# FEBS *Letters*

# Tripeptidyl peptidase II serves as an alternative to impaired proteasome to maintain viral growth in the host cells

Jingchun Zhang, Jerry Wong, Guang Gao, Honglin Luo *

*James Hogg Research Center, Providence Heart + Lung Institute, St. Paul's Hospital, University of British Columbia, Vancouver, BC, Canada*
*Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, BC, Canada*

**ABSTRACT**

The ubiquitin–proteasome system is known to be utilized by coxsackievirus to facilitate its propagation within the host cells. The present study explores the role of tripeptidyl peptidase II (TPPII), a serine peptidase contributing to protein turnover by acting downstream of the proteasome, in regulating coxsackievirus infection. Inhibition of TPPII does not affect virus replication in cells with functional proteasome. However, when the proteasome is impaired, TPPII appears to serve as an alternative to maintain low levels of virus infection. Our results suggest an important function of TPPII in the maintenance of viral growth and may have implications for anti-viral therapy.

© 2010 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

## 1. Introduction

Coxsackievirus type B3 (CVB3) is a widespread, common etiologic agent causing myocarditis and pancreatitis [1,2]. We have previously demonstrated a critical role of the ubiquitin–proteasome system (UPS) in the control of CVB3 replication within the host cells [3–6]. The UPS is the major intracellular protein degradative pathway which contributes significantly to the regulation of many essential cellular functions by removing misfolded and damaged proteins as well as recycling short-lived regulatory proteins [7,8]. We have provided evidence that CVB3 utilizes the function of the host UPS to enhance their efficiency of replication in the host cells. We showed that inhibition of the proteasome function by chemical inhibitors significantly reduces CVB3 infectivity in vitro [3,5,6], and results in attenuated myocardial damage in vivo [4].

Proteasome-independent proteolysis has emerging as an alternative pathway to the proteasome-mediated protein turnover [9,10]. Tripeptidyl peptidase II (TPPII) is a giant intracellular serine

peptidase which exhibits both exo- and endo-proteolytic activities [9,11–13]. It is believed that TPPII acts downstream of the proteasome to cleave proteasome-generated peptides into tripeptides that can then be further degraded into free amino acids by aminopeptidases [9,11–13]. In addition to its role related to general protein turnover responsible for the maintenance of numerous fundamental cellular functions, TPPII plays an essential role in MHC class I antigen processing via further digest proteasomal products into smaller peptides more suitable for antigen presentation [14,15]. Adaptive proteolytic responses to impaired proteasome function have been reported to play a compensatory role in the regulation of protein degradation. TPPII appears to make an essential contribution to such proteolytic activities that substitute for those of the proteasome in proteasome-inhibited cells [10,16,17]. In the cells with the loss of the proteasome function, TPPII has been shown to exhibit enhanced activity to complement proteasomal activities.

The objective of this study is to explore the role of TPPII in the control of CVB3 infection in the presence or absence of proteasome inhibition. We demonstrate that inhibition of TPPII does not affect CVB3 replication in cells with normal functional proteasome. However, after the loss of the proteasome activity, TPPII activities appear to serve as an alternative proteolytic pathway to maintain minimum CVB3 infection as inhibition of TPPII further reduces CVB3 replication in a synergistic fashion in combination with a proteasome inhibitor. Our results suggest an important function of TPPII in the regulation of virus infectivity.

## 2. Materials and methods

### 2.1. Cell culture and reagents

HeLa cells purchased from the American Type Culture Collection (Rockville, MD) were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 µg/ml penicillin/streptomycin (Invitrogen Life Technologies) at 37 °C in a 5% $CO_2$ humidified incubator.

The mouse monoclonal anti-VP1 antibody was obtained from DakoCytomation. The monoclonal anti-β-actin antibody was from Sigma, and the horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology. The proteasome inhibitors (MG132 and lactacystin), and the TPPII inhibitor (H-Ala-Ala-Phe-chloromethylketone, H-AAF-CMK) were obtained from Calbiochem.

### 2.2. CVB3 infection and inhibitor treatment

HeLa cells were pre-incubated with increasing concentrations of various inhibitors as specified in the figure legends for 30 min. Cells were then infected with CVB3 (Kandolf strain, from Dr. Reinhard Kandolf [18], University of Tubingen, Germany) at a multiplicity of infection of 10 or mock infected with phosphate buffered saline (PBS) for 1 h. The infected cells were then washed with PBS and replaced with new DMEM containing fresh inhibitors.

### 2.3. Western blot analysis

Equal amounts of protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes as previously described [19]. The membrane was blocked with 5% non-fat dry milk solution containing 0.1% Tween 20 for 1 h. The blots were then probed for 1 h with primary antibody followed by incubation for 1 h with secondary antibodies conjugated to horseradish peroxidase-conjugated secondary antibody. The immunoreactive protein bands were visualized by enhanced chemiluminescence (GE Healthcare).

### 2.4. Viral plaque assay

The viral titer in the supernatant was evaluated by an agar overlay plaque assay as previously described [6]. In brief, supernatant was serially diluted and overlaid on a monolayer of HeLa cells. After 1 h incubation, medium was removed and complete DMEM containing 0.75% agar was added. Three days later, cells were fixed with Carnoy's fixative (25% acetic acid, 75% ethanol) and then stained with 1% crystal violet. The plaques were counted and the viral titer was calculated as plaque forming unit per milliliter.

### 2.5. Peptidase assay

The cell lysates were prepared and collected in a lysis buffer (50 mM pyrophosphate, 50 mM NaF, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 100 µM $Na_3VO_4$, 10 mM HEPES (pH 7.4), and 0.1% Triton X-100) containing no protease inhibitors as previously described [19]. The chymotrypsin-like activity of 20S proteasome and the TPPII activity were determined using synthetic fluorogenic substrates, Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Calbiochem) and H-Ala-Ala-Phe-7-amino-4-methylcoumarin (H-AAF-AMC, Sigma), respectively. Briefly, 50 µg of cellular extracts was incubated with 75 µM of fluorogenic peptide substrate, in an assay buffer (20 mM Tris–HCl [pH 8.0], 1 mM ATP, and 2 mM $MgCl_2$) at 30 °C for 1 h. The fluorescence of the released AMC in the supernatant was measured at a 465 nm emission wavelength using a Tecan fluorometer (Männedorf, Switzerland).

## 3. Results and discussion

### 3.1. Inhibition of TPPII activities does not affect CVB3 replication

To determine the role of the TPPII in the regulation of CVB3 infection, we first examined the effect of TPPII inhibition on CVB3 protein expression and progeny virus titers. HeLa cells were pre-incubated with increasing concentrations of TPPII inhibitor (H-AAF-CMK) for 30 min, followed by CVB3 infection for 7 h. Proteasome inhibitors, MG132 and lactacystin, were used to show the effects of proteasome inhibition on CVB3 replication. The Western blot results in Fig. 1A and plaque assay data in Fig. 1B showed that treatment with TPPII inhibitor did not result in any changes of viral protein VP1 expressions and virus titers, respectively. However, proteasome inhibitor MG132 and lactacystin dramatically reduced viral protein expression and viral growth rate, in consistent with our previously reports [5,6]. Our results suggest that the activity of TPPII is not required for the maintenance of viral growth in the host cells which possess normal proteasome function.

### 3.2. Synergetic effect of treatment with TPPII and proteasome inhibitors on CVB3 replication

It has been reported that TPPII can complement proteasomal activity when proteasome function is damaged [16,17]. We next determined the role of TPPII in regulating viral infection in cells with impaired proteasome function. Specifically, we examined whether TPPII inhibition can further reduce proteasome inhibition-mediated suppression of CVB3 replication. HeLa cells were incubated with increasing doses of TPPII inhibitor in the presence
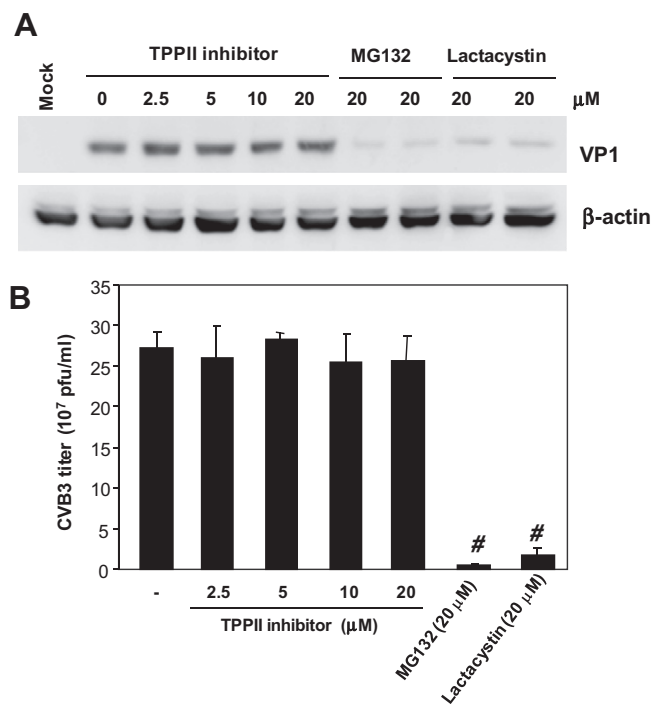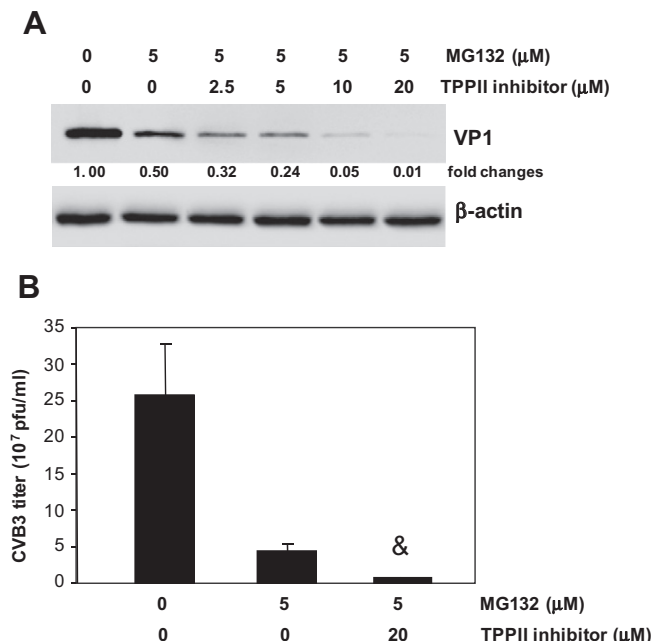


**Fig. 1.** Inhibition of TPPII activities does not affect CVB3 replication. HeLa cells were pre-incubated with various concentrations of protease inhibitors as indicated for 30 min and then infected with CVB3 for 7 h. (A) Cell lysates were collected and Western blot analysis for the expression levels of viral capsid protein VP1 and β-actin (loading control) was performed. The results are representative of three independent experiments. (B) Supernatants of infected cells were harvested for viral plaque assay. The results are presented as mean ± S.D. (n = 3). #P < 0.001 for comparison to CVB3-infected, but non-inhibitor treated cells (column 1).

of 5 μM MG132. As shown in Fig. 2, treatment with MG132 led to decreased viral protein VP1 expression and virus titers. Addition of TPPII inhibitor synergically reduced viral infectivity in a dose-dependent manner, suggesting that TPPII activity may compensate for the loss of proteasome function to maintain low levels of CVB3 propagation in the cells.
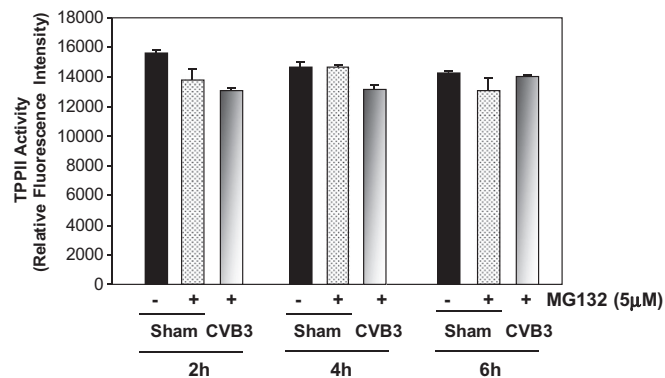
### 3.3. TPPII activities after CVB3 infection and treatment of proteasome and TPPII inhibitors

We further examined the TPPII activity during the course of viral infection. Fig. 3 showed that TPPII activity was not changed at 2, 4, and 6 h post-infection either in the presence or absence of proteasome inhibitor MG132 (5 μM). We have previously demonstrated that proteasome activities are also unaltered throughout the time courses of CVB3 infection [5,20]. Together, these results suggest that CVB3 infection does not appear to affect the activities of the proteasome and its downstream executor TPPII.

To validate the impact of proteasome inhibitor MG132 and TPPII inhibitor on chymotrypsin-like activities of the 20S proteasome and the TPPII activities, we performed proteolytic assays using two different synthetic fluorogenic peptide substrates. We found that the different doses of MG132 used in this study (i.e. 2.5, 5, 10, and 20 μM) dramatically decreased the chymotrypsin-like activity in a concentration-dependent manner as anticipated (Fig. 4A). However, treatment with different concentrations of TPPII inhibitor appeared to have no inhibitory effect on proteasome activities (Fig. 4A), but led to dose-dependent decreases of the TPPII activities (Fig. 4B). Interestingly, we found that higher doses of MG132 (i.e. 10 and 20 μM) can also inhibit TPPII activity,
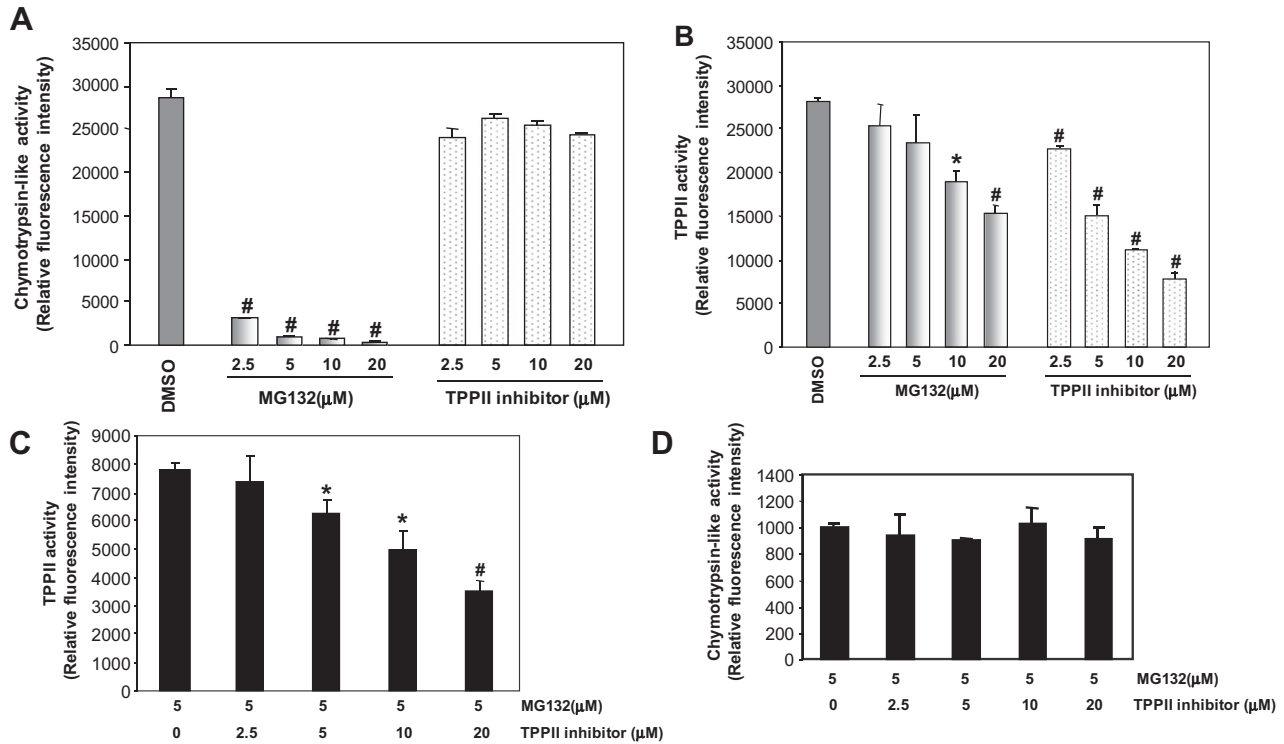
**Fig. 3.** TPPII activities after CVB3 infection. HeLa cells were mock-infected with PBS or infected with CVB3 for 2, 4, and 6 h in the presence or absence of 5 μM MG132. Cell lysates were harvested and subjected to TPPII activity assay with a fluorogenic peptide substrate (H-AAF-AMC) as described in Section 2. The results are displayed as mean ± standard deviation (S.D.) ($n$ = 3) and the statistical significance was determined using the unpaired Student's $t$-test. No statistical differences between two groups at all three time courses were observed.

suggesting that the inhibitory effect of MG132 on viral replication at these doses is a consequence of inhibition of both the proteasome and the TPPII activities. Finally, we demonstrated that a combination of 5 μM MG132 (a dose with no significant inhibition of the TPPII activity, Fig. 4B), with increasing concentrations of TPPII inhibitor significantly decreased the TPPII activity (Fig. 4C), but had no influences on chymotrypsin-like activities (Fig. 4D). Collectively, our results suggest that functional TPPII plays a compensatory role in the regulation of CVB3 infectivity when the proteasome function is lost.

## 4. Discussion

Targeting the proteasome has been suggested to be a promising therapeutic option against infections of many viruses, including coxsackieviruses [5,6], coronaviruses [21], retroviruses [22], cytomegaloviruses [23], and influenza viruses [24]. We have demonstrated that general inhibition of the proteolytic activity of proteasome results in a dramatic reduction of coxsackieviral replication and virus-induced pathogenesis [5,6]. However, the function of proteasome can to some degree be redundant. Treatment with proteasome inhibitors has been shown to be able to induce the development of adaptation conferring the cells with the resistance to proteasome inhibition [16,17]. Alternative proteases to the impaired proteasome have been identified [9]. TPPII is a large serine peptidase which contributes to both protein turnover and antigen presentation by further degrading proteasome-generated peptides [11]. Increasing evidence has suggested that TPPII, under stress conditions, may compensate and substitute for the function of the proteasome [10,16,17]. The present study explored the potential role of TPPII in regulating CVB3 replication in the presence and absence of proteasome inhibitor and demonstrated an essential function of the TPPII in the maintenance of viral latency after the loss of the proteasome activities.

The potential mechanisms by which the TPPII pathway assists in CVB3 replication in the absence of functional proteasome is likely related to its role in maintaining general protein turnover. Many host intracellular proteins can function as barriers against viral replication. For example, we have recently demonstrated that tumor suppressor protein p53 is an anti-CVB3 host protein and increased proteolysis of this protein is beneficial to CVB3 replication [3]. We thus postulate that TPPII functions to maintain CVB3 replication by facilitating the degradation of anti-viral host proteins.

**Fig. 2.** Synergetic effect of treatment with TPPII and proteasome inhibitors on CVB3 replication. HeLa cells were treated with MG132 (5 μM) alone or in combination with increasing doses of TPPII inhibitor as detailed in the figure. (A) The protein expression of VP1 and β-actin (loading control) was determined by Western blot analysis. The levels of VP1 were quantitated by densitometric analysis using NIH ImageJ V1.43, normalized to those of β-actin, and presented as fold changes compared with non-treated cells (which was arbitrarily set a value of 1.0). The data are representative of three independent experiments. (B) Supernatant was collected from CVB3 infected cells and virus titers were determined by plaque assays. The results are presented as mean ± S.D. ($n$ = 3). [&]$P < 0.005$ for comparison to MG132 treatment alone.

**Fig. 4.** Peptidase activities after the treatment with proteasome and TPPII inhibitors. HeLa cells were treated with different concentrations of vehicle (DMSO), MG132, and/or TPPII inhibitor, as indicated in the figure for 5 h. The chymotrypsin-like activities of 20S proteasome (A, C) and the TPPII activities (B, D) in the cell lysates were measured using the fluorogenic substrates as described in Section 2. The results are expressed as the amount of AMC formed by the enzymatic cleavage of substrate (mean ± S.D., $n = 3$). *$P < 0.05$; #$P < 0.001$ compared to DMSO treated control group.

MG132 is a peptide aldehyde and inhibits the function of the proteasome by acting as a proteasome substrate analog. In addition to proteasome inhibition, MG132 can inhibit lysosomal and calpain protease activities [25]. In this study, we also demonstrated a non-specific effect of MG132 on the inhibition of the TPPII activities. Thus, the striking inhibition effect of MG132 at the higher doses on CVB3 replication shown in the previous studies [5,6] and in the current study appears to be due to the blockage of both proteasome and TPPII activities.

In conclusion, out data provide the first evidence that TPPII can partially substitute for compromised proteasome function to serve as an alternative pathway to favor viral growth within the host cells. These findings provide potentially useful information for novel anti-viral drug design.

### Acknowledgements

### References

[1] Esfandiarei, M. and McManus, B.M. (2008) Molecular biology and pathogenesis of viral myocarditis. Annu. Rev. Pathol. 3, 127–155.
[2] Luo, H., Wong, J. and Wong, B. (2010) Protein degradation systems in viral myocarditis leading to dilated cardiomyopathy. Cardiovasc. Res. 85, 347–356.
[3] Gao, G. et al. (2010) Proteasome activator REG{gamma} enhances coxsackieviral infection via facilitating p53 degradation. J. Virol. 84, 11056–11066.
[4] Gao, G., Zhang, J., Si, X., Wong, J., Cheung, C., McManus, B. and Luo, H. (2008) Proteasome inhibition attenuates coxsackievirus-induced myocardial damage in mice. Am. J. Physiol. Heart Circ. Physiol. 295, H401–H408.
[5] Luo, H., Zhang, J., Cheung, C., Suarez, A., McManus, B.M. and Yang, D. (2003) Proteasome inhibition reduces coxsackievirus B3 replication in murine cardiomyocytes. Am. J. Pathol. 163, 381–385.
[6] Si, X., Gao, G., Wong, J., Wang, Y., Zhang, J. and Luo, H. (2008) Ubiquitination is required for effective replication of coxsackievirus B3. PLoS ONE 3, e2585.
[7] Glickman, M.H. and Ciechanover, A. (2002) The ubiquitin–proteasome proteolytic pathway: destruction for the sake of construction. Physiol. Rev. 82, 373–428.
[8] Pickart, C.M. and Cohen, R.E. (2004) Proteasomes and their kin: proteases in the machine age. Nat. Rev. Mol. Cell Biol. 5, 177–187.
[9] Anton, L.C. and Villasevil, E.M. (2008) Is there an alternative to the proteasome in cytosolic protein degradation? Biochem. Soc. Trans. 36, 839–842.
[10] Gavioli, R., Frisan, T., Vertuani, S., Bornkamm, G.W. and Masucci, M.G. (2001) c-myc overexpression activates alternative pathways for intracellular proteolysis in lymphoma cells. Nat. Cell Biol. 3, 283–288.
[11] Tomkinson, B. and Lindas, A.C. (2005) Tripeptidyl-peptidase II: a multi-purpose peptidase. Int. J. Biochem. Cell Biol. 37, 1933–1937.
[12] Yao, T. and Cohen, R.E. (1999) Giant proteases: beyond the proteasome. Curr. Biol. 9, R551–R553.
[13] Yewdell, J.W. and Princiotta, M.F. (2004) Proteasomes get by with lots of help from their friends. Immunity 20, 362–363.
[14] Saveanu, L., Fruci, D. and van Endert, P. (2002) Beyond the proteasome: trimming, degradation and generation of MHC class I ligands by auxiliary proteases. Mol. Immunol. 39, 203–215.
[15] Seifert, U. et al. (2003) An essential role for tripeptidyl peptidase in the generation of an MHC class I epitope. Nat. Immunol. 4, 375–379.
[16] Geier, E., Pfeifer, G., Wilm, M., Lucchiari-Hartz, M., Baumeister, W., Eichmann, K. and Niedermann, G. (1999) A giant protease with potential to substitute for some functions of the proteasome. Science 283, 978–981.
[17] Glas, R., Bogyo, M., McMaster, J.S., Gaczynska, M. and Ploegh, H.L. (1998) A proteolytic system that compensates for loss of proteasome function. Nature 392, 618–622.
[18] Huber, M., Selinka, H.C. and Kandolf, R. (1997) Tyrosine phosphorylation events during coxsackievirus B3 replication. J. Virol. 71, 595–600.
[19] Si, X., Wang, Y., Wong, J., Zhang, J., McManus, B.M. and Luo, H. (2007) Dysregulation of the ubiquitin–proteasome system by curcumin suppresses coxsackievirus B3 replication. J. Virol. 81, 3142–3150.
[20] Luo, H. et al. (2003) Ubiquitin-dependent proteolysis of cyclin D1 is associated with coxsackievirus-induced cell growth arrest. J. Virol. 77, 1–9.
[21] Ma, X.Z. et al. (2010) Proteasome inhibition in vivo promotes survival in a lethal murine model of SARS. J. Virol. 84, 12419–12428.

[22] Schubert, U. et al. (2000) Proteasome inhibition interferes with gag polyprotein processing, release, and maturation of HIV-1 and HIV-2. Proc. Natl. Acad. Sci. USA 97, 13057–13062.

[23] Kaspari, M., Tavalai, N., Stamminger, T., Zimmermann, A., Schilf, R. and Bogner, E. (2008) Proteasome inhibitor MG132 blocks viral DNA replication and assembly of human cytomegalovirus. FEBS Lett. 582, 666–672.

[24] Dudek, S.E., Luig, C., Pauli, E.K., Schubert, U. and Ludwig, S. (2010) The clinically approved proteasome inhibitor PS-341 efficiently blocks influenza A virus and vesicular stomatitis virus propagation by establishing an antiviral state. J. Virol. 84, 9439–9451.

[25] Lee, D.H. and Goldberg, A.L. (1998) Proteasome inhibitors: valuable new tools for cell biologists. Trends Cell Biol. 8, 397–403.