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Copper-binding compounds as proteasome inhibitors and apoptosis inducers in human cancer

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1. ABSTRACT

The trace element copper is vital to the healthy functioning of organisms. Copper is used in a multitude of cellular activities including respiration, angiogenesis, and immune responses. Recently, copper has become a focus in medical research ranging from Alzheimer's disease to cancer. Copper modulation has been suggested to be a potential modality for therapy in these diseases. Several copper-binding compounds have been found to spontaneously complex with copper and form active proteasome inhibitors and apoptosis inducers. This review examines compounds in the quinoline and dithiocarbamate families and from the National Cancer Institute (NCI) Diversity Set that bind with copper and act as anticancer agents. In each case, it is shown that these compounds can bind with copper, inhibit the proteasome activity, and induce apoptosis in cancer cells. These activities are absent when copper is not present. Compounds alone, clioquinol and pyrrolidinedithiocarbamate as examples, are shown to have no effects in normal breast cells. Current research suggests that a possible therapeutic modality for cancer may be developed using the difference of high copper load in tumors *versus* low copper load in normal cells. This strategy would convert tumor cellular copper into a potent, specific proteasome inhibitor and apoptosis inducer. Thus, this approach could pave the way for the development of nontoxic anticancer therapy.

2. INTRODUCTION

2.1. Copper, angiogenesis and cancer

Copper (Cu) is found in all living organisms and is a crucial trace element in redox chemistry, growth and development (1, 2). A variety of Cu-containing metalloenzymes are found in both plants and animals in which Cu ions are typically bound to nitrogen or sulfur containing ligands. These metalloenzymes are important in electron transfer, oxygen transport and oxygenation reactions, and can contain Cu(I) or Cu(II) species. Daily intake of Cu ranges from 0.6 to 1.6 mg / day with the main sources of Cu being seeds, grains, nuts, beans, shellfish, and liver (2, 3). Cu toxicity comes about from its ability to produce reactive oxygen species, displace other metal ions, peroxidize lipids, and directly cleave DNA and RNA (4, 5). Cu homeostasis is tightly regulated with excretion as the main factor in maintaining copper levels (1, 2).

Many cancer tissues contain highly elevated levels of Cu (6, 7). The reasons for this elevation are unclear but one possible result is increased angiogenesis (8-10). It was a novel concept proposed by Dr. Folkman of the Harvard School of Medicine, whose pioneering study in angiogenesis started the field, that tumors require angiogenesis for growth (11-13). There is no action of angiogenesis in normal adult tissues except for wound healing, inflammatory reaction or the menstrual cycles

(14). Then, scientists explored the essential and requisite factors for angiogenesis. It has been found that in the cornea of rabbits' eyes, the cornea could develop capillaries when effectors became rich in copper ions (15). Another group tested three molecules, ceruloplasmin, heparin and glycyl-L-histidyl-L-lysine, and results showed that these molecules were able to induce angiogenesis when they were bound to copper ion (15). Recently, more and more studies demonstrate that tumor growth and metastasis depend upon angiogenesis, the neovascularization process (16, 17) that requires growth factors, proteases, and the trace element copper (8-10, 18). Copper, but not other transition metals, is a co-factor essential for the tumor angiogenesis processes (8-10). High levels of Cu have been found in many types of human cancers (6, 7, 19, 20). Cu stimulates proliferation and migration of human endothelial cells (21, 22). A specific amount of local Cu appears to be required for angiogenesis to occur (8-10).

2.2. *D*-Penicillamine, trientine, and tetrathiomolybdate

In light of the similarity of copper accumulation in cancer to that of Wilson's disease (23), a rare autosomal recessive Cu storage disease that results in Cu accumulation (24, 25), several anti-copper drugs used in Wilson's disease have been evaluated for use in cancer. It has been shown that three anti-copper drugs used in the treatment of Wilson's Disease, *D*-penicillamine (*D*-PA), trientine, and tetrathiomolybdate (TM) have antiangiogenic effects in murine cancer models (22, 26, 27). Based on this information, several studies and clinical trials (in the case of TM) have been performed to evaluate the antiangiogenic effects of these anti-copper drugs on solid tumors (8).

D-PA is capable of binding to Cu in proteins and stabilizing the complex for elimination (26). When used in conjunction with a Cu restricted diet, *D*-PA blocked angiogenesis and gliosarcoma in F-344 rats (27). However, this same treatment failed to block lung metastasis or vascularization and tumor growth of VX2 tumors in thigh muscle (27). The failure of the treatment was unknown but possibly due to differences in Cu deposition (27). As with many cancers, hepatocellular carcinoma (HCC) cells have been shown to have significantly elevated Cu loads (26). Yoshii, *et al.*, found that high-dose *D*-PA (3,000 ppm) administered to rats bearing HCC xenografts blocked tumor development and more so when in conjunction with a Cu deficient diet (26). However, *D*-PA was not as potent as trientine, and *D*-PA treatment can result in significant side effects including auto-immune disease (26). For these reasons and others, *D*-PA has not been further pursued since other Cu chelating agents are available.

Trientine is generally more potent than penicillamine at binding Cu and having anticancer effects even without the use of a Cu deficient diet (26). In a previous study, SH-SY5Y neuroblastoma cells were cultured in the presence of trientine (27). As expected, cellular Cu was depleted. Early responses included overexpression of p53 and p21 as well as activation of caspase-9 and caspase-3 (27). In this case, Cu elimination resulted in apoptosis induction *via* the mitochondrial pathway (27). In another murine study, 1500 ppm of trientine was provided to Long-Evans cinnamon rats (a murine model for Wilson's disease) (28). Compared to untreated rats, those receiving trientine had 33% smaller HCCs and significantly lowered numbers of HCCs (0.6 average in treated animals compared to 4.1 for untreated), suggesting a strong tumor preventative effect by trientine without toxicity (28).

TM has been used in pre-clinical mouse studies and clinical trials. Earlier studies examined cancer prone Her2/*neu* transgenic mice and athymic nude mice with breast tumor xenografts, which were treated with TM (22). TM treatment (0.7 mg/day) resulted in a nearly 70% reduction in tumor volume and only minimal neovascularization in nude mice with xenografts (22). Furthermore, treatment with 0.75 mg/day TM was capable of preventing development of tumors in Her2/*neu* mice (22). While there was histological evidence of full transformed breast cells in these mice, there was a complete absence of any invasive activity or angiogenesis (22). In another study, male C3H/HeJ mice, serving as an orthotopic model of head and neck squamous cell carcinoma, received 50 mg of TM per day in drinking water, which was well tolerated (29). Control mice developed tumors 4.7 times greater in volume than TM-treated mice and had 50% increased development of microvasculature (29). These results further supported the potential role of TM as an anticancer drug that acts through prevention of angiogenesis.

Phase I clinical trials, conducted during 1998 and 1999, measured the pharmacodynamics of TM in patients with metastatic cancer (30). The trial was conducted with 18 patients receiving 90-120 mg/day TM treatment. No toxicity was found when serum ceruloplasmin (Cp), a copper carrying protein that is a marker of systemic copper load, was reduced to 15-20% of baseline (30). Reversible anemia was found when Cp levels dropped below that threshold (30). While this was not an efficacy trial, *per se*, it should be noted that 5 of 6 patients with TM induced copper deficiency displayed stable disease and reduced angiogenesis within tumor masses (30). These results strongly support the idea that copper-binding compounds and their resulting complexes can have very mild and reversible toxicity, a necessary characteristic for chemotherapeutic agents. In 2003, the results from a TM phase II clinical trial with kidney cancer patients was reported (31). The treatment consisted of 40 mg of TM three times a day with 60 mg prior to sleep. While no patients demonstrated complete or partial response, the disease stabilized in four patients during the six-month treatment (31). Overall, TM was well tolerated with mild toxicities (31). It should be noted that this was a small study with patients bearing advanced kidney cancer and this factor may have contributed to the relatively minimal effect of TM on the disease.

The above studies primarily concerned themselves with copper elimination as a therapeutic modality. However, in each case the results were limited. This might suggest that chelating and promoting elimination of copper, passive elimination, is not entirely

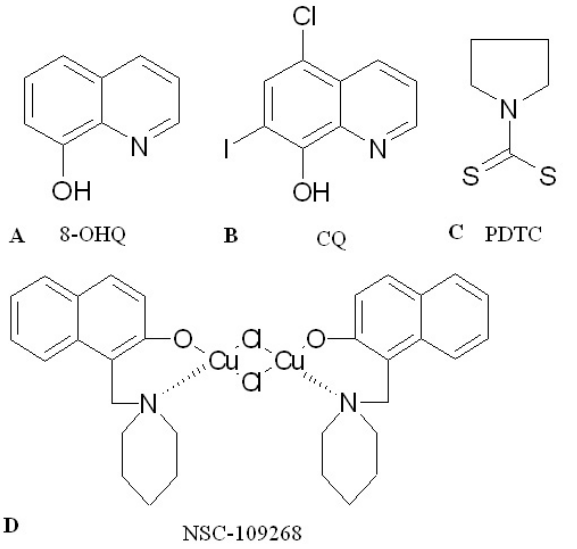

Figure 1. Structures of selected copper-binding compounds and NCI Diversity Set compound. A) 8-hydroxyquinoline (8-OHQ), B) clioquinol (CQ), C) pyrrolidine dithiocarbamate (PDTC), and D) NCI Diversity Set compound NSC-109268.

sufficient as a therapy. We have previously shown that certain classes of organic compounds seem to be capable of active chelation in which the compound binds to copper and forms a complex that inhibits proteasome activity and induces apoptosis in cultured human cancer cells (32). We hypothesize that converting the pro-angiogenic copper to an anti-angiogenic copper-based proteasome inhibitor might be an important strategy for treating human cancers.

2.3. Proteasome inhibitors for treatment of cancer

It has been found that proteasome inhibitors (*i.e.*, CEP1612 and Velcade) are able to induce apoptosis in various tumor cell lines (33-38). CEP1612, a dipeptidyl proteasome inhibitor, was able to rapidly induce apoptosis in all the human cancer cell lines tested, including breast, prostate, leukemia, lung, bone, brain, and head and neck, but not in human normal breast and normal fibroblast cells (34, 39). Velcade is a potent and selective dipeptidyl boronic acid proteasome inhibitor *in vitro* and *in vivo* (40, 41). Inhibition of the tumor cellular proteasome activity in various tumor culture models by Velcade is also associated with induction of apoptosis (33, 42, 43). In different animal studies, Velcade suppresses tumor growth and angiogenesis as either a single drug or in combination with other cytotoxic agents (33, 44). Velcade has been given FDA approval for the potential treatment of human hematological malignant neoplasms and solid tumors (40, 41). Preliminary data from Phase I and II trials confirm the antitumor activity of Velcade. However, some associated side effects were observed, such as nausea, fatigue, diarrhea, peripheral neuropathy, rapidly reversible reduction in platelets, and reversible thrombocytopenia (40, 41). Since proteasome inhibition as a therapeutic modality can lower toxicity, certain organic compounds, when complexed with copper, can inhibit the proteasome and cancer tissues contain elevated copper, it is possible that certain classes of organic compounds can be used to target copper in cancer cells for on-site proteasome inhibitor formation (32). Therefore, it can be hypothesized that such a strategy could result in highly effective and selective cancer killing that avoids toxicity. The potential use of proteasome inhibitors in caner therapies has been extensively reviewed previously, such as in refs. (40, 41) and will not be detailed here.

3. COPPER-BINDING COMPOUNDS

3.1. 8-Hydroxyquinoline and clioquinol

Many compounds in the quinoline class, such as 8-hydroxyquinoline (8-OHQ; Figure 1A) and clioquinol (CQ; 5-chloro-7-iodo-8-hydroxyquinoline; Figure 1B), have been used as antibiotics and antifungal agents (45). This class of compounds possesses an established and favorable toxicology profile with the US Pharmacopoeia (www.usp.org) (45). However, CQ is a controversial antibiotic. During the 1950s to the 1970s, CQ was used as an antibiotic (46, 47). CQ was withdrawn due to association with subacute myelo-optic neuropathy (SMON) possibly due to overdose and/or a reversible vitamin B_{12} deficiency (46, 48).

Recently, interest in CQ has reemerged due to studies involving its use, in combination with B_{12}, for treatment of Alzheimer's Disease (45, 49, 50). CQ is a lipophilic compound that is capable of forming stable complexes with Cu(II) ions (51). In a Phase II clinical trial, CQ, at starting concentration of 3.3 mg/kg, the same order of magnitude of treatment used in mice, was found to be well-tolerated and suitable for further study (49).

A previous report showed that copper chelators were capable of inhibiting the NFκB pathway (52). Most recently, we discovered that several quinoline-copper complexes potently and specifically inhibited the chymotrypsin-like activity of the proteasome *in vitro* and in cell culture (32). Inhibition of the proteasome activity by these organic copper compounds occurs very rapidly in human tumor cells (15 min), followed by induction of apoptosis. Neither proteasome inhibition nor apoptosis were found in normal or non-transformed cells under the same treatment. Most importantly, proteasome inhibition and apoptosis were also detected in copper-containing tumor cells treated with 8-OHQ. None of these events occurred in cells treated with either inorganic copper, ligand-treated cells that did not contain copper, or pretreatment with the closely related nickel followed by addition of the ligand (32). We also found that 5,7-dichloro-8-hydroxyquinoline synthesized to contain copper was a potent proteasome inhibitor and apoptosis inducer (32).

Similar to 8-OHQ, CQ was found to inhibit proteasome activity and induce apoptosis in breast cancer cells only when complexed with copper (53). Interestingly, CQ, even when complexed with copper, did not inhibit proteasome activity or induce apoptosis in normal breast cells (53), suggesting a strong selective effect for cancer

cells. As with 8-OHQ, premalignant breast cancer cells cultured to contain clinically relevant levels of Cu were highly sensitive proteasome inhibition and apoptosis induction by CQ alone (53). In both cases, the quinoline was an inactive compound in the absence of Cu at the concentrations tested. In the presence of copper or when cells containing copper were treated with each quinoline compound, proteasome inhibition and apoptosis induction followed (32, 53).

Ding, *et al.* observed that the addition of Cu, iron, or zinc did not rescue cultured tumor cells from CQ-induced cytotoxicity but enhanced its killing, arguing that chelation and elimination was unlikely to be the major mode of action for CQ (54). In addition, an ovarian and B-cell lymphoma xenograft nude mouse model was used to examine the anticancer effects of CQ *in vivo* (54). Their results showed that use of CQ dramatically enhanced the survivability of the mice from 0% in controls to 75% after 60 days (54). While the mechanisms of apoptosis induction by CQ and Cu were unclear, inhibition of super-oxide dismutase and metal depletion were ruled out, though a possible action as a zinc ionophore remained (54).

Even though the quinolines, generally, have a favorable toxicity profile, the controversial nature of CQ cannot be understated (45). This compound was implicated in approximately 8,000 cases of SMON in Japan (45). However, the uniqueness of the epidemic to Japan seems to reflect either overdosing or an association with vitamin B_{12} deficiency. When used at low concentrations and/or with supplementation with B_{12}, CQ manifests no toxic effects (45, 54).

3.2. Pyrrolidine dithiocarbamate

Dithiocarbamates are a class of metal chelating compounds. These compounds have previously been used in the treatment of bacteria and fungi as well as considered for use in the treatment of AIDS (55, 56). Pyrrolidine dithiocarbamate (PDTC; Figure 1C) is a synthetic antioxidant and inhibitor of NFκB that is capable of binding copper (57, 58). PDTC and other dithiocarbamates have been found to induce apoptosis in conjunction with copper in different types of cancer cells (57, 59).

Previously we examined the ability of PDTC to inhibit proteasome activity and induce apoptosis in human breast premalignant and cancer cells as compared to normal breast cells (53). PDTC alone had no effect on either the proteasome activity or cellular viability of any of the lines tested. However, when complexed with Cu, PDTC potently inhibited the proteasome activity in premalignant and malignant breast cells (53). Normal breast cells showed no reduction in proteasome activity by up to 24 h. PDTC, when complexed with Cu, also blocked proliferation of breast cancer cells in a dose-dependent manner with almost 40% inhibition at 1 μM and complete inhibition at 10 μM (53). Measurement of poly(ADP-Ribose) polymerase (PARP) and morphological analysis of the nucleus, two markers of apoptosis, showed that the PDTC-Cu complex induced apoptosis most strongly in malignant breast cells, less strongly in premalignant cells and did not induce apoptosis in normal breast cells (53). Importantly, similar to 8-OHQ and CQ, PDTC was capable of proteasome inhibition and apoptosis induction on its own when used in premalignant breast cancer cells containing clinical concentrations of copper (53).

We also examined the ability of PDTC to inhibit proteasome activity and induce apoptosis in prostate cancer cells (60). Similar proteasome-inhibitory and apoptosis-inducing activities of the PDTC-Cu complex were observed in cultured prostate cancer cells (60). Additionally, Positron Emission Tomography was used to examine the effects of the PDTC-Cu complex on the uptake of the radiopharmaceutical 2-[^{18}F] Fluoro-2-deoxy-D-glucose (^{18}F-FDG) as a measure of antitumor activity (60). Treatment of prostate cancer cells with the PDTC-Cu complex showed a dramatic inhibition of ^{18}F-FDG uptake, indicating potential antitumor activity (60). Importantly, PDTC alone was capable of modest inhibition of ^{18}F-FDG uptake, suggesting that the endogenous copper of cultured prostate cancer cells (significantly lower than prostate cancer *in vivo*) may complex with PDTC resulting in some complex with biological activity (60).

While it has been reported that PDTC can inhibit NFκB activity and induce apoptosis in the presence of copper, the mechanism of these effects was not known (57-59). Our data suggest a possible mechanism in which PDTC and copper form a complex that inhibits proteasome activity and induces apoptosis. Importantly, PDTC complexed with copper has no apparent toxic effect on normal breast cells at these concentrations (53).

3.3. National Cancer Institute (NCI)–Diversity Set Library

The NCI's Pure Chemicals Repository contains >140,000 synthetic and pure natural compounds of diverse structural types. From this library the NCI generated a Diversity Set containing 1,990 compounds. Although many NCI compounds have been shown to have anti-tumor activity *in vivo*, the molecular targets of most, if not all, of the compounds remain unknown. Of the 1,990 compounds in the Diversity Set, we found that NSC-109268, an organic copper compound (Figure 1D), inhibited the proteasome activity *in vitro* and in tumor cell cultures (32).

To further this investigation, we requested and received, from NCI, four copper-containing compounds, NSC-109268 (Figure 1D), -109267, -109271 & -109272 (Figure 2), and six putative organic ligands, NSC-109237, -109241, -109249, -109266, -109273 & -109276 (Figure 2), which do not contain copper but have the potential to bind with copper. For clarity, we will refer all these compounds only by their last 3 digits. We first tested the effects of the copper-containing compounds on prostate cancer cells (Figure 3). LNCaP cells were treated with 15 μM of -268 or -272 for up to 96 h, followed by measurement of proteasome inhibition and apoptosis. By 4 h accumulation of ubiquitinated proteins was apparent, which was significantly increased by 96 h (Figure 3A), suggesting inhibition of the proteasome activity in intact prostate cancer cells. Cleavage of PARP was seen at 1 and 4 h

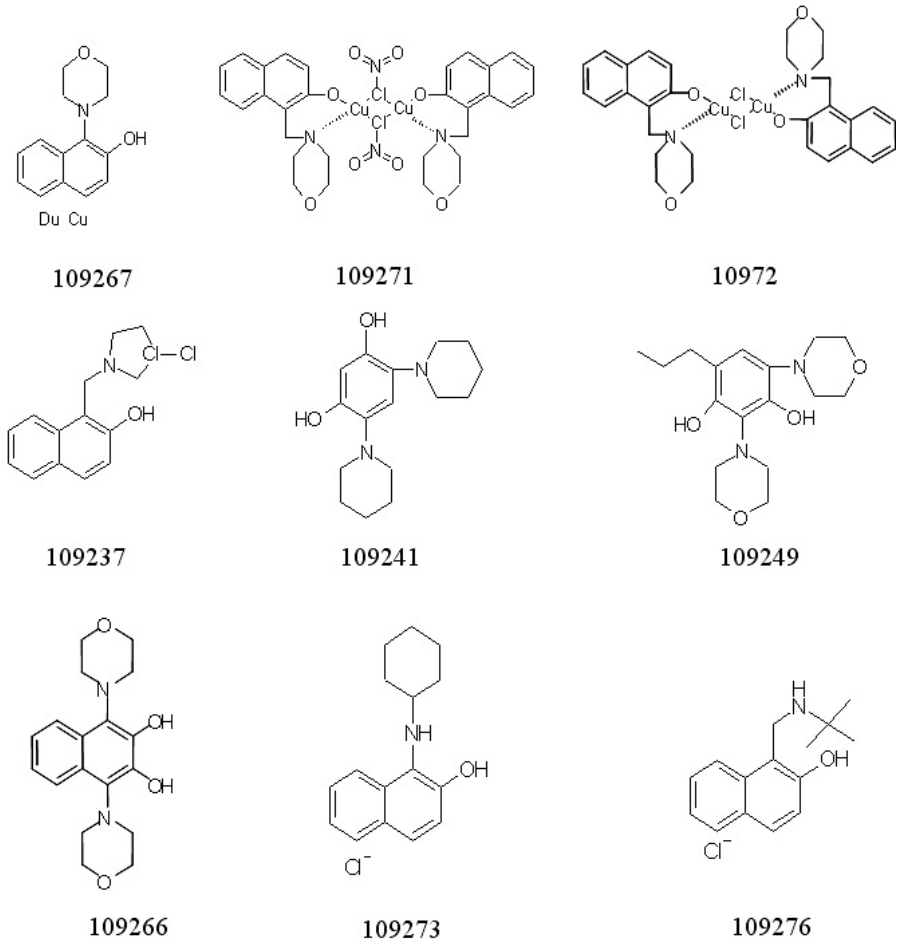

Figure 2. Structures of NCI copper-containing compounds and ligands.

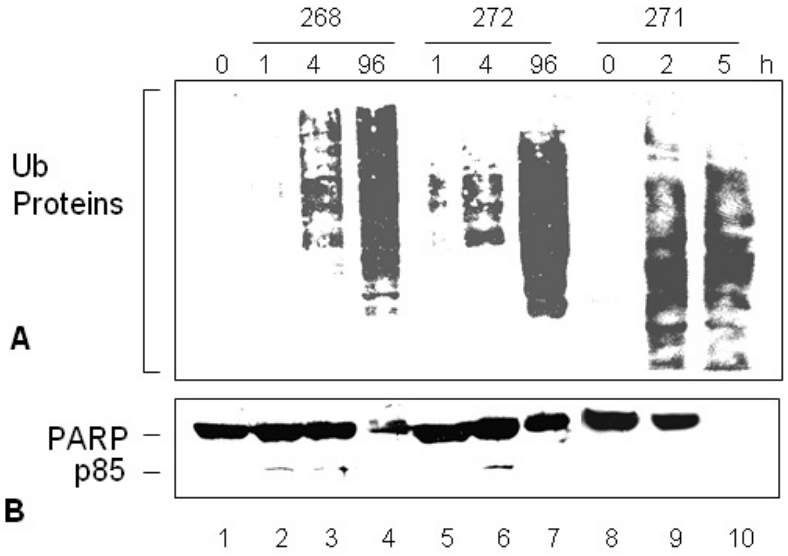

Figure 3. Accumulation of ubiquitinated proteins and induction of apoptosis in prostate cancer LNCaP and PC-3 cells by NCI copper-containing compounds -268, -272 and -271. LNCaP cells were treated with 15 μM of -268 (lanes 2 to 4) or -272 (lanes 5 to 7) for 1, 4, and 96 h, or PC-3 cells were treated with 15 μM of -271 (lanes 8 to 10) for 2 or 5 h, followed by Western blotting (50 μg per sample) for ubiquitinated protein accumulation (A) and PARP cleavage (B).

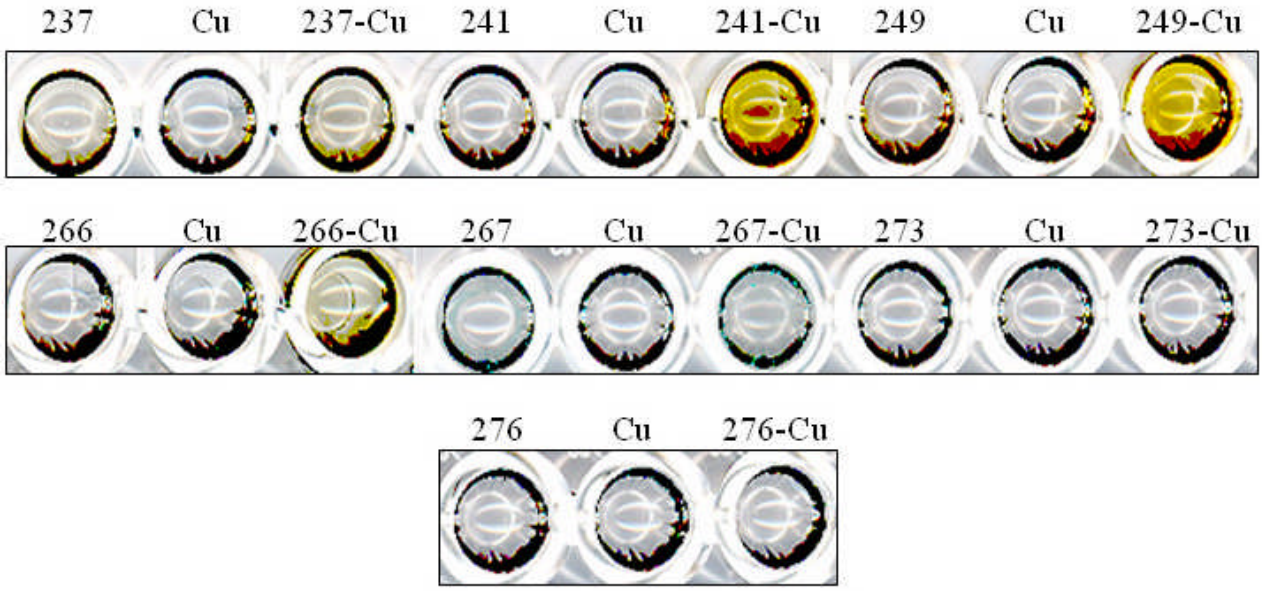

Figure 4. Color change due to reaction of a NCI ligand with copper. 10 mM of each NCI ligand was directly mixed with 10 mM $CuCl_2$ and the resulting solution was monitored for color changes indicative of a chemical reaction.

(Figure 3B), indicating induction of apoptosis. By 96 h, a further decrease in intact PARP protein was observed, suggesting occurrence of further cell death. Similar results were seen in prostate cancer PC-3 cells treated with -271 for 2-5 h (Figure 3, lanes 8-10).

To determine whether the six potential organic ligands can bind to copper, a 10 mM solution of each was mixed with 10 mM Copper(II) Chloride ($CuCl_2$). Color changes were observed in the mixtures containing several NCI organic ligands, in the order of -241, -249 > -266 > -237 > -267, -273, -276 (Figure 4), indicating a chemical reaction and possible complex formation. To further examine this idea, prostate cancer PC-3 cells were treated ligand NSC-266 or -273, $CuCl_2$, or their mixtures (Figure 5A). We found that the order of accumulated levels of the proteasome target p27 were: mixtures > ligands > $CuCl_2$, DMSO (Figure 5A). The slight increase in p27 levels by -266 or -273 alone (Figure 5A, lanes 2, 4 *vs*. 1) suggests binding of these ligands to trace amount of cellular copper. PC-3 cells were also treated with 50 μM of -241, -249, the Cu-241 mixture, the Cu-249 mixture, or DMSO (as solvent control) for 24 h. Cells were collected and lysed, and the extracts were analyzed for the proteasomal chymotrypsin-like activity by rate of AMC release. It was found that -241 and -249 showed no proteasome-inhibitory effect on PC-3 cells unless combined with copper (Figure 5B).

When added into PC-3 cells that had been pretreated with $CuCl_2$ for 48 h, -241, but not DMSO, induced proteasome inhibition (Figure 6A) and cell death (Figure 6B). A post-treatment with TM had much less effect (data not shown). Taken together, these results suggest that some NCI organic ligands could bind to tumor cellular copper, forming proteasome inhibitors and apoptosis inducers.

4. SUMMARY

Many diseases and conditions are associated with Cu, from the classic Menkes and Wilson's diseases of Cu storage to current research linking Cu to Alzheimer's disease and Cu accumulation in cancer (23). A key problem with current cancer therapies is the inability to distinguish normal cells and tissues from cancerous ones. However, an intriguing feature of cancer cells and tissues is the accumulation of high concentrations of copper (6, 7, 19, 20). This coupled with the ability of several copper ligands discussed above to inhibit the proteasome and induce apoptosis in cancer cells suggested that it may be possible to treat cancer cells, containing clinically relevant levels of copper, with a copper-binding compound to form an anticancer copper complex inside the cell.

Several different classes of copper-binding compounds have been shown to inhibit proteasome activity and induce apoptosis when introduced to a copper-enriched environment. However, the mechanism of action has been unknown. Recent publications have all shown a requirement for copper and that in the absence of copper these compounds have a substantially mitigated effect, if any at all. Therefore, copper may be required for the activity of these compounds.

It is important to note that not all copper-binding compounds have proteasome-inhibitory and apoptosis-inducing capability. For example, TM and ethylenediaminetetraacetic acid are potent copper chelators but have no proteasome-inhibitory activity when combined with copper (32, 53), thus raising the question: what characteristics do copper-binding compounds, or copper complexes, require to inhibit proteasome activity?

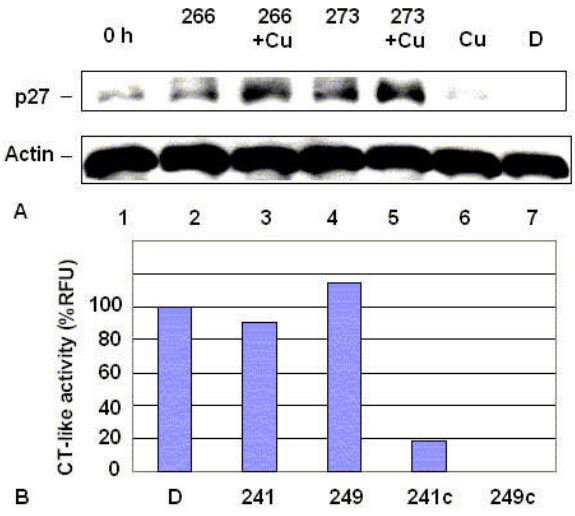

Figure 5. Accumulation of p27 in PC-3 cells by NCI ligand-copper mixtures. PC-3 cells (0 h) were treated with 50 μM of indicated NCI ligand, ligand-copper mixture, copper alone, or solvent (DMSO). (A) After 4 days, protein extracts were analyzed for Western blotting using antibodies to p27 and actin (as a loading control). (B) PC-3 cells were treated with 50 μM of -241, -249, 241-Cu mixture. 249-Cu mixture, or DMSO as solvent control for 24 h. Cells were collected and extracts analyzed for the proteasomal chymotrypsin (CT)-like activity by rate of AMC release.

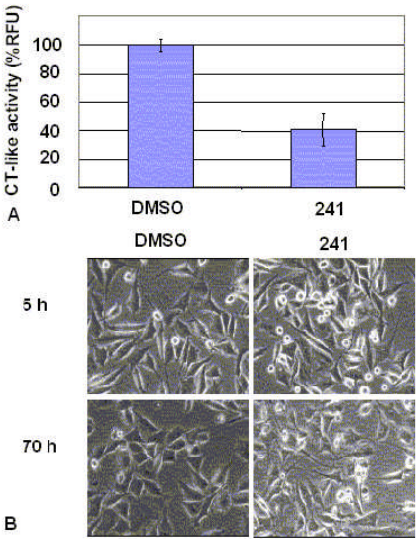

Figure 6. Inhibition of proteasome and induction of apoptotic morphology by NSC-241 in the presence of copper. PC-3 cells were cultured in 100 μM $CuCl_2$-containing media to elevate intracellular copper loads. After 48 h, copper-media was removed, and cells were washed and given standard media. Cells were then post-treated with 50 μM of 241 or DMSO. (A) Proteasomal chymotryptic (CT) activity was decreased in extracts of PC-3 cells post-treated with 241 *vs*. DMSO. (B) Cells post-treated with 241 (but not DMSO) were rounded up at 5 h and dying at 70 h.

Additionally, it has been shown that PDTC and CQ when complexed with copper had no toxic effect or proteasome inhibitory effect on normal breast cells (53). This suggests a selection mechanism by the complex or a difference in normal cell proteasome activity as compared to cancer cell proteasome activity. However, the ability of these compounds to inhibit the proteasome and induce apoptosis by themselves in copper-enriched cancer cells is promising for their development as anticancer compounds. This methodology would use a variety of checks to block toxicity: i) the compound itself should be nontoxic, ii) normal cells contain reduced levels of copper, and iii) normal cells are resistant to proteasome inhibition-based apoptosis. By using the dramatic difference in copper accumulation in normal *versus* cancer cells and tissues, it may be possible to develop nontoxic chemotherapeutic strategy.

5. ACKNOWLEDGMENTS

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