Clioquinol, a Therapeutic Agent for Alzheimer's Disease, Has Proteasome-Inhibitory, Androgen Receptor–Suppressing, Apoptosis-Inducing, and Antitumor Activities in Human Prostate Cancer Cells and Xenografts

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Abstract

Tumor growth and metastasis depend on angiogenesis that requires the cofactor copper. Consistently, high levels of copper have been found in many types of human cancers, including prostate, breast, colon, and lung. Recent studies suggest that copper could be used as a novel selective target for cancer therapies. Clioquinol is capable of forming stable complexes with copper and currently used in clinics for treatment of Alzheimer's disease. Most recently, it has been reported that clioquinol possesses antitumor effects. However, the underlying molecular mechanism is unclear. We report here that after binding to copper, clioquinol can inhibit the proteasomal chymotrypsin-like activity, repress androgen receptor (AR) protein expression, and induce apoptotic cell death in human prostate cancer LNCaP and C4-2B cells. In addition, clioquinol alone exhibits similar effects in prostate cancer cell lines with elevated copper at concentrations similar to those found in patients. Addition of dihydrotestosterone did not affect clioquinol-mediated proteasome inhibition in both prostate cancer cell lines. However, dihydrotestosterone partially inhibited clioquinol-induced AR suppression and apoptosis only in androgen-dependent LNCaP cells. Animal studies show that clioquinol treatment significantly inhibits the growth of human prostate tumor C4-2B xenografts (by 66%), associated with *in vivo* proteasome inhibition, AR protein repression, angiogenesis suppression, and apoptosis induction. Our study provides strong evidence that clioquinol is able to target tumor proteasome *in vivo* in a copper-dependent manner, resulting in formation of an active AR inhibitor and apoptosis inducer that is responsible for its observed antiprostate tumor effect. [Cancer Res 2007;67(4):1636–44]

Introduction

Prostate cancer is the most frequently diagnosed neoplasm and the second leading cause of death in men in industrialized countries (1). The androgen receptor (AR) is a transcription factor that mediates the action of androgens in target tissues and expressed in nearly all prostate cancers (2, 3). Concurrent AR overexpression was associated with a higher clinical stage, higher levels of prostate-specific antigen, an AR-transcribed gene product (2, 3), and earlier relapse after radical prostatectomy (4). It has been reported that several naturally occurring chemopreventive agents, such as isoflavones, curcumin, and (−)-epigallocatechin-3-gallate, can inhibit AR expression (5, 6). New approaches in therapies for prostate cancer are focused on down-regulating AR expression and overcoming difficulties associated with commonly used forms of antiandrogen (7, 8).

The ubiquitin/proteasome system plays an important role in the degradation of cellular proteins. This proteolytic system involves two distinct steps: ubiquitination and degradation (9, 10). The eukaryotic proteasome contains several known activities, all of which are associated with its β subunits (9, 10). We and others have reported that inhibition of the proteasomal chymotrypsin-like, but not trypsin-like activity, is associated with induction of apoptosis in tumor cells (11, 12).

Tumor growth and metastasis depend on angiogenesis that requires growth factors, proteases, and copper (13–16). The following four lines of research suggest that copper could be used as a novel selective target for cancer therapies. (*a*) Copper, but not other metals, is a cofactor essential for tumor angiogenesis (13–16). (*b*) High tissue or serum levels of copper have been found in many types of human cancers, including prostate, breast, colon, lung, and brain (17–21). (*c*) In normal organs (e.g., liver) of human and mammals, there are no known adverse effects observed when the bioavailability of copper is decreased up to 80% from the baseline (22). (*d*) Therapies using the strong copper chelator tetrathiomolybdate not only are well tolerated but also stabilize advanced kidney cancer (23, 24). It is important to note that tetrathiomolybdate therapy was found to prevent disease progression only in patients who became copper deficient. In other patients, the disease advanced before copper levels were sufficiently lowered (13, 14, 23). Thus, passive copper chelating may not be sufficient to eliminate tumors.

Clioquinol or 5-chloro-7-iodo-8-hydroxyquinoline (Fig. 1*A*), a lipophilic compound capable of forming stable complexes with copper(II) ions (25), was used as an antibiotic for treating diarrhea and skin infection (26). Although clioquinol use was thought to be associated with occurrence of subacute myelo-optic neuropathy in Japan (25–27), this conclusion was not supported by the subsequent epidemiologic analysis (28). Instead, decreased levels of vitamin B_{12} may play a role in this syndrome (26). In fact, clioquinol may be used safely in humans with vitamin B_{12}

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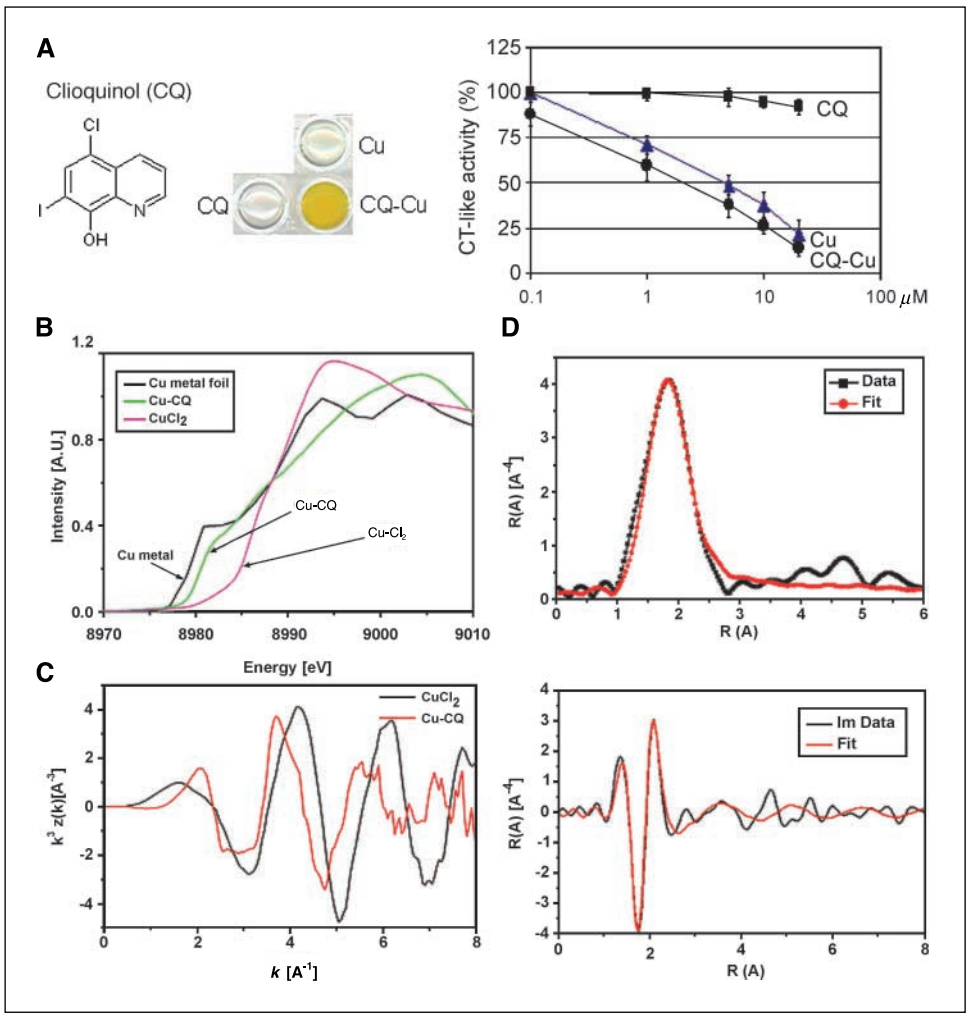

Figure 1. *In vitro* proteasome-inhibitory activity and structure analysis of clioquinol-copper complex. *A,* color change when clioquinol is mixed with $CuCl_2$ and proteasomal inhibition by the clioquinol-copper complex. Clioquinol (*CQ, colorless*) was mixed with $CuCl_2$ (*Cu, light green*), both dissolved in DMSO at 20 mmol/L, in a 1:1 molar ratio to form clioquinol-copper complex (*CQ-Cu, orange*). Inhibition of the chymotrypsin-like activity of purified rabbit 20S proteasome by $CuCl_2$, clioquinol, and clioquinol-copper was measured. Purified 20S rabbit proteasome was incubated with the peptide substrate for the proteasomal chymotrypsin (*CT*)-like activity in the presence of $CuCl_2$, clioquinol, and clioquinol-copper at indicated concentrations, as described in Materials and Methods. *B,* copper K edge XANES spectra. Copper metal foil and $CuCl_2$ are references for copper oxidation state. *Black line,* copper metal foil; *purple line,* $CuCl_2$. In contrast, the copper in clioquinol-copper mixture showed a change in its copper oxidation state (*green line*). *C,* EXAFS spectra at the copper K edge of $CuCl_2$ and clioquinol-copper mixture. *D,* Fourier transform of the clioquinol-copper EXAFS data. Magnitude and imaginary part are shown along with their respective fitting curves (*red*). The measured bond distance and coordination number of the copper site and other elements are not shown.

supplementation (29). Recent studies also show the potential use of clioquinol for treatment of Alzheimer's disease (30) and Huntington's disease (31). Two clioquinol clinical trials for Alzheimer's disease showed no toxicity in any patients and some clinical benefit in some patients (29, 32). Most recently, it was reported that in an *in vivo* lymphoma xenografts mouse model, clioquinol inhibited tumor growth (33), although the detailed mechanism of action is unclear.

We have reported that certain classes of copper-containing compounds act as potent proteasome inhibitors (34, 35). In the current study, we report that clioquinol was capable of binding copper and forming a complex, as verified by X-ray absorption near-edge spectroscopy (XANES) and by extended X-ray absorption fine structure spectroscopy (EXAFS). The clioquinol-copper complex possessed proteasome-inhibitory, AR-suppressive, and apoptosis-inducing activities in both androgen-dependent (LNCaP) and androgen-independent (C4-2B) human prostate cancer cell lines (36, 37). When used in prostate cancer cells containing a cellular copper level similar to those found in cancer patients, clioquinol alone had such activities. When administered to mice bearing C4-2B xenografts, clioquinol potently inhibited the tumor growth associated with *in vivo* proteasome inhibition, AR suppression, angiogenesis reduction, and apoptotic cell death.

Materials and Methods

Materials. Clioquinol, $CuCl_2$, ammonium tetrathiomolybdate, and Cremophor were purchased from Sigma-Aldrich (St. Louis, MO). Fluorogenic peptide substrates Suc-LLVY-AMC and Ac-DEVD-AMC were obtained from Calbiochem, Inc. (San Diego, CA). The peptide substrate Z-GGL-AMC was from Biomol International LP (Plymouth Meeting, PA). Apoptag Peroxidase *In situ* Apoptosis Detection kit was from Chemicon International, Inc. (Temecula, CA).

XAFS experiments in copper solutions. EXAFS and XANES at the copper K edge have been done at the 18-ID beamline of the Biophysics Collaborative Access Team facility (38, 39) at the Advance Photon Source, Argonne National Laboratory. The samples were 1 mmol/L of $CuCl_2$, clioquinol, and clioquinol-$CuCl_2$ solutions. Copper foil was also used as a reference. A Si(111) double crystal monochromator and a Si glass mirror comprise the main beamline optics. Both monochromator and mirror provide doubly focusing capabilities, the focused beam size being 160 μm H × 100 μm V (full width at half maximum). The excitation energy was scanned in the copper K edge energy region according to the standard procedures. To minimize X-ray dose and reduce the risk of radiation damage, samples were mounted in custom-made sample cells, quickly frozen in liquid nitrogen, and mounted in a Displex cryostat at 40K. Samples were placed in vertical and horizontal positions; thus, each sample spot was only exposed to X-rays for 60 s. The measurements were then repeated 100 times on different sample spots. Data were analyzed using IFEFFIT, Athena, and Artemis software (40, 41).

Cell cultures and whole-cell extract preparation. Human prostate cancer LNCaP and C4-2B cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and maintained at 37°C and 5% CO_2. A whole-cell extract was prepared as described previously (11, 34) for all the following procedures.

Color change reactions. Clioquinol and $CuCl_2$, each of which was dissolved in DMSO to a final concentration of 20 mmol/L, were mixed in a 1:1 ratio for color change.

Cellular morphologic analysis. A Zeiss Axiovert 25 microscope was used for microscopic imaging with phase contrast for cellular morphology.

Cell proliferation assay. LNCaP and C4-2B cells were seeded in triplicate in a 96-well plate and grown until 70% to 80% confluence, followed by treatment with indicated agents for 24 h. After that, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was done as described previously (35).

Western blot analysis. The cell or tissue extracts were separated by SDS-PAGE and transferred to a nitrocellulose blotting membrane. Western blot analysis was done using specific antibodies against ubiquitin, AR, p27, Bax, IκB-α, or β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or poly(ADP)ribose polymerase (PARP; Biomol International LP), followed by visualization using the enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ).

Inhibition of purified 20S proteasome activity by copper chloride and the clioquinol-copper mixture. The chymotrypsin-like activity of purified 20S proteasome was measured as described previously (34). Briefly, 17.5 ng of purified 20S proteasome were incubated in 100 μL of assay buffer [50 mmol/L Tris-HCl (pH 7.5)] with or without different concentrations of copper chloride, clioquinol, or the clioquinol-copper mixture and 10 μmol/L fluorogenic peptide substrate Suc-LLVY-AMC (for the proteasomal chymotrypsin-like activity) for 2 h at 37°C. After incubation, production of hydrolyzed AMC groups was measured using a Wallac Victor3 multilabel counter with an excitation filter of 365 nm and an emission filter of 460 nm.

Proteasomal chymotrypsin-like and caspase-3/7 activity assays using cell or tissue extracts. Total lysates (10 μg per reaction) of cells or tissue from human prostate tumor xenograft were incubated for 60 min at 37°C in 100 μL of assay buffer [50 mmol/L Tris-HCl (pH 7.5)] with 20 μmol/L of fluorogenic substrate Suc-LLVY-AMC (for the proteasomal chymotrypsin-like activity in cell extracts) or Z-GGL-AMC (for specific chymotrypsin-like activity in tumor tissues) or Ac-DEVD-AMC (for caspase-3/7 in tumor tissues), followed by measurement of AMC group release, as described above.

Human prostate tumor xenograft experiments. Five-week-old male athymic nude mice were purchased from Taconic Research Animal Services (Hudson, NY) and housed under pathogen-free conditions according to Wayne State University animal care guidelines. The protocols of animal experiments were reviewed and approved by Institutional Laboratory Animal Care and Use Committee of Wayne State University. C4-2B cells (5×10^6) were injected s.c. at one flank of the mice. Tumor size was measured every other day by a caliper. Tumor volume (V) was determined by the following equation $V = (L \times W^2) \times 0.5$, where L is the length and W is the width of the tumor. When xenografts reached volumes of ~200 mm^3, the mice bearing tumors were randomly assigned to control or clioquinol group administered daily using either vehicle control (mixture of Cremophor/PBS/ethanol/DMSO, 5:2:2:1) or 10 mg/kg/d of clioquinol. When the control tumors reached ~1,500 mm^3 (on day 15), the experiment was terminated and the mice were sacrificed. The tumors were removed and photographed, and the tumor tissues were then used for multiple assays to measure proteasome inhibition and apoptotic cell death.

Terminal deoxyribonucleotide transferase–mediated nick-end labeling assay. Tumor tissues were paraffin-embedded and stained according to the manufacturer's instruction. Briefly, after deparaffinization and hydration, the tissue sections were incubated with working strength terminal deoxynucleotidyl transferase enzyme and working strength stop/wash buffer, conjugated with antidigoxigenin, and then stained by peroxidase substrate. Finally, the tissue sections were mounted under a glass coverslip in Permount and viewed under microscope.

Immunohistochemistry. Tumor tissues were paraffin-embedded and sectioned. After deparaffinization and hydration, the slide was blocked by 3% hydrogen peroxide, incubated with primary antibody to p27 (1:20; Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom) or CD31, and then biotinylated with secondary antibody or anti-mouse IgG (H + L), followed by incubation in avidin and biotinylated horseradish peroxidase complex reagent (DAKO Laboratories, Cambridgeshire, United Kingdom). Finally, the slide was mounted with 3,3′-diaminobenzidine and visualized under microscope.

H&E staining assay. Paraffin-embedded tissue slides were deparaffinized and hydrated, and then stained with hematoxylin for 1 min. After rinsing, the slides were stained with eosin for 1 min, rinsed thoroughly, and mounted with Permount.

Statistical analysis. Statistical analysis was done with Microsoft Excel software. Student's t test for independent analysis was applied to evaluate differences between treatment and control.

Results

***In vitro* proteasome-inhibitory activity and structural analysis of the clioquinol-copper(II) complex.** It has been shown that clioquinol is capable of binding to copper (25). Indeed, when a solution of clioquinol was mixed with a solution of $CuCl_2$ at 1:1 molar ratio, dramatic color change was observed (Fig. 1*A*), indicating that a chemical reaction has occurred that involves formation of a clioquinol-copper complex.

To examine whether the complex of clioquinol-copper is capable of inhibiting the proteasome activity, we incubated $CuCl_2$, clioquinol alone, or the clioquinol-copper mixture at various concentrations with a purified rabbit 20S proteasome. The results showed that both $CuCl_2$ and clioquinol-copper complex inhibit the chymotrypsin-like activity of the purified 20S proteasome with IC_{50} values of 5.1 and 2.5 μmol/L, respectively (Fig. 1*A*), indicating that the complexed copper has increased proteasome-inhibitory activity. In a sharp contrast, clioquinol alone at even 20 μmol/L had little effect (Fig. 1*A*). This is consistent with our hypothesis (34, 35) that clioquinol is able to carry the copper ion into tumor cells and that the copper ion is responsible for inhibiting the proteasome activity (see below).

To further verify that clioquinol reacts with copper and forms a stable complex, a series of samples, including clioquinol, $CuCl_2$, and a mixture of clioquinol and $CuCl_2$, were analyzed by XANES and EXAFS. Clioquinol was measured to ensure it was free of copper. The result of XANES experiment (Fig. 1*B*) shows that the clioquinol-copper mixture has a different copper oxidation state (*green line*) from those of $CuCl_2$ (*purple line*) and metal copper (*black line*), confirming that copper ion interacted with clioquinol in solution and suggesting the formation of a new complex between $CuCl_2$ and clioquinol.

EXAFS data from the clioquinol-copper mixture also showed that the copper ion was coordinated to six-neighbor atoms at a distance of 2.36 Å in an octahedral configuration (Fig. 1*C* and *D*; data not shown), whereas in the $CuCl_2$ sample, four chlorine atoms were configured in a tetrahedral arrangement at a distance of 2.19 Å (Fig. 1*C* and *D*; data not shown). Therefore, both XANES and EXAFS results showed that the newly formed octahedral clioquinol-copper complex was distinct from the inorganic $CuCl_2$.

The clioquinol-copper complex inhibits the proteasome activity, reduces AR expression, suppresses cell proliferation, and induces apoptotic cell death in human prostate cancer cells. To test whether the clioquinol-copper complex was an active proteasome-inhibitory complex in intact prostate cancer cells,

androgen-dependent LNCaP and androgen-independent C4-2B cell lines were treated for 24 h with 20 μmol/L of $CuCl_2$, clioquinol, or the clioquinol-copper complex, using DMSO, tetrathiomolybdate, and tetrathiomolybdate-copper complex as controls. After the treatment, cells were collected and protein extracts were prepared for analysis of proteasome inhibition by two assays: the chymotrypsin-like activity assay (Fig. 2*A*) and the accumulation of ubiquitinated and target proteins by Western blotting (Fig. 2*B*). We found that the clioquinol-copper complex significantly inhibited the proteasome activity in both LNCaP (by 82%) and C4-2B (by 83%) cell lines (Fig. 2*A*). Consistently, the accumulation of ubiquitinated proteins and the proteasome target protein Bax was observed in both cell lines treated with the clioquinol-copper complex (Fig. 2*B*, *lanes 4* and *10*). In parallel, copper or clioquinol alone did not cause proteasome inhibition (Fig. 2*A* and *B*, *lanes 2, 3, 8*, and *9*). In addition, neither tetrathiomolybdate nor the tetrathiomolybdate-copper complex was able to inhibit the proteasome activity in both cell lines (Fig. 2*A* and *B*). Our data support that tetrathiomolybdate acts as a passive copper chelator/eliminator and that tetrathiomolybdate-copper is an inactive complex against the proteasome (35).

It has been shown that inhibition of the proteasomal chymotrypsin-like activity is associated with growth inhibition and apoptosis induction in tumor cells (11, 12). To determine whether proteasome inhibition by the clioquinol-copper complex causes suppression of cell proliferation, LNCaP and C4-2B cells were treated for 24 h with copper, clioquinol, clioquinol-copper complex, tetrathiomolybdate, the tetrathiomolybdate-copper complex (all at 20 μmol/L), or the vehicle DMSO. We found that the clioquinol-copper complex inhibited proliferation of LNCaP and C4-2B cells by 83% and 85%, respectively (Fig. 2*C*). In contrast, all the other treatments had very little or no inhibitory effect on cell proliferation of both lines (Fig. 2*C*).

To determine whether the proteasome-inhibitory effect of the clioquinol-copper complex was associated with apoptosis, two specific assays, PARP cleavage and cellular morphologic changes, were done. The cleavage of PARP was detected in both LNCaP and C4-2B cell lines treated with the clioquinol-copper complex (Fig. 2*B*, *lanes 4* and *10*). Furthermore, both cells lines treated with the clioquinol-copper complex became spherical and detached (Fig. 2*D*), indicating apoptotic cell death. In a sharp contrast, neither PARP cleavage nor cellular rounding/detachment were observed when cells were treated with copper, clioquinol alone, tetrathiomolybdate alone, or tetrathiomolybdate-copper complex (Fig. 2*B* and *D*).

For reasons that are not completely clear, inhibition of the proteasome activity has been shown to reduce AR protein (42). To determine the effects of the clioquinol-copper complex and other treatments on AR expression, aliquots of the cell lysates were analyzed by Western blotting. The AR protein was significantly decreased in cells of both lines treated with the clioquinol-copper complex (Fig. 2*B*, *lanes 4* and *10*). However, all other treatments had little or no effect on the AR protein levels (Fig. 2*B*). These results indicate that the proteasome-inhibitory clioquinol-copper complex down-regulates AR protein expression, which, in turn, may contribute to increased growth arrest and apoptosis. Indeed, a kinetic experiment showed that AR reduction (as early as 30 min) preceded apoptosis that did not start until 6 h after the clioquinol-copper treatment (data not shown).

Copper-enriched prostate cancer LNCaP and C4-2B cells are sensitive to treatment with clioquinol alone. Fundamental to the potential clinical utility of the nontoxic clioquinol ligand is its

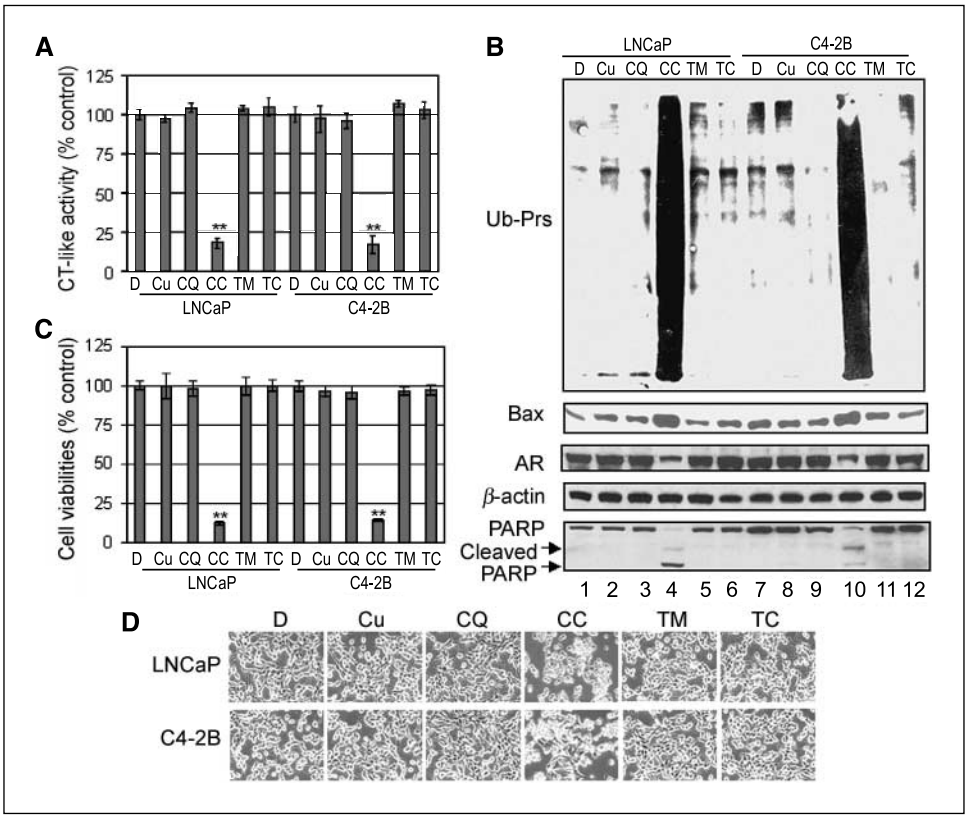

Figure 2. Inhibition of proteasome activity, cell proliferation, and AR expression as well as induction of apoptosis in LNCaP and C4-2B prostate cancer cells by clioquinol-copper complex. LNCaP and C4-2B prostate cancer cells were treated with 20 μmol/L of copper (*Cu*), clioquinol (*CQ*), clioquinol-copper complex (*CC*), tetrathiomolybdate (*TM*), and tetrathiomolybdate-copper complex (*TC*), using DMSO (*D*) as a control. After 24 h of treatment, cellular morphologic pictures were taken, and cells were collected and analyzed for proteasome inhibition and Western blotting. *A,* the proteasomal chymotrypsin-like activity was significantly decreased in both cell lines treated with clioquinol-copper complex compared with others. **, $P \leq 0.01$. *B,* Western blot analysis showed accumulation of ubiquitinated proteins (*Ub-Prs*) and Bax, decreased AR protein levels, and PARP cleavage in both cell lines treated with clioquinol-copper complex but not other treatments. The β-actin was used as loading control. *C,* MTT assay. LNCaP and C4-2B cells were treated with 20 μmol/L of copper, clioquinol, clioquinol-copper complex, tetrathiomolybdate, tetrathiomolybdate-copper complex, or DMSO (*D*) as control for 24 h, followed by MTT assay as described in Materials and Methods. *Columns,* mean of three experiments; *bars,* SD. **, $P < 0.01$. *D,* morphologic analysis. Cell death–associated morphologic changes (*spherical* and *detached*) were found in both cell lines treated only with clioquinol-copper.

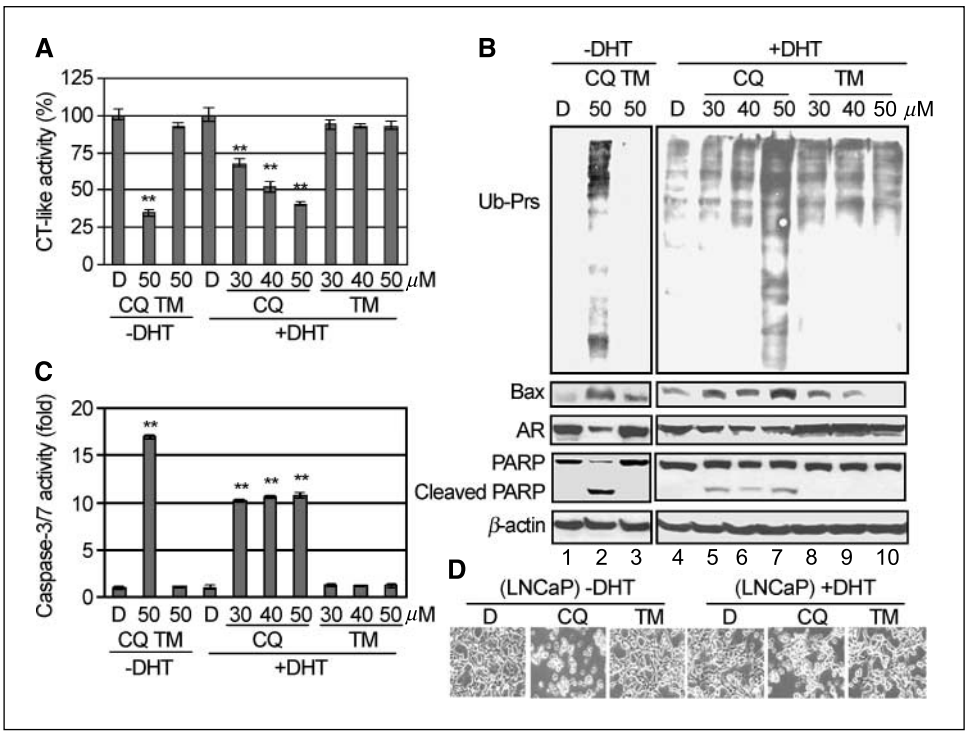

Figure 3. Copper-enriched LNCaP human prostate cancer cells were sensitive to treatment of clioquinol alone, protectable by dihydrotestosterone. LNCaP cells were cultured in medium containing 50 μmol/L of copper for 7 d, then maintained in normal growth medium containing either dihydrotestosterone (+*DHT*) or the same amount of ethanol (−*DHT*) for 12 h, followed by treatment with 30 to 50 μmol/L of clioquinol, tetrathiomolybdate, or vehicle DMSO for 24 h. *A,* the proteasomal chymotrypsin-like activity was decreased in cells treated with clioquinol alone whether pretreated with or without dihydrotestosterone. **, $P \leq 0.01$. Tetrathiomolybdate has no such effect. *B,* Western blot analysis using antibodies to ubiquitin (for ubiquitinated proteins), Bax, AR, PARP, and β-actin. *C,* caspase-3/7 activity. Fluorescent detection of caspase-3/7 activity was measured using an activity assay (see Materials and Methods). All data are representatives of independent triplicate experiments. *Columns,* fold caspase-3/7 activity; *bars*, SD. *D,* morphologic analysis. For details, see Fig. 2.

ability to bind with elevated endogenous copper in a tumor cell to form a complex that can inhibit the proteasome activity, suppress AR expression, and selectively induce tumor cell apoptosis. Although tumor tissues contain high level of copper (17–21), the cultured prostate tumor cell lines contain undetectable copper levels (34, 35). To mimic the *in vivo* situation, both LNCaP and C4-2B cell lines were cultured in medium containing 50 μmol/L $CuCl_2$ for 7 days and then cultured in normal copper-free growth medium. This $CuCl_2$ pretreatment led to increased cellular copper concentration by severalfold (34, 35), comparable with the copper levels found in patients (0.3-20 μmol/L; refs. 17, 18).

The $CuCl_2$-pretreated LNCaP (Fig. 3) and C4-2B (Fig. 4) cell lines were then treated with 50 μmol/L clioquinol or tetrathiomolybdate for 24 h, followed by measurement of proteasome inhibition, AR expression, and apoptosis induction. Both copper-enriched LNCaP and C4-2B cell lines were sensitive to treatment of clioquinol but not tetrathiomolybdate. The clioquinol treatment of the copper-enriched prostate cancer cells caused significant proteasome inhibition, as shown by the decreased proteasomal chymotryptic activity (Figs. 3*A* and 4*A*, *columns 2*) and by the increased levels of ubiquitinated proteins and Bax (Figs. 3*B* and 4*B, lanes 2*). In contrast, tetrathiomolybdate treatment had no significant effect (Figs. 3 and 4). Treatment with clioquinol, but not tetrathiomolybdate, also greatly reduced the levels of AR protein in both cell lines (by 52% and 82%, respectively; Figs. 3*B* and 4*B, lanes 2*). In addition, clioquinol, but not tetrathiomolybdate, induced apoptotic cell death of both LNCaP and C4-2B lines, as shown by PARP cleavage (Figs. 3*B* and 4*B, lanes 2*) and the cell rounding/detachment morphologic changes (Figs. 3*D* and 4*D, first three panels*). These results support the hypothesis that clioquinol is capable of binding prostate tumor cellular copper, resulting in proteasome inhibition, AR suppression, and apoptosis induction in both androgen-dependent and androgen-independent prostate cancer cells.

Androgen has greater protective effect in LNCaP than in C4-2B cells. To further study the function of AR in cell sensitivity to the proteasome inhibition–induced apoptosis, the copper-enriched LNCaP cells (Fig. 3) were treated with 10 nmol/L dihydrotestosterone for 12 h, followed by a further treatment with 30 to 50 μmol/L of clioquinol, tetrathiomolybdate, or equal volume of DMSO for 24 h. The end points, proteasome activity, AR protein level, and cell death, were then measured. Pretreatment and cotreatment with dihydrotestosterone did not affect clioquinol-induced proteasome inhibition in these LNCaP cells because similar levels of the proteasomal chymotrypsin-like activity were found after clioquinol treatment with or without dihydrotestosterone (Fig. 3*A*, *column 7* versus *column 2*). Consistently, high levels of ubiquitinated proteins and Bax protein were found in LNCaP cells cotreated with dihydrotestosterone and clioquinol, comparable with that of clioquinol treatment without dihydrotestosterone (Fig. 3*B*, *lane 7* versus *lane 2*). In parallel, tetrathiomolybdate exhibited no proteasome-inhibitory effect (Fig. 3*B*). Importantly, the AR decrease induced by clioquinol was inhibited by dihydrotestosterone treatment (52% versus 32% decrease; Fig. 3*B*, compare *lane 2* with *lane 1* versus *lane 7* with *lane 4*). This result suggests that proteasome inhibition by the clioquinol-copper complex occurs before the activation of AR by dihydrotestosterone. Consistent with the report that AR is an apoptosis inhibitor (7, 8), clioquinol-induced apoptotic cell death was also inhibited by dihydrotestosterone treatment of these cells, as shown by the decrease of PARP cleavage (73% versus 25% cleavage; Fig. 3*B*, *lane 2* versus *lane 7*), caspase activation (18-fold versus 11-fold; Fig. 3*C*), and rounding/detachment phenotypical changes (99% versus 60%; Fig. 3*D*).

Copper-enriched, androgen-independent C4-2B cells were also tested (Fig. 4). As with LNCaP cells, pretreatment and cotreatment with dihydrotestosterone did not affect clioquinol-induced proteasome inhibition, because similar low levels of the proteasomal chymotrypsin-like activity and similar high levels of ubiquitinated

proteins and Bax were found after clioquinol treatment with or without dihydrotestosterone (Fig. 4*A* and *B*). However, unlike the results with LNCaP cells (Fig. 3*B*), AR repression induced by clioquinol in C4-2B cells was not inhibited by dihydrotestosterone treatment (82% versus 78% decrease; Fig. 4*B*). In fact, in the presence of dihydrotestosterone, clioquinol still induced AR decrease (up to 78%) in C4-2B cells in a dose-dependent manner (Fig. 4*B*). These results indicated that dihydrotestosterone had less protective effect in C4-2B than in LNCaP cells against AR decrease (Figs. 4*B* versus 3*B*). This is consistent with the report that dihydrotestosterone can induce AR expression in androgen-dependent LNCaP but not in androgen-independent C4-2B cells (36). More importantly, clioquinol-induced apoptotic cell death in C4-2B cells was also uninhibited by dihydrotestosterone treatment, as shown by the comparable levels of PARP cleavage (58% versus 53%; Fig. 4*B*, *lane 7* versus *lane 2*), caspase activation (15-fold versus 18-fold; Fig. 3*C*), and the number of cells with apoptotic morphology (99% versus 99%; Fig. 3*D*) to those in the absence of dihydrotestosterone. These data further support that AR is an inhibitor of prostate cancer cell death and that the clioquinol-copper proteasome inhibitor induces apoptosis in hormone-refractory human prostate cancer cells, at least in part, by repressing AR.

Clioquinol inhibits tumor proteasome activity, suppresses AR, induces apoptosis, and inhibits angiogenesis *in vivo*. The aforementioned *in vitro* data showed that the clioquinol-copper complex (in LNCaP and C4-2B cells; Fig. 2) or clioquinol (in the copper-enriched cell lines; Figs. 3 and 4) inhibits proteasome activity, represses AR expression, and induces apoptosis. High levels of copper are found in tumor tissues and serum samples of cancer patients, including prostate, breast, colon, lung, and brain (17–21). It has also been shown that treatment with strong copper chelator tetrathiomolybdate inhibits the growth of human prostate, breast, and lung tumors in various mouse models (43, 44), indicating that human tumor xenografts in mice also contain high levels of copper.

To test whether clioquinol alone could have antitumor activity in AR-dependent, androgen-insensitive prostate cancer cells *in vivo*, we implanted C4-2B cells s.c. in male nude mice, followed by daily treatment with clioquinol at 10 mg/kg. We found that clioquinol treatment significantly inhibited tumor growth compared with the control (by 66%, $P < 0.01$; Fig. 5*A*). Proteasomal activity assay using tumor tissue extracts showed that clioquinol treatment significantly inhibited the proteasomal chymotrypsin-like activity (by 69%, $P < 0.01$, Fig. 5*B*). Consistently, Western blot analysis showed increased levels of ubiquitinated proteins and the natural proteasome target proteins, Bax, IκB-α, and p27, in clioquinol-treated C4-2B tumors (Fig. 5*D*). Immunostaining further confirmed the increase of p27 in tumors treated with clioquinol (Fig. 6*A*). Consistent with *in vitro* data, a significant decrease in AR protein levels was observed in C4-2B tumors treated with clioquinol (Fig. 5*D*).

The clioquinol-induced proteasome inhibition and AR suppression were associated with increased apoptosis, as shown by increased levels of caspase-3/7 activity (by 2.6-fold, $P < 0.01$; Fig. 5*C*), PARP cleavage (Fig. 5*D*), terminal deoxyribonucleotide transferase–mediated nick-end labeling (TUNEL) positivity (Fig. 6*B*), and condensed nuclei (Fig. 6*C*). Finally, treatment of C4-2B tumors with clioquinol significantly inhibited the expression of CD31 (Fig. 6*D*), an endothelial marker in blood microvessels (45), demonstrating the antiangiogenic effect of clioquinol. Altogether, we have shown that clioquinol treatment significantly inhibits the growth of prostate cancer C4-2B xenografts and this tumor-specific toxicity is associated with inhibition of the proteasomal activity, AR expression, and angiogenesis and induction of apoptosis *in vivo*.

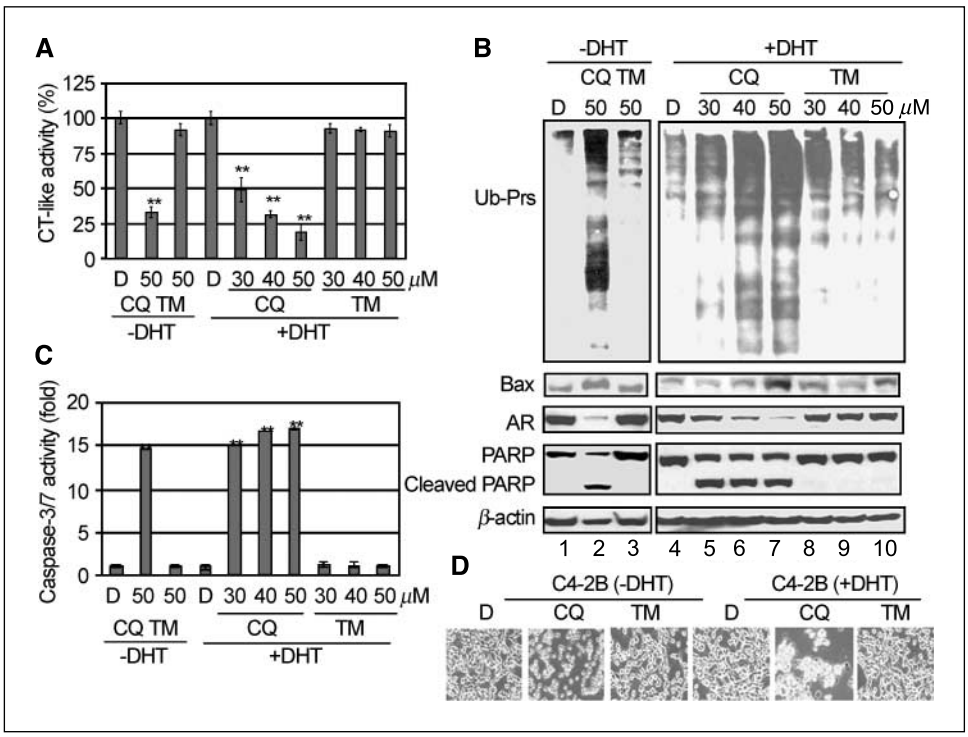

Figure 4. Copper-enriched C4-2B human prostate cancer cells were sensitive to treatment of clioquinol alone, which cannot be protected by dihydrotestosterone. Similar to Fig. 3, C4-2B cells were cultured in medium containing 50 μmol/L of copper for 7 d, then maintained in normal growth medium containing either dihydrotestosterone or the same amount of ethanol for 12 h, followed by treatment with 30 to 50 μmol/L of clioquinol, tetrathiomolybdate, or vehicle DMSO for 24 h. *A,* the proteasomal chymotrypsin-like activity. **, $P \leq 0.01$. *B,* Western blot analysis using antibodies to ubiquitin (for ubiquitinated proteins), Bax, AR, PARP, and β-actin. *C,* caspase-3/7 activity. *D,* morphologic analysis.

Discussion

Because clioquinol is a potent copper-binding compound (25), we hypothesized that the clioquinol-copper complex may act as a proteasome inhibitor. In the current study, we tested this hypothesis *in vitro* and *in vivo* by using human prostate cancer as a model. We reported that clioquinol was capable of binding copper and forming a complex, as verified by XANES and EXAFS. The clioquinol-copper complex possessed proteasome-inhibitory, AR-suppressive, and apoptosis-inducing activities in both androgen-dependent (LNCaP) and androgen-independent (C4-2B) human prostate cancer cell lines. When used in prostate cancer cells containing a cellular copper level similar to those found in cancer patients, clioquinol alone inhibited the proteasome activity and AR protein expression and induced apoptosis. Addition of dihydrotestosterone in these prostate cancer cell lines did not affect clioquinol-mediated proteasome inhibition, but inhibited clioquinol-induced AR suppression and apoptosis only in androgen-dependent LNCaP cells. Furthermore, when administered to mice bearing human prostate cancer C4-2B xenografts, clioquinol potently inhibited the tumor growth associated with *in vivo* proteasome inhibition, AR suppression, angiogenesis reduction, and apoptotic cell death. Our study shows that inhibition of the proteasome activity in human prostate cancer can be achieved by targeting tumor cellular copper and suggests the potential use of clioquinol as a novel anti-AR agent for prostate cancer prevention and treatment.

The most serious problem associated with many currently used anticancer drugs is their inability to distinguish normal cells from tumor cells. To develop novel selective anticancer agents, it is of paramount importance to explore the distinct properties or features of cancer cells from normal cells. One unique feature of tumors is the elevated levels of copper, which has been found in many types of human cancers, including prostate, breast, colon, lung, and brain (17–21). Accumulated evidence has shown that copper, but not other transition metals, is a cofactor essential for tumor angiogenesis (13, 14).

In searching for new proteasome inhibitors with high potency and low toxicity, we found that several organic copper complexes, including clioquinol-copper, possessed a high potency in inhibiting the proteasomal activity in tumor, but not normal cells (34, 35). In the current study, we found that clioquinol could interact with copper and form a new complex that was a proteasome inhibitor, an AR suppressor, and an apoptosis inducer in cultured human prostate cancer LNCaP and C4-2B cells.

Under the *in vivo* mimic condition, clioquinol alone, but not tetrathiomolybdate, could inhibit proteasome activity, suppress AR protein, and induce apoptosis in copper-enriched human prostate cancer LNCaP and C4-2B cells (Figs. 3 and 4). The results support our hypothesis that the organic ligand clioquinol could interact with tumor cellular copper and form an active, specific proteasome-inhibitory complex, which leads to AR repression and apoptosis induction (Figs. 3 and 4). Although the strong metal

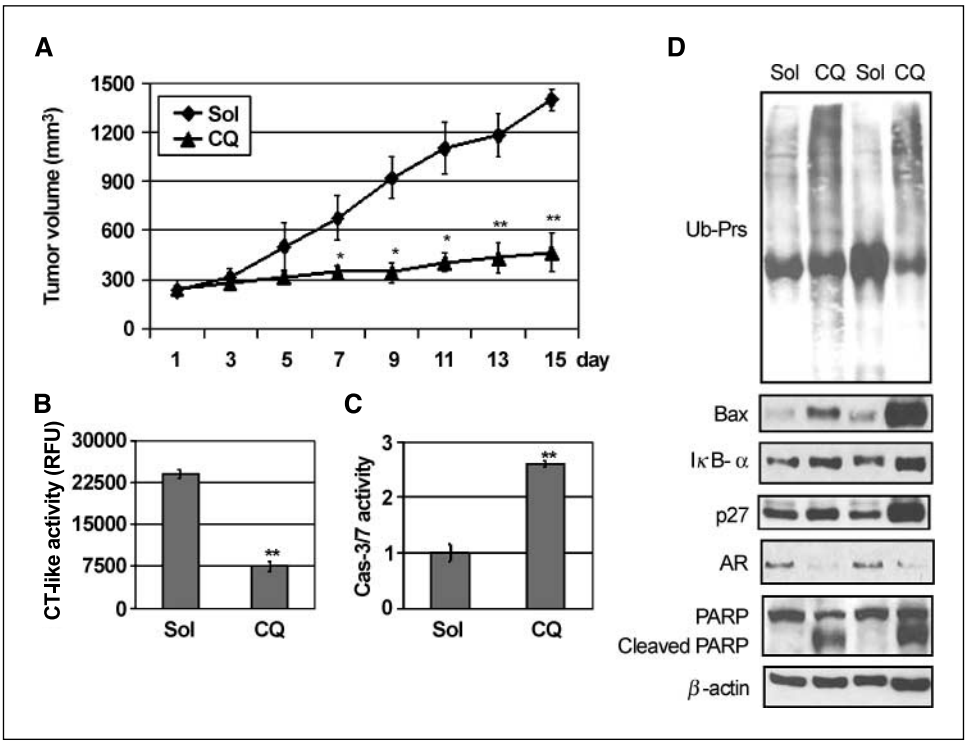

Figure 5. Clioquinol treatment inhibits tumor growth in mice bearing C4-2B xenografts, associated with inhibition of proteasome activity, suppression of AR protein expression, and induction of apoptosis *in vivo*. Male athymic nude mice were xenografted by injection of C4-2B cells. When tumor size reached to ~200 mm^3, the mice were divided into two groups and treated with either solvent (*Sol*) control or clioquinol (10 mg/kg/d; $n = 8$). Tumors were collected after 15-d treatment, and the prepared tissue samples were used for the proteasomal and caspase-3/7 activity assays and Western blotting as well as immunohistochemistry. *A,* tumor growth chart. Clioquinol inhibited tumor growth by up to 66% after 15-d treatment when compared with control. *Points,* mean of tumor volume in each experimental group; *bars,* SD. **, $P < 0.01$. *B,* proteasomal chymotrypsin-like activity assay. The chymotrypsin-like activity was inhibited by 69% in the tissue extract of tumors treated with clioquinol when compared with control. *Columns,* chymotrypsin-like activity (*RFU,* relative fluorescence unit); *bars,* SD. **, $P < 0.01$. *C,* caspase-3/7 activity assays. An increase of 2.6-fold in caspase-3/7 activity was found in the tissue extract of tumors treated with clioquinol when compared with control. *Columns,* caspase-3/7 activity; *bars,* SD. **, $P < 0.01$. *D,* Western blot analysis of tumor tissue extracts. The accumulation of ubiquitinated proteins, Bax, IκB-α, and p27, and cleavage of PARP were shown in the tissue extracts of tumors treated with clioquinol.

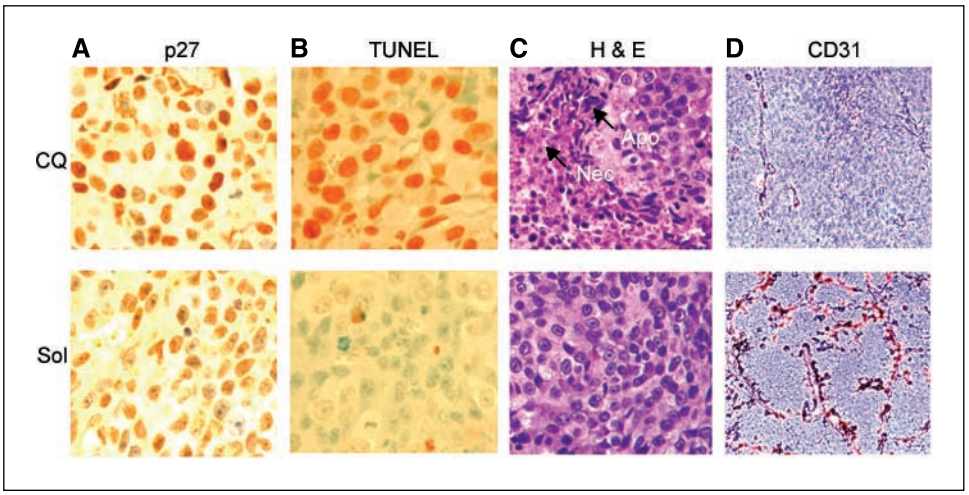

Figure 6. Immunohistochemistry, TUNEL, and H&E staining assays using mouse tumor samples. Tumors were collected after 15-d treatment (see Fig. 5 legend), and the prepared tissue slides were used for immunostaining with antibodies to p27 or CD31, and for TUNEL and H&E staining assays. The images showed stronger or/and more p27-positive cells (*A*), more TUNEL-positive nuclei (*B*), more apoptotic-condensed nuclei (*Apo with arrow*), and more necrotic tumor cells (*Nec with arrow*; *C*) in tumor tissues from mice treated with clioquinol (*A-C*; magnification, ×400). Much less formation of blood vessels, measured by CD31 staining, was also shown in tumors treated with clioquinol compared with solvent control (*D*; magnification, ×100).

chelator tetrathiomolybdate should bind to copper in cancer cells, the resulting complex seems inactive against the proteasome (Figs. 3 and 4).

AR plays an important role in both androgen-dependent and androgen-independent prostate cancer cells because AR continues to be expressed in androgen-independent tumors and the AR signaling pathway remains functional in these tumors (2, 3). In the current study, we examined the effect of the clioquinol-copper complex on both LNCaP and C4-2B cell lines and whether addition of androgen could alter clioquinol-induced effects in both cell lines. Our results showed that dihydrotestosterone played a more protective role in androgen-dependent LNCaP cells than in androgen-independent C4-2B cells on clioquinol-induced AR suppression and apoptosis induction (Figs. 3 and 4).

Our *in vivo* data support the conclusion that clioquinol can react with endogenous copper within tumor tissue and form an active complex that exhibits antitumor activity. Indeed, clioquinol significantly inhibited tumor growth in mice bearing C4-2B tumor xenografts (by 66%; Fig. 5*A*). It is important to note that the antitumor activity of clioquinol was tightly associated with its proteasome-inhibitory abilities because clioquinol treatment resulted in the inhibition of proteasomal chymotrypsin-like activity in tumors (by 69%; Fig. 5*B*) and accumulation of ubiquitinated proteins and proteasome target proteins Bax, IκB-α, and p27 (Figs. 5*D* and 6*A*). The *in vivo* results further supported the idea that inhibition of the proteasomal chymotrypsin-like activity was associated with induction of apoptosis in tumors (11, 12), as shown by the clioquinol treatment–induced apoptosis in C4-2B implant tumor (i.e., increased caspase-3/7 activity, by 2.6-fold; Fig. 5*C*), PARP cleavage (Fig. 5*D*), TUNEL positivity (Fig. 6*B*), and condensed nuclei (Figs. 6*C*). In addition, clioquinol treatment also caused repression of AR expression (Fig. 5*D*) and inhibition of angiogenesis in tumor tissue as measured by CD31 immunohistochemistry (Fig. 6*D*). Most recently, it was reported that in an *in vivo* lymphoma xenograft mouse model, clioquinol inhibited tumor growth and its role as a zinc ionophore was suggested as a potential mechanism (33). We have found that a clioquinol-zinc complex is also a proteasome inhibitor, although its potency is weaker than that of the clioquinol-copper complex.[6] Our data presented here have provided an alternative mechanistic interpretation for their findings (33) and showed the requirement of proteasome inhibition for the antitumor activity of clioquinol.

Our finding supports the novel concept of using elevated copper level and proteasome activity in cancer cells and tissues as selective targets for cancer chemotherapy. Cancer cells and tissues that contain elevated copper and more dependent on proteasome activity for their survival should be very sensitive to treatment of clioquinol. In contrast, normal cells and tissues that contain trace amounts of copper and have basal level of proteasome activity should be much more resistant to clioquinol treatment. Clioquinol should have such a tumor-selective activity, which is supported by the observation that it exhibits no toxicity in the animals. Taken together, our finding suggests that clioquinol may serve as an excellent anti-AR agent and a novel anticancer drug for prostate cancer prevention and treatment.

[6] D. Chen and Q. P. Dou, unpublished data.

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