Progress Review

Ubiquitin-Proteasome System and Proteasome Inhibition: New Strategies in Stroke Therapy

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Background and Purpose—Proteasomes are large multicatalytic protease complexes that are found in the cytosol and in the nucleus of eukaryotic cells with a central role in cellular protein turnover. The ubiquitin-proteasome system (UPS) has a central role in the selective degradation of intracellular proteins. Among the key proteins whose levels are modulated by the proteasome are those involved in the control of inflammatory processes, cell cycle regulation, and gene expression. There are now overwhelming data suggesting that the UPS contributes to cerebral ischemic injury.

Summary of Review—Proteasome inhibition is a potential treatment option for stroke. Thus far, proof of principle has been obtained from studies in several animal models of cerebral ischemia. Treatment with proteasome inhibitors reduces effectively neuronal and astrocytic degeneration, cortical infarct volume, infarct neutrophil infiltration, and NF-κB immunoreactivity with an extension of the neuroprotective effect at least 6 hours after ischemic insult. However, it is clear that the UPS represents a central pathway for the processing and metabolism of multiple proteins with critical roles in cellular function. To avoid eliciting significant side effects associated with complete inhibition of the proteasome and the possible immunosuppressive effects from persistent suppression of NF-κB activation, it is critical that we understand how to partially and temporally attenuate proteasome function to elicit the desired therapeutic effect before any large-scale use in humans.

Conclusion—This review highlights the most recent advances in our knowledge on UPS, as well as the early experience of using proteasome inhibition strategies to treat acute stroke. (Stroke. 2004;35:1506-1518.)

Key Words: cerebral ischemia ■ NF-kappa B ■ inflammation ■ ubiquitin ■ proteasome

The progression and extension of cerebral ischemia are related to several mechanisms, many of which involve an inflammatory response component.1 Recent neuroprotective strategies include targeting inflammatory mediators as a whole, although it has proved difficult because of their redundancy. Alternate inflammatory pathways may circumvent the suppression of a single targeted mediator.2 Targeting inflammatory cascades as a whole is another approach, although it has shown no benefit.3 Novel approaches have now focused on alteration of inflammatory transcriptional factors to simultaneously interfere with the upregulation of multiple inflammatory genes.

One promising approach is to suppress the activation of transcription nuclear factor-kappa B (NF-κB) by stabilizing the inhibitory protein IκB (via inhibition of the ubiquitin (Ub)-proteasome system (UPS)).4 UPS is the major nonlysosomal pathway of proteolysis in human cells and accounts for the degradation of most short-lived, misfolded, or damaged proteins, as well as long-lived proteins. This pathway is important in the regulation of a number of key biological regulatory mechanisms.5 Inhibitors that target the UPS should suppress the activation of NF-κB by stabilizing IκB, thereby reducing levels of multiple proinflammatory proteins, providing antiinflammatory effects and, ultimately, successfully attenuating the inflammatory cascade in cerebral ischemia, leading to the reduction of the ischemic damage. As such, proteasome inhibitors are a novel approach to the treatment of cerebral ischemia. They have entered clinical evaluation based on efficacy and action demonstrated in laboratory studies, uncovering insights into the pathophysiology of cerebral ischemia.

Ubiquitin and Ubiquitination
Ub is covalently attached to other proteins via either an isopeptide (via ε-amino groups of internal Lys) or peptide bond.3 Ub conjugation (ubiquitination) is performed by 1 of multiple Ub ligases (E3s). E3s transfer Ub from 1 of several Ub-conjugating enzymes (UBCs or E2s), which obtain Ub from the Ub-activating enzyme (UBA or E1). Ub activation requires energy provided by ATP hydrolysis (Figure 1). The recognition of a substrate by an E3 involves various mechanisms: the presence of a specific primary sequence, an N-terminal destabilizing amino acid, phosphorylation of a specific residue, or poorly characterized structural motifs.5

Received October 14, 2003; final revision received December 31, 2003; accepted February 9, 2004.
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Stroke is available at http://www.strokeaha.org

DOI: 10.1161/01.STR.0000126891.93919.4e

1506
Polyubiquitin chains are often trimmed by multiple deubiquitininating enzymes (DUBs), which counteract the E1-E2-E3 cascade rescuing certain substrates from degradation.6

20S and 26S Proteasomes

The 26S proteasomes are composed of the 20S proteasome core and two 19S caps (PA700 activators). The 20S proteasome is a barrel-shaped molecule made up of 4 stacked rings, 2 outer α-rings and 2 inner β-rings. Each ring is made up of 7 different subunits of the α-type and β-type, respectively. Cleavage of the peptide bonds is performed in the central catalytic chamber of the 20S proteasome by a nuclease attack of an N-terminal Thr residue of either β5, β2, or β1 subunits,6,7 displaying different substrate specificity. These activities have been named chymotrypsin-like (ChTL), trypsin-like (TL), and post-glutamyl-peptide hydrolyzing (PGPH), because they cleave peptides after hydrophobic, basic, and acidic residues, respectively.8

20S proteasomes degrade short peptides and unfolded proteins. Unfolded polypeptide chains reach the central chamber by 1 of the termini or in the form of a loop.9 PA700 has an affinity for the poly-Ub chains, bringing ubiquitinated substrates to the proteasome. Because of its “unfoldase” or “inverse chaperone” activity, the PA700 allows the degradation of folded proteins.6,5 Although the target protein itself is unfolded and fed within the central chamber of the proteasome, the poly-Ub chain is removed and disassembled.10

PA700 contains a hexameric ring of AAA ATPases binding the proteasomal α-rings: it forms the “base” together with 2 other subunits, while the remaining 9 PA700 subunits form its “lid.”11,12 The 26S enzyme has a central role in the UPS; however, the 20S core enzyme may play an important role in the degradation of unfolded proteins. Such proteins become more abundant within the cells in situations of oxidative stress. Although ATP depletion associated with ischemia impairs the 26S proteolytic activity, the 20S proteasome remains active.13,14

Localization of Proteasomes Within the Cells

Proteasomes are present within the nucleus and cytoplasm of all cells, making up to 1% of total cell protein.15 Most proteasomes diffuse freely within cytoplasm and nucleus; however, a fraction is stably associated with different structures. Proteasomes are enriched at discrete subnuclear foci called the PML bodies, whereas in the cytoplasm, proteasome subpopulations are associated with the external surface of the endoplasmic reticulum (ER) and the cytoskeleton16 and the centrosome.17 In situations of impaired UPS function, ubiquitinated proteins accumulate at PML bodies and around the centrosome, forming a single aggregate,18,19 or “aggresome.” Formation of such aggregates is achieved in vitro by the action of proteasome inhibitors and by overexpression of certain proteins usually degraded by the UPS.

Physiological Roles of Proteasomes

In yeast, the 20S proteasome subunits and several PA700 subunits are essential.20,21 Block in the cell cycle and induction of apoptosis are obvious effects of proteasome inhibition resulting from the inhibition of the degradation of regulatory proteins.22 Cells treated with proteasome inhibitors accumulate numerous polyubiquitinated proteins. Regulatory proteins are only a fraction of them, most of them being made up of aged structural and functional proteins, which are normally turned over by the UPS. Many polyubiquitinated proteins are the defective newly translated proteins.23 Aggregates of heavily polyubiquitinated proteins may be engulfed by autophagosomes and degraded by the lysosomal pathway, therefore rescuing cells from a death by suffocation with unwanted proteins. It is a matter of discussion whether the formation of protein aggregates is toxic or beneficial in serving to sequester ubiquitinated proteins from wreaking havoc within the cell.24

Peptides generated by proteasomes have from few to 20–30 amino acids. They are further degraded by cytosolic peptidases; however, a fraction of them are delivered to ER-associated TAP peptide transporter. Once in the ER lumen, they bind the assembling MHC class I molecules. Mature MHC class I molecules are exported to the cell surface, where they present the antigenic peptides to immunologically competent cells.25,26
Role of the UPS in the Inflammatory Pathways: IκBα Signaling

Inflammation can be defined as a complex set of interactions among soluble factors and cells that arise in any tissue in response to traumatic, infectious, postischemic, toxic, or autoimmune injury. The inflammatory process normally leads to recovery and healing; however, it can often lead to persistent tissue damage, caused by the infiltration and activity of inflammatory cells. An inflammatory response is also present in cerebral ischemia and after an acute ischemic stroke as sustained and persistent inflammatory response.

Inflammatory pathways are regulated by a limited number of transcription factors, the most important being NF-κB. NF-κB is a collective name for dimeric transcription factors of the Rel family. Its most abundant form is the cytoplasmic p65/p50 dimer, bound to IκBα. On stimulation of various cell types by several cytokines (IL-1, TNF-α), bacterial lipopolysaccharide, UV radiation, ionizing radiation, or oxidative stress, a signal transduction cascade is activated, leading to the phosphorylation of IκBα on Ser 32 and 36 by the multimeric IKK (IκB kinase) complex.

IKK-mediated phosphorylation triggers the ubiquitination of IκBα by the E3 ligase SCFβTRCP. Ubiquitinated IκBα is targeted to the 26S proteasome. Once IκBα is degraded, the nuclear localization signal of NF-κB is unmasked, allowing its translocation to the nucleus where it binds to specific promoter sequences initiating transcription of NF-κB-dependent genes, many of them mediators of the inflammatory response.

The p50 subunit of NF-κB is generated from the p105 precursor by limited proteolytic cleavage mediated by the 26S proteasome. The intensity of NF-κB activation depends on various factors, including the variable E3 activity of the SCFβTRCP complex, which is regulated by a reversible covalent modification with the Ub-like protein NEDD8.

Finally, the activity of the IKK kinase depends on the formation of unusual poly-Ub chains linked by Lys63.

UPS in the Central Nervous System

UPS components are among the most abundant proteins of the central nervous system (CNS), but the relative levels of individual components show high variability between different regions and cell types. Ub immunoreactivity is normally diffuse; however, in various pathological conditions, it concentrates in neuronal inclusion bodies, suggesting that neurodegenerative disorders can involve an impairment of the UPS function.

Neuronal differentiation in vitro is accompanied by increased levels of Ub conjugates, decreased levels of free Ub, enhanced capacity of ubiquitination, increased proteasome activity, and induction of immunoproteasome subunits. Intense Ub immunoreactivity correlates with neuronal differentiation, involving dendrite outgrowth and arborization in vivo. Ub is also involved in the sodium-dependent uptake of various neurotransmitters in the cerebral cortex. Curiously, while dipeptide inhibitors of the N-end rule prevent neurite outgrowth, proteasome inhibitors induce neurite outgrowth, indicating that UPS role in the CNS...
goes far beyond a simple degradative role. At higher doses and longer exposures, proteasome inhibitors induce neuronal cell death.47

The C-terminal Ub hydrolase UCH-L1 (PGP 9.5) constitutes 1% to 2% of total soluble brain protein and is used as a marker of neurons and neuroendocrine cells. UPS activity is required for experience-dependent remodeling of the postsynaptic densities in cultured rat hippocampal neurons.48 UPS at the synapse appears to operate at the presynaptic and at the postsynaptic level. The net outcome of inhibiting the UPS is to enhance synaptic transmission.49

Brain proteasomes display the same basic enzymatic activities.50 They have been detected in the cytoplasm, nuclei, dendrites, axons, and synaptic buttons of various CNS cell types, including pyramidal cells, granular cells of the hippocampus, Purkinje cells, and glial cells.51-53 Activity and expression of brain proteasomes decrease in neurodegenerative disorders and with age, contributing to the elevations in protein oxidation, protein aggregation, and neurodegenerative diseases.54-56

Activity and expression of brain proteasomes decrease in neurodegenerative diseases.56-58

General Properties and Chemistry of Proteasome Inhibitors

Several natural and synthetic compounds that act as proteasome inhibitors have been reported.59 Their chemical classes, chemical structures, and mechanisms of action are summarized in Table 1. One of these compounds, the MLN-519, is a synthetic analog of the bacterial metabolite lactacystin,60 is under clinical evaluation for inhibiting reperfusion injury after ischemic CNS injury.61 Other natural proteasome inhibitors include epnomycin,62 epoxomycin,63 aclacinomycin A64 and PR-39, an Arg- and Pro-rich porcine polypeptide.65 Additional synthetic proteasome inhibitors are aldehyde derivatives (CEP-161267,25 or MG13266), dipeptide benzamide derivatives (CVT-63467), and dipeptide boronic acid substituents (Bortezomib,68 NVP-AFB340, and NVP-AFD31449). Another group of vinyl sulfone tripeptide proteasome inhibitors has been described by Bogyo et al.60 Finally, the HIV-1 protease inhibitor, ritonavir, is also a competitive micromolar inhibitor of the proteasome.71

Changes in the UPS During Ischemia/Reperfusion Injury in the Brain

The UPS plays a complex and unambiguous role in the etiopathology of cerebral ischemia/reperfusion, both directly and indirectly, because of its pivot role in many intracellular pathways.59

Adjusted Changes of Ubiquitin-Proteasome System to Hypoxia

Although brief and acute hypoxia does not impair proteasome function,72 a clear inhibitory effect of hypoxia on proteasome function is evident after prolonged hypoxic periods73 and in the presence of inflammatory mediators.74,75 Repeated and intermittent episodes of hypoxia decrease markedly proteasomal activity in aged Sprague-Dawley rat brain.76 Hypoxia stabilizes the HIF-1α component of the dimeric hypoxia inducible factor (HIF) transcription factor. Under normoxic conditions, HIF-1α is ubiquitinated by the Von Hippel-Lindau (VHL) E3 and degraded by the proteasome, whereas the other HIF subunit—HIF-1/βARNT (aryl hydrocarbon receptor nuclear translocator)—is constitutively expressed.73 HIF-1α coordinates the response to prolonged hypoxia, which pertains to glycolysis, glucose transport, vaso dilatation, and angiogenesis.73 Proteasome inhibitors prevent HIF-1α degradation,77,78 resulting in accelerated angiogenesis in vitro.79 This mechanism could contribute to the rescue of the penumbra of an ischemic lesion.

Adjusted Changes of Ubiquitin-Proteasome System to Ischemia

In the postischemic hippocampus, conjugated Ub accumulates and free Ub is depleted.72,80,81 The accumulation of conjugated Ub may reflect hypofunction of downstream proteasome activity that normally degrades ubiquitinated proteins. Moreover, direct injection of a proteasome inhibitor into the lateral ventricles of the rat-induced DNA fragmentation in various CNS areas, suggesting that suppression of proteasome is able to induce neuronal apoptosis.82 Therefore, it is reasonable to speculate that proteasome malfunction may in part underlie the molecular events of the ischemia-induced neuronal death. Decreased proteasome activity at the ischemic core and the surrounding tissues allows accumulation of oxidized proteins, resulting in formation of protein aggregates, ER stress, impairment of cell function, and eventually cell death. In an experimental ischemia of rat brains, a 60% elevation of Ub conjugate levels in the ischemic compared with the nonischemic animals was observed within 1 hour of recovery. The conjugate immunoreactivity remained at this level for 6 hours but eventually decreased to control levels by 24 hours of recovery.72,80 Increased formation of poly-Ub conjugates was accompanied with a significant increase in the transcription levels of poly-Ub genes.83

Adjusted Changes of Ubiquitin-Proteasome System to ATP Depletion

Ischemic ATP depletion affects the ubiquitination cascade itself.84-86 One-hour transient focal cerebral ischemia induces marked depletion of the E3 parkin protein levels but does not affect the levels of several E2s. Upregulation of the expression of parkin protects cells from injury induced by ER stress; therefore, parkin depletion may increase the sensitivity of neurons to ER stress and the aggregation of ubiquitinated proteins during the reperfusion period.87 At the ischemic core, the ATP-dependent and Ub-dependent degradation mediated by the 26S proteasome is impaired, whereas the ATP-independent and Ub-independent degradation mediated by 20S proteasome proceeds without obstacles. Many 26S proteasomes dissociate under these conditions into 20S proteasomes and PA700 caps. After ischemia in the gerbil cortex, the 26S proteasome ChTL activity decreases, whereas the 20S proteasome ChTL activity increases.88 Moreover, while in most regions the 26S proteasome activity is recovered after reperfusion, in certain regions (eg, the CA1 region of the hippocampus) PA700 and 20S proteasomes are not fully able to reassociate, indicating the occurrence of irreversible bio-
<table>
<thead>
<tr>
<th>Naturally Occurring</th>
<th>Class of Compound</th>
<th>Mechanism of Action</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactacystin</td>
<td>Prodrug for the (\beta)-lactone structure</td>
<td>binds covalently to subunit (\beta_5) of mammalian proteasomes; also inhibits cathepsin A and tripeptidyl peptidase II</td>
<td><img src="image1" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>Aclacinomycin Aklavinone</td>
<td>(\alpha^\prime)-(\beta^\prime)-epoxyketone</td>
<td>inhibits the ChTL activity of the proteasome without effects on cathepsin B, stimulated trypsin, and inhibited chymotrypsin, and, to a lesser extent, calpain</td>
<td><img src="image2" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>Eponemycin</td>
<td>(\alpha^\prime)-(\beta^\prime)-epoxyketone</td>
<td>binds covalently the (\beta_5), (\beta_5), and (\beta_1) catalytic subunits of the 20S proteasome and selectively inhibits the 3 major proteasome proteolytic activities at different rates</td>
<td><img src="image3" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>Epoxomycin</td>
<td>(\alpha^\prime)-(\beta^\prime)-epoxyketone</td>
<td>binds covalently to the (\beta_5), (\beta_5), and (\beta_2) catalytic subunits of the 20S proteasome and inhibits primarily the ChTL activity; does not inhibit other proteases (ie, calpain, cathepsin B, papain, trypsin, chymotrypsin) at concentrations up to 50 \text{mM}</td>
<td><img src="image4" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>PR-39</td>
<td>Cathelicidin</td>
<td>a highly basic arginine/proline-rich peptide; reversibly binds to the (\alpha_7) subunit of the proteasome</td>
<td><img src="image5" alt="Chemical Structure" /></td>
</tr>
</tbody>
</table>
### TABLE 1. Continued

<table>
<thead>
<tr>
<th>Proteasome Inhibitors</th>
<th>Class of Compound</th>
<th>Mechanism of Action</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Synthetic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peptide mimetic</td>
<td>No specific</td>
<td>no specific proteasome inhibitors</td>
<td></td>
</tr>
<tr>
<td>Peptidyl aldehyde</td>
<td>Synthetic agent</td>
<td>reacts with the proposed catalytic nucleophile O(^\gamma)/H(^{9253}) of Thr(^{1}) on the (\beta5) subunit</td>
<td></td>
</tr>
<tr>
<td>MG-132</td>
<td>Tripeptidyl aldehyde</td>
<td>commonly used reversible inhibitor of the ChTL activity of the proteasome; also inhibits cathepsins and calpains</td>
<td></td>
</tr>
<tr>
<td>CEP-1612</td>
<td>Dipeptidyl aldehyde</td>
<td>forms reversible covalent adducts with the proposed catalytic nucleophile, O(^\gamma)/H(^{9253}) of Thr(^{1}) on the (\beta)-subunit; also inhibits lysosomal and Ca(^{2+})-activated proteases.</td>
<td></td>
</tr>
<tr>
<td>CVT-634</td>
<td>Dipeptide benzamide</td>
<td>inhibits ChTL activity</td>
<td></td>
</tr>
<tr>
<td>Bortezomib (formerly Velcade, PS-341)</td>
<td>Dipeptidyl boronic acid inhibitors</td>
<td>inhibits ChTL activity, approved by FDA for treatment of multiple myeloma and other malignancies</td>
<td></td>
</tr>
<tr>
<td>2-aminobenzylstatine, formerly NVP-AFB340 and NVP-AFD314</td>
<td>Boronic acid derivatives</td>
<td>a structure-based optimization approach improved the potency of this series with the most potent compound achieving an IC(<em>{50}) value of 7 nmol/L against the ChTL activity; in addition, these compounds demonstrated good selectivity against the PGPH and TL proteasomal activities (all IC(</em>{50}) values&gt;20 (\mu)mol/L)</td>
<td></td>
</tr>
<tr>
<td>Vinyl sulfone tripeptides</td>
<td>Vinyl sulfone moieties of tripeptides</td>
<td>competitive inhibitors that are largely specific for individual (\beta) subunits of the 20S proteasome but also inhibit intracellular cysteine proteases</td>
<td></td>
</tr>
<tr>
<td>Ritonavir</td>
<td>HIV-1 protease inhibitor</td>
<td>also a weak, low-micromolar inhibitor of the chymotryptic activity of the 20S proteasome by binding the proteasome subunit (\beta5) and (\beta5i)</td>
<td></td>
</tr>
</tbody>
</table>
chemical changes. This probably underlies the delayed neuronal cell death in such regions, which has many features common with neurodegeneration.

Adjusted Changes of Ubiquitin-Proteasome System to Intracellular pH Levels
The cellular site of action of low pH is not completely resolved. Effects on UPS that appear to exist but have not yet been extensively investigated appear the most likely and are quite reasonable. Proteasome inhibition is correlated with hypoxia-evoked decreases in extracellular and intracellular pH.94 Certainly, less specifically, pH may act by altering proteasome subunits at critical times, by direct transient denaturation or indirectly by enhancement of free radical formation (via iron delocalization and the Fenton reaction),99 or more specifically by altering subunit displacement of the proteosomal complex catalytic activity and altering the action of Ub-protein-ligase complexes.90,91

Adjusted Changes of UPS to Intracellular Ca\(^{2+}\) Levels
The effects of proteasome inhibitors on intracellular Ca\(^{2+}\) levels were tested in murine neocortical cultures and resulted in widespread neuronal death associated with a reduction in intracellular free calcium associated with intracellular calcium starvation.92

UPS and Mitochondrial Function
Proteasome inhibition induces multiple effects on mitochondria: mitochondrial membrane potential (Δψm) reduction, dense mitochondrial deposition, cytochrome-c release into the cytosol with secondary dilated rough ER, formation of cytoplasmic vacuoles, and caspase activation inducing neuronal apoptosis.38,47,93,94

Adjusted Changes of UPS to Reactive Oxygen Species
After cerebral ischemia reperfusion injury, there is a time-dependent decrease in proteasome activity in the affected area that is associated with posttranslational changes and not with decreased expression of proteasome subunits.38,55 Indeed, reactive oxygen species (ROS) are known to modify several proteasome subunits (α1, α2, and α4) and impair proteasome activity.95,96 20S proteasomes degrade mildly oxidized proteins without previous ubiquitination; however, they are unable to degrade extensively oxidized proteins.97 Moreover, oxidative damage enhances the effects of proteasome inhibition, leading to protein aggregation and cell death.98 Because metal ions appear to be delocalized from the proteins in the ischemic and postischemic phase, the effect of oxidative stress induced by neurotoxic metal ions on the properties of the brain 20S proteasome has also been studied, showing that metal-catalyzed oxidation strongly affects the functions of the brain 20S proteasome: TL activity showed gradual activation whereas ChTL and PGPH activities were substantially inhibited.99 At the same time, the intracellular redox status, probably through the level of oxidized proteins, is an important element that can either activate or downregulate the 20S proteasome ChTL activity acting by a feedback mechanism, because the antioxidant system is also subjected to the proteasome-dependent proteolysis.100

Relationship of UPS and Glutamate Excitotoxicity
The early phase of necrosis induced as a result of glutamate neurotoxicity apparently does not require proteasome activation.101,102 Proteasome inhibitors do not affect the NF-κB activation in rat striatal neurons by NMDA receptor stimulation involving IκBα degradation by a caspase-dependent mechanism.102 Proteasome inhibition can prevent cytochrome-c release in cerebellar granular cells undergoing apoptosis, thus improving cell survival but not necrosis.101 However, glutamate receptor antagonists might also exacerbate proteasome inhibition-induced neuronal death.92

UPS and Protein Synthesis
There is very little known about rates of protein degradation after ischemia. There is a marked decrease in Ub immunoreactivity in gerbil and rat hippocampus within hours after global ischemia, which then recovers over the next 1 to 3 days in all cell types except CA1 cells that are destined for death.80,103 During focal ischemia, blocking proteasome activity was extremely protective to the core of the lesion.104 One explanation for this is that generalized protein degradation makes a major contribution to ischemic core damage. Indeed, protein content within the core of a focal lesion is severely reduced. However, another possible explanation is that proteasome activity is damaging because it allows NF-κB activation.105 Cell death might not result from a functional defect in 1 or more key processes; rather, it may result from continued activation of perpetrators set in motion by the ischemia, with ultimate breakdown of the cell as a unit. Besides the UPS, during ischemia there may be an activation of calpains and lysosomal cathepsins, which degrade material delivered by autophagy. Thus, at the doses used in vivo, it is possible that proteasome inhibitors are also blocking activity of other proteolytic systems, either directly or indirectly.

UPS and the Damage to the Cytoskeleton
The microtubule dissolution contributes significantly to apoptotic or necrotic cell death.106 Proteasome inhibitors prevent Wallerian degeneration in vitro and in vivo, stabilizing microtubular cytoskeleton in the axons.107,108 Because increased proteolysis of different cytoskeletal elements is one of the early events in the penumbra of an ischemic lesion, it is likely that such a mechanism also contributes to the neuroprotection in stroke.

UPS and Protein Kinases
Permanent or long-term inactivation of protein kinases or phosphatases could lead to initiation of apoptosis or could lead to the permanent alteration of proteins involved in cell membrane or mitochondrial function, the cytoskeleton, or protein synthesis. Such effects could thus make a major contribution to ischemic cell damage.106 The UPS has been implicated in regulating the levels of many cellular proteins of the signal transduction pathways.109 There is a direct relationship between the phosphorylation/dephosphorylation cascade of the signal transduction pathways and the targeting
of the regulatory proteins for ubiquitination. These interacting systems are seen for protein kinase C,\textsuperscript{110–112} Ca\textsuperscript{2+}–calmodulin–dependent protein kinase,\textsuperscript{113,114} MAP kinases,\textsuperscript{115,116} cyclin-dependent kinases,\textsuperscript{117–119} and calcineurin (calmodulin-dependent phosphatase).\textsuperscript{120} Proteasome inhibitors demonstrate that many proteins of the signal transduction pathways are regulated by degradation via the UPS, and their use is associated with multiple perturbations in expression/activation of signaling-related and survival-related proteins.

**UPS and Gene-Mediated Effects Acting on NF-κB**

Free radical damage after ischemia is probably partially mediated by NF-κB. Although the NF-κB pathway can be antiapoptotic in some conditions,\textsuperscript{121,122} there is evidence that it is damaging after ischemia. NF-κB is activated in the core and penumbra 1 day after 90-minute temporary focal ischemia of the cortex.\textsuperscript{123} MLN-519 strongly attenuates damage measured 24 hours after 2-hour ischemia,\textsuperscript{104} which may reflect prevention of damage via increased global proteolysis, or it may reflect the prevention of NF-κB activation. However, because the proteasome pathway is required for NF-κB activation, the result may reflect the importance of NF-κB in focal damage. If so, it shows that the ischemic core is most susceptible to damage via this system. NF-κB drives the transcription of many proinflammatory cytokines (IL-1β and TNF-α), enzymes (COX-2, iNOS), which are damaging in focal and global ischemia, and also of cell adhesion molecules, such as ICAM-1 and selectins of endothelial cells, fibronectin, and laminin of the extracellular matrix, and integrins and L-selectins of neutrophiles.\textsuperscript{122,124} Both these responses were blunted by proteasome inhibitors.\textsuperscript{104,123,125}

**UPS and Heat Shock Protein**

The pattern of heat shock protein (Hsp) expression after ischemia is very similar to that of the immediate early genes. Messenger RNA for Hsp-70 and Hsp-90 begin to rise within a few minutes of the ischemic insult in all regions and persist.\textsuperscript{106} Hsps may confer resistance to ischemia by preserving proteasome function and attenuating the toxicity of proteasome inhibition reducing oxidative stress.\textsuperscript{38} It is suggested that Hsp-90 induces conformational changes that affect the ChT-L and PGPH activities,\textsuperscript{126} depending on the activation state of the proteasome.\textsuperscript{127} The accumulation of misfolded proteins in the cytosol leads to increased Hsps expression, whereas accumulation of such proteins in the ER (ER stress) triggers the unfolded protein response, stimulating the expression of many ER resident chaperones. Proteasome inhibitors block the rapid degradation of abnormal cytosolic and ER-associated proteins,\textsuperscript{22} therefore inducing the expression of various Hsps and ER chaperones.

**UPS and Ischemic Core Damage**

Ischemic core damage does seem to be prevented by proteasome inhibitors. This is a somewhat surprising result, because the inhibitor actually extended penumbral damage somewhat (possibly because of the prevention of normally antiapoptotic effect of NF-κB). It seems quite unlikely that the effect at the core is caused by NF-κB blockade. Damage there seems unlikely to require synthesis of new proteins. Thus the result suggests that the activation of 20S proteasome contributes to cell death by causing breakdown of specific proteins.\textsuperscript{104,125}

**Proteasome Inhibitors in Animal Models of Focal Ischemia and Reperfusion Injury**

It is clear that there are numerous mechanisms of action for proteasome inhibitors in protecting neurones and glia from ischemic damage. Reduction of cerebral infarct volume by proteasome inhibitors may depend on a combination of effects and from the net balance of their positive and negative effects in modulating the cellular metabolic pathways. Proteasome inhibitors have been tested in different stroke models (Table 2), although not all their possible mechanisms of action are clearly established. Only for MLN-519 is a relatively complete evaluation of neuroprotective properties available.\textsuperscript{59,61} It has demonstrated consistently reduction of cerebral infarct volume in several rat models of focal brain ischemia.\textsuperscript{61,104} with evidence of a dose-response effect within readily achieved serum levels and a prolonged time window of up to 6 hours after onset of ischemia, which is highly favorable for a neuroprotective drug.\textsuperscript{104,125} In rodent models of ischemia, MLN-519 attenuated the expression of the inflammatory cascade acting on NF-κB pathway, reduced the invasion of leukocytes, and, hence, limited tissue damage.\textsuperscript{104,125} When MLN-519 was combined with tissue plasminogen activator in a rodent embolic stroke model, it could not only reduce infarct volume and improve neurological outcome 1 week after the ischemic episode but also could eliminate the hemorrhage associated with tissue plasminogen activator treatment given 6 hours after vessel occlusion.\textsuperscript{126} The apparent neuroprotective effect was also evident in reducing inflammatory response in a model of cerebral hemorrhage.\textsuperscript{61}

**Time-Dependent and Cell-Dependent Effects of Proteasome Inhibitors**

At the present, it is difficult to outline a single and clearly defined role of the UPS in cerebral ischemia and to establish what are the exact reasons why in stroke models the proteasome inhibitors have an apparently well-defined neuroprotective effect. Some reasons can be postulated.

First, proteasome inhibition occurs during cerebral ischemia reperfusion injury and is mediated, at least in part, by oxidative stress, which also directly activates NF-κB.\textsuperscript{38,92,129} Proteasome inhibition may be the means by which oxidative stress mediates neuronal cell death. After cerebral ischemia reperfusion injury, there is a time-dependent decrease in proteasome activity that is not associated with decreased expression of proteasome subunits. At the same time, a time- and dose-dependent proteasome inhibition promotes neuronal survival after stroke and helps neurons to maintain their physiological functions. Probably, proteasome activity plays a double role in ischemic damage. Posts ischemic impairment of proteasome activity leads to accumulation of Ub conjugates, contributing to loss of neuronal function; however, proteasome activity is associated with a developing inflammatory response by activation of NF-κB–mediated transcription in neuronal and non-neuronal cells.\textsuperscript{130} Although neurons can withstand relatively long periods with intracellular accu-
mulations of ubiquitinated proteins such as found in neurodegenerative disorders, they are very sensitive to damage elicited by an inflammatory response. Therefore, proteasome inhibitors are considered to be of interest in stroke medicine, because they are able to prevent NF-κB activation and therefore reduce the ischemic damage after stroke.

Second, probably, proteasome inhibition prevents the death of neurons immediately after cerebral ischemia but may start to kill them thereafter. It is demonstrated that prolonged proteasome inhibition has detrimental effects in cultured neuronal cells. This phenomenon is observed in different systems. Proteasome inhibition also induces a time-dependent and dose-dependent increase in protein poly-ADP-ribosylation in the neural PC6 cell line and in primary hippocampal neuron cultures and dopamine neurotoxicity increases in the presence of proteasome inhibitors in a neural PC12 cell line. By contrast, repair mediated by UPS appears to be long-lasting. This is in agreement with the physiological function of proteasome in the nervous system during development.

Third, proteasome inhibitors have, probably, a cell specificity of effect because regulated protein degradation mediated by the proteasomes evidently play distinct and well-defined roles on the various pathways: some cells are sensitive to proteasome inhibition and others are not. Hypoxic endothelia showed a >10-fold increase in sensitivity to inhibitors of proteasome activation.

Fourth, a relevant effect of proteasome inhibitors is the inhibition of gene-mediated effects acting on NF-κB. However, the role of NF-κB in the brain is unclear. In vitro, NF-κB activation can be either protective or deleterious. Cell culture studies have clearly shown that activation of NF-κB in neurons protects them against excitotoxic and metabolic insults relevant to the pathogenesis of stroke. Data from studies of mice lacking the p50 subunit of NF-κB suggest that, overall, NF-κB activation enhances ischemic neuronal death, but its effects differ between cell types such that, whereas activation of NF-κB in microglia promotes ischemic neuronal degeneration, activation of NF-κB in neurons may increase their survival after a stroke. The neuroprotective effects of proteasome inhibitors in vivo probably involve some non-neuronal mechanisms, primarily in the vasculature within the ischemic area by the downregulated expression of genes in microvascular endothelial cells that encode for inflammatory cytokines and adhesion molecules. Radiolabeled proteasome inhibitors did not show any evidence of brain penetration when administered at times when blood–brain barrier integrity was weakest (at 2 and 24 hours after injection) in an ischemic stroke model. At the same time, proteasome inhibitors prevent the disruption of the integrity of the microvascular beds, partially based on their inhibitory action on matrix metalloproteinas.

**Limitations, Future Areas, and New Perspectives:**

**Hypothesis of the Dual Role of the UPS in Stroke**

It is clear that the proteasome represents a central target for the processing and metabolism of multiple proteins whose critical roles in cellular function are being elucidated through the use of selective inhibitors. To avoid eliciting the significant side effects associated with complete inhibition of the proteasome (because of its central role in many cellular functions) and the possible immunosuppressive effects (with increased risk of infection and cancer) from persistent suppression of NF-κB activation, it is, however, critical that we understand how to partially and temporally attenuate proteasome function to elicit the desired therapeutic effect before any large-scale use in humans. Perhaps attention to specific aspects may provide more promise for neuroprotective efficacy than the simpler and less specific proteasome inhibition.

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**TABLE 2. Animal Models of Cerebral Ischemia and Proteasome Inhibitor Treatment**

<table>
<thead>
<tr>
<th>Study</th>
<th>Year</th>
<th>Treatment</th>
<th>Model of Ischemia/Strain</th>
<th>Results</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buchan AM et al</td>
<td>2000</td>
<td>CVT-634</td>
<td>90 minutes MCAo/SH, rats</td>
<td>Smaller infarct of 13%±2% (P&lt;0.01) and 2%±2% (P&lt;0.001) of hemispheric volume at 1 day and 7 days</td>
<td>regional cerebral blood flows were not affected</td>
</tr>
<tr>
<td>Phillips JB et al</td>
<td>2000</td>
<td>MLN-519</td>
<td>Temporary MCAo/Sprague-Dawley, rats</td>
<td>Neuroprotection approached 60%; neutrophil infiltration at 24 h was significantly decreased (63% to 70%, P&lt;0.05)</td>
<td>Neuroprotective effect, is in part, caused by a reduction in the leukocyte inflammatory response</td>
</tr>
<tr>
<td>Zhang L et al</td>
<td>2001</td>
<td>MLN-519</td>
<td>Cardioembolic stroke model/Wistar, rats</td>
<td>Combination treatment with t-PA even at 6 h significantly (P&gt;0.05) reduced infarct volume, improved neurological recovery, and did not increase the incidence of hemorrhagic transformation</td>
<td>Combination treatment extends the neuroprotective effect to at least 6 h after embolization</td>
</tr>
<tr>
<td>Williams AJ et al</td>
<td>2003</td>
<td>MLN-519</td>
<td>Temporary MCAo/Sprague-Dawley, rats</td>
<td>Treatment up to 6 h after MCAo (4 h after reperfusion) reduced neuronal and astrocytic degeneration, decreased cortical infarct volume (−48%), and increased neurologic recovery (+51%), with a reduced neutrophil infiltration (−38%) and a decrease in activated NF-κB immunoreactivity (−45%)</td>
<td>These effects were related to a &gt;80% reduction in blood proteasome levels</td>
</tr>
</tbody>
</table>

MCAo indicates middle cerebral artery occlusion.
The interaction of these events is complex, and the outcome of therapeutic interventions aimed at these elements of cellular injury is uncertain without more rational and specific targeting of these mechanisms and knowledge of the underlying state of the organism with respect to these factors. Cytoprotective therapies, based on blockade of proteasome, are suitable for use in human emergency medicine. However, an excessive inhibition could counterbalance the apparent positive effect of experimental data and produce a negative result in clinical practice with a strict therapeutic window for the protective effect of proteasome inhibition in humans.

In conclusion, proteasome inhibitors are promising neuroprotective agents. The preclinical profile is superior to many previously investigated compounds and is robust in the hands of different investigators. However, more data on their pharmacokinetics, safety profile, and toxicity are necessary before entering in a more rigorous test of clinical efficacy.

Acknowledgments
C.W. is a recipient of the American Heart Association—Texas Affiliate beginning grant-in-aid 0365148Y.

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*Stroke*. 2004;35:1506-1518; originally published online April 29, 2004; doi: 10.1161/01.STR.0000126891.93919.4e

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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