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Cupriphilic compounds to aid in proteasome inhibition

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ABSTRACT

It has been found that tumor cells and tissues, compared to normal cells, have higher levels of copper and possibly other metal ions. This presents a potential vulnerability of tumor cells that can serve as a physiological difference between cancer cells and normal cells and allows design of compounds that selectively target tumor cells while sparing normal cells. Recently we have identified compounds that have potential to inhibit the proteasome in tumor cells and induce cell death by mobilizing endogenous tumor copper resulting in in cellulo activation of the compound. These compounds hence act as pro-drugs, becoming active drugs in tumor cells with high copper content but remaining essentially inactive in normal cells, thereby greatly reducing adverse effects in patients. Such use would be of significant benefit in early detection and treatment of cancers, in particular, aggressive cancers such as pancreatic cancer which is usually not detected until it has reached an advanced stage. Six compounds were identified following virtual screening of the NCI Diversity Set with our proteasome computer model followed by confirmation with a biochemical assay that showed significant inhibition of the proteasome by the compounds in the presence of copper ions. In a dose response assay, NSC 37408 (6,7-dihydroxy-1-benzofuran-3-one), our best compound, exhibited an IC_{50} of 3 μM in the presence of 100 nM copper.

In cancer, therapy can be as debilitating as the disease itself considering that current treatments are often accompanied by severe toxicities in patients. These toxicities prompt continuing investigation into new therapies with reduced, or preferably, no toxic effects. Whereas treating cancer cells without toxicity in normal cells is the ultimate goal of drug discovery, this task has met with limited success due in large part to the difficulty of finding therapeutics that can distinguish cancer cells from normal cells.

Copper, which has the ability to adopt both oxidized (Cu^{2+}) and reduced (Cu^{1+}) states, is an essential trace element for various metabolic processes in living organisms.[1] There are several enzymes that use copper for processes necessary for carcinogenesis such as extracellular matrix degradation, endothelial cell proliferation, and migration mediated by integrins.[2,3] Due to its role in important physiologic processes, including metabolism, the concentration of copper in organisms is tightly regulated.[4,5] Copper is an element that plays an essential role in tumor development, angiogenesis, and metastasis.[6–10] Experimental evidence exists that shows tumor tissues possess both elevated copper and altered copper/zinc ratios in a stage dependent manner across multiple types of carcinomas.[11] However, these studies of the disposition of trace metals within cancer patients comparing normal tissues to cancerous tissues have been limited. In most of the studies focusing on copper, zinc, iron, and selenium, it has been observed that the copper concentrations, as opposed to other metals, were almost always found to be elevated compared to age matched samples from normal tissue.[12–15] For example, elevated serum copper levels in cancer patients have been reported in a wide variety of tumors in the following tissues: breast, cervical, ovarian, lung, prostate, and stomach.[16–19]

The biomolecular target of the present study, the proteasome, contains a 700 kDa barrel-shaped core particle formed by four axially stacked heptameric rings. These rings include the β subunits that possess six proteases whose active sites face an interior lumen where proteolysis occurs. These active sites further possess a catalytic region (S1) which includes the catalytic THR1 residue and recognition region (S3). The 20S proteasome, which is the proteolytic core of the 26S proteasome complex, contains multiple peptidase activities including the chymotrypsin-like (CTL), trypsin-like (TL), and peptidylglutamyl peptide hydrolyzing (PGPH) or caspase-like activities. The proteasome is involved in oncogenic events such as up-regulation of cellular proliferation, angiogenesis, down regulation of apoptosis, and drug resistance.[20–24] Pursuing proteasome inhibition is a clinically validated therapeutic strategy.[25,35] For example, proteasome inhibition is believed to result in buildup of abnormal or damaged proteins that triggers apoptosis. Figure 1 depicts the 20S proteasome containing β subunits 4, 5, and 6 which

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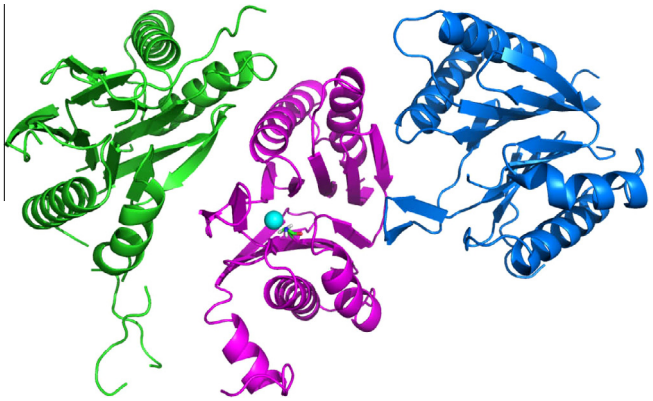

Figure 1. β4 (green), β5 (magenta) and β6 (blue) subunits of 20S proteasome. Copper is depicted in cyan. The three subunits play an important role in mediating the CTL activity in the proteasome.

are important in mediating the CTL activity of the proteasome. Copper is placed here in cyan based on quantum calculations discussed later. Note that copper is not required for proteasome activity.

In prior studies, we have shown that addition of copper complexation agents (independent of the salt form) to copper, had similar potency to inorganic copper salts in purified protein assays.[26] However, these compounds displayed substantially increased potency on human leukemia cancer cell lines. Thus, treatment of Jurkat T-cells with an 8-hydroxyquinoline copper mixture induced loss of viability in cell cultures. This effect was not observed upon treatment of Jurkat T-cells with 8-hydroxyquinoline alone or copper alone.

We previously also performed experiments on immortalized MCF-10A, premalignant MCF10AT1K.cl2, malignant MCF10DCIS.com and normal MDA-MB-231 breast cell lines, which were treated with copper complexing compounds clioquinol or pyrrolidine dithiocarbamate, in the presence and absence of copper, followed by measurement of cell survival rate. CTL activity of proteasome inhibition and apoptosis were also determined by levels of the ubiquitinated proteins in protein extracts of the treated cells and showed that clioquinol or pyrrolidine dithiocarbamate become active proteasome inhibitors and breast cancer cell killers in the presence of copper. These prior literature and experimental facts have brought to light the possibility of targeting tumor growth, angiogenesis, and metastasis with cupriphilic compounds.[27] In an attempt to realize this goal, our lab is using small molecules with a propensity to bind copper, cost effective synthesis, and a high potential for rational synthesis of analogues. With elevated copper levels as a marker, we hypothesize that our compounds can be used specifically against tumor cells with minimal impact on normal cells and may alleviate toxicities seen in current chemotherapeutic treatments.

Previous studies show that in cellulo assembled copper-activated proteasome inhibitors have apoptosis-inducing effects on a wide array of solid tumors and no measurable effect on normal cells.[27] Yet, the field of copper-activated proteasome inhibitors has stalled due to lack of therapeutically suitable compounds. Only a very small number of organic scaffolds have been studied with respect to complexation with copper for proteasome inhibition in cancer cells, including: pyrrolidine dithiocarbamate,[27] 8-hydroxyquinoline (8-HQ),[27] clioquinol (CQ),[26] and disulfiram.[28] Prior studies have shown that these compounds have differential effects in immortalized, pre-malignant, and malignant breast cancer cells.[27]

In the present study, our lab has utilized computational screening followed by experimental testing in an in vitro biochemical assay to discover a new set of compounds that, according to the hypothesized mode of action, should be selective for induction of apoptosis in tumor cells via proteasome inhibition. The compounds identified presumably could act as pro-drugs, becoming an active drug in the presence of copper in tumor cells, and then inducing apoptosis by proteasome inhibition.

Virtual screening was performed on compounds contained within the NCI Diversity Set 3[36] against the 20S proteasome model. Schrödinger's Maestro 9.3.5 was used as the primary graphical user interface for molecule structure preparation and Schrödinger applications were used for analysis. Quantum mechanical refinement of copper interactions with the THR1 in the active site using Q-site[29] and Jaguar[30] with B3LYP/LACVP* allowed for placement of copper and the assignment of partial charges on THR1 and the copper ion. The virtual screening method employed the modified yeast 20S proteasome crystal structure derived from PDB ID: 1IRU.[31] Ligands from the NCI Diversity Set 3 were prepared with LigPrep[32] and metal binding sites were added for generation of appropriate ligand states to interact with the copper ion. The standard precision (SP) setting in GLIDE was used for docking to incorporate metal binding sites.[33] Out of 1597 compounds, 62 were selected by the virtual screening method, which were then tested at 10 μM in the presence of 1 μM copper.

A Beckman Coulter Biomek FX^P Lab Automation Workstation was used for the automated assay runs. The assay was performed in 384-well black Nunc plates. The process involved the addition of 2 μL compound in DMSO added to 28 μL buffer (50 mM Tris; pH 7.6, 37 °C), with 20 μM, 10 μL 20S proteasome, and 10 μL of suc-leu-leu-val-tyr-AMC substrate and the rate of substrate cleavage/20S proteasome activity was determined. The assay in the presence of copper chloride solution involved addition of 3 μL of the copper solution and decrease in the amount of buffer solution to 25 μL. To allow chelation of copper to the compound, the plate was allowed to sit for 40 min with gentle shaking. The overall volume per well was kept constant at 50 μL. The compounds and substrate were initially dissolved in 100% DMSO, but the final concentration of DMSO per well plate was brought down to below 2% following subsequent dilutions. Plates were incubated at 37 °C for 2 h. A Perkin Elmer 2102 multi-label plate reader was used for fluorescence measurement. The plates were read using 340 nm excitation and 460 nm emission filters. All liquid transfers to the plates were performed using the Biomek workstation. Six lead compounds, depicted in Figure 2, exhibited greater than 80% proteasome inhibition in the presence of 1 μM copper. Disulfiram, a well-known copper chelating compound, and copper alone were used as controls in the experiment. For dose-response curves, the concentrations of copper chloride and compound were varied to find the greatest percent inhibition and results have been reported herein.

To understand whether other metals were interacting with the compound, a solution of metals such as nickel, calcium, iron, zinc and copper chloride were taken and tested in the same way as mentioned above with the compound. The inhibition of proteasome was to the same order as that for copper chloride for the same conditions suggesting that this is a copper specific phenomenon.

A series of novel copper-activated proteasome inhibitors was identified from NCI Diversity Set 3 that exhibited greater than 80% inhibition of the 20S proteasome when assayed at 10 μM in the presence of 1 μM copper. In a dose-response assay, NSC 37408 gave the best results with an IC_{50} of 3 μM in presence of 100 nM copper.

Figure 3 depicts the dose-response curves for proteasome inhibition with copper alone, compound alone, and compound with 100 nM copper. This shows a dramatic improvement with the compound in the presence of 100 nM copper, while copper alone or compound alone at that concentration exhibits nominal activity.