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Systemic Proteasome Inhibition Induces Sustained Post-stroke Neurological Recovery and Neuroprotection via Mechanisms Involving Reversal of Peripheral Immunosuppression and Preservation of Blood–Brain–Barrier Integrity

Thorsten R. Doeppner[1,2] · Britta Kaltwasser[1] · Ulrike Kuckelkorn[3] · Petra Henkelein[3] · Eva Bretschneider[4] · Ertugrul Kilic[2] · Dirk M. Hermann[1]

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Abstract In view of its profound effect on cell survival and function, the modulation of the ubiquitin-proteasome-system has recently been shown to promote neurological recovery and brain remodeling after focal cerebral ischemia. Hitherto, local intracerebral delivery strategies were used, which can hardly be translated to human patients. We herein analyzed effects of systemic intraperitoneal delivery of the proteasome inhibitor BSc2118 on neurological recovery, brain injury, peripheral and cerebral immune responses, neurovascular integrity, as well as cerebral neurogenesis and angiogenesis in a mouse model of transient intraluminal middle cerebral artery occlusion. Systemic delivery of BSc2118 induced acute neuroprotection reflected by reduced infarct volume when delivered up to 9 h post-stroke. The latter was associated with reduced brain edema and stabilization of blood–brain–barrier integrity, albeit cerebral proteasome activity was only mildly reduced. Neuronal survival persisted in the post-acute stroke phase up to 28 days post-stroke and was associated with improved neurological recovery when the proteasome inhibitor was continuously delivered over 7 days. Systemic proteasome inhibition prevented stroke-induced acute leukocytosis in peripheral blood and reversed the subsequent immunosuppression, namely, the reduction of blood lymphocyte and granulocyte counts. On the contrary, post-ischemic brain inflammation, cerebral HIF-1α abundance, cell proliferation, neurogenesis, and angiogenesis were not influenced by the proteasome inhibitor. The modulation of peripheral immune responses might thus represent an attractive target for the clinical translation of proteasome inhibitors.

Keywords Cerebral ischemia · Stroke · Proteasome · Neuroprotection · Neuroregeneration · Inflammation

Introduction

The proteasome is a multicatalytic protease complex, which is a major site of protein turnover in eukaryotic cells. Proteasomes containing the catalytic 20S core are ubiquitously expressed in mammalian tissues [1, 2], controlling protein abundance under physiological conditions and degrading misfolded proteins under pathophysiological conditions. In view of its profound influence on cell cycle and signaling, inhibition of the proteasome has been used as therapeutic strategy in malignant tumors and vascular diseases as well [3–8].

Over short observation periods, the neuroprotective effects of proteasome inhibitors have repeatedly been reported in animal models of ischemic stroke [9–16]. These studies were hampered by severe side effects and drug resistance, which precluded restorative studies in the post-acute stroke phase

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✉ Thorsten R. Doeppner
thorsten.doeppner@uk-essen.de

[1] Department of Neurology, University of Duisburg-Essen, Essen, Germany

[2] Regenerative and Restorative Medical Research Center, Istanbul Medipol University, Istanbul, Turkey

[3] Department of Biochemistry, Charité-Universitätsmedizin, Berlin, Germany

[4] Department of Otorhinolaryngology, Johannes Wesling Klinikum, Minden, Germany

with repeated drug delivery [17]. More recently, the proteasome inhibitor BSc2118 has become available, which is well tolerated and exerts both anti-melanoma and anti-myeloma activities [18–20]. Using a mouse model of cerebral ischemia, we have previously shown that the intracerebral delivery of BSc2118 induces sustained neuroprotection, cerebral neurogenesis, and angiogenesis via mechanisms that involve stabilization of the transcription factor HIF-1α [21].

In view of its invasive route of application, the local intracerebral delivery of proteasome inhibitors can hardly be translated to human patients. Since BSc2118 poorly penetrates the blood–brain–barrier (BBB), the efficacy of systemically delivered proteasome inhibitors was hitherto unknown. In the present study, we evaluated the effects of intraperitoneal BSc2118 delivery on post-stroke brain injury, neurological deficits, immune responses, and neurovascular remodeling, demonstrating that systemic proteasome inhibition induces sustained neuroprotection via mechanisms involving reversal of post-ischemic immunosuppression and stabilization of BBB integrity.

Materials and Methods

Study Design and Animal Procedures

All studies were performed according to local government authorities. Animals had free access to food and water and were kept under circadian rhythm. In vivo studies were performed on male C57BL6 mice (Harlan, Germany; 22–27 g). Animals were strictly randomized to experimental groups, and both experimenters and analysts were blinded at all stages of the study. Induction of stroke was performed as stated below, and mice were allowed to survive for 1 h (analysis of proteasome activity), 1 day (Evans blue extravasation analysis, zymography, Western blots, flow cytometry, and proteasome activity studies), 2 days (analysis of proteasome activity), 4 days (analysis of infarct volume and brain edema), 7 days (flow cytometry), or 28 days (behavioral tests and immunohistochemistry). The number of animals used for statistical analyses is given in the figure legends.

Focal cerebral ischemia was induced as previously described [21]. Briefly, the left common carotid artery (CCA) was prepared and a silicon-coated nylon filament (Doccol, USA) was inserted into the CCA. The filament was carefully moved forward until the proximal branch of the left middle cerebral artery (MCA). Under constant laser Doppler flow (LDF) control, the filament stayed in situ for 45 min in order to induce transient focal cerebral ischemia in the left MCA territory. After filament removal, LDF control was continued for an additional 15 min in order to ensure adequate reperfusion. Sham animals underwent the same surgical procedure but without inserting the filament. Application of the proteasome inhibitor BSc2118 (synthesized at the Department of Biochemistry, Charité-Universitätsmedizin, Berlin, Germany; 30 mg/kg solved in DMSO) was done via intraperitoneal (i.p.) injection (injection volume 50 μl). The proteasome inhibitor was delivered as single injection (for the majority of studies 9 h post-stroke) or as chronic delivery, i.e., first injection was given 9 h post-stroke with additional daily injections until day 7. Control animals received 50 μl of DMSO only. The potential clinical relevance of a systemic BSc2118 treatment against stroke was further evaluated using an intravenous injection of rt-PA (10 mg/kg body weight) or NaCl during the beginning of the reperfusion followed by treatment with BSc2118 (or DMSO as control) 9 h post-stroke and infarct volume analysis on day 4. For analysis of proteasome activity in brain lysates, a volume of 5 μl of BSc2118 (30 mg/kg) was stereotactically injected into the left striatum as previously described [21]. Post-stroke neurogenesis and angiogenesis were assessed by means of daily i.p. injections of bromodeoxyuridine (BrdU, 50 mg/kg; Sigma-Aldrich, Germany) on days 8–18 with subsequent histochemical analysis as stated below.

Behavioral Tests

Neurological recovery was evaluated using the rotarod, tight rope, corner turn, and balance beam tests, as described before by our group (e.g., see [22]). Prior to stroke induction, all mice were trained in order to ensure adequate performance, i.e., rotarod test performance >300 s, tight rope test score >19, corn turn test performance <0.6, and balance beam test performance <20 s. In the rotarod test, which evaluates motor coordination deficits, the time until the animal dropped off the accelerating rotating drum (velocity 4–40 rpm with maximal testing time of 300 s) was measured. In the tight rope test, which evaluates coordinated climbing abilities as a means to assess motor coordination as well, a validated score from 0 (min) to 20 (max) was obtained. In the corner turn test, in which the mice were placed into an apparatus consisting of two vertical boards with an angle of 30°, the laterality index, defined as number of right turns/ten measurements, was evaluated. A score of 1 indicated severe movement preference as a result of stroke-induced neurological impairment, whereas a score of 0.5 indicated no neurological impairment. In the balance beam test, the time until the mice reached the opposite platform was analyzed. All tests were performed twice on occasion of each time point. Mean values were calculated for both measurements.

Analysis of Infarct Volume and Brain Edema

For analysis of infarct volume and brain edema, 2-mm-thick coronal brain slices obtained from animals sacrificed at 4 days post-stroke were stained with 2 % 2,3,5-triphenyltetrazolium

chloride (TTC). Healthy and injured tissues in both hemispheres were outlined using ImageJ software. For analysis of infarct volume, edema-corrected infarct areas were determined by subtraction of healthy tissue in both hemispheres. Brain edema was calculated as relative increase of ipsilateral volume compared with contralateral hemispheric volume.

Evaluation of BBB Permeability

As previously described [21, 23], BBB permeability was evaluated in mice receiving Evans blue injections at 22 h post-stroke, followed by transcardiac perfusion with 0.1 M phosphate-buffered saline (PBS) 2 h later. Extravasated Evans blue was measured with a luminescence spectrophotometer with $\lambda_{exc.}$ at 620 nm and $\lambda_{em.}$ at 680 nm.

Immunohistochemistry

Immunohistochemical stainings were performed on 2-μm-thick coronal paraffin sections obtained from mice sacrificed 28 days post-stroke by transcardial perfusion with 4 % paraformaldehyde in 0.1 M PBS. The following primary antibodies were used: monoclonal mouse anti-BrdU (1:400; Roche, Switzerland), monoclonal rat anti-BrdU (1:400; Abcam, UK), polyclonal goat anti-doublecortin (1:50; Santa Cruz Biotechnology, Germany), monoclonal mouse anti-NeuN (1:1000; Millipore, UK), and monoclonal rat anti-CD31 (1:200; BD Biosciences, Germany). Secondary antibodies included goat anti-mouse Cy-3 (1:400; Dianova, Germany), goat anti-rat Alexa-594 (1:400; Dianova), donkey anti-goat Alexa-488 (1:250; Invitrogen, Germany), goat anti-mouse Alexa-488 (1:100; Jackson ImmunoResearch, Germany), and goat anti-rat Alexa-488 (1:250; Invitrogen). Sections were evaluated in three regions of interest (ROIs) of the striatum at coordinates AP +0.14 mm, ML ±1.5–2.25 mm, and DV −2.5–3.25 mm in four sections per animal. Mean values were calculated for all ROIs.

Zymography of Matrix Metalloprotease 9

As described [24], matrix metalloprotease 9 (MMP-9) activity was evaluated by zymography in brain homogenates obtained from left (ischemic) hemispheres of mice sacrificed at 1 day post-stroke. For homogenization, a lysis buffer (referred to as basic buffer below) containing 50 mmol/l Tris–HCl (pH 7.6), 150 mmol/l NaCl, 5 mmol/l $CaCl_2$, 0.05 % BRIJ-35, 0.02 % NaN_3, and 1 % Triton X-100 was used. After centrifugation, pellets were resuspended in elution buffer (10 % DMSO and 20 % volume of basic lysis buffer). Samples were incubated in a non-reducing sample buffer (0.4 mol/l Tris, pH 6.8; 5 % SDS, 20 % glycerol, 0.05 % bromophenol blue) and loaded onto commercially available 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels containing 0.1 % gelatin (Novex Zymogram Gels; Invitrogen, USA). After electrophoresis, samples were further processed in 2.5 % Triton X-100, equilibrated with developing buffer (Novex), and incubated over 18 h at 37 °C. Coomassie blue was used for protein staining. As standards, 0.1 ng of human pro-MMP-9 and 0.01 ng of activated MMP-9 (both from Merck Biosciences, Germany) were used. Gels were scanned and densitometrically analyzed.

Western Blot Analysis of HIF-1α Abundance

As previously reported [21], HIF-1α was detected by Western blotting in brain homogenates obtained from left (ischemic) hemispheres of mice sacrificed at 1 day post-stroke. For homogenization, a lysis buffer containing 50 mmol/l Tris, pH 8.0; 150 mmol/l NaCl; 1 % Triton X-100; and protease inhibitors was used. Equal amounts of protein (40 μg) were plotted for SDS-PAGE. After transfer onto PVDF membranes, proteins were incubated with a polyclonal rabbit anti-HIF-1α antibody (1:1000; Abcam, UK) that was detected with a peroxidase-coupled goat anti-rabbit antibody (Santa Cruz Biotechnology). Blots were scanned and densitometrically analyzed.

Analysis of Proteasome Activity

Proteasome activity was determined in brain homogenates obtained from left (ischemic) hemispheres at 1, 24, and 48 h post-stroke or at corresponding time points after sham surgery [20, 21]. For homogenization, a lysis buffer containing 100 mM Tris–HCl, 145 mM NaCl, 10 mM EDTA, and 0.5 % Triton X-100 at pH 7.5 was used. Chymotrypsin-like activity was evaluated using Suc-Leu-Leu-Val-Tyr-AMC (Sigma-Aldrich; 50 μM) in a reaction buffer consisting of 50 mM Tris, 20 mM KCl, 1 mM magnesium acetate, 2 mM dithiothreitol, 1 mM leupeptin (Sigma-Aldrich), and 1 mM phenylmethylsulfonyl fluoride (PMSF; Merck). Protease activity was fluorimetrically measured at $\lambda_{exc.}$ 355 nm and $\lambda_{em.}$ 460 nm and was evaluated as relative activity in comparison to corresponding control tissues set as 100 %. Protein concentrations were determined using the Bradford assay.

Flow Cytometry

Absolute numbers of leukocytes ($CD45^{+high}$), relative amounts of lymphocytes ($CD45^{+high}$, SSC^{low}, $CD11b^{-}$, $CD11c^{-}$) and relative amounts of granulocytes ($CD45^{high}$, SSC^{high}) were analyzed in ischemic hemispheres and peripheral blood by flow cytometry 1 or 7 days post-stroke with slight modifications to a protocol previously described by our group [25, 26]. For lysis of erythrocytes, blood samples were processed with lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 3 mM EDTA). Brain tissue samples were

mechanically homogenized using a buffer containing collagenase type XI (125 U/ml), hyaluronidase (60 U/ml), and collagenase (450 U/ml) in Ca^{2+}/Mg^{2+}-supplemented 0.1 M PBS. After centrifugation and resuspension in 30 % Percoll (GE Healthcare, Sweden), cells were stained with rat anti-CD45 (BioLegend, Fell, Germany), rat anti-mouse CD11b FITC (eBiosciences, Germany), and hamster anti-mouse CD11c APC (BD Biosciences, Germany) antibody and sorted.

Statistics

Data is presented as mean±standard deviation (SD) values. For comparisons between two groups, Student *t* tests and, for comparisons between multiple groups, one-way ANOVAs followed by the Tukey's post hoc tests were used. *p* values <0.05 were considered statistically significant.

Results

Systemic Delivery of BSc2118 Induces Post-ischemic Neuroprotection

To evaluate neuroprotective effects of BSc2118, we performed a detailed analysis of its therapeutic window using infarct volumetry. Analysis at 4 days post-stroke revealed a significant reduction of infarct volume after single administration of BSc2118 when the proteasome inhibitor was delivered up to 9 h after intraluminal MCA occlusion (Fig. 1a). Of note, a single systemic BSc2118 delivery was also effective in combination with rt-PA treatment when BSc2118 was again given at 9 h post-stroke. Infarct volumes on day 4 were 59.7±6.2 mm^3 (control+NaCl), 83.9±11.0 mm^3 (control+rt-PA), 33.5±9.7 mm^3 (BSc2118+NaCl), and 35.8±7.0 mm^3 (BSc2118+rt-PA). However, analysis of surviving neurons in the ischemic striatum revealed that this survival-promoting effect was lost in the post-acute ischemic phase at 28 days post-stroke, as long as BSc2118 was administered as single bolus injection (Fig. 1b). On the other hand, sustained promotion of neuronal survival was noted at 28 days post-stroke in animals receiving repeated BSc2118 delivery over 7 days with the first injection 9 h after MCA occlusion (Fig. 1b). Under no conditions did the authors observe any clinical signs of toxicity under BSc2118 treatment, as has been reported before [21].

Systemic BSc2118 Delivery Stabilizes BBB Integrity but Does Not Influence Cerebral HIF-1α Abundance

As previously reported following intracerebral BSc2118 delivery [21], neuroprotection by BSc2118 was associated with reduced brain edema at 4 days after MCA occlusion (Fig. 2a). Likewise, systemic BSc2118 delivery reduced BBB permeability assessed by Evans blue extravasation analysis (Fig. 2b) and reduced MMP-9 activity (Fig. 2c). The latter is critically involved in post-ischemic BBB breakdown [27] at 24 h post-stroke. Contrary to intracerebral BSc2118 delivery [21], HIF-1α abundance was not influenced by systemic BSc2118 delivery (Fig. 2d).

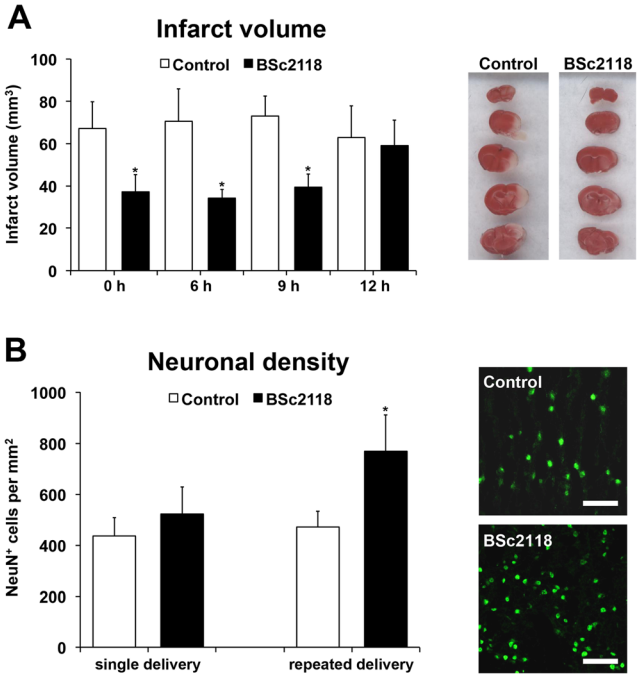


Fig. 1 Systemic delivery of proteasome inhibitor BSc2118 protects against focal cerebral ischemia. **a** Infarct volume in mice receiving single intraperitoneal (i.p.) injections of vehicle (control) or BSc2118 (30 mg/kg) at various time points after intraluminal middle cerebral artery (MCA) occlusion followed by animal sacrifice at 4 days post-stroke. Representative 2,3,5-triphenyltetrazolium chloride (TTC) stainings are shown from mice receiving single vehicle or BSc2118 injections 9 h after reperfusion. **b** Neuronal survival in the striatum evaluated by NeuN immunohistochemistry in mice receiving single i.p. injections of vehicle or BSc2118 (30 mg/kg) 9 h after MCA occlusion (single delivery) or daily i.p. injections of vehicle or BSc2118 over 7 days starting 9 h after MCA occlusion (repeated delivery). Representative microphotographs of $NeuN^+$ cells are shown from mice receiving repeated vehicle or BSc2118 injections. *Scale bars*=50 μm. *$p<0.05$ compared with corresponding control mice (n=7 mice per group (in **a**)/n=12–14 mice per group (in **b**))

Systemic BSc2118 Delivery Enhances Post-ischemic Neurological Recovery

Rotarod, tight rope, corner turn, and balance beam tests revealed that repeated BSc2118 delivery over 7 days induced sustained improvement of motor and coordination recovery that persisted up to 28 days post-stroke (Fig. 3). Conversely, single BSc2118 delivery 9 h after stroke did not result in sustained motor coordination improvement (data not shown).

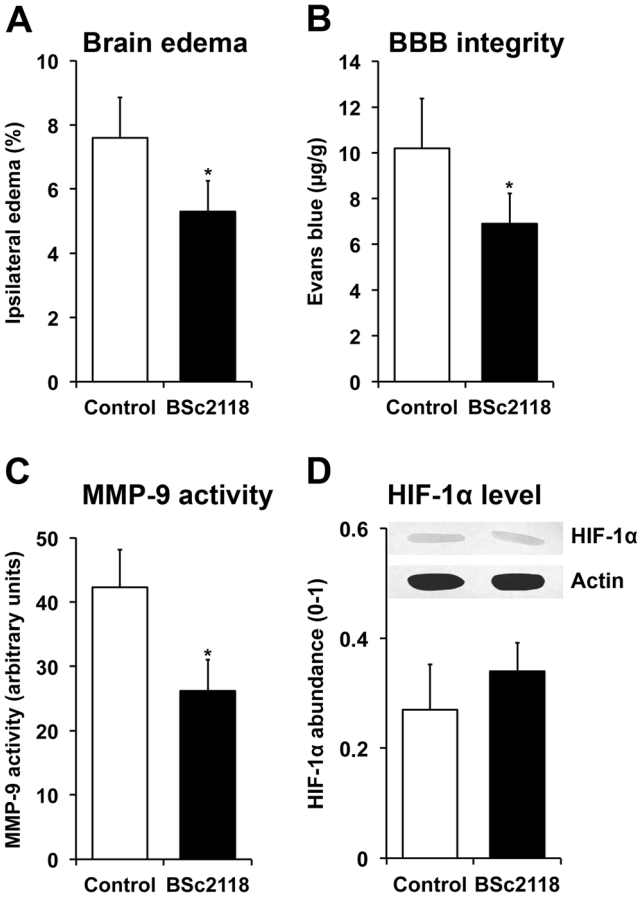


Fig. 2 Systemic delivery of BSc2118 reduces post-ischemic brain edema and blood-brain-barrier permeability without affecting HIF-1α abundance. **a** Brain edema as evaluated by TTC staining in mice receiving single intraperitoneal (i.p.) injections of vehicle (control) or BSc2118 (30 mg/kg) 9 h after intraluminal MCA occlusion followed by animal sacrifice at 4 days post-stroke. **b** Blood-brain-barrier (BBB) permeability, revealed by extravasation of Evans blue in mice receiving i.p. injections of vehicle (control) or BSc2118 (30 mg/kg) 9 h after MCA occlusion followed by animal sacrifice at 24 h post-stroke. **c** Matrix metalloprotease-9 (MMP-9) activity assessed by gelatinase zymography in ischemic brain tissue of mice receiving i.p. injections of vehicle (control) or BSc2118 (30 mg/kg) 9 h after MCA occlusion followed by animal sacrifice at 24 h post-stroke. **d** HIF-1α abundance analyzed by Western blots in ischemic brain tissue of mice receiving i.p. injections of vehicle (control) or BSc2118 (30 mg/kg) 9 h after MCA occlusion followed by animal sacrifice at 24 h post-stroke. *$p<0.05$ compared with corresponding control group ($n=7$ mice per group (in **a**)/$n=4$ mice per group (in **b**–**d**))

Systemic BSc2118 Delivery Does Not Influence Cerebral Proteasome Activity to a Major Extent

Since BBB permeability of BSc2118 is low [20, 21], we next compared cerebral proteasome activity after intracerebral (i.e., intrastriatal) and systemic (i.e., i.p.) BSc2118 delivery. While a single intracerebral bolus injection of BSc2118 resulted in near-complete proteasome inhibition in sham-operated and ischemic mice that persisted over up to 48 h (Fig. 4a), systemic BSc2118 delivery only mildly affected cerebral proteasome activity (Fig.4b).

Systemic BSc2118 Delivery Attenuates Acute Post-ischemic Leukocytosis and Reverses Subsequent Peripheral Immunosuppression

Considering that focal cerebral ischemia induces inflammatory responses in the brain [28, 29] and peripheral blood, namely, acute leukocytosis followed by subsequent immunosuppression [30, 31], we next evaluated immune responses induced by BSc2118. Interestingly, systemic BSc2118 delivery did not affect cerebral post-stroke leukocyte infiltration, neither 24 h nor 7 days post-stroke (Fig. 5), but attenuated the acute stroke-induced peripheral leukocytosis 24 h post-stroke (Fig. 6a). Furthermore, repeated but not single BSc2118 delivery reversed the stroke-induced immunosuppression, restoring total leukocyte counts, relative lymphocyte amounts and relative granulocyte amounts in the blood at 7 days post-stroke (Fig. 6a–c).

Systemic BSc2118 Delivery Does Not Influence Post-ischemic Angioneurogenesis

Since intracerebral BSc2118 delivery enhances post-ischemic neurogenesis and angiogenesis [21], we finally examined whether systemic BSc2118 administration induced post-stroke neuroregeneration despite limited brain bioavailability. Analysis of proliferating cells by BrdU immunohistochemistry within the ischemic lesion site did not reveal any difference between vehicle-treated control mice and mice receiving BSc2118 over 7 days (Fig. 7a). Likewise, differentiation analysis of $BrdU^+$ cells did not detect any differences in the co-expression of the neuronal markers Dcx and NeuN and the endothelial marker CD31 (Fig. 7b–d). Of note, analysis of $BrdU^+$ cells (47.1±12.5 in controls vs. 43.3±9.4 per mm^2 in BSc2118-treated mice), determination of co-expression between BrdU and Dcx (8.8±4.3 % in controls vs. 7.9±6.2 % in BSc2118-treated mice), as well as co-expression between BrdU and NeuN (no co-expression observed in both groups) within the ipsilateral subventricular zone did not show any significant difference, either.

Discussion

The present study demonstrates for the first time that repeated, systemic delivery of the proteasome inhibitor BSc2118 induces sustained neuroprotection after focal cerebral ischemia, characterized by reduced infarct volume, decreased brain edema, and enhanced BBB integrity, resulting in neurological recovery that persists in the post-acute stroke phase. Albeit intracerebral proteasome activity was only slightly reduced

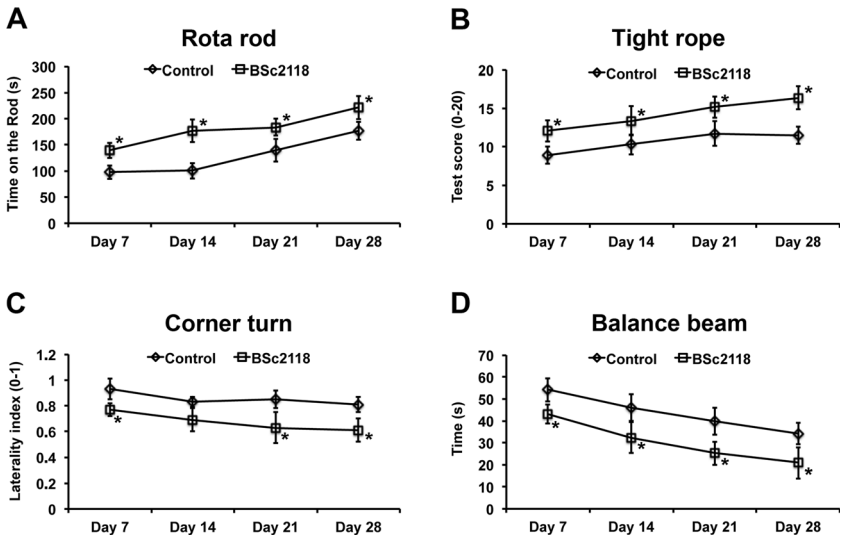


Fig. 3 Systemic BSc2118 delivery induces sustained motor coordination recovery after focal cerebral ischemia. **a** Rotarod, **b** tight rope, **c** corner turn, and **d** balance beam test in mice receiving daily intraperitoneal injections of vehicle (control) or BSc2118 (30 mg/kg) over 7 days starting at 9 h after intraluminal MCA occlusion. *$p<0.05$ compared with corresponding control group ($n=12$–14 mice per group)

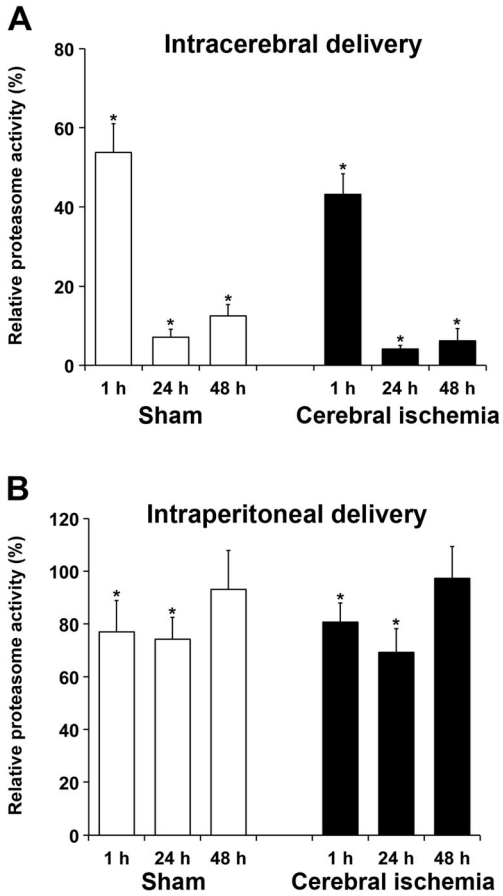
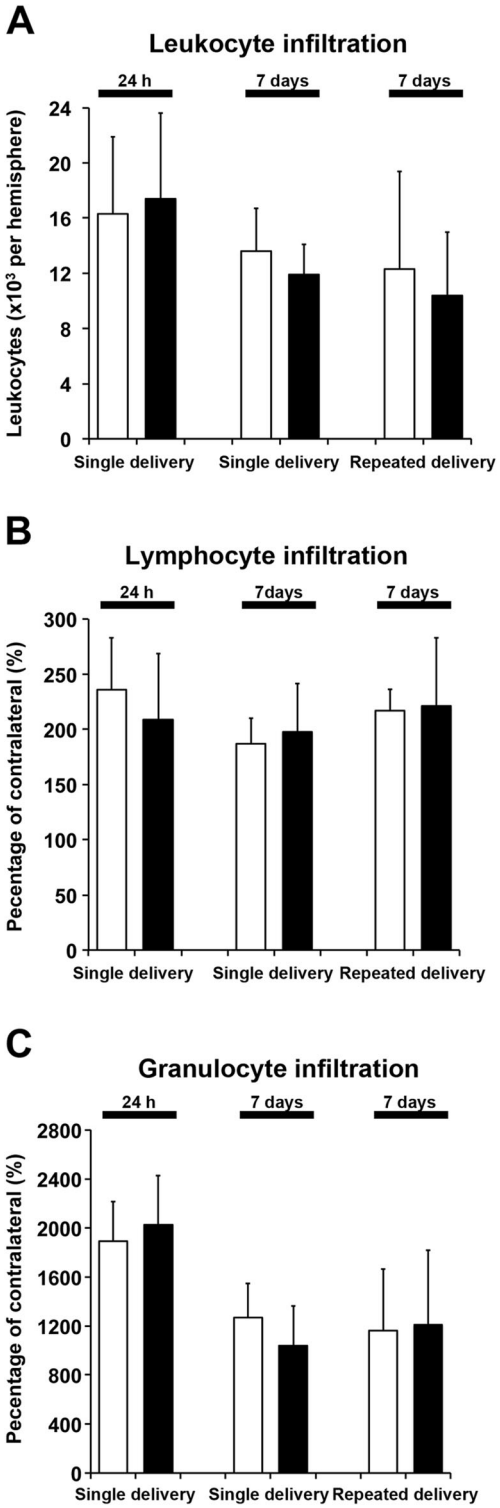


Fig. 4 Systemic BSc2118 delivery does not influence cerebral proteasome activity to a major extent. Proteasome activity in brain lysates of sham operated (*open columns*) and ischemic (*closed columns*) mice 1 to 48 h after single **a** intracerebral (i.e., intrastriatal) or **b** intraperitoneal (i.p.) BSc2118 (30 mg/kg) delivery. Note the near-complete inhibition of cerebral proteasome activity after local intracerebral but not systemic i.p. delivery. *$p<0.05$ compared with baseline ($n=4$ mice per group)

by systemic BSc2118 delivery, distinct changes of peripheral immune responses were noted after systemic BSc2118 delivery, namely, the prevention of stroke-induced acute leukocytosis and reversal of subsequent immunosuppression in peripheral blood. Cerebral neurogenesis and angiogenesis were not influenced by BSc2118 delivery, arguing in favor of a neuroprotective rather than true restorative action. Our study provides a new mechanism of action of proteasome inhibitors in models of ischemic stroke.

Although proteasome inhibition is a double-edged sword [32], neuroprotection induced by proteasome inhibitors has previously been described in experimental models of focal cerebral ischemia [9–16]. The clinical relevance of these findings, however, was limited. Due to severe side effects, pharmacological inhibitors could not repeatedly be delivered, and as such, observation periods of these studies were limited to a maximum of 7 days only. More recently, we locally administered the later generation proteasome inhibitor BSc2118 that lacks such side effects using an intracerebral delivery approach, demonstrating that proteasome inhibition protected against stroke when administered up to 12 h after focal cerebral ischemia [21]. These experiments extended therapeutic windows of proteasome inhibitors in earlier studies, which had been in the range of a maximum of 4–6 h. The local intracerebral BSc2118 delivery yielded almost total proteasome inhibition, resulting in stabilization of HIF-1α followed by increased neurogenesis and angiogenesis that translated into neurological recovery which still persisted after as long as 3 months post-stroke.

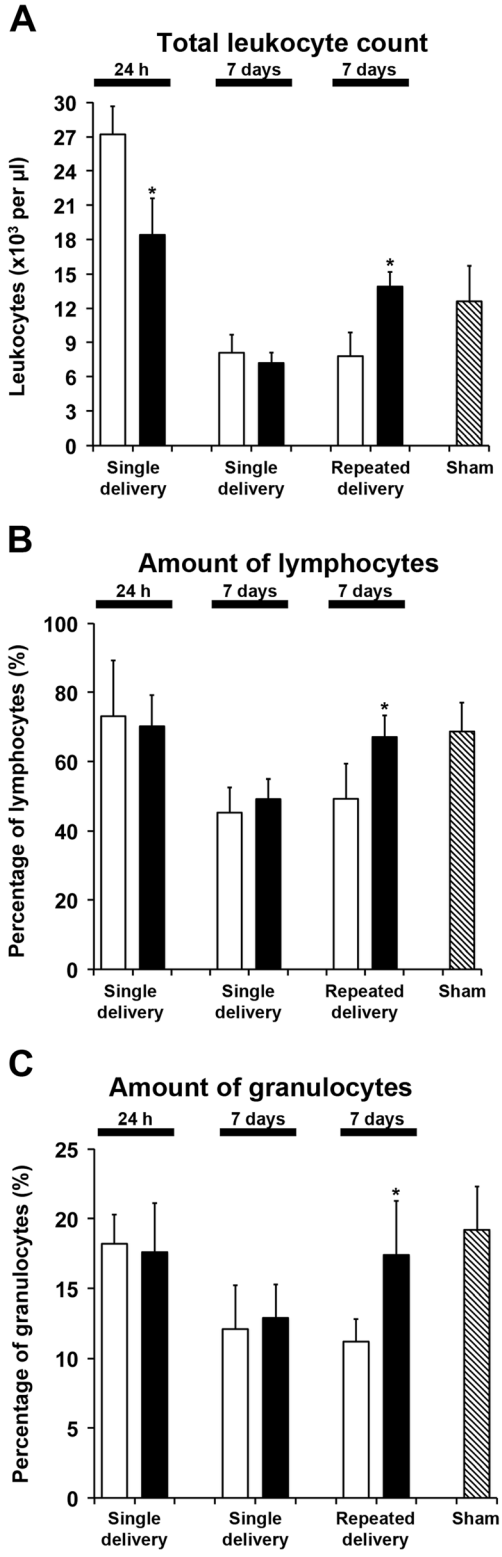
Since intracerebral application is not feasible in the clinical setting, we now evaluated the effects of systemic BSc2118 delivery. Previous data from our group showed that BSc2118 only has limited bioavailability in the brain after systemic application [20]. In line with this, cerebral proteasome activity was only mildly inhibited in the present study



◀ **Fig. 5** Systemic BSc2118 delivery does not influence post-ischemic cerebral inflammatory response. *Open columns* indicate controls and *closed columns* indicate treatment with BSc2118. Number of brain **a** leukocytes, **b** lymphocytes, and **c** granulocytes, evaluated by flow cytometry in ischemic hemispheres of mice receiving single i.p. injections of vehicle (control) or BSc2118 (30 mg/kg) at 9 h after MCA occlusion (single delivery) or daily i.p. injections of vehicle or BSc2118 (30 mg/kg) over 7 days starting at 9 h after MCA occlusion (repeated delivery), followed by animal sacrifice at 24 h (single delivery only) or 7 days post-stroke (single or repeated delivery). No differences were detected between groups (n=6 animals per group)

following i.p. delivery with a maximum inhibition 24 h after stroke. This is in line with in vitro data, demonstrating a decline of BSc2118 inhibition potency in microsomal fractions after 24 h by approximately 75 % despite stability in aqueous solution [20, 21]. The latter observations prompted us to use once daily injections for repeated BSc2118 delivery. We were surprised to see that systemic BSc2118 delivery induced sustained neuroprotection when delivered up to 9 h post-stroke. Yet, sustained neuroprotection associated with neurological recovery was observed only after repeated and not after single BSc2118 delivery. Since systemic BSc2118 delivery only slightly reduced cerebral proteasome activity, it is not surprising that cerebral HIF-1α abundance was not affected in this study. Our data indicate that systemic BSc2118 delivery acts via a different mode of action than intracerebral application. Notably, brain edema, BBB permeability, and cerebral MMP-9 activity were reduced by systemic BSc2118 administration. These findings argue in favor of a stabilization of the neurovascular unit that accompanies the BSc2118-induced promotion of neuronal survival.

Furthermore, distinct alterations of peripheral immune responses were noted after systemic BSc2118 delivery, namely, a prevention of acute post-stroke leukocytosis and reversal of subsequent immunosuppression in the peripheral blood. Peripheral immune responses are a well-established component in the progression of ischemic injury [28, 29]. Rapid activation of the immune response associated with leukocytosis in peripheral blood is followed by a transient phase of immunosuppression [30, 31, 33]. Although proteasome inhibition is known to modulate inflammation under various pathological conditions [34], the here-reported prevention of acute post-stroke leukocytosis and reversal of subsequent immunosuppression, i.e., the normalization of total leukocyte counts and more specifically lymphocyte and granulocyte amounts, is new. It is noteworthy that unlike in other proteasome inhibition studies [9, 13, 14, 21, 35], alterations in the brain inflammatory response were not noticed after systemic BSc2118 delivery. Evaluating precise immune mechanisms underlying BSc2118-induced neuroprotection was beyond the scope of this work. Thus, we did not analyze subsets of immune cells in the brain. It is conceivable that BSc2118 induced changes in the composition of brain immune cell infiltrates, which might explain its neuroprotective action. Further studies are needed on this issue. Moreover, the present study was done on adolescent and healthy animals not precisely reflecting the clinical situation. As a matter of fact, age and/or diseases critically affect post-ischemic immune responses, which has been shown for hypercholesterolemic mice by our group before



◀ **Fig. 6** Systemic BSc2118 delivery attenuates acute post-ischemic leukocytosis and reverses subsequent peripheral immunosuppression in the blood. *Open columns* indicate controls and *closed columns* indicate treatment with BSc2118. **a** Total leukocyte count, **b** relative amount of lymphocytes, and **c** relative amount of granulocytes in peripheral blood of mice receiving single i.p. injections of vehicle (control) or BSc2118 (30 mg/kg) at 9 h after MCA occlusion (single delivery) or daily i.p. injections of vehicle or BSc2118 (30 mg/kg) over 7 days starting at 9 h after MCA occlusion (repeated delivery), followed by animal sacrifice at 24 h (single delivery only) or 7 days post-stroke (single or repeated delivery). Cell counts and percentages determined in sham operated mice are also shown. *$p<0.05$ compared with corresponding control group ($n=6$ animals per group)

[26]. Studies using the latter mice strain were, however, beyond the scope of the present work and might have to be performed in the future.

In line with an increased HIF-1α abundance due to inhibition of cerebral proteasome activity, the local intracerebral delivery of BSc2118 was previously shown to stimulate cerebral cell proliferation, neurogenesis, and angiogenesis after focal cerebral ischemia [21]. In contrast to local intracerebral BSc2118 delivery, systemic BSc2118 administration did not

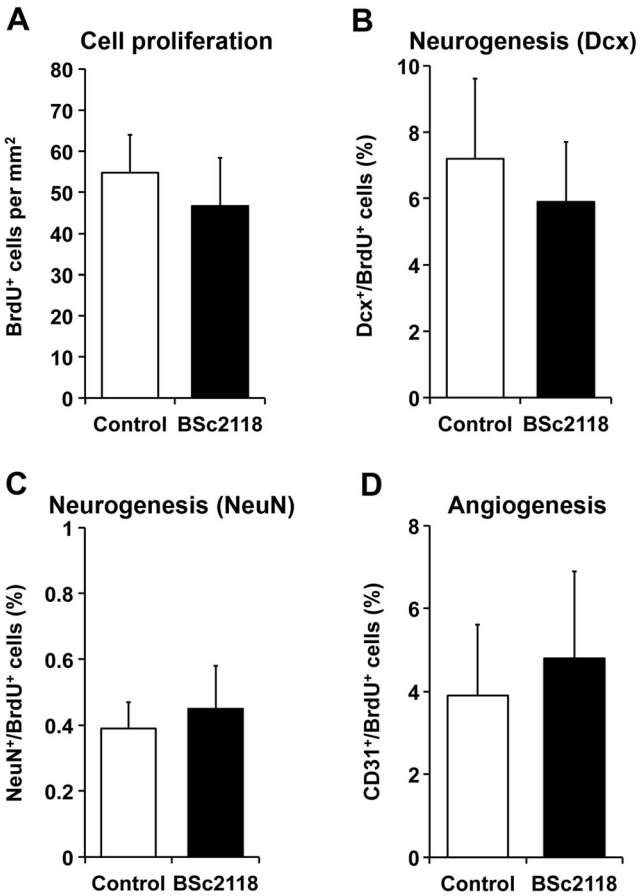


Fig. 7 Systemic BSc2118 delivery does not influence post-ischemic angioneurogenesis. **a** Cell proliferation evaluated by BrdU immunohistochemistry, **b** neurogenesis assessed by BrdU co-expression with the immature neuronal marker doublecortin (Dcx), **c** neurogenesis analyzed by BrdU co-expression with the mature neuronal marker NeuN, and **d** angiogenesis examined by BrdU co-expression with the endothelial marker CD31 in the ischemic striatum of mice receiving daily i.p. injections of vehicle (control) or BSc2118 (30 mg/kg) over 7 days starting at 9 h after MCA occlusion, followed by animal sacrifice at 28 days post-stroke. No differences were detected between groups ($n=12$–14 mice per group)

stimulate post-ischemic cell proliferation, neurogenesis, and angiogenesis, most likely as a consequence of lack of HIF-1α stabilization. HIF-1α is known to stimulate post-ischemic neurogenesis and angiogenesis in otherwise hostile cellular microenvironments [36–39]. Although neurogenesis and angiogenesis are stimulated by ischemia [40–43], their significance for neurological recovery is still a matter of debate due to low survival and differentiation rates of newborn cells [44]. Considering that systemic BSc2118 delivery promoted neuronal survival without affecting neurogenesis and angiogenesis, this delivery paradigm appears to represent a neuroprotective rather than true neurorestorative strategy. The modulation of peripheral immune responses might represent an attractive target for the clinical translation of proteasome inhibitors.

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Compliance with Ethical Standards All studies were performed according to local government authorities.

Conflict of Interest The authors declare that they have no competing interests.

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