproenzymes. At high Cu contents, Cu^{+1} binds to the metal-binding domain, inducing conformational changes and phosphorylation of several sites on the molecules by kinases. Hyper-phosphorylated ATPases then exit *trans*-Golgi and is present in endosomal/lysomal vesicles and trafficking to cell membrane where Cu is eliminated [41, 42]. Thus, both ATP7A and ATP7B play important roles in regulating cellular Cu homeostasis by eliminating excess Cu. The N-terminal CXXC motifs are also involved in Pt binding in cDDP treatments [43, 44]. However, whether the platination involves Atox1-bound cDDP is not clear. Moreover, whether elimination of cDDP by ATPases involves vesicle trafficking is not clear.

These observations collectively suggest that Cu^{+1} and cDDP share similar gross mechanisms in metal acquisition, distribution, and elimination, although differences in terms of transport kinetics and other parameters may exist.

2.2. Roles of copper transporter in cDDP sensitivity

Many studies have demonstrated that expression of hCtr1 and ATP7A/ATP7B affect cellular sensitivity of cDDP in cultured cell systems [45, 46]. In clinical settings, several studies have reported that expression levels of hCtr1 in tumors are significantly correlated with better treatment outcomes in terms of increased progression-free survival (PFS) time and overall survival (OS) times in patients treated with Pt-based therapeutics [47–50]. In some tumor types, elevated expression of hCtr1 is intrinsically sensitive to Pt-based treatment, suggesting that hCtr1 levels may be a predictive marker for effectiveness of Pt drug in cancer treatment. (see below). However, correlations between PFS or OS and ATP7 and ATP7B expression in ovarian cancer patients [50] and lung cancer patients were not as significant [47]. Moreover, expression of hCtr1, but not ATP7A or ATP7B, can be readily upregulated by manipulations of cellular Cu contents (see below). These results suggest that hCtr1 may be a valid target for improving Pt drug treatment efficacy through inducing its regulation for enhanced drug transport.

3. Regulation of cDDP sensitivity through modulation of hCtr1 expression

Copper is an essential micronutrient for cell survival but is poisonous when in excess. Therefore, cellular Cu levels have to be adequately regulated. Although the steady-state cellular Cu levels are regulated by the balancing acts among hCtr1, Cu chaperons, and Cu exporters, hCtr1 expression is regulated by acute Cu concentration changes. Under Cu-limited conditions, hCtr1 expression is upregulated, whereas under Cu-replete conditions, hCtr1 expression is repressed. Two major mechanisms have been described for hCtr1 regulation by Cu concentration variations as described below.

3.1 Regulation of hCtr1 by endocytosis

It has been reported that under Cu-replete conditions, hCtr1 is rapidly internalized by endocytosis where hCtr1 may [51] or may not be degraded [52]. In contrast, under Cu-deplete conditions, the internalized hCtr1 is recycled back to the plasma membrane [52].

This post-translational regulation model depicts that Cu and hCtr1 are mutually regulated and suggest that the sensing mechanism of Cu bioavailability that triggers hCtr1 endocytosis may reside on hCtr1 itself (Fig. 1A).

It must be noted that this two-component mutual regulation model was mainly carried out using anti-hCtr1 antibodies in cultured cells stably overexpressed hCtr1 by transfection of *hCtr1* recombinants. The Ctr1 family is an evolutionally conserved group of proteins, and it has been difficult to obtain high affinity anti-hCtr1 antibodies for research. Polyclonal anti-hCtr1 antibodies produced by different laboratories using different portions of hCtr1 as antigens detected hCtr1 with different molecular masses by Western blotting, *i.e.* 28 kDa [53], 24 kDa [54], 35 kDa [55], 24 kDa and 36 kDa [56]. The 23- to 24-kDa proteins correspond to the unmodified hCtr1 monomer of 190 amino acid residues, whereas the 28-, 35- and 36-kDa proteins are considered as glycosylated hCtr1 [55, 57]. We produced a polyclonal anti-hCtr1 antibody using the N-terminal 50 amino acids ecto-peptide sequence as an antigen. This antibody recognizes the 23 kDa hCtr1 by Western blotting and stains cytoplasmic membrane localized hCtr1 in un-transfected cancer cells [50, 58]. It is unclear how the modified and unmodified hCtr1 may contribute to endocytosis under Cu stressed conditions.

Because of the difficulty of obtaining high quality anti-hCtr1 antibodies, current evidence showing Cu-induced hCtr1 endocytosis was mostly carried out using genetically engineered cell lines overproducing hCtr1 by transfection [41]. These hCtr1-overproducing cells therefore had been pre-loaded with Cu, causing reduction of endogenous hCtr1 levels [50, 59–61], much like cells that had been treated with Cu. As a consequence, these cells may have altered buffered bioavailable Cu capacities that control hCtr1 regulation under copper stressed conditions (see below).

3.2 Transcriptional regulation of hCtr1 by Cu bioavailability

Transcriptional regulation as an important mechanism of Ctr1 regulation has been well demonstrated in multiple organisms. In the yeast *Saccaromyces cerevisiae*, low Cu conditions activate the transcription factor Mac for upregulation of *yCtr1* and *yCtr3* and metal reductase *Fre1*, together encoding a high-affinity Cu import system [62]. At high Cu concentrations, Mac is inactive and unstable. Another transcription factor AceI is activated and induces the expression of Cu-chelating metallothionein encoded by *CUP1* and *CRS5* and the antioxidant superoxide dismutase encoded by *SOD1* for detoxifying Cu overload [63]. In *Drosophila* under conditions of Cu starvation, Cu transporter *dCtr1B* expression is transcriptionally upregulated by metal-responsive transcription factor-1 (MTF-1). Strikingly, MTF-1 is a well-known metal-responsive transcriptional factor involved in upregulation of *metallothionein* genes for the detoxification of Cu and other heavy metals [64]. Thus, dMTF-1 responds to both excessive and limiting Cu conditions and regulates different genes accordingly. But how dMTF-1 senses these two different conditions and activates its target genes is not known. In *Arabidopsis*, transcription factor *SQUAMOSA*-promoter like binding protein 7 (SPL-7) upregulates the three Cu transporters, *COPT1, COPT2* and *COPT6* in response to Cu-limiting conditions [65–67].

We demonstrated that regulation of *hCtr1* expression is controlled by transcription factor specific protein 1 (Sp1) which binds the GC boxes located at the *Sp1* and *hCtr1* promoter regions. Cu deficiency conditions, induced either by treatment with Cu chelators or by transfection with dominant-negative *hCtr1* recombinant, upregulates *Sp1* which in turn upregulates *hCtr1* expression through promoter binding. In contrast, Cu sufficiency either by treating cells with $CuSO_4$ or by overexpressing the wild-type *hCtr1* recombinant, results in downregulation of Sp1 expression which in turn downregulates *hCtr1* expression [46, 60]. These observations showed that human Cu homeostasis is controlled by the three-way mutually regulatory loop consisting of Cu, Sp1, and hCtr1 [46] (Fig. 2). This regulatory loop highlights the dynamic mechanism of Cu homeostatic regulation: changing any one component in the loop will affect the levels of the other two, resulting in a new homeostatic balance among all three. These findings have led to the development of using Cu chelators to upregulate hCtr1 expression, thereby enhancing cDDP transport capacity for its cell-killing activity (see below).

4. The sensing mechanisms of Cu bioavailability by Sp1

Many transcription factors mediate Ctr1 regulation through DNA binding therefore, these transcription factors may function as sensors of Cu bioavailability. For Ace1, excess Cu promotes its DNA binding [68]. In contrast, for Mac1 and SPL-7 [66], excess Cu inhibits their DNA-binding activities and corresponding promoter activities. These transcriptional regulators contain metal-binding motifs that respond to metal ions for their transcriptional activities. It appears that sensing of Cu bioavailability relies on Cu interactions between transcriptional regulators and the promoters of their target genes.

Sp1 is a ubiquitous transcription factor consisting of a DNA-binding domain that contains three zinc fingers (ZF) and a transactivation domain that contains two serine/threonine-rich and two glutamine-rich (Q-rich 1 and Q-rich 2) subdomains. Each ZF consists of $Cys_2{\cdot}His_2$ that is coordinated by one Zn molecule. We previously demonstrated that both ZF and Q-rich 2 domains are essential for Sp1's Cu homeostatic regulation [60]. Sp1 is constitutively bound by Zn ions because unliganded Sp1 is very unstable [69]. Excess Cu^{2+} competes the bound Zn^{2+}, resulting in distortion of its DNA-binding activities and downregulation of target genes *hCtr1* and *Sp1*. In contrast, Cu-limiting conditions enhance the stabilization of interactions between Sp1 and the *Sp1* and *hCtr1* promoters. These explanations are consistent with the ranked order of stability of metal complexes known as the Irving-Williams series [70] as follows: $Cu^{+2} > Ni^{+2} > Co^{+2} > Zn^{+2} > Fe^{+2} > Mn^{+2} > Mg^{+2}$.

The effects of Cu^{+2}, Zn^{+2}, and Cu chelators (bathocuproine disulfonic acid (BCS)) on the stability of Sp1-DNA binding can be determined by using gel electrophoretic mobility shift (GEMS) assay which employs ^{32}P-labeled double-stranded DNA probe with a Sp1 binding sequence [60] in the presence of increased concentrtions of Cu^{+2} or Zn^{+2} according to the published procedures [71]. This analysis revealed that Cu^{+2} ions indeed are more potent than Zn^{+2} in destabilization of the Sp1-DNA complex, consistent with the Irving and Williams' series. In contrast, BCS increases Sp1-DNA complex stabilization (Z.D.L, and MTK, unpublished data).

Cupric ions (Cu^{+2}) are at the top of the Irving-Williams series, suggesting that Cu^{+2} ions have a strong competitive edge over Zn^{+2} in binding to the same binding site [72]. Cuprous ions (Cu^{1+}) are also highly competitive, and hence both Cu ions have the potential to displace less competitive metal ions from metellocomplexes [42, 73]. Cu ions in eukaryotic cytosol are predominantly in Cu^{+1} form, and is intracellularly trafficked via Cu^{1+}-binding carrier proteins, because of mostly reduced environment. However, Cu^{+1} can be converted into Cu^{+2} by the Fenton reaction under oxidative stressed conditions, but Cu^{+2} can be converted back to Cu^{+1} by metal reductase (*Fre*1 from the yeast *S. cerevisiae*, which is homologous to the $gp91^{Phox}$ in the NADPH redox regulating complex [74]). Naturally occurred Cu is mostly in the form of Cu^{+2}. It has to be converted to Cu^{+1} before transport by hCtr1.

We also found that the Q-rich 2 subdomain within the Sp1-TAD is also involved in Cu regulation of Sp1 and hCtr1 expression [60]. Cu can interact with the carboxylate group of glutamate (Q). The Q-rich 2 sequence interacts with $TAF_{II}110$ in the TFIID, a core protein complex in the basal transcriptional machinery of RNA polymerase II [75, 76]. This domain is also involved in Sp1 self-multimerization resulting in DNA looping in the supra-activation of transcription [77, 78]. High concentrations of Cu may also disrupt Sp1 multimerization and its cross-talk with basal transcriptional machinery resulting in attenuation of Sp1's transcription activity.

4.1. The cellular bioavailable Cu sensing capacity

It has been proposed that virtually all cellular Cu ions are tightly bound and that only a small fraction is "labile" or bioavailable for regulation [73]. The physiologically bioavailable Cu ion pools have to be kept within a window with lower and upper limits. The inter-regulatory loop of Cu-Sp1-hCtr1 in Cu homeostasis regulation (Fig. 2) is also consistent with this notion. Using fluorescent probes for AceI and Mac activations that sense opposite bioavailable Cu limits, it was demonstrated that the upper and lower limits of available Cu^{1+} concentrations in yeast are ~8.9×10^{-17} M and ~5.1×10^{-21} M, respectively [79]. In higher organisms, measurement of the buffered bioavailable Cu windows is more difficult. It was estimated that cellular buffered Cu^{+1} capacities are at about 10^{-15} M ranges whereas Zn^{+2} are at 10^{-12} M [72].

4.2. Cellular bioavailable Cu pool and hCtr1 regulation

The bioavailable buffered Cu pool, although still ill-defined in human cells, plays an important role in controlling hCtr1 regulation in response to Cu variations. The pool sizes may vary according to cell types at various physiological states. In addition, like cDDP, Cu can bind to many cellular constituents including small molecules such as amino acids, organic and inorganic ligands, and to the side-chain ligands on the surface of macromolecules such as cuproenzymes. Cu can also bind to sulfate-containing molecules such as glutathione (GSH) and metallothionein (MT) which are considered as the major Cu sink. Overexpression of GSH by elevated expression of γ-glutamylcysteine synthetase resulted in reduced bioavailable Cu pools, upregulation of hCtr1 and *increased* cellular cDDP sensitivity [80]. Cu can also displace Zn binding to MT and the released Zn may affect the transcription activities of ZF-containing transcription factors.

The capacities of the bioavailable Cu pool may also differ among different tissues/organs, reflecting in differential effectiveness for the induction of Ctr1 expression by copper-lowering agents. Intestinal epithelia are the main tissue involved in acquiring Cu from foodstuff in animals. Mice with intestinal epithelial ablation of both *mCtr1* alleles (*mCtr1*$^{int/int}$) show substantial variations in the severity of Cu reduction among different organs in the body as compared with their counterparts in *mCtr1*$^{flox/flox}$ mice [57]. Previously study showed that rats fed a Cu-deficient diet failed to show increased Ctr1 mRNA levels in livers and small intestines despite a substantial loss in Cu levels in these organs (69 ~ 89% reduction), compared with those in animals fed Cu-adequate diet [59]. These observations revealed that different organs have different responsivenesses of mCtr1 mRNA expression to Cu stress.

Elevated Cu levels have been frequently found in many human malignancies, including those of prostate, breast, colon, lung, and brain [81]. Cu can stimulate tumor cell growth and Cu chelation suppresses tumor growth in experimental animal tumor model [82]. Recent study demonstrated Cu is required for the oncogenic pathway elicited by the BRAF^{V600E} mutation which occurs in approximately 50 % of malignant melanoma, by interacting with MEK1 which phosphorylates ERK1/2 growth signal [83]. These results demonstrated that the window of buffered Cu contents may differ between normal and neoplastic sources.

Sp1 is an essential enzyme that is ubiquitously expressed. Sp1 expression is transcriptionally self-regulated in the Cu homeostatic cycle [60]. More than 40 interacting proteins can associate with Sp1 and many of them are coregulators that either synergize or suppress Sp1's transcription activity [84]. Moreover, posttranslational regulation such as that by glycosylation, acetylation, phosphorylation, SUMOylation, and ubiquitination can also affect its activity and stability [84]. Elevated expression of Sp1 are frequently found in tumors [85]. However, the roles of Sp1 in tumorigenesis are complex. For example, Sp1 upregulation is involved in Kras-induced lung tumorigenesis, however Sp1 expression is decreased during tumor progression, and reduced Sp1 levels is associated with lung tumor progression and metastasis [86]. Sp1 is a downstream effector of oncogenic Kras signaling. We found that expression of expression of Sp1 and hCtr1 is increased in the oncogenic Ras-transformed, SV40 T/t and telomerase reverse transcriptase-immortalized human fallopian tube epithelial (FTE) cells. FTE has been suggested as origin of ovarian cancers [87]. Furthermore, expression of hCtr1 and Sp1 can be induced in Kras-transformed FTE by Cu chelation but not in the non-transformed immortalized FTE cells cannot (L.Y., J. L, MTK, unpublished results). These findings are consistent with the notion that regulation of Sp1/hCtr1, and therefore Cu homeostasis, may differ between non-cancerous and cancerous cells.

5. Overcoming cDDP resistance by modulating hCtr1 expression

5.1. Preclinical studies

Induction of hCtr1 expression in cultured cells by Cu chelation occurs at about 30 minutes after treating with a Cu chelator, but takes 2 – 3 days to return to basal levels after Cu chelator is removed [50, 58]. Neither ATP7A nor ATP7B levels is changed under these conditions. Because of tight regulation of Sp1 and hCtr1 in the Cu homeostasis regulation

loop, levels of regulation in regular cells are moderate. These moderate levels may also be contributed by the weak promoter of *hCtr1,* which is shared by another transcription unit encoding FK506 binding protein in the opposite direction located 201 nucleotides upstream from the transcription start site of *hCtr1*. This inter-genic sequence contains no enhancer-like sequence.

On the other hand, because cDDP-resistant variants often exhibit reduced hCtr1 levels, we demonstrated that these $hCtr1^{low}$ cells showed greater magnitudes of Sp1 and hCtr1 upregulation than $hCtr1^{high}$ cells by Cu chelation that are associated with greater re-sensitization to Pt drugs [50]. Moreover, cDDP by itself, like Ag^{+1} or Zn^{+2}, can be considered as a Cu-lowering agent because it functions as a competitor of hCtr1-mediated Cu transport. cDDP upregulates Sp1 and hCtr1 expression, facilitating cDDP transport and cell killing capacity [58]. The preferential upregulation of hCtr1 levels in cDDP-resistant cells provides as a mechanistic basis of using Cu-lowering agents for overcoming cDDP resistance in clinical evaluation.

5.2 Clinical evaluation of Cu-lowering agents in overcoming Pt drug resistance

Cu-lowering agents such as trientine and *D*-pencillamine have been used for more than four decades for treating Cu toxicosis resulting from Cu accumulation in Wilson's disease, a genetic disorder caused by defective *ATP7B*. A phase I exploratory study using carboplatin plus trientine in patients with advanced malignancies (*n=55*, 45 failed prior Pt drug treatment) has recently been completed at MD Anderson Cancer Center [88, 89]. These results demonstrated that (a) the combination of trientine with carboplatin was well-tolerated and associated with improved antitumor activity in a subset of Pt-refractory cancer patients who experienced substantial decreases of serum Cu levels, and (b) reduction of serum ceruloplasmin and/or Cu levels may be a prognostic marker for the treatment efficacy, *i.e.,* patients who achieve rapid reduction in serum Cu levels are likely to respond better to the chemotherapy than those that did not show reduction. (c) No dose-limiting toxicity or treatment-related deaths were observed. The common grade 2 adverse events included anemia, thrombocytopenia, and neutropenia, fatique, nausea/vomiting, anorexia and neuropathy.

The response rate of the current Cu chelation therapy remains low (19%) and improvement is warrant from the following considerations: (a) This exploratory study included a heterogeneous cohort of patients with different tumor lineages. Most of the patients had unsuccessful prior Pt-based treatment. Because multiple mechanisms are known for cDDP resistance, it is imperative to determine whether the response to Cu chelation therapy in a subset of patients was related to reduced hCtr1 expression. (b) Moreover, different tumor types may have different buffered capacities for hCtr1 induction by Cu chelation as discussed above, much as cDDP shows favorable treatment efficacy for one type of tumor over others. The possible tumor type-related effectiveness of Cu-lowering agents in reducing Cu levels need to be investigated. (c) This current study measured serum Cu and ceruloplasmin levels as surrogate references for Cu chelation. However, the utility of these markers in monitoring tumors' Cu levels remains to be critically evaluated..

6. Conclusion

The discovery that the Cu transporter systems also transport Pt drugs [15] has provided new insights into how modulation of cellular Cu levels can affect Pt drug sensitivity in cancer chemotherapy. Studies of the molecular bases of Cu homeostasis regulation by hCtr1 have resulted in an exploratory clinical investigation using a Cu-lowering agent to enhance Pt drugs transport. Although current results showed that about 20% of advanced cancer patients benefited from this treatment, however, this strategy remain attractive and the overall efficacy may be improved through better understanding the molecular mechanisms of hCtr1 regulation. Further studies are needed to improve the results of using Cu-chelation approach in improving the treatment efficacy of Pt drugs in cancer chemotherapy.

7. Expert Opinion

Since its approval by FDA in 1978, cDDP has been a highly prescribed antineoplastic agent and has become a pillar of cancer chemotherapy in treating many types of human malignancies. cDDP can be used in front-line therapy as well as in savage therapy after failure with targeted agents. cDDP resistance has been an important problem associated with cDDP failure because once resistance develops, other effective treatment options are limited. Mechanisms of cDDP resistance are multifactorial due to multiple cellular targets in its mode of toxicities [90, 91]. Over the years, substantial efforts have been devoted in developing effective agents to circumvent Pt drug resistance. Classical studies have been concentrated on DNA damages induced by cDDP, identifying genes and pathways that repair DNA damages and developing small molecules that target these pathways. Along this line, olaparib, rucaparib, niraparib, and BMN673, inhibitors of poly (ADP-ribose) polymerase targeting the repair system of cDDP-induced DNA damages [92], have shown promise for improving cDDP therapy although its clinical utility requires further studies [93].

Platinum drug transporters have been an important area of Pt research for the last decade. However, translating this research into clinical benefit remains a largely uncharted domain. The observations that vast majority of cDDP resistant variants are associated with reduced drug accumulation underscore the likelihood that cDDP transport systems are important targets for overcoming cDDP resistance. Of particular attractive is hCtr1 which controls the import of cDDP and carboplatin and to much reduced extent oxaliplatin drugs, particularly, its expression is correlated with clinical sensitivity of Pt drugs [47–50]. The exploratory clinical trial using a copper-lowering agent in overcoming cDDP resistance represents the first-in-human study and has obtained an incremental success. Current results showed about 20% of Pt-refractory patients with advanced malignancies experienced response to Pt drug in combination with Cu chelator treatment. Admittedly, clinical investigations targeting hCtr1 upregulation using Cu chelation strategy remain at their infancy, although Cu-lowering agents have been used for treating other indications for a long time. This strategy, upon further development, would likely to become a low cost treatment option. Another strength of this strategy lies on the differential sensitivity of hCtr1 induction by Cu chelation between drug-resistant tumor cells and normal cells, providing a favorable “therapeutic index” in cancer chemotherapy. Given the complexity of Cu homeostasis in controlling hCtr1 expression, more work is imperative. Another area that is equally important is the

exploration of drug retention mechanisms regulated by ATP7A and ATP7B. Specifically, agents that can suppress activities and/or expression levels of these drug exporters are likely to be useful. This area of research remains understudied.

Since Pt transport mechanisms are intrinsically tied into the Cu homeostasis system, better understanding on global regulation mechanisms of Cu metabolism under various challenges will be needed to identify interacting pathway networking that affects the bioavailability of cellular Pt drugs. Moreover, global analyses of transcriptional and post-transcriptional regulators, epigenetic modifiers, and interferences of small molecules including metal ions such as Zn^{+2} and Fe^{+2} [94] that would likely regulate the expression of these transporters will need to be integrated into the core regulators described here. These studies may provide an integrated therapeutic rationale for improving the overall treatment efficacy of Pt drugs in cancer chemotherapy.

List of Abbreviations used in the manuscript

ATP7A and ATP7B	two trans-Golgi PIB-type ATPases
Atox1 and CCS	two copper chaperones
BCS	bathocuproine disulfic acid
cDDP	cis-diamminedichloroplatinum (II), cisplatin
Ctr1	high-affinity copper transporter
Cu	copper
GEMS	gel electrophoretic mobility shift
MTF-1	metal-responsive transcription factor
MT	metallothionein
Q-rich	glutamine-rich
SCLC	small-cell-lung cancer
Sp1	specific protein 1
SPL-7	SQUAMOSA-promoter like-binding protein 7
ZF	zinc finger

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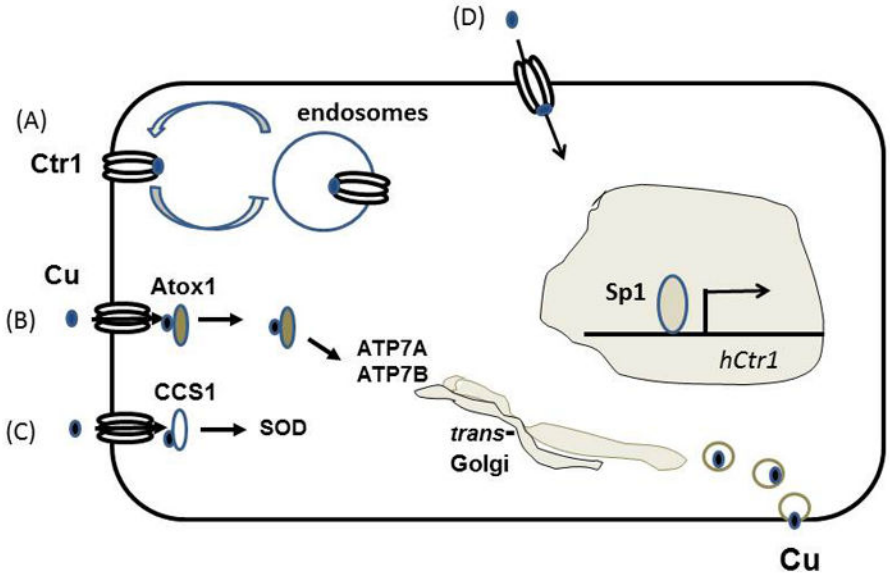

Figure 1. The shared import and export mechanisms of Cu and platinum drugs

(A) hCtr1 transports Cu^{+1} by endocytosis, (B) The Cu chaperone Atox1 captures Cu by interacting with hCtr1. Cu is then transferred to ATP7A/ATP7B for eliminating by secretory vesicles. (C) The Cu chaperone CCS captures Cu by interacting with hCtr1. (D) hCtr1 may directly transport Cu into the cells.

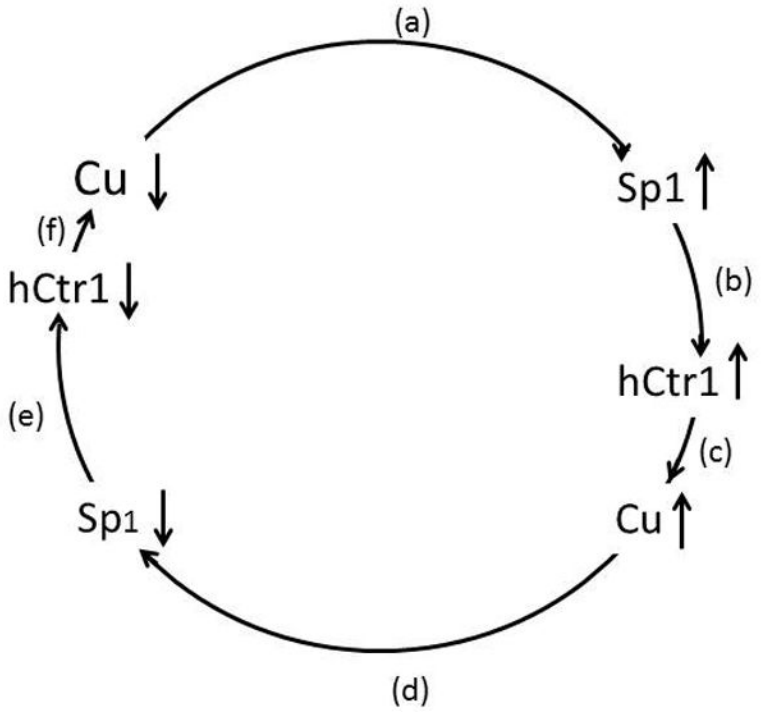

Figure 2. Schematics of Cu homeostasis regulation

(a) Cu depletion upregulates Sp1. (b) Upregulated Sp1 increases hCtr1 expression, (c) Increased hCtr1 enhances Cu transport. (d) Increased Cu suppresses hCtr1 expression. (e) Reduced Sp1 down-regulates hCtr1, resulting in reduced Cu (f).