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Research paper

The cell-penetrating peptide octa-arginine is a potent inhibitor of proteasome activities

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ABSTRACT

Oligo-arginines are cell-penetrating peptides and find use as carriers for transportation of various membrane-impermeable biopharmaceuticals into target cells. We have found that oligo-arginines of a length of 4–10 amino acids, but especially $(Arg)_8$, are able to inhibit the major intracellular proteolytic system, the proteasome, with mixed-type inhibition characteristics. The IC_{50} values of $(Arg)_8$ for the proteasomal chymotrypsin-like and caspase-like activities are approximately 100 and 200 nM, respectively. The inhibition of the trypsin-like activity never exceeds 50% even at micromolar concentrations. $(Arg)_8$ also inhibits 20S proteasome/PA28 complexes as well as 26S proteasomes, although with a decreased efficiency. Due to its cell membrane-penetrating capability, incubation of HeLa cells in the presence of $(Arg)_8$ resulted in an impaired activity of proteasomes going along with an accumulation of high-molecular mass ubiquitin-conjugated proteins, the preferred substrates of 26S proteasomes. The *in vivo* susceptibility of the three proteasome activities resembles that found *in vitro* with chymotrypsin-like > caspase-like > trypsin-like activities. Since inhibition of the proteasome system might affect fundamental basic cellular processes but on the other side might also prevent the degradation of a proteinacous cargo, we suggest that this proteasome inhibitory activity should be taken into account when oligo-arginines are being considered for use as vectors for the intracellular delivery of pharmaceuticals.

1. Introduction

One obstacle to the successful delivery of biopharmaceuticals in the drug-based therapeutic interventions is the impermeability of biological membranes. A great step forward in this area came with the discovery and development of cell-penetrating peptides (CPPs) [1,2]. One important group of CPPs is oligo-arginine vectors, the membrane permeability of which was identified fortuitously in the arginine-rich sequence responsible for the internalization of the HIV Tat protein [3]. Oligo-arginines have been used to transfer various compounds and proteins into cells and tissues [4]. Although the mechanism of uptake of oligo-arginines into the cell is not entirely clear, interaction with negatively charged membrane proteoglycans, macropinocytosis, and actin rearrangement seems to be involved [5,6]. Even less well understood is the fate of oligo-arginine peptides within the cell. Whilst one fraction might enter lysosomes, another seems to escape from endosomes via the Golgi by retrograde transport, or by other undefined mechanisms, to end up in the cytosol [7]. Membrane transduction of oligo-arginines directly into the cytosol and not mediated by macropinocytosis was observed in certain cases [8,9]. On its transition through the various cell compartments, oligo-arginine is exposed to many proteases and peptidases and, thus, may constantly be subject to degradation. In contrast, oligo-arginines have been found to be potent inhibitors of proteases located within the endosomal-lysosomal compartment, with examples including furin [10,11] and cathepsin C [12]. This property may ensure that considerable amounts of oligo-arginine peptides survive administration and, when applied intravascularly, allow them to penetrate deep into the tissue [13]. Oligo-arginine-mediated inhibition of furin has been used to suppress productive infection of T-cell lines by HIV-1, since furin is essential for the correct processing of the HIV envelope protein gp160 [14].

Abbreviations: Arg, arginine; Bz-VGR, benzoyl-Val-Gly-Arg; CPP, cell-penetrating peptide; AMC, 7-amino-4-methylcoumarin; Suc-LLVY, succinyl-Leu-Leu-Val-Tyr; Z-LLE, benzyloxycarbonyl-Leu-Leu-Glu.

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Once located within the cytosolic compartment, the oligo-arginine peptide encounters the major intracellular proteinase, the proteasome. This can degrade substrate proteins in an ubiquitin-dependent or ubiquitin-independent fashion, and is not only responsible for the general turnover of most cellular proteins but is also involved in proteolytic regulation of cellular processes such as cell cycle, cell growth, differentiation, antigen processing, and regulation of metabolic pathways to name just a few [15]. The proteasome has been shown to be inhibited by a proline/arginine-rich polypeptide, designated PR39 [16,17]. PR39 derives from the C-terminus of a precursor protein that belongs to the family of cathelicidins. These proteins have preprosequences almost identical to cathelin, a protein isolated from porcine leukocytes, but with variable C-terminal sequences demonstrated to possess antimicrobial activity [18]. The N-terminal 11 amino acids of PR39, designated PR11, have been shown to inhibit proteasomal activity [19], with the three N-terminal arginyl residues being mandatory for the inhibitory effect in both cases. As arginyl oligomers are widely used as transportation carriers, we were interested whether these peptide vectors *per se* might exhibit proteasome inhibitory activity. In the present investigation, we demonstrate that oligo-arginine peptides, especially the cell-penetrating peptide $(Arg)_8$, exhibit strong proteasome-inhibiting properties both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Peptides

Peptides were synthesised using Fmoc/*tert*-butyl-based solid-phase synthetic procedures, were purified by reverse-phase HPLC, and were analysed by reverse-phase HPLC and mass spectrometry. Data obtained were fully consistent with the desired structures and peptide purities of $\geqslant$95%. Peptide content was determined by amino acid analysis following acid hydrolysis. Net peptide content was taken into account when preparing all peptide solutions for use and thus peptide molarities stated are absolute. The peptides $(Lys)_8$, $(Orn)_8$, $(His)_6$, $(Arg)_8$, $(Arg)_9$, $(Arg)_{10}$, AARRRRRR, RAARRRRR, RRAARRRR, RRRAARRR, RRRRAARR, RRRRRAAR, RRRRRRAA as well as $(D\text{-}Arg)_8$ were prepared in house. The peptides $(Arg)_3$, $(Arg)_4$, $(Arg)_6$, $(Lys)_3$, $(Ala)_3$, $(Ala)_6$, and $(Asp)_4$ were purchased from Bachem (Switzerland).

2.2. Purification of proteasomes and PA28

As described elsewhere, 20S proteasomes were purified from rat skeletal muscle tissue and human erythrocytes [20]. 26S proteasomes as well as PA28 were purified from human erythrocytes [21,22].

2.3. Determination of proteasome activity

Suc-LLVY-AMC and Bz-VGR-AMC were purchased from Bachem, Z-LLE-AMC from Calbiochem, Darmstadt. The activities of purified proteasomes (50–100 ng/10 μl) were measured routinely using fluorogenic peptides dissolved in 20 μl of TEAD buffer (20 mM Tris/HCl, 1 mM EDTA, 1 mM DTT, 1 mM NaN_3, pH 7.0). Unless stated otherwise, Suc-LLVY-AMC was used at a final concentration of 100 μM to test the chymotrypsin-like activity, Bz-VGR-AMC at 200 μM for measuring the trypsin-like activity, and Z-LLE-AMC at 200 μM for determination of the caspase-like activity. Degradation assays were performed at 37 °C for 30 min and the released AMC was measured fluorimetrically as described elsewhere [20]. Activity of 26S proteasome was measured as described in [23]. To investigate potential inhibition, oligo-arginines and other peptides were dissolved in 10 μl H_2O and pre-incubated with proteasomes for 5 min at room temperature before substrates were added. For measurements of the effect of $(Arg)_8$ on proteasome activity in cell extracts, 10 μl of cell extract (0.2 mg protein/ml) was mixed with 10 μl TEAD buffer or (Arg)8 solution and then incubated for 10–20 min at 37 °C with 20 μl of substrate solution before AMC release was measured.

2.4. Cell experiments

The human cervix carcinoma cells (HeLa) were seeded at a concentration of 5×10^5 cells in each well of a six-well plate and grown in 1.5 ml of DMPI medium overnight under standard conditions. To study the influence of oligo-arginines on proteasome activity, cells were treated with different concentrations of $(Arg)_8$ dissolved in growth medium or growth medium alone as a control. For the measurement of proteasome activity, HeLa cells were washed with PBS, collected by rubber policeman, and then lysed by sonication in TEAD buffer. Insoluble material was precipitated by centrifugation for 10 min at 11,000*g*. The protein concentrations of cell lysate preparations were determined by Bradford assay. Cell extracts were diluted with TEAD buffer to similar protein concentrations (200 μg/ml) and then used for measurement of proteasome activity by incubation of 20 μl of cell extract with 20 μl of substrate solution for 10 min at 37 °C before AMC release was measured fluorimetrically.

For detection of ubiquitinated proteins, aliquots of the cell extracts containing 5 μg protein each were subjected to SDS–PAGE and Western blotted onto PVDF membranes. Poly-ubiquitinated proteins were detected by use (dilution 1:4000) of a polyclonal antibody to ubiquitin (DAKO, Denmark) and visualized by enhanced chemiluminescence technique [20]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was probed as a protein loading control. Quantitative determination of ubiquitinated proteins was performed by densitometric analysis using the NIH ImageJ programme.

3. Results

3.1. Effect of oligo-arginyl peptides on proteasome activity

In preliminary experiments, we corroborated findings by other investigators [19] that the proteasome inhibitory activity of PR39 was solely due to its N-terminal region (PR11), while the C-terminal region showed an activating effect (data not shown). Since replacement of each of the N-terminal three arginine residues of PR11 by alanine abolished the inhibitory effect on the chymotrypsin-like activity of 20S proteasomes [19] and on proteasome-mediated IκBα degradation [16], we investigated whether arginyl-oligo-peptides *per se* have inhibitory activity on 20S proteasomes. If not stated otherwise, tests were performed with 20S proteasomes purified from rat skeletal muscle. $(Arg)_3$ had no effect at nanomolar and low micromolar concentrations, while at high micromolar concentrations the tripeptide exhibited an activating effect on the chymotrypsin-like activity of 20S proteasomes (Fig. 1A). However, arginyl-oligopeptides of chain length $\geqslant$4 exerted considerable proteasome inhibitory activity. This effect increased with peptide length, as was clearly observed when $(Arg)_4$ and $(Arg)_6$ were compared. Among the compounds tested, $(Arg)_8$ had the optimum chain length with respect to proteasome inhibition, since $(Arg)_9$ and $(Arg)_{10}$ showed a comparatively lower inhibitory effect. Even at a concentration of 10 μmol/l, inhibition by $(Arg)_9$ and $(Arg)_{10}$ did not exceed 80% (Fig. 1B). To investigate whether the inhibitory effect was confined to arginyl-oligopeptides, several peptides containing amino acids other than arginine were tested for their capacity to affect the chymotrypsin-like activ-

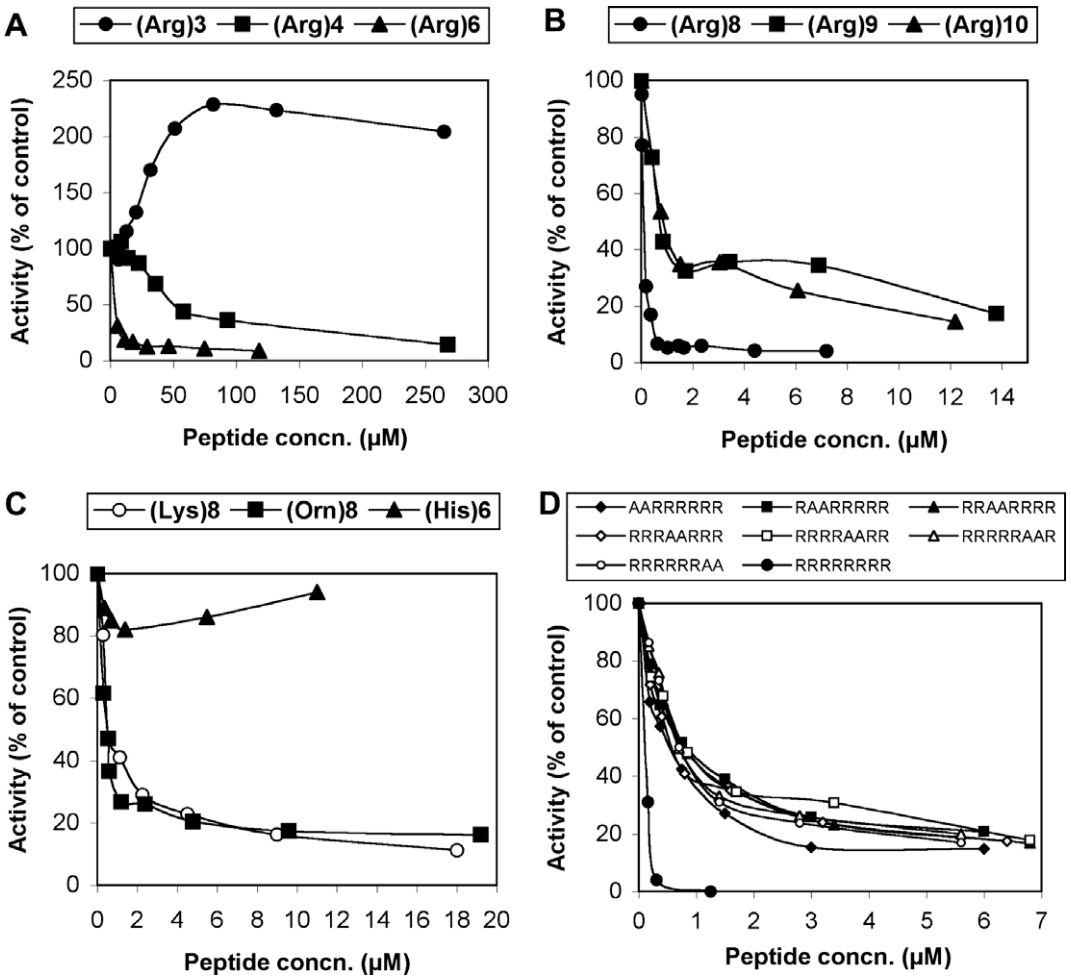

Fig. 1. Effect of oligo-arginine and non-arginine peptides on proteasome activity. 20S proteasome (100 ng) was incubated with increasing amounts of different peptides for 5 min at 21 °C before the chymotrypsin-like activity of proteasomes was measured by the addition of substrate. All data shown are mean values of two separate experiments.

ity of 20S proteasomes. No effect was observed with $(Ala)_3$, $(Ala)_6$, $(Lys)_3$ (data not shown) or with $(His)_6$, even at high micromolar concentrations (Fig. 1C). On the other hand, oligo-peptides of other positively charged amino acids such as $(Lys)_8$ and $(Orn)_8$ inhibited 20S proteasomes, but only to the extent seen with $(Arg)_9$ and $(Arg)_{10}$ (Fig. 1C). These results suggest that a prerequisite for effective inhibition is an accumulation of positively charged amino acids within a peptide comprising a minimum of six amino acids.

To investigate whether, in order to exhibit proteasome inhibitory activity, the accumulation of positively charged amino acids must be located at the N-terminus of the octapeptide, we replaced two arginine residues by alanine at a time and then tested their inhibitory activity. As shown in Fig. 1D, all peptides tested inhibited the chymotrypsin-like activity of 20S proteasomes similarly; however, their IC_{50} value was higher (~800 nM) than that of $(Arg)_8$, with complete inhibition not being obtained within the range of peptide concentrations tested (up to 10 μM).

3.2. Inhibition of multiple proteolytic activities of 20S proteasomes by $(Arg)_8$

Three sites with differing proteolytic specificity are located within the barrel-shaped 20S proteasome complex and catalyse amide bond hydrolysis. Therefore, it was of interest to examine whether $(Arg)_8$ inhibited all three activities equally. Fig. 2 summarizes the results obtained when the three activities were measured by the use of specific fluorogenic peptide substrates in the presence of increasing amounts of $(Arg)_8$. IC_{50} for inhibition of the chymotrypsin-like activity by $(Arg)_8$ is about 100 nM, whilst some 200 nM was required for 50% inhibition of the caspase-like activity and the inhibition did not exceed 80% even at a concentration of 8 μM (Fig. 2A). The trypsin-like activity was also inhibited but never exceeded 50% and almost no inhibition occurred at concentrations below 2 μM (Fig. 2A). Additionally, we have found that inhibition by $(Arg)_8$ did not depend on the time of pre-incubation with proteasomes (data not shown). To scrutinize the mechanism of inhibition by $(Arg)_8$, we used a direct linear plot (V_{max}^{app}/K_M^{app}) analysis. As shown for both chymotrypsin-like (Fig. 2B) and caspase-like (Fig. 2C) activities, intersections occur in the third quadrant indicating that $(Arg)_8$ affects the proteasome by multisite or mixed-type inhibition [24] .

3.3. Susceptibility of 26S proteasomes and 20S proteasome/PA28 complexes to inhibition by $(Arg)_8$

As the mechanism of inhibition of proteasomes by $(Arg)_8$ is based on multisite interactions (Fig. 2B and C), it is possible that the susceptibility of proteasomes to $(Arg)_8$ may be restricted when regulator/activator complexes, such as the 19S regulator or the PA28 activator, are attached to the 20S core. These regulator/activator complexes are associated with the outer α-rings of the 20S proteasome core and thereby may affect the ability of 20S protea-

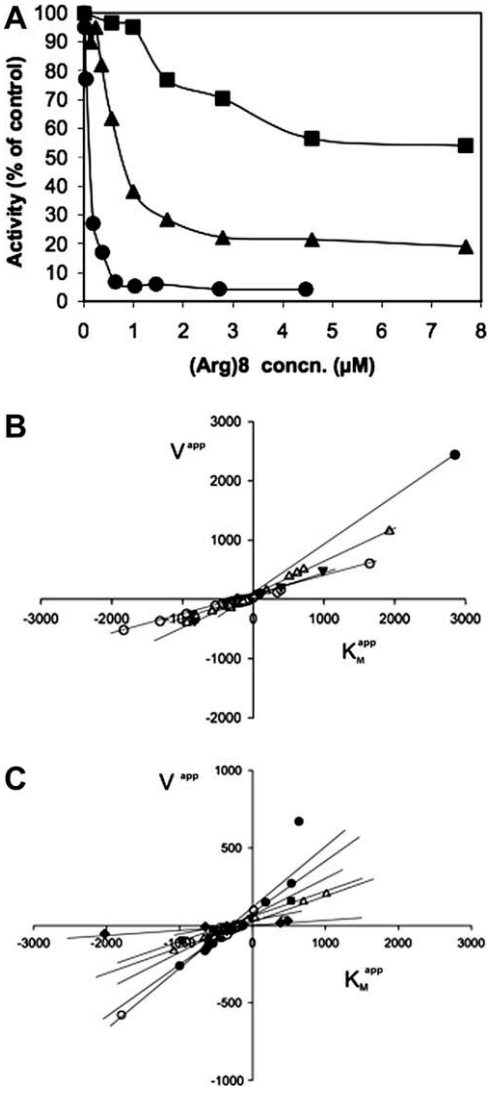

Fig. 2. Effect of $(Arg)_8$ on 20S proteasome activities. (A) 20S proteasome (100 ng) was incubated with increasing amounts of $(Arg)_8$ for 5 min at 21 °C before the three activities of proteasomes were measured by addition of the substrates Suc-LLVY-AMC (●), Z-LLE-AMC (▲), Bz-VGR-AMC (■). Data are mean values of two experiments. (B) 20S proteasomes were incubated with different amounts of $(Arg)_8$ (●, 0 nM; △, 750 nM; ▼, 1000 nM; ○, 3000 nM) before the chymotrypsin-like activity was measured with eight different concentrations (25–200 μM) of substrate Suc-LLVY-AMC. (C) 20S proteasomes were incubated with different amounts of $(Arg)_8$ (●, 0 nM; ○, 66 nM; ▼, 133 nM; △, 266 nM; ■, 317 nM; □, 625 nM; ♦, 1250 nM) before the caspase-like activity was measured with six different concentrations (98–250 μM) of substrate Z-LLE-AMC. Data (mean values of two experiments) in C and D are depicted as direct linear plots (V^{app} vs. K_M ^{app}) calculated by using SigmaPlot software. Scale unit for K_M is in nM, V is given in pmol/min.

somes to bind $(Arg)_8$. Therefore, we tested whether $(Arg)_8$ is also able to inhibit the activity of 26S proteasomes. As shown in Fig. 3, inhibition of the chymotrypsin-like activity of 26S proteasomes occurred, but remained incomplete, even at micromolar concentrations of the $(Arg)_8$-peptide. To test the susceptibility of 20S proteasome/PA28 complexes to the inhibition by $(Arg)_8$, 20S proteasomes and PA28 were mixed to allow complex formation and maximum activation [22] before increasing the concentrations of $(Arg)_8$ were added and the remaining proteolytic activities were determined. Fig. 3 shows that all three peptide-hydrolysing activities of the 20S/PA28 proteasome complexes were markedly diminished by $(Arg)_8$, albeit with inhibition of the chymotrypsin-like

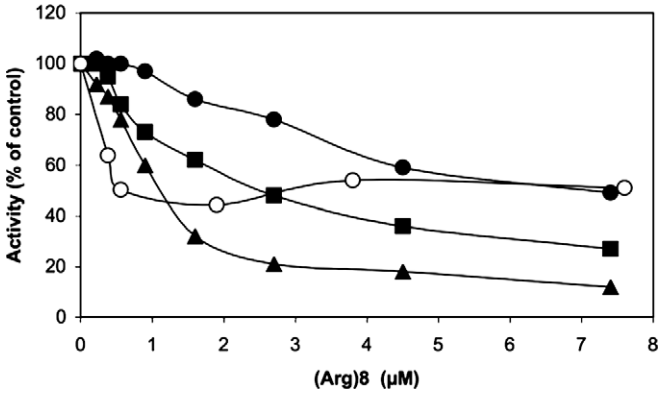

Fig. 3. Effect of $(Arg)_8$ on the activities of 26S- and 20S/PA28-proteasome complexes. 26S proteasome from human erythrocytes was incubated with increasing amounts of $(Arg)_8$ (O) for 5 min at 21 °C before chymotrypsin-like activity was tested by addition of the substrate Suc-LLVY-AMC. 20S proteasome from human erythrocytes mixed at a molar ratio of 1:2 with PA28 from human erythrocytes was incubated with increasing concentrations of $(Arg)_8$. After 5 min of incubation at 21 °C, the proteolytic activities of the complexes were measured by addition of the substrates Suc-LLVY-AMC (●), Z-LLE-AMC (▲), and Bz-VGR-AMC (■). Data are mean values of two separate experiments.

activity being lower when compared to the effect of $(Arg)_8$ on 20S proteasomes alone (Fig. 2A).

3.4. Efficacy of $(Arg)_8$ under in vivo conditions

All inhibition tests of proteasomes with $(Arg)_8$ so far described have been performed in low salt buffer at concentrations far below those of physiological relevance. Since binding of a basic, positively charged peptide such as $(Arg)_8$ to proteasomes having an isoelectric point of 5.2 might depend on electrostatic interactions, it was of interest to know whether stable inhibition of proteasomes might also occur under physiological ionic concentrations. Therefore we tested whether intracellular concentrations of NaCl, KCl and $MgCl_2$ affected the inhibitory activity of $(Arg)_8$. Although both Na^+ and K^+ ions inhibited proteasome activity in isolation, this effect was abrogated by the stimulating effect of $MgCl_2$, resulting in an 80% higher proteasomal activity under physiological salt concentrations when compared to values measured in TEAD buffer alone (Table 1). Addition of $(Arg)_8$ under physiological salt concentrations led to ~63% inhibition of the chymotrypsin-like activity. The degree of inhibition did not increase even if the $(Arg)_8$ concentration was increased 40-fold; however, it remained stable when the inhibited complex was dialysed against buffer at physiological salt concentration (Table 1). Thus, $(Arg)_8$ has the ability to affect proteasomes under ionic salt concentrations similar to those experienced under physiological conditions.

To validate this further, we tested the effect of $(Arg)_8$ on proteasome activity in HeLa cells. For inhibition of proteasomes in extracts of HeLa cells addition of $(Arg)_8$ at concentrations higher than 10 μM were required. Maximum inhibition of the chymotrypsin-like activity was 60% irrespective of whether L- or D-$(Arg)_8$ was added (Fig. 4A). A similar range of inhibition was observed when (Arg)8 was added to the culture medium. As shown in Fig. 4B, culturing HeLa cells for 2 h in the presence of 30 or 60 μM $(Arg)_8$ before determination of proteasome activity was performed resulting in inhibition of the chymotrypsin-like activity of proteasomes to about 60% and inhibition of the caspase-like activity to about 50%; no significant effect on the trypsin-like activity was detectable. Longer incubation periods of HeLa cells with $(Arg)_8$ did not increase its inhibitory effect. In accordance with the findings by other investigators [25,26], we found that $(Arg)_8$ causes no cell toxicity as long as its concentration remained below 100 μM. Since we have

Table 1
Effect of intracellular salt concentrations on proteasome activity and inhibition by $(Arg)_8$. 20S proteasomes were dialysed against TEAD buffer (20 mM Tris/HCl, 1 mM EDTA, 1 mM NaN_3, 1 mM DTT, pH 7.0) or TEAD buffer containing NaCl, KCl and $MgCl_2$ at the concentrations detailed below for 2 h at 4 °C. Afterwards, Suc-LLVY-AMC hydrolysing activity of the proteasomes was measured with or without prior incubation (5 min, 21 °C) with $(Arg)_8$ at different concentrations. 15 mM NaCl/140 mM KCl/15 mM $MgCl_2$ is designated as 'intracellular salt'. Data are given as mean ± SD of two experiments.

Buffer components and $(Arg)_8$ concn.	Proteasome activity		
	(pmol/min ml)	%	%
TEAD	70.9 ± 4.9	100	
15 mM NaCl/TEAD	43.6 ± 0.6	62	
140 mM KCl/TEAD	45.8 ± 0.2	65	
15 mM $MgCl_2$/TEAD	191.2 ± 17.8	273	
'intracellular salt'/TEAD	125.9 ± 8.4	180	100
'intracellular salt'/TEAD + 0.76 μM $(Arg)_8$	47.0 ± 3.1		37
'intracellular salt'/TEAD + 7.6 μM $(Arg)_8$	50.2 ± 0.3		39
'intracellular salt'/TEAD + 31 μM $(Arg)_8$	43.6 ± 2.6		34
'intracellular salt'/TEAD + 31 μM $(Arg)_8$ and then 4 h dialysis against 'intracellular salt'/TEAD	46.1 ± 0.9		36

shown *in vitro* that $(Arg)_8$ affects the activities not only of 20S proteasomes but also of 26S proteasomes, we investigated whether this holds true also under *in vivo* conditions. Ubiquitin-conjugated proteins are the preferred substrates of 26S proteasomes and their rapid degradation can be inhibited by proteasome inhibitors such as MG132 [27]. Therefore, we inspected the ubiquitinated proteins in extracts of HeLa cells (Fig. 4B) and measured their concentration in dependence of previous treatment of the cells with $(Arg)_8$ (Fig. 4C). As high-molecular mass ubiquitin-conjugated proteins accumulated in the cells treated with $(Arg)_8$ at concentrations significantly inhibiting the proteasome activities, we conclude that the inhibition of 26S proteasomes by (Arg)8 resulted in a considerable decrease in breakdown of ubiquitin-conjugated proteins.

4. Discussion

The bioavailability of many drugs and other bioactive compounds is limited due to their low absorption and permeability through biological membranes [28]. Similarly, gene transfer, as well as introduction of proteins into target cells, is very often hampered by the impermeability of biological membranes for these macromolecules. This obstacle was effectively removed by the discovery of protein transduction domains that are present in several proteins, *e.g.* the HIV transactivator protein, where the domain consists of a cationic stretch of amino acids [29,30]. Based on these findings, several other positively charged peptides were tested for their ability to translocate across biological membranes, and oligoarginines were found to have a high potential to carry various compounds into mammalian cells [25,31–33].

The capacity for inhibition of the major intracellular proteinase, the proteasome, by a arginine-rich biological processing product of a membrane-penetrating cathelicidin-like antimicrobial precursor protein of leucocytes, PR39, is based on its N-terminal three arginine residues. Consequently, we investigated whether membrane-penetrating arginine-rich carrier peptides also possess proteasome-inhibiting activity and, thus, may influence cell metabolism and viability.

Surprisingly, the arginyl tripeptide, $(Arg)_3$, does not inhibit but stimulates the proteolytic activities of proteasomes and, thus, behaves like hydrophobic peptides that promote proteasomal peptide hydrolysis [34]. However, with the addition of a further single arginyl residue, stimulation is abrogated and an inhibitory effect is observed, as seen with $(Arg)_4$. Both of these effects are observed at peptide concentrations in excess of 30 μM. However, stepwise elongation of the peptide by a further two arginyl residues leads to an exponential reduction in IC50: ~110 μM, ~7 μM, and ~0.1 μM for $(Arg)_4$, $(Arg)_6$, and $(Arg)_8$, respectively. Beyond $(Arg)_8$, further chain elongation only serves to heighten the IC50 again to about 1 μM for $(Arg)_9$ and $(Arg)_{10}$. The finding that neither $(Lys)_8$ nor $(Orn)_8$, which also possess cell-penetrating properties [35,36], is as effective as $(Arg)_8$, and that exchange of arginine by alanine at any given position in $(Arg)_8$ diminishes its activity, shows that the homo-oligomeric octa-arginine is of an optimal size and charge to inhibit 20S proteasomes. Interestingly, it is also of the optimal length for cell membrane penetration [25]. Octa-arginine was also found to inhibit the lysosomal protease cathepsin C with inhibition not being improved by elongation [12]. However, the IC_{50} for this enzyme is about 30 μM and is thus 200-fold higher when compared to proteasomes. Nona-arginine derivatives have been shown to be potent inhibitors of the calcium-dependent, membrane-bound serine protease furin. In a similar fashion to that of cathepsin C, inhibition of furin by $(Arg)_9$ occurs in a competitive manner with longer incubation times leading to substrate degradation to $(Arg)_7$, $(Arg)_6$, and $(Arg)_5$; although these fragments still exert an inhibitory effect on furin [11]. We have incubated proteasomes with $(Arg)_8$ for up to 6 h with subsequent mass spectrometric analysis showing no more than 1–3% degradation of the peptide to truncated forms (data not shown). Thus, the inhibitory effect of $(Arg)_8$-peptide is essentially not degraded by proteasomes during the incubation period. This fits with the results of our kinetic analysis showing that the mechanism of inhibition of proteasomes by $(Arg)_8$ is not of a competitive type but rather of a mixed-type due to multisite binding of the peptide to proteasomes.

Peptides are known to affect the proteasome activity due to the peculiarity of the architecture of proteasomes. The 20S proteasome is built up of four stacked seven-membered rings, with α7β7β7α7 stoichiometry and the catalytic sites being located in the β-subunits rings within the interior of the barrel-like protein complex. To enter the proteolytic cavity, substrate proteins have first to gain access through a narrow central channel in the two outer rings composed of the α-subunits [37]. These channels may be occluded by an N-terminal tail sequence of one of the α-subunits thereby preventing entry of bulky substrates [38]. It has been suggested that opening and closing ('gating') of these channels go along with a conformational transition from a barrel – to a cylinder-shape of the proteasome complex; this change appears to occur equally well in the presence of peptide or protein substrates [39]. For example, hydrophobic tri- and tetrapeptides, which bind to non-catalytic modifier sites, have been found to promote peptide hydrolysis possibly by opening these channels [34]. On the other hand, small peptides that are degraded by the caspase-like activity of the proteasome have also been shown to bind to non-catalytic modifier sites and cause inhibition, particulary of the chymotrypsin-like activity [40,41]. Since hexapeptides containing either one basic amino acid residue or those containing one acidic amino acid residue inhibit proteasomes by different kinetic mechanisms, it has been suggested that there is more than one modifier site in 20S proteasomes [42].

In a similar fashion to that of $(Arg)_8$, PR39 and HIV Tat proteins have been shown to inhibit 20S proteasomes in a non-competitive way, most probably by interaction with α-subunits [43]. It was suggested that inhibition is due to the presence of a sequence of basic amino acids (-RKKRRQRRR-) within the viral Tat protein [44,45] that binds to 20S proteasomes in such a way that it competes with the binding of the proteasome activator PA28. $(Arg)_8$ inhibits the activity of 20S proteasome/PA28 complex as well as that of 20S proteasome/19S regulator complexes (26S proteasome), although the effects are attenuated, indicating that binding of $(Arg)_8$ is hampered in 20S proteasome-activator/regu-

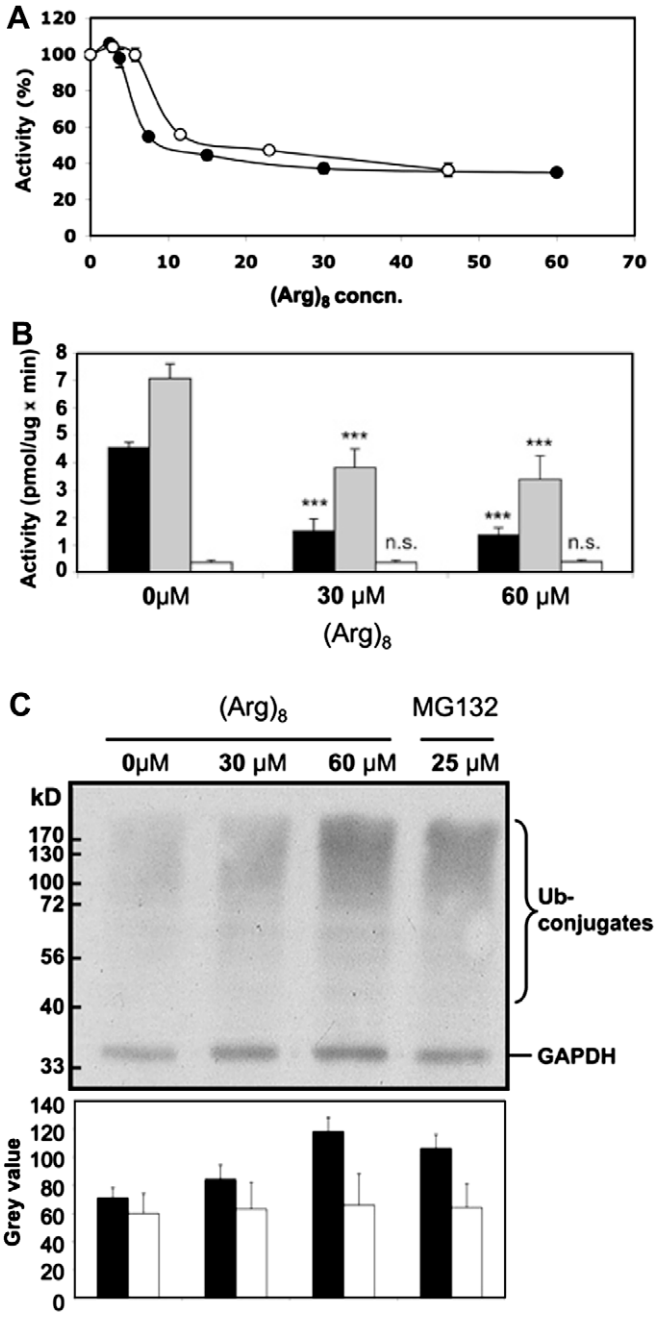

Fig. 4. Effect of $(Arg)_8$ on proteasome activity in HeLa cells. (A) HeLa cells were cultured, harvested, washed, homogenized and cell extract prepared by centrifugation. The chymotrypsin-like activity was measured in the cell extract with Suc-LLVY-AMC as a substrate 5 min after addition of (D-$Arg)_8$(O) and (L-$Arg)_8$ (●) at the concentrations indicated (means ± SD of a single experiment performed in triplicate). (B) HeLa cells were cultured in the absence or presence of 30 and 60 µM $(Arg)_8$, respectively. After 2 h, cells were harvested, washed and extracts prepared for measurement of the chymotrypsin-like (black columns), caspase-like (grey columns) and trypsin-like (white columns) activities. Specific activities are given as means ± SD of three experiments. Statistically significant differences compared to controls (0 µM) were calculated by *t*-test, $^{***}p < 0.001$; n.s., not significant. (C, upper panel) Aliquots (5 µg protein) from the cell extracts prepared under (B) were subjected to SDS–PAGE, Western blotted and then probed with an antibody raised against ubiquitin. For comparison an extract of HeLa cells grown in the presence of MG132 was run in parallel. The blot was reprobed with an antibody to glycerin-aldehydephosphate-dehydrogenase (GAPDH) as loading control and the blot quantitated by densitometric analysis (C, lower panel). Black bars represent the amount of ubiquitinated proteins and white bars the amount of GAPDH.

lator complexes. Since a large proportion of proteasomes *in vivo* are complexed with activators and regulators [46], it was important to examine the ability of $(Arg)_8$ to affect the proteasomal activity under physiological conditions. Although we have shown that physiological salt concentrations slightly attenuate the interaction between $(Arg)_8$ and proteasomes, the peptide significantly suppresses the proteasome activity in HeLa cells. Even though concentrations of $(Arg)_8$ as high as 30–60 µM effect only a 50–60% inhibition of proteasome activity in HeLa cells, the susceptibility of the three activities to the inhibition by $(Arg)_8$ followed a similar order to that observed *in vitro*, where the chymotrypsin- and caspase-like activities are very sensitive to the peptide, whereas the trypsin-like activity is not. Since more than 90% of the chymotrypsin-like and of the caspase-like activities measurable in HeLa cell extracts are due to the activity of proteasomes [47], the reduced *in vivo* effectiveness of $(Arg)_8$ is not due to the presence of other $(Arg)_8$ insensitive proteases, but may rather be due to the lower susceptibility to $(Arg)_8$ of 26S proteasomes and proteasome/PA28 complexes, constituting about 50% of all proteasomes in HeLa cells [46]. Another reason may be that the route of cellular-uptake of arginine-peptides proceeds *via* the endolysosomal compartment, where a considerable part of the peptides were found to be degraded [48] before eventually somehow being released to the cytoplasmic compartment containing the majority of proteasomes. However, the mechanism of uptake of CPPs is still a matter of debate [49,50]. The uptake of oligo-arginine has recently been scrutinized especially in HeLa cells and found not to be mediated by macropinocytosis [8] supporting data obtained in previous investigations [9,51]. Our finding on the inhibition of proteasome activity by $(Arg)_8$ can also be interpreted as indicative for a direct transduction of $(Arg)_8$ through the cell membrane into the cytoplasm, thus offering the possibility to deliver cells by (Arg)8 with cargos that otherwise are membrane-impermeable [1,2,52]. Even if – due to a minor uptake pathway by macropinocytosis – $(Arg)_8$ should partially be degraded to smaller peptides, one has to consider that $(Arg)_6$ and $(Arg)_4$ remain able to inhibit proteasome activities, especially when present at high concentrations. A clear indication of the *in vivo* inhibitory efficacy of $(Arg)_8$ on proteasomes is the increase in the concentration of ubiquitin-conjugated proteins that are well known to accumulate after inhibition of 26S proteasomes [27]. As 26S proteasomes are involved in the regulation of many fundamental processes for maintaining cell viability, for example regulation of cell cycling, apoptosis, transcription, signal transduction, metabolism, and antigen processing [15], inhibition of this enzyme system as a side-effect of an oligo-arginyl-carrier entering a cell may have unpredictable consequences. Whether these are deleterious or beneficial effects may depend on the cell type and on the extent of proteasome inhibition in the target tissues [53]. Proteasome inhibition might be detrimental to a cell if it provokes induction of apoptosis, and is therefore used as a strategy for therapeutic treatment of neoplastic and rapidly growing cells [54]. On the other hand, prevention of unwanted, premature proteasomal degradation of a CPP and especially its cargo if it is of proteinaceous nature, could be a desired effect by using $(Arg)_8$ as a carrier. Therefore, we suggest that it is important to take due consideration of such matters when oligo-arginine peptides are being considered as cell-penetrating carriers.

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