Title page

Title:

Deubiquitinase Inhibition of 19S Regulatory Particles by 4-Arylidene Curcumin Analogue AC17 Causes NF-κB Inhibition and p53 Reactivation in Human Lung Cancer Cells

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Running title:

Deubiquitinase Inhibition of 19S RP by AC17

Key words:

curcumin analogue; deubiquitinase inhibitor; NF-κB; p53; lung cancer

Abbreviations:

deubiquitinase DUB

19S regulatory particles 19S RP
IkB kinase
ubiquitin-proteasome pathway
20S proteolytic core particle
peptidylglutamyl peptide hydrolyzing-like
trypsin-like
chymotrypsin-like
deeubiquitinating enzymes
ubiquitin C-terminal hydrolase 5
ubiquitin specific proteases 14
proteasomal deubiquitinase 1
N-ethylmaleimide
ubiquitin-7-amido-4-methylcoumarin
reverse transcription
propidium iodide
hematoxylin and eosin
cycloheximide
inhibitory κB
reduced glutathione

**Disclosure of potential conflicts of interest**

The authors disclose no potential conflicts of interest.
Abstract

Proteasome inhibitors have been suggested as potential anti-cancer agents in many clinical trials. Recent evidence indicates that proteasomal deubiquitinase (DUB) inhibitors, bearing a different mechanism from that of traditional proteasome inhibitors, would be appropriate candidates for new anti-cancer drug development. In the present study, we describe the DUB inhibition of 19S regulatory particles (19S RP) by AC17, a 4-arylidene curcumin analogue synthesized in our laboratory. Although 4-arylidene curcumin analogues were reported to act as IκB kinase (IKK) inhibitors, AC17 instead induced a rapid and marked accumulation of ubiquitinated proteins without inhibiting proteasome proteolytic activities. In contrast to its parent compound curcumin that is a proteasome proteolytic inhibitor, AC17 serves as an irreversible DUB inhibitor of 19S RP, resulting in inhibition of NF-κB pathway and reactivation of pro-apoptotic protein p53. Additionally, in a murine xenograft model of human lung cancer A549, treatment with AC17 suppresses tumor growth in a manner associated with proteasome inhibition, NF-κB blockage, and p53 reactivation. These results suggest that 4-arylidene curcumin analogues are novel 19S DUB inhibitors with great potential for anti-cancer drug development.
Introduction

The ubiquitin-proteasome pathway (UPS) is responsible for the non-lysosomal degradation of most intracellular proteins and plays a crucial role in the regulation of numerous cellular and physiological functions, including protein quality control, cell cycle progression, proliferation, differentiation, angiogenesis and apoptosis (1, 2). In UPS, proteins targeted for degradation are first covalently modified by a poly-ubiquitin chain. Three classes of enzymes are involved: ubiquitin-activating enzyme E1 (3), ubiquitin-conjugating enzyme E2 (4), and ubiquitin-protein ligase E3 (5). E1 activate ubiquitin monomers and transfer them to E2, which then work in conjunction with E3 to specifically recognize and recruit substrates of the ubiquitination reaction. The polyubiquitinated substrates are then recognized by 26S proteasome (a large multi-subunit and ATP-dependent proteolytic complex) and rapidly degraded into small peptides (6).

Thus 26S proteasome is the key component of UPS. Based on both structure and function, 26S proteasome consists of a hollow cylindrical 20S proteolytic core particle (20S CP) and one or two 19S regulatory particles (19S RP) (7). At the middle of 26S proteasome is 20S CP, which is formed by four stacked rings made of different α (structural) and β (catalytic) subunits, with the β1, β2, and β5 subunits accounting for peptidylglutamyl peptide hydrolyzing-like (PGPH-like), trypsin-like (T-like) and chymotrypsin-like (CT-like) activities, respectively (8). Capping at either end of 20S CP is 19S RP that controls ubiquitin-tagged substrates for proteolysis in the catalytic
chamber. In human body, 19S RP is associated with three deubiquitinating enzymes (DUBs): ubiquitin C-terminal hydrolase 5 (UCHL5), ubiquitin specific proteases 14 (USP14) and proteasomal deubiquitinase 1 (POH1) (9-11). Though the physiological functions of 19S DUBs are not completely clear, all of them have been proved to dynamically regulate 26S proteasome activity (12, 13).

Since the US Food and Drug Administration approved the 20S proteolytic inhibitor bortezomib (VELCADE®) for treatment of multiple myeloma in 2003 (14), UPS has been considered an appropriate therapeutic target for cancer therapy and caught increasing academic. In the past several decades, many structurally diverse inhibitors of 20S CP have been discovered from both synthetic and natural product, in which at least five are at different stages of clinical development (15). However, not all multiple myeloma patients respond to bortezomib treatment (16, 17). The molecular mechanisms of resistance to bortezomib are multiple, involving increased expression of anti-apoptotic proteins or mutated status of pro-apoptotic factors, such as overexpression of Bcl-2 (18-20) or disruption of p53 (21). Therefore, the development of novel proteasome inhibitors for second-line treatment is required.

AC17, a 4-arylidene curcumin analogue, was initially synthesized and identified as an IKK inhibitor in our preliminary study (22). Compared to the parent compound curcumin, AC17 shows improved oral bioavailability, metabolic stability (23) and moderately potent anti-cancer activities against several different cancer cell lines, including lung cancer, colon cancer, breast cancer, and hepatocellular carcinoma cells.
(Supplementary Fig. S1). As we have previously described, IKK blockage by 4-arylidene curcumin analogues only partly reveals the mechanism of the anti-cancer activity of AC17 (22). Although the exact mode of action of AC17 remains unclear, AC17 most likely is a pleiotropic molecule such as curcumin, which modulates numerous targets (24-26). In the present study, AC17 serves as a non-classical proteasome inhibitor that blocks the DUB activity of 19S RP without inhibiting the proteolytic activities of 20S CP, inducing a rapid and noticeable accumulation of protein-ubiquitin conjugates, resulting in inhibition of NF-κB pathway and reactivation of p53 function. Furthermore, treatment of human lung cancer-bearing BALB/c-nu mice with AC17 resulted in tumor growth suppression, correlating with \textit{in vivo} ubiquitinated proteins accumulation, transcription factor NF-κB inhibition, and pro-apoptotic protein p53 reactivation. These results show that AC17 represents a novel class of proteasome inhibitors through inhibiting 19S DUB activity but not 20S proteolytic activities, which could potentially be used for the treatment of human cancers.

\textbf{Materials and methods}

\textbf{Cell culture, chemical reagents, and enzymes}

Human lung carcinoma cell lines A549 and NCI-H1299 were obtained from the cell bank of the Shanghai Institute of Biochemistry and Cell Biology, in which they were tested and authenticated for genotypes by DNA-fingerprinting analysis. The cell lines were not passaged over 6 months, and thus no authentication was done by the authors.
Cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% FBS (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin. All cells were cultured and maintained at 37°C under humidified atmosphere with 5% CO₂.

4-Arylidene curcumin analogue AC17, (1E,6E)-1,7-bis(3,4-dimethoxyphenyl)-4-(4-hydroxy-3-methoxybenzylidene)hepta-1, 6-diene-3,5-dione), was synthesized in an analogous manner as previously reported (22). Other reagents were obtained from the following sources: TNFα, NEM (N-ethylmaleimide), MG-132, Nutlin-3a, DMSO (Sigma); Ub-AMC (ubiquitin-7-amido-4-methylcoumarin), human 19S proteasome, human 20S proteasome, human 26S proteasome, Suc-LLVY-AMC, Z-LLE-AMC (BostonBiochem); Z-ARR-AMC (Calbiochem); Vectors (pRL-TK and pNF-κB-luc; Promega).

**Western blotting and immunoprecipitation analysis**

Whole-cell lysates were prepared by using cell lysis buffer (Beyotime) and boiling in 1 x Laemmli reducing sample buffer. Equal protein amounts were electrophoresed on SDS-PAGE gels, transferred to membranes, and immunoblotted. For immunoprecipitation, cellular lysate (500 μg) was used to immunoprecipitate, and Western blotting was performed to examine ubiquitination or interaction of proteins.

Antibodies were purchased from the following sources: anti-β-actin, anti-MDM2, anti-GAPDH, anti-ubiquitin, goat anti-mouse/rabbit IgG-conjugated horseradish peroxidase (Santa Cruz Biotechnology); anti-IκBα, anti-p-IκBα (Ser32), anti-p65,
anti-p53, anti-p21 (Cell Signaling Technology).

Levels of mRNA by real-time reverse transcriptase PCR

To determine mRNA expression, 500 ng of total RNA extracted from each sample were used for reverse transcription (RT) reaction in 10 μL of reaction volume using a reverse transcription system (TaKaRa) according to the manufacturer’s instructions. SYBR *Premix Ex Taq*™ Kit (TaKaRa) was used for real-time PCR reaction. After normalization to GAPDH gene, expression levels for each target gene were calculated using the comparative threshold cycle method.

Sequences of PCR primers were as follows:

- **p53**, forward, 5´- GCGCACAGAGGAAGAATCTCG-3´,
  and reverse, 5´- TTTGGCTGGGGAGAGGAGCTG-3´;

- **p21**, forward, 5´- TGTACCCTTGTGCCTCGCTC-3´,
  and reverse, 5´- TGGAGAAGATCAGCCGGCGT-3´;

- **IκBα**, forward, 5´- CTGAGCTCCGAGACTTTCGAGG-3´,
  and reverse, 5´- CGTCCTCTGTGAACTCCGTG-3´;

- **MDM2**, forward, 5´- ATCTTGGCCAGTATATTATG-3´,
  and reverse, 5´- GTTCCTGTAGATCATGGTAT-3´;

- **GAPDH**, forward, 5´- CACCCAGAAGACTGTGGATGG-3´,
  and reverse, 5´- GTCTACATGGCAACTGTGAGG-3´.

Reporter gene assay

Cells at approximate 70% confluency were transfected with 0.2 μg DNA/cm² per
pNF-κB-luc plasmid using Lipofectamine 2000 (Invitrogen). pRL-TK was co-transfected as a control for transfection efficiency. At 24 h after transfection, AC17 was added for 1 h prior to exposure to TNFα for another 2 h. Then cells were lysed and luciferase activity was determined with a dual-luciferase assay kit (Promega).

**Proteasome activity assay**

Fluoregenic peptide substrates Suc-LLVY-AMC, Z-LLE-AMC, Z-ARR-AMC were used to assay for the proteasomal CT-like, PGPH-like, and T-like activities. To assess cellular proteasome activity, 20 μg whole-cell extracts were incubated at 37°C with 40 μM fluorogenic substrates in 200 μL assay buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% (v/v) glycerol, 0.03% (w/v) SDS. For the evaluation of T-like activity, SDS was omitted from the assay buffer. To assess proteasome activity in vitro, purified proteasome was incubated with compounds for 30 mins at 37°C before addition of substrates. Fluorescence intensity was measured using a Flex Station 3 microplate reader (Molecular Devices) at λ<sub>ex</sub> = 380 nm and λ<sub>em</sub> = 460 nm after compensation for the buffer background and compound quenching fluorescence according to the Stern-Volmer equation.

**Ub-AMC protease assay**

Cells were lysed in an assay buffer of 50 mM Tris-HCl (pH 7.5), 5 mM MgCl<sub>2</sub>, 1 mM DTT, 2 mM ATP, and 250 mM sucrose. 10 μg of clarified lysate were incubated with 400 nM Ub-AMC at 37°C, and fluorescence intensity was recorded at excitation/emission of 380/460 nm using a spectrofluorometer.
Purified proteasome was incubated in assay buffer containing AC17 (indicated concentration), vehicle (DMSO), or positive control (NEM) at 37°C for 30 mins, following addition of 400 nM Ub-AMC. The reaction was quantified at excitation/emission of 380/460 nm after compensation for the buffer background and compound quenching fluorescence according to the Stern-Volmer equation.

**Cell cycle analysis**

Both adherent and floating cells were harvested and fixed with 75% ethanol at -20°C overnight. Then cells were incubated with 5 μg/mL propidium iodide (PI) and 5 μg/mL RNase A at 37°C for 30 mins. Cell cycle distribution and sub-G1 DNA content cells were measured with an EPICS XL analyzer (Beckman Coulter).

**Cellular and nuclear morphology analysis**

A Nikon Eclipse Ti-S microscope was used for all microscopic imaging with either phase contrast for cellular morphology or fluorescence for nuclear morphology. For fluorescent nuclear morphology analysis, cells were washed with ice-cold PBS, fixed in 4% paraformaldehyde for 1 h, stained with 50 μM Hoechst 33342 in the dark at 4°C for 10 mins, and then observed.

**Animal experiments**

All animal experiments complied with the Zhongshan School of Medicine Policy on the Care and Use of Laboratory Animals. Female BALB/c-nu mice (5 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd and maintained in pathogen-free conditions. A549 cells were harvested during log phase growth and
re-suspended in RPMI-1640 medium at $5 \times 10^7$ cells/mL. Each mouse was injected subcutaneously in the right flank with $1 \times 10^7$ cells. When the tumor volume reached approximate 120 mm$^3$, mice were randomized into three groups and treatment was initiated (at day 7). AC17 (1 and 5 mg/kg body weight) dissolved in 200 µL solution (polyethylene glycol 400: Cremophor EL: Physiological Saline, 8:1:11) was administered intraperitoneally every 5th day for 4 times. Control group was treated with an equal volume of vehicle. The tumor volume was estimated according to the formula: tumor volume = $L \times W^2/2$, where $L$ is the length and $W$ is the width of tumor. One day after the last injection, tumors and organs were rapidly frozen in liquid nitrogen and stored at -70°C for protein extraction, and another portion was fixed in formalin for histology.

**Immunohistochemistry**

Formalin-fixed tissue sections were evaluated by hematoxylin and eosin (H&E) staining and immunohistochemistry. Immunofluorescence staining of formalin-fixed paraffin-embedded tissue was performed as previously described (27).

**Statistical analysis**

Cell data were derived from at least three independent experiments, and animal data were derived from the xenograft study described above. Student’s $t$ test was used to assess the differences between sets of data. Probability values below 0.05 were considered significant.

**Results**
Compared with curcumin, AC17 displayed much greater growth inhibitory activities against several types of cancer cell lines (Fig. 1A, Supplementary Fig. S1). Notably, AC17 was more toxic to lung cancer cell lines such as A549 and NCI-H1299 cells compared to mouse primary hepatocytes (Supplementary Fig. S2). Our previous study showed that AC17 inhibited TNFα-induced NF-κB activation in an NF-κB translocation assay with an IC50 of 1.0 ± 0.55 μM (Fig. 1B) (22). Dual-Glo® luciferase analysis affirmed that AC17 blocked TNFα-induced transcriptional activation of NF-κB (Fig. 1C). Although NF-κB pathway inhibition is partially responsible for the potent anti-cancer activity of AC17 (Supplementary Fig. S3), the underlying mechanism remains unclear. The effects of AC17 both in vitro and in vivo were therefore investigated in details.

**Inhibition of IκBα degradation by AC17 suppresses NF-κB activation**

Following treatment with the protein translational inhibitor (cycloheximide, CHX), the decay rate of IκBα was much slower than that without AC17 pretreatment (Fig. 1D). No significant change of IκBα mRNA level in a time-dependent manner was observed, as indicated by RT-PCR analysis of IκBα mRNA expression in AC17-treated A549 cells (Fig. 1E). These results strongly imply that the regulation of IκBα expression by AC17 occurs at post-translational level, not at transcriptional level. The degradation of inhibitory κB (IκB) in UPS is required for the release of NF-κB, which after translocation to the nucleus will activate genes involved in cell proliferation and survival. To determine whether AC17 inhibits IκB degradation,
IkBα was immunoprecipitated from the extracted whole-cell proteins, and the ubiquitination state of IkBα was measured by Western blotting. Compared with the well-known 20S proteolytic inhibitor MG-132, AC17 resulted in an accumulation of polyubiquitinated IkBα after treating A549 cells for 12 h (Fig. 1F). The interaction between IkBα and NF-κB p65 subunit was detected in AC17- or MG-132-treated A549 cells, indicating that there was no dissociation of IkBα from p65 after AC17 or MG-132 exposure (Fig. 1G), and thus prevented NF-κB transcriptional activity. In short, NF-κB activation could be inhibited by AC17 by blocking UPS of IkB degradation.

**Rapid ubiquitination is induced by AC17 by blocking the DUB activity of 19S RP**

Western blot analysis of whole-cell extracts from AC17-treated A549 cells demonstrated a marked concentration-dependent accumulation of ubiquitinated proteins after 2 h of treatment (Fig. 2A, *left*). A similar increase in protein ubiquitination was also observed in AC17-treated NCI-H1299 cells (Fig. 2A, *right*). As shown in Fig. 2B, a rapid, time-dependent accumulation of ubiquitinated proteins was induced in A549 cells by AC17 treatment. Similar to AC17, MG-132 induced an increase of protein ubiquitination in A549 cells (Fig. 2C), suggesting similar downstream effectors for AC17 and MG-132. However, further investigation of the effect of AC17 on 20S proteolytic activities showed no significant decline of any of proteasome proteolytic activities following AC17 incubation in A549 cells, purified human 20S or 26S proteasome, whereas MG-132 exhibited substantial inhibitory
effects in all assays (Fig. 2D). These results show that, unlike MG-132, AC17 does not directly block proteasome proteolytic activities in vitro.

Molecular analysis has showed that a cross-conjugated α,β-unsaturated dienone compound with two sterically accessible electrophilic β-carbons is a molecular determinant of DUB inhibitors (28), and the inhibition of cellular DUB activity leads to an increase in ubiquitinated proteins. Based on the chemical structure of AC17 (Fig. 1A), the possibility of DUB inhibition in AC17-treated cells was assessed. However, no distinct reduction of total DUB activity was detected using Ub-AMC as a substrate in A549 or NCI-H1299 cells following AC17 treatment (Fig. 2E). As a positive control, NEM completely suppressed total DUB activity in both lung carcinoma cell lines (Fig. 2E). Given the similar pharmacological effect of a novel class of proteasome inhibitors, which block the 19S DUB activity without inhibiting the 20S proteolytic activities (29), we hypothesize that AC17 blocks 19S DUB activity indirectly for its proteasome inhibition. To assess the effect of AC17 on the DUB activity of 19S RP, purified human 19S or 26S proteasome was incubated with AC17 for 30 mins, and then the substrate Ub-AMC was added. The fluorescence intensity change as a consequence of substrate cleavage showed that AC17 significantly inhibited the 19S DUB activity (Fig. 2F), and IC\textsubscript{50} value is 4.23 ± 0.010 μM (Supplementary Fig. S4). 19S DUB inhibition by AC17 was further confirmed by K63-linked ubiquitin tetramer chain disassembly in vitro (Supplementary Fig. S5). In addition, AC17 was found to be an irreversible 19S DUB inhibitor (Supplementary
Fig. S6), and the inhibitory activity of AC17 was completely lost in the presence of GSH (Supplementary Fig. S7). These together suggest that AC17 may covalently bind to cysteine residues of target proteins. These observations show that AC17 is not a general DUB inhibitor such as NEM; instead, AC17 selectively inhibits 26S proteasome by blocking the 19S DUB activity (necessary for efficient cellular protein degradation), resulting in the accumulation of ubiquitinated proteins.

**AC17 reactivates wild-type p53 by inhibiting p53 degradation**

For tumors expressing wild-type p53, p53 reactivation can be achieved in the context of a general disruption of UPS as a therapeutic goal (30). Our previous studies indicated that A549 cells were more sensitive to AC17 treatment than NCI-H1299 cells (Supplementary Fig. S2). Since p53 is partially responsible for the cytotoxicity of AC17 (Supplementary Fig. S3) and there is an obvious difference between A549 cells and NCI-H1299 cells in the p53 status (wild-type p53 in A549 cells and null p53 in NCI-H1299 cells), it is hypothesized that wild-type p53 is reactivated by AC17 through inhibiting 19S DUB activity in A549 cells. Exposing A549 cells to AC17 for 12 h noticeably increased p53 expression, as well as its downstream targets (MDM2 and p21) expression in a dose-dependent manner (Fig. 3A). MDM2 is a key negative regulator of p53, which promotes p53 ubiquitination and targets p53 for proteasomal degradation (31). p21 is a well-characterized cyclin-dependent kinase inhibitor that plays an important role in cell cycle control. p21 overexpression results in cell cycle arrest, which contributes to its tumor suppressor function. Cell cycle analysis
demonstrated that sub-G1 cell populations of A549 cells increased in a concentration-dependent manner after a 24-h incubation with AC17 (Fig. 3B), indicating that apoptosis was induced by AC17 treatment. AC17-induced apoptosis was further demonstrated by apoptosis-associated cellular and nuclear morphologic changes. Compared with the control group, cellular morphology changes (spherical shape and detachment) were observed in AC17-treated A549 cells (Fig. 3C, top). After Hoechst staining, apoptotic nuclear changes (punctate or granular and bright nuclei) were visualized only in cells treated with AC17, not cells treated with DMSO (Fig. 3C, bottom). These results support the hypothesis that AC17 reactivates wild-type p53 and induces apoptosis in a concentration-dependent manner in A549 cells.

To investigate whether 19S DUB inhibitory activity of AC17 is associated with wild-type p53 reactivation, p53 mRNA expression and the interaction between MDM2 and p53 were studied. RT-PCR analysis revealed that after incubation A549 cells with AC17, p53 mRNA decreased, while MDM2 and p21 mRNA increased in a concentration-dependent manner (Fig. 3D). Presumably, the reactivation of wild-type p53 by AC17 occurs at post-translational level. Since the tumor suppressor activity of p53 is effectively inhibited by its cellular inhibitor MDM2 through direct interaction with p53, blocking MDM2-p53 interaction is an important strategy for anti-cancer drug design (32). To determine whether the MDM2-p53 interaction is inhibited by AC17 treatment, aliquots of A549 cells after AC17, MG-132, and positive control
(Nutlin-3a) treatment were subjected to co-immunoprecipitation analysis. As shown in Fig. 3E, treatment with AC17 or MG-132 did not dissociate p53 from MDM2, whereas Nutlin-3a caused the dissociation of p53-MDM2 complex. If 19S DUB inhibition by AC17 is responsible for wild-type p53 reactivation, the levels of ubiquitinated p53 should be increased after AC17 treatment of A549 cells. Compared with the control, accumulation of ubiquitinated p53 was detected after 12 hours of AC17 treatment (Fig. 3F). MG-132 treatment had a similar effect, but Nutlin-3a treatment prevented the accumulation of poly-ubiquitinated p53 (Fig. 3F). Collectively, these results show that, as a 19S DUB inhibitor, AC17 reactivates endogenous wild-type p53 by inhibiting UPS of p53 degradation.

**Suppression of human lung adenocarcinoma A549 xenograft proliferation by AC17 is associated with proteasome inhibition, p53 reactivation, and NF-κB blockage**

AC17 is a novel proteasomal DUB inhibitor that blocks NF-κB pathway and reactivates wild-type p53 in the cultured lung cancer cell line A549. Therefore, we further investigated the anti-tumor effects of AC17 in vivo. To do so, A549 cells were subcutaneously injected into the flanks of female BALB/c nude mice. When the tumors reached approximately 120 mm³, mice were randomized into 3 groups and treated by intraperitoneal injection with either vehicle control or AC17 at doses of 1 and 5 mg/kg. During treatment, the tumor sizes in these groups were measured two times per week (Fig. 4A). At the end of the experiment, tumor tissues were removed...
from the mice, weighed, and photographed (Fig. 4B and C). The inhibition of tumor growth by AC17 at 1 and 5 mg/kg compared with the vehicle-administered control group were 34.5% and 45.6%, respectively. These results imply that AC17 possesses effective anti-tumor activity \textit{in vivo}.

To determine whether the observed anti-tumor effect of AC17 \textit{in vivo} is associated with its proteasome-inhibitory, p53-reactivation, and NF-κB-blocking activities, samples of the control or AC17-treated tumors were subjected to multiple assays. Fig. 4D shows that the accumulation of ubiquitinated proteins was obvious in AC17-treated tumors, indicating that AC17 inhibited proteasome activity \textit{in vivo}. Accompanying proteasome inhibition, p53 was reactivated in the A549 xenografts, as shown by increased p53, MDM2, and p21 expression in tissue extracts (Fig. 4D). Increased p53 expression compared to vehicle-treated mice was further verified by immunostaining in tumors from AC17-treated mice (Fig. 5A). The interaction between IκBα and p65 in tumor tissues was analyzed to determine whether AC17-induced proteasomal inhibition induces a consequent inhibition of NF-κB pathway, which is constitutively activated in human lung cancer cell lines (33), and plays a key role in tumor proliferation, invasion, and metastasis (34, 35). Indeed, the activated NF-κB p65 subunit in the corresponding vehicle-treated control tumors was mostly dissociated from IκBα (Fig. 4E), which was in sharp contrast to the cultured A549 cells (Fig. 1G). At 24 hours after the last treatment, tumors from AC17-treated mice showed dose-dependent increase of the interaction between IκBα and p65,
suggesting that NF-κB pathway was inhibited by AC17 in vivo (Fig. 4E). Immunofluorescence staining of tumor tissues further demonstrated that p65 was localized in the nucleus in the vehicle-treated control group, whereas mostly in the cytoplasm in the AC17-treated groups (Fig. 5B). Taken together, these observations indicate that AC17 has the ability to inhibit proteasomal activity, resulting in reactivation of tumor suppressor p53 and blockade of NF-κB pathway within the tumor, most likely accounting in part for the observed anti-tumor activity of AC17 in vivo.

In addition, histological examination of H&E-stained lung, liver, and kidney tissues from the treatment groups was performed. The lungs of AC17-treated mice appeared normal with less consolidation compared to the control group (Fig. 5C, top). Livers from control mice demonstrated hepatomegaly and necrosis, while AC17-treated mice showed normally structured hepatocytes (Fig. 5C, bottom). There were no detectable changes in kidneys after treatment with AC17 compared to the vehicle control (data not shown). These results indicate that AC17 is considerably less toxic to normal cells than tumor cells, in agreement with several previous studies of proteasome inhibitors (36, 37).

**Discussion**

Curcumin has attracted much attention because of its surprisingly wide range of beneficial properties, including anti-inflammatory, anti-oxidative, and anti-cancer activities, as well as its pharmacological safety and potency as a chemopreventive
agent by regulating a variety of molecular targets (38, 39). Unfortunately, therapeutic potential of curcumin is limited by its relatively low potency and poor cellular bioavailability (40). Therefore, there is a need to develop highly active and clinically promising curcumin analogues. In the present study, we demonstrated that our synthetic 4-arylidene curcumin analogue (AC17) was a potent 19S DUB inhibitor that was much more effective in suppressing the proliferation of several human cancers than its parent compound curcumin (Supplementary Fig. S1) and with improved cellular bioavailability and metabolic stability (22, 23).

Due to the emerging role in tumor cell proliferation and resistance, UPS appears to be an ideal target for the development of novel cancer therapies (2, 41-43). The success of the 20S proteolytic inhibitor bortezomib for the treatment of multiple myeloma and mantle cell lymphoma has verified this and stimulated interest in the development of UPS inhibitors. As a natural medicinal component, curcumin has been confirmed to inhibit proteolytic activities of purified rabbit 20S with low IC$_{50}$ values (44). Surprisingly, our current results showed that AC17, a 4-arylidene curcumin analogue, had little effect on proteasomal CT-like, T-like, and PGPH-like activities in vitro (Fig. 2D). Interestingly, AC17 caused a rapid and marked accumulation of polyubiquitinated proteins in both A549 and NCI-H1299 cells (Fig. 2A and B). Moreover, it was envisaged that AC17 was a DUB inhibitor based on molecular structure analysis (28). Again unexpectedly, the total cellular DUB activity was not dramatically inhibited in AC17-treated cells (Fig. 2E). Therefore, the exact
mechanism by which AC17 induces the accumulation of ubiquitinated proteins is drastically different from its parent compound curcumin.

Recent advances have revealed that the 19S RP is considered a novel anti-cancer drug target (29, 41). Functions of 19S RP include binding ubiquitinated proteins, recycling ubiquitin, unfolding proteins and threading them into the 20S CP (45). Therefore, compounds that inhibit one or more functions of 19S RP are potential 19S inhibitors. The first reported 19S inhibitors were ubistatins, which block crucial interaction of ubiquitinated proteins and the 19S RP (46). As a dual reversible inhibitor of USP14 and UCHL5 of the 19S RP, b-AP15 has been shown to induce accumulation of protein-ubiquitin conjugates similar to the traditional proteasome inhibitors, and yet there are some differences between b-AP15 and bortezomib in sensitivity to Bcl-2 overexpression and p53 status as well as their anti-cancer activity against solid tumors (29, 41). Herein, we reported that AC17 irreversibly inhibited 19S DUB activity selectively, without inhibiting total DUB activity in cells (Fig. 2E and F, Supplementary Fig. S4-6). AC17 treatment increased the levels of p53 and p21 expression, reactivated p53 (which is associated with the induction of apoptosis in A549 cells) (Fig. 3A-D), all of which are consistent with previous reports on proteasome inhibitors (47, 48). Although p53 partially contributed to the cytotoxic effects of AC17, p53 depletion did not prevent the AC17-induced cytotoxicity completely (Supplementary Fig. S3 and 8). In contrast, bortezomib-induced cytotoxic activity was much more sensitive to p53 depletion (Supplementary Fig. S8) (21, 29).
These findings have important implications because p53 is involved in mediating bortezomib resistance (49); a therapeutic approach using 19S DUB inhibitors would potentially overcome bortezomib resistance.

Furthermore, previous studies have shown that NF-κB activation, regulated by UPS (50), is important for tumor proliferation, invasion, and metastasis, accounting for radiation and chemotherapy resistance in cancer cells (34, 51). Chemotherapeutic drugs activate NF-κB through diverse pathways, leading to resistance to and failure of chemotherapy (52). Inhibition of NF-κB activation would therefore contribute to the anti-cancer activity of UPS inhibitors. We have reported that 4-arylidene curcumin analogues inhibit NF-κB activation in part by blocking IKK activity (22). Herein we showed that AC17 induced the accumulation of polyubiquitinated IκBα and IκBα binding to NF-κB p65 subunit (Fig. 1D and E), resulting in inhibition of NF-κB activation by stabilizing the intrinsic inhibitor, IκB. The two mechanisms of AC17 NF-κB inhibitory activity are cooperative and shed some light on the mechanism of AC17-induced cancer cell death.

Using an A549 xenograft mouse model, treatment of AC17 resulted in the accumulation of ubiquitinated proteins, reactivation of tumor suppressor protein p53 (Fig. 4D), and an enhanced interaction between IκBα and p65 in vivo (Fig. 4E). Immunostaining and immunofluorescence assays of tumor tissues further confirmed that p53 was overexpressed and that NF-κB translocation to the nucleus was inhibited in AC17-treated mice (Fig. 5A and B). Consistent with these, AC17 treatment
effectively inhibited tumor growth \textit{in vivo} (Fig. 4A-C). Notably, mice treated with AC17 under the present experimental conditions showed no renal toxicity, hepatotoxicity, or pulmonary consolidation (Fig. 5C). These results strongly suggest that 4-arylidene curcumin analogues could be used as new agents for cancer chemoprevention, cancer chemotherapy, or both.

In the present study, the exact molecular mechanism by which AC17 inhibits the DUB activity of 19S RP was not completely elucidated. The $\alpha,\beta$-unsaturated dienone in AC17 may serve as a Michael acceptor that theoretically interacts with the thiol groups of cysteine residues in DUBs (53). This may also explain how AC17 acts as an irreversible 19S DUB inhibitor (Supplementary Fig. S6) and entirely loses its 19S DUB inhibition in the presence of GSH (Supplementary Fig. S7). However, the type of DUBs specifically inhibited by AC17 remains unclear. Therefore, the full explanation of the activity of AC17 requires further study. Additionally, structure-activity relationship studies of 4-arylidene curcumin analogues with 19S DUB inhibitory activity may help to improve such proteasome DUB inhibitors and aid new anti-cancer agent development in clinical settings.
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**Figure Legends**

**Figure 1.** AC17 inhibits NF-κB activation by inhibiting degradation of IκBα.

A, the chemical structural design of curcumin to AC17. B, A549 cells were treated with DMSO (control) or AC17 for 30 mins, followed by stimulation with TNFα (10 ng/mL) for 30 mins, and then NF-κB subcellular localization was verified by immunofluorescence staining with p65 antibody. C, A549 cells were co-transfected with pNF-κB-luc and pRL-TK and treated as indicated with TNFα (10 ng/mL), AC17 (1 μM), and the combination prior to determining NF-κB transcriptional activity by the dual-luciferase reporter assay system. The results present mean ± SD of three independent experiments. *p < 0.05. D, A549 cells were treated either with or without 4 μM AC17 for 1 h, followed by incubation with CHX (5 μM) for different time points as indicated, and then whole-cell extracts were analyzed by Western blotting for IκBα expression. The intensity of immunoblots digitized by ImageJ software was normalized to β-actin, and then arbitrarily normalized with the intensity value for IκBα at time 0 to 1.00. E, A549 cells were treated with AC17 for different time as indicated, and total RNA was isolated and subjected to RT-PCR analysis for IκBα mRNA levels. Columns, mean (n = 3); bars, SD. F, G, A549 cells were treated with either AC17 or MG-132 for 12 h, then the extracted whole proteins were immunoprecipitated with IκBα or p65 antibody. The levels of ubiquitinated IκBα (F) and interaction of p65 with IκBα (G) were detected by Western blot analysis. TNFα and IgG were used as comparison. β-actin protein amounts were used to verify equal
protein for immunoprecipitation.

**Figure 2.** AC17 induces a rapid accumulation of polyubiquitinated proteins with the 19S DUB inhibition.

*A*, A549 cells (left) and NCI-H1299 cells (right) were incubated with the indicated concentration of AC17 for 2 h before whole-cell extracts were subjected to Western blot with ubiquitin antibody. **B**, A549 cells were treated with 4 μM AC17 for the different hours, and the extracts were analyzed by Western blot for the accumulation of ubiquitinated proteins. **C**, A549 cells were treated with AC17 (4 μM) or MG-132 (10 μM) for 2 h, then whole-cell lysates were examined for ubiquitinated proteins. GAPDH and β-actin were used as a loading control. **D**, purified human 20S (left) and 26S proteasome (middle) were incubated with AC17 (50 μM) or MG-132 (10 μM) for 30 mins before analysis of three proteasomal proteolytic activities; Lysates (right) from the control (DMSO), AC17-treated, MG-132-treated A549 cells (10 μM, 2h) were analyzed for these activities. **Columns**, average of triplicate experiments; **bars**, SD. **E**, A549 cells (left) and NCI-H1299 cells (right) were treated with 4 μM AC17 for 4 h, and total DUB activity was determined from whole-cell extracts by measuring Ub-AMC cleavage. NEM (10 mM) was a control. **F**, purified human 19S (left) and 26S proteasome (right) were incubated with AC17 (50 μM) or NEM (10 mM) for 30 mins, following DUB activity was measured as described in Materials and Methods. The results were from one representative in three independent experiments. Similar results were observed in additional two assays.
**Figure 3.** AC17 reactivates pro-apoptotic protein p53 by inhibiting p53 degradation.

*A*, extracts from A549 cells treated with different concentrations of AC17 for 12 h was analyzed by Western blot for p53, p21, and MDM2 expression. *B*, A549 cells were treated with AC17 for 24 h, stained with PI, and analyzed by flow cytometry. Cell cycle distributions were analyzed using FlowJo software. *C*, A549 cells were treated for 24 h with AC17, followed by photographing of cellular morphologic changes (× 200), and apoptotic nuclear changes (× 400) after staining with Hoechst 33342. *D*, total RNA was isolated from A549 cells after treatment of AC17 for 12 h and subjected to RT-PCR analysis for p53, p21 and MDM2 mRNA levels. Values obtained from three separate experiments and expressed as mean ± SD, *p* < 0.05. *E*, *F*, A549 cells were treated for 12 h with AC17 or MG-132, and the extracted proteins was immunoprecipitated with p53 or MDM2 antibody. The p53 and MDM2 complexes (*E*) or levels of ubiquitinated p53 (*F*) were determined by Western blotting using p53, MDM2, and ubiquitin antibodies. Nutlin-3a and IgG were used as comparison, and GAPDH was a control for immunoprecipitation.

**Figure 4.** AC17 suppresses tumor growth in the A549 xenograft mouse model, associated with proteasomal inhibition, NF-κB blockage, and p53 reactivation.

Female nude mice were xenografted by subcutaneous injection of A549 cells (1 × 10^7) at one flank. When tumor size reached ~120 mm^3, mice were divided into 3 groups and treated with vehicle solvent or AC17 (1 and 5 mg/kg). Tumors were collected and weighted after 23-day treatment (*B*, *C*), and the prepared tissue extracts were analyzed...
for Western blotting (D) and immunoprecipitation analysis (E). A, inhibition of A549 tumor growth by AC17. Points, mean tumor volume in each experimental group containing five mice; bars, SD. *p < 0.05. B, the effect of AC17-induced growth inhibition on tumor weight at the endpoint. Columns, mean (n = 5); bars, SD. *p < 0.05. C, example images of tumor excised from mice of each group at the endpoint. D, Western blot analysis of tumor tissue extracts with antibodies of ubiquitin, p53, p21, MDM2, and GAPDH. E, the association of p65 and IκBα in tumor tissue extracts was verified by immunoprecipitation analysis. GAPDH was used as a loading control.

**Figure 5.** Immunofluorescence p65, immunohistochemistry p53 and H&E staining assays using tissue samples.

Tumors, lungs, and livers were collected after 23-day treatment and the prepared tissue slides (4 μm) were used for immunohistochemistry with p53 antibody (A), immunofluorescence staining with p65 antibody (B), and H&E staining assays (A, C). A, slides of tumor tissues were used for immunostaining with p53 antibody and H&E staining assays. Magnifications, × 200 and × 400 as indicated. B, NF-κB subcellular localization in tumor tissues was stained with p65 antibody, followed DyLight 549-labeled anti-rabbit IgG (H+L), and nuclear staining was used by Hoechst 33342. Magnifications, × 630. C, microscopical pictures of lungs and livers from different treated groups were obtained by H&E staining. Magnifications, × 100.
Figure 1

A  
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\text{Curcumin} \quad \xrightarrow{\text{Knoevenagel condensation}} \quad \text{AC17}
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E  
![Graph showing relative amount of IκBα mRNA](image_11)

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Binhua Zhou, Yinglin Zuo, Baojian Li, et al.

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