FEBS Letters 413 (1997) 354–358 FEBS 19078

Cyclosporine A is an uncompetitive inhibitor of proteasome activity and prevents NF-κB activation

Stephanie Meyer, N. Gail Kohler, Alison Joly*

CV Therapeutics, Inc., 3172 Porter Drive, Palo Alto, CA 94304, USA

Received 4 June 1997

Abstract Cyclosporine A is an immunosuppressive agent that is used clinically in the prevention of transplant rejection and development of graft-versus-host disease. Recently, cyclosporine A has been shown to possess anti-inflammatory properties and is capable of inhibiting lipopolysaccharide-induced NF-κB activation. Ubiquitin-mediated proteasomal proteolysis plays a critical role in signal-induced NF-κB activation since it regulates both IκB degradation and p105 processing, it is also involved in the production of peptides for the assembly of MHC class I molecules. We report here that cylcosporine A acts as an uncompetitive inhibitor of the chymotrypsin-like activity of the 20S proteasome in vitro and that it suppresses lipopolysaccharide-induced IκB degradation and p105 processing in vivo demonstrating that inhibition of proteasome proteolysis is the mechanism by which cyclosporine A prevents NF-κB activation. A structurally unrelated immunosuppressant, rapamycin, did not inhibit the 20S proteasome in vitro.

Key words: Proteasome proteolysis; NF-κB activation; Cyclosporine A; Immunosuppressant; Inflammation

1. Introduction

The multicatalytic proteinase, or the 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 complex and has been crystallized from the archaebacteria *Thermoplasma acidophilum* [2]. The archaebacterial proteasome contains 14 copies of two distinct types of subunits, α and β, which form a cylindrical structure consisting of four stacked rings. The top and bottom rings contain seven α subunits each whilst the inner rings contain seven β subunits. A pore extends through the middle of the structure and contains the proteolytic active sites and proteins destined for degradation pass through this channel. The eukaryotic 20S proteasome is structurally similar to its bacterial counterpart; however, a major distinction between the bacterial and mammalian systems is related to its catalytic activities. Unlike the archaebacterial proteasome that primarily exhibits chymotrypsin-like proteolytic activity [3,4], the eukaryotic proteasome contains at least five identifiable protease activities. Three of these activities are similar in specificity to chymotrypsin, trypsin and peptidylglutamyl peptidase. Two other activities have also been described, one exhibiting a preference for cleavage of peptide bonds on the carboxyl side of branched chain amino acids and the other toward peptide bonds between short-chain neutral amino acids [5].

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 bigger 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 [1]).

Two types of inhibitors that inhibit the proteolytic activity of the proteasome have been described. Certain peptide aldehydes have been reported that inhibit the chymotrypsin-like activity of the proteasome [6–8]. These are *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN) and a closely related compound, *N*-acetyl-L-leucinyl-L-leucinyl-methional (LLM) with a K_i of 0.14 mM. The most potent inhibitor of this type is a structurally related compound, *N*-carbobenzoxyl-L-leucinyl-L-leucinyl-L-norvalinal (MG115), which exhibits a K_i of 0.021 mM. Although these peptide aldehydes are most effective against the chymotrypsin-like proteolytic activity of the proteasome, other studies have shown that they are non-specific protease inhibitors. More recent reports have described a series of potent dipeptide inhibitors that have IC_{50} values in the range of 10–100 nM range [9]. A series of α-ketocarbonyl and boronic ester derived dipeptides [10] have been described that are also potent inhibitors of the proteasome.

Another report describes a different class of compounds that exhibit specificity in inhibiting proteasome activity [11]. Lactacystin is a *Streptomyces* metabolite that specifically inhibits the proteolytic activity of the proteasome complex. This molecule was originally discovered for its ability to induce neurite outgrowth in a neuroblastoma cell line [12] later it was shown to inhibit the proliferation of several cell types [13]. By using radiolabelled lactacystin binding studies [14] have shown that lactacystin binds irreversibly to a threonine residue located at the amino terminus of the β subunit of proteasomes.

It is now well established that the proteasome is a major extralysosomal proteolytic system which is involved in the proteolytic pathways essential for diverse cellular functions [8,14,15]. 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 signals such as lipopolysaccharide (LPS) activate NF-κB by

*Corresponding author. Fax: (415) 858-0390

Abbreviations: ALLN, *N*-acetyl-leucinyl-leucinyl-norleucinal; CsA, cyclosporine A; LPS, bacterial lipopolysaccharide; MHC, major histocompatability complex; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TNF, tumor necrosis factor

PII S0014-5793(97)00930-7

initiating the signalling pathway leading to the degradation of IκB. These signals can also stimulate the processing of p105 into p50. Another important function of proteasomal proteolysis is to generate small peptides that are presented to T-lymphocytes which initiate immune responses. Inhibitors of the proteasome have been shown to inhibit the proteolytic processes that generate these peptides and thus prevent the assembly of MHC class I molecules [8].

Cyclosporine A (CsA) inhibits T-cell responses and is used as an immunosuppressive agent in organ transplantation and in preventing graft-versus-host disease. Mechanistic studies have shown that CsA binds to the intracellular protein, cyclophilin, and this binary complex inhibits the activity of calcineurin, a calcium- and calmodulin-dependent serine/threonine phosphatase [16]. Calcineurin is required for activation of the transcription factor, NF-AT, that binds to the IL-2 promotor and activates its transcription. Inhibition of calcineurin activity results in the inhibition of *IL-2* gene activation in T-cells [16].

Anti-inflammatory effects of CsA have also been described [17]. Using animal models of IgE-dependent cutaneous inflammation, it has been shown that CsA inhibits the synthesis of IgE-dependent TNF synthesis by mast cells. A recent report [18] describes the inhibitory effect of CsA upon LPS-induced NF-κB activation and tissue factor expression in human peripheral blood mononuclear cells. Due to the integral role that the proteasome plays in NF-κB activation and the peptide nature of CsA we decided to explore the possibility that CsA exherts its anti-inflammatory action, at least in part, by inhibition of proteasome proteolysis. We report here that CsA acts as an uncompetitive inhibitor of the chymotrypsin-like activity of the 20S proteasome in vitro and that it acts to suppress LPS-induced IκB degradation and p105 processing in vivo thereby preventing NF-κB activation. A structurally unrelated immunosuppressant, rapamycin, did not inhibit the 20S proteasome in vitro. These results support the view that the anti-inflammatory properties of CsA may, at least in part, be due to inhibition of the chymotryptic activity associated with the proteasome.

2. Materials and methods

2.1. 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 [19]. The chymotryptic-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 purified 20S proteasome protein in 200 μl of reaction buffer (50 mM HEPES, 0.1% sodium dodecyl sulfate, pH 7.5). The proteolytic reaction was initiated by the addition of 50 μM fluorogenic peptide substrate and allowed to progress for 15 min at 37°C. The reaction was terminated by the addition of 100 μl of 100 mM acetate buffer, pH 4.0. The rate of proteolysis is directly proportional to the amount of liberated aminomethyl coumarin which was measured by fluorescent spectroscopy (EX_{370nm}/EM_{430nm}) using an Hitachi model F-2000 fluorescent spectrophotomer. The tryptic-like and peptidylglutamyl peptidase activities of the 20S complex [20] were assayed as described above using *N*-carbobenzoxyl-(D)-alanine-leucine-arginine- tyrosine-7-amino-4-methyl coumarin and *N*-carbobenzoxyl-leucine-leucine-glutamine-tyrosine-7-amino-4-methyl coumarin substrates respectively. Peptide substrates were custom synthesized by SynPep Corp. (Dublin, CA). CsA and rapamycin (Sigma) were dissolved in methanol, *N*-acetyl-leucinyl-leucinyl-norleucinal (Sigma) was dissolved in dimethylsulfoxide.

2.2. Cell culture

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

Monolayers of RAW cells were pretreated with CsA (25 μg/ml) for 1 h prior to exposure to bacterial LPS serotype 0111:B4 (100 ng/ml) (Sigma). One hour after LPS exposure, the monolayers were washed 3 times with phosphate-buffered saline. Whole-cell lysates for Western blot analysis were prepared by lysing the cell monolayer with RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 100 μg/ml PMSF, 3 U/ml aprotinin, 1 mM sodium orthovanadate) (Sigma). Nuclear extracts were prepared using the high-salt buffer extraction procedure as described [21].

2.3. Western blots

Cell lysates (10 μg protein) for were first subjected to electrophoresis using a 10–20% gradient readygel and a mini-protean II gel electrophoresis unit (Bio-Rad). Following separation, proteins were transferred to nitrocellulose using a mini-protean II transfer unit. Immunoblots were probed with either IκB (MAD 3) or NF-κB p50 (Santa Cruz Biotechnology) antibody and developed with the BM Chemilunimescence Western Blotting Kit (Boehringer Mannheim).

2.4. Electrophoretic mobility shift assay

10 pmol of either double-stranded NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) or OCT-1 (5′-TGTCGATCGGGGCGGGGCGAGC-3′) consensus oligonucleotide (Promega) was 5′-end labeled with 5 μCi [γ-^{32}P]ATP (> 5000 Ci/mmol, New England Nuclear Corp, Boston, MA) by incubation with T4 polynucleotide kinase for 1 h at 37°C. 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 1 h and consisted of 10 μg nuclear extract protein, 1 μg salmon sperm DNA, and 5×10^4 cpm of ^{32}P-labeled consensus oligonucleotide in the presence and absence of 50-fold unlabeled oligonucleotide. DNA–protein complexes were resolved by 8% non-denaturing polyacrylamide gel electrophoresis, the gels were dried onto filter paper and visualized by autoradiography.

2.5. LPS-induced TNF synthesis

The effect of CsA on TNF synthesis by RAW cells was examined. Triplicate cultures of RAW cells were pretreated with varing amounts of CsA for 1 h prior to the addition of LPS (100 ng/ml). One hour after LPS addition, the spent medium was assayed for TNF using ELISA (Genzyme).

3. Results and discussion

We have attempted to investigate a potential additional mechanism of action of the immunosuppressant, CsA since this agent is capable of inhibiting signal-induced NF-κB activation [18]. Due to the integral role that the proteasome plays in NF-κB we explored the possibility that CsA exherts its anti-inflammatory action, in part, by inhibition of proteasome proteolysis. Since the proteasome is also involved in the production of peptides for antigen presentation on MHC class I molecules [8] it was attractive to postulate an additional mechanism for the immunosuppressive properties of CsA. CsA is a cyclic undecapeptide and many of the previously described inhibitors of proteasome proteolysis are themselves peptide in nature.

We first asked whether CsA was capable of inhibiting the 20S proteasome proteolytic activities in vitro. Fig. 1a shows the effect of CsA upon three of the major proteolytic activites of the 20S proteasome. It was found that at the two concentrations tested (10 and 50 μg/ml) CsA had no effect upon

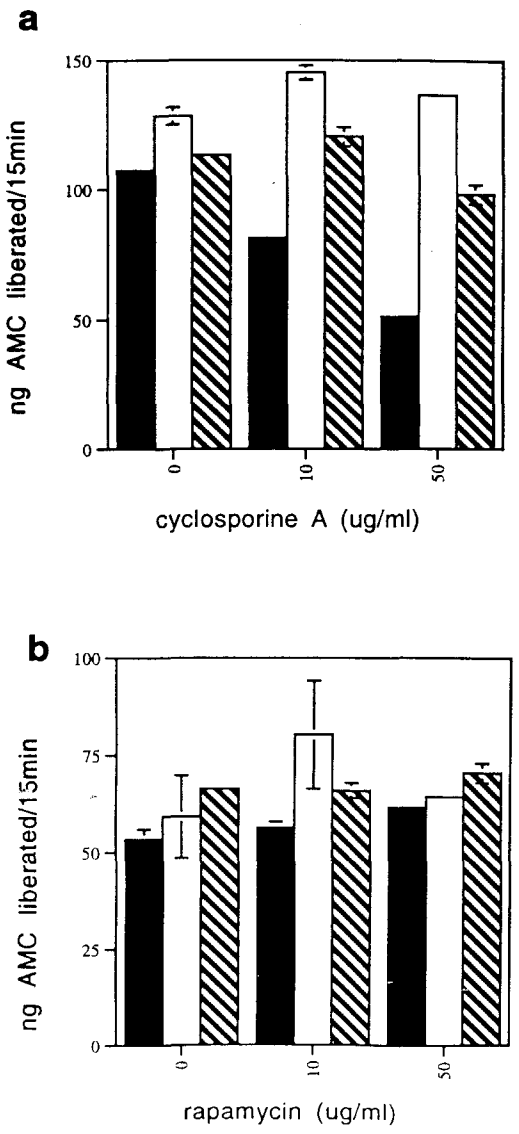

Fig. 1. Effect of CsA and rapamycin on 20S proteasome activity. The effect of (a) CsA and (b) rapamycin on the three major 20S proteasome activities were investigated at 0, 10 and 50 μg/ml. Chymotryptic (solid bars), tryptic (open bars) and peptidylglutamyl peptidase (hatched bars). Assays were performed as described in Section 2.

either the tryptic or the peptidylglutamyl peptidase activities of the 20S proteasome; however, CsA did inhibit the chymotryptic activity of the 20S proteasome in vitro with an IC_{50} of 50 μg/ml. Fig. 1b shows the same analysis performed using rapamycin an immunosuppressant which is structurally unrelated to CsA showing no inhibition of the 20S proteasome activities.

Fig. 2 shows the double reciprocal (Lineweaver-Burk) plot of $1/v_0$ versus 1/[S] for the 20S chymotryptic assay in the absence or presence of CsA at 10 and 50 μg/ml. The appearance of parallel lines in this plot is characteristic of an uncompetitive enzyme inhibitor whereby the presence of the inhibitor increases the intercept on the $1/v_0$ ordinate without changing the slope of the lines.

Next we wanted to investigate the contributing role of inhibition of the chymotryptic activity of the 20S proteasome in signal-induced NF-κB activation cells. Fig. 3a shows an immunoblot, probed with antibodies directed toward IκB, of whole-cell lysates which had been pretreated with or without CsA or a known inhibitor of the 20S proteasome, *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) for 1 h prior to the administration of LPS (100 ng/ml) to the cultures. In the absence of CsA or ALLN no detectable IκB is present whilst addition of either CsA or ALLN is seen to prevent the LPS-induced degradation of IκB since IκB is clearly detectable as the major immunoreactive band migrating with an apparent molecular weight of 43 kDa. An extended exposure of the autoradiogram, as shown here, also reveals the presence of high molecular weight immunoreactive species in the presence of the inhibitor molecules corresponding to the ubiquitinated forms of IκB indicating that the target of inhibition, in this case, is with proteasomal proteolysis and not with the signal-induced phosphorylation and ubiquitination of IκB. Fig. 3b shows an immunoblot, with antibodies directed toward the p50 subunit of NF-κB. Cells that were exposed to LPS alone clearly show the presence of both p50 and its high molecular weight precursor, p105, at both protein loading concentrations whilst those cells that had been exposed to CsA contained quantitatively more p105 than p50 indicating that Csa had inhibited the signal-induced proteasome processing of p105 into p50. Fig. 3c shows a gel mobility shift assay using nuclear extracts that had been prepared from cells that had been pretreated in the presence and absence of CsA for 1 h prior to the administration of LPS. In cells exposed to LPS alone, NF-κB binding activity is readily demonstrated. Specificity of this binding activity is demonstrated by competition with excess cold NF-κB consensus oligonucleotide in the binding reactions and inclusion of an NF-κB p50 subunit antibody which causes the specific binding activity to shift to a higher molecular

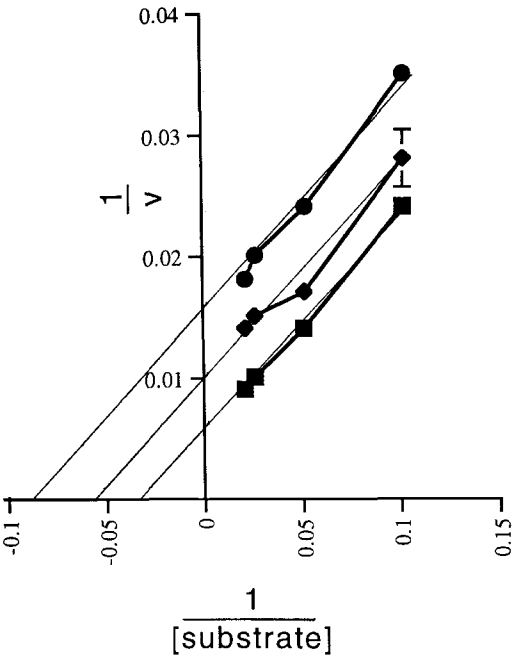

Fig. 2. CsA is an uncompetitive inhibitor of the chymotrypsin-like activity of the 20S proteasome. Double reciprocal Lineweaver-Burke plots were used to determine the inhibition characteristics of CsA on the chymotrypsin-like activity of the 20S proteasome. Assays were performed using variable substrate concentrations (10–50 μM) with either no (■), 10 μg/ml (♦) or 50 μg/ml (●) CsA as described in Section 2.

weight migrating species. In nuclear extracts prepared from cells that had been exposed to CsA prior to LPS, very little nuclear NF-κB binding activity could be demonstrated. Control electrophoretic mobility shift assays for both nuclear extracts using the OCT-1 consensus oligonucleotide demonstrate uniformity of protein content in the binding reactions. Taken together these data suggest that CsA inhibits NF-κB activation by directly inhibiting the proteolytic processes involved in signal-induced activation and translocation of NF-κB to the nucleus.

Many studies have shown that NF-κB is involved in the regulation of genes that are involved in inflammatory and immune responses including the synthesis of proinflammatory cytokines such as TNF, IL-1 and IL-8 [22]. Since the data shown in Fig. 3 indicate that CsA inhibits NF-κB activation, the effect of this drug on TNF synthesis by LPS stimulated

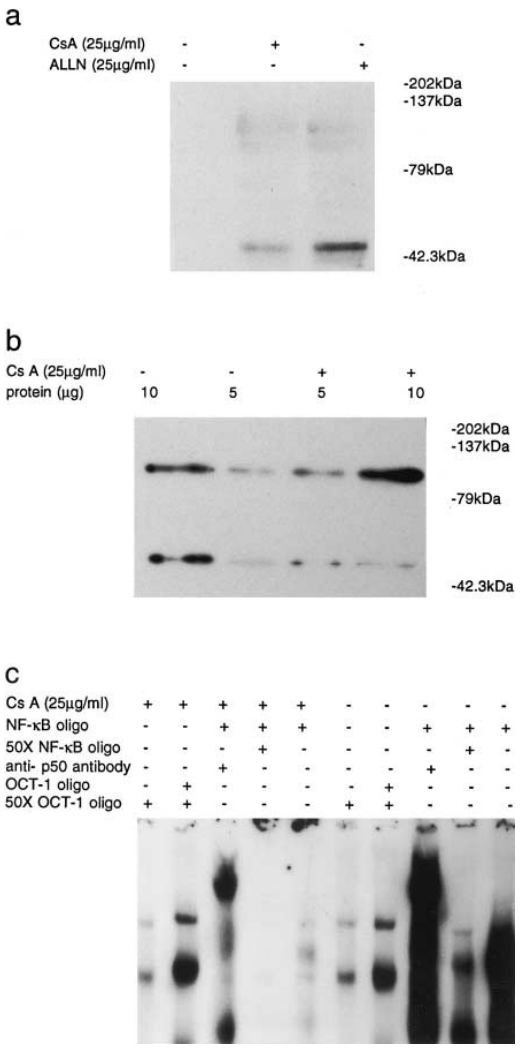

Fig. 3. Inhibition of LPS-induced proteasomal proteolysis. RAW cels were incubated for 1 h in the presence and absence of 25 μg/ml CsA or 25 μg/ml ALLN then treated for 15 min with LPS (100 ng/ml). Immunoblots of cell lysate protein were prepared and analysed with antibodies directed toward (a) IκB (10 μg protein) and (b) p50 (5 or 10 μg protein). For gel mobility shift assay (c) nuclear extracts and binding reactions were prepared from cell monolayers that had been incubated for 1 h in the presence and absence of CsA (25 μg/ml) then treated for 30 min with LPS (100 ng/ml) as described in Section 2.

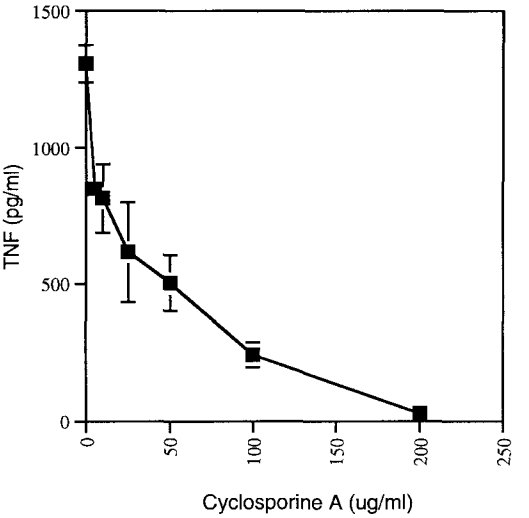

Fig. 4. Inhibition of LPS-induced TNF synthesis by CsA. RAW cells were incubated for 1 h in the presence of the indicated concentration of CsA then treated for 1 h with LPS (100 ng/ml). Cell culture supernatants were harvested and analysed for TNF content by ELISA according to the manufacturers instructions.

RAW cells was examined. Fig. 4 shows that TNF synthesis was inhibited by CsA in a dose-dependent manner and the IC_{50} for CsA was approximately 25 μg/ml.

Since CsA exhibits anti-inflammatory properties and can inhibit NF-κB activation and TNF synthesis in mast cells stimulated with IgE [17], we have attempted to investigate its mechanism of action in this respect. Chymotryptic activity associated with the proteasome is inhibited by CsA and consistent with this observation, activation of NF-κB is prevented by CsA. Thus, in addition to the inhibitory effect of CsA on T-cell responses to antigen, this imunosuppressive agent also inhibits proteasome activity and thereby prevents NF-κB activation in stimulated cels. Expression of adhesion molecules (CAMs, selectins) and tissue factor, synthesis of inflammatory cytokines (IL-1, TNF, IL-8, etc.) are all dependent on NF-κB activation. These proteins are an integral part of the inflammatory process and it is tempting to speculate that inhibiton of proteasome-dependent proteolysis by CsA is responsible, at least in part, for the anti-inflammatory effects of this drug.

It should be noted that the concentration of CsA required to inhibit proteasome-dependent proteolysis in vitro (IC_{50} = 50 μg/ml) appears to be higher than that required to see an effect upon LPS-induced proteasome proteolysis (25 μg/ml) and NF-κB activation in the cell based assays described here. TNF synthesis by mast cells stimulated with IgE was suppressed by CsA and the IC_{50} for inhibition was approximately 1 μg/ml [17]. In our studies with LPS stimulated RAW cells, the IC_{50} for inhibition of TNF synthesis was approximately 10 μg/ml. Thus depending on the cell source and the stimulant used, concentrations of CsA required to inhibit a specific response can vary. The concentration of CsA needed to inhibit IL-2 synthesis by a T-cell hybridoma that is stimulated with ionomycin and phorbol ester is approximately 1 ng/ml [23]. In human organ transplantation, peak blood levels needed to prevent transplant rejection are estimated at 2.5 μg/ml when the dose of intravenous CsA used was 2.7 mg/kg. When an oral dose is used (4.5 mg/kg), sustained peak blood levels ranged between 1 and 1.5 μg/ml [24]. Thus it appears that

the dose of CsA required to inhibit the chymotryptic activity of the proteasome is considerably higher than that required for transplant rejection. CsA is extremely hydrophobic and its bioavailability in vivo when bound to lipoproteins and to other binding proteins may be different. In the in vitro assay used to measure effects on proteasome activity, these in vivo factors are absent and this may account for the higher values needed to inhibit the in vitro proteolytic activity.

Acknowledgements: We thank Drs. S. Kerwar and M. Wick for their support and critical reading of the manuscript.

References

[1] Coux, O., Tanaka, K. and Goldberg, A. (1996) Ann. Rev. Biochem. 65, 801–847.
[2] Lowe, J., Stock, D., Jap, B., Zwickl, P., Baumeister, W. and Huber, R. (1995) Science 268, 533–539.
[3] Dahlmann, B., Kopp, F., Kuehn, L., Niedel, B. and Pfeifer, G. (1989) FEBS Lett. 251, 125–131.
[4] Seemuller, E., Lupas, A., Zuhl, F., Zwickl, P. and Baumeister, W. (1995) FEBS Lett. 359, 173–178.
[5] Orlowski, M. (1990) Biochemistry 29, 10289–10297.
[6] Vinitsky, A., Michaud, C., Powers, J. and Orlowski, M. (1992) Biochemistry 31, 9421–9428.
[7] Tsubuki, S., Hiroshi, K., Saito, Y., Miyashita, N., Inomata, M. and Kawashima, S. (1993) Biochem. Biophys. Res. Commun. 196, 1191–1201.
[8] Rock, K.I., Gram, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D. and Goldberg, A.L. (1994) Cell 78, 761–771.
[9] Iqbal, M., Chatterjee, S., Kauer, J.C., Das, M., Messina, P., Freed, B., Biazzo, W. and Siman, R. (1995) J. Med. Chem. 38, 2276–2277.
[10] Iqbal, M., Chatterjee, S., Kauer, J.C., Mallamo, J.P., Messina, P.A., Reiboldt, A. and Siman, R. (1996) Bioorg. Med. Chem. Lett. 6, 287–290.
[11] Fenteany, G., Standaert, R.F., Lane, W.S., Choi, S., Corey, E.J. and Schreiber, S.L. (1995) Science 268, 726–731.
[12] Omura, S., Matsuzaki, K., Fujimoto, T., Kosuge, K., Furuya, T., Fujita, S. and Nakagawa, A. (1991) J. Antibiot. 44, 117–118.
[13] Fenteany, G., Standaert, R.F., Reichard, G.A., Corey, E.J. and Schreiber, S.L. (1994) Proc. Natl. Acad. Sci. USA 91, 3358–3362.
[14] Ciechanover, A. (1994) Cell 79, 13–21.
[15] V.J. Palombella, O.J. Rando, A.L. Goldberg, and Maniatis, Cell 78 (1994) 7737–7785.
[16] Bierer, B.E., Hollander, G., Fruman, D. and Burakoff, S.J. (1993) Curr. Opin. Immunol. 5, 763–773.
[17] Wershil, B.K., Furuta, G.T., Lavigne, J.A., Choudhury, A.R., Wang, Z.S. and Galli, S.J. (1995) J. Immunol. 154, 1391–1398.
[18] Holschermann, H., Durfeld, F., Maus, U., Bierhaus, A., Heidinger, K., Lohmeyer, J., Nawroth, P.P., Tillmanns, H. and Hberbosch, W. (1996) Blood 88, 3837–3845.
[19] Wilk, S. and Orlowski, M. (1983) J. Neurochem. 40, 842–849.
[20] Orlowski, M., Cardozo, C. and Michaud, C. (1993) Biochemistry 32, 1563–1572.
[21] Dignam, J.D., Lebovitz, R.M. and Roeder, R. (1983) Nucl. Acids Res. 11, 1475–1488.
[22] Wulczyn, F.G., Krappmann, D. and Scheidereit, C. (1996) J. Mol. Med. 74, 749–769.
[23] Fruman, D.A., Klee, C.B., Bierer, B.E. and Burakoff, S.J. (1992) Proc. Natl. Acad. Sci. USA 89, 3686–3690.
[24] Fahr, A. (1993) Clin. Pharmacokinet. 24, 472–496.