The novel proteasome inhibitor BSc2118 protects against cerebral ischaemia through HIF1A accumulation and enhanced angioneurogenesis

Thorsten R. Doeppner,1,2 Izabela Mlynarczuk-Bialy,3 Ulrike Kuckelkorn,4 Britta Kaltwasser,1,2 Josephine Herz,1 Mohammad R. Hasan,1 Dirk M. Hermann1 and Mathias Bähr2,5

1 Department of Neurology, University of Duisburg-Essen Medical School, Essen, Germany
2 Department of Neurology, University of Göttingen Medical School, Göttingen, Germany
3 Department of Histology and Embryology, Centre of Biostructure Research, Medical University of Warsaw, Warsaw, Poland
4 Department of Biochemistry, Charité-Universitätsmedizin Berlin, Germany
5 DFG Research Centre for the Molecular Physiology of the Brain (CMPB), Göttingen, Germany

Correspondence to: Thorsten R. Doeppner, MD, MSc, Department of Neurology, University of Duisburg-Essen Medical School, Hufelandstr. 55, 45147 Essen, Germany
E-mail: thorsten.doeppner@uk-essen.de

Only a minority of stroke patients receive thrombolytic therapy. Therefore, new therapeutic strategies focusing on neuroprotection are under review, among which, inhibition of the proteasome is attractive, as it affects multiple cellular pathways. As proteasome inhibitors like bortezomib have severe side effects, we applied the novel proteasome inhibitor BSc2118, which is putatively better tolerated, and analysed its therapeutic potential in a mouse model of cerebral ischaemia. Stroke was induced in male C57BL/6 mice using the intraluminal middle cerebral artery occlusion model. BSc2118 was intrastriatally injected 12 h post-stroke in mice that had received normal saline or recombinant tissue-plasminogen activator injections during early reperfusion. Brain injury, behavioural tests, western blotting, MMP9 zymography and analysis of angioneurogenesis were performed for up to 3 months post-stroke. Single injections of BSc2118 induced long-term neuroprotection, reduced functional impairment, stabilized blood–brain barrier through decreased MMP9 activity and enhanced angioneurogenesis when given no later than 12 h post-stroke. On the contrary, recombinant tissue-plasminogen activator enhanced brain injury, which was reversed by BSc2118. Protein expression of the transcription factor HIF1A was significantly increased in saline-treated and recombinant tissue-plasminogen activator-treated mice after BSc2118 application. In contrast, knock-down of HIF1A using small interfering RNA constructs or application of the HIF1A inhibitor YC1 (now known as RNA-binding motif, single-stranded-interacting protein 1 (RBMS1)) reversed BSc2118-induced neuroprotection. Noteworthy, loss of neuroprotection after combined treatment with BSc2118 and YC1 in recombinant tissue-plasminogen activator-treated animals was in the same order as in saline-treated mice, i.e. reduction of recombinant tissue-plasminogen activator toxicity through BSc2118 did not solely depend on HIF1A. Thus, the proteasome inhibitor BSc2118 is a promising new candidate for stroke therapy, which may in addition alleviate recombinant tissue-plasminogen activator-induced brain toxicity.

Keywords: cerebral ischaemia; thrombolytic therapy; neurogenesis; neuroregeneration; neuroprotective agents

Abbreviations: rt-PA = recombinant tissue-plasminogen activator; Suc-LLVY-AMC = suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin; TTC = 2,3,5-triphenyltetrazolium chloride
Introduction

Thrombolytic therapy of ischaemic stroke is limited by a narrow time window and by side effects of the thrombolytic recombinant tissue-plasminogen activator (rt-PA) (Lees et al., 2010). Novel approaches that might overcome these side effects and extend the therapeutic time window of the latter are, therefore, urgently needed. However, most neuroprotective drugs that were successful in animal models have so far failed in clinical trials (Dinarel, 2006). As modulation of single specific pathways is inferior in experimental stroke therapy, interference with multimodal cell cascades is advantageous (Rogalewski et al., 2006). Thus, the ubiquitin proteasome system is an ideal candidate for drug therapy, as it is involved in multiple cellular pathways (Dahlmann, 2007).

The proteasome is a multicatalytic protease complex that contains the catalytic 20S core complex with three distinct activities (Rivett, 1993; Goldberg, 1995). Proteasomes are found in the cytosol and the nucleus of mammalian cells, being the predominant non-lysosomal machinery for protein turnover. As such, proteasomes are critically involved in various physiological and pathological processes, including the cell cycle, apoptosis, transcription, cell signalling and inflammation. Pharmacological proteasome inhibition for the treatment of various diseases, such as ischaemia reperfusion injury, has gained increasing interest in recent years (Elliott and Ross, 2001; Kukan, 2004; Di Napoli and McLaughlin, 2005; Dahlmann, 2007; Petroski, 2008).

Proteasome inhibition has been shown to induce short-term neuroprotection against cerebral ischaemia in rodents. Among the different proteasome inhibitors used, CVT-634 was the first to achieve neuroprotection for as long as 7 days, albeit underlying mechanisms were not analysed at that time (Buchan et al., 2000). On the other hand, the lactacystin derivative MLN519 (also known as PS-519) induced neuroprotection by inhibiting pro-inflammatory nuclear factor-κB when given no later than 4–6 h post-stroke (Phillips et al., 2000; Zhang et al., 2001; Berti et al., 2003; Williams et al., 2003). Likewise, the well-known proteasome inhibitor bortezomib (Velcade®, also known as PS-341) reduced infarct size and post-ischaemic inflammation when given as monotherapy 2–4 h after stroke (Henninger et al., 2006; Zhang et al., 2006, 2010).

Although proteasome inhibition against stroke has not yet reached clinical application, the (partially) reversible proteasome inhibitor bortezomib has been approved by the Food and Drug Administration for the treatment of multiple myeloma and mantle cell lymphoma. However, treatment with bortezomib is limited because of severe side effects and drug resistance (Ruschak et al., 2011), indicating the urgent need to introduce new and better tolerable proteasome inhibitors. Consequently, we have recently developed the novel proteasome inhibitor BSc2118, which shows significant anti-myeloma activity associated with a broad therapeutic range (Braun et al., 2005; Sterz et al., 2010).

In the present work, we applied BSc2118 in a model of transient focal cerebral ischaemia in mice and analysed mechanisms underlying BSc2118-induced neuroprotection.

Materials and methods

Animals and injection protocols

All experimental procedures were performed according to European Union guidelines for the care and use of laboratory animals and were approved by local authorities. Experiments were performed on adult male C57BL/6J mice (Charles River). Treatment of mice was blinded to investigators. The number of animals used, including non-ischaemic mice, was 444. Numbers of animals used for each experiment are given in the figure legends. Survival rate was 100% for animals surviving a maximum of 4 days, whereas survival rate was 84.6–100% in animals that survived for 28–84 days after induction of stroke. An outline of the in vivo experiments is given in Supplementary Fig. 1.

Injection of the novel proteasome inhibitor BSc2118 (dissolved in 100% dimethyl sulphoxide), which was synthesized and provided by Petra Henklein (Institute for Biochemistry of the Charité, Berlin, Germany), was performed on anaesthetized animals that were injected with ketamine (10 mg/kg body weight) and xylazine (25 mg/kg body weight). Thereafter, mice were placed in a stereotactic device (Kopf Instruments) and fixed accordingly. The skull was exposed and a hole was drilled in the skull overlying the left hemisphere at 0.4 mm rostral and 1.8 mm lateral to bregma. Mice received 5 μl of either BSc2118 (30 mg/kg body weight) or dimethyl sulphoxide (control animals) at 3.5 mm ventral to bregma. Non-treated animals underwent the same treatment paradigm, including insertion of the Hamilton syringe, but received no injection of drug or solvent. To achieve knock-down of hypoxia inducible factor 1α (HIF1α), injections for intracerebral delivery of small interfering RNAs were performed on Day 3 before stroke induction following the same protocol as for BSc2118 injection. Animals received either Accell SMARTpool mouse HIF1α small interfering RNA (4 mg/kg body weight; Dharmacon), which consists of a mixture of four small interfering RNA constructs provided as a single reagent, thereby ensuring high-transfection rates (www.dharmacon.com), whereas control animals received Accell non-targeting small interfering RNA (4 mg/kg body weight; Dharmacon). For each animal, two injections (5 μl volume each) of small interfering RNAs were done at the same time point in the left hemisphere at 1.5 and 2.2 mm lateral to bregma. Ventral and rostral coordinates were identical as stated previously for BSc2118 injection. Cerebral ischaemia was induced 3 days after stereotactic injection of small interfering RNAs.

For studies on rt-PA-induced brain toxicity, rt-PA (10 mg/kg body weight, Boehringer Ingelheim) was injected into the right femoral vein at the beginning of the reperfusion. Although 10% of rt-PA contents was given as bolus injection, the remaining 90% was given within the following 15 min. Control animals received saline.

Experiments using the HIF1α inhibitor YC1, which is equivalent to 3-(5-hydroxymethyl-2-furyl)-1-benzylindazole (Enzo Life Sciences), were performed at dosages of 10 mg/kg body weight (dissolved in 100% dimethyl sulphoxide) per injection. Mice received continuous daily intraperitoneal injections of 50 μl beginning on Day 1 until the end of the experiment. Dimethyl sulphoxide served as solvent control.

Detection of fluorescence labelled BSc2118

Non-ischaemic animals were stereotactically injected with fluorescence-labelled BSc2118, using the same coordinates as for
further experiments (see earlier in the text). At 24 h after injection, mice were intraperitoneal injected with chloral hydrate (7% in 0.1 M phosphate buffered saline (PBS), 420 mg/kg body weight) and transcardially perfused with paraformaldehyde (4% in PBS). Brains were removed, shock frozen in liquid nitrogen, and cryosections were prepared. Fluorescence was analysed using an epifluorescence microscope (Zeiss).

**BSc2118 inhibitor assays on isolated 20S proteasomes**

The chymotrypsin-like activity of 20S proteasomes was estimated by hydrolysis of Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC; Bachem) as described previously (Braun et al., 2005). Briefly, proteasomes (100 ng) were preincubated with 0.01–1 μM of BSc2118 in 20 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.2) for 10 min at room temperature. The measurement of proteasome activity was initiated by addition of 50 μM Suc-LLVY-AMC in the same buffer and was incubated for 30 min at 37°C. The released AMC is proportional to the proteasome activity and is detected by fluorescence emission at 460 nm (excitation at 360 nm) using a BioTek Synergy fluorimeter. Fluorescence release by proteasomes without inhibitor preincubation was set as 100%. For estimation of inhibition velocity of BSc2118, kinetics were performed without 10 min of preincubation. Rather, substrate and BSc2118 (0–10 μM) were preincubated as described previously. The reaction was started by adding 205 proteasomes (100 ng), followed by immediate recording of AMC cleavage for as long as 30 min.

To analyse the stability and bioavailability of BSc2118, an 0.1 mM BSc2118 solution in an aqueous solution, such as Tris (pH 7.4) containing 10% foetal calf serum was made and stored for up to 3 days at 4°C. From these stock solutions, the inhibitor concentrations (0.05–1 μM) for the proteasome assay (see earlier in the text) were prepared. To analyse the interaction of the inhibitor with the proteasome in terms of reversibility, 20S proteasomes were incubated with BSc2118, and stability of interaction was tested by dilution and dialysis assays. For dilution assays, 10 μg of human 20S proteasomes derived from erythrocytes were incubated with 1 μM BSc2118 or with 0.1 μM bortezomib (Velcade®, Millenium) for 1 h at room temperature. Thereafter, incubated proteasomes were diluted (1:2, 1:4, 1:8 and 1:16) and incubated again for 1 h. The remaining proteasome activity was measured with calculated 100 ng proteasomes per assay as described previously. For the dialysis assay, 10 μg of 20S proteasomes were incubated with 1 μM of BSc2118 or 0.1 μM of bortezomib in 100 μl for 1 h. The samples were diluted with 1 ml of 20 mM Tris (pH 7.2) and were incubated for 30 min. The unbound inhibitor was removed by centrifugation with Vivasin® 500 (3000 molecular weight cut-off). The dilution/centrifugation step was repeated twice. The volume of dialysed proteasomes were adjusted to 100 μl, and the proteasome activity was measured as described previously.

**Assessment of BSc2118-induced toxicity after systemic application**

To rule out major toxic effects of the proteasome inhibitor, BSc2118 was given intraperitoneally at dosages of 30 or 60 mg/kg body weight to non-ischaemic C57BL/6 mice. BSc2118 was given as single intraperitoneal injections for seven consecutive days, i.e. Days 0–6. Animals were allowed to recover from injections on Days 7–14. Dimethyl sulphoxide (100%) was administered as control. Parameters analysed included weight loss and peripheral blood analysis. On Days 0, 7 and 14, 50 μl of blood was taken from the tail vein, which was then haematologically analysed. Each peripheral blood analysis was done within 2 h after blood sampling. Quantitative examinations were made on a veterinary haematological analyser (BC-2800Vet, Mindray) using an impedance method.

**Fluorometric measurement of proteasome activity in brain lysates**

Proteasome activity was determined in brain homogenates of left non-ischaemic hemispheres 24 h after intrastriatal injection using a lysis buffer containing 100 mM Tris-HCl, 145 mM NaCl, 10 mM EDTA and 0.5% Triton® X-100 at pH 7.5, as described previously (Doeppner et al., 2003). The substrate Suc-LLVY-AMC (50 μM; Bachem) was used for detection of the chymotrypsin-like activity of the proteasome. Briefly, 200 μl of buffer containing 90 μl of a buffer containing 50 mM Tris, 20 mM KCl, 1 mM magnesium acetate, 2 mM dithiothreitol, 1 mM leupeptin, 1 μg/ml aprotinin (Sigma-Aldrich) and 1 mM phenylmethylsulphonyl fluoride (Merck). Substrate cleavage was determined at 37°C in a fluorescence microtitre plate reader with Iexcitation = 355 nm and Iemission = 460 nm; AMC (Sigma Aldrich) was used for initial calibration. Protease activities are given as arbitrary units per min per mg of protein. The protein content of each sample was determined using the Bradford assay.

**Induction of transient focal cerebral ischaemia**

Stroke was induced using the intraluminal middle cerebral artery occlusion model as described previously (Doeppner et al., 2010). Briefly, mice were anaesthetized (1–1.5% isoflurane, 30% O2, remainder N2O), and body temperature was maintained at 37°C using a rectal thermometer feedback-controlled heating system. Cerebral blood flow was continuously assessed by recording laser Doppler flow to ensure appropriate ischaemia and reperfusion. Middle cerebral artery occlusion was induced using a 7-0 silicon rubber coated monofilament (coating length 4–5 mm, tip diameter 180 μm; Doccol), which was withdrawn after 45 min of ischaemia to allow reperfusion of the middle cerebral artery.

**Analysis of post-ischaemic brain injury and of tissue responses by immunohistochemistry**

Infarct volumes were analysed on Day 4, for which brains were removed and cut into slices of 2 mm each. Slices were stained with 2,3,5-triphenyltetrazolium chloride (TTC, 2%), and a computer-based analysis of post-ischaemic brain injury was performed using an impedance method. The lesioned areas were determined using NeuN-staining, counting the number of NeuN-positive neurons in the affected hemisphere. The lesioned area was expressed as a percentage of the contralateral hemisphere.
for which sections were exposed to blocking solution and subsequently stained with a mouse monoclonal anti-NeuN antibody (1:200, 18 h, 4 °C; Chemicon). After washing, sections were incubated for 1 h at room temperature with a secondary goat anti-mouse Alexa Fluor® 488 antibody (1:400; Molecular Probes). Quantitative analyses were performed defining four regions of interest within the ischaemic striatum located 0.14 mm anterior, 2.5–3.75 mm ventral and 1.5–2.25 mm lateral from bregma (see also Supplementary Fig. 1). For each region of interest, three sections per animal were analysed in a blinded manner.

For analysis of BSc2118-mediated effects on post-ischaemic cell proliferation and angiogenesis, mice received daily intraperitoneal injections of 5-bromo-2-deoxyuridine (BrdU, 50 mg/kg body weight; Sigma) on Days 8–18. Brain sections (see earlier) were stained with a rat monoclonal anti-BrdU antibody (1:50; 18 h, 4 °C; Serotec) in combination with a goat polyclonal anti-doublecortin antibody (1:50; Santa Cruz Biotechnology) or a mouse monoclonal anti-NeuN antibody (see earlier in the text). For analysis of post-ischaemic vessel density and angiogenesis, a rat anti-cluster of differentiation (CD)31 antibody (1:200, BD Biosciences) was used. The following secondary antibodies were applied: goat anti-rat Alexa Fluor® 594 (1:400; Dianova) for BrdU and CD31 staining, donkey anti-goat Alexa Fluor® 488 (1:250; Molecular Probes) for doublecortin (Dcx) staining and goat anti-mouse Alexa Fluor® 488 (see earlier in the text) for NeuN staining. Quantitative analysis implied the same regions of interest as stated previously.

Analysis of post-stroke functional deficits

Behavioural tests were performed on the same animals that were also used for immunohistochemical analysis on Day 84. Experimenters involved in behavioural tests were blinded to the treatment paradigm of animals used. All mice were trained 1 day before stroke. Motor coordination deficits were analysed using the rotarod test, tight rope and the corner turn test on Days 7, 14, 28, 56 and 84, as has been described by us in detail (Doepppner et al., 2010, 2011b). Briefly, mice used for the rotarod test were put on an accelerating treadmill (TSE Systems), and the time spent on the rod (maximum 300 s) was determined. For the tight rope test, animals were placed on a 60 cm long rope, which was attached to two platforms, and were scored from 0 (minimum) to 20 (maximum) depending on the time they spent on the rope and whether they reached the platform. Both tests were performed twice at each time point, and means were calculated. For the corner turn test, each mouse was tested for the side chosen > 10 trials per test day, i.e. right or left rearing in the corner. Although healthy animals leave the corner without side preference, mice suffering from stroke preferentially turn to the left, non-impaired body side (Zhang et al., 2002). The laterality index was calculated according to the following formula: (number of left turns – number of right turns)/10.

Deficits in learning and memory were assessed by means of a modified water maze test on Days 82–84 post-stroke (Doepppner et al., 2010). Each experiment consisted of 24 trials, i.e. four trials in the morning and four trials in the afternoon of each test day. For Trials 1–16 and 21–24, the transparent plexiglass platform was always located in the centre of the same quadrant, and animals were always put into the same of one of the remaining quadrants. For assessment of new learning strategies (task switch), the platform was set into another quadrant for Trials 17–20 in the morning of Day 84. Thereafter, the platform was relocated at its original position for the remaining four trials (21–24). The time (maximal 90 s per trial) needed to reach the platform was statistically analysed using a computer-based system (TSE Systems). Data are given as means of four trials each.

Assessment of blood–brain barrier permeability

Blood–brain barrier permeability was analysed using Evans Blue extravasation as previously described by Chiba et al. (2008). Evans Blue (2 %, 2 ml/kg body weight) was injected 22 h post-stroke followed by transcardiac perfusion with PBS 2 h later. Brains were removed. Left hemispheres were weighed, homogenized in 2 ml of 50 % trichloroacetic acid and centrifuged at 10 000 g for 20 min. The extracted Evans Blue dye was further diluted with ethanol and the fluorescence signal was measured with a luminescence spectrophotometer (λ excitation = 620 nm, λ emission = 680 nm). An external standard (62.5–500 ng/ml) was used for calculation of Evans Blue contents.

Matrix metalloprotease 9 zymography

Matrix metalloprotease 9 (MMP9) zymography has been performed as previously described (Doepppner et al., 2011a). Briefly, left hemispheres were homogenized in cold lysis buffer (basic buffer) containing 50 mM/l Tris–HCl (pH 7.6), 150 mM/l NaCl, 5 mM/l CaCl2, 0.05 % Brij-35, 0.02 % Na3 and 1 % Triton X-100 followed by centrifugation and subsequent resuspension of pellets in elution buffer (basic buffer containing 10 % dimethyl sulphoxide and 20 % volume of lysis buffer). Protein concentrations were determined by the bicinchoninic acid method (BCA kit, Thermo Scientific).

Separation of MMP9 as pro-form and active form was done using Novex® Zymogram Gels (Invitrogen) according to the manufacturer’s instructions. Samples were incubated in non-reducing sample buffer (0.4 M/l Tris, pH 6.8, 5 % sodium dodecyl sulfate (SDS), 20 % glycerol, 0.05 % bromophenyl blue) for 10 min at room temperature. Thereafter, the samples were loaded onto 10 % SDS-PAGE gels containing 0.1 % gelatin. After electrophoresis, samples were incubated with 2.5 % Triton X-100 twice for 20 min, equilibrated with developing buffer (Novex) and incubated for > 18 h at 37 °C. Gels were stained with Coomassie blue for 30 min and were destained in washing solution (30 % methanol, 10 % acetic acid). As standards, 0.1 ng of human pro-MMP9 and 0.01 ng of activated MMP9 (both from Merck Biosciences) were used. Gels were scanned and densitometrically analysed.

Measurement of haemoglobin content

Analysis of haemoglobin content followed a protocol described by Su et al. (2008). Animals were sacrificed on Day 4 after stroke, followed by transcardiac perfusion with PBS. Left hemispheres were homogenized in 475 μl PBS at 25 000 g for 30 s, with subsequent addition of 25 ml of 10 % Triton X-100. After thorough mixing, samples were centrifuged at 25 000 g for 30 min (4 °C). A volume of 50 μl from each supernatant was used for photometric analysis at an absorbance of 410 nm, using purified haemoglobin (Sigma-Aldrich) as standard.

Western blotting for HIF1A

For analysis of HIF1A abundance 24 h post-stroke, left (ischaemic) hemispheres of individual mice were complemented with lysis buffer (50 mM/l Tris, pH 8.0, 150 mM/l NaCl, 1 % Triton X-100, protease inhibitors), homogenized and centrifuged. Lysates were centrifuged, and...
supernatants were used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Equal amounts of protein (40 μg) were diluted in 6 × sample buffer, boiled and loaded onto 12% polyacrylamide gels. Proteins were transferred onto polyvinyl difluoride membranes, which were immersed in blocking solution (5% milk in 0.1% Tween 20 + Tris-buffered saline; 1 h at room temperature) and incubated with a rabbit anti-HIF1A antibody (1:1000, Abcam). Thereafter, membranes were incubated with a peroxidase-coupled goat anti-rabbit secondary antibody (1:2000; Santa Cruz Biotechnology), washed several times, immersed in enhanced chemiluminescence (ECL) solution and exposed to ECL-Hyperfilm (Amersham).

**Determination of thiobarbituric acid reactive substances**

Oxidative stress was assessed 24 h after stroke in brain homogenates (see ‘western blotting’ section). Formation of reactive oxygen species leads to peroxidation of fatty acids of phospholipids contained within the cell membrane. During peroxidation, formation of thiobarbituric acid reactive substances is generated, such as malondialdehyde. The latter reacts with thiobarbituric acid yielding a chromogenic compound whose absorption can be photometrically measured at λ = 532 nm as described previously (Noll et al., 1987). The extent of thiobarbituric acid reactive substance formation is expressed as malondialdehyde equivalents, using 1,1,3,3-tetramethoxypropane as standard.

**Enzyme-linked immunosorbent assays**

For ELISA, brain samples were collected as described previously on Days 28 and 84. Levels of vascular endothelial growth factor (R&D Systems), nerve growth factor (Promega), brain-derived neurotrophic factor (Promega), glial cell line-derived neurotrophic factor (Promega), basic fibroblast growth factor (R&D Systems), erythropoietin (Abnova) and epidermal growth factor (R&D Systems) were measured using commercial mouse ELISA kits according to the manufacturers’ instructions.

**Statistics**

All data are given as mean ± standard deviation (SD). For comparison between two groups, the Student t-test was used, whereas for a multi-group comparison, a one-way or a multi-way ANOVA was performed. For post hoc analysis, the Tukey’s test was used. P-values of <0.05 were considered to indicate statistical significance.

**Results**

**Characterization of the novel proteasome inhibitor BSc2118**

Before the in vivo experiments, the inhibitor profile of BSc2118 was analysed on isolated 20S proteasomes (Fig. 1A–D). Kinetics of BSc2118-induced inhibition of 20S proteasome activities without further preincubation of the inhibitor revealed immediate inhibition of proteasome activity, which was functionally complete at a BSc2118 concentration of 500 nM (Fig. 1A). Fluorescence intensity in arbitrary units after 30 min of measurement was 137.5 ± 10.4 (control), 92.8 ± 5.0 (BSc2118, 50 nM), 54.5 ± 13.9 (BSc2118, 100 nM) and 13.7 ± 8.1 (BSc2118, 500 nM). Inhibitor potency of BSc2118 (0.05–1 μM) on isolated 20S proteasomes was preserved at high concentrations (1 μM) for as long as 3 days despite storage in aqueous solution, which was chosen to better mimic the (extra-) cellular compartment of the brain (Fig. 1B). Relative proteasomal activity after treatment with BSc2118 (1 μM) was 18.9 ± 1.3% (Day 0), 24.8 ± 1.0% (Day 1), 31.3 ± 2.0% (Day 2) and 27.9 ± 9.7% (Day 3). The reduction of inhibitory potency as seen for intermediate concentrations, i.e. 0.1–0.5 μM, is most likely because of unspecific binding on proteins within the buffer. Remarkably, a steady state was observed on Days 2 and 3, where the inhibitory potency was no further reduced, suggesting that BSc2118 is relatively stable under these experimental conditions. Assessment of reversibility of BSc2118-induced inhibition of the 20S proteasome was assessed using dilution and dialysis assays (Fig. 1C and D). Proteasome activity after repeated dilution of the proteasome-inhibitor complex did not significantly enhance proteasome activity when proteasomes were initially incubated with the novel proteasome inhibitor BSc2118 (Fig. 1C). Applying 1 μM of BSc2118, we observed a proteasome activity of 51.9 ± 6.2% before dilution and an activity of 66.6 ± 6.7% after 16 × dilution compared with non-inhibited proteasomes. Of note, proteasomal activity after treatment with the reference inhibitor bortezomib significantly increased after 16 × dilution (35.7 ± 5.0 versus 84.6 ± 23.0%). In another experimental setting, proteasome activities with/without presence of inhibitors were analysed after having removed the unbound inhibitor from the proteasome-inhibitor interaction by means of dialysis. Measurement of residual proteasome activities after dialysis, however, did not significantly differ from values obtained before dialysis (Fig. 1D), i.e. proteasome activity after BSc2118 treatment was 41.0 ± 17.1% before dialysis and 53.0 ± 13.9% after dialysis. These data suggest that BSc2118 irreversibly binds to 20S proteasomes, thus, inhibiting enzyme activities.

To ensure that BSc2118 is well tolerated in mice, toxicity studies after systemic (intraperitoneal) injection of BSc2118 for seven consecutive days were performed. Although BSc2118 at a dosage of 60 mg/kg body weight induced temporary weight loss closely related with the treatment phase, animals completely recovered at the end of the observation period (Fig. 1E). Minimal weight observed for animals being treated with BSc2118 (60 mg/kg body weight) was measured on Day 5, with a weight of 14.3 ± 1.4 g compared with mice treated with 30 mg/kg body weight of BSc2118 (16.4 ± 1.1 g) or to control animals (15.8 ± 0.9 g). In line with this, treatment with BSc2118 (30 and 60 mg/kg body weight) did not affect haematological parameters, albeit white blood cells were reduced solely on Day 14 in animals treated with BSc2118 in a dosage of 30 mg/kg body weight (Supplementary Table 1). However, no leukopaenia was observed in these animals. Taken further into account that all animals behaved normally during and after treatment with BSc2118, the novel proteasome inhibitor is well tolerated, even when given systemically.

To ensure sufficient bioavailability in the brain, a fluorescently labelled BSc2118 construct was injected into the peritoneum and the striatum. Intrastriatal injection of the proteasome inhibitor into the non-ischaemic left striatum yielded a significant fluorescence signal at 24 h (Fig. 1F–H), whereas intraperitoneal injection did not
result in detection of any intracerebral fluorescence signal (data not shown). Likewise, proteasome activity in brain lysates was significantly reduced in non-ischaemic animals that were treated with BSc2118 at dosages of 5 to 10 mg/kg body weight, albeit inhibition of enzyme activity was more prominent with inhibitor dosages of 30 and 60 mg/kg body weight (Fig. 1I). After 20 min of continuous measurement, we observed an AMC release of 882.0 ± 99.7 (control), 602.0 ± 56.2 (10 mg/kg body weight BSc2118), 156.0 ± 25.6 (30 mg/kg body weight BSc2118) and 119.0 ± 46.1 (60 mg/kg body weight BSc2118). On the contrary, systemic injection of BSc2118 (30 and 60 mg/kg body weight) did not affect cerebral proteasome activity (data not shown). In light

---

Figure 1 Characterization of the novel proteasome inhibitor BSc2118. (A) Kinetics of BSc2118-induced inhibition of isolated 20S proteasome activity (n = 4 experiments). Using different concentrations of BSc2118 (0–500 nM), measurements were started when 20S proteasomes were added to the incubation buffer containing the substrate Suc-LLVY-AMC and BSc2118. No preincubation was performed. (B) To assure sufficient stability for later in vivo experiments, BSc2118 at different concentrations ranging from 0.05 to 1 μM was incubated with 20S proteasomes in an aqueous solution (Tris buffer), mimicking (extra-) cellular compartments of the brain (n = 4 experiments for each group). Proteasome activity was measured for as long as 3 days. (C) The reversibility of proteasome-inhibitor interactions after treatment with either BSc2118 (1 μM) or bortezomib (0.1 μM) was analysed by continuous dilution of the proteasome-inhibitor complex and compared with non-inhibited proteasomes thereafter (n = 4 experiments). (D) 20S proteasomes were incubated in Tris buffer (pH 7.2) either without inhibitor, with 1 μM of BSc2118, or with 0.1 μM of bortezomib. The proteasome-inhibitor interaction was investigated by removing the unbound inhibitor through centrifugation through Vivaspin21 filters (dialysis). Proteasome activities before dialysis were compared with that after dialysis (n = 4 experiments). (E) Assessment of weight loss as indicator for BSc2118-induced toxicity after systemic application in mice (n = 7 mice per group). Mice were intraperitoneally treated with single injections of BSc2118 or dimethyl sulphoxide for seven consecutive days. Thereafter, mice were observed for additional 8 days without further treatment. Arrows indicate treatment paradigm. (F–H) Labelled BSc2118 (F, green, 4 mg/kg body weight) was stereotactically injected into the left striatum. Mice (n = 4) were sacrificed 24 h after injection and cryostat sections of the brain were prepared. Nuclear DAPI staining (G) served for orientation. (H) Merged photograph from F and G. (I) Measurement of proteasome activities (n = 4 animals per group) in left non-ischaemic brain homogenates 24 h after injection of dimethyl sulphoxide (‘control’) or BSc2118 (1–60 mg/kg body weight) using Suc-LLVY-AMC as substrate. Data are given as arbitrary fluorescence units. Scale bar = 50 μm. Data are given as means (SD). *Significantly different from controls (A, C–E and I) or Day 0 (B), P < 0.05.
of lack of cerebral fluorescence signal and lack of inhibition of cerebral proteasome activity after systemic application, these data suggest that BSc2118 in its current form does not pass the intact blood–brain barrier, prompting further experiments to be undertaken through the intrastriatal delivery route.

**BSc2118 is neuroprotective against cerebral ischaemia**

When given intrastriatally 12 h before induction of stroke, BSc2118 yielded significant reduction of infarct volumes on Day 4 (Fig. 2A and B), starting at a dose of 10 mg/kg body weight (46.2 ± 5.7 mm$^3$) when compared with non-treated mice (61.9 ± 10.2 mm$^3$). Although no further difference with respect to brain injury between dosages of 30 mg (40.1 ± 7.5 mm$^3$) and 60 mg (37.6 ± 9.5 mm$^3$) was observed, a dosage of 100 mg/kg body weight was lethal in all animals (data not shown). Of note, injection of dimethyl sulphoxide solvent did not affect brain injury when compared with non-treated animals (71.4 ± 6.1 versus 61.9 ± 10.2 mm$^3$), which had received no intrastriatal injection. All animals receiving intrastriatal injections of either BSc2118 or solvent showed no signs of clinical impairment as had been stated for the systemic delivery route previously. To reflect a clinically relevant situation, BSc2118 (30 mg/kg body weight) was given at different time points before and after induction of stroke, resulting in significant neuroprotection when given up to 12 h post-ischaemia (Fig. 2C). With a 6 h post-stroke treatment paradigm, infarct volumes were 42.3 ± 7.2 mm$^3$ (BSc2118) and 73.9 ± 4.8 mm$^3$ (control), whereas treatment after 12 h post-stroke yielded an infarct volume of 44.7 ± 6.3 mm$^3$ (BSc2118) and 80.5 ± 11.3 mm$^3$ (control). In line with this, injection of BSc2118 (30 mg/kg body weight) at 12 h post-stroke induced long-term neuroprotection for as long as 3 months (Fig. 2D). Neuronal density on Day 84 was 774.3 ± 73.8 (BSc2118) and 489.1 ± 57.2 (control) per mm$^2$. Reduced brain injury in animals treated with BSc2118 was

---

**Figure 2** BSc2118 induces neuroprotection after focal cerebral ischaemia. (A) Dose-response assessment of infarct volume by means of TTC staining on Day 4 post-stroke in mice treated with the proteasome inhibitor BSc2118 12 h before the stroke. Non-treated mice were exposed to cerebral ischaemia, but did not receive any treatment, whereas control mice received intrastriatal injections of dimethyl sulphoxide (DMSO) as solvent control. (B) Representative TTC stainings depicting infarcts from non-treated, dimethyl sulphoxide-treated and BSc2118-treated (30 mg/kg body weight) mice (obtained from studies in A). (C) Analysis of therapeutic time window by means of TTC staining on Day 4 post-stroke in mice receiving dimethyl sulphoxide (open columns) or BSc2118 (filled columns; 30 mg/kg body weight) at various time points. (D) Neuronal survival examined by NeuN immunohistochemistry on Days 28 and 84 post-stroke in mice treated with dimethyl sulphoxide (open columns) or BSc2118 (filled columns; 30 mg/kg body weight, single injection at 12 h post-stroke). Data are means (SD); n = 7 (A–C) or n = 11 (D) mice per group. *Significantly different from corresponding (non-treated and dimethyl sulphoxide treated) control mice, P < 0.05.
associated with significant reduction of motor coordination and cognitive deficits (Fig. 3). Test performance on Day 84 of BSc2118-treated mice in the rotarod test was 284.0 ± 20.4 s, in the tight rope test 17.3 ± 1.7, in the corner turn test 0.57 ± 0.06 and in the water maze test (runs XXI–XXIV) 29.5 ± 4.5 s, whereas control animals achieved test results of 219.0 ± 22.9 s (rotarod), 13.2 ± 1.5 (tight rope), 0.75 ± 0.09 (corner turn) and 53.8 ± 2.9 s (water maze). These data suggest that single injections of the novel proteasome inhibitor are sufficient to induce long-term neuroprotection and improve functional outcome after stroke.

**BSc2118 stabilizes blood–brain barrier and reduces activation of MMP9**

Cerebral ischaemia involves multiple deleterious events, among which blood–brain barrier breakdown is one key factor. As such, we first analysed whether BSc2118 when given 12 h post-stroke reduces brain swelling. Analysis of oedema formation on Day 4 post-stroke revealed reduced brain swelling in mice that had been treated with the proteasome inhibitor (Fig. 4A; 3.3 ± 0.6%) compared with dimethyl sulfoxide control animals (6.2 ± 0.8%). Likewise, the same treatment paradigm resulted in reduced extravasation of Evans Blue 24 h post-stroke (Fig. 4B), i.e. Evans Blue extravasation was 5.6 ± 0.8 µg/g in BSc2118-treated animals versus 9.7 ± 1.6 µg/g in control mice. As activated MMP9 is critically involved in post-ischaemic blood–brain barrier breakdown (Candelario-Jalil et al., 2009), MMP9 activity was assessed 24 h after induction of stroke using gelatin zymography (Fig. 4C and D). Although non-ischaemic control animals showed no relevant MMP9 activation (0.30 ± 0.05), the latter was critically enhanced in animals that were exposed to cerebral ischaemia (14.2 ± 1.6 in non-treated and 12.8 ± 1.4 in dimethyl sulfoxide-treated control animals). Treatment with BSc2118, however, significantly reduced activation of MMP9 in animals that had been exposed to cerebral ischaemia (4.9 ± 0.6), suggesting that BSc2118 stabilizes blood–brain barrier integrity—among others—through reduced MMP9 activation.

**HIF1A is critically involved in BSc2118-mediated neuroprotection**

The transcription factor HIF1A is regulated through proteasomal degradation, playing a decisive role in cerebral ischaemia (Shi, 2009). As such, we analysed HIF1A protein abundance 24 h after stroke in mice that had received intravenous injection of rt-PA or normal saline as control (Fig. 5A and B). Although HIF1A
expression was hardly detectable in non-ischaemic mice (0.04 ± 0.03), cerebral ischaemia induced upregulation of HIF1A in rt-PA-treated (0.39 ± 0.11) and saline-treated (0.36 ± 0.09) animals. Additional application of BSc2118 at 12 h post-stroke, however, resulted in significantly increased HIF1A levels in saline-treated (0.75 ± 0.07) and rt-PA-treated mice (0.79 ± 0.05). Consequently, enhanced protein abundance of HIF1A in BSc2118-treated animals also resulted in reduced thio-barbituric acid reactive substances formation at 24 h post-stroke (Fig. 5C), i.e. the proteasome inhibitor induced formation of reactive oxygen species in mice treated with saline (0.92 ± 0.13 nM/mg) or rt-PA (0.84 ± 0.09 nM/mg) compared with control mice (1.36 ± 0.15 and 1.48 ± 0.18 nM/mg). As proteasome inhibition is an unspecific tool, which can result in modulation of different cell cascades, we analysed whether BSc2118-induced effects on HIF1A expression and brain injury can be reversed by inhibition of HIF1A itself. Using the HIF1A inhibitor YC1 in combination with BSc2118 (Fig. 5D), infarct volumes significantly increased on Day 4 in animals that had been treated with either saline (62.5 ± 7.3 mm³) or rt-PA (59.1 ± 8.1 mm³) compared with control animals (38.6 ± 5.8 mm³ for saline-treated and 37.1 ± 4.7 mm³ for rt-PA-treated mice). To further strengthen our hypothesis that HIF1A is a critical mediator of BSc2118-induced neuroprotection, a small interfering RNA construct was intrastriatally given 3 days before induction of stroke, resulting in a knock-down of HIF1A in these animals (Fig. 5E). In line with the aforementioned inhibition of HIF1A through YC1, knock-down of HIF1A was associated with a lack of neuroprotection despite application of BSc2118 (Fig. 5F), as shown by infarct volumes of 51.9 ± 4.9 and 55.3 ± 3.1 mm³ for saline-treated and rt-PA-treated animals, respectively.

BSc2118 reduces recombinant tissue-plasminogen activator-induced post-ischaemic toxicity

Although rt-PA induces excitotoxic cell injury and enhances secondary haemorrhage, it remains the gold standard for causative therapy of ischaemic stroke. Therefore, we analysed BSc2118-mediated effects on infarct size, Evans Blue extravasation, MMP9 activation and haemorrhage formation after rt-PA treatment. Intravenous delivery of rt-PA at the beginning of reperfusion resulted in aggravation of brain injury on Day 4 post-stroke (86.9 ± 9.3 mm³ in non-treated mice and 89.1 ± 8.5 mm³ in dimethyl sulphoxide control mice), which was prevented by BSc2118 (41.7 ± 5.8 mm³; Fig. 6A). Such rt-PA-induced aggravation of injury was not observed after BSc2118 treatment (34.2 ± 6.2 versus 41.7 ± 5.8 mm³). Notably, infarct volumes of animals that had received intravenous saline injection and subsequent intracerebral treatment with or without dimethyl sulphoxide (66.2 ± 7.2 and 61.9 ± 11.5 mm³; Fig. 6A) were similar to animals that had received intravenous injection of saline and a combined treatment with BSc2118 and YC1 (Fig. 5D), suggesting that YC1 completely reversed BSc2118-induced neuroprotection in saline-treated mice. Although BSc2118-induced neuroprotection...
after rt-PA injection was also reversed after combined treatment with BSc2118 and YC1 (Fig. 5D), the extent of brain injury in these animals was still significantly (*P* < 0.05) lower than in animals treated with rt-PA with (89.1 ± 8.5 mm³) or without (86.9 ± 9.3 mm³) dimethyl sulphoxide (Fig. 6A). In other words, BSc2118-induced reduction of rt-PA-induced aggravation of brain injury does not completely depend on HIF1A.

In line with this, analysis of blood–brain barrier integrity revealed that increased extravasation of Evans Blue after rt-PA treatment (12.6 ± 0.7 and 13.8 ± 0.9 μg/g, respectively, for non-treated and dimethyl sulphoxide treated mice) was significantly reduced when BSc2118 was injected into the ischaemic brain (5.7 ± 0.8 μg/g; Fig. 6B). Enhanced post-ischaemic MMP9 activity, which was highly increased in animals that had received rt-PA (23.6 ± 3.5 and 24.7 ± 1.7), was also significantly reduced after treatment with BSc2118 (10.6 ± 3.3; Fig. 6C). Analysis of haemoglobin content (Fig. 6D) revealed enhanced secondary haemorrhage in animals that had been treated with rt-PA (59.2 ± 4.1 and 65.1 ± 7.1 μg, respectively, for non-treated and dimethyl sulphoxide treated mice). Combined treatment with rt-PA and BSc2118, however, significantly reduced haemoglobin content in these animals (43.8 ± 6.1 μg), when compared with control animals. These data suggest that application of the proteasome inhibitor BSc2118 reduces rt-PA-induced post-ischaemic toxicity.
Application of BSc2118 is associated with enhanced post-ischaemic angioneurogenesis

As single treatment with BSc2118 resulted in long-term neuroprotection (Fig. 2D), we wondered whether the latter was a consequence of profound acute neuroprotection (Fig. 2A–C) or implied other mechanisms as well. As BSc2118 affected HIF1A protein abundance (Fig. 5A and B), which modulates differentiation of neural precursor cells and angiogenesis (Shi, 2009; Mazumdar et al., 2010; Tsai et al., 2011; Cunningham et al., 2012), we analysed post-ischaemic angioneurogenesis and growth factor contents for as long as 3 months after induction of stroke. Assessment of post-ischaemic cell proliferation revealed an increased number of BrdU+ proliferating cells within the subventricular zone (e.g. 89.1 ± 10.3 versus 49.8 ± 11.3 cells per mm² on Day 84) and within the ischaemic striatum (e.g. 581.3 ± 72.8 versus 297.2 ± 57.2 cells per mm² on Day 84) of mice that had been treated with the proteasome inhibitor (Fig. 7A–F). Likewise, we found a significantly enhanced number of proliferating cells expressing the immature neuronal marker Dcx in the subventricular zone and the striatum of animals treated with BSc2118 (Fig. 7G–K). Within the subventricular zone of BSc2118-treated animals, we found 4.9 ± 0.4% (Day 28) and 5.6 ± 1.1% (Day 84) Dcx+/BrdU+ cells, whereas cell numbers in control animals were 1.3 ± 0.2% (Day 28) and 1.8 ± 0.5% (Day 84). As for the striatum, numbers of Dcx+/BrdU+ cells in BSc2118-treated animals were 17.2 ± 2.8% (Day 28) and 24.8 ± 3.2% (Day 84) compared with 9.2 ± 1.9% (Day 28) and 13.1 ± 2.4% (Day 84) in control animals (Fig. 7G). Analysis of co-expression patterns of proliferating cells with the mature neuronal marker NeuN revealed enhanced numbers of NeuN+/BrdU+ cells located in the ischaemic striatum of mice treated with BSc2118 (e.g. 4.9 ± 0.8 versus 1.7 ± 0.5% cells per mm² in control animals on Day 84), albeit numbers of NeuN+/BrdU+ cells were low (Fig. 7G, L and M). However, no co-expression between BrdU and NeuN within the subventricular zone of any experimental group was observed (data not shown).

Treatment with BSc2118 also affected vessel density as indirectly assessed using quantification of endothelial cells expressing the marker CD31 within the ischaemic striatum (Fig. 8A and B). We found an increased number of CD31+ cells in animals treated with BSc2118, on Day 28 (43.1 ± 6.4 versus 21.8 ± 4.8 cells per mm²) and on Day 84 (49.2 ± 8.1 versus 25.9 ± 5.6 cells per mm²). Analysis of post-ischaemic angiogenesis using BrdU/CD31 double staining (Fig. 8C) revealed an enhanced percentage of BrdU+ cells co-expressing CD31 in animals that had been
**Figure 7** BSc2118 enhances post-ischaemic neurogenesis. Quantitative analysis of proliferating cells as indicated by means of BrdU staining within (A) the ischaemic striatum and (D) the ipsilateral subventricular zone (SVZ) of mice that were treated with intrastrial injections of either BSc2118 (30 mg/kg body weight) or dimethyl sulphoxide (DMSO) at 12 h after stroke. (B, C, E and F) Representative microphotographs from the ischaemic striatum (B1–B3 and C1–C3) and the ipsilateral subventricular zone (E1–E3 and F1–F3) taken on Day 84 after BrdU (red) and DAPI (blue) staining. Scale bars = 20 μm. 3D reconstructions of merged microphotographs are depicted in B4, C4, E4 and F4. (G) Quantitative differentiation analysis of BrdU+ cells in the ischaemic striatum examining the expression of the immature and mature neuronal markers Dcx and NeuN. (H–K) Representative images on Day 84 showing BrdU (red) and Dcx (green) staining in the ischaemic striatum (H1–H4 and I2–I5) and the ipsilateral subventricular zone (J1–J4 and K2–K5). For orientation purposes, DAPI (blue) was used for nuclear counterstaining. Scale bars = 10 μm (J1–J4 and K2–K5) and 20 μm (H1–H4 and I2–I5). 3D reconstructions of merged microphotographs are depicted in H5, I1, J5 and K1. Representative images in the ischaemic striatum showing BrdU (red) and NeuN (green) staining (DAPI in blue) taken from the same animals that were used for BrdU/Dcx staining are depicted in L1–L4 and M2–M5. Scale bars = 20 μm. 3D reconstruction of merged microphotographs are shown in L5 and M1. Data are means (SD); n = 11 animals per group. *Significantly different from dimethyl sulphoxide treated control animals, P < 0.05.
treated with the proteasome inhibitor (e.g. 14.2 ± 3.7 versus 6.4 ± 2.2% in control animals on Day 84).

Taken into account that enhanced angioneurogenesis can orchestrate sustained changes within the ischaemic milieu through bystander effects (Hermann and Chopp, 2012), we analysed the contents of selected growth factors (Fig. 8D). We found elevated contents of erythropoietin, vascular endothelial growth factor and brain-derived neurotrophic factor in mice treated with the novel proteasome inhibitor (e.g. 641.7 ± 73.1, 341.2 ± 38.9 and 149.3 ± 20.6 pg/ml on Day 84) compared with control mice (e.g. 192.8 ± 47.9, 189.1 ± 31.4 and 64.6 ± 12.7 pg/ml on Day 84). These data suggest that therapeutic application of BSc2118 does not only yield acute neuroprotection but also induces enhanced stimulation of angioneogenesis, which might be critically involved in the sustained neuroprotection noticed.

**Discussion**

The present study shows that a single intrastriatal injection of the novel proteasome inhibitor BSc2118 induces long-term neuroprotection after cerebral ischaemia in mice, which is associated with reduced functional neurological deficits, decreased blood–brain barrier breakdown and enhanced angioneurogenesis. Along with this, protein abundance of HIF1A is significantly increased by BSc2118, making proteasome inhibition a promising strategy for stroke therapy.

Proteasome inhibitors have successfully been used before to induce post-ischaemic neuroprotection when given no later than 4–6 h post-stroke as described previously. The herein presented novel proteasome inhibitor BSc2118 further prolongs the aforementioned time window for successful neuroprotective therapy towards 12 h post-stroke. In this context, BSc2118 application was not associated with evident signs of toxicity when given systemically or intrastriatally in mice, which is a limiting factor for therapies using proteasome inhibitors like bortezomib (Richardson et al., 2003). Although observation periods from the aforementioned studies were limited to a maximum of 7 days, our study shows for the first time that single injections of a proteasome inhibitor are sufficient to induce sustained neuroprotection for up to 3 months, which go along with functional neurological improvements, as evidenced by motor coordination, spatial learning and memory capabilities. The need for single injections only is a consequence of high BSc2118 stability in aqueous solution and...
irreversible binding of BSc2118 to the 20S proteasome, inducing long-term effects in the brain tissue. As the latter is likely a result of BSc2118s actions on acute cell injury and post-ischæmic tissue reorganization (see also later in the text), successful single injections of BSc2118 should not be given later than 12 h post-stroke, albeit 20S proteasome activity is immediately inhibited by BSc2118. Clinical relevance for future translational approaches, however, is temporarily hampered, as BSc2118 in its current chemical form does not pass the intact blood–brain barrier after systemic application. Although the therapeutic range of BSc2118 is likely to be superior to bortezomib, as suggested by us through in vitro experiments (Sterz et al., 2010) and the herein presented in vivo data, dosages of 100 mg/kg of BSc2118 were lethal in vivo (unpublished observations). This observation indicates that proteasome inhibition has a two-sided activity, which is in line with Ge et al. (2007) who described proteasome malfunction after cerebral ischaemia resulting in agglomeration of misfolded proteins and ultimately cell death.

As proteasome inhibitors, such as MLN519 and bortezomib, successfully reduce inflammation after haemorrhagic and ischaemic stroke by helping to maintain blood–brain barrier integrity (Berti et al., 2003; Williams et al., 2003; Zhang et al., 2006; Sinn et al., 2007); blood–brain barrier permeability was assessed after BSc2118 treatment. Animals treated with the proteasome inhibitor showed significantly enhanced blood–brain barrier stability and decreased MMP9 activation, which is critically involved in post-ischæmic blood–brain barrier disruption (Candelario-Jalil et al., 2009). Yet, the actions of BSc2118 were not restricted to the preservation of the neurovascular unit, but also involved effects on the brain parenchyma itself. Among important key mediators of cerebral ischaemia, HIF1A—an important regulator of hypoxia being regulated by proteasomal degradation—has gained increasing interest (Shi, 2009). Induction of cerebral ischaemia leads to up regulation of HIF1A in animals treated with rt-PA and saline, whereas HIF1A protein abundance was just above detection threshold in non-ischæmic mice, as was expected. Treatment with BSc2118, however, significantly increased HIF1A levels in rt-PA-treated and saline-treated animals, suggesting that BSc2118 successfully inhibited secondary degradation of the transcription factor. Indeed, inhibition of the latter by means of the HIF1A inhibitor YC1 reversed BSc2118-induced neuroprotection. Similarly, knock-down of HIF1A by means of small interfering RNA constructs successfully reversed BSc2118-induced neuroprotection, further supporting the hypothesis that effects of BSc2118 is indeed causatively mediated by HIF1A, perhaps rather than by HIF1A independent pathways, such as mammalian target of rapamycin (mTOR) inhibition, which has been described before (Sun et al., 2007). Although the role of HIF1A in cerebral ischaemia remains complex (Chavez and LaManna, 2002; Shi, 2009; Yan et al., 2011), the role of HIF1A as mediator of BSc2118-induced neuroprotection is intriguing based on the data presented here.

Thrombolytic therapy with rt-PA does not only induce beneficial effects through recanalization of the occluded vessel, but also yields aggravation of brain injury because of intrinsic properties of rt-PA itself (Klic et al., 2001, 2005; b; Crome et al., 2007; Copin et al., 2011), albeit the underlying mechanisms have not been fully elucidated, yet. On the other hand, application of BSc2118 reduces rt-PA-mediated aggravation of brain injury, such as secondary intracerebral haemorrhage. Notably, brain injury in rt-PA-treated mice was still significantly smaller after inhibition of HIF1A by means of YC1 compared with animals that had received rt-PA only. In other words, BSc2118-induced inhibition of rt-PA-mediated aggravation of brain injury is likely to be not solely dependent on HIF1A. In this context, Chopp and colleagues also observed an extended thrombolytic time window (up to 6 h after stroke onset) when rt-PA was applied with proteasome inhibitors (Zhang et al., 2001, 2006, 2010). The authors inferred that proteasome inhibition potentiated rt-PAs beneficial effects on cerebral perfusion, thus, explaining the enhanced outcome in their cerebral thromboembolism model.

As HIF1A stimulates angiogenesis and differentiation of neural precursor cells (Shi, 2009; Mazumdar et al., 2010; Tsai et al., 2011; Cunningham et al., 2012), we analysed post-ischæmic angiogenesis after BSc2118-treatment. Stroke itself yielded stimulated angiogenesis and neurogenesis, with the latter being tightly linked to the former as has been described previously (Arvidsson et al., 2002; Greenberg and Jin, 2005; Hess and Bolongan, 2008; Xiong et al., 2010). On the other hand, treatment with BSc2118 further increased angiogenesis. As endogenous neural precursor cells rather act through indirect bystander effects than by neural cell replacement of lost tissue (Hess and Bolongan, 2008), contents of selected growth factors within the ischaemic hemisphere were determined, showing increased levels of erythropoietin, brain-derived neurotrophic factor and vascular endothelial growth factor after treatment with BSc2118. Sustained post-ischæmic neuroprotection because of proteasome inhibition is, therefore, most likely a multimodal result of acute neuroprotection and enhanced brain remodelling, with the latter being most likely a consequence of HIF1A stimulated angiogenesis and neuronal differentiation, which has recently been described through Notch and Wnt/β-catenin signalling pathways (Cunningham et al., 2012). On the other hand, BSc2118 itself might also act directly on endogenous neural precursor cells and endothelial precursor cells, providing enhanced survival of neural precursor cells and endothelial precursor cells to guarantee sufficient secretion of protecting factors into the cellular microenvironment. Based on these multiple actions, proteasome inhibition is a promising strategy for stroke treatment. Future studies should focus on chemical formulations of proteasome inhibitors suitable for systemic application.

**Supplementary material**

Supplementary material is available at Brain online.

**Funding**

This study was in part supported by the DFG Research Centre for the Molecular Physiology of the Brain (CMPB), Göttingen, Germany (to M.B.)
References

Arvidsson A, Collin T, Kirk D, Kokaia Z, Lindvall O. Neuronal replacement from endogenous precursors in the adult brain after stroke. Nat Med 2002; 8: 963–70.

Berti R, Williams AJ, Velarde LC, Moffett JR, Elliott PJ, Adams J, et al. Effect of the proteasome inhibitor MLN519 on the expression of inflammatory molecules following middle cerebral artery occlusion and reperfusion in the rat. Neurotox Res 2003; 5: 505–14.

Braun HA, Umbreen S, Groll M, Kuckelkorn U, Mlynarczuk I, Wigand ME, et al. Tripeptide mimetics inhibit the 20S proteasome by covalent bonding to the active threonines. J Biol Chem 2005; 280: 28394–401.

Buchan AM, Li H, Blackburn B. Neuprotection achieved with a novel proteasome inhibitor which blocks NF-kappaB activation. Neureport 2000; 11: 427–30.

Candelario-Jalil E, Yang Y, Rosenberg GA. Diverse roles of matrix metalloproteinases and tissue inhibitors of metalloproteinases in neuroinflammation and cerebral ischaemia. Neuroscience 2009; 158: 983–94.

Chavez JC, LaManna JC. Activation of hypoxia-inducible factor-1 in the rat cerebral cortex after transient global ischaemia: potential role of insulin-like growth factor-1. J Neurosci 2002; 22: 8922–31.

Chiba Y, Sasayama T, Miyake S, Koyama J, Kondoh T, Hosoda K, et al. Anti-VEGF receptor antagonist (VGA1155) reduces infarction in rat permanent focal brain ischaemia. Kobe J Med Sci 2008; 54: E136–46.

Copin JC, Bengaidal D, Da Silva RF, Kargiotsis O, Schaller K, Gasche Y. Recombinant tissue plasminogen activator induces blood-brain barrier breakdown by a matrix metalloproteinase-9-independent pathway after transient focal cerebral ischaemia in mouse. Eur J Neurosci 2011; 34: 1085–92.

Crome O, Doepnner TR, Schwarting S, Muller B, Bahr M, Weise J. Enhanced poly(ADP-ribose) polymerase-1 activation contributes to recombiant tissue plasminogen activator-induced aggravation of ischaemic brain injury in vivo. J Neurosci Res 2007; 85: 1734–43.

Cunningham LA, Candelario K, Li L. Roles for HIF-Talpha in neuronal cell function and the regenerative response to stroke. Behav Brain Res 2012; 227: 410–17.

Dahmann B. Role of proteasomes in disease. BMC Biochem 2007; 8 (Suppl 1): S3.

Di Napoli M, McLaughlin B. The ubiquitin-proteasome system as a drug target in cerebrovascular disease: therapeutic potential of proteasome inhibitors. Curr Opin Investig Drugs 2005; 6: 686–96.

Dirmagl U, Benck to bedside: the quest for quality in experimental stroke research. J Cereb Blood Flow Metab 2006; 26: 1465–78.

Doepnner TR, Grune T, de Groot H, Rauen U. Cold-induced apoptosis of rat liver endothelial cells: involvement of the proteasome. Transplantation 2003; 75: 1946–53.

Doepnner TR, Kaltwasser B, Ellai A, Zechariah A, Hermann DM, Bahr M. Acute hepatocyte growth factor treatment induces long-term neuroprotection and stroke recovery via mechanisms involving neuronal precursor cell proliferation and differentiation. J Cereb Blood Flow Metab 2011a; 31: 1251–62.

Doepnner TR, El Aanbouri M, Dietz GP, Weise J, Schwarting S, Bahr M. Transplantation of TAT-Bcl-xl-transduced neural precursor cells: long-term neuroprotection after stroke. Neurobiol Dis 2010; 40: 265–76.

Doepnner TR, Bretschneider E, Doehring M, Segura I, Senturk A, Acker-Palmer A, et al. Enhancement of endogenous neurogenesis in ephrin-B3 deficient mice after transient focal cerebral ischaemia. Acta Neuropathol 2011b; 122: 429–42.

Elliott PJ, Ross JS. The proteasome: a new target for novel drug therapies. Am J Clin Pathol 2001; 116: 637–49.

Ge P, Luo Y, Liu CL, Hu B. Protein aggregation and proteasome dysfunction after brain ischaemia. Stroke 2007; 38: 3230–6.

Goldberg AL. Functions of the proteasome: the lysis at the end of the tunnel. Science 1995; 268: 522–3.

Greenberg DA, Jin K. From angiogenesis to neuropathology. Nature 2005; 438: 954–9.

Henninger N, Sicard KM, Bouley J, Fisher M, Stagliano NE. The proteasome inhibitor VELCADE reduces infarction in rat models of focal cerebral ischaemia. Neurosci Lett 2006; 398: 300–5.

Hermann DM, Chopp M. Promoting brain remodelling and plasticity for stroke recovery: therapeutic promise and potential pitfalls of clinical translation. Lancet Neurol 2012; 11: 369–80.

Hess DC, Borlongan CV. Stem cells and neurological diseases. Cell Proli 2008; 41 (Suppl 1): 94–114.

Klic E, Bahr M, Hermann DM. Effects of recombinant tissue plasminogen activator after intraluminal thread occlusion in mice: role of hemodynamic alterations. Stroke 2001; 32: 2641–7.

Klic E, Klic U, Bahr M, Hermann DM. Tissue plasminogen activator-induced ischaemic injury is reversed by NMDA antagonist MK-801 in vivo. Neurodegener Dis 2005a; 2: 49–55.

Klic E, Klic U, Matter CM, Lüscher TF, Bassetti CL, Hermann DM. Aggravation of focal cerebral ischaemia by tissue plasminogen activator is reversed by 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor but does not depend on endothelial NO synthase. Stroke 2005b; 36: 332–6.

Kukan M. Emerging roles of proteasomes in ischaemia-reperfusion injury of organs. J Physiol Pharmacol 2004; 55: 3–15.

Lees KR, Bluhmki E, von Kummer R, Brott TG, Toni D, Grotta JC, et al. Time to treatment with intravenous alteplase and outcome in stroke: an updated pooled analysis of ECASS, ATLANTIS, NINDS, and EPITHET trials. Lancet 2010; 375: 1695–703.

Mazumdar J, O’Brien WT, Johnson RS, LaManna JC, Chavez JC, Klein PS, Simon MC. O2 regulates stem cells through Wnt/beta-catenin signalling. Nat Cell Biol 2010; 12: 1007–13.

Noll T, de Groot H, Sies H. Distinct temporal relation among oxygen uptake, malondialdehyde formation, and low-level chemiluminescence during microsomal lipid peroxidation. Arch Biochem Biophys 1987; 252: 284–91.

Petroski MD. The ubiquitin system, disease, and drug discovery. BMC Biochem 2008; 9 (Suppl 1): 57.

Phillips JB, Williams AJ, Adams J, Elliott PJ, Tortella FC. Proteasome inhibitor PS519 reduces infarction and attenuates leukocyte infiltration in a rat model of focal cerebral ischaemia. Stroke 2000; 31: 1686–93.

Richardson PG, Barlogie B, Berenson J, Singhal S, Jagannath S, Irwin D, et al. A phase 2 study of zotarolimus in relapsed, refractory myeloma. N Engl J Med 2003; 348: 2699–701.

Rivett AJ. Proteasomes: multicatalytic protease complexes. Biochem J 1993; 291 (Pt 1): 1–10.

Rogalewski A, Schneider A, Schabelitz WR. Toward a multimodal neuroprotective treatment of stroke. Stroke 2006; 37: 1129–36.

Ruschak AM, Berti R, Williams AJ, Vellarde LC, Moffett JR, Elliott PJ, Adams J, et al. Acute hepatocyte growth factor treatment induces long-term neuroprotection and stroke recovery via mechanisms involving neuronal precursor cell proliferation and differentiation. J Cereb Blood Flow Metab 2011a; 31: 1251–62.

Doepnner TR, El Aanbouri M, Dietz GP, Weise J, Schwarting S, Bahr M. Transplantation of TAT-Bcl-xl-transduced neural precursor cells: long-term neuroprotection after stroke. Neurobiol Dis 2010; 40: 265–76.

Doepnner TR, Bretschneider E, Doehring M, Segura I, Senturk A, Acker-Palmer A, et al. Enhancement of endogenous neurogenesis in ephrin-B3 deficient mice after transient focal cerebral ischaemia. Acta Neuropathol 2011b; 122: 429–42.

Elliot PJ, Ross JS. The proteasome: a new target for novel drug therapies. Am J Clin Pathol 2001; 116: 637–49.

Ge P, Luo Y, Liu CL, Hu B. Protein aggregation and proteasome dysfunction after brain ischaemia. Stroke 2007; 38: 3230–6.

Goldberg AL. Functions of the proteasome: the lysis at the end of the tunnel. Science 1995; 268: 522–3.
expression and reverses spatial memory deficits in rats. PLoS One 2011; 6: e24001.

Wacker BK, Park TS, Gidday JM. Hypoxic preconditioning-induced cerebral ischaemic tolerance: role of microvascular sphingosine kinase 2. Stroke 2009; 40: 3342–8.

Williams AJ, Hale SL, Moffett JR, Dave JR, Elliott PJ, Adams J, et al. Delayed treatment with MLN519 reduces infarction and associated neurologic deficit caused by focal ischaemic brain injury in rats via antiinflammatory mechanisms involving nuclear factor-kappaB activation, gliosis, and leukocyte infiltration. J Cereb Blood Flow Metab 2003; 23: 75–87.

Xiong Y, Mahmood A, Chopp M. Angiogenesis, neurogenesis and brain recovery of function following injury. Curr Opin Investig Drugs 2010; 11: 298–308.

Yan J, Zhou B, Taheri S, Shi H. Differential effects of HIF-1 inhibition by YC-1 on the overall outcome and blood-brain barrier damage in a rat model of ischemic stroke. PLoS One 2011; 6: e27798.

Zhang L, Zhang ZG, Zhang RL, Lu M, Adams J, Elliott PJ, et al. Postischaemic (6-Hour) treatment with recombinant human tissue plasminogen activator and proteasome inhibitor PS-519 reduces infarction in a rat model of embolic focal cerebral ischaemia. Stroke 2001; 32: 2926–31.

Zhang L, Schallert T, Zhang ZG, Jiang Q, Amiego P, Li Q, et al. A test for detecting long-term sensorimotor dysfunction in the mouse after focal cerebral ischaemia. J Neurosci Methods 2002; 117: 207–14.

Zhang L, Zhang ZG, Liu X, Hozeska A, Stagliano N, Riordan W, et al. Treatment of embolic stroke in rats with bortezomib and recombinant human tissue plasminogen activator. Thromb Haemost 2006; 95: 166–73.

Zhang L, Zhang ZG, Buller B, Jiang J, Jiang Y, Zhao D, et al. Combination treatment with VELCADE and low-dose tissue plasminogen activator provides potent neuroprotection in aged rats after embolic focal ischaemia. Stroke 2010; 41: 1001–7.