Chapter 4.1

PROTEASOME INHIBITORS AS COMPLEMENTARY OR ALTERNATIVE ANTIVIRAL THERAPEUTICS

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Abstract: The ubiquitin-proteasome system (UPS) catalyses the orderly degradation of abnormal or short-lived regulatory proteins and thus is critical for cell cycle regulation, signal transduction, transcription regulation, protein sorting, apoptosis as well as for the generation of antigenic peptides for the T-cell mediated immune response. Numerous studies suggest that efficient replication of many, if not all, viruses essentially depends on a functional UPS. Consequently proteasome inhibitors (PI) have been found to interfere with efficient replication of many viruses. The mechanisms by which the UPS is involved in regulation of replication of individual viruses are different. Depending on the virus the UPS has been shown to be important for entry, transport, gene expression, assembly or egress of the virus. The introduction of proteasome inhibitors in the clinical praxis as anti-cancer or anti-inflammatory drugs now rise the question whether these compounds present a new class of supplementary or even alternative antiviral drugs. The following article will give an short overview on the structure and function of the UPS, the currently available PI and the various aspects of virus UPS interaction. Finally the potential of different PI as antiviral approaches for the treatment of virus infections is discussed.

1. INTRODUCTION

Viruses are obligate intracellular parasites and thus widely depend on the cellular machinery for replication and transcription as well as protein and energy metabolism. Options for antiviral therapy are limited to drugs

targeting virus-encoded enzymes like DNA- or RNA-polymerases which control replication of the viral genome (e.g. inhibitors of herpesvirus polymerase, of HIV reverse transcriptase or RNA-dependent RNA polymerases), proteases which catalyse processing of viral (poly)proteins (e.g. inhibitors of HIV protease) or viral proteins which are involved in uncoating and viral egress (e.g. inhibitors of Influenza virus neuraminidase or M2/hemagglutinin fusion). These drugs are highly specific for certain viruses or virus families and show a satisfying overall *in vivo* tolerance. However, prophylaxis or even broad therapeutic treatment over a long period of time favours evolution of drug resistant mutants, particularly when the drug dose or the drug itself is suboptimal and reduces virus replication only marginally. The molecular basis for this phenomenon is a high mutation rate of viral genomes (especially of RNA viruses) combined with a high reproduction rate leading to millions of progeny viruses in one generation. Consequently, drug-mediated modulation of specific cellular pathways essentially involved in virus replication represents an attractive complementary or even alternative strategy to block virus multiplication. Cellular genes have much lower mutation rates so that the problem of drug-resistance should be limited. However, drugs modulating cellular pathways may have a higher toxicity and thus undesirable side effects could limit their *in vivo* utility.

The ubiquitin-proteasome system (UPS) is the central cellular mediator of regulated protein turnover in living cells. Apart from degradation of misfolded proteins, the proteasome also functions in defined processing of inactive precursor proteins (Ciechanover and Schwartz, 1998). Furthermore, by catalysing generation of immunogenic peptides for MHC-I presentation, the UPS plays an important role for the MHC-I-restricted cellular immune response against viruses (Kloetzel, 2004). Moreover, the UPS is essentially involved in regulation of transcription, signalling, cell-cycle, apoptosis and protein sorting which represent crucial processes for viral replication. Therefore, intervention into these elementary cellular processes may have more or less severe consequences for replication of certain viruses. Therefore, it seemed legitimate to investigate whether proteasome inhibitors (PI) are useful complementary or alternative therapeutics for treatment of viral infections for which no or only suboptimal drugs are currently available. A new impetus for these studies was the development of pharmacologically potent PI like PS-341 (Bortezomib) which recently has been introduced in clinical use as an anti-cancer drug in relapsed myeloma patients that are refractory to conventional chemotherapy (Adams, 2002). The clinical data support that partial and time-limited inhibition of the UPS is a calculable option for therapeutic intervention, even though certain

adverse and toxic effects depending on the dose and the individual patient situation might occur (Richardson *et al.*, 2003).

The following article will give a short overview concerning the structure of the UPS, its function in cellular metabolism and in replication of certain viruses. Furthermore, currently available PI for research and clinical practice as well as their influence on viral replication will be addressed.

2. THE UBIQUITIN-PROTEASOME SYSTEM AND ITS FUNCTIONS IN CELLULAR METABOLISM AND IMMUNE RESPONSE

2.1 Structure of the ubiquitin-proteasome system

The ubiquitin/adenosine triphosphate (ATP)-dependent proteolytic pathway comprises a cascade of enzymes that first catalyse poly-ubiquitinylation of substrates – ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin ligases (E3) and ubiquitin chain-assembly factor (E4). The poly-ubiquitinated proteins are finally recognised by the proteasome for degradation or processing (for details see Fig. 1, Ciechanova and Schwartz, 1998). Additionally, the UPS catalyses mono-ubiquitinylation of certain proteins. Mono-ubiquitinylation has been shown to regulate protein function and serves as a signal in protein sorting and membrane receptor recycling but does not target the protein for proteasomal degradation (Fig.1). Interestingly, processes depending on mono-ubiquitinylation of proteins can be suppressed by PI indicating that they directly or indirectly require proteasome activity (Dupre *et al.*, 2001). present in the cytoplasm and the nucleus of all eukaryotic cells. There are two functionally distinct proteasome types – the 20S proteasome and the 26S proteasome. The barrel-shaped 20S proteasome comprises four stacked rings each composed of 7 α or β homodimeric subunits that enclose a central chamber. The two central β -rings harbour three main proteolytic activities, a chymotrypsin-like (CT-L) activity that cleaves after large hydrophobic residues, a trypsin-like (T-L) activity that cleaves after acidic residues and a peptidylglutamyl-peptide hydrolysing (PGPH) or caspase-like activity that also cleaves after acidic residues. There are two further activities that cleave after branched-chain residues or small neutral amino acids. The primary sequence of these proteolytically active β -subunits and their mechanisms of action differ from those of other known cellular proteases.

Figure 1: The ubiquitin-proteasome system (UPS). The ubiquitin-proteasome system involves a cascade of enzymes which catalyse mono- or poly-ubiquitylation of substrates starting with ubiquitin-activating enzyme (E1), followed by ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3) which finally mediate covalent conjugation of ubiquitin to the target protein. Repeated cycles of ubiquitin conjugation result in generation of insoluble poly-ubiquitinated proteins which are then recognised by the proteasome as mis-folded and damaged proteins and are degraded or processed. Eukaryotic cells contain one E1, several E2 and more than 100 different E3 enzymes. The E3 enzymes are suggested to determine the specificity of ubiquitylation. Mono-ubiquitylation of proteins is associated with changes in protein function and plays a role in protein transport and sorting, as well as transcription regulation.

Proteasomes are highly conserved multimeric peptidase complexes that are The β -subunits are flanked by two α -rings that guide the entrance of unfolded polypeptide chains into the 20S core (Arendt and Hochstrasser, 1997; Baumeister *et al.*, 1998; Bochtler *et al.*, 1999).

The 26S proteasome is composed of the 20S core particle and two additional 19S regulatory complexes that associate with the outer α -rings and contain the substrate recognition and binding sites. The 19S complex harbours six ATPases and catalyses de-polymerisation of poly-ubiquitin chains as well as unfolding of the protein substrate thus facilitating its entry into the 20S proteasome (Braun *et al.*, 1999; Ferrell *et al.*, 2000). A minimum of four ubiquitin molecules is required for recognition by the

26S proteasome. The ubiquitin chains are then removed from the substrate by de-ubiquitinating enzymes and re-enter the pool of free ubiquitin.

The 20S proteasome can also associate with the proteasome activator 28/11S regulator (PA28) in an ATP-independent manner thus allowing degradation of non-ubiquitinated proteins (Baumeister *et al.*, 1998; Rechsteiner *et al.*, 2000; Hoffman *et al.*, 1992). PA28 activates the 20S proteasome by induction of conformational changes which presumably open the core maximally and thus facilitate substrate entry and cleavage product exit without affecting the active sites of the proteases (Stohwasser *et al.*, 2000; Hill *et al.*, 2002). In vivo, the 20S proteasome also forms so-called hybrid proteasomes with one 19S and one PA28 regulatory subunit. However, these hybrid proteasomes are as active as the 19S-20S-19S proteasome (Kopp *et al.*, 2001; Tanahashi *et al.*, 2000).

In the presence of interferon (IFN)- γ (or other cytokines like TNF α and IFN- β) the catalytic β -subunits of the proteasome are replaced by the subunits LMP-2, LMP-7 and MECL-1 thus generating the immuno-proteasome. The immuno-proteasome plays a central role for the proteolytic generation of antigenic peptides for presentation via MHC class I molecules (Kloetzel, 2004). These IFN-inducible subunits modify the cleavage specificity profile of the proteasome by altered cleavage site preference and enhanced generation of specific peptides.

2.2 Function of the proteasome in cellular metabolism

Originally, the proteasome was discovered due to its function in degradation of aged or misfolded proteins into small peptides of 3-20 amino acid residues. However, the proteasome also controls specific degradation of regulatory proteins and processing of inactive precursor proteins.

The UPS is involved in regulation of key cellular processes like cell-cycle progression, transcription, translation, signal transduction, stress response, apoptosis, receptor function and protein sorting by catalysing degradation, processing or translocation of several proteins involved in regulation of these processes, e.g. cyclins and cyclin-dependent kinases, p53, c myc, bcl-2, Bax, Nuclear factor (NF)- κ B precursor p100 and p105, I- κ B (Inhibitor of NF- κ B), JNK and c-jun (Rolfe *et al.*, 1997; Salghetti *et al.*, 1999; Breitschopf *et al.*, 1999; Strous and Govers, 1999; Longva *et al.*, 2002; Baldwin, 1996; Karin and Ben-Neriah, 2000; Dupre *et al.*, 2001; Adams, 2002; Stahl and Barbieri, 2002; Beinke and Ley, 2004). Additionally, the proteasome plays an important role in the cellular immune

response by generating peptides for presentation via MHC class I molecules (Kloetzel, 2004).

The most intensively studied proteasome substrate is the transcription factor NF- κ B which is crucial for transcription regulation of genes involved in pro-inflammatory processes (e.g. TNF- α , IL-1 and -6, MIP-1 α , RANTES, E-selectin and VCAM-1), immune response (e.g. T-cell activation by up-regulation of MHC molecules and CD80/86 on antigen presenting cells), anti-apoptosis (e.g. c.IAP-1/2, AI, Bcl-2 and Bcl-XL) and cell cycle progression (cell-cycle regulator cyclin D1) (Zhang and Ghosh, 2001, Li and Verma, 2002; Karin and Li, 2002; Karin *et al.*, 2002). Inhibition of NF- κ B activation therefore represents the main mechanism by which PI induce apoptosis and block cell cycle progression or inhibit inflammatory processes.

The most dominant form of NF- κ B is a heterodimer consisting of p50 and p65. The p50/p65 heterodimer remains in an inactive state by association with its inhibitors I κ B α or I κ B β in the cytoplasm. Various stimuli are able to trigger activation of NF- κ B by induction of proteasome-dependent I κ B degradation following its phosphorylation by the IKK complex and ubiquitinylation by the SCF (Skp1/Cu11/F-box) E3 ubiquitin ligase complex (Baldwin, 1996; Karin and Ben-Neriah, 2000). The active p50/p65 heterodimer is then translocated into the nucleus where it binds to the promoters of responsive genes. Apart from I κ B degradation, the 26S proteasome also controls processing of the NF- κ B p105 and p100 precursors to generate the NF- κ B p52 and p50 subunits. Furthermore, p50 can be retained in the cytoplasm by binding to its precursor p105. Following stimulation of the cells p105 can be completely degraded by the UPS allowing translocation of p50 into the nucleus (Beinke and Ley, 2004).

3. PROTEASOME INHIBITORS

In the last few years, various low-molecular-weight compounds have been identified that more or less selectively inhibit the UPS through interaction with the active site subunits of the 20S proteasome. These compounds are either synthetic products or naturally occurring substances; the most relevant compounds are listed in Tab. 1. Synthetic PI consist of linear di- or tripeptides linked to different pharmacophores such as benzamides, α -ketoamides, aldehydes, α -ketoaldehydes, vinyl sulfones or boronic acids (Fig. 2). Natural product PI display a broad structural heterogeneity containing both linear as well as non-linear scaffold structures (Fig. 3).

In the early 1980s, serine/cysteine protease and calpain inhibitors belonging to the family of peptide aldehydes were initially discovered to interfere with T-L and CT-L activity of the 20S proteasome, respectively (Wilk *et al.*, 1983; Figueiredo-Pereira *et al.*, 1994). Today, a variety of

peptide aldehydes with higher potency and increased selectivity towards the 20S proteasome like MG132, MG115 and PSI have been developed. Binding of peptide aldehydes to the active site β -subunits results in formation of a reversible hemiacetal adduct between the aldehyde group and the hydroxyl group of the N-terminal threonine residue. Due to the highly reactive functional aldehyde group, cross-reactivity with cellular proteases remains a major drawback of this class of inhibitors that limits their *in vivo* utility.

Figure 2: Synthetic proteasome inhibitors

Figure 3: Naturally occurring proteasome inhibitors

Table 1: Proteasome inhibitors

CLASS	COMPOUND	NATURE OF INHIBITION	OTHER TARGETS	APPLICATION
Peptide aldehydes	MG132	reversible	Calpains,	<i>In vitro</i> studies
	MG115		Cathepsins	<i>In vitro</i> studies
Boronic acid peptides	MG262	reversible	-	<i>In vitro</i> studies
	PS-341		-	Multiple Myeloma
Lactacystins	β -Lacton	irreversible	Cathepsin A	<i>In vitro</i> studies
	PS-519		-	Stroke, asthma, psoriasis, multiple sclerosis (rodents)
Epoxyketones	Epoxomicin	irreversible	-	Cutaneous inflammation
	Eponemycin		-	B16 melanoma (mouse)
Macrocyclic PI	TMC-95	reversible	-	<i>In vitro</i> studies
Vinyl sulfones	NLVS	irreversible	Cathepsins	<i>In vitro</i> studies
Epipolythioperazin toxins	Gliotoxin	reversible	(-)	Graft rejection (mouse)

As a strategy to overcome these limitations, boronic acid derivatives of peptide aldehydes with improved selectivity and higher affinity were developed (Adams *et al.*, 1998; Gardner *et al.*, 2000). Studies on their mode of action revealed that the empty p-orbital of boron accepts the oxygen lone pair of the N-terminal threonine residue to form a stable tetrahedral intermediate. Boronic acid-based inhibitors can be truncated to dipeptides, thus improving important therapeutic characteristics like solubility and membrane permeability. Apart from that, slower dissociation rates of boronic acid peptides lead to a prolonged inhibition of the proteasome. The most common representatives of this class in the context of *in vivo* studies and clinical applications are MG262 and PS-341 (see below).

Peptide vinyl sulfones irreversibly modify the hydroxyl group of the active site N-terminal threonine (Bogyo *et al.*, 1997). However, since they inhibit both the 20S proteasome and cysteine proteases, these compounds are not regarded as suitable for *in vivo* applications.

The first naturally occurring proteasome inhibitor identified was the *Streptomyces lactacystinaeus* metabolite lactacystin (Omura *et al.*, 1991). In aqueous solutions, lactacystin is spontaneously converted into clasto-lactacystin β -lactone that reacts with the hydroxyl group of the β -subunit

N-terminal threonine to form a covalent ester adduct (Fenteany *et al.*, 1995). Thus, lactacystin irreversibly inhibits the CT-L and T-L activity of the proteasome. Although it was originally believed to exclusively target the 20S proteasome, there is increasing experimental evidence that lactacystin also inhibits cathepsin A (Ostrowska *et al.*, 2000). With the aim to reduce non-specific interactions, a synthetic analogue of lactacystin termed PS-519 was designed that has already demonstrated effectiveness against inflammatory diseases in clinical studies (Elliott *et al.*, 1999; Kondagunta *et al.*, 2004).

Screening for anti-tumour agents in mice led to the discovery of peptide α' , β' -epoxyketones, another class of natural product PI (Hanada *et al.*, 1992). Epoxomicin and eponemycin, the most common representatives of this class, were isolated from *Actinomycetes* strain No. Q996-17 and *Streptomyces hygroscopicus*, respectively. They inhibit the 20S proteasome by forming a morpholino ring complex with the active site threonine residue. Cross-reactions with other cellular targets have not been reported for this class of inhibitors. However, due to the inhibition of all catalytic activities of the 20S proteasome, α' , β' epoxyketones might be highly cell toxic and thus of limited therapeutic potential.

A family of non-linear PI was isolated from the fermentation broth of *Apiospora montagnei* Sacc. TC 1093 (Koguchi *et al.*, 2000). Compounds of this class like the TMC-95 stereoisomers are characterised by a macrocyclic scaffold structure and bind non covalently to the proteasome.

Another non-linear PI class termed epipolythiopiperazin toxins was discovered in *Aspergillus fumigatus*. Representatives of this class such as gliotoxin consist of a heterobicyclic core containing bisulfide bridges. Proteasome inhibition by gliotoxin can be reversed by dithiothreitol (Kroll *et al.*, 1999).

Generally, PI target the catalytic activities of the proteasome, thus preventing degradation and cleavage of proteins. Consequently, poly-ubiquitinated proteins accumulate in the cell, precursor proteins remain in their inactive configuration and several proteins are mis-sorted. Signalling pathways are blocked (e.g. the NF- κ B pathway, see above) or constitutively activated (e.g. the JNK/c-jun pathway, Nakayama *et al.*, 2001) causing either a decreased or increased expression of responsive genes. The most extensively studied inhibitors are PS-341 (Bortezomib, Velcade) and PS-519. Both compounds have been tested as potential therapeutics for oncological and inflammatory conditions. Bortezomib has demonstrated activity in phase II trials as a treatment option for renal cell cancer, lung cancer, sarcoma and lymphoma (Richardson *et al.*, 2003; Davis *et al.*, 2004; Kondagunta *et al.*, 2004; Maki *et al.*, 2005). Since 2003, bortezomib has been approved for clinical use in patients with multiple myeloma. PS-519 is

the most advanced inhibitor in the context of inflammatory diseases like psoriasis, rheumatoid arthritis, asthma, multiple sclerosis, sepsis and vascular restenosis and has entered phase II clinical studies for the indications of acute stroke and myocardial infarction (Phillips *et al.*, 2000; Palombella *et al.*, 1998; Elliott *et al.*, 1999; Kondagunta *et al.*, 2004).

The anti-cancer effect of bortezomib and other PI is mainly based on their ability to stabilise cell cycle inhibitory proteins. Inhibition of NF- κ B transcriptional activity furthermore down-regulates expression of various growth, survival and angiogenic factors (Sunwoo *et al.*, 2001). The ability of PI to increase tumour suppressor p53 levels and to activate c-Jun N-terminal kinase (JNK) leading to Fas up-regulation and caspase activation also contributes to the anti-neoplastic, pro-apoptotic effect (Hideshima *et al.*, 2001; Mitsiades *et al.*, 2002; Drexler, 1997, Shinohara *et al.*, 1996). Additionally, it has also been shown that proteasome inhibition enhances the sensitivity of cancer cells for traditional anti-cancer agents (Bold *et al.*, 2001). The anti-inflammatory effect of PS-519 and other PIs is probably based on inhibition of NF- κ B activation that plays an important role in the pathogenesis of many inflammatory diseases by controlling expression of cytokines, adhesion molecules and pro-inflammatory molecules.

Given the fundamental metabolic role of the proteasome for all eukaryotic cells, undesirable adverse effects of proteasome inhibition might occur. However, most somatic cells are in a quiescent state and are thus less susceptible for proteasome inhibitors than actively dividing cells like cancer or immune cells. In accordance with this, bortezomib displayed a satisfying overall tolerance in clinical testing. The reported side-effects include fatigue and/or low fever, thrombocytopenia, gastrointestinal symptoms, electrolyte disturbances, headache, anemia, arthralgia, low diarrhea, frequent skin rashes and peripheral neuropathy (Richardson *et al.*, 2003; Davis *et al.*, 2004). Autoradiography experiments in rats showed that bortezomib is present in all organs except CNS, spinal cord, testes and eyes thus sparing these organs adverse effects of proteasome inhibition. Apart from that, proteasome inhibition by bortezomib is reversible and it could be shown that the inhibitor is rapidly metabolised (Adams, 2002). Similarly, PS-519 showed a good bioavailability in all organs except brain tissue. Side-effects have not been reported yet.

4. INTERACTION BETWEEN VIRUSES AND THE UPS

Interactions between viruses and the UPS are highly complex and variable. Certain viruses essentially require the proteasome for different

steps of their replication cycle. For other viruses, however, the proteasome represents a barrier and a degradative factor. In vitro studies also revealed that the interaction between the UPS and the virus presumably depends on the cell type. Most interestingly, the UPS may play different and even opposing roles during different stages of virus replication. Consequently, virus replication is either PI-sensitive or -insensitive and proteasome inhibition either suppresses or even supports replication of the virus depending on the specific situation. So far, antiviral effects of PI have been demonstrated in vitro for members of seven virus families - retroviridae, herpesviridae, parvoviridae, picornaviridae, orthomyxoviridae, paramyxoviridae and coronaviridae. For other viruses it has been shown that certain steps in virus replication are controlled by the UPS, however, an antiviral effect of PI has not been demonstrated yet. Involvement of the UPS in the interaction between the virus and cellular metabolism does not necessarily imply that proteasome inhibition blocks virus replication as shown for Adenovirus (Corbin-Lickfett and Bridge, 2003). The so far described antiviral effects of PI target entry/egress of the virus or interfere with viral transcription/translation which depend on the cellular gene expression machinery. Data arise exclusively from in vitro experiments using virus-infected cell cultures. However, preliminary data from animal experiments performed in our laboratory raise the hope that PI do not only suppress virus replication in vitro but also in vivo (Gille, Prösch., unpublished observation, see below).

4.1 Involvement of the UPS in virus entry

Several studies indicate that proteasome inhibition may block entry of viruses which essentially involve the endocytotic pathway and in particular the late endosome, as has been demonstrated for Influenza virus and a strain of Coronaviruses (Khor *et al.*, 2003; Sieczkarski and Whittaker, 2003; Yu and Lai, 2005). This observation is in accordance with the finding that sorting of proteins into the late endosome via “inwards” budding of membranes is coupled with the protein trafficking and ubiquitin-vacuolar sorting system and requires the functional UPS (Stahl and Barbieri, 2002; Strous and Govers, 1999; Longva *et al.*, 2002).

Productive infection by influenza viruses essentially depends on late endosomes and is thus highly sensitive to proteasome inhibition. Treatment of cells with PI caused a mis-targeting of the virus into so-called sorting and recycling endosomes which are different from early and late endosomes. Accordingly, the virus did not enter into the late endosomes and into the nucleus where replication occurs (Khor *et al.*, 2003; Sieczkarski and

Whittaker, 2003; Yu and Lai, 2005). Since PI do not influence the entry of Vesicular Stomatitis Virus (VSV) and Semliki Forest Virus (SFV) which only require the early and not the late endosome, the target for PI might be localised beyond the early endosome. The exact mechanism of PI-dependent inhibition of influenza virus entry, however, remains unclear up to date. A cellular target for proteasomal degradation seems more likely because viral proteins have not been found to be ubiquitinated.

Similar to influenza virus, PI also suppress entry and nuclear translocation of Mouse Hepatitis Virus (MHV) strain JHM, a murine Coronavirus, into mouse astrocytoma cells (DBT cell line) (Ros and Kempf, 2004). Treatment of MHV strain JHM infected cells with PI caused a strong accumulation of incoming virus in the early endosome and led to a missorting into the lysosome. MHV may enter its target cells either by plasma membrane fusion or by endocytosis depending on the virus strain and the cell type (Nash and Buchmeier, 1997). Correspondingly, replication of MHV strain JHM which enters DBT cells via the endocytotic pathway was highly sensitive to proteasome inhibition, while MHV strain A59, which enters the cells by fusion, was less efficiently inhibited by PI.

Interestingly, the UPS has also been found to be involved in the pH dependent but endosome-independent entry and nuclear translocation of certain parvoviridae. Replication of at least two members of this virus family, the Minute Virus of Mice (MVM) and the Canine Parvovirus (CPV), but not the distantly related Bovine Parvovirus (BPV), were inhibited by PI. In the presence of PI virus particles aggregated in ring-like structures around the nuclei but were unable to enter into the nucleus (Ros *et al.*, 2002, Ros and Kempf, 2004). The underlying mechanism remains unclear because the proteasome does not seem to be involved in cleavage/processing of capsid protein VP and the virus did not co-localise with the early or late endosomal or the lysosomal compartment. Furthermore, uncoating of parvoviruses occurs in the nuclear core or in the nucleus but not in the cytosol.

4.2 Involvement of UPS in virus maturation and budding

Generally, virus budding at the outer plasma membrane follows the same strategy of “inwards” budding of membranes into so-called multivesicular bodies and association with the protein trafficking and ubiquitin-vacuolar sorting system. Accordingly, budding of certain retroviruses including Human Immunodeficiency Virus (HIV) type 1 and 2, Rous Sarkoma Virus (RSV), Murine Leukemia Virus (MuLV) and Mazon Pfizer Monkey Virus (MPMV) as well as the rhabdovirus VSV and the paramyxovirus SV5 was strongly inhibited by PI (Patniak *et al.*, 2000; Schubert, *et al.*, 2000; Strack

et al., 2000; Harty *et al.*, 2001; Ott *et al.*, 2003, recently reviewed in Klinger and Schubert, 2005; Schmitt *et al.*, 2005). Intensive studies on HIV-1 revealed in the absence of a functional UPS retarded budding structures, which accumulated and the released HIV-1 particles were not infectious. It was also shown that processing of the Gag-polyprotein but not the env-precursor, was strongly impaired. As a consequence Gag processing intermediates accumulated in the cell and the intracellular and virus-associated levels of the capsid protein p24 were strongly reduced. Interestingly, the viral protease which normally cleaves the Gag-polyprotein was not targeted by PI (Schubert *et al.*, 2000; Klinger and Schubert, 2005). Despite intensive investigations, the mechanism of PI-dependent inhibition of HIV budding is not completely understood. Several studies demonstrated that the C-terminal portion of Gag proteins of different retroviruses (e.g. p6 of HIV-1 and SIV Gag, p12 of Mo-MLV Gag, p9 of EIAV Gag) is mono-ubiquitinated. The ubiquitin residue was suggested to play a role in function of the L-domains that are present in these proteins and are required for budding (Ott *et al.*, 1998, 2000, 2002a and b and 2003; Pornillos *et al.*, 2002). Since treatment with PI causes an accumulation of poly-ubiquitinated proteins, limitation of the endogenous pool of free ubiquitin may prevent Gag ubiquitination. However, HIV-1 p6 Gag mutants deficient for ubiquitination showed an unchanged phenotype, indicating that ubiquitination alone is not sufficient for virus budding (Ott *et al.*, 2000 and 2003). Moreover, mono-ubiquitination of Gag alone does not provide an explanation why budding of some retroviruses like Equine Infectious Anemia Virus (EIAV) and Mouse Mammary Tumour Virus (MMTV) are insensitive to PI (Patniak *et al.*, 2002; Ott *et al.*, 2003). The structure of the Gag L-domains and their interaction with different components of the UPS-dependent vacuolar protein-sorting system seems to be more crucial for virus budding. Only viruses with proteins containing an L-domain of the PPXY-type (e.g. MuLV, RSV, Ebola virus) or the PTAP type (e.g. HIV-1 and-2) but not of the YXDL-type (e.g. EIAV) are sensitive to PI (Shehu-Xhilaga *et al.*, 2004; Klinger and Schubert, 2005). By binding to different “bridging molecules”, L-domains mediate interaction between the viruses and the vacuolar protein-sorting system required for budding. L-domains of the PPXY-type are able to bind different HECT ubiquitin ligases like WWP1, WWP2 or Itch, while L-domains of the PTAP-motif bind Tsg 101 (e.g. HIV, Ebola virus VP40) which then recruits other components of the endosomal sorting system like ESCRT-I, -II, -III and thus connects the Gag protein to the vacuolar protein sorting system (Garrus *et al.*, 2001; Amit *et al.*, 2004; Martin-Serrano *et al.*, 2001, 2003 and 2005).

Since VP40 of Ebola Virus and Human T-cell Lymphotropic virus (HTLV-1) Gag also contain L-domain motifs of the PPXY type it has been speculated that Ebola virus and HTLV-1 are also sensitive to proteasome inhibition. However, this still has to be proved in an infection model (Harty *et al.*, 2000; Bouamr *et al.*, 2003; Heidecker *et al.*, 2004; Martin-Serrano *et al.*, 2005).

In the paramyxovirus SV5 matrix protein, very recently, a new type of L-domains (FPIV) has been identified which may functionally substitute the PTAP motif in HIV-1 Gag and cause the PI-sensitivity of this virus (Schmitt *et al.*, 2005). The precise role of ubiquitinylation of either viral or cellular targets or of the ubiquitin ligases in virus budding remains unclear, however. Most interestingly, HIV protease inhibitors like nelfinavir and saquinavir display proteasome inhibitory activity (Piccinini *et al.*, 2002 and 2005). This unexpected activity may be responsible for some side effects of these drugs, whether they contribute to the anti-HIV activity, e.g. by inhibiting NF- κ B activation which is involved in positive regulation of HIV gene expression or by inhibiting the budding – is unclear.

An alternative model to explain the PI-sensitivity of HIV suggests that PI facilitate the production of Gag-related defective ribosomal products (DriPs) which are encapsidated together with the normal Gag polyprotein and which may act as competitive non-cleavable substrate inhibitors of the viral protease during Gag-polyprotein processing (Schubert *et al.*, 2000). This hypothesis, however, contradicts the finding that PI are selective only for certain retroviruses.

4.3 Involvement of the UPS in viral gene transcription and translation

The UPS is not only involved in entry and budding of viruses but also in regulation of viral gene expression. Very recently, this effect was demonstrated for Coxsackievirus B3, the most prevalent virus associated with the pathogenesis of myocarditis and cardiomyopathy (McManus *et al.*, 1991; Martino *et al.*, 1994). In vitro treatment of virus-infected murine cardiomyocytes with different PIs caused a strong reduction in virus replication. Inhibition occurred at the level of viral RNA transcription and protein synthesis but did not affect virus entry or proteolytic activity of the virus-encoded protease (Luo *et al.*, 2003). However, the mechanism by which PI inhibit coxsackievirus transcription/translation remains unclear so far.

As a further example, Human Cytomegalovirus (HCMV) as well as Rat Cytomegalovirus (RCMV) in vitro-replication in fibroblasts is efficiently suppressed by different reversible as well as irreversible PI (Prösch *et al.*,

2003; Kaspari, Prösch unpublished data). Inhibition of HCMV replication occurs at the immediate early (IE) stage of infection by suppression of the IE gene expression. Virus adsorption as well as entry and trafficking of the capsid/tegument complex into the nucleus are not influenced. Several lines of evidence support the hypothesis that PI specifically block generation of the IE2 messenger while generation of the IE1 messenger is not affected. Both IE1 and IE2 messengers are generated by different splicing of the IE locus, suggesting that the proteasome may be involved in regulation of IE2 mRNA splicing. In accordance with this it was found that expression and localisation of several splice factors are altered in the absence of a functional UPS (Rockel and Mikecz, 2002). Since the IE2 protein is essential for induction of early and late viral proteins and for virus-dependent cellular gene expression, PI are detrimental to HCMV replication. The structure of the IE gene locus of RCMV is very similar to that of HCMV regarding its modular composition and splicing processes, thus, it might be assumed that PI inhibit replication of RCMV and HCMV by the same mechanism.

Remarkably, in a first experiment in which we treated RCMV-infected immunosuppressed rats with MG262, the viral load in all tested organs was clearly reduced (Gille, Prösch, unpublished data). These results raise the hope that the in vitro observed anti-CMV effect of PI is also present in vivo and that PI represent an alternative treatment option against GCV-resistant strains or a complementary drug to ganciclovir/valganciclovir which block virus replication only at the late stage (Prösch *et al.*, 2003).

A third example for involvement of the proteasome in regulation of viral gene expression are Adenoviruses (Corbin-Lickfett and Bridge, 2003). Adenoviruses require the proteasome for initiation of late gene expression by the E4-34kDa protein, subsequently, PI block expression of late genes. However, in vitro replication of Adenovirus was only marginally reduced in the presence of PI, indicating that the UPS function is not essentially required for efficient replication.

Furthermore, transfection experiments suggest that the UPS influences translation of hepatitis C virus, however, it has not been proved whether PI inhibit HCV replication (Kruger *et al.*, 2001).

Table 2: Viruses for which in vitro antiviral activity of proteasome inhibitors has been demonstrated

Virus	Target of antiviral activity	References
Coronaviridae		
Mouse Hepatitis Virus	Virus entry	Yu and Lai, 2005
Enteroviridae		
Coxsackievirus B3	Transcription	Luo <i>et al.</i> , 2003
Filoviridae *		
Ebola Virus	Virus budding	Harty <i>et al.</i> , 2000 Martin-Serrano <i>et al.</i> , 2005
Herpesviridae		
Human Cytomegalovirus	Transcription	Prösch <i>et al.</i> , 2003
Rat Cytomegalovirus	IE2 protein expression undefined	Kaspari, Prösch, unpubl. Gille, Prösch, unpubl.
Orthomyxoviridae		
Influenza Virus A	Virus entry	Khor <i>et al.</i> , 2003
Paramyxoviridae		
SV5	Virus budding	Schmitt <i>et al.</i> , 2005
Parvoviridae		
Minute Virus of Mice Canine Parvovirus	Entry	Ros <i>et al.</i> , 2002
Rhabdoviridae		
Vesicular Stomatitis Virus	Virus budding	Harty <i>et al.</i> , 2000
Retroviridae		
Human Immunodeficiency Virus	Gag-protein processing,	Patniak <i>et al.</i> , 2000
Rous Sarkoma Virus	assembly, budding,	Strack <i>et al.</i> , 2000
Murine Leukemia Virus	maturation	Schubert <i>et al.</i> , 2000
Mazon Pfizer Monkey Virus		Ott <i>et al.</i> , 2003
HTLV*		Heidecker <i>et al.</i> , 2004

* The antiviral effect has only been shown for the budding of virus-like particles, not for infectious virus.

4.4 The UPS in first line defence against viruses - proteasome inhibition and virus (re)activation

The use of PI as antiviral drugs may be contraindicated by the fact that under certain circumstances some viruses may benefit from proteasome inhibition. One example is the Herpes Simplex Virus (HSV) type 1. Establishment and maintenance of latency in neuronal cells has been found to depend on the proteasomal degradation of the viral UL9 protein which may facilitate reactivation of the virus by binding to and unwinding of the origin 3. UL9 interacts with the neurone specific NFB24 protein and is thus recruited to an E3 ubiquitin ligase complex facilitating its ubiquitinylation and proteasomal degradation. Proteasome inhibition allows accumulation of UL9 and thus induce viral replication/reactivation (Eom and Lehmann, 2003). Similarly, reactivation of latent integrated HIV may benefit from proteasome inhibition (Krishnan and Zeichner, 2004; Schwartz *et al.*, 1998). As a reason it has been speculated that key factors required to effectively activate the HIV-1 LTR are degraded or repressed in latently infected cells and that their stabilisation by PI then promotes transcription. Accordingly, latently infected cells show increased expression of various proteasome subunits and inhibition of the UPS may restore this process.

Additionally, for some HIV strains and the Adeno-associated Virus (AAV), a parvovirus, it has been shown that the proteasome may limit their infectivity. Consequently, PI increased their infectivity by preventing viral degradation during entry. This effect is especially relevant for HIV strains entering the cell by fusion, while strains using the endocytotic pathway are less successible (Wei *et al.*, 2005; Schwartz *et al.*, 2001). One explanation might be that the proteasome degrades Gag proteins within the cytoplasm. Similarly, PI support transduction of the recombinant AAV (rAAV). Normally, in less efficiently transduced cells incoming virus is effectively degraded by the proteasome (Douar *et al.*, 2001; Hansen *et al.*, 2000; Duan *et al.*, 2000). Inhibition of the proteasome enhances nuclear accumulation of the virus and viral gene expression (Duan *et al.*, 2000; Jennings *et al.*, 2004). Since rAAV is of great interest as a vector for gene therapy, proteasome inhibition has demonstrated to promote transgene expression (Yan *et al.*, 2002).

5. CONCLUSIONS

During the last years it became more clearly that the UPS is essentially involved in different steps of virus replication. For some viruses, inhibition of the UPS by PI has been shown fatal at least under certain conditions. This makes PI highly interesting candidates for alternative or complementary treatment of infections with these viruses. All studies so far have been carried out with PI developed as research tools only which are not suitable for clinical use. To evaluate PI as antiviral therapeutics, these experiments have to be confirmed with clinically approved PI like PS-341 and PS-519 and have to be verified in animal settings. Our first in vivo data raise the hope that PI display their antiviral activity not only in vitro but also in vivo.

The therapeutic index of PI as observed in our lab for HCMV and RCMV is low compared to classical antiviral drugs which target virus-specific genes. This emphasises the need to develop PI with increased specificity and reduced toxicity. Alternatively, drugs which target the more specific E3 ubiquitin ligases involved in virus UPS interaction should be more specific and less toxic. Development of such compounds, however, requires the knowledge of viral and cellular factors involved in the interaction between virus and UPS. A further question which has to be addressed is the influence of PI on (re)activation of highly prevalent latent or persistent viruses to exclude that treatment of an acute infection with one virus does not trigger reactivation of another virus. This question is also of substantial interest for the use of PI in anti-cancer therapy because persistent viruses like EBV or HCMV are co-factors for tumour genesis.

Reduced resistance should be one advantage of PI compared to classical antiviral drugs, however, it can not be guaranteed as already shown for other drugs targeting cellular factors. Apart from that, PI are an ideal alternative option for the treatment of viruses resistant to classical antiviral drugs (e.g. GCV-resistant HCMV, Prösch *et al.*, 2003) since they target a cellular factor thus excluding or minimising cross-resistance.

In summary, the present data raise the hope that inhibitors of the UPS support successful treatment or even prevention of severe viral infections as caused e.g. by HIV-1, HCMV or Coxsackievirus because PI are detrimental to replication of these viruses.

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