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Advances in Brief

CEP1612, a Dipeptidyl Proteasome Inhibitor, Induces $p21^{WAF1}$ and $p27^{KIP1}$ Expression and Apoptosis and Inhibits the Growth of the Human Lung Adenocarcinoma A-549 in Nude Mice[1]

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Abstract

The ubiquitin proteasome system is responsible for the proteolysis of important cell cycle and apoptosis-regulatory proteins. In this paper we report that the dipeptidyl proteasome inhibitor, phthalimide-$(CH_2)_8$CH-(cyclopentyl) CO-Arg(NO_2)-Leu-H (CEP1612), induces apoptosis and inhibits tumor growth of the human lung cancer cell line A-549 in an *in vivo* model. In cultured A-549 cells, CEP1612 treatment results in accumulation of two proteasome natural substrates, the cyclin-dependent kinase inhibitors $p21^{WAF1}$ and $p27^{KIP1}$, indicating its ability to inhibit proteasome activity in intact cells. Furthermore, CEP1612 induces apoptosis as evident by caspase-3 activation and poly(ADP-ribose) polymerase cleavage. Treatment of A-549 tumor-bearing nude mice with CEP1612 (10 mg/kg/day, i.p. for 31 days) resulted in massive induction of apoptosis and significant (68%; $P < 0.05$) tumor growth inhibition, as shown by terminal deoxynucleotidyltransferase-mediated UTP end labeling. Furthermore, immunostaining of tumor specimens demonstrated *in vivo* accumulation of $p21^{WAF1}$ and $p27^{KIP1}$ after CEP1612 treatment. The results suggest that CEP1612 is a promising candidate for further development as an anticancer drug and demonstrate the feasibility of using proteasome inhibitors as novel antitumor agents.

Introduction

The proteasome is a high molecular weight multicatalytic protease complex. This 20S proteasome (M_r 700,000) associates with activators to form a 26S proteasome (M_r 2,000,000), which catalyzes the ATP-dependent degradation of polyubiquitinated protein substrates (1–3). The multicatalytic complex has several proteolytic activities including chymotrypsin-like, trypsin-like, peptidylglutamylpeptide-hydrolyzing, small neutral amino acids-preferring, and branched chain amino acid-preferring activities. Among the proteasome substrates are proteins intimately involved in the regulation of several important pathways including those of programmed cell death (apoptosis), cell division cycle, signal transduction, and antigen presentation (reviewed in Refs. 4–8). Aberrant proteasome activity has been implicated in several pathological conditions such as cancer, muscular dystrophy, emphysema, leprosy, and Alzheimer's disease (7, 9). Of particular interest to us is the involvement of the proteasome in the degradation of proteins pivotal to the cell division cycle and apoptosis in human cancer cells. For example, the degradation by the proteasome of cyclins B and E is required for exit out of mitosis and entry into the S-phase of the cell cycle, respectively. The proteasome is also responsible for the degradation of proteins involved in the regulation of cell survival/apoptosis (*i.e.*, p53 and nuclear factor-κB) as well as those involved in the interface between cell cycle and apoptosis (*i.e.*, the cyclin-dependent kinase inhibitors $p21^{WAF1}$ and $p27^{KIP1}$, Refs. 4–8).

The fact that the proteasome has been implicated in many disease states suggested that proteasome inhibitors have therapeutic potential. Furthermore, these inhibitors would provide outstanding tools for determining the role of the proteasome in various physiological functions. This has prompted many to design, synthesize, and biologically evaluate proteasome inhibitors.

Several groups have made a variety of structurally unrelated proteasome inhibitors (6, 10). These include peptide aldehydes such as PSI (*N*-benzyloxycarbonyl-Ile-Glu-(*O-t*-butyl)-Ala-Leu-H (11) or CbzLLnV-H and CbzLLnL-H (12), which inhibit the chymotrypsin-like activity of the proteasome. Others have made tripeptide epoxyketones (13), dipeptidyl boronic acid (14), indanylamide derivatives (15), and vinyl sulfone derivatives (16).

Iqbal *et al.* (17) designed a series of dipeptidyl aldehydes that inhibited potently the chymotrypsin-like activity of the proteasome. One of these, CEP1612,[4] was highly selective (>500-fold) for chymotrypsin-like over trypsin-like activity of the proteasome. CEP1612 was also shown to be cell permeable and inhibited the proteasome in intact cells with an IC_{50} of 1 μM. Initially, CEP1612 was shown to block MCH-1 antigen processing (18). Subsequently, An *et al.* (19) demonstrated that CEP1612 induces apoptosis in human prostate, breast, tongue, and brain cancer cell lines. Furthermore, they demonstrated that CEP1612 induced apoptosis in SV40-transformed but not the parental normal human fibroblasts WI-38 (19). In addition, inhibition of the proteasome activity is sufficient to overcome Bcl-2- or Bcr-Abl-mediated drug resistance of tumor cells (19, 20). In this study, human lung adenocarcinoma A-549 cells were implanted s.c. in nude mice to evaluate whether CEP1612 can induce apoptosis and inhibit human tumor growth *in vivo*.

Materials and Methods

Synthesis of Dipeptidyl Proteasome Inhibitor CEP-1612. CEP1612 was prepared according to the procedure reported previously (17), except for a key modification in the synthesis of the NH_2-terminal region. Benzyl cyclopentylacetate was monoalkylated with 1,8-diiodooctane in tetrahydrofuran by sodium hexamethyldisilazide at −78°C. Substitution with potassium phthalimide and deprotection of the benzyl ester produced the NH_2-terminal derivative. This was coupled with a COOH-terminal moiety using an acid chloride

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[4] The abbreviations used are: CEP1612, phthalimide-$(CH_2)_8$CH(cyclopentyl) CO-Arg(NO_2)-Leu-H, CKI, cyclin-dependent kinase inhibitor, TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling; PARP, poly(ADP-ribose) polymerase; RFU, relative fluorescent unit.

activation method. Final deprotection by 1 N HCl produced the aldehyde, CEP1612.

Cell Culture and Drug Treatment. Human lung adenocarcinoma A-549 cells were purchased from American Type Culture Collection (Rockville, MD) and grown in Ham's F-12K Nutrient Mix, containing 10% FBS, at 37°C in a humidified incubator containing 10% CO_2. Treatment of cells with the proteasome inhibitor CEP1612 was performed as described previously (19).

Proteasome Activity Assay. Chymotrypsin-like substrate suc-Leu-Leu-Val-Tyr-AMC and 20S proteasome (recombinant, *Methanosarcina thermophila, Escherichia coli*) were purchased from Calbiochem (La Jolla, CA). For measurement of 20S proteasome activity *in vitro*, Suc-Leu-Leu-Val-Tyr-AMC (20 μM), 0.5 μg of 20S proteasome, and inhibitor CEP1612 (0.05–10 μM) were incubated in 100 μl of assay buffer (20 mM HEPES, 0.5 mM EDTA, and 0.035% SDS, pH 8.0) for 1.5 h at 37°C. Reaction samples were diluted to 200 μl with assay buffer. Free AMC liberated by the substrate hydrolysis was quantified on a fluorometer (VersaFluor fluorometer; Bio-Rad, Richmond, CA) with an excitation filter of 380 nm and an emission filter of 460 nm. For estimation of proteasome activity in tumor tissue extracts from nude mice treated and untreated with CEP1612, tumor extracts (30 μg) and 20 μM Suc-Leu-Leu-Val-Tyr-AMC were incubated in 200 μl of assay buffer (20 mM HEPES, 0.5 mM EDTA, pH 8.0) at specific time intervals at 37°C. Free AMC was quantified as described above.

Western Blotting. Whole-cell extraction and the enhanced chemiluminescence Western blot assay were performed as we described previously (21). Briefly, proteins were resolved by 15 or 8% SDS-PAGE gel and immunoblotted with antibodies against caspase-3 (CPP32), $p21^{WAF1}$ (SX118), $p27^{KIP1}$ (G173-524; PharMingen, San Diego, CA), and PARP (Boehringer Mannheim). The ECL blotting system (NEN Life Science Products, Boston, MA) was used for detection of positive antibody reactions.

Antitumor Activity in the Nude Mouse Tumor Xenograft Model. Nude mice (Charles River, Wilmington, MA) were maintained in accordance with the Institutional Animal Care and Use Committee procedures and guidelines. A-549 cells were harvested, resuspended in PBS, and injected s.c. into the right and left flank (10×10^6 cells/flank) of 8-week-old female nude mice as reported previously (22). When tumors reached about 100–150 mm^3, animals were dosed i.p. with 0.2 ml once daily. Control animals received a saline vehicle, whereas treated animals received injections of CEP1612 (10 mg/kg/day). The tumor volumes were determined by measuring the length (l) and the width (w) and calculating the volume ($V = lw^2/2$), as described previously (22). Statistical significance between control and treated groups was evaluated by using Student's t test ($P < 0.05$).

Effects of CEP1612 on Organ Proteasome Activity. The ability of CEP1612 to reach mice organs was determined by injecting nude mice with CEP1612 (10 mg/kg i.p. once) and sacrificing the mice at times 0, 5, 15, 60, and 120 min after injection. Three mice were collected per time point. Livers, kidneys, and lungs were then collected and processed for proteasome activity assay as described above for tumor proteasome activity measurements.

TUNEL Assay and Immunostaining. Apoptosis was determined by TUNEL using an *in situ* cell death detection kit (Boehringer Mannheim). Frozen sections were prepared from the treated and untreated tumors. The slides were fixed in paraformaldehyde (4% in PBS, pH 7.4). After rinsing with PBS and incubation in permeabilization solution, the tissues were cross reacted with TUNEL reaction mixture (for 60 min at 37°C in a humidified chamber), with converter-alkaline phosphatase solution (for 30 min at 37°C in a humidified chamber), and with alkaline phosphatase substrate solution (Vector Laboratories, Burlington, MA, for 5–10 min). For the immunostaining of $p21^{WAF1}$ and $p27^{KIP1}$, the tumor frozen sections and slides were prepared as described for TUNEL assay; the anti-$p21^{WAF1}$ or anti-$p27^{KIP1}$ antibody was applied to the slide. The reactions were analyzed by light microscopy.

Results and Discussion

CEP1612, a cell permeable dipeptidyl aldehyde proteasome inhibitor, was initially designed and synthesized by Iqbal *et al.* (17) and subsequently shown to block MCH-1 antigen processing (18). More recently, An *et al.* (19) demonstrated that CEP1612 induced apoptosis in several human cancer cell lines and that normal cells were resistant to CEP1612-induced apoptosis. In this report, we present evidence for the ability of CEP1612 to induce the expression of $p21^{WAF1}$ and $p27^{KIP1}$ and apoptosis as well as inhibit tumor growth of the human lung adenocarcinoma A-549 *in vivo* using the nude mouse xenograft model.

To determine the *in vivo* apoptotic and antitumor activity of CEP1612, we synthesized this molecule as described previously by Iqbal *et al.* (17), using modifications of the route as described in "Materials and Methods," and confirmed its ability to inhibit proteasome activity (Fig. 1). CEP1612 inhibition of the chymotrypsin-like proteasome activity was demonstrated using a recombinant 20S proteasome and Suc-leu-leu-val-Tyr-AMC as substrate as described in "Materials and Methods." Fig. 1*B* shows that CEP1612 inhibited the proteasome activity in a concentration-dependent manner with an IC_{50} of 60 nM. Our IC_{50} is higher than Iqbal *et al.* (17), and this could be because of the fact that they used human brain 20S proteasomes and methoxysuccinyl-Glu-Val-Lys-Met-para-nitroanilide as substrate. More important, however, is the fact that CEP1612 synthesized by us was as potent as that synthesized by Iqbal *et al.* (17) when used in whole cells against the breast carcinoma MCF-7 and SV40-transformed WI-38 (see below).

We next investigated whether CEP1612 inhibits proteasome activity and induces apoptosis in cultured A-549 cells. A-549 cells were treated with CEP1612 (30 μM) for 24 h and lysed, and the lysates were immunoblotted with antibodies to $p21^{WAF1}$ and $p27^{KIP1}$ as described in "Materials and Methods." $p21^{WAF1}$ and $p27^{KIP1}$ are two cyclin-dependent kinase inhibitors that are known natural substrates for the chymotrypsin-like activity of the proteasome (23, 24). Fig. 2*A* shows that treatment with CEP1612 resulted in the accumulation of both $p21^{WAF1}$ and $p27^{KIP1}$. These data confirmed that CEP1612 inhibits

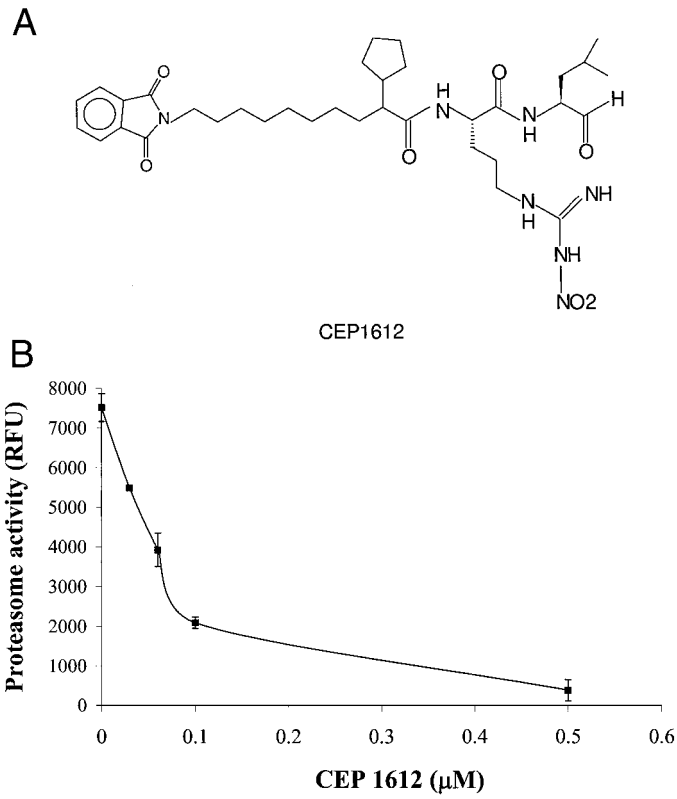

Fig. 1. *A*, structure of the dipeptidyl proteasome inhibitor, CEP1612. *B*, CEP1612 inhibits the 20S proteasome activity *in vitro*. To determine whether the dipeptidyl proteasome inhibitor CEP1612 blocks proteasome activity *in vitro*, a cell-free system was used by incubation of the inhibitor with a recombinant 20S proteasome from *E. coli* and chymotrypsin-like substrate. The proteasome activity was determined as RFU by measuring with a fluorometer the fluorescence from the AMC cleaved product as described in "Materials and Methods." Data are representative of three independent experiments. *Bars*, SD.

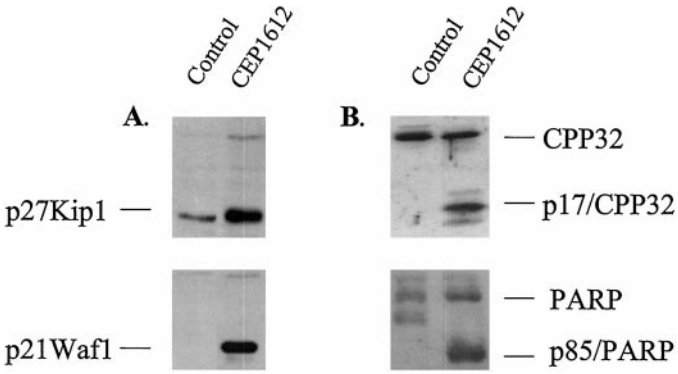

Fig. 2. The proteasome inhibitor CEP1612 results in accumulation of cyclin-dependent kinase inhibitors $p21^{WAF1}$ and $p27^{KIP1}$ and induces apoptosis *in vitro*. *A*, accumulation of the cyclin-dependent kinase inhibitors $p21^{WAF1}$ and $p27^{KIP1}$ in A-549 cells treated either with DMSO vehicle or 30 μM CEP1612 for 24 h is shown. Whole-cell extracts were prepared and analyzed by Western blot using anti-$p21^{WAF1}$ and anti-$p27^{KIP1}$ antibodies. *B*, activation of caspase-3 and stimulation of PARP cleavage in A-549 cells treated with 30 μM CEP1612 for 24 h. Immunoblotting was performed using anti-CPP32 and anti-PARP antibodies. The full-length PARP and M_r 85,000 fragment *(p85/PARP)* as well as pro-CPP32 *(CPP32)* and activated form *(p17/CPP32)* are indicated. Data are representative of two independent experiments.

proteasome activity in A-549 cells. Previously, An *et al.* (19) reported that CEP1612 induces apoptosis in human breast, prostate, brain, and head and neck cancer cell lines. To determine whether CEP1612 induces programmed cell death in A-549 cells, we exposed these cells to CEP1612 and determined its ability to induce processing and activation of caspase-3 and cleavage of PARP as described in "Materials and Methods." Fig. 2*B* shows that treatment with CEP1612 resulted in the processing of caspase-3, as evident from the CEP1612-dependent generation of the p17 form from the CPP32 form. Similarly, CEP1612 treatment also resulted in the cleavage of PARP to its p85 proteolytic fragment (Fig. 2*B*). The IC_{50} of CEP1612 to induce PARP cleavage in A-549, MCF-7, and SV40-transformed WI-38 was 1 μM and similar to what An *et al.* (19) reported previously.

The data described above clearly demonstrate that the proteasome inhibitor CEP1612 induces apoptosis in cultured A-549 cells. To determine CEP1612 antitumor efficacy and its ability to induce apoptosis *in vivo*, we implanted A-549 cells s.c. in nude mice, and when the tumors were palpable (100–150 mm^3), the mice were either treated with vehicle control or CEP1612 (10 mg/kg/day, i.p.). After 1 month of daily treatment, the A-549 tumors were removed and processed for inhibition of proteasome activity and TUNEL as well as $p21^{WAF1}$ and $p27^{KIP1}$ immunostaining as described in "Materials and Methods." Fig. 3*A* shows that tumors from control animals grew to an average size of 285.3 $\pm$ 26.0 mm^3. In contrast, tumors from CEP1612-treated animals grew to an average size of only 176.4 $\pm$ 36.6 mm^3. Thus, treatment with CEP1612 resulted in a statistically significant ($P < 0.05$), 68% tumor growth inhibition (Fig. 3*A*). To demonstrate that the proteasome activity of A-549 tumor cells was inhibited *in vivo*, tumor extracts were incubated with purified peptide substrate for various periods of time, and the chymotrypsin-like activity of the proteasome was assayed as described in "Materials and Methods." Fig. 3*B* shows that tumor extracts from control animals contained proteasome activity that reached 2000 RFUs over 120 min. In contrast, tumor extracts from CEP1612-treated animals contained only 1050 RFUs, demonstrating that CEP1612 treatment resulted in 43% inhibition of proteasome activity *in vivo* (Fig. 3*B*). To further document that CEP1612 inhibited the proteasome activity, we next determined by immunostaining whether treatment of nude mice with this molecule resulted in the accumulation in the A-549 tumors of two of its natural substrates, $p21^{WAF1}$ and $p27^{KIP1}$. Fig. 4*A* shows that tumors from control animals have low levels of $p21^{WAF1}$ and $p27^{KIP1}$. However, tumors from CEP1612-treated animals contained much higher levels of these cyclin-dependent kinase inhibitors. Consistent with our *in vitro* results of Fig. 2*B*, CEP1612 treatment *in vivo* was also able to induce accumulation of $p21^{WAF1}$ and $p27^{KIP1}$. Thus, CEP1612 injected i.p. was able to reach the s.c.-implanted A-549 tumors and inhibit the intended target *in vivo*. We next determined whether CEP1612 also inhibited the proteasome activity in mice organs. To this end, we have injected i.p. 15 nude mice with CEP1612 (10 mg/kg) and sacrificed the mice after 5, 15, 60, and 120 min (3 mice/time point). Livers, lungs, and kidneys were then processed for proteasome activity assays as described in "Materials and Methods." CEP1612 inhibited potently (80%) liver proteasome activity within 5 min of treatment. In contrast, kidney proteasome activity was inhibited by only 50% 2 h after CEP1612 injection. Lung proteasome activity was not affected by CEP1612 treatment (Fig. 4*C*).

The above results demonstrated that CEP1612 reaches its target and inhibits human tumor growth *in vivo*. Whether this inhibition of tumor growth is attributable to induction of apoptosis was next determined. Tumor specimens from control and CEP1612-treated animals were processed for TUNEL assay as described in "Materials and Methods."

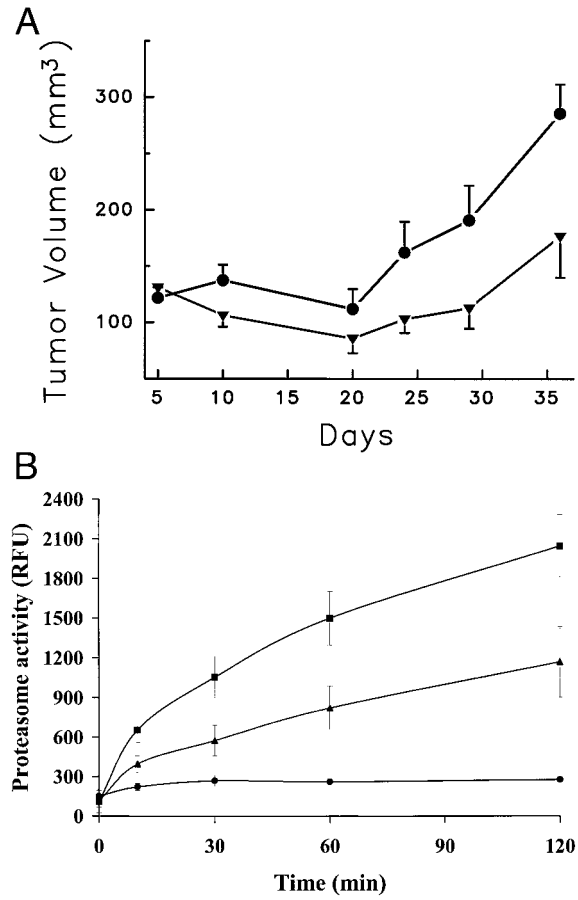

Fig. 3. *A*, antitumor efficacy of CEP1612 against human lung adenocarcinoma A-549 xenografts in nude mouse. A-549 cells were injected s.c. at day 0 into the right and left flanks (10×10^6 cells/flank) of 8-week-old female nude mice. When the tumor size reached an average of 125 mm^3, animals (five mice/group) were treated i.p. daily either with vehicle (●) or CEP1612 (10 mg/kg; ▼) for 31 days, and tumor volumes were determined as described in "Materials and Methods." Data are representative of two independent experiments. Statistical significance between control and treated groups were evaluated by using Student's *t* test ($P < 0.05$). *Bars*, SE. *B*, CEP1612 inhibits SE proteasome enzyme activity *in vivo*. Nude mice were treated as described in *A*. At the end of experiment, tumor specimens were dissected, frozen, and sectioned. Then tumor extracts from CEP1612 treated (▲), untreated (■), or BSA control (●) were incubated with purified chymotrypsin-like substrates at various times (0–120 min), and the proteasome activity was determined using a fluorometer as described in "Materials and Methods." Data are representative of two independent experiments; *Bars*, SD.

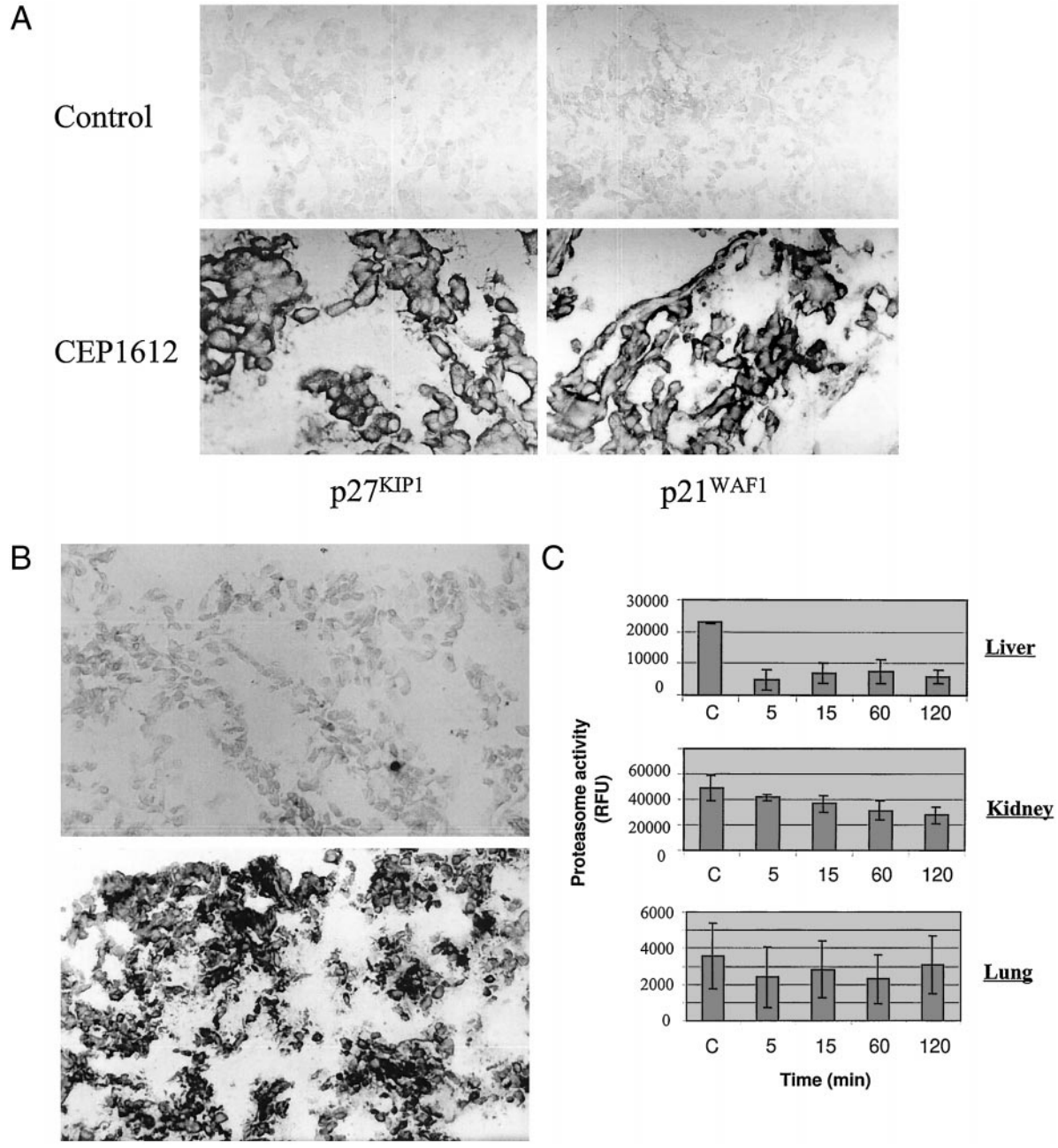

Fig. 4. CEP1612 accumulates the cyclin-dependent kinase inhibitors $p21^{WAF1}$ and $p27^{KIP1}$ and induces apoptosis *in vivo*. *A*, A-549 xenografts in nude mice were treated and prepared as described in the Fig. 3 legend, and immunostaining was performed by using anti-$p21^{WAF1}$ and anti-$p27^{KIP1}$ antibodies. *B*, to determine whether administration of CEP1612 induces apoptosis of A-549 cells *in vivo*, nude mice bearing A-549 tumors were treated either with vehicle (DMSO) or CEP1612 (10 mg/kg/day, i.p.) for 31 days and sacrificed, and tumor specimens were dissected, frozen, and sectioned; apoptosis was detected using an *in situ* TUNEL assay as described in "Materials and Methods." Areas of the tumor that are apoptotic show dark nuclear staining, and $p21^{WAF1}$ and $p27^{KIP1}$ also show intense staining. The fields shown are × 100. *C*, to determine whether CEP1612 inhibits the intense proteasome activity in mice organs, we treated the mice with CEP1612 for various periods of time and collected lungs, livers, and kidneys and measured their proteasome activity as described in "Materials and Methods." Data are representative of two independent experiments; *Bars*, SD.

TUNEL analysis demonstrated that A-549 tumors from vehicle control-treated animals contain no apoptotic cells (Fig. 4*B*). In contrast, tumor cells from animals treated with CEP1612 were apoptotic. Thus, CEP1612 treatment resulted in the induction of massive apoptosis in A-549 tumors *in vivo*.

Taken together, our data demonstrate that treatment of A-549 tumor-bearing nude mice i.p. with CEP1612 resulted in inhibition of the proteasome activity in these tumors, accumulation of cyclin-dependent kinase inhibitors, induction of programmed cell death, and significant inhibition of tumor growth. However, despite induction of apoptosis, no tumor regression was observed, suggesting possibly that greater plasma levels may be required. Alternatively, a subpopulation of the tumor cells may be resistant to the proteasome inhibitor. Therefore, the long-term effect on tumors may be tumor growth delay rather than tumor regression. With regard to side effects, over the 31-day period of daily i.p. treatment, no overall gross toxicity was observed. Indeed, animals treated with CEP1612 (10 mg/Kg/day; 31 days) showed no weight loss, decreased activity, or anorexia. This is consistent with previous studies from An *et al.* (19) that demonstrated that in cultured cells, human cancer cells were sensitive whereas normal cells were resistant to CEP1612-induced apoptosis. However, our pharmacodynamic studies demonstrated that the proteasome in-

hibitor was able to reach several organs and inhibit their proteasome activity. Therefore, more detailed microscopic and macroscopic pathology studies are required to document the lack of toxicity of this proteasome inhibitor.

Recently, Orlowski *et al.* (25) and Adams *et al.* (26), using structurally unrelated molecules, also demonstrated the ability of proteasome inhibitors to inhibit tumor growth. Orlowski *et al.* (25) showed that treatment of Burkitt's lymphoma-bearing nude mice with Z-LLF-CHO inhibited tumor growth by 42%. Similarly, Adams *et al.* (26) showed that the dipeptide boronic acid PS-341 inhibited by 60% the growth in nude mice of the prostate cancer cell line PC-3. The fact that three structurally unrelated proteasome inhibitors can inhibit human tumor growth *in vivo* gives strong support for proof-of-concept of using proteasome inhibitors as novel anticancer drugs. The remaining challenge is to design more potent and selective proteasome inhibitors, with optimal pharmacokinetic profiles to suppress human tumor growth without toxicity in clinical settings.

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