Curcumin Inhibits the Proteasome Activity in Human Colon Cancer Cells *In vitro* and *In vivo*

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Abstract

Curcumin (diferuloylmethane) is the major active ingredient of turmeric (*Curcuma longa*) used in South Asian cuisine for centuries. Curcumin has been shown to inhibit the growth of transformed cells and to have a number of potential molecular targets. However, the essential molecular targets of curcumin under physiologic conditions have not been completely defined. Herein, we report that the tumor cellular proteasome is most likely an important target of curcumin. Nucleophilic susceptibility and *in silico* docking studies show that both carbonyl carbons of the curcumin molecule are highly susceptible to a nucleophilic attack by the hydroxyl group of the NH_2-terminal threonine of the proteasomal chymotrypsin-like (CT-like) subunit. Consistently, curcumin potently inhibits the CT-like activity of a purified rabbit 20S proteasome (IC_{50} = 1.85 μmol/L) and cellular 26S proteasome. Furthermore, inhibition of proteasome activity by curcumin in human colon cancer HCT-116 and SW480 cell lines leads to accumulation of ubiquitinated proteins and several proteasome target proteins, and subsequent induction of apoptosis. Furthermore, treatment of HCT-116 colon tumor–bearing ICR SCID mice with curcumin resulted in decreased tumor growth, associated with proteasome inhibition, proliferation suppression, and apoptosis induction in tumor tissues. Our study shows that proteasome inhibition could be one of the mechanisms for the chemopreventive and/or therapeutic roles of curcumin in human colon cancer. Based on its ability to inhibit the proteasome and induce apoptosis in both HCT-116 and metastatic SW480 colon cancer cell lines, our study suggests that curcumin could potentially be used for treatment of both early-stage and late-stage/refractory colon cancer. [Cancer Res 2008;68(18):7283–92]

Introduction

Curcumin (diferuloylmethane) is the active ingredient of turmeric, derived from the rhizome of *Curcuma longa*. Besides its traditional use as a food coloring and flavoring agent, curcumin also has a well-documented history in medicine in India and Southeast Asia. Curcumin is used to treat various diseases, including respiratory conditions, inflammation, liver disorders, diabetic wounds, cough, and certain tumors (1).

Although curcumin has been shown to inhibit the growth of transformed cells and all three stages of colon carcinogenesis (initiation, promotion, and progression) in carcinogen-induced rodent models, the underlying mechanisms are not fully understood. Many molecular targets have been suggested including various transcription factors, inflammatory enzymes, cytokines, adhesion molecules, and cell survival proteins (2).

The ubiquitin-proteasome pathway is essential for many fundamental cellular processes, including the cell cycle, apoptosis, angiogenesis, and differentiation (3, 4). The proteasome contributes to the pathologic state of several human diseases including cancer and AIDS, in which some regulatory proteins are either stabilized due to decreased degradation or lost due to accelerated degradation (5). The 20S proteasome, the proteolytic core of 26S proteasome complex, contains multiple peptidase activities including the chymotrypsin like (CT-like), trypsin like (T-like), and peptidylglutamyl peptide hydrolyzing like (PGPH-like; ref. 6). It has been shown that inhibition of tumor cellular CT-like activity is a strong stimulus that induces apoptosis (7, 8). The possibility of therapeutically targeting the ubiquitin-proteasome pathway was initially met with great skepticism because this pathway plays an important role in normal cellular homeostasis as well. However, after the demonstration that actively proliferating cancer cells are more sensitive to apoptosis-inducing stimuli, including proteasome inhibition, proteasome inhibitors became the subject of further investigation (9–11). With the finding that proteasome inhibitors were well-tolerated and had activity in experimental models of human malignancies *in vivo* (12), the proteasome inhibitor bortezomib (Velcade/PS-341) was introduced into a phase I clinical trial (13). Although the data from the bortezomib trials showed significant clinical benefit, some toxicity was observed (14). The most common side effects include nausea, fatigue, and diarrhea, whereas more adverse events are thrombocytopenia, peripheral neuropathy, neutropenia, lymphopenia, and hyponatremia. Among them, peripheral neuropathy is the one that often causes an early cessation of the treatment (4). Therefore, there is a need to search for other proteasome inhibitors with less or no toxic side effects, e.g., from naturally occurring or nutritional compounds, mostly because they are generally more tolerable in human body (15).

The benefit and chemopreventative role of dietary curcumin was observed in a variety of rodent models of carcinogenesis. As a logical consequence, curcumin is currently in clinical trials for treatment of various cancers, including multiple myeloma, pancreatic cancer, and colon cancer (16). The fact that colon cancer is one of the leading cancer-related deaths in Western countries and that current treatment options have only limited efficacy against advanced colon cancer contributes to the increasing interest for the use of curcumin in colon cancer prevention (17). Moreover, curcumin has been shown to inhibit the

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expression of cyclooxygenase-2 (COX-2) that plays a significant role in colon carcinogenesis (18). It is also important to emphasize that to date, no toxicity associated with curcumin in either experimental animals or humans, even at very high doses, has been observed (2).

Curcumin-induced suppression of carcinogenesis is thought to be due to inhibition of nuclear factor-κB (NF-κB), which is controlled by the proteasome-mediated proteolytic degradation pathway (19), and subsequent inhibition of proinflammatory pathways (20). Additionally, curcumin was shown to down-regulate cyclin D1, cyclin E, and MDM2; up-regulate p21, p27, and p53; and to induce apoptosis (2).

A recent report revealed that curcumin enhanced the antitumor activity of celecoxib, indicating their synergistic effect on the growth of colorectal cancer cells (21). Trials currently underway include an evaluation of colon cancer patients treated with gemcitabine in combination with curcumin and celecoxib (NCT00295035; Tel-Aviv Sourasky Medical Center). Recently, we have reported that in colon cancer cells, curcumin acts synergistically with 5-fluorouracil, oxaliplatin, and leucovorin (FOLFOX), a chemotherapeutic regimen that, together with 5-fluorouracil, irinotecan, and leucovorin, remains the backbone of colon cancer treatment (22). Taken together, the results suggest that chemotherapeutics in combination with curcumin may provide a superior therapeutic strategy in the clinical setting for treatment of refractory tumors.

In the present study, we have studied the potential molecular target of curcumin in human colon cancer cells. We first show that a computer docking model predicts curcumin to inhibit proteasomal chymothypsin-like activity. We then show that curcumin indeed directly inhibits the CT-like activity of a purified rabbit 20S proteasome (IC_{50} = 1.85 μmol/L) and 26S proteasome in human colon cancer HCT-116 and SW480 cell lines. After proteasome inhibition, apoptotic cell death was induced. Furthermore, treatment of colon cancer–bearing ICR SCID mice with curcumin (500 mg/kg/d, intragastric, for 21 days) resulted in tumor growth suppression, associated with *in vivo* proteasome inhibition, growth arrest, and apoptosis induction. Our results suggest that curcumin has proteasome-inhibitory activity *in vitro* and *in vivo* and could effectively be used for the prevention and treatment of human colon cancer.

Materials and Methods

Materials. Curcumin, ethanol, and DMSO were purchased from Sigma-Aldrich. DMEM/F12, McCoy's 5A, penicillin, and streptomycin were purchased from Invitrogen. Purified rabbit 20S proteasome and fluorogenic peptide substrates Suc-LLVY-AMC, Z-LLE-AMC, Z-ARR-AMC (for the proteasomal CT-like, PGPH-like, T-like activities, respectively), and Ac-DEVD-AMC (for the caspase-3/7 activity) were from Calbiochem. Mouse monoclonal antibody against human poly(ADP-ribose) polymerase (PARP) was purchased from BIOMOL International LP. Mouse monoclonal antibodies against Bax (B-9), p27 (F-8), ubiquitin (P4D1), rabbit polyclonal antibody against IκB-α (C-15), goat polyclonal antibody against actin (C-11), and secondary antibodies were from Santa Cruz Biotechnology, Inc.

Nucleophilic susceptibility analysis and computational modeling. Molecules were constructed using the CAChe Workstation (Fujitsu, Inc.) as described previously (23). The AutoDock 3.0 suite of programs was used for the docking calculations and the output from AutoDock was rendered with PyMOL as described previously (23).

Cell cultures and whole cell extract preparation and Western blot analysis. The human colon carcinoma HCT-116 (a generous gift from Dr. Charles D. Lopez, Oregon Health & Science University, Portland, OR) and metastatic SW480 cell lines (a generous gift from Prof. Satya Narayan, UF Shands Cancer Center, Gainesville, FL, USA) were grown in DMEM/F12 and McCoy's 5A medium, respectively, both supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin at 37°C in a humidified incubator with an atmosphere of 5% CO_2. A whole cell extract preparation and Western blot analysis were done as previously described (24). Densitometry was quantified using AlphaEase FC software (Alpha Innotech Corporation).

Inhibition of purified 20S proteasome activity. Purified rabbit 20S proteasome (17.5 ng) was incubated with 10 μmol/L of the various substrates in 100 μL of assay buffer [25 mmol/L Tris-HCl (pH 7.5)] in the presence of curcumin at various concentrations or equivalent volume of solvent ethanol as a control. After 2 h of incubation at 37°C, inhibition of each proteasomal activity was measured by the release of hydrolyzed AMC groups, as previously described (24).

Inhibition of the proteasomal CT-like activity in intact cells. HCT-116 or SW480 cells were cultured in 96-well plates (1×10^4 cells per well) and treated for various time points with different concentrations of curcumin or MG132. After the additional 2-h incubation with the fluorogenic peptide substrate specific for the proteasomal CT-like activity, production of hydrolyzed AMC groups was measured, as described above.

Caspase-3/-7 activity assay. Proteins extracted from treated cells were incubated in 100 μL of assay buffer [25 mmol/L Tris-HCl (pH 7.5)] with 20 μmol/L fluorogenic substrate specific for caspase-3/-7 activity. After 3 h incubation at 37°C, release of hydrolyzed AMC groups was measured as described above.

Cellular morphology analysis and terminal deoxynecleotydyl transferase–mediated dUPT-biotin nick end-labeling assay. A Zeiss Axiovert 25 microscope was used for all microscopic imaging with phase contrast for cellular morphology as described previously (25). Terminal deoxynecleotydyl transferase–mediated dUPT-biotin nick end-labeling assay (TUNEL) was performed using the BD Biosciences PharMingen, APO-DIRECT kit. Cells were treated with various curcumin concentrations for 24 h, harvested, fixed, and stained according to the protocol.

Mice and tumor cell implantation. Six-week-old female homozygous ICR SCID mice were purchased from Taconic Farms. The mice were adapted to animal housing and HCT-116 xenografts were developed as described earlier (26). Before treatment, curcumin was dissolved in DMSO (final volume 0.05%) and further diluted in sesame oil. Tumor growth deduced as volume of the tumor in each group was determined by twice weekly caliper measurements. All mice were euthanized 1 d after the last dose of treatment. H&E staining confirmed the presence of tumor.

Determination of proliferating cell nuclear antigen immunoreactivity, apoptosis by TUNEL assay, immunostaining, and H&E assays using tumor tissue samples. Determination of proliferating cell nuclear antigen (PCNA) immunoreactivity, as a measure of proliferative activity, was done as described previously (27). TUNEL assay was done to detect apoptotic cells using the *In situ* Cell Death Detection kit from Roche Applied Science according to the manufacturer's instructions. Immunostaining of p27 was done as described previously (28). H&E staining in tumor tissues were performed following manufacturer's protocol.

Statistical analysis. Data are represented as mean ± SD for the absolute values as indicated in the vertical axis legend of figures. The statistical significance of differential findings between curcumin-treated and control mice was determined by Student's *t* test as implemented by Excel 2000 (Microsoft Corp.). *P* values smaller than 0.05 were considered statistically significant.

Results

Curcumin adopts a proteasome-inhibitory pose in an *in silico* model. We have reported that the ester bond carbon of β-lactone, the green tea polyphenol (−)-epigallocatechin-3-gallate [(−)-EGCG], (−)-EGCG analogues, and tannic acid is responsible for the potent and specific proteasomal CT-like inhibition (29–31). Moreover, our recent data suggest that the carbonyl carbon of tea polyphenols

and flavonoids confers their proteasome-inhibitory potencies (32, 33). Because curcumin contains two carbonyl carbons (Fig. 1*A*), we hypothesized and found both of them to be the sites of nucleophilic susceptibility (Fig. 1*B*).

We then proposed that curcumin is susceptible to nucleophilic attack by NH_2-terminal threonine (Thr 1) of the β5 CT-like subunit of the proteasome and is able to be oriented in an inhibitory pose within this subunit. To test this, curcumin was docked to the proteasomal β5 subunit, and cluster analysis was performed. The predictability of binding was determined based on the frequency of curcumin to adopt an inhibitory pose (a pose that places the curcumin carbonyl carbon within 4.0 Å of Thr 1). Curcumin docked with an average of 80 of 100 poses (80%) that placed at least one of the carbonyl carbons in a suitable position to undergo nucleophilic attack and the most representative pose is shown in Fig. 1*C*. Furthermore, due to exposed hydroxyl groups, curcumin could potentially form a hydrogen bonds with the surrounding amino acids, such as Serine 96 with a distance of 2.18 Å in the β5-subunit (Fig. 1*C*), strengthening its binding potential. Therefore, the computer docking results predict that curcumin binds to Thr 1 of the proteasomal β5-subunit, which might cause inhibition of the proteasomal CT-like activity.

Inhibition of purified 20S proteasome CT-like activity by curcumin. To provide direct evidence for proteasome inhibition by curcumin, we carried out a cell-free proteasome activity assay by incubating a purified rabbit 20S proteasome with different concentrations of curcumin. As predicted by the *in silico* model, we found that the β5-mediated CT-like activity of the purified 20S proteasome was significantly inhibited by curcumin with an IC_{50} value of 1.85 μmol/L (Fig. 1*D*). To investigate whether curcumin specifically inhibits the proteasomal CT-like activity, its effects on the T-like and PGPH-like activities of the purified 20S proteasome were also examined. When used at 1 μmol/L, curcumin inhibited the CT-like, T-like, and PGPH-like activities of the purified 20S proteasome by 46%, 20%, and 34%, respectively (Fig. 1*D*), whereas at 5 μmol/L, it inhibited these three proteasomal activities by 73%, 46%, and 59%, respectively (Fig. 1*D*). The IC_{50} values of curcumin to the CT-like, T-like, and PGPH-like activities of the purified 20S proteasome were determined to be 1.85, 6.23, and 3.68 μmol/L, respectively. Taken together, our results show that curcumin possesses the ability to inhibit all the three proteasomal activities but with the highest potency to the CT-like activity.

Curcumin inhibits proteasomal activity and induces apoptosis in human colon cancer HCT-116 and SW480 cells in a dose-dependent manner. After demonstrating that curcumin inhibits the purified 20S proteasome (Fig. 1), the next set of experiments was performed to determine if curcumin would also target and inhibit the tumor cellular 26S proteasome. Human colon cancer HCT-116 and SW480 cells were plated in 96-well plates and treated with various concentrations of curcumin for 24 hours, followed by 2 hours of additional incubation with a specific peptide substrate for the proteasomal CT-like activity. We found a dose-dependent inhibition induced by curcumin in both colon cancer cell lines (Fig. 2*A*). When used at a 10 μmol/L concentration, curcumin induced 32% and 42% inhibition in HCT-116 and SW480 cells, respectively, whereas at 30 μmol/L concentration, 65% and 81% inhibition was induced in HCT-116 and SW480 cells, respectively (Fig. 2*A*).

We also compared curcumin to a well-known proteasome inhibitor MG132. When used at 10 or 30 μmol/L concentrations in HCT-116 cells, MG132 induced 53% and 70% inhibition, respectively (Fig. 2*A*), showing similar potency to curcumin.

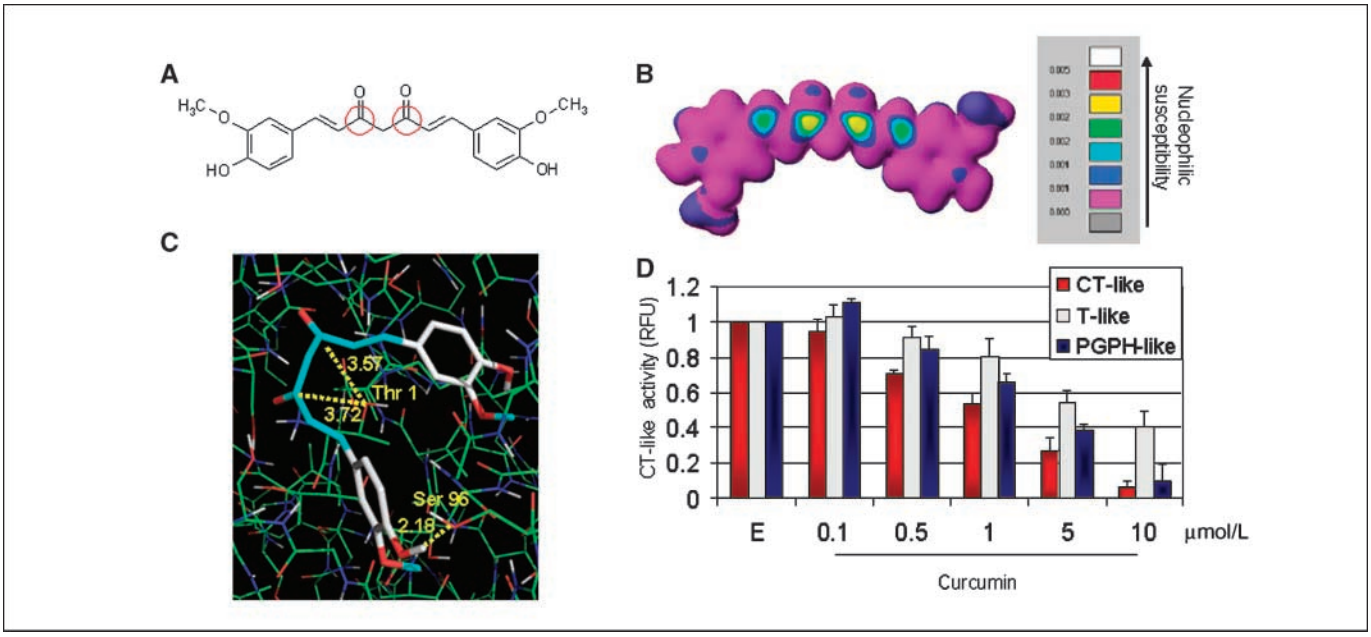

Figure 1. *In silico* and *in vitro* proteasome inhibition by curcumin. *A,* the chemical structure of curcumin. The regions with carbonyl carbons that have the structure activity relationship are marked with red circles. *B,* molecular orbital energy analysis is shown by drawing and electron density isosurface and coloring by nucleophilic susceptibility. *Yellow center,* the highest area of susceptibility. *C,* docking analysis of curcumin, which is represented by the stick structure. The colors are representative of atom type (*gray,* carbon; *red,* oxygen; *white,* hydrogen; *light blue,* methyl). *Yellow dotted line,* the distance, in angstroms, of each of the carbonyl carbons to Thr 1, indicative of potential nucleophilic attack, and to Ser 96, indicative of potential hydrogen bonding. *D, in vitro* analysis using a 20S proteasome indicates that curcumin inhibits CT-like, T-like, and PGPH-like activities with IC_{50} values of 1.85 ± 0.35, 6.23 ± 0.22, and 3.68 ± 0.19 μmol/L, respectively. Ethanol was used as a control (*E*). *Columns,* mean of representative independent triplicate experiments; *bars,* SD.

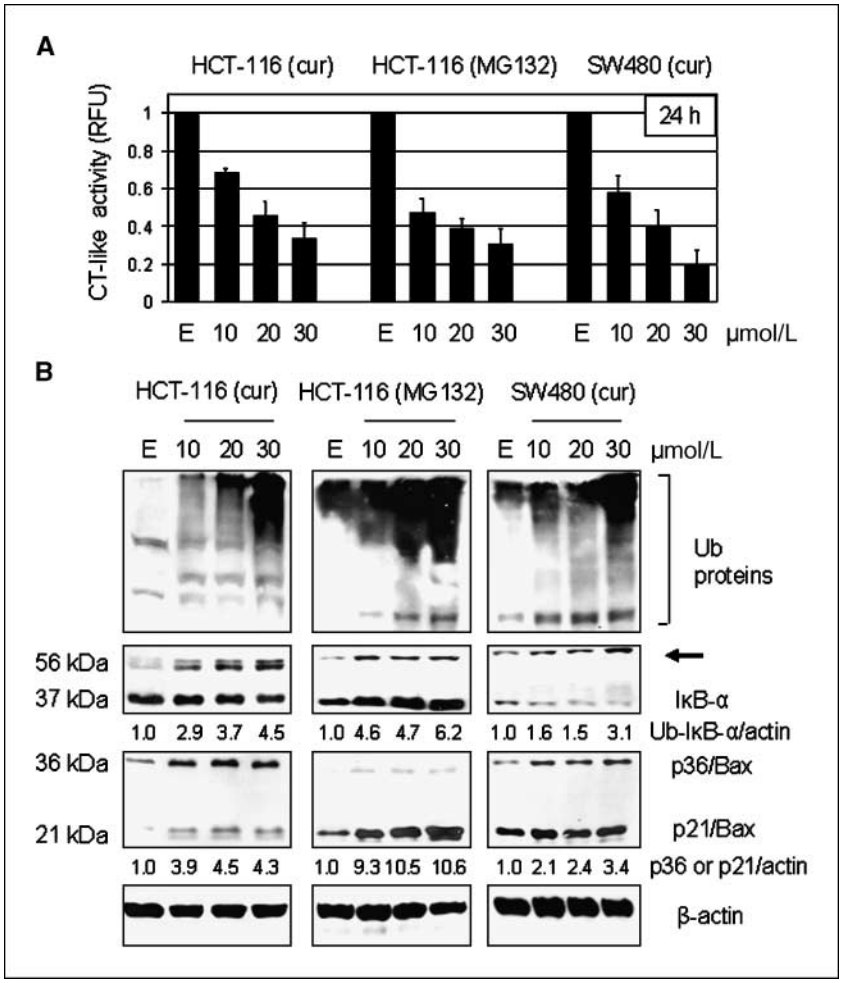

Figure 2. Inhibition of proteasomal CT-like activity in human HCT-116 and SW480 colon cancer cells by curcumin (*cur*). *A,* inhibition of proteasomal CT-like activity by curcumin in intact HCT-116 and SW480 cells. HCT-116 and SW480 cells were treated with different curcumin concentrations (10, 20, and 30 μmol/L) for 24 h, followed by measurement of proteasomal CT-like activity. Proteasome inhibitor MG132 was used as comparison with curcumin and the solvent, ethanol, was used as a control. *B,* Western blot analysis. HCT-116 and SW480 cells were treated with different curcumin concentrations (10, 20, and 30 μmol/L) for 24 h, and used for whole cell extract preparation. Cell extract was analyzed by Western blot for accumulation of ubiquitinated proteins, IκB-α, and Bax (*right* and *left*). Proteasome inhibitor MG132 was used as comparison to curcumin (*middle*), and the solvent, ethanol, was used as a control. Actin was used as a loading control. Change in the levels of ubiquitinated IκB-α and p36/Bax proteins in the cells treated with curcumin, and the levels of ubiquitinated IκB-α and p21/Bax proteins in the cells treated with MG132 were analyzed by densitometry and quantified using AlphaEase FC software. *RFU,* relative flourescence unit.

To further confirm the proteasomal inhibition caused by curcumin, HCT-116 and SW480 cells were treated for 24 hours, harvested, and used for cell extraction, followed by Western blot analysis. We found a dose-dependent accumulation of ubiquitinated proteins in both cell lines (Fig. 2*B, left* and *right*). We have previously reported an ubiquitinated form of IκB-α protein with molecular weight of ~56 kDa (34). Dose-dependent accumulation of a similar p56 band was detected in both cell lines, after 24 hours of treatment (Fig. 2*B, left* and *right*). It is well-known that ubiquitinated IκB-α protein is recognized and degraded by the proteasome leading to release of NF-κB and its translocation to the nucleus, where it activates genes involved in cell proliferation and survival. However, when the proteasomal activity is inhibited, ubiquitinated IkB-α stays bound to NF-κB, preventing its transcriptional activity. Therefore, accumulation of ubiquitinated form of IkB-α upon curcumin treatment confirms proteasomal inhibition. As a comparison, treatment of HCT-116 cells with MG132 had similar effect on accumulation of ubiquitinated proteins and ubiquitinated IkB-α (Fig. 2*B, middle*).

To investigate whether the proteasomal inhibition by curcumin is associated with apoptosis induction, caspase-3/-7 activation and PARP cleavage were measured, and TUNEL assay was performed. After 24 hours of treatment with curcumin, a dose-dependent increase in caspase-3/-7 activity was detected in both HCT-116 and SW480 cells (Fig. 3*A*). At 10 μmol/L concentration, curcumin induced a 6.4-fold and 8.2-fold increase in HCT-116 and SW480 cells, respectively, whereas at 30 μmol/L, it induced 8.4-fold and 16.6-fold increase in HCT-116 and SW480 cells, respectively (Fig. 3*A*). It has been shown that caspase-3/-7 activation is associated with production of the p85 cleaved PARP fragment (35), which we detected by Western blot analysis, together with a p65 PARP fragment (Fig. 3*B, left* and *right*), which was previously shown to be a product of calpain cleavage (36). The calpain involvement is further supported by the accumulation of p36/Bax, a homodimer of p18/Bax (a calpain cleaved product of p21/Bax; refs. 37, 38), found in the cells treated with all three concentrations of curcumin in both cell lines (and to some extent, in the cells treated with MG132; Fig. 2*B*). Our results show that curcumin is able to inhibit the proteasomal CT-like activity and activate caspase-3/-7 and calpain, thereby inducing apoptosis in human colon cancer HCT116 and SW480 cells. Treatment with MG132 had similar apoptosis-inducing effect in HCT-116 cells (Fig. 3*A* and *B, middle*). Dose-dependent apoptosis induction by curcumin was confirmed with TUNEL assay (Fig. 3*C*; data not shown). At the highest concentration used (30 μmol/L), curcumin induced production of 69.1% apoptotic HCT-116 and 90.7% apoptotic SW480 cells (Fig. 3*C*). Curcumin also induced apoptosis-related changes in cell morphology (shrunken cells and blebbing) in a concentration-dependent manner (data not shown). Therefore, SW480 cells are slightly more sensitive to curcumin treatment than HCT-116 cells.

Because curcumin has been previously shown to induce cell cycle arrest (16), we wanted to investigate if under our experimental

conditions, in addition to apoptosis induction, curcumin also affected cell cycle progression. Western blot analysis was used to examine the levels of Cyclin D1, which is essential for G_1-S transition. Twenty-four hour treatment with either concentration of curcumin did not significantly affect Cyclin D1 protein expression (Fig. 3*B, left* and *right*), indicating that under these conditions, curcumin-mediated proteasome inhibition mainly triggers apoptosis. Under the same experimental condition, 30 μmol/L MG132 induced ~2-fold increase in Cyclin D1 protein level (Fig. 3*B, middle*). MG132 was also found to have stronger antiproliferative effect against HCT-116 cells compared with curcumin, whereas the ability of curcumin to inhibit proliferation of HCT-116 and SW480 cells was very similar, as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown).

Curcumin-induced proteasome inhibition and apoptosis induction are time dependent. To further study the effect of curcumin in human colon cancer cells, HCT-116 cells plated in 96-well plate were treated with 20 μmol/L curcumin for various time points (0.5–12 hours). We found that 60% of the proteasomal CT-like activity was inhibited as early as half an hour after the addition of curcumin and remained inhibited at about the same extent for the rest of the experimental period (Fig. 4*A*). Proteasomal inhibition was confirmed with Western blot analysis that showed an increased level of ubiquitinated proteins after 6 hours of treatment, together with the accumulation of the proteasomal target proteins, IκB-α, p27, and p21/Bax (Fig. 4*B*).

In the same kinetic experiment, apoptosis-associated morphologic changes were observed after the first 6 hours of treatment, and these changes were greatly increased after 48 hours (Fig. 4*D*). To confirm apoptosis induction, we measured the activity of caspase-3/-7 and found a 4-, 6.5-, 11-, and 13-fold increase at 6, 16, 24, and 48 hours, respectively (Fig. 4*C*). Apoptosis was also confirmed with Western blot analysis by the presence of PARP cleavage (Fig. 4*B*). The appearance of p65-cleaved PARP fragment and accumulation of p36/Bax were also detected during the treatment (Fig. 4*B*), again supporting activation of calpain. Additionally, slight down-regulation (~30%) of Cyclin D1 by curcumin was found at the later time point (48 hours; Fig. 4*C*).

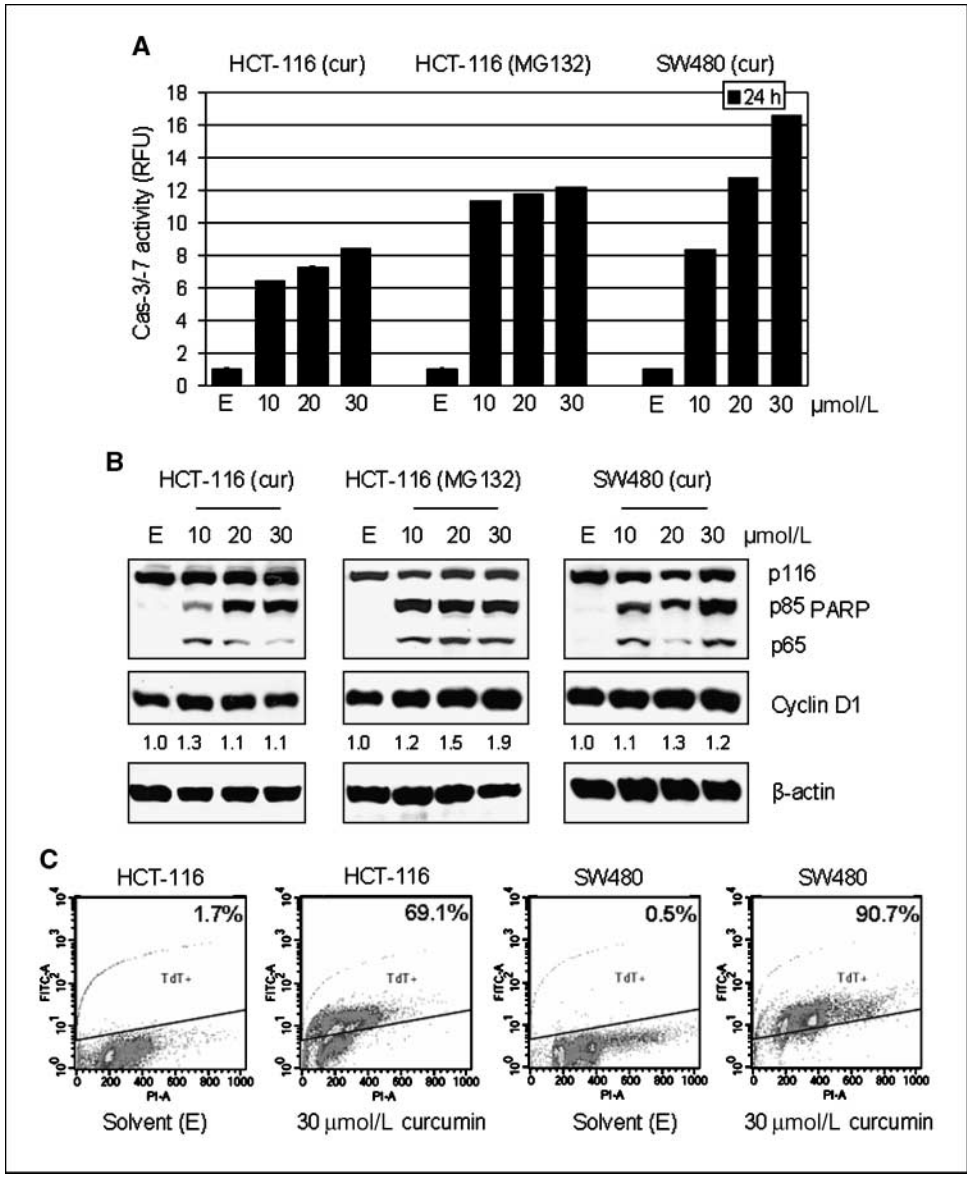

Figure 3. Induction of apoptosis in human HCT-116 and SW480 colon cancer cells by curcumin. *A,* a cell-free caspase-3/-7 activity assay. HCT-116 and SW480 cells were treated with different curcumin concentrations (10, 20, and 30 μmol/L) for 24 h, harvested, and prepared whole cell extract was used for fluorescent detection of caspase-3/-7 activity (see Materials and Methods). Proteasome inhibitor MG132 was used as comparison with curcumin, and the solvent, ethanol, was used as a control. *Columns,* mean of representative independent triplicate experiments; *bars,* SD. *B,* Western blot analysis. Cell extract from HCT-116 and SW480 cells treated with curcumin was analyzed by Western blot for PARP cleavage and Cyclin D1 detection (*left* and *right*). Proteasome inhibitor MG132 was used as comparison with curcumin (*middle*), and the solvent, ethanol, was used as a control. Actin was used as a loading control. Change in Cyclin D1 protein level was analyzed by densitometry and quantified using AlphaEase FC software. *C,* for TUNEL assay, HCT-116 and SW480 cells were treated for 24 h with 30 μmol/L curcumin or ethanol (solvent) as a control. The TUNEL assay was performed by using an APO-DIRECT kit and the number of apoptotic (TdT positive) cells is indicated.

Curcumin suppresses the growth of human colon cancer HCT-116 xenografts, associated with proteasome inhibition, proliferation suppression, and apoptosis induction. After we showed that curcumin inhibited proteasomal activity and induced apoptosis in cultured colon cancer cells (Figs. 3 and 4), we investigated whether the antitumor activity of curcumin could, at least partly, be attributed to inhibition of the proteasomal activity and induction of apoptosis *in vivo*. To do so, we implanted HCT-116 cells (3×10^6) s.c. into ICR SCID mice (both flanks). When the tumors began to enlarge (50 mm^3), mice were randomized into the 2 groups for treatment of vehicle control and curcumin (500 mg/kg body weight given intragastric every day for 3 weeks). Inhibition of tumor growth by curcumin was observed after a 21-day treatment, confirming the antitumor activity of curcumin (Fig. 5*A*). Control tumors grew to an average size of 1,587 mm^3, and curcumin-treated tumors grew to 959 mm^3, corresponding to 40% inhibition ($P < 0.01$; Fig. 5*A*).

To determine if the observed antitumor effect of curcumin was associated with its proteasome-inhibitory activity, we extracted proteins from the tumor remnants and used them for multiple assays. We found that the proteasomal CT-like activity was inhibited by 55% in the tumors from mice treated with curcumin, compared with vehicle-treated mice (Fig. 5*B*). This was associated with the accumulation of ubiquitinated proteins and the proteasome target proteins p27, IκB-α, and p21/Bax (Fig. 5*C*). Increased expression of p27 was confirmed with immunostaining only in the tumors from mice treated with curcumin (Fig. 6*A*). The results show that curcumin is able to inhibit tumor proteasome activity *in vivo*.

The formalin-fixed tumor tissue was analyzed to determine whether curcumin-induced proteasomal inhibition found in colon cancer caused a consequent inhibition of proliferation and stimulation of apoptosis. There was a 3-fold reduction in PCNA immunoreactivity (a marker of cell proliferation) in the tumor remnants of curcumin-treated ICR SCID mice compared with the vehicle-treated controls (Fig. 5*D, top*). Consistent with growth inhibition *in vivo* by curcumin, Western blot analysis showed down-regulation of Cyclin D1, another marker of cell proliferation, in the tumors from mice treated with curcumin, compared with

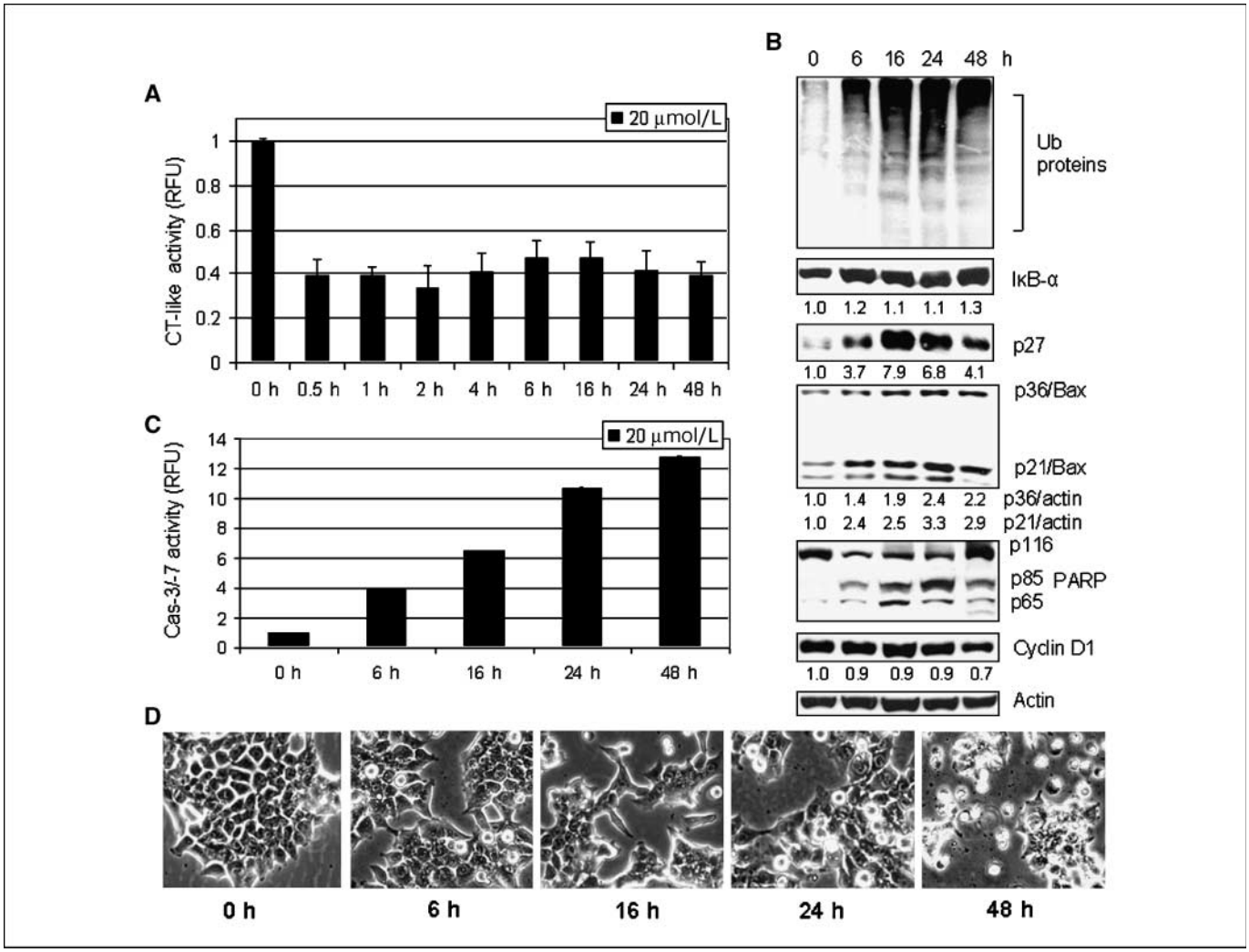

Figure 4. Kinetic effect of curcumin on HCT-116 cells. *A,* HCT-116 cells were treated with 20 μmol/L curcumin for 0.5 to 48 h, followed by the proteasomal CT-like activity assay using Z-GGL-AMC. *B,* HCT-116 cells treated with 20 μmol/L curcumin for 6 to 48 h were used for whole cell extract preparation. *Columns,* mean of representative independent triplicate experiments; *bars,* SD. Cell extract analyzed by Western blot confirmed proteasomal inhibition by accumulation of ubiquitinated proteins, and proteasome target proteins IκB-α, p27, and p21/Bax. Apoptosis induced by curcumin treatment was confirmed by PARP cleavage (*B*), caspase-3/7 activation (*C*), and apoptotic morphologic changes (*D*). Actin was used as a loading control. *Columns,* mean of independent triplicate experiments; *bars,* SD. Change in the level of IκB-α, p27, p21/Bax, p36/Bax, and Cyclin D1 proteins was analyzed by densitometry and quantified using AlphaEase FC software.

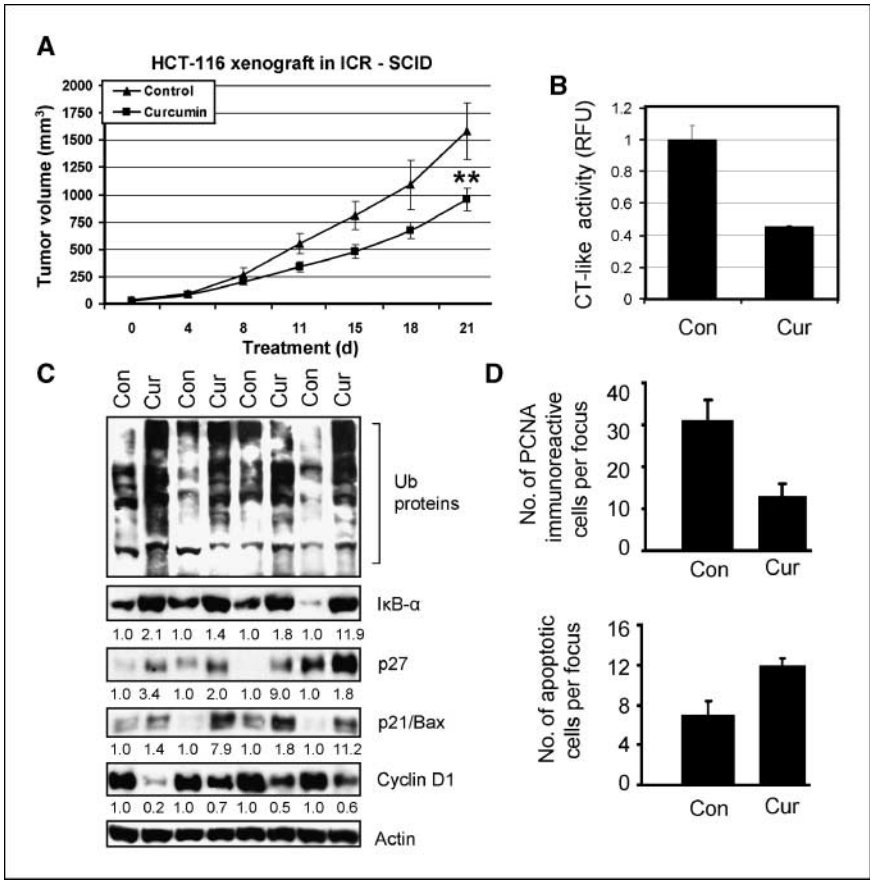

Figure 5. Curcumin treatment leads to inhibition of the proteasomal CT-like activity, proliferation suppression, and apoptosis induction *in vivo*. Female homozygous ICR SCID mice bearing HCT-116 tumors were treated with either control solvent or curcumin at 500 mg/kg/d to day 21. *A,* inhibition of HCT-116 tumor growth by curcumin. *Points*, mean tumor volume in each experimental group containing six mice; *bars*, SD; **, $P < 0.01$. *B* to *D,* effects of curcumin at the end point of the experiment. Tumors were collected after 21 d of treatment, and the prepared tissues were analyzed by the proteasomal CT-like activity assay (*B*), Western blotting (*C*), and immunohistochemistry (*D*). Inhibition of proteasome activity (*B*); accumulation of ubiquitinated proteins, p27, IκB-α, and p21/Bax proteins; and down-regulation of Cyclin D1 (*C*) were found in all four tumors treated with curcumin, compared with control mice (*con*) treated with the control solvent alone. The slides prepared from the tumors treated with the control solvent or curcumin were used for PCNA immunostaining and TUNEL (*D*). *Columns,* mean numbers of PCNA-immunoreactive cells per focus; *bars,* SD (*D, top*); *columns,* mean numbers of apoptotic cells per focus; *bars,* SD (*D, bottom*). Change in protein level was analyzed by densitometry and quantified using AlphaEase FC software.

control (Fig. 5*C*), which was not observed in cultured cells treated with curcumin (Figs. 3 and 4).

Apoptosis was then determined using the TUNEL assay, which showed a 2-fold increase in the number of apoptotic cells in the tumors from mice treated with curcumin compared with the corresponding vehicle-treated control (Fig. 5*D, bottom*). High levels of condensed apoptotic nuclei, another apoptotic feature, together with necrotic tumor cells were observed in tumors from curcumin-treated animals after H&E staining (Fig. 6*B*). As a comparison, in tumor tissue from mice treated with solvent, only few apoptotic cells and much more nonapoptotic cells were found. Taken together, these data show that curcumin has the ability to regress colon cancer growth in ICR SCID mice bearing HCT-116 xenografts by inhibiting the proteasomal CT-like activity, resulting in proliferation inhibition and apoptosis induction.

Discussion

Interest in potential cancer chemopreventive and therapeutic properties of dietary ingredients, which are generally more tolerable in the human body (15), has increased in recent years, especially for the cancers that usually do not respond well to currently available therapies (17). Therefore, it is not surprising that chemoprevention of colon cancer, the leading cancer-related death in Western countries, is becoming more attractive. One of the dietary ingredients that possess anti-inflammatory and/or anti-oxidant properties is curcumin. Epidemiologic data suggest that curcumin may be responsible for the lower colon cancer rate in India and Southeast Asia (39). Based on that and its long history of consumption without any adverse health effects, curcumin is considered to be a safe chemopreventative agent (15).

Although it was suggested previously that curcumin-induced apoptosis is mediated through the impairment of the ubiquitin-proteasome pathway (40), the exact mechanism was not fully elucidated. Although the antioxidative property of curcumin is well-documented, it has also been shown that at higher concentrations (50 and 100 μmol/L), curcumin could induce apoptosis through production of reactive oxygen intermediates (41). Therefore, it was hypothesized that curcumin could induce proteasomal malfunction through the oxidative stress generation (40). Another possible mechanism for the proteasomal inhibition induced by curcumin was proposed based on its chemical structure. Because it belongs to a class of compounds with α,β-unsaturated ketones and two sterically accessible β carbons, curcumin was suggested to inhibit the ubiquitin isopeptidase activity located at the 19S regulatory subunit of the 26S proteasome (42).

On the other hand, the chemical structure of curcumin led us to the hypothesis of the direct binding of curcumin to the 20S proteasome. By using an *in silico* model, we found that both carbonyl carbons are indeed susceptible to nucleophilic attack by Thr 1 within the β5 subunit of the proteasome (Fig. 1*B* and *C*), suggesting that curcumin binds to Thr 1 with high predictability. The proteasomal inhibition by curcumin was confirmed by an *in vitro* experiment that showed a significant inhibition of the CT-like activity of a purified 20S proteasome with an IC_{50} value of 1.85 μmol/L (Fig. 1*D*). Taken together, these data indicate that inhibition of proteasomal CT-like activity induced by curcumin is mediated through its direct binding.

In our study, we used physiologic concentrations of curcumin (10–30 μmol/L), thereby excluding the possibility that curcumin-induced reactive oxygen species production is involved in proteasomal inhibition. We have further confirmed that this inhibition is physiologically functional by demonstrating accumulation of ubiquitinated proteins and proteasome target proteins IκB-α, p27, and p21/Bax. More importantly, our data show that intragastric treatment of HCT-116 tumor-bearing IRA SCID mice with curcumin resulted in the inhibition of proteasomal CT-like activity in the tumors (Figs. 5*B* and *C* and 6*A*), associated with a 40% inhibition of tumor growth (Fig. 5*A*), suggesting that the effective plasma levels of curcumin have been reached. Consistent with previous reports (7, 8), we found that inhibition of the proteasomal CT-like activity, induced by curcumin in colon cancer cells and xenografts, was associated with the induction of apoptosis (Figs. 3–6). We have also found that inhibition of tumor growth was caused not only by apoptosis induction but also by induction of cell cycle arrest, most probably G_1 arrest, as shown by decreased levels of two proliferation markers PCNA (Fig. 5*D*) and Cyclin D1 (Fig. 5*C*), and accumulation of p27 (Figs. 5*C* and 6*A*). However, this G_1 arrest was not observed in cultured cells treated with curcumin (Figs. 3 and 4).

When comparing the *in vitro* and *in vivo* potencies of curcumin, we found that although 1.85 μmol/L curcumin was sufficient to inhibit 50% of the CT-like activity of purified 20S proteasome (Fig. 1*D*), a much higher concentration of curcumin (10 μmol/L) produced only 32% to 42% inhibition of the CT-like activity in colon cancer cells (Fig. 2*A*). This finding is similar to previous reports with other natural compounds with proteasome-inhibitory activities (30), suggesting that natural compounds have more than one cellular target and/or might not be very stable in cells. Furthermore, our *in silico* data show that two hydroxyl groups could potentially form hydrogen bonds with surrounding amino acids in the β5-subunit, strengthening the potential of curcumin to bind the proteasome. Modification of these two hydrogen groups, such as methylation, could also explain the lower potency of curcumin to bind and inhibit the proteasome within the cells, as opposed to the purified 20S proteasome.

We have also shown that curcumin inhibited ~60% of the proteasomal activity within the first 30 minutes of the treatment, which did not change much until the end of 48 hour treatment (Fig. 4*A*). However, due to the cumulative effect of this continuous inhibition, the level of proteasomal target proteins did increase

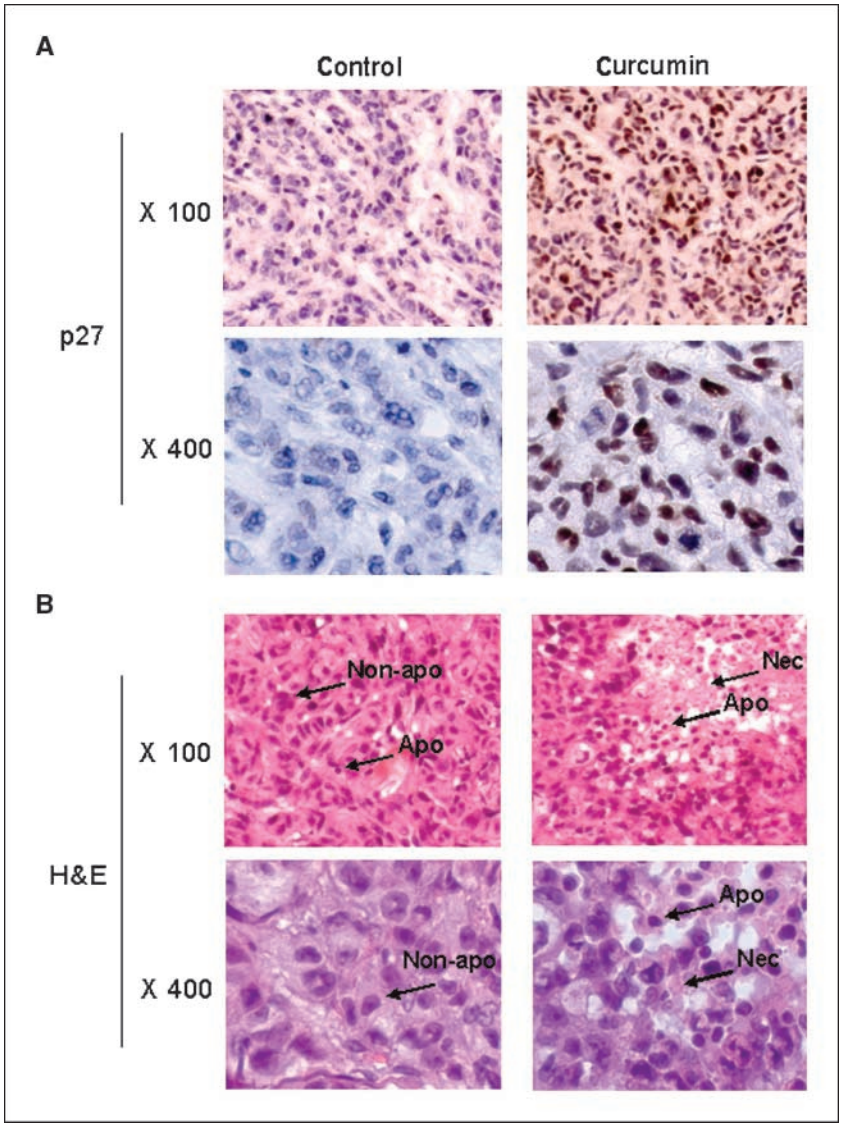

Figure 6. Immunohistochemistry p27 and H&E staining assays using mouse tumor samples. Tumors were collected after 21-d treatment (see Fig. 5 legend), and the prepared tissue slides were used for immunostaining with p27 antibody (*A*) and H&E staining assays (*B*). Stronger or/and more p27-positive cells (*A*), more apoptotic-condensed nuclei (*Apo with arrow*), and more necrotic tumor cells (*Nec with arrow*) were found in tumor tissue from mice treated with curcumin. Only few apoptotic cells and much more nonapoptotic cells (*Non-apo with arrow*) were found in tumor tissue from mice treated with solvent. Magnifications, ×100 and ×400 as indicated.

after 6 hours of treatment. Once the level of accumulated proapoptotic proteins reached the critical level for apoptosis induction and caspase-3/-7 activation, the cells became committed to apoptosis, resulting in time-dependent caspase-3/-7 activation. We also found that although the proteasome activity was not completely inhibited in cultured tumor cells (30–50% inhibition), apoptosis was still induced by curcumin, supporting the idea of the proteasome being an important cellular target for curcumin. Moreover, curcumin was shown to be a highly pleiotropic molecule that can interact with numerous molecular targets, thereby affecting various biochemical cascades and pathways, including caspase cascade and apoptosis (2).

We have published that some natural compounds with proteasome-inhibitory activity such as (−)-EGCG are unstable under physiologic conditions and could be rapidly degraded or metabolized through interactions with the hydroxyl groups on the phenol rings (43). Knowing that curcumin contains two hydroxyl groups, it is possible that the same mechanism could be applied on curcumin. Indeed, a number of studies over the past three decades have shown that poor absorption, rapid metabolism, and rapid systemic elimination of curcumin are responsible for its poor bioavailability (44). In an attempt to improve the bioavailability of curcumin, different approaches have been undertaken, such as combining curcumin treatment with piperine, a known inhibitor of hepatic and intestinal glucuronidation. This combinational treatment was found to significantly enhance serum concentration, absorption level, and bioavailability of curcumin in both rats and humans with no adverse effects (45).

Because curcumin undergoes a rapid and efficient metabolism and biotransformation during absorption in the intestinal tract (17), it would be expected that different parts of the intestine, including the colon, would accumulate a higher concentration of curcumin in the metabolically active form, compared with other organs. Numerous trials investigating the chemopreventative and therapeutic effects of curcumin alone or in combination with other natural or synthetic compounds have been and continue to be carried out (2). Curcumin has been shown to potentiate the antitumor effects of gemcitabine in pancreatic cancer cells growing *in vitro* or *in vivo* (46) and synergize with FOLFOX in colon cancer cells *in vitro* (22). It has also been shown that curcumin potentiates the effect of gemcitabine, paclitaxel, tumor necrosis factor, and TRAIL against bladder cancer cells (47). These results are consistent with that proteasome inhibition could chemosensitize human cancer cells (48). On the other hand, curcumin has also been found to significantly inhibit the chemotherapy-induced effect of camptothecin, mechlorethamine, and doxorubicin in breast cancer cells *in vitro*, and cyclophosphamide-induced tumor regression *in vivo* (1). Therefore, to design a better and more efficient combination treatment that includes curcumin, it is necessary not only to identify its molecular targets but also to show that curcumin could reach them *in vivo*.

It has been shown that overexpression of COX-2 in intestinal epithelium is associated with increased colon cancer incidence and progression, and that curcumin has potential to specifically inhibit COX-2 expression at the RNA and protein level (49). Although the underlying mechanism of COX-2 inhibition by curcumin is not well-understood, it seems to be different from the class of COX-2 inhibitors that act through direct binding to the COX-2 enzyme (50). Different mechanisms of action are supported by the fact that no toxicity associated with curcumin treatment, even when used at very high doses, has been observed (2). On the other hand, the observed toxicity associated with COX-2 inhibitor treatment seems to be result of their specific chemical properties and are unrelated to COX-2 binding and inhibition (51). Because both colon cancer cell lines used in our study (HCT-116 and SW480) do not express COX-2, our finding is consistent with previously published data, which indicate that growth inhibition of colon cancer cells by curcumin is independent of COX-2 expression (49) and supported by recently published finding that docosahexaenoic acid stimulates degradation of β-catenin and induces apoptosis in the same cell lines independently of COX-2 inhibition (52). Our data showing that curcumin is able to inhibit the proteasome and induce apoptosis in both, HCT-116 and metastatic colon cancer SW480 cells, suggests its possible use for treatment of both early stage and late stage/refractory colon cancer.

In summary, our data reveal the proteasome as an important cellular target of curcumin *in vitro* and *in vivo*. We have shown that inhibition of the proteasomal activity (especially, the CT-like activity) by curcumin is a strong apoptotic stimulus for both colon cancer HCT-116 and SW480 cell lines *in vitro* and that SW480 cells are slightly more sensitive to curcumin treatment than HCT-116 cells. We have also shown that intragastric administration of curcumin to ICR SCID mice bearing xenografts of colon cancer HCT-116 cells resulted in significant inhibition of tumor growth, as a consequence of proteasomal inhibition, inhibition of cell proliferation, and stimulation of apoptosis. The fact that curcumin is able to target the human colon tumor proteasome and inhibit human colon tumor growth *in vivo* provides a strong impetus for using curcumin as a chemopreventative and/or chemotherapeutic agent for human colon cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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