The copper *bis*(thiosemicarbazone) complex Cu^{II}(atsm) is protective against cerebral ischemia through modulation of the inflammatory milieu

Mikko T Huuskonen[1,*], Qing-zhang Tuo[2,3,*], Sanna Loppi[1], Hiramani Dhungana[1], Paula Korhonen[1], Paul S Donnelly[4], Alexandra Grubman[5], Sara Wojciechowski[1], Katarina Lejavova[1], Yuriy Pomeshchik[1], Laura Periviita[1], Lotta Kosonen[1], Martina Giordano[1], Frederick R Walker[6], Rong Liu[2], Ashley I Bush[3], Jari Koistinaho[1], Tarja Malm[1], Anthony R White[3,5,8,*], Peng Lei[3,7,*], Katja M Kanninen[1]

[1]Department of Neurobiology, A.I.Virtanen Institute for Molecular Sciences, University of Eastern Finland, Kuopio, Finland

[2]Key Laboratory of Ministry of Education of China for Neurological Disorders, Department of Pathophysiology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

[3]Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville, Victoria, Australia

[4]School of Chemistry and Bio21 Institute for Molecular Science and Biotechnology, The University of Melbourne, Victoria, Australia

[5]Department of Pathology, The University of Melbourne, Parkville, Victoria, Australia

[6]School of Biomedical Sciences and Pharmacy, University of Newcastle, Callaghan, NSW, Australia

[7]Department of Neurology, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, and Collaborative Innovation Center for Biotherapy, Sichuan, China

[8]QIMR Berghofer Medical Research Institute, Herston, Queensland, Australia (current address).

[*] These authors contributed equally to the work.

Address correspondence to:

Katja M. Kanninen
Department of Neurobiology
A.I.Virtanen Institute for Molecular Sciences
University of Eastern Finland
Ph: +358 40 135 4377
Email: katja.kanninen@uef.fi

Anthony White
Cell and Molecular Biology
QIMR Berghofer Medical Research Institute
Locked Bag 2000
Royal Brisbane Hospital QLD 4029
T: +61 (7) 3362 0360
E: tony.white@qimrberghofer.edu.au

Peng Lei
Department of Neurology and State Key Laboratory of Biotherapy
West China Hospital
Sichuan University
Ph: +86 28 87636824
Email: peng.lei@scu.edu.cn; peng.lei@florey.edu.au

ABSTRACT

Developing new therapies for stroke is urgently needed, as this disease is the leading cause of death and disability in the aged population, and the existing treatment is only available for a small subset of patients. The interruption of blood flow to the brain during ischemic stroke launches multiple immune responses, which are characterized by infiltration of peripheral immune cells, the activation of resident brain microglial cells, and the accumulation of immune mediators. Copper is an essential trace element that is required for many critical processes in the brain. Copper homeostasis is disturbed in chronic neurodegenerative diseases and altered in stroke patients, and restoration of brain copper homeostasis has been shown to be protective against chronic neurodegeneration. This study was undertaken to assess whether the copper *bis*(thiosemicarbazone) complex, Cu^{II}(atsm), is beneficial in acute brain injury, in preclinical mouse models of ischemic stroke. We demonstrate that the copper complex Cu^{II}(atsm) protects neurons from excitotoxicity *in vitro,* and is protective in permanent and transient ischemia models in mice as measured by functional outcome and lesion size. Restoration of copper homeostasis in the ischemic brains modulates the inflammatory response, specifically affecting the resident microglial cells. It reduces CD45 and Iba1 immunoreactivity, and alters the morphology of Iba1 positive cells in the ischemic brain. Cu^{II}(atsm) also protects endogenous microglia against ischemic insult and reduces the proportion of invading monocytes. These results demonstrate that the copper complex Cu^{II}(atsm) is an inflammation-modulating compound with high therapeutic potential in stroke and is a strong candidate for the development of therapies for acute brain injury.

INTRODUCTION

Ischemic stroke, caused by blockage of blood flow in the brain, is a leading cause of death and long-term disability because of limited therapeutic interventions that are available in clinics. Despite significant efforts, drugs that have the potential to limit neuronal damage after the onset of ischemic stroke have not been discovered. The lack of efficient therapeutics for stroke is largely related to the poor translation of preclinical results into clinics. Due to the heterogeneity of stroke types, this disorder is challenging to model in laboratory settings, and results gained from one preclinical model rarely represent stroke in a broad number of patients.

The interruption of blood flow to the brain during ischemic stroke launches multiple immune responses, which are characterized by infiltration of peripheral immune cells, the activation of resident brain immune cells, and the accumulation of immune mediators (Lakhan, Kirchgessner et al. 2009). Microglia, the resident immune cells of the brain, are a heterogeneous group of cells that have multiple roles in promoting and modulating brain functions under physiological conditions, including phagocytosis of debris or dying cells, neurogenesis and synaptic modulation (Solano Fonseca, Mahesula et al. 2016, Paolicelli, Bolasco et al. 2011). Microglia also have the potential to contribute to the neurological outcome of brain injury (Lalancette-Hebert, Swarup et al. 2012). During acute brain injury, microglia rapidly undergo dramatic morphological and phenotypic changes. Considering the heterogeneous nature of microglia, it is not surprising that studies assessing the effect of microglia on stroke outcome have yielded controversial results. In any case, neuroinflammation and blood brain barrier disruption are critical steps in the loss of the neurogliavascular network integrity and exacerbation of ischemic damage, and activated microglia are a driving factor in these phenomena (Jolivel, Bicker et al. 2015). They up-regulate the expression of particular cell surface antigens and produce inflammatory mediators such as tumor necrosis factor (TNF), which can promote neurotoxicity (Ma, Wang et al. 2016). However, microglia also possess several beneficial functions in stroke, including the promotion of neurogenesis and production of neurotrophic factors (Thored, Heldmann et al. 2009). Furthermore, ablation of microglia has been shown to exacerbate ischemic injury in the brain (Lalancette-Hebert, Gowing et al. 2007).

Copper is an essential trace element that is required for many critical processes in the brain (Zucconi, Cipriani et al. 2007). While copper homeostasis is disturbed in chronic neurodegenerative diseases such as Alzheimer's disease (AD) (Schrag, Crofton et al. 2011), little is known about copper homeostasis in acute ischemic stroke. Several years ago Kodali et al. reported an increase in the plasma copper concentrations of ischemic stroke patients (Kodali, Chitta et al. 2012). This observation was supported

by a recent paper demonstrating that serum levels of both total copper and copper loosely bound to small molecules are elevated in serum of ischemic stroke patients (Lai, Wang et al. 2016). These papers provide evidence for copper dyshomeostasis upon ischemic damage in patients. In mouse models, chronic intake of copper has been reported to aggravate ischemic damage, an effect attributed to reduction of angiogenesis (Jiang, Wang et al. 2015).

Restoration of brain copper homeostasis is protective against chronic neurodegeneration in animal models of disease (Grubman, White et al. 2014). The copper *bis*(thiosemicarbazones) are stable, lipophilic neutral copper(II) complexes that are capable of crossing both cell membranes and the blood brain barrier (Donnelly, Caragounis et al. 2008, Torres, Andreozzi et al. 2016). These metal delivery and redistribution agents are neuroprotective in animal models of AD, Parkinson's disease, and amyotrophic lateral sclerosis (Crouch, Hung et al. 2009, Soon, Donnelly et al. 2011, McAllum, Lim et al. 2013, Hung, Villemagne et al. 2012) Furthermore, we have recently demonstrated that Cu^{II}(atsm) reduces brain inflammation caused by peripheral administration of bacterial lipopolysaccharide, and that copper delivery is anti-inflammatory in the chronic neuroinflammatory milieu of AD model mice (Choo et al. submitted). To our knowledge, therapeutic approaches targeted to modulation of copper homeostasis in ischemic stroke, where significant neuroinflammation takes place, have not previously been tested.

To address the issue of poor translation of stroke therapeutics into human patients, in this study we tested the copper complex Cu^{II}(atsm) in both permanent and transient ischemia mouse models, and characterized the inflammatory responses after restoration of copper homeostasis in the ischemic brains. We demonstrate that Cu^{II}(atsm) is protective in both permanent and transient ischemia models, and that restoration of copper homeostasis in the ischemic brains modulates the inflammatory response, specifically affecting the resident microglial cells. These results demonstrate that Cu^{II}(atsm) is a compound with high therapeutic potential in stroke and is a strong candidate for the development of therapies for acute brain injury.

MATERIALS AND METHODS

Mice

All animal experiments were approved by the National Animal Experiment Board of Finland and followed the Council of Europe Legislation and Regulation for Animal Protection, or by the Florey

Institute animal ethics committee (15-019) and were performed in accordance with the National Health and Medical Research Council Australia guidelines. Mice were housed in individual cages in conditions of controlled humidity, temperature, and light conditions. Water and food were provided *ad libitum*. Adult male Balb/cOlaHsd mice (Harlan Laboratories B.V., An Venrey, Netherlands) were used for the permanent ischemia studies and C57Bl/6 mice (Animal Resources Centre, Western Australia) were used for the transient ischemia studies. Mice were randomized into treatment groups using GraphPad QuickCalcs (GraphPad Software, San Diego, CA, USA) and all the analyses were performed blinded to the experimental groups. The exclusion criteria were predetermined: mice with hemorrhages visible in magnetic resonance imaging (MRI), bleeding during the surgery, and unsuccessful induction of ischemia were excluded. The numbers of mice used for each experiment are indicated in the figure legends.

Ischemia surgeries and treatment of mice with Cu^{II}(atsm)

Transient acute focal cerebral ischemia was induced by the intraluminal middle cerebral artery occlusion (MCAO) as described previously (Tu, Xu et al. 2010, Longa, Weinstein et al. 1989). Mice were deeply anaesthetized with 5 % isoflurane in 30% O_2/70% N_2O using the Anesthesia system (Mediquip, Australia). The level of isoflurane was then reduced and maintained at 1%. A 4-mm distal nylon monofilament (30 mm in length, 0.16 mm in diameter, Amber, Japan) segment was coated with 0.21-0.22 mm diameter silicone (Henkel, Australia). MCAO was performed by insertion of the monofilament via the common carotid artery into the left internal carotid artery, advanced 9-10 mm past the carotid bifurcation until a slight resistance was felt. The filament was left in place for 60 min, and then withdrawn for reperfusion. In the sham-operated animals, the occluding filament was inserted only 5 mm above the carotid bifurcation. We excluded mice from further studies if excessive bleeding occurred during surgery, if the operation time exceeded 90 min, if the mouse failed to recover from anesthesia within 15 min, or if hemorrhage was found in the brain slices or at the base of the circle of Willis during postmortem examination.

For permanent occlusion of the MCA, mice were deeply anesthetized with 5 % isoflurane in 30% O_2/70% N_2O and the anesthesia was maintained at 2 % during the surgery. The left MCA was permanently occluded as previously described (Dhungana, Malm et al. 2013). Briefly, first the temporal bone was first exposed and a 1-mm-diameter hole was drilled to expose the artery. The dura was removed, after which the artery was lifted and occluded by a thermocoagulator (Aaron Medical Industries Inc., Clearwater, FL, USA). MCA occlusion was confirmed by cutting the artery, after which the temporal muscle was replaced and the wound was sutured.

The body temperatures of animals were maintained at 37 ± 0.5 °C throughout the procedures using a heat-pad. The temperature and respiratory rates were monitored during the surgery. The sham mice went through all the same procedures except the occlusion of the MCA.

Cu^{II}(atsm) was prepared according to published procedures (Gingras, Suprunchuk et al. 1962). The mice were treated with Cu^{II}(atsm) dissolved in standard suspension vehicle (SSV) solution containing 0.9% (w/v) NaCl, 0.5% (w/v) sodium carboxymethylcellulose, 0.5% (v/v) benzyl alcohol, 0.4% (v/v) Tween 80, or SSV alone by oral gavage. For the transient MCAO mice were treated daily at a dose of 15 mg/kg while for the permanent MCAO the mice were treated daily at a dose of 60 mg/kg. Mice were sacrificed at 1 or 3 dpi and tissues were collected as described below.

Post-surgery evaluation of outcome

After 24 h of transient MCAO induction, the neurological deficit of each mouse was evaluated by a simple scale (five-point scale) as described previously (Bederson, Pitts et al. 1986): 0, no observable deficit; 1, right forelimb flexion; 2, decreased resistance to left lateral push (and right forelimb flexion) without circling; 3, same behavior as grade 2, with circling to right; 4, severe rotation progressing into barreling, loss of walking or righting reflex. Two investigators blinded to the experimental groups performed the neurological assessment post-surgery.

At 1 day post injury (dpi) the locomotor activity of the mice subjected to permanent MCAO was assessed by the latency to move test as previously described (Bargiotas, Krenz et al. 2012). The mice were placed on a flat surface and the time to move one body length (7 cm) was recorded by an investigator blinded to the treatment groups. Each mouse received two trials.

Measurement of lesion volume

TTC staining was used to quantify the lesion size following transient ischemia. 24 hours after the induction of MCAO the mice were euthanized with an overdose of sodium pentobarbitone (Lethabarb, 100mg/kg). The brain was removed rapidly and frozen at -20°C for 20 min. Coronal slices were made at 2 mm intervals from the frontal poles, and sections were immersed in 0.5% 2,3,5-tripenyltetrazolium chloride (TTC, Sigma-Aldrich) in phosphate buffered saline (PBS) at 37°C for 20 min. The presence or absence of infarction was determined by examining TTC-stained sections for the areas on the side of infarction that did not stain with TTC. The brain slices were fixed in 4% paraformaldehyde at 4°C until

imaging. Serial sections were photographed using a digital camera and the area of infarct was quantified with Image J (1.49m, NIH) by an investigator blinded to the experimental groups. The area of infarct, the area of ipsilateral hemisphere, and the area of the contralateral hemisphere were measured for each section by a blinded operator. The volume was calculated by summing the representative areas in all sections and multiplying by the slice thickness, then correcting for edema, as previously described (Lin, He et al. 1993): *Corrected Infarct Volume (CIV) = contralateral hemisphere volume – (ipsilateral hemisphere volume – infarct volume).*

MRI imaging was utilized to determine the lesion volume following permanent MCAO. A vertical 9.4 T Oxford NMR 400 magnet (Oxford Instrument PLC, Abington, UK) was used for visualizing the lesion as previously described (Dhungana, Malm et al. 2013). The mice were anesthetized with 5 % isoflurane in 30% O_2/70% N_2O and multislice T2-weighted images (repetition time 3000 ms, echo time 40 ms, matrix size 128 × 256, field of view 19.2 × 19.2 mm^2, slice thickness 0.8 mm and number of slices 12) were obtained. The images were analyzed using in-house made software Aedes in the Matlab environment (Math-works, Natick, MA, USA). The infarction volume was calculated as previously described (Shuaib 2002) by using the following formula: *Infarct volume = (volume of left hemisphere − (volume of right hemisphere − measured infarct volume)) / volume of left hemisphere.*

Immunohistochemistry

Mice were euthanized at 1 or 3 dpi for tissue collection. Mice were anesthesized with 250 mg/kg Avertin and perfused transcardially with heparinized (2500IU/L) saline. The brains were removed and post-fixed in 4% paraformaldehyde for 20 h, after which they were cryoprotected for 48 h in 30% sucrose solution, frozen in liquid nitrogen and cut into 20 μm thick sections with a cryostat (Leica Microsystems, Wetzlar, Germany). Six sections at an interval of 400 μm were selected for immunohistological staining.

The brain sections were incubated overnight at room temperature with primary antibodies (CD45, 1:100 dilution, Bio-Rad, Hercules, CA, USA; CD68, 1:2000 dilution, Bio-Rad, Hercules, CA, USA; GFAP, 1:500 dilution, ABR Affinity BioReagents, Golden, CO, USA; Iba-1, 1:250 dilution, Wako Chemicals, Tokyo, Japan; Ly6-G neutrophil, 1:100 dilution, BioLegend, San Diego, CA, USA; phospho-p38, 1:100 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA). All stainings except the Ly6-G neutrophil stain required antigen retrieval in 10 mM aqueous solution of sodium citrate dihydrate (pH 6, preheated to 92°C) before application of primary antibodies. Secondary antibodies were applied on sections after three washes in 0.05% Tween20 in PBS. Fluorescent Alexa

568-conjugated secondary antibody (1:200 dilution, Abcam, Cambridge, UK) was used with CD45 and CD68, and 488-conjugated secondary antibody with Iba-1, Ly6G and GFAP. With the phospho-p38 staining, biotinylated secondary antibody (1:200 dilution, Vector Laboratories, Burlingame, CA, USA) was used instead of a fluorescent one, followed by reaction with avidin-biotin complex reagent (1:200 dilution, Vector Laboratories, Burlingame, CA, USA) according to manufacturer's instructions. The bound immunoreactivity was then visualized by development with nickel-enhanced 3,3'-diaminobenzidine.

For quantification of CD68, GFAP, Iba-1 immunoreactivities in the peri-ischemic area, a 718 x 532 µm cortical area adjacent to the infarct border was imaged using 10x magnification on an AX70 microscope (Olympus corporation, Tokyo, Japan) with an adjacent digital camera (Color View 12 or F-View, Soft imaging system, Muenster, Germany) running AnalySis software (Soft Imaging System). For CD45 and neutrophil stainings, images of equal size were taken from the lesion area, where the majority of the immunoreactivity was observed. Quantification of the immunoreactivities was performed blinded to the study groups using ImagePro Plus software (Media Cybernetics, Rockville, MD, USA) at a predefined range and presented as relative immunoreactive area.

Measurement of cell morphology

The morphological reconstitution of Iba1 positive cells was carried out by Matlab v2013b as described (Kongsui, Johnson et al. 2015). Several characteristics of the segmented cell processes and cell bodies were analyzed, including area, perimeter and diameter. The images for morphological assessment were taken by a Zeiss LSM 700 confocal microscope (Zeiss Inc., Maple Grove, USA) with an attached digital camera (Color View 12 or F-View; Soft Imaging System, Munster, Germany) running Zen 2012 Image analysis Software (Zeiss inc., Maple Grove, USA).

Cytokine measurement

Cytokine concentrations were measured in the brain tissues of mice subjected to permanent MCAO. The mice were sacrificed as described above and following dissection of the peri-ischemic brain area, the samples snap frozen in liquid nitrogen were first homogenized in 20 mM Tris–HCl (pH 7.4), 8.56% sucrose, 0.5 mM EDTA, 0.5 mM EGTA and protease inhibitor cocktail (Complete, Roche Applied Science). The protein levels of IL-6, IL-10, MCP-1, IFN-γ, TNF and IL-12p70 were then measured using the mouse anti-inflammatory cytokine bead array (CBA) kit (BD Biosciences, San Jose, CA, USA), running the samples on a FACS Calibur flow cytometer (BD Biosciences). The results were

analyzed using FCAP Array 2.0 software (Soft Flow Hungary Ltd, Pecs, Hungary). Total protein concentrations of the brain samples were determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc, Hercules, CA, USA) and the results of the CBA assay were normalized to the total protein concentrations.

Measurement of brain copper content

Inductively coupled mass spectrometry (ICP-MS) was used to measure copper levels in peri-ischemic brain areas as reported previously (Grubman, James et al. 2014). Briefly, cell pellets collected for copper analysis were digested overnight in concentrated nitric acid (Aristar, BDH, Kilsyth, VIC, Australia), after which samples were heated for 20 min at 90°C. The volume of each sample was reduced to approximately 40–50 µl then 1 ml of 1% (v/v) nitric acid diluent was added to the samples. Measurements were made using an Agilent ICPMS 7700x series ICP-MS instrument. Results were expressed as micromole per liter concentrations of copper (µmol/l).

Cortical neuron cultures and MTT viability assay

Primary cortical neuron cultures were prepared from E15 embryos. Cortices were dissected, after which tissues were dissociated with trypsin (0.0125% for 15 min at 37°C, Sigma-Aldrich, St. Louis, MO, USA). Neurons were counted and plated on poly-d-lysine (Sigma-Aldrich, St. Louis, MO, USA) coated 48-well plates at a density of 150 000 cells/well in Neurobasal media containing 2% B27, 500 µM l-glutamine and 1% penicillin–streptomycin (all ThermoFisher Scientific, Waltham, MA, USA). 50% of the medium was changed four days after plating and cultures were used for experiments on days 6-7 *in vitro*.

Treatment of cortical neurons was carried out in 50 % fresh Neurobasal medium containing 2% B27, 500 µM l-glutamine and 1% penicillin–streptomycin and 50 % media collected from the cells. Neurons were treated with 1 µM Cu^{II}(atsm) prepared as above and/or 400 µM glutamate (Sigma-Aldrich, St. Louis, MO, USA) for 24 h prior to measurement of cell viability by the MTT assay.

The MTT reduction assay was performed 24 hours after treatment with Cu^{II}(atsm) and glutamate according to Denizot & Lang (Denizot, Lang 1986) with the following modifications. Briefly, following removal of the media (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) was added to cells at a concentration of 120 µM, after which the cells were incubated for 2 hours at 37°C at 5 % CO_2. Following removal of the media cells were dissolved in DMSO. The absorbances were read

at 585 nm with a Wallac Victor^2 1420 multilabel counter (PerkinElmer Inc, Waltham, MA, USA). The results were calculated as relative absorbances compared to the control wells.

Brain cell isolation and flow cytometry

At 3 dpi the mice that underwent pMCAO were transcardially perfused with heparnized saline as above and the brains were isolated in HBSS on ice and the cerebellum was removed. The tissue was minced on ice with forceps in petri dishes in digest buffer containing 0.5 mg/ml collagenase type 4 (Worthington, Lakewood, NJ, USA) DNAse 1, 25 U/ml, and RPMI-1640 (both from Sigma-Aldrich) then incubated at 37°C, 5% CO2 for 20 min. Samples were put directly back on ice, triturated, and sequentially filtered through 70 µm and 40 µm cell strainers (Falcon, Corning, Germany). Homogenates were centrifuged 450 x *g* for 5 min and resuspended in Miltenyi Myelin Debris Removal Beads II (Miltenyi, Cologne, Germany). Samples were incubated for 15 min at 4°C then washed with cold MACS buffer (PBS and 0.5% BSA, both from Sigma) and centrifuged at 400 x *g* for 10 min. Pellets were resuspended in cold MACS buffer and applied to magnetic LD columns (Miltenyi) with 50 µm CellTrics pre-filters (Sysmex, Norderstedt, Germany). The flow-through was collected on ice, counted then spun 400 x *g* for 10 min and resuspended in RPMI-1620.

Cell surface staining was done on 200 000-300 000 cells per mouse in round bottom polypropylene 96-well plates (Corning). Cells were washed with PBS, stained with Zombie NIR fixable viability dye (BioLegend, San Diego, CA, USA) for 15 min at RT, then blocked with CD16/32 (clone 24G2, BD Biosciences, San Jose, CA, USA) and stained with the following antibodies: CD45 PerCP-Cy5.5 (clone 30F11, eBioscience, San Diego, CA, USA) CD11b PECy7 (clone M1/70, eBioscience), Ly6G FITC (clone 1A8, BioLegend), F4/80 PE (clone A3-1, AbD Serotec, Oxford, UK) Ly6C APC (clone AL-21, BD Biosciences) for 30 min at 4°C. Cells were washed twice in PBS and fixed in 0.5 % Ultra Pure formaldehyde (ThermoFisher). Samples were acquired on BD FACSAria III equipped with 488 and 633nm lasers with standard configuration. Data were analyzed using FCSExpress v5 (DeNovo Software).

Statistics

Statistical analyses were performed in GraphPad Prism software 5.03 (GraphPad Software, La Jolla, CA, USA) using either unpaired 2-tailed t-test, 1-way ANOVA or 2-way ANOVA as appropriate. The statistical test used and n-numbers are indicated in each figure legend. p-values < 0.05 were considered statistically significant. All data are presented as mean ± S.D.

RESULTS

1. The copper complex Cu^{II}(atsm) protects neurons from glutamate-induced excitotoxicity *in vitro.*

Excitotoxicity is a primary mechanism of neuronal injury following stroke. To determine the neuroprotective potential of Cu^{II}(atsm) against excitotoxic insult, primary cortical neurons were treated with Cu^{II}(atsm) in the presence of glutamate. Treating neurons with glutamate for 24 h resulted in approximately 40 % reduction in cell viability as measured by the MTT assay (Fig 3). Co-treatment with Cu^{II}(atsm) significantly improved the viability of glutamate-treated neurons, although the treatment did not entirely rescue the cells from excitotoxicity. At the tested concentrations, neuronal viability was not affected by Cu^{II}(atsm) treatment alone.

2. Cu^{II}(atsm) reduces ischemic injury in a transient model of cerebral ischemia

Cu^{II}(atsm) was tested firstly in the transient middle cerebral artery occlusion (MCAO) mouse model of cerebral ischemia. We found that Cu^{II}(atsm) treatment (15 mg/kg, by oral gavage) both prior to MCAO, and at the start of reperfusion reduced infarct sizes 24 h later (Fig 1A-B). MCAO-induced functional impairment indexed by neuroscore was also prevented by pre-treatment of Cu^{II}(atsm) (Fig 1C) 24 h later.

3. Cu^{II}(atsm) confers a beneficial effect on the outcome of ischemic stroke in a permanent model of cerebral ischemia

Cu^{II}(atsm) was administered to mice by oral gavage immediately after permanent middle cerebral artery occlusion and the lesion volume was measured 24 h later by MRI. Cu^{II}(atsm) administered at a dose of 60 mg/kg caused a significant reduction in the lesion volume (Fig 2A-B). The locomotor activity of the ischemic mice was assessed using the latency to move test, which is reported to be affected for several days after ischemia (Lubjuhn, Gastens et al. 2009). Ischemic mice showed a significant reduction in the latency to move at 1 dpi, which was corrected to the levels of sham mice by Cu^{II}(atsm) treatment (Fig 2C). To confirm that the observed beneficial effects were related to copper

delivery by Cu^{II}(atsm), the ischemic brain tissues were analysed for copper content by ICP-MS. When compared to vehicle treated mice, the concentration of copper in the peri-ischemic area of the Cu^{II}(atsm) treated mice was 57 % higher (Fig 2D), indicating efficient delivery of copper by the complex.

4. Cu^{II}(atsm) reduces CD45 levels in the ischemic core

Previous studies have suggested that copper delivery may have anti-inflammatory effects in neurodegenerative diseases (Malm, Iivonen et al. 2007). To assess the effect of copper delivery by Cu^{II}(atsm) on neuroinflammation in the context of ischemic stroke, we first assessed astrocytic and myeloid cell activation in the ischemic brains by immunohistochemistry. Astrocytic activation as assessed by GFAP immunohistochemistry was not affected by Cu^{II}(atsm) treatment in the peri-infarct area (data not shown). However, analyses of brains subjected to permanent ischemia revealed significant Cu^{II}(atsm) induced alterations to the levels of CD45. CD45 is a marker expressed highly by monocytes and to a lower extent by microglia, in which expression is up-regulated after injury (Ritzel, Patel et al. 2015). Copper delivery with Cu^{II}(atsm) reduced the amount of CD45 immunoreactivity in the ischemic core both at 1 (Fig 4A-B) and 3 dpi (Fig 4C-D).

5. Cu^{II}(atsm) reduces Iba1 immunoreactivity and alters the morphology of Iba1-positive cells in the ischemic brain

To extend our analyses of myeloid cell involvement in Cu^{II}(atsm)-mediated protection from ischemic injury we undertook a panel of immunohistological stainings in order to determine which cell types are affected by the treatment. While the immunoreactivities for CD68 (a marker of active phagocytosis) and Arginase-1 (a marker of alternatively activated cells) remained unaltered (data not shown), Iba1 levels in the peri-ischemic brains were altered by copper delivery. Iba1 is expressed by microglia and monocytes and its expression is reported to increase during ischemia (Ito, Tanaka et al. 2001). It is considered one of the useful proteins for distinguishing microglia through immunostaining especially in stroke studies (Ito, Tanaka et al. 2001). The Iba1 staining revealed a significant reduction of immunoreactivity by the Cu^{II}(atsm) treatment at 3 dpi in mice subjected to pMCAO (Fig 5A-B).

While measuring immunoreactivity following histological staining provides information about changes in the cells, it does not give detailed information about whether the change is related to the presence of more or less cells, larger or smaller cells or more or less intensively stained cells. Moreover, as

microglial morphology is tightly coupled to their function we next assessed the effect of copper delivery on the structure of the Iba1-positive cells. The morphology of the Iba1 positive cells was analyzed as reported (Kongsui, Johnson et al. 2015). The digital reconstruction (Fig 5C-D) revealed that the morphology of Iba1-positive cells was altered by copper delivery in ischemic mice. Cu^{II}(atsm) did not affect cellular branching or processes (data not shown). However, despite the reduction in immunoreactivity (Fig 5A-B) the treatment significantly increased the cellular area of the Iba1-positive cells (Fig 5F).

6. Cu^{II}(atsm) increases the proportion of resident microglia in the ischemic brain

To create a multiparameter characterization of the cell types affected by Cu^{II}(atsm) during ischemia we isolated immune cells from the brain and applied flow cytometry for several cellular markers. The gating strategies are shown in Fig 6A. The cells were discriminated based on the expression of CD45, CD11b, F4/80, Ly6C and Ly6G (Fig 6A-B). In comparison to sham mice, ischemia induced a 59 % increase in $CD45^{+}CD11b^{-}$ lymphocytes ($p=0.001$), and a 97 % increase in $Ly6G^{+}$ neutrophils ($p<0.001$) (data not shown). Ischemia also induced a 95 % increase in $CD45^{high}$ $CD11b^{+}$ $Ly6G^{-}$ infiltrating myeloid cells ($p<0.001$), a 53 % increase in the $Ly6C^{high}$ $CD45^{low}$ cells ($p=0.007$), a 41 % increase $Ly6C^{high}$ $CD45^{high}$ cells, and a 70 % increase in $CD45^{high}$ $F4/80^{+}$ cells ($p<0.001$) (data not shown).

The numbers of resident, $CD45^{low}$ $CD11b^{+}$ $Ly6G^{-}$ microglia were reduced by 86 % ($p<0.001$) in ischemic brains (data not shown). Cu^{II}(atsm) treatment had a significant effect on the resident microglia. These microglia were $Ly6G^{low}$ and $F4/80^{+}$ (Fig 6B). When compared to vehicle-treated ischemic mice, the percentages of resident $CD45^{low}$ $CD11b^{+}$ $Ly6G^{-}$ microglia were increased by 30%, while invading $CD45^{high}$ $CD11b^{+}$ $Ly6G^{-}$ cells were reduced by 30% in the Cu^{II}(atsm) treated mice at 3 dpi (Fig 6B-C).

7. Copper delivery by Cu^{II}(atsm) reduces additional markers of inflammation in the ischemic brain

To further assess the effect of copper delivery on the inflammatory milieu following ischemia, the immunoreactivity of p38 MAPK was analyzed by histochemical staining and the levels of 6 brain cytokines were analyzed by CBA. p38 MAPK is a key serine/threonine protein kinase that is an important contributor to increased microglial production of proinflammatory cytokines (Bachstetter, Xing et al. 2011). Staining for the active form of p38 MAPK in the brains of ischemic mice revealed that Cu^{II}(atsm) reduced its expression at 1 dpi (Fig 7A-B).

CBA analyses showed that Cu^{II}(atsm) treatment at 1 dpi did not affect the amount of IL-6, IL-10, MCP-1, IFN-γ or TNF in the ischemic brain. However, copper delivery by Cu^{II}(atsm) reduced the protein level of IL-12 by 54 % in the peri-ischemic area of the brain at 1 dpi (Fig 7C). In contrast, IL-10 levels were increased by Cu^{II}(atsm) treatment at 3 dpi (Fig 7D).

DISCUSSION

Cu^{II}(atsm) has been shown to have protective effects in multiple animal models of chronic neurodegenerative diseases, such as ALS and PD (Soon, Donnelly et al. 2011, Hung, Villemagne et al. 2012). The compound is about to be tested in clinical trials in ALS patients. Interestingly, Cu^{II}(atsm) has a tendency to deliver copper into cells that suffer from a dysfunctional electron transport chain, indicating optimal pharmacokinetics in the ischemic brain (Donnelly, Liddell et al. 2012). Here, we report for the first time that Cu^{II}(atsm) is protective in acute brain injury, in two *in vivo* models of ischemic stroke. The protective effect is accompanied by beneficial modulation of the inflammatory milieu during stroke.

We report that Cu^{II}(atsm) reduces lesion volume and improves functional outcome in both transient and permanent models of ischemic stroke in mice. Demonstration of the therapeutic effect in two different models shows that protection is not dependent on mouse strain, duration of ischemia, or location of lesion. In the transient model the doses sufficient to elicit therapeutic effects were about half of that required in the permanent model of cerebral ischemia. The difference in effective dose may be connected to access to the ischemic area, as the reperfusion period in the transient model opens a circulatory connection to the injured site, mimicking recanalization therapy received by a human patient. The fact that Cu^{II}(atsm) reduced lesion volume and promoted functional recovery in both transient and permanent ischemia models suggests that it is a potent approach given the heterogeneous stroke subtypes observed in humans. In addition, using both permanent and transient ischemia is a recommendation mentioned in STAIR criteria aiming to improve translation of preclinical stroke research (Fisher, Feuerstein et al. 2009).

As previously mentioned, studies with human patients have suggested an imbalance in copper homeostasis after cerebral ischemia (Lai, Wang et al. 2016, Kodali, Chitta et al. 2012). Elevated levels of copper were observed in the circulation of stroke patients in both studies, suggesting that copper is redistributed due to stroke-induced damage. Moreover, chronic intake of copper in the form of copper

sulfate in the drinking water has been shown to exacerbate ischemic damage in mice, thereby indicating that increases in free copper exert toxic effects in the brain (Jiang, Wang et al. 2015). Here, we report that Cu^{II}(atsm) increased peri-ischemic concentrations of copper coincidentally with reduced lesion size and behavioural improvements. Formerly known as a hypoxia-imaging agent that releases copper mainly in the presence of a dysfunctional electron transport chain (Donnelly, Liddell et al. 2012) we propose that short-term treatment with Cu^{II}(atsm) helps to restore peri-ischemic disturbances of copper homeostasis occurring as a result of ischemic damage.

Neuronal excitotoxicity is one of the key components of stroke pathology leading to mitochondrial dysfunction (Stanika, Pivovarova et al. 2009). It has been earlier described that copper has an essential function in protecting neurons against excitotoxicity through NMDAR receptor modulation (Gasperini, Meneghetti et al. 2015). In agreement with this, we demonstrate that the copper complex Cu^{II}(atsm) improved the viability of primary cortical neurons exposed to excitotoxic injury. However, it is possible that Cu^{II}(atsm) also affected secondary damage in the cells, rather than merely receptor activation, since copper delivery by Cu^{II}(atsm) is improved in the presence of mitochondrial dysfunction (Donnelly, Liddell et al. 2012). In addition, lack of complete protection against excitotoxic insult might indicate a more complex scheme of protection *in vivo*, involving the interplay between neurons and microglia. Nevertheless, it is plausible that the neuroprotective effect seen in animal models of ischemia was at least partly caused by Cu^{II}(atsm)-mediated reductions in excitotoxicity.

We have earlier reported that the copper *bis*(thiosemicarbazonato) complexes mediate anti-inflammatory effects in neurodegenerative diseases (Choo et al. submitted). Thus, we next characterized this anti-inflammatory effect in acute injury during stroke. Indeed, quantification of CD45 immunoreactivity revealed a significant reduction in this cell type in the ischemic core at 1 and 3 days after pMCAO, coinciding with reduced lesion volume and improved functional outcome. The cells in the ischemic core appeared $CD45^{high}$-expressing, indicating that Cu^{II}(atsm) reduced the amount of infiltrating myeloid cells into the ischemic area (Greter, Lelios et al. 2015). Because reduced infiltration of myeloid cells indicated altered initiation of post-stroke inflammation, we used multiple markers to specify the effect of Cu^{II}(atsm) on different inflammatory cell types in the pMCAO model. Based on immunohistochemical analyses of the peri-ischemic brain area, we report a Cu^{II}(atsm)-mediated reduction in Iba1 immunoreactivity. While previous studies have shown that Iba1 can be expressed by both microglia and invading peripheral inflammatory cells, the early time point of 3 days after ischemia used in the current study suggests that the observed reduction may be caused by reduced activation of resident microglia (Ito, Tanaka et al. 2001, Miro-Mur, Perez-de-Puig et al. 2016).

Since microglial morphology and function are usually linked to each other (Zhang, Chopp et al. 1997), we next used a computer algorithm to assess the morphology of the Iba1 positive cells following ischemia. Cu^{II}(atsm) increased the size of the somas of Iba1 positive microglia without affecting their processes or branching. Increased soma sizes have been reported to correlate with increased microglial activation (Kozlowski, Weimer 2012). It appears that there may be separate phenotypic populations of microglial cells responding to Cu^{II}(atsm): those at a more naïve state displaying low Iba1 expression, and those with high Iba1 expression. Morphological assessment of the $Iba1^{high}$ cells revealed that the $Iba1^{high}$ cells are actually in a more activated state in Cu^{II}(atsm)-treated animals. These kinds of spatiotemporal differences in microglial responses to experimental treatments have been described earlier in the context of ischemic stroke, and are thought to arise from the heterogeneous nature of microglia (Ruscher, Inacio et al. 2012, Schroeter, Jander et al. 1999).

To calculate the true proportions of each cell type in the ischemic hemisphere, we used flow cytometry for multiple cellular markers. We report that 3 days after permanent MCAO, there was a strong infiltration of $CD45^{high}$ $CD11b^{+}$ $Ly6G^{-}$ monocytes, CD45+CD11b- lymphocytes and Ly6G+ neutrophils. Infiltration of monocytes and lymphocytes is known to take place at this time point, whereas some controversy remains about the latency of neutrophil infiltration (Zhou, Liesz et al. 2013, Chu, Kim et al. 2014). In line with results from immunohistochemistry, Cu^{II}(atsm) significantly increased the amount of resident microglia, characterized by $CD45^{low}$ $CD11b^{+}$ $Ly6G^{-}$, and reduced the proportion of invading $CD45^{high}$ $CD11b^{+}$ $Ly6G^{-}$ cells 3 days after permanent MCAO (Greter, Lelios et al. 2015). These results suggest that Cu^{II}(atsm) reduces the proportion of invading monocytes, and protects the endogenous microglia against ischemic insult. This may be related to the cellular protection observed in the quantification of the lesion size in MRI images.

P38 MAPK is a key mediator of microglial production of proinflammatory cytokines in the presence of multiple stressors (Bachstetter, Xing et al. 2011). In addition, inhibition of this kinase is protective in experimental models of stroke through multiple other pathways (Zhang, Zhang et al. 2015). In the current study, we show that immunoreactivity of the active form of p38 was reduced in the ischemic Cu^{II}(atsm)-treated animals. Based on morphology, these cells were microglial cells, giving further evidence of a reduced inflammatory state of the cells in the ischemic brain. At the same time, we also saw a reduction in IL-12 levels and a later increase in IL-10 levels. These alleviations in inflammatory signaling might be an important feature in Cu^{II}(atsm)-mediated protection of higher brain functions, as IL-12 has been associated with cognitive decline in stroke patients (Narasimhalu, Lee et al. 2015). In addition, IL-10 deficiency has been previously shown to exacerbate ischemic brain damage, whereas common protectants such as minocycline increase IL-10-signaling (Yang, Salayandia et al. 2015,

Perez-de Puig, Miro et al. 2013). Taken together, these results suggest that Cu^{II}(atsm) has several inflammation—modulating actions in the ischemic brain.

Available treatment options for stroke are inadequate and those available have limited efficacy. This study reports the therapeutic actions of Cu^{II}(atsm) in two preclinical *in vivo* models of stroke, and characterizes the effects of copper modulation on immune responses occurring during experimental stroke. These result suggest that similarly to models of AD and peripheral inflammation (Choo et al. submitted), the copper *bis*(thiosemicarbazonato) complexes confer their beneficial effects at least partly through the modulation of inflammation, and specifically microglia, in ischemic brain injury. Since a copper *bis*(thiosemicarbazonato) complex is going to tested clinically for ALS, it may shed a light for the complex to be tested against neuronal damage after ischemic stroke clinically. This study also provides further support for the neurotherapeutic potential of copper modulation in brain disorders.

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COMPETING FINANCIAL INTERESTS

Dr. Bush is a shareholder in Prana Biotechnology Ltd, Cogstate Ltd, Mesoblast Ltd, NextVet Ltd, Brighton Biotech LLC, and Cogstate Ltd, and a consultant for Collaborative Medicinal Development Pty Ltd.

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FIGURE LEGENDS

Figure 1. Cu^{II}(atsm) is protective against excitotoxicity in primary neurons. Primary cortical neurons were treated with Cu^{II}(atsm) in the presence of 400 μM glutamate for 24 h, after which neuronal viability was measured by MTT reduction. Data show means ± S.D. from one representative experiment. 1-way ANOVA, *p<0.05, ***p<0.001. n=6 in all groups.

Figure 2. Cu^{II}(atsm) is protective in transient ischemia. A) Representative images of TTC stained brain sections at 1 dpi. B) Quantitative analysis of infarct volume at 24 h after tMCAO. Mean ± SD. One-way ANOVA with Tukey post hoc test, *p<0.05, **p<0.01 compared to vehicle, n=3-4/treatment group. C) MCAO-induced functional impairment indexed by neuroscore 1 dpi. Mean ± SD, one-way ANOVA with Tukey post hoc test, ***p<0.001. n= 3-6/treatment group.

Figure 3. Cu^{II}(atsm) is protective in permanent ischemia and increases the brain copper content. A) Representative MRI images of ischemic brains at 1 dpi. B) Quantitative analysis of infarct volume at 24 h after pMCAO. Unpaired 2-tailed t-test, *p<0.05. n=10 in vehicle group, n=8 in Cu^{II}(atsm)

group. C) The latency to move was measured at 1 day after ischemia in sham operated and in mice subjected to pMCAO. Each mouse was tested twice and the data shows the average of 2 trials for each mouse. 2-way ANOVA, *p<0.05, **p<0.01. n=12 in sham vehicle group, n=12 in sham Cu^{II}(atsm) group, n=8 in ischemic vehicle group, n=8 in ischemic Cu^{II}(atsm) group. D) The copper concentrations in the peri-ischemic brain areas were measured by ICP-MS at 3 dpi and are shown normalized to mass of the tissue ± SD. Unpaired 2-tailed t-test, *p<0.05. n=6 in vehicle group, n=4 in Cu^{II}(atsm) group.

Figure 4. Cu^{II}(atsm) reduces CD45 immunoreactivity in ischemic mice. A) Typical example images of CD45 immunoreactivity in the ischemic lesion in vehicle-treated and Cu^{II}(atsm) treated mouse brains at 1 day after pMCAO. Scale bar=20μm. B) Quantitative analysis of CD45 immunoreactivity in the lesion at 1 dpi. Data are shown as means ± S.D. Unpaired 2-tailed t-test, *p<0.05. n=7 in both groups. C) Typical example images of CD45 immunoreactivity in the ischemic lesion in vehicle-treated and Cu^{II}(atsm) treated mouse brains at 3 dpi. Scale bar=20μm. D) Quantitative analysis of CD45 immunoreactivity in the lesion at 3 dpi. Data are shown as means ± S.D. Unpaired 2-tailed t-test, *p<0.05. n=5 in both groups.

Figure 5. Cu^{II}(atsm) reduces Iba1 immunoreactivity and alters the morphology of Iba1-positive cells in ischemic mice. A) Typical example images of Iba1 immunoreactivity in the peri-ischemic brain area in vehicle-treated and Cu^{II}(atsm) treated mouse brains at 3 days after pMCAO. Scale bar=20μm. B) Quantitative analysis of Iba1 immunoreactivity in the peri-ischemic brain area at 3 dpi. Data are shown as means ± S.D. Unpaired 2-tailed t-test, *p<0.05. n=5 in both groups. C) Typical example images of the algorithm used to determine the morphology of Iba1 positive cells. Scale bar=50μm. D) Quantification of the cellular area of the Iba1 positive cells in the peri-ischemic brain areas of mice treated with Cu^{II}(atsm) at 3 dpi. Data are shown as means ± S.D. Unpaired 2-tailed t-test, *p<0.05. n=8 in both groups.

Figure 6. Cu^{II}(atsm) alters the ratios of myeloid cell in the ischemic hemisphere. A) Gating strategy for FACS. B) Representative plots of $CD45^{low}CD11b^{+}$ $Ly6G^{-}$ and $CD45^{high}CD11b^{+}$ $Ly6G^{-}$ cells in the ischemic hemibrain at 3 days after ischemia. C) Quantified ratios of $CD45^{high}$ and $CD45^{low}$ $CD11b^{+}$ $Ly6G^{-}$ microglia in sham operated animals, and in mice that underwent pMCAO with either vehicle or Cu^{II}(atsm) treatment. Mean ± SD, 1-way ANOVA, *p<0.05, ***p<0.001. n=6 in stroke groups.

Figure 7. Copper delivery by Cu^{II}(atsm) reduces markers of inflammation in the ischemic brain. A) Typical example images of phosphorylated p38 MAPK immunoreactivity in the peri-ischemic brain

area in vehicle-treated and Cu^{II}(atsm) treated mouse brains at 1 day after pMCAO. Scale bar=20μm. B) Quantitative analysis of phosphorylated p38 MAPK immunoreactivity in the peri-ischemic brain area at 1 dpi. Data are shown as means ± S.D. Unpaired 2-tailed t-test, *$p<0.05$. n=8 in both groups. C) The concentration of IL-12 in the peri-ischemic brain area was measured by CBA at 1 dpi. Data are shown as means ± S.D. Unpaired 2-tailed t-test, *$p<0.05$. n=5 in vehicle and n=6 in Cu^{II}(atsm) group. D) The concentration of IL-10 in the peri-ischemic brain area was measured by CBA at 3 dpi. Data are shown as means ± S.D. Unpaired 2-tailed t-test, *$p<0.05$. n=5 in vehicle and n=6 in Cu^{II}(atsm) group.

Author/s:
Huuskonen, MT; Tuo, Q-Z; Loppi, S; Dhungana, H; Korhonen, P; McInnes, LE; Donnelly, PS; Grubman, A; Wojciechowski, S; Lejavova, K; Pomeshchik, Y; Periviita, L; Kosonen, L; Giordano, M; Walker, FR; Liu, R; Bush, AI; Koistinaho, J; Malm, T; White, AR; Lei, P; Kanninen, KM

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