

New applications of old metal-binding drugs in the treatment of human cancer

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TABLE OF CONTENTS

1. Abstract
2. Introduction
 - 2.1. The use of metal complexes for cancer treatment
 - 2.2. Ruthenium-based complexes as potential anticancer agents
3. Importance of metals in the growth and progression of cancer
 - 3.1. Copper
 - 3.2. Zinc
4. Ubiquitin-proteasome pathway
5. Dithiocarbamates
 - 5.1. Gold Dithiocarbamates
 - 5.2. Copper and zinc-containing dithiocarbamates
 - 5.2.1. Disulfiram, EtDTC, and PyDTC
 - 5.2.2. Synthetic EtDTC copper and zinc complexes
 - 5.2.3. Synthetic PyDTC copper and zinc complexes
6. Hydroxyquinolines
 - 6.1. Clioquinol
 - 6.2. 8-Hydroxyquinoline analogs
7. Conclusions
8. Acknowledgements
9. References

1. ABSTRACT

Significant advances in the use of metal complexes, precipitated by platinum, have fostered a renewed interest in harnessing their rich potential in the treatment of cancer. In addition to platinum-based complexes, the anticancer properties of other metals such as ruthenium have been realized, and ruthenium-based compounds are currently being investigated in clinical trials. Since the process of drug development can be expensive and cumbersome, finding new applications of existing drugs may provide effective means to expedite the regulatory process in bringing new drugs to the clinical setting. Encouraging findings from laboratory studies reveal significant anticancer activity from different classes of metal-chelating compounds, such as disulfiram, clioquinol, and dithiocarbamate derivatives that are currently approved for the treatment of various pathological disorders. Their use as coordination complexes with metals such as copper, zinc, and gold that target the ubiquitin-proteasome pathway have shown significant promise as potential anticancer agents. This review discusses the unique role of several selected metals in relation to their anti-cancer properties as well as the new therapeutic potential of several previously approved metal-chelating drugs. *In vitro* and *in vivo* experimental evidence along with mechanisms of action (e.g., *via* targeting the tumor proteasome) will also be discussed with anticipation of strengthening this exciting new concept.

2. INTRODUCTION

2.1. The use of metal complexes for cancer treatment

The development of metal-based complexes for the treatment of cancer began with the discovery of the anti-cancer properties of cisplatin in the early 1960s (Figure 1). Over 90% of testicular cancer cases have been cured by cisplatin, and it has also been important in the treatment of several other types of cancer, including ovarian, cervical, bladder, head and neck, melanoma, and lymphomas (1). Cisplatin interacts with DNA and forms adducts which interfere with the replication and transcription processes, and ultimately triggers apoptosis (2). These cisplatin-DNA interactions have been extensively studied, and it has been clearly shown that a (Pt)-GG intrastrand cross-link is responsible for the cytotoxicity of cisplatin (3). However, the toxicities associated with cisplatin, coupled with intrinsic and acquired drug resistance, have hampered its widespread clinical use (4-5).

The limitations of cisplatin have stimulated the search for new, less toxic platinum-based drugs. Second and third generation platinum-based drugs, including carboplatin and oxaliplatin (Figure 1), have been developed as alternatives to increase efficacy and offset the toxicity associated with cisplatin (6). Carboplatin is an effective treatment for ovarian, lung, and head and neck cancers (7), and oxaliplatin is clinically approved for the treatment of cisplatin-resistant colorectal cancer (8). The investigation

Metal compounds as tumor proteasome inhibitors

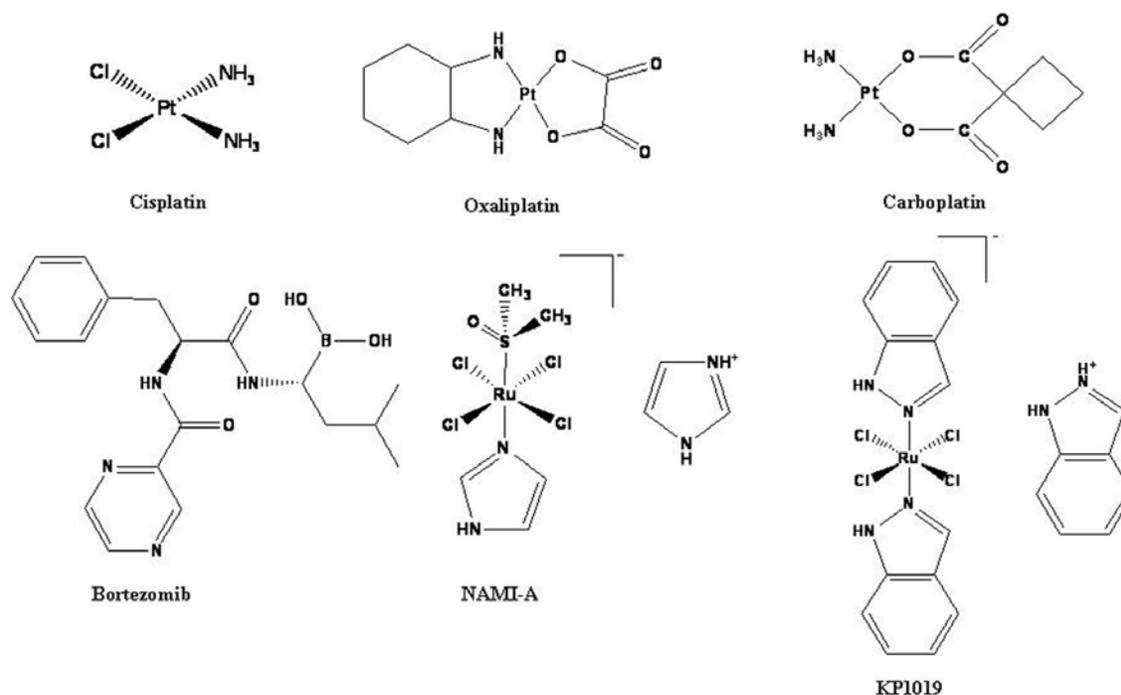


Figure 1. Chemical structures of platinum- and ruthenium-based drugs, as well as the proteasome inhibitor bortezomib, used in the treatment of human cancers. Chemical structure of bortezomib, the first proteasome inhibitor clinically tested is shown. Cisplatin, oxaliplatin, and carboplatin are platinum-containing drugs used for the treatment of human cancers. NAMI-A and KP1019 are ruthenium-based drugs under investigation for the potential treatment of human cancers.

into other metal-based drugs began by the rational design of complexes that are structurally similar to cisplatin, the theory being that similar structure leads to similar function (Figure 1) (9). However, the activities of these metal compounds are not solely governed by the presence of the metal itself, but can be highly influenced by the oxidation state, number and type of ligand bound, and the coordination geometry of the complex. Other properties that can play a pivotal role in the biological activity of metal complexes include kinetic lability, redox behavior, and electric charge. These unique properties have prompted investigation into metal-based complexes as potential anticancer agents that present with various mechanisms of action.

2.2. Ruthenium-based complexes as potential anticancer agents

The severe side effects and resistance associated with platinum-based drugs have spurred a search for non-platinum metals that may decrease the toxicities associated with metal-based drugs. Because it is a transition metal of group 8B of the periodic table, the same group as platinum, ruthenium's anticancer effects were originally believed to be exerted by direct binding to DNA, the same mechanism by which platinum agents cause cell death. However, it has been demonstrated that ruthenium exhibits many distinct properties at the cellular level compared to platinum. First, ruthenium seems to preferentially accumulate in malignant cells rather than normal cells, possibly by utilizing a transferrin-mediated mode of transport (10). Additionally, prior to reaching the tumor mass, ruthenium remains in its

inactive Ru (III) oxidation state. The lower oxygen level and higher acidity of the tumor environment reduce the ruthenium to its more reactive Ru (II) state (11). It has also been found that some ruthenium complexes display greater efficacy toward tumor cell metastases rather than acting on primary tumors. This is believed to be due to inhibition of tumor cell detachment, invasion and migration, and re-adhesion to new growth substrate (12). In light of these properties, ruthenium is predicted to show distinct patterns of anti-tumor activity that diverge from those demonstrated with platinum.

Currently, two ruthenium-containing complexes are undergoing clinical trials, NAMI-A and KP1019 (Figure 1) (13-14). Despite their structural similarities, these ruthenium (III) complexes differ drastically in their antitumor activities. Pre-clinical studies have demonstrated that NAMI-A is able to inhibit the formation of metastases in various animal tumor models but shows no direct cytotoxic effects (15-16). KP1019 however, has shown direct antitumor effects against a wide variety of tumor xenografts through induction of apoptosis (17-18). Despite encouraging preclinical and clinical data, the complete mechanisms of action still remain unresolved.

3. IMPORTANCE OF METALS IN THE GROWTH AND PROGRESSION OF CANCER

3.1. Copper

The discovery that copper levels in tumor-bearing mice and humans are altered (19-20) led to extensive

Metal compounds as tumor proteasome inhibitors

studies regarding the role of copper in carcinogenesis. High serum and tissue levels of copper have been observed in a variety of human tumors including brain (21), breast (22-23), colon (24), lung (25), and prostate (24, 26). In 1980, it was first noticed that Cu played a critical role in angiogenesis (27). Gullino *et al* found that in the corneas of rabbits' eyes, new capillaries developed when angiogenesis effectors became rich in Cu ions (28). Results from cell culture studies showed that Cu could stimulate proliferation and migration of human endothelial cells (29). Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis. VEGF can stimulate growth promotion, migration and differentiation of endothelial cells from existing blood vessels (30). Studies in cell cultures and animal models have demonstrated that Cu is able to induce VEGF mRNA transcription and protein expression (31-32). Copper, but not other metals, is a co-factor required for several angiogenic mediators including VEGF (32), basic fibroblast growth factor (bFGF) (33), interleukin 1 (IL-1) and interleukin 8 (IL-8) (34), all of which are essential regulators for tumor angiogenesis (35-38). Tumors are dependent on angiogenesis for their growth, invasion and metastasis (39-40), and Cu plays an important role in this process. Due to the importance of angiogenesis and copper to tumor development, the use of copper chelators for antiangiogenic therapy has emerged as an interesting concept in cancer therapeutics (41-42).

3.2. Zinc

Like copper, zinc also plays an important role in many cellular processes, including proliferation and differentiation, as well as defense against free radicals (43-44). Zinc is also a structural component in various proteins and enzymes such as transcription factors, cell signaling proteins, and DNA repair enzymes (45-46). Additionally, a critical role has been suggested for zinc in apoptosis (47-49). However, this effect appears to be complex, and no firm conclusions have been established. For example, in prostate and ovarian epithelial, as well as glial cells, zinc is pro-apoptotic, while in breast, HeLa, renal, and lung epithelial cells, as well as macrophages, zinc is anti-apoptotic (49-50). Altered Zn levels have been found to be associated with certain systemic abnormalities such as the development of cancer (51). Although Zn levels are often compromised in cancer patients, a firm relationship between cancer development and Zn has not been proven, and seems dependent on tumor type (49, 52-53). Low levels of zinc have been observed in several malignancies, such as those of the liver, gallbladder, digestive tract, and prostate (54-56). Conversely, both high and low levels of zinc have been found in breast cancers (54, 57-58).

Consequently, it is no surprise that an association between zinc transporter levels and cancer progression has also been proposed (51, 59). Multiple zinc transporters, including ZIP4, ZIP6, ZIP10, and ZIP1, have been identified as factors in the progression of various types of cancer. ZIP4 has been reported to increase cell proliferation through zinc transport, resulting in tumor growth, most specifically in pancreatic cancer (44, 55). Both ZIP6 and ZIP10 have roles in the progression and metastasis of breast cancer (46, 57-58) and ZIP1 has been suggested as a

tumor suppressor of prostate cancer (56). Thus, by disrupting the distribution of zinc in tissues, altered levels of zinc transporters may enhance the development of various tumors, indicating the potential of zinc as an anticancer agent.

4. UBIQUITIN-PROTEASOME PATHWAY

The ubiquitin-proteasome pathway (UPP) is so important to normal cellular function that, in 2004, the Nobel Prize in Chemistry was awarded to its discoverers (60-61). The ubiquitin-proteasome pathway (Figure 2) is responsible for selective proteolytic processing of proteins involved in various biological processes, such as development, differentiation, proliferation, signal transduction, and apoptosis (62). There are two critical steps in the ubiquitin-proteasome pathway: (i) conjugation of multiple ubiquitin molecules to the target protein, and (ii) degradation of the tagged protein by the 26S proteasome (63). The 26S proteasome is a large (2.5 MDa), multi-subunit complex found in the nucleus and cytosol of cells and consists of a catalytic core, the 20S proteasome, and two recognition sites, the 19S regulatory caps (Figure 2) (64-65). The 20S core is made up of four stacked rings, two non-catalytic alpha rings (seven subunits each) outside of two catalytic beta rings (seven subunits each), that form a barrel-like structure, consisting of 28 subunits total (66-68). While the function of the alpha subunits is to block direct access to the proteasomal active site by allowing access only to unfolded proteins, the beta subunits are responsible for the proteolytic activities of the proteasome (68). The active beta subunits are beta-1, beta-2, and beta-5, which are responsible for the caspase or peptidyl-glutamyl peptide-hydrolyzing (PGPH)-like, trypsin-like, and chymotrypsin (CT)-like activities, respectively (68-69). Each active subunit contains a Thr1 active residue at the amino terminal, which is responsible for catalysis. It is this active site that can be targeted by some proteasome inhibitors (such as Bortezomib) (Figure 1) through nucleophilic attack (67, 70). Additionally, the 19S regulatory particle (700 kDa) contains six ATPase and at least eight non-ATPase subunits, which are required for recognition, deubiquitination, unfolding, and translocation of tagged proteins prior to degradation by the 20S proteasome (71-72).

The ubiquitination step of the UPP is typified by three different enzymes, E1, E2, and E3. The UPP pathway is initiated by ATP-dependent E1-mediated activation of ubiquitin, a 76 amino acid protein that is expressed ubiquitously and serves as a tag for target proteins destined for degradation by the UPP (Figure 2). Transfer of activated ubiquitin from E1 to E2, which is responsible for conjugation of ubiquitin, and E3, the ubiquitin-ligating enzyme, which then facilitates the transfer of active ubiquitin to lysine residues of the target protein (73-74). The ubiquitin-tagged target protein is then transported to the 26S proteasome, where degradation occurs and the ubiquitin is released for recycling (Figure 2) (75). This is a tightly regulated process and is important for the regulation of several cellular processes, including those involved in tumorigenesis (76).

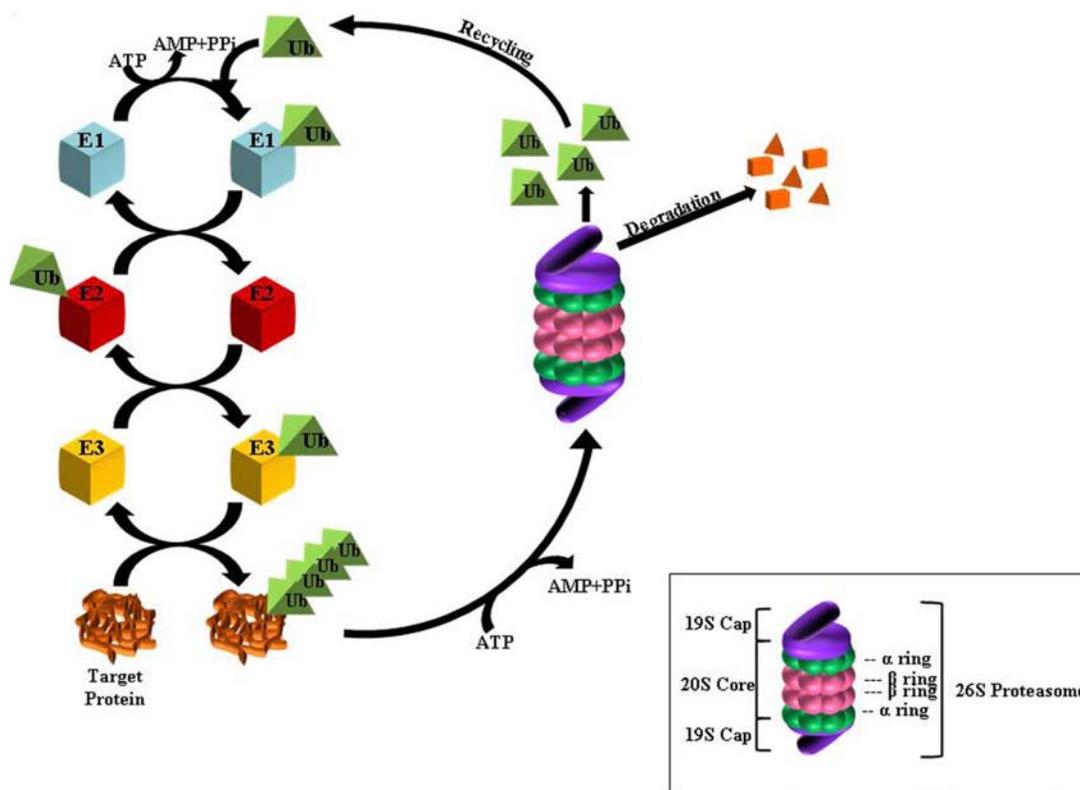


Figure 2. Schematic representation of the ubiquitin-proteasome pathway (UPP). The UPP is a highly regulated ATP-dependent pathway, which is vital for the processing of intracellular proteins. It is also a promising target for anticancer therapeutics, and many metal-based or metal-binding compounds, such as CQ, DSF and Au-complexes, have been shown to be potent inhibitors of the proteasomal activity.

Because of the essential role that unbalanced protein homeostasis plays in the development, growth, and survival of cancer (77), targeting factors responsible for the synthesis and degradation of proteins as an anticancer strategy has been investigated (78). Increased proteasome activity has been observed in various malignancies, such as prostate (79), colon (80), and leukemia (81), indicating that cancer cells are more dependent on the ubiquitin-proteasome pathway than normal cells and that targeting the UPP is a viable option in the treatment of human cancer. Importantly, inhibition of the CT-like activity of the tumor proteasome is associated with cell cycle arrest and induction of apoptosis (82-83), suggesting that proteasome inhibition may be effective in not only selectively killing cancer cells with minimal effect on healthy cells but also in sensitizing resistant cancer cells to chemotherapy or radiotherapy (84).

Bortezomib (Velcade, PS-341), a dipeptide boronic acid derivative, was the first proteasome inhibitor approved for clinical use by the FDA (Figure 1). It demonstrates potent apoptosis-inducing ability in various cancer cell lines and animal models (85-87) and is currently used for the treatment of multiple myeloma and mantle cell lymphoma as well as other cancers (88-89). Bortezomib is a slowly reversible inhibitor that induces cell death through direct inhibition of the beta-5 proteasomal subunit (90),

which leads to suppression of NF- κ B activity, causing down-regulation of its target genes (85, 91). In a series of animal studies, bortezomib was shown to inhibit tumor growth and angiogenesis in various solid tumors, including prostate (92), lung, breast (93), mesothelioma (94), and neuroblastoma (95). Phase I, II, and III clinical trials showed favorable responses in NHL, AML, and MM patients to treatment with bortezomib alone or in combination with various chemotherapeutics and indicated a significant clinical benefit (96-98). While bortezomib is highly effective against several hematological malignancies, it has exhibited little activity toward solid tumors, and development of resistance has been observed. Furthermore, most recently, it has been found that the proteasome-inhibitory and anticancer activity of bortezomib and other boronic acid-based proteasome inhibitors can be blocked by green tea polyphenols *via* direct drug-drug interactions (99-100). These observations, along with the toxicities associated with bortezomib, have prompted the development of next generation proteasome inhibitors with a more favorable therapeutic profile and a broader spectrum of activity. Given the significant achievement of platinum-based anticancer therapy, the use of different metals, especially when complexed with previously approved medicinal compounds that target the ubiquitin-proteasome pathway has received considerable attention as a viable route in cancer treatment.

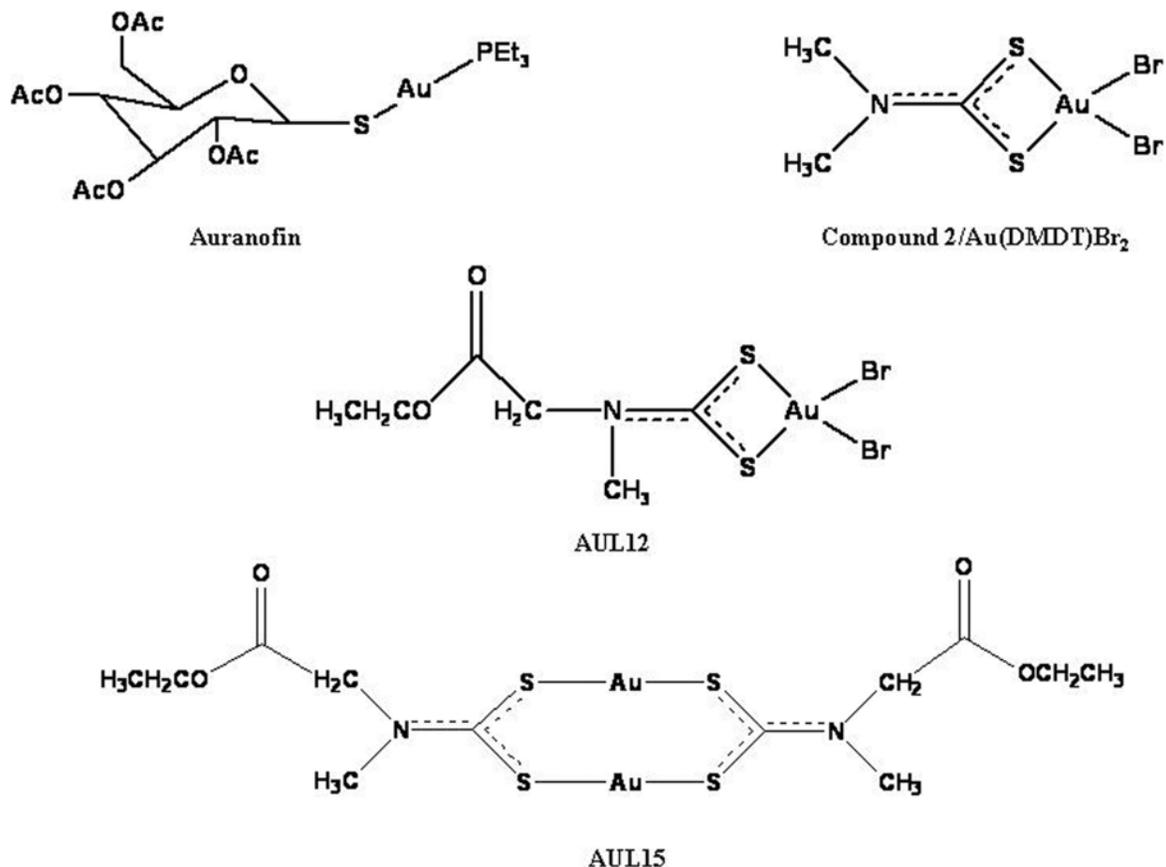


Figure 3. Chemical structures of gold-based compounds. Auranofin, one of the first clinically used gold-based drugs, prompted the search for novel gold- compounds that may be effective in the treatment of various pathological disorders such as cancer. Au(DMDT)Br₂, AUL12, and AUL15 are gold compounds with proteasome-inhibitory activity.

5. DITHIOCARBAMATES

One class of medicinally important metal-chelating compounds is dithiocarbamates. This class of medicine includes several drugs that have previously been approved for the treatment of various ailments, such as bacterial and fungal infections, as well as AIDS (101-102).

5.1. Gold dithiocarbamates

The medicinal applications of gold date back thousands of years, but its rational use did not begin until the early twentieth century when Robert Koch found that K (Au (CN)₂) could kill the tuberculosis bacteria. However, serious side effects were observed and the treatment for tuberculosis was changed to the less-toxic Au (I)-thiolate complexes. Jacque Forestier also used these gold complexes for the treatment of rheumatoid arthritis, and they remained the drug of choice for many years (103). The severe toxicity of these gold-based drugs prompted the search for novel, less toxic gold compounds. Because the coordination of gold (I) with phosphine ligands stabilizes the 1+ oxidation state, Au (I)-phosphine complexes have been investigated. This led to the discovery of auranofin (Figure 3), which, until only recently, was the drug of choice to treat rheumatoid arthritis (104).

In an effort to broaden the medicinal applications of gold, gold compounds have been investigated for potential anticancer activity. Gold (I) complexes, including auranofin analogs, were synthesized and potent cytotoxic activity against B16 melanoma and P388 leukemia cells was observed (105). Phosphine-gold (I) thiosugars were the most potent, and while anti-tumor activity against leukemia was seen *in vivo*, these analogs were completely inactive against solid tumors (106). Digold (I) phosphine complexes were also found to confer cytotoxic activity in cisplatin-resistant cells *in vitro*. This activity is thought to be due to the ability of these complexes to alter mitochondrial function and inhibit protein synthesis (107). Like the previous gold (I)-phosphine thiolate sugars, these complexes were also inactive against solid tumors. Digold (I)-phosphines did not enter clinical trials due to their severe cardiotoxicity (108).

Although their use as anticancer drugs was originally questioned due to high redox activity and poor stability, gold (III) complexes were also investigated. Because the cellular environment is generally reducing, Au (III) is expected to be reduced to Au (I) and metallic Au, making Au (III) complexes less effective (109). Interest in these complexes increased after Pt (II) complexes showed

Metal compounds as tumor proteasome inhibitors

positive results, because Au (III) and Pt (II) are isoelectronic and tetracoordinate gold (III) complexes share the same square planar geometry as cisplatin (110). Many complexes were synthesized and tested against a variety of human cancers, including cisplatin-resistant cell lines (108). Importantly, recent evidence suggests that the cellular proteasome is a molecular target for gold complexes (see the next section) and the mechanism of action underlying their activity is only beginning to emerge.

Recently, various gold (III) compounds exhibiting greater stability have been synthesized using ligand platforms containing nitrogen atoms as donor groups (111). These newer compounds exhibit a superior chemotherapeutic index than cisplatin due to greater bioavailability, increased cytotoxicity, and fewer toxic side effects (109). Dithiocarbamates have been evaluated for their efficacy as inhibitors of cisplatin-induced nephrotoxicity (112-113) and have since been tested for *in vitro* cytotoxicity toward a variety of human tumor cell lines. Most, particularly derivatives of N,N-dimethyldithiocarbamate and ethylsarcosinedithiocarbamate such as (Au (DMDT)Cl₂), (Au (DMDT)Br₂), (Au (ESDT)Cl₂), and (Au (ESDT)Br₂), were demonstrated to be 1-4 fold more potent than cisplatin and were able to overcome intrinsic and acquired cisplatin resistance (109). These dithiocarbamates act fast to inhibit RNA and DNA synthesis and show only minimal cross-resistance with cisplatin (110), suggesting a different mechanism of action.

In an attempt to discern a possible mechanism of action, our lab selected Au (DMDT)Br₂ (Figure 3) and tested its proteasome-inhibitory potential. We reported that the CT-like activity of the purified 20S proteasome (IC₅₀= 7.4 microM) and 26S proteasome in intact MDA-MB-231 breast cancer cells (10-20 microM) was significantly inhibited by Au (DMDT)Br₂. PGPH-like and trypsin-like activities were also inhibited, but the CT-like inhibition was the most significant, indicating that this complex preferentially binds to and inhibits the CT-like beta-5 subunit of the proteasome. Associated with proteasomal inhibition, an accumulation of ubiquitinated proteins and p27 as well as induction of apoptosis was observed in the breast cancer cells. Considerable inhibition of proliferation (80-90%) was also seen in several breast cancer cell lines, including MCF10AT1K.c12, MCF10dcis.com, MCF-7, and MDA-MD-231. Additionally, Au (DMDT)Br₂ was able to potently inhibit tumor growth (~50%) associated with inhibition of proteasomal CT-like activity (40%) in breast cancer xenografts (114).

Our lab has also investigated the effect of two gold compounds with different oxidation states (Figure 3) toward the cellular proteasome, and endeavored to gain insight into their potential mechanism of action. We first compared the effects of a gold (I) compound (Au (ESDT)₂), AUL15, to a gold (III) compound (AuBr₂ (ESDT)), AUL12, toward growth inhibition of breast cancer cells (Figure 3). We found that while both inhibited the growth of MDA-MB-231 breast cancer cells, AUL12 was much more potent (IC₅₀= 4.5 microM, 70% inhibition) than AUL15 (IC₅₀= 13.5 microM, 35% inhibition). We also

observed that both complexes were able to inhibit purified 20S proteasome (AUL12 IC₅₀= 1.13 microM; AUL15 IC₅₀= 17.7 microM) as well as intact 26S proteasome, again with AUL12 exhibiting much higher activity (115). Additionally, we observed that AUL15 inhibited the cellular proteasome much later (> 24 hr) compared to AUL12 (4 hr) in intact breast cancer cells. Associated with these effects was the accumulation of ubiquitinated proteins and IκB-alpha as well as induction of cell death as demonstrated by PARP cleavage and increased levels of p36 Bax protein. These death-associated changes appeared much later in AUL15-treated cells compared to in AUL12-treated cells. In an effort to gain insight into the mechanism of action responsible for their biological effects, we investigated whether these gold compounds could induce the production of reactive oxygen species. Interestingly, we found that treatment with AUL12 (Au (III)), but not AUL15 (Au (I)), was associated with redox processes, suggesting that induction of oxidative stress may be partially responsible for the cytotoxic activity of gold (III) compounds (115).

5.2. Copper and zinc-containing dithiocarbamates

5.2.1. Disulfiram, EtDTC, and PyDTC

Copper and zinc are not only indispensable metals involved in critical biological processes such as tumorigenesis, they have also gained considerable interest as potential anticancer drug targets. An exciting new concept is ongoing in the field that takes advantage of the regulatory approval of drugs used for treatment of different pathological conditions which have also been shown to function as suitable metal chelators. The formation of metal complexes from previously approved drugs, such as Disulfiram (DSF), diethyldithiocarbamate (EtDTC), and pyrrolidinedithiocarbamates (PyDTC) (Figure 4), has been rigorously investigated as potential novel anticancer agents that target the ubiquitin-proteasome pathway.

One such drug selected and tested is an irreversible inhibitor of aldehyde dehydrogenase, disulfiram (tetraethylthiuram disulfide, DSF) (Figure 4). DSF is one of only two drugs approved for the treatment of alcoholism, due to its efficacy without toxicity (116-118). The structure of DSF contains an R1R2NC (S)SR3 functional group, with sulfhydryl groups that afford it the ability to react with Cu (II) (119). This reaction was confirmed through the observation of an intense color change when DSF and CuCl₂ were mixed at a 1:1 ratio (120). Although DSF is not suitable for binding various other biological metal ions such as Fe (II or III) or Mn (III) (119), it has been reported that DSF is able to interact with Zn (II). One group reported that DSF treatment of melanoma and hepatic cancer could be potentiated by Zn (II) supplementation (121) and we have also found that a DSF-Zn complex is able to inhibit the proteasome, though at a weaker potency than DSF-Cu (unpublished data). Studies have shown that DSF is rapidly converted to its copper complex during its absorption into the gastrointestinal system (116).

A potential target of disulfiram is superoxide dismutase, the inhibition of which may be associated with

Metal compounds as tumor proteasome inhibitors

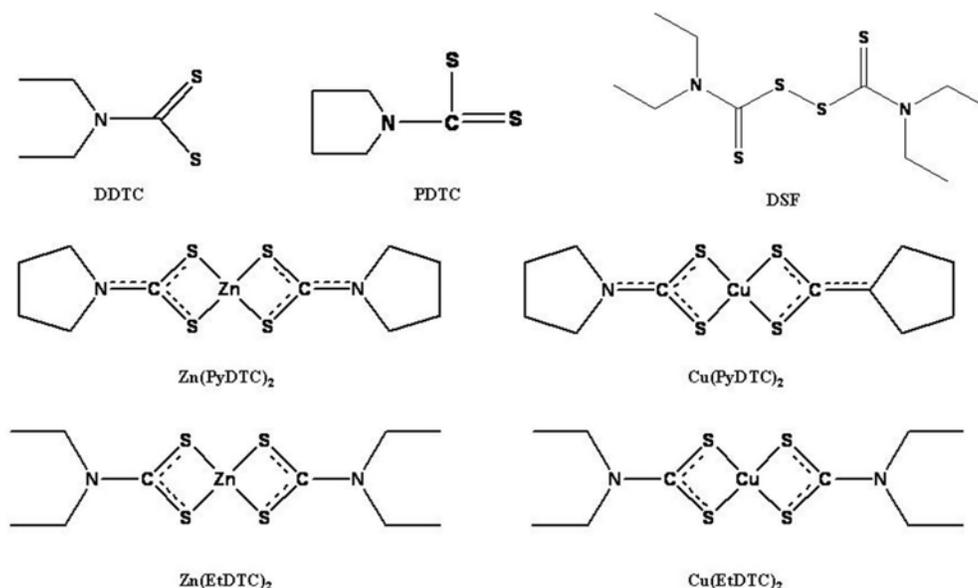


Figure 4. Chemical structures of dithiocarbamates. DDTC, PDTC, and DSF have all been used previously in the clinical setting for a variety of diseases. These complexes are able to chelate copper, and DSF-Cu complexes are active against human tumor cells. EtDTC and PyDTC are metal chelating compounds from the dithiocarbamate family that have been investigated for their proteasome-inhibitory activity.

the inhibition of angiogenesis (122). Our lab has demonstrated that the DSF-Cu complex was also able to inhibit purified 20S proteasome ($IC_{50}=7.5$ microM) and 26S proteasome in intact MDA-MB-231 breast cancer cells (20 microM). The CT-like activity was inhibited by >95% and proliferation was inhibited by up to 85%. An increase in levels of ubiquitinated proteins, as well as apoptotic PARP cleavage and morphological changes were apparent. DSF alone had no visible effect in cultured cells, but an increase in efficacy was observed in breast cancer cells cultured in copper-enriched conditions. Importantly, normal breast MCF-10A cells exhibited no response to DSF, indicating a lack of toxicity, as well as a therapeutic strategy that utilizes heightened levels of copper as a tumor-targeting mechanism (120).

We have also reported the ability of disulfiram to inhibit the proteasome under *in vivo* conditions. Daily treatment of mice bearing MDA-MB-231 xenografts with 50 mg/kg DSF for 30 days resulted in significant tumor growth inhibition (74%). Associated with this growth inhibition, a significant decrease in proteasomal chymotrypsin-like activity (87%) and accumulation of ubiquitinated proteins, p27, and Bax were visible. Furthermore, apoptosis-associated increases in caspase-3 activity and PARP cleavage were also observed (120). Interestingly, phase I/II clinical trials investigating the effects of DSF on metastatic melanoma have been completed, but the results are not yet available (NCT00256230; Chao Family Comprehensive Cancer Center). Patients are also currently being recruited for a phase I study determining the safety and toxicity profile of DSF and copper gluconate co-treatment in refractory malignancies with liver metastasis (NCT00742911; Huntsman Cancer Institute). Taken together, these results

demonstrate a novel application for the use of DSF in the presence of copper for the potential treatment of cancer.

5.2.2. Synthetic EtDTC copper and zinc complexes

Because DSF can be converted to diethyldithiocarbamate (EtDTC) in the body, it is believed that the ability of EtDTC to complex with copper gives DSF its anticancer activity (116). EtDTC complexes have previously been shown to promote T cell maturation and reduce lymphadenopathy in animal models (123-124), and for this reason we investigated the anti-tumor activities of Zn (II) and Cu (II) EtDTC complexes (Figure 4). We treated MDA-MB-231 breast cancer cells with Cu (EtDTC)₂ and Zn (EtDTC)₂ and found that both complexes caused cell death associated with 26S proteasome inhibition (125). Inhibitory activity against purified 20S proteasome was much lower (125), suggesting that the effect of these complexes may be due to inhibition of the JAMM domain in the 19S particle of the proteasome (126). The JAMM domain, a metalloisopeptidase with a coordinated zinc ion (126), is necessary for the deubiquitinating activity of the 19S particle and has been proposed as a possible target for anticancer drugs (127).

5.2.3. Synthetic PyDTC copper and zinc complexes

We have also examined the possible chemotherapeutic properties of another group of dithiocarbamate complexes, pyrrolidinedithiocarbamates (PyDTC), when coupled with copper and zinc (Figure 4). We found that both the Zn (PyDTC)₂ and Cu (PyDTC)₂ complexes exhibited proteasome inhibitory activity against purified 20S proteasome as well as intact 26S proteasome in MDA-MB-231 cells (128). Accumulation of ubiquitinated proteins and proteasomal target proteins I κ B- α and p27, associated with proteasome inhibition, and apoptosis associated morphological changes and PARP

Metal compounds as tumor proteasome inhibitors

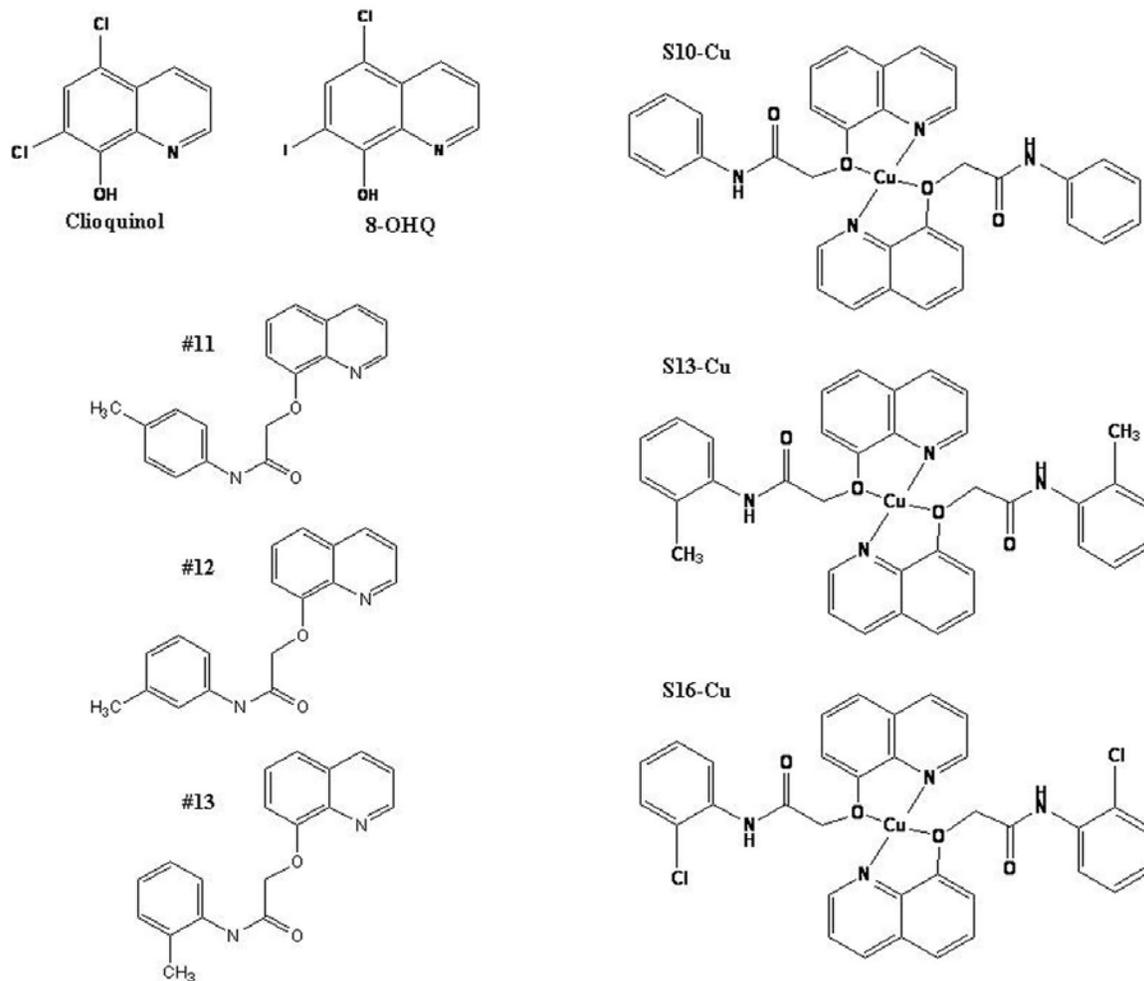


Figure 5. Chemical structures of hydroxyquinolines. CQ and 8-OHQ are members of the hydroxyquinoline family, and are able to bind copper and actively inhibit the proteasome. Analogs of 8-OHQ with methyl group additions were investigated, and found to exhibit proteasome-inhibitory activity when complexed with copper.

cleavage occurred in cells treated with either Zn (PyDTC)₂ or Cu (PyDTC)₂. We also observed that the effects of these PyDTC complexes were time-dependent, with >50% inhibition at early time points (128). To further examine the results observed with PyDTC complexes, we synthesized PyDTC:metal complexes in a 2:1 ratio (Zn (PyDTC)₂ and Cu (PyDTC)₂). We determined that these synthetic complexes were much less potent toward purified 20S proteasome (40% inhibition at 50 μM), but more potent toward intact 26S proteasome in MDA-MB-231 cells, with Cu (PyDTC)₂ exhibiting higher activity than Zn (PyDTC)₂. These synthetic complexes were also effective in other cell lines, including breast cancer DCIS and MCF7, and prostate cancer PC-3 cells (128). Additionally, partial inhibition of Cu/Zn (PyDTC)₂-induced cell death occurred when cells were pretreated with a calpain inhibitor. However, addition of a calpain inhibitor did not affect proteasome inhibition, suggesting that calpain involvement is important in apoptotic cell death induced by synthetic Cu/Zn (PyDTC)₂ complexes (128).

6. HYDROXYQUINOLINES

6.1. Clioquinol

Clioquinol (5-chloro-7-iodo-8-hydroxyquinoline, CQ) (Figure 5) is a compound in the hydroxyquinoline family and has been shown to reduce or prevent the formation of amyloid plaques in the brains of Alzheimer's disease transgenic mice (129). This discovery led to the initiation of two clinical trials which showed that CQ is beneficial in treating Alzheimer's disease with no visible toxicity (130-131). Consequently, CQ is currently in use clinically for the treatment of Alzheimer's and Huntington's diseases (132-133). Prior to the discovery of its efficacy in treating Alzheimer's disease, CQ was used successfully to treat and prevent *shigella* and *entamoeba histolytica* infections (134).

Although CQ use was thought to be associated with occurrence of subacute myelo-optic neuropathy in Japan, this conclusion was not supported by the subsequent epidemiologic analysis. Instead, decreased levels of vitamin

Metal compounds as tumor proteasome inhibitors

B₁₂ may play a role in this syndrome. In fact, CQ may be used safely in humans with vitamin B₁₂ supplementation.

Clioquinol is a lipophilic compound that is able to form stable complexes with copper (II) ions (135). To test this, we mixed CQ and CuCl₂ in a 1:1 molar ratio and an observable color change occurred, indicating that a chemical reaction had taken place (136). This was further confirmed by the use of X-ray absorption near-edge spectroscopy (XANES) and extended X-ray absorption fine structure spectroscopy (EXAFS), which showed that the CQ-Cu mixture had a different copper oxidation state than CQ or CuCl₂ alone, which verified that a coordination complex with copper had indeed been formed (136).

We examined the effects of the CQ-Cu complex on the purified 20S proteasome, and inhibition of chymotrypsin-like activity was observed (IC₅₀= 2.5 microM). Human LNCaP and C4-2B prostate cancer cells were treated with the CQ-Cu complex (20 microM) to determine proteasome inhibitory activity in intact cells. We discovered that the complex is able to potently inhibit proteasomal chymotrypsin-like activity (82% and 83%), suppress androgen receptor expression, suppress cell proliferation, and induce apoptosis. PARP cleavage and cellular morphologic changes, associated with apoptosis-induction, were also detected (136). As observed with DSF, CQ alone (not in complex with Cu) was ineffective in intact cultured cells, due to the low levels of Cu present in cultured cell lines, contradictory to clinical or animal tumors, which contain high copper levels. Therefore, copper-enriched LNCaP and C4-2B cells were examined and found to be sensitive to treatment with CQ alone (136). Additionally, C4-2B xenograft-bearing mice were used to investigate the *in vivo* effects of CQ. In addition to significant tumor growth inhibition (66%) as compared to controls, proteasome inhibition, apoptosis induction, suppression of AR expression, and inhibition of angiogenesis (indicated by decreased CD31 expression) were also observed (136). These results present a compelling rationale for further investigation into the use of CQ in clinical trials as a potential anticancer agent.

6.2. 8-hydroxyquinoline analogs

Based on our encouraging findings with CQ, we selected and tested an analog, 8-hydroxyquinoline (8-OHQ) (Figure 5). We found that 8-OHQ is able to inhibit the proteasomal chymotrypsin-like activity when complexed with copper at a 1:1 molar ratio in purified 20S proteasome as well as in intact Jurkat leukemia T cells (10 microM) (137). Additionally, loss of cell viability and PARP cleavage were observed in the treated Jurkat cells and accumulation of ubiquitinated proteins occurred after treatment with 1 microM 8-OHQ. Importantly, no apoptosis was observed in non-transformed immortalized natural killer (YT) cells (137). To simulate *in vivo* tumor conditions, PC-3 prostate cancer cells were grown in copper (CuCl₂) enriched media followed by treatment with 8-OHQ, which resulted in proteasome inhibition and PARP cleavage associated with induction of apoptosis (137).

Based on these positive results, seven new 8-OHQ analogs were synthesized and labeled #10-#16 (Figure 5). We treated MDA-MB-231 breast cancer cells with complexes of each analog and copper (1:1 molar ratio) at 25 microM (138). Measurement by MTT showed that the copper mixtures of #11, #12, and #13 (Figure 5) were able to inhibit ~90% of proliferation, while the other compounds showed little inhibitory activity. This suggests that the attachment of a methyl group to the phenyl ring does not affect the activity of the copper complexes (138). The copper mixtures of analogs #11, #12, and #13 also inhibited proteasome activity by 45-55%. The highest potency analog, #13, was chosen and found to inhibit proliferation of MDA-MB-231 cells, when mixed with copper, in a dose-dependent manner. Inhibition of proteasomal activity also occurred in a dose- and time-dependent manner. Consistent with this effect, accumulation of ubiquitinated proteins and IκB-α were observed, as well as morphological changes and PARP cleavage associated with apoptosis induction (138).

To substantiate the anticancer properties of these analogs and to gain insight into the roles of the metal and ligand, we synthesized complexes 10, 13, and 16 with copper at a 2:1 molar ratio (S10-, S13-, S16-Cu) (Figure 5). We observed that S13-Cu induced >90% growth inhibition at 15 microM, and S10- and S16-Cu were almost as potent at 25 microM. At the highest concentration all three complexes inhibited only ~20% activity in purified 20S proteasome. In intact MDA-MB-231 cells, S13-Cu at 15 microM inhibited >70% proteasomal activity after four hours and almost 90% after 24 hours (138). S10- and S16-Cu, however, showed minimal activity toward MDA-MB-231 cells. Proteasome inhibition by S13-Cu was confirmed by Western blot, which showed accumulation of ubiquitinated proteins, IκB-α, and p36 Bax. Apoptotic morphological changes and PARP cleavage also occurred in S13-Cu treated cells. Importantly, S13-Cu had no effects in non-malignant MCF-10A cells (138). Additionally, to determine whether the position of the methyl group affects the potency, we compared activities of the three methyl-containing analogs, #11, #12, and #13. Complex 11 inhibited ~90% proliferation at 10 microM, but complex 12 and 13 showed very little activity at this concentration. Complex 11 was also the most potent inhibitor of 26S proteasome activity with observable apoptosis-related changes at 10 microM (138). These data indicate the possibility that both the ligand and the metal are important to the activity of these metal compounds, and that the use of analogs of old previously used drugs is a viable option in the search for novel anticancer agents.

Importantly, we have also investigated the necessity of copper to the anticancer properties of CQ and 8-OHQ. Synthetic chemical probe molecules that mimic the structures of 8-OHQ and CQ, but have no copper-binding capability, were tested for their anti-tumor activities (139) (Figure 6). In contrast to both CQ and 8-OHQ, these inactive analogs were unable to inhibit growth of human breast cancer DCIS cells, either alone or in combination with CuCl₂. Similarly, neither cell death nor proteasome inhibition was observed in cells treated with these synthetic

Metal compounds as tumor proteasome inhibitors

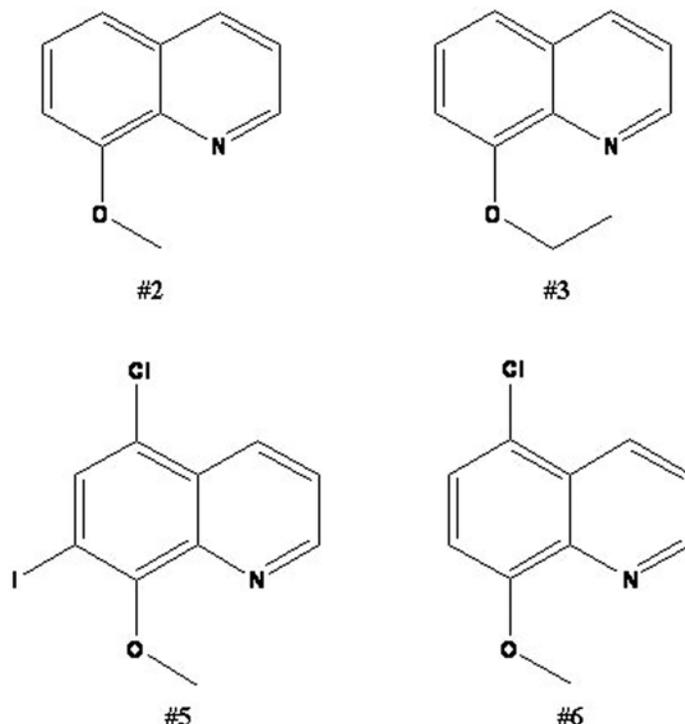


Figure 6. Chemical structures of synthetic non-copper binding CQ and 8-OHQ analogs. Four molecules similar in structure to CQ and 8-OHQ without the ability to bind copper were synthesized. In contrast to CQ and 8-OHQ, these analogs exhibited no proteasome-inhibitory or apoptosis-inducing activity alone or in combination with copper.

molecules plus copper (139). Additionally, accumulation of ubiquitinated proteins and Bax, associated with proteasome inhibition did not occur in cells treated with the analogs-copper mixtures. These data demonstrate that copper-binding is necessary for CQ and 8-OHQ to be transported into breast cancer cells, and to subsequently exert their proteasome-inhibiting and apoptosis-inducing activities (139).

7. CONCLUSIONS

The clinical use of platinum-containing drugs in the treatment of a variety of human tumors represented a landmark achievement in metal-based cancer chemotherapy. Efforts to develop novel anticancer agents based on different metals and ligand platforms have been prompted by exciting new preclinical and clinical evidence. Investigation of different metal complexes was stimulated not only by attempting to overcome shortcomings of platinum-based compounds, but that foster mechanisms of action not realized by these conventional drugs. In recent years, ruthenium complexes have been investigated as both antitumor and antimetastatic agents and are currently being investigated in clinical trials. The unique properties of ruthenium as it relates to tumor uptake and reduction potential, present the possibility of a more selective tumor targeting strategy.

The clinical use of proteasome inhibitors, such as bortezomib was validation of the importance of the

cellular proteasome as a critical molecular target to be exploited for therapeutic purposes. Different metals and metal compounds that target the ubiquitin-proteasome pathway have received considerable attention as potential anticancer drugs. Essential metals such as copper and zinc are not only critical components in cellular metabolism, but have displayed potential as anticancer drug targets via proteasome inhibition. Along this line, since the medicinal applications of gold and gold complexes have been known throughout human history, their potential as anticancer agents, with proteasome-inhibitory activity, are only beginning to be fully appreciated. The physiochemical properties of metal complexes are not only dictated by the nature of the metal, but also the type and number of ligands involved. Two prominent classes of metal-chelating compounds investigated for anticancer activity with different metals are dithiocarbamates and hydroxyquinolines. Their applications consist of being used as either coordination complexes or by targeting increased levels of tumor-associated copper, leading to tumor proteasome inhibition and subsequent tumor cell death. These represent significant findings since representative examples from these classes, i.e. disulfiram and clioquinol are already clinically approved for pathological disorders, so the findings that these drugs harbor anticancer activity in the presence of metals may help expedite the regulatory process as novel anticancer drugs. Since the drug development process can be burdensome replete with regulatory demands, this concept could represent a significant achievement in establishing

Metal compounds as tumor proteasome inhibitors

positive momentum in generating further lead candidates in anticancer drug discovery. Overall, the interesting properties of metal-based complexes, coupled with the significant progress made in elucidating their mechanisms of action, will help facilitate these entities into the clinical setting as drug candidates.

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Metal compounds as tumor proteasome inhibitors

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Metal compounds as tumor proteasome inhibitors

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Abbreviations: Platinum: Pt, Ruthenium: Ru, Copper: Cu, Zinc: Zn, Vascular Endothelial Growth Factor: VEGF, Ubiquitin-proteasome pathway: UPP, peptidyl-glutamyl peptide-hydrolyzing: PGPH, chymotrypsin:CT, Disulfiram: DSF, poly-ADP ribose polymerase: PARP, diethyldithiocarbamate: EtDTC, pyrrolidinedithiocarbamate: PyDTC, Clioquinol: CQ, X-ray absorption near-edge spectroscopy: XANES, extended X-ray absorption fine structure spectroscopy: EXAFS, 8-hydroxyquinoline: 8-OHQ

Key Words: Disulfiram, Clioquinol, Dithiocarbamates, Copper, Zinc, Ubiquitin-Proteasome Pathway, Proteasome Inhibitor, Chymotrypsin-Like Activity, Review

Metal compounds as tumor proteasome inhibitors

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