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New uses for old copper-binding drugs: converting the pro-angiogenic copper to a specific cancer cell death inducer

Di Chen and Q Ping Dou[†]

Wayne State University, The Prevention Program, Barbara Ann Karmanos Cancer Institute, Department of Pathology, School of Medicine, Detroit, Michigan, USA

Abstract

Background—The conventional approach toward anticancer drug development is a time-consuming and expensive procedure.

Objective/methods—One approach to expedite this process and achieve more affordable means is to discover new applications of existing drugs, since their pharmacokinetics and pharmacological profiles are well known.

Results—Our encouraging findings in recent studies reveal anticancer activities of several copper-binding ligands including disulfiram (an antialcoholism drug), clioquinol (used to treat Alzheimer's and Huntington's diseases) and diethyldithiocarbamate (an agent for HIV-1 infection treatment).

Conclusion—These *in vitro* and *in vivo* studies have demonstrated that these archaic drugs can target and react with tumor cellular copper, forming complexes that act as potent proteasome inhibitors and apoptosis inducers.

Keywords

Antitumor activity; clioquinol; copper; disulfiram; dithiocarbamate; proteasome inhibitors

1. Introduction

Copper (Cu) is one of the essential trace metals for all organisms from yeast to human beings [1,2]. Cu plays an important role in eukaryotes as a redox cofactor for many Cu-dependent enzymes and proteins since it exists at both oxidized [Cu(II)] and reduced [Cu(I)] states [2]. On the other hand, Cu can also be very toxic due to generation of hydroxyl radicals, free radical species that can damage DNA, proteins and other cellular components [3]. Because Cu is both essential for life and potentially highly toxic, it is critical for all organisms to acquire sufficient levels of Cu for their biological functions but not to accumulate it at levels that are potentially toxic [4,5].

Cu homeostasis in humans is tightly regulated by different systems. After absorption from the gastrointestinal system, more than 90% of Cu in serum is carried by the Cu carrier ceruloferrin and < 10% is associated with albumin [6]. Generally, Cu transport in the blood is its primary mode of transit, where it is destined for cells that require Cu. Cu uptake by cells is through Cu transporter, Ctr1 [7], which is located on the cell membrane and

recognizes only reduced Cu (Figure 1). Therefore, in order to be transported into the cells by Ctrl, Cu(II) has to be reduced to Cu(I) by metalloreductase, Steap protein [8]. Within a cell there are various Cu chaperones that receive Cu and deliver it to specific locations [9]. In the cytosol, the copper chaperone for superoxide dismutase (Ccs1) delivers Cu to superoxide dismutase 1 (SOD1) [10,11]. The chaperone COX17 delivers Cu to mitochondria for binding to subunits of cytochrome C oxidase [12]. The efflux of Cu is via the Cu-transporting ATPases, ATP7A and ATP7B [13] on the Golgi and cell membrane (Figure 1). The extra Cu in the body is predominantly eliminated through the bile and urine [14].

2. Role of Cu in angiogenesis and cancer progression

High serum and tissue levels of Cu were found in many types of human cancers including breast [15–17], prostate [18,19], colon, lung [20], brain [21] and carcinomas of the digestive organs, especially those in advanced stages and metastasis [22]. However, the reasons for this elevation were unclear. In 1980, it was first noticed that Cu played an important role in angiogenesis [23]. In normal adult tissues, there is no action of angiogenesis except for wound healing, inflammatory reaction or the menstrual cycles [24]. Tumors are dependent on angiogenesis for their growth, invasion and metastasis, a novel concept proposed by Dr Folkman in 1971 [25,26]. It has been shown that tumors can not grow larger than 1 to 2 mm³ without forming new blood vessels [27]. Encouraged by these findings, scientists started to explore the essential factors for angiogenesis and found that in the cornea of rabbits' eyes, the cornea could develop new capillaries when the action of angiogenesis effectors became rich in the Cu ions [28]. Furthermore, three molecules, ceruloplasmin, heparin and glycyl-L-histidyl-L-lysine were tested and results showed that these molecules could induce angiogenesis when they were bound to the Cu ion [28]. Moreover, the results from the cell culture studies showed that Cu could stimulate proliferation and migration of human endothelial cells [29].

Vascular endothelial growth factor (VEGF) is a glycoprotein and a key regulator of angiogenesis. VEGF pathway activation results in growth promotion, migration and differentiation of endothelial cells from existing blood vessels (Figure 1) [30]. Cu could dramatically induce VEGF protein and its mRNA expression that was demonstrated in cell cultures and animal models [31,32]. Recently, additional findings demonstrate that tumor growth and metastasis depend upon angiogenesis [33,34] that requires growth factors, proteases, and trace element Cu. Copper, but not other transition metals, is a co-factor required for several angiogenic mediators including VEGF [32], basic fibroblast growth factor (bFGF) [35], IL-1 (IL-1) and IL-8 (IL-8) [36], which are essential for tumor angiogenesis processes (Figure 1) [37–40].

Based on the biological function of Cu in tumor progression, the effort to block angiogenesis by Cu chelators was performed on animal models. Two Cu chelators, trientine and penicillamine, were used to treat mice bearing hepatocellular carcinoma xenografts [41]. The results showed that both Cu chelators significantly suppressed the tumor growth associated with suppression of tumor angiogenesis [41]. Another Cu-chelator, tetrathiomolybdate (TM) developed as an effective anticopper remedy for the treatment of Wilson's disease, also showed significant antiangiogenic and antitumor effect in animal models with human squamous cell carcinoma xenografts [42].

In clinical trials, the approach to block angiogenesis via Cu chelation by TM showed positive results. In order to evaluate whether the reduction of serum Cu level could result in anemia, the most frequent side effects of Cu deficiency, a Phase I clinical trial with TM administrated to the patients with metastatic cancer was performed. TM was nontoxic since when serum ceruloplasmin (Cp), a biomarker for total body Cu, was reduced to 15 – 20% of

baseline. Meanwhile, the hematocrit was maintained without reducing below 80% of baseline [43]. One of the Phase II clinical trials with TM was carried out in thirty four patients with cytoreduced malignant pleural mesothelioma (MPM) in Karmanos Cancer Institute (Detroit). After 34 days of treatment with TM (180 mg/day), the level of Cp in all patients was reduced from 45.2 ± 2 to 13 ± 2 mg/dl, and VEGF decreased significantly from an average of 2086 to 1250 pg/ml [44]. Another Phase II trial demonstrated that TM could lead to stable disease for a median of 34.5 weeks in 31% of the advanced kidney cancer patients and TM was well tolerated and consistently decreased the copper levels in the patients. At the time of reaching Cu deficiency, the levels of VEGF, bFGF, and IL-8 were significantly reduced from pretreatment levels, which may correlate with Cu depletion but not with disease stability [45].

3. Ubiquitin/proteasome pathway and proteasome inhibitors for cancer treatment

The major function of the ubiquitin/proteasome pathway is to degrade intracellular unneeded proteins that are crucial for the control of many processes, including gene transcription, signal transduction, cellular differentiation, proliferation, cell cycle progression and apoptosis [46–49]. Since the milestone discovery of the ubiquitin-proteasome pathway, the 2004 Nobel Prize in Chemistry was awarded to Drs Aaron Ciechanover, Avram Hershtko and Irwin Rose for their discovery of ubiquitin-mediated protein degradation [50,51].

A protein marked for degradation by attaching a chain of multiple ubiquitin molecules is recognized by 26S proteasome, which is a large multisubunit complex localized in the cytosol and nucleus [52]. The 26S proteasome is composed of a 20S catalytic core and two 19S regulatory caps on both ends of the 20S core. The 19S proteasome is involved in binding and unfolding of ubiquitinated proteins, and opening the alpha subunit gate of the 20S proteasome to allow for entry into the catalytic core [53–55]. The 28 subunits in the 20S core are arranged in four heptameric rings (α , β , β , α) to form a cylindrical structure, in which the α -subunits make up the two outer rings, and the β -subunits make up the two inner rings. At least three primary proteolytic activities are confined to the β -subunits including chymotrypsin-like (cleavage after hydrophobic amino acids, mediated by the β_5 subunit), peptidylglutamyl peptide hydrolyzing-like or PGPH-like (cleavage after acidic residues, mediated by the β_1 subunit), and trypsin-like (cleavage after basic residues, mediated by the β_2 subunit) activities [53]. It is important to mention that inhibition of the proteasomal chymotrypsin-like activity is associated with induction of apoptosis in cancer cells [56,57]. It has been reported that since cancer cells are more dependent on the ubiquitin-proteasome pathway for their increased proliferation, the protein, mRNA and activity levels of proteasome are much higher in malignant cells than in normal cells [58]. Based on these differences, it becomes increasingly more important to develop proteasome inhibitors as selective anticancer drugs [46,59].

Bortezomib (Velcade, PS-341) is the first proteasome inhibitor approved by the US Food and Drug Administration (FDA) for the treatment of multiple myeloma [60–62]. In different animal studies, Bortezomib suppresses tumor growth and angiogenesis in solid tumors, including breast cancer, prostate cancer, lung cancer, neuroblastoma and mesothelioma [63–66]. The clinical trials using Bortezomib alone or in combination with various anticancer agents in Phase I, II and III showed favorable responses. There were encouraging results in non-Hodgkin lymphoma, acute myeloid leukemia, and multiple myeloma patients, in terms of response rate, time to progression, and survival [67–69].

4. New applications of Cu binding compounds for potential cancer treatment

A novel idea was born based on the findings that high plasma and tissue levels of Cu were found in many types of cancer patients, and Cu was a co-factor essential for the processes of tumor angiogenesis. It was a promising example and exciting beginning in this field that TM was used in clinical trials to reduce Cu level and inhibit tumor progression in cancer patients [44]. However, the results showed that TM was a strong Cu chelator but its formed complex was inactive in cell killing ability for cancer cells [70].

Our laboratory started to search for novel proteasome inhibitors several years ago. Through screening National Cancer Institute (NCI)–Diversity Set Library that contains 1,990 compounds generated from more than 140,000 synthetic and pure natural compounds in The NCI's Pure Chemicals Repository, we found that NSC-109268, an organic Cu compound (Figure 2), was able to inhibit the proteasome activity in purified 20S proteasome and human prostate cancer cells [71]. Based on the chemical structure of NSC-109268, we tested more Cu-binding ligands and found that the tested ligands could react with Cu forming complexes that induce proteasome inhibition and apoptosis in a variety of human cancer cells. These ligands include 8-Hydroxyquinoline (8-OHQ), tetraethylthiuram disulfide or disulfiram (DSF), diethyldithiocarbamate (DDTC), clioquinol (CQ), and pyrrolidine dithiocarbamate (PDTC). Their chemical structures are shown in Figure 2.

4.1 Drugs in dithiocarbamate family

DSF, DDTC and PDTC are members of the dithiocarbamate family and Cu chelators. DSF, an irreversible inhibitor of aldehyde dehydrogenase, is one of the two drugs approved by the FDA for treatment of alcoholism [72,73]. Clinical trials have shown the efficacy of DSF without toxicity [72]. The chemical structure shows that DSF possesses an ability to react to Cu with its thiol groups. The redox conversion of DSF is unique to Cu(II) but not suitable to other common biological metal ions Fe(II or III) and Mn(III) [74]. It has been reported that DSF could be rapidly converted to its bis (diethyldithiocarbamate) Cu complex during its absorption in gastrointestinal system [72]. Therefore, during absorption into blood, DSF might involve both the parent drug, DSF, and its Cu complex and both forms are degraded to DDTC rapidly [72]. DDTC is a Cu chelator as well [75]. In clinic trials, DDTC was used in patients with HIV-1 infection and found to delay progression to AIDS. DDTC could promote T cell maturation in animal models, reduce lymphadenopathy and improve survival in a murine AIDS model [76,77]. PDTC is a stable pyrrolidine derivative of dithiocarbamates and an antioxidant. Previous studies have shown that PDTC strongly inhibits replication of human rhinoviruses [78] and coxsackie-virus B3, one of the most common pathogens for human viral myocarditis [79]. PDTC also showed inhibitory ability against murine colon adenocarcinoma bearing mice through the inhibition of nuclear factor B in the tumor tissue [80].

To verify the hypothesis that Cu can be used as a novel, selective target for human cancer therapies, we examined the effect of DSF, DDTC and PDTC on human breast and prostate cancer cells and found that in the presence of Cu, all of them could inhibit proteasomal activity and induce apoptosis in cancer cells. However, without Cu present in the same testing systems the effect of these ligands was only minor.

We tested DSF in *in vitro* and *in vivo* studies using human breast cancer MDA-MB-231 and MCF10DCIS.com cell lines and compared the effect of both Cu chelators DSF and TM. We observed inhibition of proteasome activity and induction of apoptotic cell death only after

the treatment of DSF–Cu complex, but not with DSF alone, TM alone or TM–Cu complex [70].

It should be mentioned that tumor tissues contain high levels of Cu, while cultured cancer cells contain very low to undetectable Cu levels. In order to mimic the Cu-enriched *in vivo* condition of cancer tissues, we cultured MDA-MB-231 cells in medium containing 25 $\mu\text{mol/l}$ CuCl_2 for three days. The medium was then replaced with regular medium without additional Cu before the cells were treated with DSF or TM alone. Similar to DSF–Cu complex, DSF alone but not TM, was able to inhibit the proteasome activity and induced apoptotic cell death in Cu-enriched MDA-MB-231 cells [70]. To answer the question whether the DSF–Cu complex could inhibit the proteasome activity and induce apoptosis selectively in breast cancer over normal cells, we chose MCF-10A, a spontaneously immortalized human breast epithelial cell line, as a comparison to MCF10DCIS.com line. It was found that DSF–Cu complex had little proteasome-inhibitory and apoptosis-inducing effect in MCF-10A cells [70].

Next question we set out to answer was whether DSF could react with Cu in tumor and convert the pro-angiogenic Cu to an anticancer complex *in vivo*. We treated the mice bearing human breast MDA-MB-231 xenografts with DSF (50 mg/kg) and found that DSF indeed significantly inhibited the tumor growth by 74%. The inhibition of proteasome activity and induction of apoptosis were observed in the tumor tissues treated with DSF [70]. Our findings demonstrated that i) DSF was a potent proteasome inhibitor and apoptosis inducer *in vitro* and *in vivo* only when Cu was present; ii) DSF–Cu complex selectively inhibited proteasome activity and induced apoptosis in cancer but not normal cells; and iii) DSF had advantage over TM because DSF–Cu but not TM–Cu, complex had proteasome inhibitory and antitumor activities.

It has been reported that DSF could inhibit cancer cell invasion by interacting with matrix metalloprotease 2 (MMP-2) and MMP-9 and inhibiting their proteolytic activities via chelating zinc [81]. An observation from another group suggested that antitumor activity of DSF in melanoma and hepatic tumor could be potentiated by Zn^{2+} supplementation [82]. We found that a DDTC-zinc complex was also a proteasome inhibitor although its potency was weaker than the DDTC–Cu complex [83]. Our results have further confirmed the findings of other researchers and also demonstrated the requirement of proteasome inhibition for the antitumor activity of DSF. We also tested human breast cancer cells with PDTC–Cu [84] and both breast and prostate cancer cells with DDTC–Cu [75]. The results showed that both Cu complexes were potent proteasome inhibitors and apoptosis inducers.

4.2 Drugs in hydroxyquinoline family

Clioquinol (5-chloro-7-iodo-8-hydroxyquinoline, CQ) (Figure 2), an 8-hydroxyquinoline derivative, is a therapeutic drug for treatment of Alzheimer's disease [85] and Huntington's disease [86]. Two clinical trials using CQ for Alzheimer's disease showed no overall toxicity in all patients and clinical benefit in some patients [87,88]. Early in 1964, CQ was successfully used in treatment and prevention of shigellosis, caused by *Shigella* infection, and amebiasis that was an *entamoeba histolytica* infection [89]. This report included 4000 individuals and over a 4-year period, and showed few side effects [89]. Although CQ use was thought to be associated with the occurrence of subacute myelo-optic neuropathy in Japan [90–92], this conclusion was not supported by the subsequent epidemiologic analysis [93]. Instead, decreased levels of vitamin B_{12} may play a role in this syndrome [92]. CQ is also a lipophilic compound capable of forming stable complexes with Cu(II) ions [90]. Indeed, when we mixed a solution of CQ with a solution of CuCl_2 at 1:1 molar ratio, dramatic color change was observed, indicating that a chemical reaction had occurred and resulted in CQ–Cu complex formation had occurred [94]. To further verify that the color

change of the CQ–Cu mixture indicates a formation of a stable CQ–Cu complex, a series of samples including CQ, CuCl₂ and a mixture of CQ and CuCl₂, were analyzed by X-ray absorption near-edge spectroscopy (XANES) and extended X-ray absorption fine structure spectroscopy (EXAFS). The results showed that the CQ–Cu mixture had a different Cu oxidation state from those of CuCl₂ and metal Cu, confirming that a chemical reaction occurred between CQ and Cu [94].

We tested the CQ–Cu complex in human prostate cancer LNCaP (androgen-dependent) and C4-2B (androgen-independent) cells. The results showed that inhibition of proteasome activity, reduction of androgen receptor (AR) expression, suppression of cell proliferation, and induction of apoptotic cell death were all observed in both cell lines treated with CQ–Cu complex but not with CQ alone [94]. Furthermore, animal studies of mice bearing human C4-2B xenografts showed that treatment with CQ (10 mg/kg) for 30 days resulted in: i) significant inhibition of tumor growth (66%) compared to the control; ii) proteasome inhibition in CQ-treated C4-2B tumors tissues; iii) induction of apoptosis; iv) suppression of AR expression; and v) inhibition of angiogenesis indicated by decreased immunohistochemistry of the cluster of differentiation 31 (CD31), an endothelial marker in blood microvessels, in CQ-treated tumor tissues [94].

5. Conclusion and future direction

It is an important strategy to discover new applications for old drugs because development of a new drug takes approximately 15 years and \$800 million [95]. Like Chong *et al.* addressed in their article ‘the most fruitful basis for the discovery of a new drug is to start with an old drug. Because existing drugs have known pharmacokinetics and safety profiles and are often approved by regulatory agencies for human use, any newly identified use can be rapidly evaluated in Phase II clinical trials, which typically last two years and cost \$17 million’ [96].

High proteasome activity and high concentration of Cu are unique features of cancer cells. Our studies have demonstrated that these features can be used as specific targets for cancer chemotherapy. Our findings opened a new avenue for researchers to discover more novel Cu binding ligands from varieties of compounds and existing drugs based on: i) the compound itself should be cell permeable and nontoxic; ii) it has ability to react with cellular Cu; and iii) the formed complex with Cu has antiproteasome and/or antitumor effect. DSF, DDTC, PDTC and CQ have presented their great application in potential treatment of cancer since they are able to suppress tumor development in multiple ways. We have studied these old copper-binding drugs in various human tumor cell systems and animal models and our next goal is to further verify their anticancer efficacy and mechanisms of action in clinical trials.

6. Expert opinion

The following four lines of research suggest that Cu could be used as a novel, selective target for human cancer therapies: i) Cu but not other metals, is a co-factor essential for the processes of tumor angiogenesis; ii) high tissue levels of Cu have been found in many types of human cancers, including breast, prostate, colon, lung and brain; iii) significant decrease in Cu levels in mammalian organs does not cause detectable side effects; and iv) in clinical trials using Cu chelator TM for patients suffering from metastatic cancers achieved the Cu-deficiency and stabilization of disease in a large portion of the patients, demonstrating the clinical feasibility.

We have found that some organic Cu complexes can selectively inhibit the activities of purified 20S proteasomes and cancer cellular 26S proteasomes. Inhibition of tumor cellular proteasome activity by these Cu complexes results in induction of apoptosis. Furthermore, a

Cu-binding ligand alone can induce proteasome inhibition and apoptosis in Cu-enriched human cancer cells that mimic *in vivo* situations of many human tumors. Some of the Cu ligands that we tested include four old Cu-binding drugs, DSF, DDTC, PDTTC and CQ. All of them are able to interact with Cu, forming complexes with potent proteasome inhibitory and apoptosis-inducing abilities in tumor cells *in vitro* and *in vivo*. This identified mechanism of action of DSF and CQ may be responsible for their observed anticancer activities. Therefore, such organic Cu compounds form a new class of proteasome inhibitors. It is known that the Cu chelator drug TM, designed to reduce Cu levels, is a relatively effective chemotherapeutic agent for cancer patients. However, the formed TM–Cu complex is an inactive proteasome inhibitor, which is different from the DSF–Cu, DDTC–Cu or CQ–Cu complexes.

The potential advantage for using the old Cu-binding drugs DSF, DDTC and CQ for cancer therapies is apparent. Due to the fact that Cu concentrations are elevated in cancer but not normal cells, DSF or CQ should have more selective effect against cancer and can bind the endogenous Cu in tumors to form a Cu-based proteasome inhibitor. Due to the difference of Cu levels in tumor and normal tissues, it is possible that these compounds may have little or no toxicity to normal cells while maintaining their anticancer activity.

These studies using old Cu-binding drugs provided strong support for proof-of-concept of converting the pro-angiogenic cofactor Cu in cancer cells to the antiangiogenic proteasome inhibitor and a cancer cell death inducer. These studies also helped identifying the new mechanism of action of the old Cu-binding agents as potential anticancer drugs, which should have great significance in developing novel strategies for the prevention and treatment of human cancer. If successful, these old Cu-binding drugs could be immediately moved to clinical trials to determine their efficacy and toxicity.

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Declaration of interest

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Figure 1.

A diagram to show that copper (Cu) is transported into a cell by Cu transporter and targets a variety of chaperones, and the stimulation of angiogenesis by Cu can be overcome by certain Cu ligands through inhibition of proteasome activity.

NCI-109268

8-OHQ

DSF

DDTC

CQ

PDTC

Figure 2. Chemical structures of NSC-109268

8-OHQ: 8-Hydroxyquinoline; CQ: Clioquinol; DDTC: Diethyldithiocarbamate; DSF: Disulfiram; PDTC: Pyrrolidine dithiocarbamate.