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A New Structural Class of Proteasome Inhibitors that Prevent NF-κB Activation

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ABSTRACT. The multicatalytic proteinase or proteasome is a highly conserved cellular structure that is responsible for the ATP-dependent proteolysis of many proteins involved in important regulatory cellular processes. We have identified a novel class of inhibitors of the chymotrypsin-like proteolytic activity of the 20S proteasome that exhibit IC_{50} values ranging from 0.1 to 0.5 μg/mL (0.1 to 1 μM). In cell proliferation assays, these compounds inhibit growth with an IC_{50} ranging from 5 to 10 μg/mL (10–20 μM). A representative member of this class of inhibitors was tested in other biological assays. CVT-634 (5-methoxy-1-indanone-3-acetyl-leu-D-leu-1-indanylamide) prevented lipopolysaccharide (LPS), tumor necrosis factor (TNF)-, and phorbol ester-induced activation of nuclear factor κB (NF-κB) *in vitro* by preventing signal-induced degradation of IκB-α. In these studies, the IκB-α that accumulated was hyperphosphorylated, indicating that CVT-634 did not inhibit IκB-α kinase, the enzyme responsible for signal-induced phosphorylation of IκB-α. *In vivo* studies indicated that CVT-634 prevented LPS-induced TNF synthesis in a murine macrophage cell line. In addition, in mice pretreated with CVT-634 at 25 and 50 mg/kg and subsequently treated with LPS, serum TNF levels were significantly lower (225 ± 59 and 83 ± 41 pg/mL, respectively) than in those mice that were treated only with LPS (865 ± 282 pg/mL). These studies suggest that specific inhibition of the chymotrypsin-like activity of the proteasome is sufficient to prevent signal-induced NF-κB activation and that the proteasome is a novel target for the identification of agents that may be useful in the treatment of diseases whose etiology is dependent upon the activation of NF-κB. BIOCHEM PHARMACOL **55**;9:1391–1397, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. proteasome; NF-κB; inflammation; proteolysis; proliferation

The multicatalytic proteinase or proteasome is a highly conserved cellular structure that is responsible for the ATP-dependent proteolysis of most cellular proteins [1]. The 20S proteasome contains the catalytic core of the proteolytic complex and has been crystallized from the archaebacterium *Thermoplasma acidophilum* [2] and from yeast [3]. Unlike the archaebacterial proteasome, which primarily exhibits chymotrypsin-like proteolytic activity [2, 4, 5], the eukaryotic proteasome contains at least five identifiable proteolytic activities. Three of these activities are similar in specificity to chymotrypsin, trypsin, and peptidylglutamyl peptidase. The other two activities described exhibit a preference for the cleavage of peptide bonds on the carboxyl side of branched chain amino acids (BrAAP) and toward peptide bonds between short chain neutral amino acids (SnAAP) [6].

Although the 20S proteasome contains the proteolytic core, it cannot degrade proteins *in vivo* unless it is complexed with a 19S cap, at either end of its structure, which itself contains multiple ATPase activities. This larger structure is known as the 26S proteasome and will rapidly degrade proteins that have been targeted for degradation by the addition of multiple molecules of the 8.5-kDa polypeptide ubiquitin [reviewed in Ref. 1].

A large number of substrate-derived functionalities have been used as potential serine and thiol protease inhibitors. Several of these motifs have been described as inhibitors to the proteasome. These include the peptide aldehydes [7–9] ALLN¶ and *N*-acetyl-L-leucinyl-L-leucinyl-methional (LLM), with the most potent inhibitor of this type being *N*-carbobenzoxyl-L-leucinyl-L-leucinyl-L-norvalinal (MG115). Other reports have described a series of dipeptide inhibitors that have IC_{50} values in the 10–100 nM range [10]. A series of α-ketocarbonyl and boronic ester-derived dipeptides [11] and epoxyketones [12] that are potent inhibitors of the proteasome have also been described.

A different compound that exhibits specificity in inhibiting proteasome activity is lactacystin [13], which is a *Streptomyces* metabolite. This molecule was originally discovered for its ability to induce neurite outgrowth in a

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¶ *Abbreviations:* ALLN, *N*-acetyl-leucinyl-leucinyl-norleucinal; CVT-634(5-Methoxy-1-indanone-3-acetyl-leu-D-leu-1-indanylamide; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; TNF, tumor necrosis factor; and PMA, phorbol-12-myristate-13-acetate.

neuroblastoma cell line [14] and later was shown to inhibit the proliferation of several cell types [15].

It is now well established that the proteasome is a major extralysosomal proteolytic system that is involved in the proteolytic pathways essential for diverse cellular functions such as cell division, antigen processing, and the degradation of short-lived regulatory proteins such as oncogene products, cyclins, and transcription factors [16, 17]. For example, the active form of NF-κB is a heterodimer consisting of a p65 and a p50 subunit. The latter is present in the cytosol as an inactive precursor (p105). The proteolytic processing of p105 to generate p50 occurs via the ubiquitin-proteasome pathway. Additionally, processed p50 and p65 are maintained in the cytosol as an inactive complex bound to the inhibitory protein IκB. Inflammatory stimuli, such as LPS, activate NF-κB by initiating the signaling pathway that leads to the phosphorylation, ubiquitination, and ultimate degradation of IκB. These signals also stimulate the processing of p105 into p50. Hence, two proteolytic events, both controlled by the ubiquitin-proteasome pathway, are required for signal-induced activation of NF-κB.

The observation that ubiquitin-mediated proteasomal proteolysis plays a critical role in the activation of NF-κB could be exploited clinically by the use of inhibitors directed toward the proteasome. Abnormal activation of NF-κB followed by the stimulation of cytokine synthesis has been observed in a variety of inflammatory and infectious diseases. Activation of NF-κB is also essential for angiogenesis and for expression of adhesion molecules (CAMs and selectins); thus, proteasome inhibitors may also have utility in the treatment of diseases associated with the vascular system.

It is well documented that the ubiquitin-proteasome pathway is critical for the regulated destruction of cyclins that govern the exit from mitosis and allow cells to progress into the next phase of the cell cycle [18]. Thus, inhibiting the degradation of cyclins by using proteasome inhibitors causes growth arrest. Therefore, another potential utility of proteasome inhibitors is their use in the treatment of diseases that result from aberrant cell division.

Little is known at this time of the relative importance of the different proteolytic activities of the proteasome. Specific inhibition of the chymotrypsin-like activity of the proteasome by the peptide aldehyde Z-LLF-al appears sufficient to induce neurite outgrowth in a neuroblastoma cell line [19]. Thus, selective inhibition of a single peptidase activity, as opposed to general inhibition of the proteasome, appears sufficient to affect specific cellular processes. We report here the effects of a novel class of specific inhibitors of the chymotrypsin-like activity of the proteasome and show that these agents are capable of inhibiting cell proliferation and NF-κB activation, as demonstrated by suppression of LPS-induced TNF synthesis *in vitro* and *in vivo*.

MATERIALS AND METHODS

Synthesis of CVT-600 Series

Compounds reported here were synthesized using commercially available reagents and standard FMOC solid phase peptide conditions. A more detailed description of the preparation and structure–activity will be forthcoming [20].

Purification and In Vitro Assay *of the 20S Proteasome from Bovine Brain*

The 20S catalytic subunit complex of the proteasome was purified to homogeneity from the soluble fraction of bovine brain and assayed according to previously published methods [21]. The chymotrypsin-like activity of the complex was measured by monitoring the increase in fluorescence following cleavage of the substrate peptide succinyl-leucine-leucine-valine-tyrosine-7-amino-4-methyl coumarin (Calbiochem-Novabiochem). The standard *in vitro* assay contained 1 μg of purified 20S proteasome protein in 200 μL of reaction buffer (50 mM of HEPES, 0.1% sodium dodecyl sulfate, pH 7.5). The proteolytic reaction was initiated by the addition of 50 μM of fluorogenic peptide substrate and allowed to progress for 15 min at 37°. The reaction was terminated by the addition of 100 μL of 100 mM of acetate buffer, pH 4.0. The rate of proteolysis is directly proportional to the amount of liberated aminomethyl coumarin that was measured by fluorescent spectroscopy (EX 370 nm/EM 430 nm) using an Hitachi model F-2000 fluorescent spectrophotometer. The trypsin-like and peptidylglutamyl peptidase activities of the 20S complex [22] were assayed as described above using *N*-carbobenzoxyl-(*d*)-alanine-leucine-arginine-7-amino-4-methyl coumarin and *N*-carbobenzoxyl-leucine-leucine-glutamine-7-amino-4-methyl coumarin substrates, respectively. The BrAPP and SnAAP activities were assayed using a coupled enzyme system [22] with aminopeptidase (Sigma) and the substrates *N*-methoxysuccinyl-alanine-alanine-proline-valine-7-amino-4-methyl coumarin and *N*-succinyl-alanine-alanine-alanine valine-7-amino-4-methyl coumarin, respectively. Peptide substrates were custom synthesized by the SynPep Corp. ALLN was purchased from Sigma. Peptide substrates and inhibitor molecules were dissolved in DMSO such that the final concentration of solvent in the assay did not exceed 1%.

Calpain I Assay

The assay system in a total volume of 1.5 mL contained 100 mM of Tris buffer, pH 7.5, 5 mM of $CaCl_2$, 0.05% 2-mercaptoethanol, 0.2 units of calpain I (Sigma), 4 μg of fluorescein isothiocyanate (FITC)-casein and various amounts of CVT-634 (5-methoxy-l-indanone-3-acetyl-leu-D-leu-l-indanylamide). Proteolysis of FITC-casein was monitored fluorometrically (EM 513 nm/EX 490 nm). The details of this assay have been described elsewhere [23].

Cell Culture

The RAW 264.6 murine macrophage cell line used in these studies was purchased from the American Type Culture Collection and was maintained in RPMI-1640:Dulbecco's modified Eagle's medium (1:1, v/v) supplemented with 10% fetal bovine serum and penicillin/streptomycin (Life Technologies) at 37° in a tissue culture incubator.

Cell Proliferation Assay

To ascertain the effect of the proteasome inhibitors on cell growth, semiconfluent replicate cultures of RAW cells were treated with various amounts of the inhibitor. After 16 h, cell growth was monitored by using the CellTiter 96 nonradioactive cell proliferation assay (Promega). In this assay, viable cells reduce MTS (Owen's reagent) in the presence of an electron coupling reagent (phenazine methosulfate) to produce formazan. The absorbance of formazan is monitored colorimetrically. The absorbance of the cell cultures at 490 nm is directly proportional to the number of viable cells in the culture.

Preparation of Cell Extracts

Monolayers of RAW cells were treated with the test compound for 1 hr prior to exposure to LPS (100 ng/mL; Sigma), TNF (10 ng/mL; Calbiochem), or PMA (10 ng/mL; Calbiochem) for the indicated time. The monolayers were washed three times with phosphate-buffered saline. Cytosolic and nuclear extracts were prepared using the high-salt buffer extraction procedure as described [24].

Immunoblots

Cytosolic extracts (5 μg of protein) were separated by electrophoresis using 10% SDS-polyacrylamide gel and a Mini-Protean II gel electrophoresis unit (Bio–Rad). To achieve sufficient resolution to separate hypo- and hyperphosphorylated forms of IκB-α, it was necessary to pour the gel using a freshly prepared acrylamide solution. Following separation, proteins were transferred to nitrocellulose using a Mini-Protean II transfer unit. Immunoblots were probed with IκB (MAD 3) (Santa Cruz Biotechnology) antibody and developed with the BM Chemiluminescence Western Blotting Kit (Boehringer Mannheim).

Electrophoretic Mobility Shift Assay

Ten picomoles of double-stranded NF-κB (5′-AGTTGAG GGGACTTTCCCAGGC-3′) or OCT-1 (5′-TGTCGAA TGCAAATCACTAGAA-3′) consensus oligonucleotide (Promega Corp.) was 5′ end labeled with 5 μCi of [γ-^{32}P]ATP (>5000 Ci/mmol, New England Nuclear Corp. by incubation with T4 polynucleotide kinase for 1 hr at 37°. Unincorporated nucleotides were removed by passing the reaction mixture over a 1-mL Sephadex G-50 spin column. Binding assays were performed at room temperature for 30 min and consisted of 5 μg of nuclear extract protein, 1 μg of salmon sperm DNA, and 5×10^4 cpm of ^{32}P-labeled consensus oligonucleotide in the presence and absence of 20-fold unlabeled oligonucleotide. DNA–protein complexes were resolved by 8% nondenaturing polyacrylamide gel electrophoresis, and the gels were dried onto filter paper and visualized by autoradiography.

LPS-induced TNF synthesis

The effect of CVT-634 on TNF synthesis by RAW cells was examined. Replicate cultures of RAW cells were treated with the indicated amount of CVT-634 for 1 hr prior to the addition of LPS (100 ng/mL). Ninety minutes after LPS addition, the spent medium was assayed for TNF using ELISA (BioSource International).

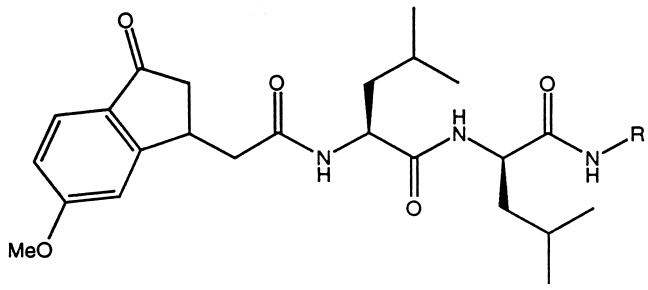
Animal Studies

The effect of CVT-634 on TNF synthesis in mice treated with LPS was also determined. Female Swiss Webster mice (30–35 g, Simonsen Laboratories, $N = 5$/group) were treated (i.p.) with CVT-634 dissolved in 0.2 mL of polyethylene glycol 300 containing 1% DMSO; the control group received vehicle alone. One hour later, all animals were treated (i.p.) with 0.2 mL of saline containing LPS (1 mg/kg) and D-galactosamine (60 mg/kg) [25]. Blood was collected from each group after 90 min by retro-orbital bleeding and then centrifuged; the plasma was assayed for TNF by ELISA (BioSource International). All animals were housed in the Animal Care Facility at CV Therapeutics, Inc., and all studies were conducted in accordance with National Institutes of Health guidelines using protocols that were approved by the CV Therapeutics, Inc. Animal Welfare Committee.

RESULTS AND DISCUSSION

The structures of the CVT-600 series of proteasome inhibitors are shown in Fig. 1. The core structure is dipeptide based and uses a 5-methoxy-1-indanone-3-acetamide as a "head group." Unlike the aldehydes and trifluoromethyl ketone groups present in other proteasome inhibitors, indanones are not known to be hydrating transition state mimetics and, therefore, represent a unique motif for the development of serine and threonine protease inhibitors.

The effects of these compounds on the chymotrypsin-like activity of the 20S proteasome are shown in Fig. 2a. The IC_{50} values for these inhibitors ranged between 0.1 and 0.5 μg/mL (0.1 to 1 μM). The effects of the CVT-600 series of inhibitors on the other four 20S proteolytic activities [22] were also examined (Fig. 2b). Included in this analysis is ALLN, which has been reported, and is shown here, to be a potent inhibitor of both the chymotrypsin and peptidyl-glutamyl peptidase activities and a weaker inhibitor of the trypsin-like activity of the 20S proteasome [9]. Addition-

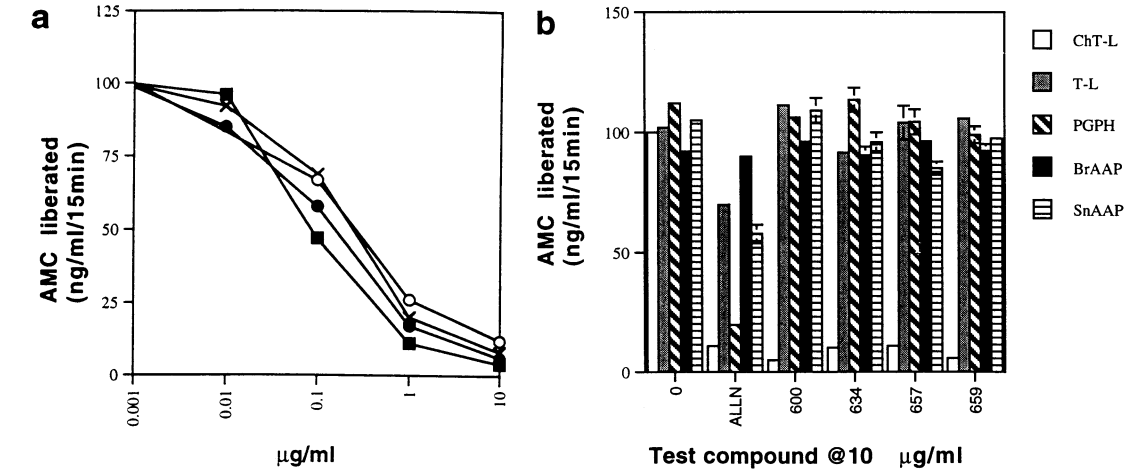


CVT Number	R
600	Benzyl
634	1-indanyl
657	piperonyl
659	2,6-difluorobenzyl

FIG. 1. Structures of the CVT-600 series of 20S proteasome inhibitors.

ally, ALLN was shown in the present study to have weak inhibitory activity (60% inhibition at 10 μg/mL) against SnAAP and was inactive against BrAAP. Figure 2b also shows that when the CVT-600 series of compounds were tested at concentrations (10 μg/mL) that inhibited the 20S proteasome chymotrypsin-like activity by greater than 90%, the other four 20S activities were unaffected. Figure 2c shows that, unlike ALLN, which inhibits the calcium-dependent cysteine protease (calpain I), representative members of the series, CVT-600 and CVT-634, were inactive against this enzyme (Fig. 2c). Thus, the CVT-600 series of proteasome inhibitors exhibit an inhibitory profile that is quite different from ALLN, a widely used proteasome inhibitor. Unlike ALLN, the CVT 600 series of compounds have no effect on the calcium-dependent cysteine protease calpain I, and, in addition, these compounds are specific inhibitors of the chymotryptic activity of the 20S proteasome. This difference in biological profile can be useful to elucidate the relative importance of the different proteolytic activities of the 20S proteasome upon various biological processes.

A property associated with known proteasome inhibitors is their ability to induce growth arrest. The CVT-600 series of compounds were evaluated for their effect on the growth of RAW cells, a murine macrophage cell line. The IC_{50}



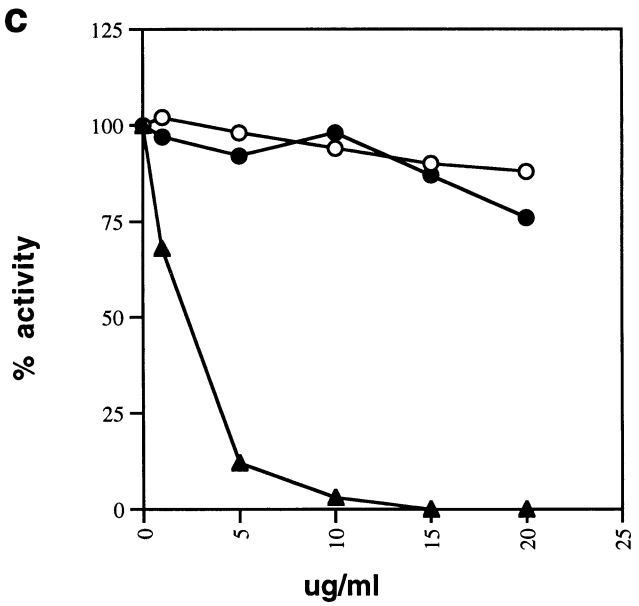


FIG. 2. *In vitro* inhibition of bovine brain 20S proteasome. Test compounds were dissolved in DMSO and included in the *in vitro* 20S proteasome assay, as described in Materials and Methods. The concentration of DMSO in the assay did not exceed 1%. AMC = 7-amino-4-methyl coumarin. (a) Inhibition curves against the chymotrypsin-like activity for CVT-600 (●), CVT-634 (○), CVT-657 (X), and CVT-659 (■). Each value is the average of three analyses. (b) Effects of the CVT-600 compound series and ALLN tested against the chymotrypsin-like (open bars), trypsin-like (gray bars), peptidylglutamyl peptidase (cross hatched bars), BrAAP (closed bars), and the SnAAP (horizontal hatched bars) activities of the 20S proteasome at a single concentration (10 μg/mL). Values are means ± SD, N = 3. (c) Effect of ALLN (▲), CVT-600 (●), and CVT-634 (○) on the *in vitro* activity of calpain I by measuring the change in fluorescence (EX 490 nm, EM 513 nm) with time [23]. One hundred percent activity corresponded to 1.8 fluorescence units/min/unit calpain I. Each value is the average of three analyses.

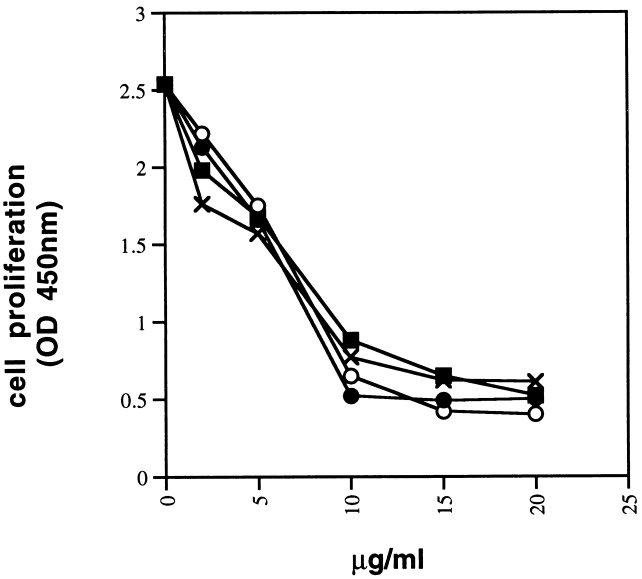


FIG. 3. Inhibition of macrophage cell proliferation. Monolayers of RAW 264.6 cells were exposed to the indicated concentration of CVT-600 compounds for 16 hr prior to the cell proliferation assay, as described in Materials and Methods. Key: CVT-600 (●), CVT-634 (○), CVT-657 (X), and CVT-659 (■). Each value is the average of three analyses.

values for inhibition of cell growth ranged from 5–10 μg/mL (10–20 μM; Fig. 3). The effects of CVT-634 and CVT-600 (representative members of the CVT-600 series) on the growth of a variety of human tumor cells were also examined. The IC_{50} values were dependent upon the cell type. CVT-600 had IC_{50} values of 17, 10, 8, and 17 μg/mL against CaCo2 (human colon carcinoma), MCF7 (human breast carcinoma), OVCAR (human ovarian carcinoma), and PANC-1 (human tk;1pancreatic carcinoma), respectively, while CVT-634 was slightly more potent, exhibiting IC_{50} values of 8, 6, 5, and 7 μg/mL, respectively, against the same cell types.

Earlier studies from this laboratory [26] have shown that proteasome inhibitors such as ALLN can prevent NF-κB activation by inhibiting the proteolytic processing of p105 and inhibiting IκB degradation. The effect of CVT-634 on NF-κB activation was investigated. Cytosolic and nuclear extracts were prepared from RAW cells that had been incubated in the presence of CVT-634 prior to exposure to a variety of stimuli. Figure 4a shows an immunoblot analysis of cytosolic extracts of RAW cells that had been treated with either LPS (100 ng/mL), TNF (10 ng/mL), or PMA (10 ng/mL) for 20 min with or without preincubation with CVT-634 (5 μg/mL, 1 hr). Figure 4a shows that in the

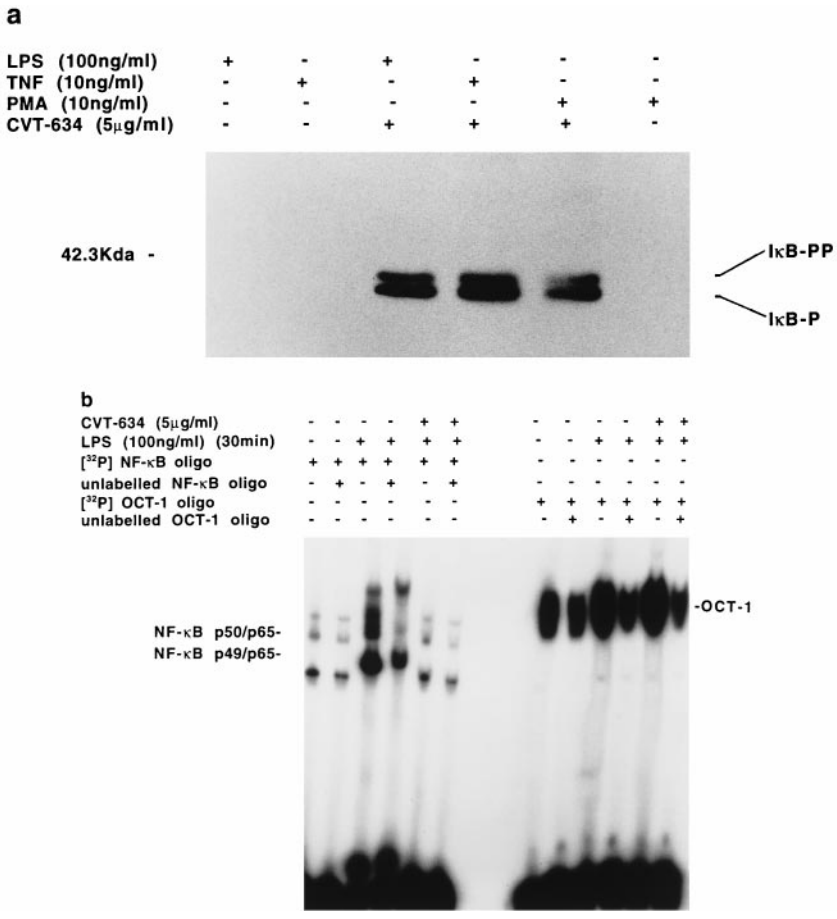


FIG. 4. Inhibition of LPS-induced NF-κB activation. (a) RAW cells were incubated for 1 hr in the presence or absence of 5 μg/mL of CVT-634 and then were treated for 20 min with either LPS (100 ng/mL), TNF (10 ng/mL), or PMA (10 ng/mL). Cytosolic extracts (5 μg of protein) were prepared and analyzed by immunoblot with antibody directed toward IκB. (b) For the electrophoretic mobility shift assay, nuclear extracts were prepared from RAW cells that had been incubated for 1 hr in the presence or absence of 5 μg/mL of CVT-634 and then were treated for 30 min with LPS (100 ng/mL). Binding reactions contained nuclear extract (5 μg of protein) and NF-κB or OCT-1 consensus oligonucleotides.

absence CVT-634 no IκB protein is detected by immunoblot, while monolayers of RAW cells that had been preexposed to CVT-634 had clearly detectable levels of hypo- and hyperphosphorylated IκB, irrespective of the source of stimulation. Hence, CVT-634 prevents the degradation of IκB in cells that have been exposed to LPS, TNF, and PMA, but does not interfere with the phosphorylation of IκB, which is a prerequisite for signal-induced ubiquitin-mediated IκB degradation [17].

To ascertain the effect of CVT-634 on the nuclear accumulation of NF-κB, an electrophoretic mobility shift assay was performed. As shown in Fig. 4b, NF-κB was present in the nuclear extracts of those cells that were stimulated with LPS. When unlabeled NF-κB consensus oligonucleotide was added to the binding assay, the radioactivity associated with this band was reduced. Nuclear extracts prepared from either unstimulated or stimulated cells that had been preexposed to CVT-634 contained much less NF-κB (Fig. 4b). The observations in Fig. 4 are consistent with earlier results [17, 26] and confirm that the activation of NF-κB occurs via the proteasome pathway. Additionally, we conclude that specific inhibition of the chymotrypsin-like activity of the proteasome is sufficient to prevent LPS-induced NF-κB activation.

Since the induction of TNF synthesis in macrophages by LPS is dependent upon NF-κB activation, the effect of CVT-634 on TNF synthesis was examined. Results shown in Fig. 5a indicate a concentration-dependent inhibition of TNF synthesis in LPS-stimulated RAW cells that had been pretreated with CVT-634. The IC_{50} for inhibition of TNF synthesis was 7 μg/mL for CVT-634. Earlier studies have shown that the IC_{50} for ALLN in this assay is approximately 40 μg/mL [26]. Thus, CVT-634 is approximately 5-fold more potent than ALLN in inhibiting LPS-dependent TNF synthesis.

Finally, the effect of CVT-634 on TNF synthesis in mice treated with LPS was investigated. Results shown in Fig. 5b indicate that in mice pretreated with CVT-634 at 25 and 50 mg/kg, circulating TNF levels were markedly lower than in control mice that had been treated with LPS alone. In this assay, CVT-634 is approximately 2-fold more potent than ALLN [26].

In summary, the novel indanone-substituted dipeptides described here are specific inhibitors of the chymotrypsin-like activity of the proteasome and may be useful in biological studies where specific inhibition of this activity of the proteasome is desirable. We are continuing the chemistry effort to fully understand the structure–activity relationship and how it may translate into small molecule mimetics to give specific inhibitors of proteasome proteolysis. In addition, results reported here support the view that targeting the proteasome may be a viable approach for preventing NF-κB activation and that inhibitors of proteasome proteolysis may be useful in the treatment of diseases that are induced by aberrant NF-κB activation.

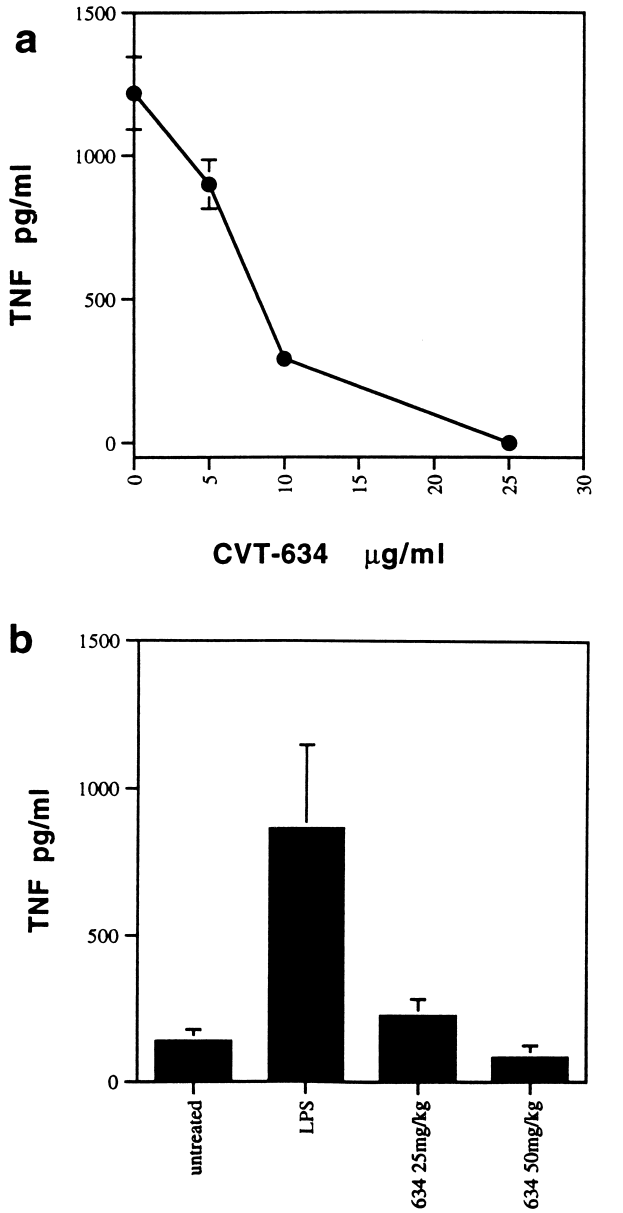


FIG. 5. Inhibition of LPS-induced TNF synthesis. RAW cells were incubated for 1 hr in the presence of the indicated concentration of CVT-634 and then were treated for 90 min with LPS (100 ng/mL). (a) Cell culture supernatants were harvested and analyzed for TNF content by ELISA according to the manufacturer's instructions. (b) For *in vivo* analysis, mice were treated with vehicle or CVT-634 at 25 or 50 mg/kg by i.p. injection 1 hr prior to the administration of LPS and D-galactosamine, as described in Materials and Methods. Plasma was prepared from blood samples harvested via retro-orbital bleed, and the level of TNF was determined by ELISA. Values are means ± SD of three analyses.

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