

Proof of concept and feasibility studies examining the influence of combination ribose, adenine and allopurinol treatment on stroke outcome in the rat

Brain and Neuroscience Advances

Volume 1: 1–13

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DOI: 10.1177/2398212817717112

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Abstract

Background: Cerebral ischaemia results in a rapid and profound depletion of adenosine triphosphate (ATP), the energy currency of the cell. This depletion leads to disruption of cellular homeostasis and cell death. Early replenishment of ATP levels might therefore have a neuroprotective effect in the injured brain. We have previously shown that the ATP precursors, D-ribose and adenine (RibAde), restored the reduced ATP levels in rat brain slices to values similar to those measured in the intact rodent brain. The aim of this study was to assess whether RibAde, either alone or in combination with the xanthine oxidase inhibitor allopurinol (RibAdeAll; to further increase the availability of ATP precursors), could improve outcome in an in vivo rodent model of transient cerebral ischaemia.

Methods: After 60 min occlusion of the middle cerebral artery, and upon reperfusion, rats were administered saline, RibAde, or RibAdeAll for 6 h. Baseline lesion volume was determined by diffusion-weighted MRI prior to reperfusion and final infarct volume determined by T2-weighted MRI at Day 7. Neurological function was assessed at Days 1, 3 and 7.

Results: Ischaemic lesion volume decreased between Days 1 and 7: a 50% reduction was observed for the RibAdeAll group, 38% for the RibAde group and 18% in the animals that received saline. Reductions in lesion size in treatment groups were accompanied by a trend for faster functional recovery.

Conclusion: These data support the potential use of ribose, adenine and allopurinol in the treatment of cerebral ischaemic injury, especially since all compounds have been used in man.

Keywords

Adenosine triphosphate, stroke, cerebral ischaemia, neuroprotection, magnetic resonance imaging, D-ribose, adenine, allopurinol, purine salvage pathway

Received: 30 March 2017; accepted: 31 May 2017

Introduction

Stroke is a major medical and economic burden being the fourth leading cause of mortality in the United Kingdom and leaving almost two-thirds of stroke survivors with a disability (Stroke Association, 2017). Despite extensive research for neuroprotective strategies, therapeutic options for acute ischaemic stroke remain limited and consist of recanalisation of the occluded vessel thereby improving revascularisation to the ischaemic territory. This can be achieved either pharmacologically, through the use of tissue plasminogen activator (rt-PA), or via surgical removal of the blood clot (e.g. endovascular thrombectomy) (Asadi et al., 2016). Recanalisation treatments are limited by a very narrow therapeutic time window (<4.5 hr for rt-PA or <6 hr for endovascular thrombectomy), thereby reducing treatment options for many patients. Therapies aimed at improving the survival of brain tissue located within the at-risk, but potentially salvageable penumbra are still desperately needed.

Ischaemia results in a rapid and profound depletion in adenosine triphosphate (ATP), the energy currency of the cell (Phillis et al., 1996a; Xing et al., 2012). The inability to fuel the activity of the Na⁺/K⁺ ATPase results in neuronal depolarisation leading

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to pathological changes in ionic homeostasis and acute cell death in the ischaemic core (Doyle et al., 2008; Pulsinelli, 1992). Beyond the core, the adjacent penumbra is at risk via hypoperfusion and waves of ischaemic depolarisation – also known as peri-infarct depolarisations – that impose high energetic demands upon poorly perfused and energetically compromised brain tissue. This results in recruitment of the otherwise salvageable penumbra into an expanding ischaemic infarct (Heiss, 2012). The loss of cellular ATP is thus the primary consequence of cerebral ischaemia and the cause of subsequent pathological cascades.

This initial ATP depletion is prolonged by virtue of the metabolism of ATP to the purine nucleosides and nucleobases, adenosine, inosine and hypoxanthine which leave the brain and enter into the general circulation (Kjellmer et al., 1989; Phillis et al., 1996a, 1996b; Weigand et al., 1999; Zur Nedden et al., 2013). This loss from the brain deprives one branch of the purine salvage pathway (Figure 1) of the hypoxanthine necessary for the formation of the adenine nucleotide backbone of ATP. In addition, the loss of adenosine from the brain and depletion of the primary reservoir of adenosine, ATP, deprives the brain of this neuroprotective and anticonvulsant purine nucleoside (Dale and Frenguelli, 2009; Pedata et al., 2016; Ribeiro et al., 2016). Consequently, for ATP levels to be replenished, resynthesis of the adenine nucleotide AMP is a prerequisite.

We have previously shown that a combination of ribose and adenine (RibAde) restored ATP levels in the *in vitro* brain slice to levels similar to those observed *in vivo*, likely by promoting the branch of the purine salvage pathway catalysed by adenine phosphoribosyltransferase (APRT; Figure 1) (Zur Nedden et al., 2011, 2014). In addition, we have also demonstrated that this enhanced ATP pool translated into greater release of adenosine in response to both electrical stimulation of afferent fibres, and oxygen/glucose deprivation (OGD), with increased inhibitory effects on synaptic plasticity and synaptic transmission, respectively. Moreover, we observed protective effects of RibAde when given after 6 h of OGD in cerebellar granule cells (Zur Nedden et al., 2014).

Given the effects observed in our *in vitro* experiments, we aimed to determine whether RibAde was effective in an *in vivo* model of stroke. From a translational point of view, this is of particular interest since both ribose and adenine have previously been used in man – ribose as an unregulated nutritional supplement and potential therapy for cardiac disorders (Dhanoa and Housner, 2007; Pauly and Pepine, 2000; Pliml et al., 1992; Shechter et al., 2010) and adenine in blood transfusion products (Hess and Greenwalt, 2002; Paglia et al., 2016; Peck et al., 1981). These compounds thus have strong translational potential in the treatment of brain injuries associated with cerebral ischaemia and ATP depletion. An additional manipulation of the purine salvage pathway was to test the anti-gout medication, allopurinol, to establish if, in combination with adenine and ribose, allopurinol could further enhance the actions of RibAde, potentially via preventing degradation of salvageable hypoxanthine to the non-salvageable xanthine (Figure 1).

The aim of this study was therefore to assess for the first time *in vivo* the potential of RibAde and RibAde/allopurinol (RibAdeAll) in protecting the ischaemic brain in a transient middle cerebral artery occlusion (tMCAO) model in rats. Our observations reveal that, at appropriate doses, RibAde and RibAdeAll are well tolerated and show an encouraging trend for reducing cerebral infarction and promoting neurological recovery after stroke.

Materials and methods

Animals and study design

All experiments were performed under a licence from the UK Home Office and subjected to the Animals (Scientific Procedures) Act 1986, incorporating European Directive 2010/63/EU. The report was written in accordance with the ARRIVE guidelines (<http://www.nc3rs.org.uk/arrive>). All experiments were performed on male Wistar rats (12–16 weeks, 290–330 g body weight) obtained from Harlan, UK. Animals were randomly allocated to the study group using a random list generator (<http://www.random.org>). The investigator was blinded to treatment until after the analysis was completed.

Preparation of experimental compounds

All reagents were sourced from Sigma–Aldrich. Ribose was freely soluble in physiological saline and was delivered intravenously (IV) as an infusion (200 mg/kg/h), which has been used previously in studies attempting to restore cardiac adenine nucleotides after myocardial ischaemia (Zimmer et al., 1989; Zimmer and Ibel, 1984; Zimmer and Schneider, 1991). For adenine, we initially aimed for an IV infusion of 50 mg/kg/h based on previous *in vivo* studies (Zimmer and Schneider, 1991), but encountered solubility issues with the free-base of adenine. As the HCl salt of adenine could not be used due to the acidity of its solution, we initially used the highest soluble dose of the free-base of adenine, which, in our hands, was 36 mg/kg/h. Our initial studies were based on this dose, but as toxicity issues were subsequently identified, this dose was replaced with an infusion of 10 mg/kg/h. The allopurinol solution was given intraperitoneally (IP) as a bolus and was prepared at a concentration of 5 mg/mL by dissolution in 1 mM NaOH, and then brought to a pH of 10 by controlled addition of 1 mM HCl and distilled water. Initially, 100 mg/kg allopurinol was administered, but this too was reduced to 10 mg/kg for the main stroke study.

Effects of RibAde on blood pressure

To determine the acute effects of RibAde on cardiovascular parameters, we first assessed the effect of RibAde on blood pressure. For that purpose, general anaesthesia was induced with 5% isoflurane in nitrous oxide (70%) and oxygen (30%). This was followed by oropharyngeal intubation and maintenance of anaesthesia through mechanical ventilation with 2% isoflurane at 3.5 mL stroke volume of nitrous oxide/oxygen mix and 60 breaths/min. Body temperature was maintained at $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$, and the right femoral artery was cannulated with a polythene cannula (ID=0.58 mm and OD=0.96 mm, Portex Fine Bore polythene tubing; Smiths Medical International Ltd, Kent, England) for continuous arterial blood pressure and heart-rate monitoring (AcqKnowledge; Biopac Systems, Goleta, CA, USA). Animals were randomly assigned to two groups ($n=4$ per group) and the investigator was blinded to treatment allocation. Group 1: IV saline infusion for 3 h at 5 mL/kg/h and group 2: IV RibAde infusion for 3 h (ribose 200 mg/kg/h and adenine 36 mg/kg/h) at 5 mL/kg/h. One rat assigned to the RibAde group died during the procedure due to a technical issue. General anaesthesia was maintained throughout the whole protocol. At the end of the 3 h measurement, the rats were killed by cervical dislocation.

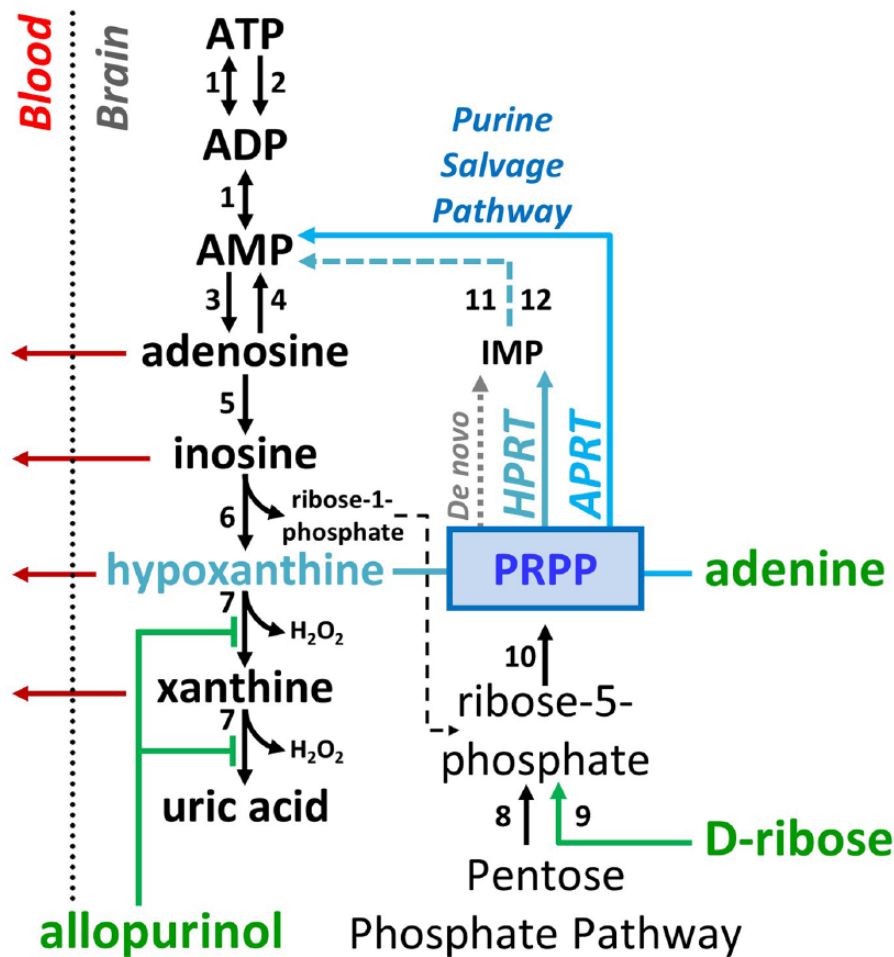


Figure 1. Degradation and resynthesis of ATP via the purine salvage pathway. ATP is degraded to metabolites such as adenosine, inosine or hypoxanthine, which can, via equilibrative transporters, leave cells and enter the blood stream. Purine salvage (blue arrows) restores adenine nucleotide levels via hypoxanthine phosphoribosyltransferase (HPRT; hypoxanthine to IMP) and adenine phosphoribosyltransferase (APRT; adenine to AMP). This reaction (as does de novo purine synthesis) requires 5-phosphoribosyl-1-pyrophosphate (PRPP), a product of the pentose phosphate pathway from which emerges ribose-5-phosphate. An additional source of ribose-5-phosphate is from the isomerisation of inosine-derived ribose-1-phosphate by phosphopentomutase (black dashed line). Adenine, hypoxanthine and D-ribose are precursors, which enter the salvage pathway. Allopurinol inhibits the metabolism of hypoxanthine to xanthine and from xanthine to uric acid. This reduces the production of non-salvageable xanthine, makes more hypoxanthine available for salvage and limits the production of hydrogen peroxide (H_2O_2) and subsequent reactive oxygen species. (1) adenylylase, (2) ATPase, (3) 5'-nucleotidase, (4) adenosine kinase, (5) adenosine deaminase, (6) purine nucleoside phosphorylase, (7) xanthine oxidase, (8) ribulose 5-phosphate isomerase, (9) ribokinase, (10) phosphoribosylpyrophosphate synthetase, (11) adenylosuccinate synthetase and (12) adenylosuccinate lyase.

Effects of RibAde and RibAdeAll on stroke outcome

The rats were randomly allocated to one of the following groups: Control group (6 h IV infusion of saline at 5 mL/kg/h and a single IP injection of saline); RibAde group (6 h IV infusion of D-ribose (200 mg/kg/h) and adenine (10 or 36 mg/kg/h) in saline delivered at 5 mL/kg/h, and single IP injection of saline); and RibAdeAll group (as before for the RibAde solution, and a single 10 or 100 mg/kg IP injection of allopurinol).

The rats were anaesthetised, intubated and ventilated as described above. Buprenorphine was injected subcutaneously (0.03 mg/kg) at induction and a follow-up dose was administered after 8 h for post-stroke analgesia. The right femoral vein was

cannulated with silicone tubing (ID=0.51 mm and OD=0.94 mm, Dow Corning Silastic Q7-4750) for the continuous infusion of the RibAde solution or saline. tMCAO was induced for 60 min with an intraluminal filament (diameter: 0.37–0.39 mm; Doccol, CA, USA) as previously described (Longa et al., 1989). The rats were then transferred under general anaesthesia for MRI scanning to assess the tissue-at-risk of infarction using diffusion-weighted imaging (DWI) 50 min after occlusion of the middle cerebral artery.

The animals were then removed from the MRI scanner, and 60 min after induction of the transient ischaemia, the filament was removed to restore blood flow through the MCA. At the time of reperfusion, the RibAde or saline infusion was started. A single bolus injection of allopurinol (or saline depending on the group) was given intraperitoneally at the same time. Infusions

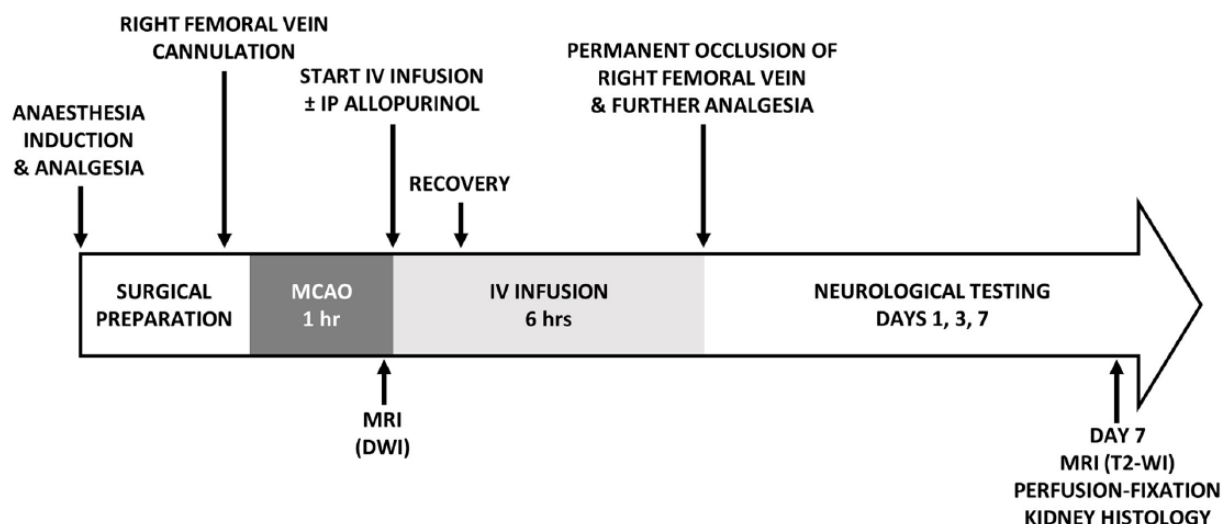


Figure 2. Time line of the main stroke study.

and injections were coded so that the investigator was blind to treatment allocation. The rats were allowed to recover, and IV infusion was continued for a total period of 6 h while the rats were conscious.

After this time, and since the animal was to recover over the following week, the intravenous line was tied off and left in place under the skin so that the animal was not able to remove it and cause a bleed. This is less invasive than surgical removal, did not require re-anaesthetising the animal and did not lead to any complications in terms of the use of the limb.

Neurological testing using an 18 point scale (Garcia et al., 1995) was carried out 24 h, 72 h and 7 days after tMCAO by an investigator blind to group identity. At 7 days, the rats were re-anaesthetised and T2-weighted MRI scanning was performed to assess the final infarct size. The rats were then killed by perfusion/fixation and the kidneys collected for histological examination. The time line of the main stroke study is given in Figure 2.

MRI scanning

MRI was performed on a Bruker Pharmascan 7T/16 cm system with a gradient insert (90 mm ID, 300 mT/m) and a 72 mm bird-cage resonator. A four-channel phased-array rat head surface coil was used for brain imaging. DWI was performed at 50 min after tMCAO (10 min prior to reperfusion and any treatment) to generate quantitative apparent diffusion coefficient (ADC) maps and to allow assessment of the acute ischaemic injury (i.e. the tissue-at-risk of infarction). The parameters used were TE 22.8 ms, TR 4000 ms, 4 shots EPI, 2 averages, 3 directions with $b=0$, 1000 s/mm², 96 × 96 matrix, 260 μm × 260 μm in-plane resolution, slice thickness 1.5 mm, 8 slices. At 7 days post-tMCAO, a RARE T2-weighted sequence was used to assess the final infarct size (TE 100 ms, TR 5000 ms, Rare factor 8, 2 averages, 256 × 256 matrix, 98 μm × 98 μm in-plane resolution, slice thickness 0.75 mm). Quantitative ADC maps were generated from raw DWI images, and T2-weighted images were subsequently processed using Image J software (<http://rsb.info.nih.gov/ij/>). The tissue-at-risk and final infarct volumes were calculated by manual drawing of the area on each slice. The area generated was

summed over the number of slices and multiplied by slice thickness for volume calculation as previously described (Baskerville et al., 2016), and the lesion size on the T2-weighted images was corrected for oedema (Gerriets et al., 2004).

Neurological testing

Animals underwent neurological testing based on an 18 point scale (Garcia et al., 1995). Testing was performed prior to tMCAO, and on Days 1, 3 and 7 after tMCAO. The scale measures six parameters assessing motor and sensory functions. The scores for each parameter were summed; the minimal score achievable (worse neurological status) is 3 and the maximum (normal rat) is 18.

Postmortem examinations

Postmortem examinations were conducted by Board-Certified veterinary pathologists at various stages of the investigation at both the gross and histological level from organs preserved in 10% formalin. Organ samples were embedded in paraffin wax prior to section and staining with haematoxylin and eosin. Samples of kidneys, lungs, heart, liver, spleen, pancreas, stomach, small intestine, caecum, colon, brain and head (multiple sections including ears, eyes, Hardarian glands, salivary glands, nasal cavity and skeletal muscle) were analysed in the rats administered the higher doses of compounds (adenine: 36 mg/kg/h and allopurinol: 100 mg/kg), whereas only the kidneys were examined in the rats which had received the lower doses (adenine: 10 mg/kg/h and allopurinol: 10 mg/kg).

Statistical analysis

Data were analysed using SPSS V24 (IBM Corp, Armonk, NY, USA). A two-way repeated measures (RM) analysis of variance (ANOVA; time and treatment) with Bonferroni post-hoc test to correct for multiple comparisons were used to assess the effect of treatment on blood pressure, body weight, lesion volume and on

neurological status. A one-way ANOVA with Bonferroni post-hoc tests was used to compare the percentage change in weight loss and lesion volume over time. After pooling of the two treatment groups, a Student's unpaired t-test (two-tailed) was used to compare the effect of treatment on the evolution of stroke lesion volume (% change over time). A linear regression analysis was used to compare acute ischaemic injury (ADC-derived lesion) with neurological status at Day 1 post-stroke. 'n' refers to the number of animals used in the experiment. This study was designed as an exploratory investigation aiming to translate, for the first time, our previous *in vitro* results in brain slices to a preclinical *in vivo* model of stroke. As such, sample size calculations were not performed as there were no comparable previous studies from which to obtain estimates of effect size.

Results

Effects of RibAde on blood pressure

An initial pilot series of experiments was carried out in order to determine the influence of RibAde on acute changes in blood pressure. For this purpose, we continuously monitored, under general anaesthesia, the mean arterial blood pressure (MABP) of two groups of rats infused with either a solution of RibAde (ribose 200 mg/kg/h and adenine 36 mg/kg/h) or with saline for 3 h. RibAde administration did not result in a significant difference in arterial blood pressure compared to the control group (two-way RM ANOVA, treatment effect $F(1, 5)=0.023$; $p=0.886$; Figure 3).

RibAde and RibAdeAll dose selection

Given the lack of effect of this combination of RibAde on blood pressure, we performed an initial series of tMCAO experiments using adenine at 36 mg/kg/h IV and allopurinol at 100 mg/kg IP. However, we observed an unexpectedly high rate of mortality or a need for humane killing after tMCAO in both treated groups. Postmortem examination revealed significant renal changes characterised by severe diffuse tubular degeneration, and necrosis with hyaline and cellular tubular casts (data not shown). There was also evidence of intrapelvic and intratubular calculi, mild neutrophilic pyelonephritis and moderate transitional cell hyperplasia (data not shown). Both adenine and allopurinol are known nephrotoxic agents when administered at high doses (Lindblad et al., 1973; Suzuki et al., 1984; Yokozawa et al., 1986) and their concurrent administration may have exacerbated their toxic effect.

Following these initial experiments, we then carried out further experiments using a lower dose of adenine (10 mg/kg/h IV), which maintained the 20:1 ratio of RibAde shown to improve tissue ATP levels in brain slices (1 mM ribose and 50 μ M adenine) (Zur Nedden et al., 2011). The duration of infusion (6 h) was based on previous cardiac studies (Zimmer and Schneider, 1991) and the dose of allopurinol (10 mg/kg) is one commonly used in the rat literature (Suzuki et al., 1984).

We subjected three rats to 200 mg/kg/h IV ribose and 10 mg/kg/h IV adenine. Two of the rats also received an IP bolus injection of allopurinol (10 mg/kg) and one received an injection of saline. These rats, which were not subjected to transient MCAO, were killed 3 days after administration of the compounds, at which point there was no discernable effect on the gross condition of the animals. Postmortem analysis (data not shown)

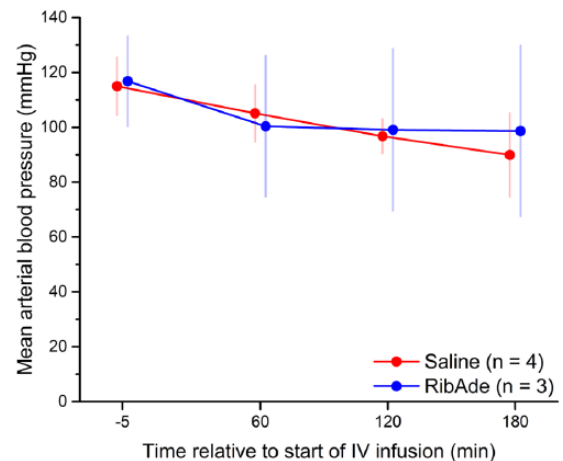


Figure 3. RibAde had no effect on blood pressure. Mean arterial blood pressure prior to and following intravenous infusion of ribose and adenine (200 and 36 mg/kg/h IV, respectively; $n=3$, blue lines and symbols) or saline ($n=4$, red lines and symbols) for 3 h, while the animals were under general anaesthesia. The graph shows mean data \pm SD for each group at each time point before (-5 min) and after (60, 120 and 180 min) saline or RibAde infusion. Treatment had no influence on blood pressure (two-way RM ANOVA, treatment effect $F(1, 5)=0.023$; $p=0.886$) over and above the progressive decrease in blood pressure seen with time under general anaesthesia. Data points have been offset for clarity.

indicated that at these doses of adenine and allopurinol, only mild renal tubular degenerative changes, with tubular dilation and epithelial atrophy were noted. Consequently, this adenine dose was considered safe to assess the effect of RibAde \pm allopurinol following tMCAO.

Main study: the effect of RibAde and RibAdeAll on stroke outcome

Three groups of animals underwent tMCAO followed by saline (IV, $n=6$), RibAde (200 mg/kg/hr and 10 mg/kg/hr IV, respectively; $n=8$) or RibAdeAll (200 mg/kg/hr and 10 mg/kg/hr IV and 10 mg/kg IP, respectively; $n=8$). As anticipated, the rats in all three groups lost weight following surgery due to expected decrease in food intake during the recovery period (Figure 4). A two-way RM ANOVA (treatment and time) with post-hoc Bonferroni correction did not show any interaction between treatment and time in terms of changes in body weight between groups ($F(2, 19)=0.776$, $p=0.474$). A treatment effect was observed ($F(2, 19)=5.045$; $p=0.017$) in which a Bonferroni post-hoc test indicated a difference between the RibAde group and the RibAdeAll group ($p=0.032$), but not in the more relevant comparisons between the saline and RibAde groups ($p=1.000$) and the saline and RibAdeAll groups ($p=0.055$). The reason for the difference between the RibAde and RibAdeAll groups is unclear. In terms of the percentage of body weight lost, a one-way ANOVA and post-hoc Bonferroni comparison of weight loss between Day 0 (before tMCAO) and Day 7 (after tMCAO) did not show any differences in percentage weight loss across the three experimental groups ($F(2, 19)=0.832$, $p=0.450$), with the saline-, RibAde- and RibAdeAll-treated groups losing (mean \pm SD) $9.1\% \pm 4.4\%$,

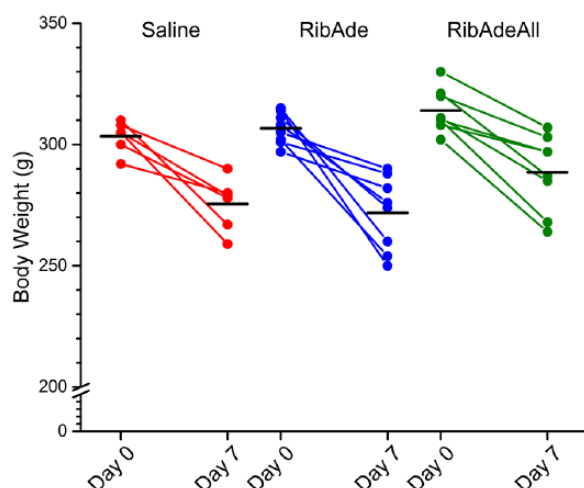


Figure 4. RibAde or RibAdeAll had no effect on stroke-induced weight loss. Body weight immediately before (Day 0) and 7 days after tMCAO (final time point) with mean value for each group and time point shown as a horizontal black bar. All animals show a decrease in body weight secondary to general anaesthesia and stroke surgery. While a two-way RM ANOVA identified a significant effect of treatment ($F(2, 19)=5.04$; $p=0.017$), post-hoc Bonferroni comparison showed no difference between the saline and RibAde ($p=1.000$) groups, nor between the saline and RibAdeAll groups at Day 0 and Day 7 ($p=0.055$). There were no differences in the percentage weight loss across the three experimental groups (mean \pm SD; n): saline ($9.1\pm4.4\%$ of initial body weight; 6), RibAde ($11.3\pm6.2\%$; 8), RibAdeAll ($8.1\pm3.8\%$; 8); one-way ANOVA ($F(2, 19)=0.832$, $p=0.450$).

$11.3\pm6.2\%$ and $8.1\pm3.8\%$ of body weight, respectively. No abnormal clinical signs were noted in the treated rats (with the exception of the expected stroke-induced neurological deficits; see below). Histopathological analysis of the kidneys ($n=12$; four randomly selected animals from each group) from these rats at Day 7 revealed no abnormality associated with RibAde or RibAdeAll treatment compared to animals administered saline (Figure 5 for saline and RibAdeAll comparison).

The initial volume of tissue-at-risk of infarction was determined immediately prior to reperfusion and the onset of treatment (ADC map, Figure 6(a); Day 0). Final infarct volume was assessed by T2-weighted imaging at Day 7 (Figure 6(b)). Each animal was therefore used as its own control, allowing us to assess the effect of reperfusion with or without treatment on the extent of tissue salvage over time (Figure 6(c)). A two-way RM ANOVA across all animals did not reveal an effect of treatment on raw lesion volume ($F(2, 19)=0.206$; $p=0.816$) or an interaction between treatment and time ($F(2, 19)=2.053$; $p=0.156$). However, an effect may have been obscured by the variability in the initial lesion sizes. Accordingly, we compared the change in lesion volume over time by expressing the lesion volume at Day 7 as a percentage of that at Day 0.

Treatment with RibAde or RibAdeAll reduced lesion volume by $38\pm10\%$ (mean \pm SEM) and $50\pm9\%$, respectively, whereas in the saline-treated animals, the reduction in lesion size following reperfusion was $18\pm12\%$ (Figure 6(d)). These values take into account all animals used in the study, including the one animal in the RibAdeAll group that had an unusually large

lesion at Day 0 and which, in contrast to the other RibAdeAll-, and indeed RibAde-treated animals, showed no reduction in lesion volume over time (Figure 6(c)). Table 1 provides a statistical analysis of the percentage change in lesion volume over time for all the animals, either comparing all three groups (one-way ANOVA), or when the saline group is compared to the combined RibAde and RibAdeAll groups (unpaired t-test). These summary statistics, together with the individual animal lesion volume trajectories between Day 0 and Day 7 are, on balance, suggestive of a positive effect of RibAde-based treatment.

Effect of RibAde and RibAdeAll on neurological recovery after stroke

To assess the impact of tMCAO and RibAde-based treatment on neurological function, we conducted a neurological assessment based on an 18 point scale (Garcia et al., 1995). Plotting of ADC lesion volume (prior to both reperfusion and initiation of treatment) against early neurological score (24 h) showed a significant inverse relationship between initial lesion volume and neurological score measured 24 h after tMCAO (Figure 7(a)). This indicated that the 18 point scale reflected stroke-induced brain damage. Moreover, segregation by treatment group showed that, by chance, animals from the treatment groups displayed larger lesions prior to treatment initiation and this was associated with greater neurological deficits at 24 h post-stroke; median neurological scores of 9 and 11 were recorded in the RibAde and RibAdeAll groups, respectively, versus a median score of 13 in the saline group (maximum deficit=3; normal animal=18; Figure 7(b)). Over the 7-day time course, neurological function improved in all groups. However, despite being more affected initially, both the RibAde and RibAdeAll treatment groups demonstrated an accelerated recovery when compared to the saline-treated animals when considering daily median neurological scores (Figure 7(b)) or the percentage change in mean daily neurological score (Figure 7(c)).

Discussion

In this proof-of-concept study, we provide evidence that RibAde \pm allopurinol shows promise as a therapy for ischaemic brain injury and warrants further investigation in a fully-powered pre-clinical study. At the doses and infusion duration used, RibAde \pm allopurinol showed a strong tendency to increase tissue salvage when given in combination with reperfusion and to improve functional recovery, while having no major side-effects.

Cerebral ATP during ischaemia: quick to go, slow to recover

Depletion of cellular ATP is the initial consequence of an ischaemic insult. It is to this that the subsequent pathology that ensues can be attributed. While attempts have been made to improve brain energy metabolism and cerebral bioenergetics after brain injury, these have revolved around targeting mitochondria and oxidative phosphorylation via the provision of substrates that feed into the tricarboxylic acid cycle such as glucose, lactate and pyruvate (Dienel, 2014), as well as ketones such as β -hydroxybutyrate (Prins and Matsumoto, 2014). These studies, albeit with occasional successes, have not led to treatments for the injured brain.

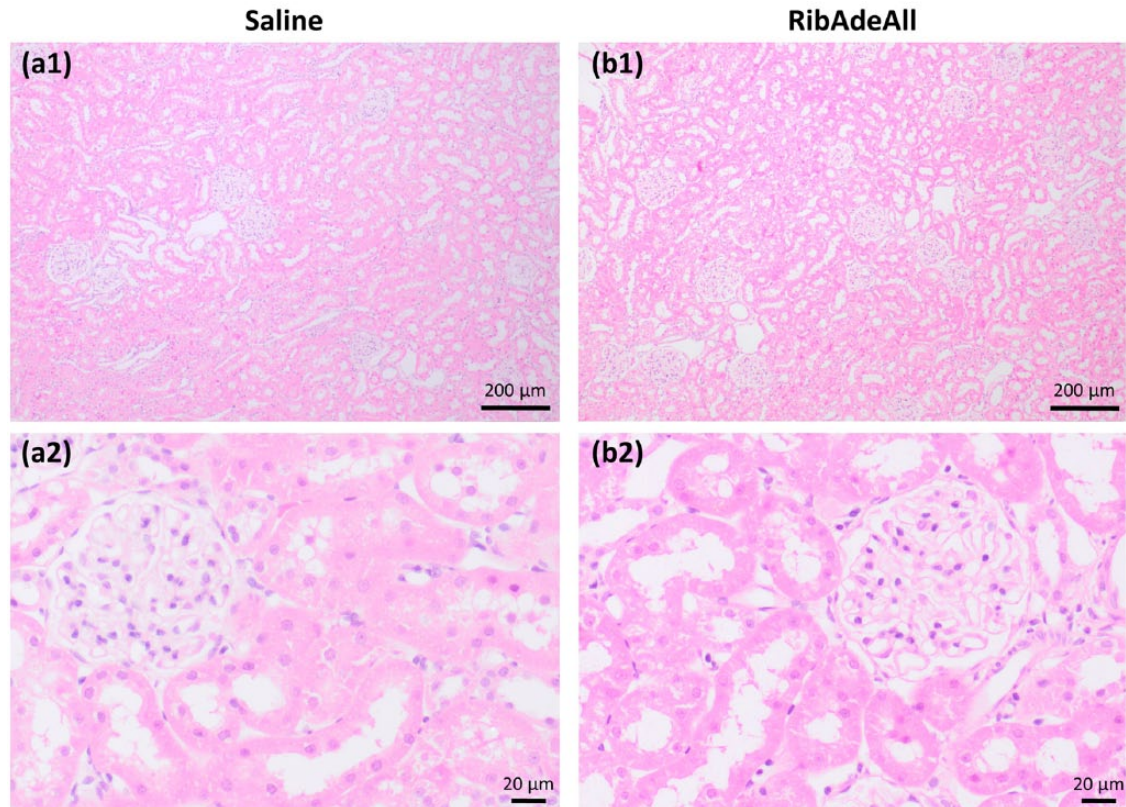


Figure 5. Histological examination of the kidney of one representative saline control animal (a1 and a2) and one RibAdeAll-treated animal (200 mg/kg/hr, 10 mg/kg/hr IV and 10 mg/kg IP, respectively; b1 and b2) at low (a1 and b1) and high magnification (a2 and b2). No structural abnormalities were detected in RibAde- or RibAdeAll-treated animals 7 days after infusion. Light microscopy images of H&E staining.

This is likely due to the fact that mitochondria are damaged after trauma/ischaemia, and the activity of the electron transport chain, upon which the synthesis of ATP depends, will be severely impaired, if not harmful in the production of reactive oxygen species (Bakthavachalam and Shanmugam, 2017). In addition, since the mitochondrial ATP synthase converts ADP to ATP, it thus absolutely relies upon the cytosolic purine salvage pathway to provide it with the necessary adenine nucleotide backbone upon which to affix additional phosphate groups. Unfortunately, the substrates for the purine salvage pathway are lost to the general circulation after cerebral ischaemia, which likely explains the slow recovery of cerebral ATP after injury.

Therapies to restore ischaemia-depleted ATP: lessons from the heart

This fundamental issue has been appreciated in the cardiac literature and there have been, and indeed continue to be, attempts to redress the basis of ATP depletion (loss of nucleosides and nucleobases) seen in myocardial ischaemia, which similarly arises through the loss to the general circulation of substrates for the purine salvage pathway. Initially, these studies in the 1980s revolved around the use of pentose sugars such as ribose, which, via the action of ribokinase is converted to ribose-5-phosphate (Figure 1). Ribose-5-phosphate is then incorporated into the phosphoribosyl pyrophosphate pool, from which a phosphoribose group is used by cytosolic APRT and HPRT to generate

purine mononucleotides, including AMP (Figure 1). Through the actions of adenylate kinase, several isoforms of which are cytosolic and found in brain, AMP can be converted to ATP (Panayiotou et al., 2014). Importantly, adenylate kinases are promiscuous in that they can utilise nucleotide triphosphate donors other than ATP, such as GTP, which are largely preserved during cerebral ischaemia (Hagberg et al., 1987); this avoids the futile cycle of robbing ATP to pay ADP. Such a cytosolic ATP-generating system is necessary in highly polarised cells such as neurons where mitochondria may be some distance away, and indeed essentially absent from most excitatory synapses on dendritic spines (Kasthuri et al., 2015).

In the perfused heart, administration of ribose increased the rate of recovery of adenine nucleotides in acute models of high energy demand such as isoproterenol challenge (Zimmer, 1983; Zimmer et al., 1980, 1984; Zimmer and Gerlach, 1978) or ischaemia-reperfusion insult (Zimmer and Ibel, 1984) while studies in chronic models show more conflicting results (Faller et al., 2013; Lamberts et al., 2007; Zimmer et al., 1989). Additional benefits for ATP production were observed via the inclusion of adenine (Zimmer, 1998). However, the nephrotoxic actions of adenine following its xanthine oxidase-mediated conversion to the insoluble 2,8 dihydroxyadenine, together with the high doses administered in early studies (Zimmer and Schneider, 1991) and the prolonged (24–48 h) intracardiac perfusion of ribose/adenine solution (St Cyr et al., 1986; Ward et al., 1983, 1984) has not led to a continuation of this approach.

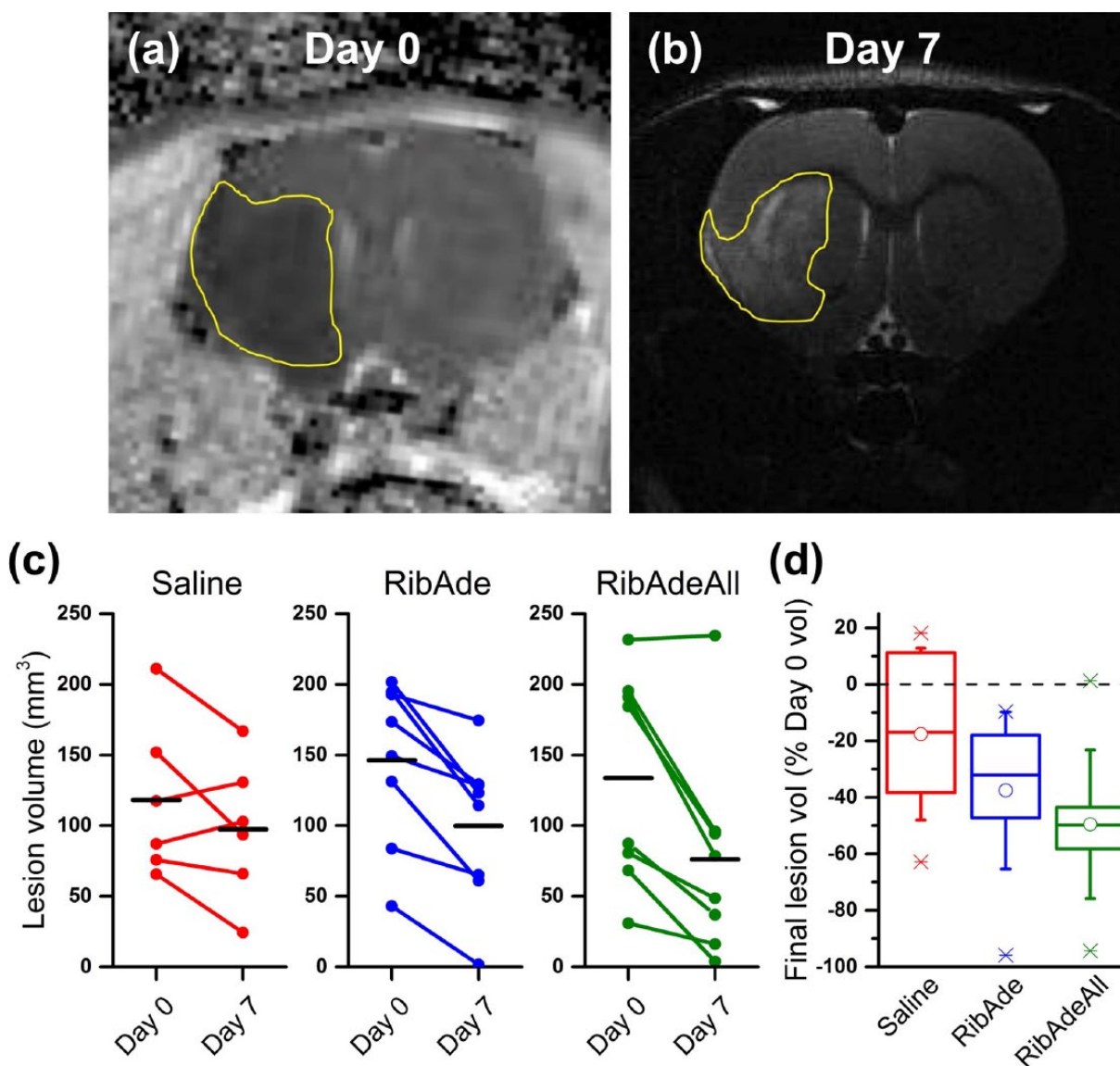


Figure 6. RibAde and RibAdeAll show a tendency to reduce stroke lesion volume. (a) Representative coronal MRI scans of the same animal (RibAdeAll group) at the level of the caudate nuclei during ischaemia (a) and 7 days after reperfusion (b). The manually determined lesion boundaries are highlighted in yellow. (a) The ADC (apparent diffusion coefficient) map derived from the DWI scan acquired 50 min after tMCAO, just prior to reperfusion, shows the tissue-at-risk of infarction as a hypo-intense signal. (b) A T2-weighted MRI scan shows the final infarct area (hyper-intense) assessed 7 days later. In this RibAdeAll-treated animal, there was a 50% reduction in lesion volume between the two time points. (c) Individual paired measurements of lesion volume at Day 0 (DWI) and Day 7 (T2-weighted imaging) in the three experimental groups of animals showing a consistent trend for substantial lesion shrinkage in the RibAde (blue; $n=8$) and RibAdeAll (green; $n=8$) groups compared to saline controls (red; $n=6$). Black bars show mean lesion volume by day. (d) Change in lesion volume between Days 0 and 7 normalised to lesion size on Day 0. Mean lesion size (open circles) decreased by 38% in the RibAde group, 50% in the RibAdeAll group and 18% in the saline group. $p=0.065$ between untreated and treated groups (t-test). Horizontal line, median; box 25%–75% range; whiskers, ± 1 SD; X, min and max values.

Cardiac ATP repletion strategies in ATP-depleted brain tissue

Given that no work on cerebral bioenergetics had been done in brain tissue with RibAde, we initially examined the influence of RibAde in acutely-prepared rat brain slices; tissue that is known to have lower levels of ATP than the intact brain, likely due to the ischaemia and trauma associated with their preparation. Indeed,

this has led some to describe the brain slice as a model for the post-traumatic, post-ischaemic brain (Hossmann, 2008).

Incubation of neocortical/hippocampal slices in modest concentrations of ribose (1 mM) and adenine (50 μ M) for 2–3 h resulted in stable elevations in cellular ATP, which, when the dead edges of brain slices were taken into account, reached values of ATP comparable to those reported in the literature for the in vivo rat brain (Zur Nedden et al., 2011). The elevation in tissue

Table 1. Statistical comparison of the percentage change in lesion volume between Day 0 and Day 7 across the groups of animals used in the study.

Test	Group	N	Mean lesion volume at Day 7 (% Day 0)	SD	Statistics (F or t)	DF	p-value	Sig.
One-way ANOVA	Saline	6	82.4	30.4	2.247	2, 19	0.133	NS
	RibAde	8	62.5	27.7				
	RibAdeAll	8	50.5	26.3				
Unpaired t-test	Saline	6	82.4	30.4	1.951	20	0.065	NS
	RibAde/All	16	56.5	26.8				

A one-way ANOVA was conducted across the three groups (Saline, RibAde and RibAdeAll). An unpaired t-test was conducted between saline and the combined treatment groups (RibAde and RibAdeAll).

Number of animals (N); standard deviation (± 1 SD); degrees of freedom (DF); not significant (NS).

ATP translated into greater release of adenosine into the extracellular space during electrical stimulation of afferent fibres (Zur Nedden et al., 2011) and in response to OGD (Zur Nedden et al., 2014). This increased adenosine release exerted an enhanced inhibitory action on excitatory synaptic transmission. Considering these two actions in the context of the energetically compromised intact brain, the enhancement of tissue ATP would allow better restoration and maintenance of cellular ionic homeostasis and reduce the prevalence and severity of anoxic depolarisation invasion of the adjacent penumbra. This would be complemented by the enhanced inhibitory action of greater adenosine release on neuronal function in terms of both reducing glutamate release and neuronal excitability. To test the potential benefits of RibAde *in vivo*, we thus adopted a model of cerebral ischaemia in which ATP depletion is known to occur (Hata et al., 1998).

In vivo treatment with RibAde \pm allopurinol produced no major side effects

We initially strove to assess the safety of the treatment. First, we evaluated the effect of RibAde on blood pressure. Prior studies suggested that infusion of adenine (at 50 mg/kg/h IV) could decrease MABP (Zimmer and Schneider, 1991). A significant decrease in MABP would compromise cerebral blood flow, increasing the severity of the ischaemic insult and the amount of permanent brain damage. At a higher dose of adenine (36 mg/kg/h IV) than the one used in the main stroke study (10 mg/kg/h IV), we detected no significant effect of RibAde on blood pressure. Second, having encountered mortality and kidney damage associated with the 36 mg/kg/h infusion, it was necessary to confirm that the lower infusion dose chosen for the main study (10 mg/kg/h IV) had no major consequences on general health.

Using adenine at 10 mg/kg/h, not only did the treated animals recover and survive for 7 days following tMCAO, the treatment also had no effect per se on body weight or the general condition of the animals compared to saline-treated controls. Although we did not measure blood markers of renal function, the former suggests that the rats did not suffer from clinical renal failure, which results in decreased food intake (Lindblad et al., 1973). Minor transient histopathological kidney changes attributed to adenine, which were noted 3 days after infusion in the preliminary study, had resolved by Day 7 in kidney histology data from the main study.

Similar doses of adenine (10 mg/kg IV) have been administered to humans, albeit over shorter durations (up to an hour), with no effect on renal function (Bartlett, 1977; Roth et al., 1975) and a higher acute dose (25 mg/kg in 1 h) was administered to non-human primates with no apparent consequences (Siegel et al., 1971). This highlights the significant potential for rapid translation to human studies as all compounds used in this study have already been used in man for various purposes. Adenine has been used for decades for the preservation of blood products (Hess and Greenwalt, 2002; Paglia et al., 2016; Peck et al., 1981), most likely for the maintenance of erythrocyte ATP, while allopurinol, a xanthine oxidase inhibitor, is a common treatment for gout and has been since the 1960s. Finally, D-ribose has been used as an ergogenic aid (Dhanoa and Housner, 2007) or a supplement for cardiac disease (Bayram et al., 2015; Pauly and Pepine, 2000; Pliml et al., 1992; Shecterle et al., 2010) and can be tolerated in man for prolonged periods at intravenous doses higher than those used in this study (Gross and Zollner, 1991).

RibAde \pm allopurinol: trend towards decreased infarct size and improved functional outcome in a pre-clinical stroke model

Our results constitute the first *in vivo* proof of concept of the potential of RibAde to protect penumbral tissue. Following a 1h ischaemia period, reperfusion reduced lesion size by 18% in the control group, by 38% in the RibAde group and by 50% in the RibAdeAll group. In addition, this appears to be linked to a faster neurological recovery. The rank order of these benefits is consistent with our hypothesis concerning the targeting of the purine salvage pathway in the direct (RibAde) and indirect (allopurinol leads to greater availability of endogenous hypoxanthine) provision of substrates with which the brain can make ATP in a manner that is independent of mitochondria.

These findings thus vindicate our extensive *in vitro* evidence of the beneficial effects of RibAde enhancing cellular ATP levels, promoting increased adenosine release and reducing cell death after OGD (Zur Nedden et al., 2011, 2014). Beneficial effect of allopurinol have been described in various animal models of brain injury including cerebral ischaemia (Akdemir et al., 2001; Ansari et al., 2013; Dong et al., 2015; Isik et al., 2005; Martz et al., 1989), but have been mostly described in terms of its antioxidant properties, although maintenance of the cerebral adenine nucleotide pool during ischaemia has been reported (Phillis et al.,

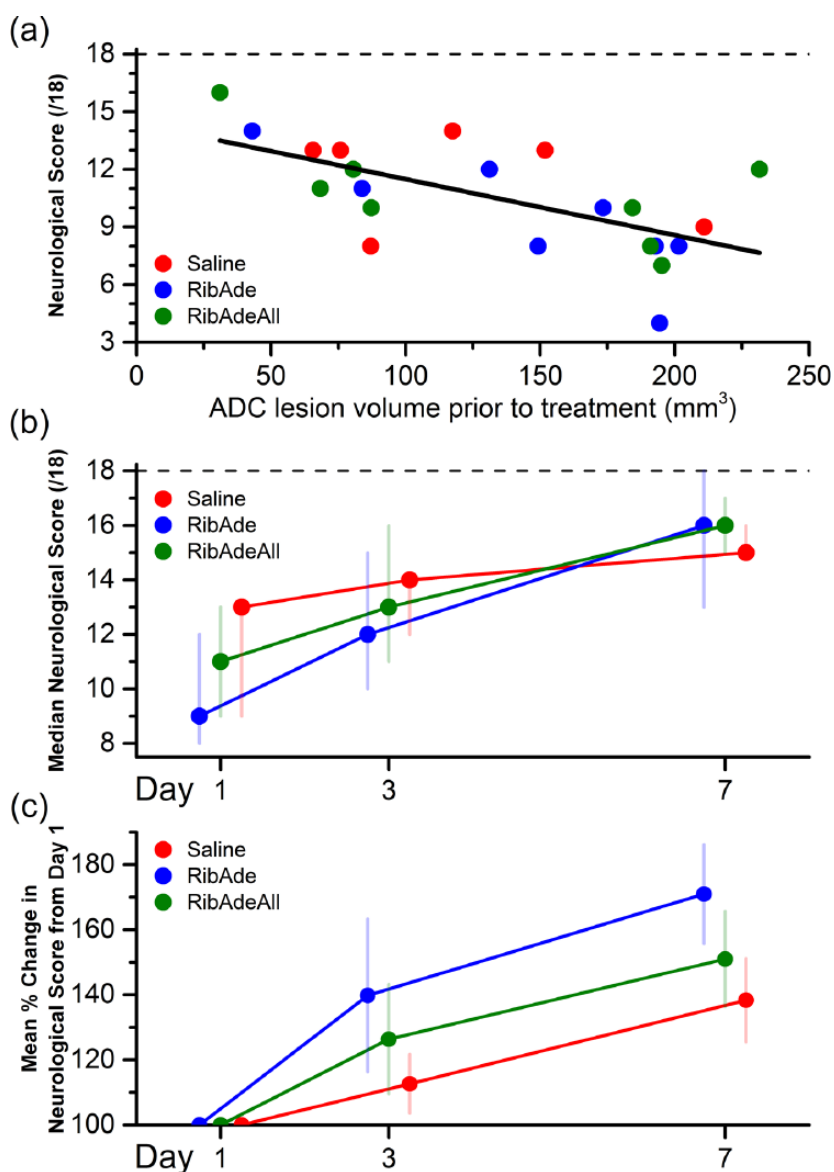


Figure 7. Trend towards an improvement in neurological outcome after stroke in RibAde and RibAdeAll groups. All animals scored the maximum value of 18 prior to tMCAO (dashed line on panels (a) and (b)). (a) Correlation between neurological score at 24 h after tMCAO (out of 18; 18=normal animal, dashed line; 3 is the lowest possible score) and volume of tissue-at-risk of infarction assessed on ADC maps at the end of tMCAO and prior to reperfusion and the start of saline (red circles), RibAde (blue circles) or RibAdeAll (green circles) treatment. Considering all animals, there was a significant inverse correlation between neurological score and lesion volume prior to treatment (linear regression, $p=0.0015$, $R^2=0.40$). Note the coincidental clustering of RibAde/RibAdeAll-treated animals with larger pre-treatment lesion volumes. (b) Median neurological scores (out of 18; dashed line) per group on Days 1, 3 and 7 after induction of stroke ($n=8$ in RibAde and RibAdeAll groups and $n=6$ in saline group). Vertical lines indicate the first and third quartiles for each data point. Median and quartile values are rounded to nearest whole number. Data points for each group have been offset for clarity. (c) Mean neurological scores (\pm SEM) for each day normalised to Day 1 revealed a trend to faster recovery, despite larger initial lesions in the treatment groups, consistent with the greater reduction in lesion size (Figure 6). However, statistical analysis of the effect of treatment on the evolution of the neurological score did not reach significance (two-way repeated-measures ANOVA with treatment and time effect; treatment effect $F(2, 19)=0.678$; $p=0.519$). Data points for each group have been offset for clarity.

1995). A further advantage of the inclusion of allopurinol is that it would reduce the xanthine oxidase-mediated conversion of adenine to the insoluble and nephrotoxic 2,8-dihydroxyadenine.

The reduction of the injury in the treated groups likely reflects sparing of penumbral tissue. This would be consistent with assessment of neurological deficit over the first week following stroke, which demonstrated a trend to faster recovery in the treatment

groups despite having larger lesions prior to the start of treatment. We hypothesise that RibAde in conjunction with allopurinol improves cellular metabolism of the ischaemic brain by allowing quicker replenishment of ATP levels by its purine saving effect as suggested by our *in vitro* experiments (Zur Nedden et al., 2011). Maintenance of ATP levels would be beneficial for multiple reasons. First, a large amount of energy is required by the neuronal

Na^+/K^+ ATPase in order to maintain cellular homeostasis and viability (Harris et al., 2012). Following ischaemia, failure of energy-dependent membrane ion pumps leads to cytotoxic oedema, depolarisation and excitotoxicity, which ultimately leads to cell death (Doyle et al., 2008). Second, adenine nucleotides are a source of adenosine, a known neuroprotective, anticonvulsant and vasodilator metabolite of ATP (Pedata et al., 2016; Ribeiro et al., 2016). Adenosine would thus be expected to protect neural tissue from excitotoxic damage, prevent peri-infarct depolarisations from the ischaemic core, suppress seizures and further brain damage. Finally, by its vasodilatory properties, adenosine release could also improve blood supply to the penumbral area allowing further protection (Dale and Frenguelli, 2009; Williams-Karnesky and Stenzel-Poore, 2009).

Study limitations

The main limitation of this study resides in the low number of animals in each group. This is compensated for to a certain extent by serial MRI scanning and sequential neurological testing, which allowed each animal to be used as its own control, and thereby decreasing markedly the number of animal required in such a variable model to draw valid conclusions. However, despite this and the trend towards a reduction in lesion volume in a manner consistent with our hypothesis, the results did not reach statistical significance. Similarly, the trend towards enhanced neurological recovery in RibAde- or RibAdeAll-treated animals was compelling, but lacked clear statistical support. Although the 18 point neurological testing used here assessed both sensory and motor function, it remains a crude way to assess function. More refined tests, including of balance, such as the staircase or cylinder tests (Schaar et al., 2010), or the modified Neurological Severity Score (mNSS) (Chen et al., 2001) could potentially have detected more subtle improvements in the RibAde/All-treated groups.

Future work will assess whether RibAde \pm allopurinol prove to be beneficial in various models of focal cerebral ischaemia including permanent middle cerebral artery occlusion models, and in aged animals with or without co-morbidities such as hypertension and diabetes. Based on data from this study, 13 animals per group would be required to show a significant difference ($p < 0.05$; power of 0.8) in lesion volume between saline- and RibAdeAll-treated animals of the magnitude reported in this study ($\sim 32\%$ difference) and an SD of 28%. Confirmation of the mode of action of RibAde will be sought by studying cerebral metabolism with ^{31}P -MRS, allowing quantification of ATP over time in vivo, or to use NMR to follow the metabolic fate of labelled RibAde in microdialysate samples. The duration of treatment, dose of allopurinol and the potential inclusion of other neuroprotective compounds, such as uric acid (Llull et al., 2016), are additional variables that could be explored.

Conclusion

In this study, we have obtained encouraging preliminary evidence, based upon extensive in vitro studies, that ribose, adenine and allopurinol have the potential to protect the ischaemic brain in a rat model of tMCAO. Following further validation, we believe that it would be a readily translatable treatment for acute injuries of the human brain such as traumatic brain injury and

stroke. The tolerability of the compounds at the doses used, plus their prior use in various indications, together with a defined scientific rationale and identified mechanism of action should facilitate translational studies, not just in the clinic, but at the point of injury by attending paramedics.

Acknowledgements

The authors are grateful to Dr Christopher McCabe for his constructive comments on the manuscript and Dr Elliot Ludvig for valuable statistical advice. The authors would also like to thank Lindsay Gallagher, Linda Carberry, Lisa Roy and James Mullin for their technical support, and Dr Francesco Marchesi for his assistance with the histological analysis.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

Funding

This work was funded by grants from the Rosetrees Trust and from Warwick University Alumni Fund.

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