Purines: from biomarkers to therapeutic agents in brain injury

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Abstract

The purines constitute a family of inter-related compounds that serve a broad range of important intracellular and extracellular biological functions. In particular, adenosine triphosphate (ATP) and its metabolite and precursor, adenosine, regulate a wide variety of cellular and systems-level physiological processes extending from ATP acting as the cellular energy currency, to the adenosine arising from the depletion of cellular ATP and responding to reduce energy demand and hence to preserve ATP during times of metabolic stress. This inter-relationship provides opportunities for both the diagnosis of energy depletion during conditions such as stroke, and the replenishment of ATP after such events. In this review we will address these opportunities and the broad potential of purines as diagnostics and restorative agents.

Introduction

Adenosine triphosphate (ATP) is an ancient molecule that serves an absolutely fundamental role in the life of cells[1]. It is universally used as the energy source to power cellular processes, from the maintenance of the membrane potential to muscular contraction. This arises, not by virtue of a high energy bond between the terminal phosphates, but instead via the extent to which the concentration of ATP is held away from the equilibrium concentration with ADP (and Pi) - a remarkable factor of 10^{10}[2]. This chemical gradient, which is maintained by mitochondrial oxidative phosphorylation, is both necessary and sufficient to power the myriad biochemical reactions required for cellular viability and intercellular communication. Accordingly, conditions where mitochondrial function is compromised, for example during cerebral ischemia, have profound implications for this gradient and indeed for cellular- and organism-level viability.

ATP: From energy source to neurotransmitters

In addition to this pivotal intracellular role as the universal energy currency, ATP acts as a neurotransmitter, a concept that was initially regarded as heretical, at both ionotropic P2X receptors and G protein-coupled P2Y receptors (Figure 1)[3]. While ATP activates all P2X receptors (P2X1-7), the subunits of which form both hetero- and homotrimeric receptors[4, 5], ATP's activation of P2Y receptors is limited to a small number of the eight known mammalian subtypes ($P2Y_{2,11}$). The other P2Y receptors are primarily activated by ADP ($P2Y_{1,12,13}$), the pyrimidine nucleotides UTP ($P2Y_{2,4}$), UDP ($P2Y_{6,14}$), UDP-glucose ($P2Y_{14}$)[6, 7] and dinucleoside polyphosphates, which may also activate some P2X receptors[8].

The release of ATP into the extracellular space to activate these receptors occurs via a variety of means, and from many cell types. Thus, exocytosis of ATP-containing vesicles has been described from both neurones and astrocytes, while additional release mechanisms include damage to the plasma membrane, lysosome-mediated release, and channels such as pannexin/connexins, and indeed P2X receptors themselves[9, 10]. Once released into the extracellular space ATP is rapidly degraded by a series of ectonucleotidases[11] to give rise to two further signalling molecules, ADP ($P2Y_{1,12,13}$)

and adenosine (Figure 1). Adenosine acts via P1 receptors comprising four G protein-coupled A_1, A_{2A}, A_{2B}, A_3 receptors[12]. Adenosine can be released directly into the extracellular space via both concentrative (SLC28) and equilibrative (SLC29) nucleoside transporters[13], while the release of cAMP via SLC22 transporters[14, 15] constitutes another source of extracellular adenosine[10, 16]. The metabolism of adenosine to inosine provides yet another, albeit less potent, agonist at adenosine receptors[17-21]. The actions of intracellular, and predominately astrocytic adenosine kinase[22], which phosphorylates adenosine to AMP, is key to maintaining an inward gradient for the removal of adenosine from the extracellular space under normal conditions[23].

Receptors (P0) for adenine have been described[10, 24, 25], and, until very recently the source of adenine in eukaryotes was thought to be via metabolism of S-5'-methylthioadenosine, a product of polyamine synthesis, via S-methyl-5'-thioadenosine phosphorylase[26, 27]. However, in an astonishing recent paper the orphan protein FAMIN (Fatty Acid Metabolism-Immunity Nexus) was shown to possess adenosine phosphorylase activity (as well as adenosine deaminase, purine nucleoside phosphorylase, and S-methyl-5'-thioadenosine phosphorylase activity) thereby liberating adenine (and ribose-1-phosphate) from adenosine[28]. Thus, adenosine may serve as a source of adenine for the activation of P0 receptors, finalising a series of at least seven signalling molecules (ATP, ADP, AMP, cAMP, adenosine, inosine, adenine) from one primordial compound – ATP (Figure 1).

When ATP demand outstrips ATP supply

The reliance upon mitochondrial oxidative phosphorylation to supply ATP brings with it challenges when the substrates for oxidative phosphorylation are in short supply. Thus, reductions in the supply of oxygen (hypoxia), glucose (hypoglycaemia) or both (ischemia), compromise the ability of mitochondria to generate ATP. Unfortunately, these conditions are all too common and can be observed during stroke, insulin overdose, birth asphyxia and traumatic brain injury, as well as cardiac arrest and compartment syndrome. Under these circumstances the ability to synthesise ATP is impaired, and, while the cellular store of phosphocreatine acts as an immediate buffer to preserve cellular ATP via the substrate-level phosphorylation of ADP to ATP by creatine kinase, ATP levels,

especially in highly metabolically-active tissue such as brain and heart, rapidly decline. In the brain this depletion of cellular ATP results in an inability to maintain neuronal membrane potential due to the reduction in the activity of the ATP-dependent Na^+/K^+ATPase resulting in depolarisation, Ca^{2+} influx and the release of neurotransmitters such as glutamate. Furthermore, the loss of neuronal and glial membrane ionic gradients impedes the ability to remove glutamate from the synaptic cleft such that high and neurotoxic levels of glutamate results in excessive activation of ionotropic (AMPA, NMDA and kainate) receptors, as well as metabotropic glutamate receptors (mGluRs). This over-stimulation of glutamate receptors results in cellular swelling, Ca^{2+} influx and the initiation of damaging intracellular enzymatic pathways, including the activation of lipases and caspases, and the liberation of damaging chemical agents such as nitric oxide and reactive oxygen species[29]. Additionally, glutamate release and K^+ efflux provoke spreading depolarisation, which expands the primary lesion into adjacent brain tissue[30, 31]. Accordingly, there is much interest in targeting both glutamate receptors and the intracellular signalling cascades initiated by their activation. While there has been no evidence of clinical efficacy of glutamate receptor antagonists in acute ATP-depleting conditions in the brain[32], preventing NMDA receptor-mediated activation of nitric oxide synthase with a membrane permeable interfering peptide does show some promise[33, 34].

Purine-based retaliatory mechanisms in the injured brain

The brain, however, is not a passive victim to the depletion of ATP and responds through a series of measures to both limit energy demand and enhance nutrient supply. Initially the metabolism of ATP is sensed via a change in the ATP:ADP or ATP:AMP ratio by AMP-activated protein kinase (AMPK), an intracellular enzyme exquisitely sensitive to both cellular energy status and intracellular glucose levels[35, 36] and which readily responds to metabolic stress in brain[37]. Under these conditions AMPK inhibits energy-consuming cellular pathways such as protein synthesis and stimulates alternative energy sources such as fatty acid oxidation and glucose uptake[35] and may be neuroprotective[38]. Downstream of AMP, when the final phosphate group has been removed, is adenosine.

Adenosine has been described as a retaliatory metabolite[39]. That is to say it is released from tissue under conditions of metabolic stress, either because of a lack of mitochondrial substrates (eg during cardiac or cerebral ischemia) or because demand for ATP outstrips the ability to synthesise it, for example during epileptic seizures[40]. During these conditions adenosine initiates a series of receptor-dependent actions to inhibit energy-sapping neurotransmitter release and neuronal firing (via A_1 receptors), and increase nutrient supply via A_2 receptor-mediated vasodilation of blood vessels[12]. About 80% of the brain's energy budget is spent pumping Na^+ out of neurons to restore the transmembrane Na^+ gradient following action potential firing and postsynaptic excitation[41]. Thus, adenosine-mediated inhibition of these activities will have an immediate benefit for the energy budget of metabolically-stressed neurons. Moreover, adenosine retaliates very promptly: an examination of the blood flow thresholds for adenosine and glutamate during ischemia revealed the release of adenosine at reduced levels of blood flow that did not evoke the release of glutamate[42]. Whether adenosine is released per se or as ATP during ischemia is quite controversial. Using highly time-resolved measurements of adenosine release using enzyme-based microelectrode biosensors[43] we were only able to discern adenosine release in the early stages of *in vitro* ischemia (oxygen/glucose deprivation). ATP release was only observed during the anoxic depolarisation[44], an event akin to spreading depolarisation[45] and which has been shown by others to evoke both ATP[46] and adenosine release[47, 48].

The accumulation of adenosine in the extracellular space occurs rapidly (within seconds) in response to a variety of insults to the mammalian, including human, brain (for a review see[40]). This accumulation is initiated by ATP depletion, but may be facilitated by the inhibition of adenosine kinase by conditions found during metabolic stress (hypoxia, ATP depletion, ADP, AMP, high levels of intracellular adenosine and low cellular K^+)[49]. This inhibition of the enzyme largely responsible for the inward gradient promoting adenosine uptake, likely contributes to the prolonged presence of extracellular adenosine observed *in vitro* after oxygen/glucose deprivation, and which has a corresponding inhibitory influence on excitatory synaptic transmission[50]. Once in the extracellular

space the high density of nucleoside transporters in the rodent, porcine[51] and human[52] blood-brain barrier[13] facilitates the efflux of adenosine from the brain into the blood[53] (Figure 2).

Purines as biomarkers of brain injury

This brain to blood efflux of adenosine and its metabolites, inosine and hypoxanthine, all of which share the same transporter (SLC29)[13] results in the appearance in blood of purines above their usual baseline levels during cerebral metabolic stress and trauma[54].

Purines are highly labile in blood, being metabolized ultimately to uric acid and also taken up into red blood cells via equilibrative transporters[55]. The half-life of adenosine in blood is of the order of 20-60 s[56-58]. Inside red blood cells adenosine can be converted to AMP or inosine and hypoxanthine, the latter of which can enter the purine salvage pathway, the primary means by which erythrocytes, the heart and indeed the brain reconstitute adenine nucleotides (Figure 2)[36, 59-61]. This complexity of dynamic processing makes the purines hard to measure in clinical samples, and has been a barrier to their adoption as biomarkers to provide clinical information. Indeed, studies that have used analytical methods such as HPLC use extensive pre-processing of blood to preserve the purine signal. These often include blockers of adenosine deaminase, and inhibitors of the equilibrative transporters in a "stopping" solution prior to separation of plasma for analysis. The widespread adoption of purines as a diagnostic tool thus needs rapid point of care measurements[54] .

To gain an appreciation of how the dynamics of purine cycling might alter such measurements we have produced a simple model of adenosine release from the brain and its conversion to the downstream purines along with uptake and processing in red blood cells (Figure 3). This simple model ignores potential release of inosine and/or hypoxanthine from brain and also metabolism of these purines inside red blood cells. Nevertheless, it captures some important features. Firstly, during a stroke, purines will rapidly rise in blood and reach a plateau in which a mixture of adenosine, inosine and hypoxanthine are present. Following resolution of the event, the purines will return to baseline with kinetics that are largely determined by how adenosine release from the brain falls with time.

This analysis suggests therefore that the blood purines can be a real-time indicator of ischemic events within the brain. The analysis further shows the time constraints on sampling of whole blood purines for point of care measurement. We have simulated purine turnover in an *ex vivo* blood sample (Figure 3). This shows that within a 3-minute measurement period almost all of the purines have been converted to hypoxanthine, and that virtually no adenosine remains. At three minutes, a hypoxanthine measurement would capture about 70% of the true purine signal *in vivo*. Obviously, shortening the time taken for the purine measurement would provide a measurement closer to the true *in vivo* value.

Having developed and used purine biosensors in the past[43, 44, 62, 63], we optimised them to be suitable for use in whole blood, a challenging electrochemical environment with many interferences that can prevent accurate electrochemical measurements[64]. Exploiting carotid endarterectomy[65] as a clinical procedure during which a timed ischemic insult is applied to the brain, we measured purines in arterial blood before, during and after clamping of the carotid artery. In awake, sedated patients a reversible elevation of purines in whole blood was detected with our biosensors (Figure 4) that was surprisingly similar in profile to the modelled purine release presented in (Figure 3)[66]. These data confirm that purines are sensitive real-time indicators of brain ischemia. A further study using biosensors to measure purines in blood of stroke victims showed that purine levels were elevated in patients that had suffered an ischemic or hemorrhagic stroke[67, 68].

Restoring the building blocks of ATP in the injured brain

While the loss of purines into the blood stream serves as a biomarker of metabolic crisis and may be useful in the diagnosis of an acute cerebral emergency, their loss deprives the brain of the substrates necessary to synthesise ATP. This is because the brain, like the metabolically highly active heart, utilises the purine salvage pathway (PSP; Figure 2) as the primary means by which to synthesise adenine nucleotides, in contrast to the slower, and metabolically more expensive de novo synthesis route[36, 59-61]. The PSP, which is present in both neurones and glia, comprises two branches, one catalysed by adenine phosphoribosyltransferase (APRT), and the other by hypoxanthine phosphoribosyltransferase (HPRT), the enzyme deficient in Lesch-Nyhan syndrome[69]. The

phosphorylation of adenosine by adenosine kinase is another route by which the pool of adenine nucleotides can be restored, and indeed very early[70] and more recent work[71] showed that the provision of adenosine could elevate ATP levels in brain tissue. However, the profound cardiovascular and respiratory effects of systemic adenosine, noted as far back as 1929[72], preclude adenosine as a means to replenish the substrates for the cerebral PSP. While hypoxanthine is a potential route to replenishment of PSP substrates, the xanthine oxidase-mediated conversion of hypoxanthine to xanthine and hence to uric acid, generates hydrogen peroxide and potentially injurious reactive oxygen species. This, together with the requirement for three enzymes in the conversion of hypoxanthine to AMP, reduces the potential for hypoxanthine as a means to restore the pool of substrates for the PSP.

In contrast, adenine itself is a viable option for the restoration of PSP substrates. Adenine is readily transported into brain via equilibrative nucleoside transporters (ENTs; SLC29)[13], is found in blood transfusion products as it supports ATP synthesis via the PSP in erythrocytes, which lack mitochondria[73], and has a 20-fold lower affinity for xanthine oxidase than xanthine[74], which would limit production of both free radical-generating hydrogen peroxide, and the insoluble adenine metabolite 2,8-dihydroxyadenine. Additionally, there is evidence that adenine has the potential to elevate brain tissue ATP[70, 71], albeit at high concentrations, and not by very much on its own. This observation suggests that some factor is lacking for the full functioning of the PSP to restore adenine nucleotides. That factor is likely to be ribose. Ribose-1-phosphate emerges from the recently-discovered metabolism of adenosine to adenine by FAMIN[28], and from the conversion of inosine to hypoxanthine by purine nucleoside phosphorylase (Figure 2). Isomerisation of ribose-1-phosphate to ribose-5-phosphate renders this molecule, which also emerges from the pentose phosphate pathway, suitable for incorporation into the cellular pool of PRPP (phosphoribosyl pyrophosphate; Figure 2). PRPP is the reservoir of the phosphoribose moiety that is required to convert adenine and hypoxanthine into AMP and IMP via the actions of APRT and HPRT, respectively. The application of exogenous D-ribose, which by-passes the pentose phosphate pathway, is known to elevate the

cellular PRPP pool[75], initially via the ribokinase-mediated conversion of D-ribose to ribose-5-phosphate and hence to PRPP via phosphoribosyl pyrophosphate synthetase (Figure 2). A series of high profile papers in the early 1980s demonstrated the ATP-replenishing and cardioprotective actions of D-ribose[76-78]. A complementary approach, to include adenine in the cardioprotective solution[79, 80] also yielded functional benefits in whole animal models of cardiac injury. However, the combined approach was not pursued, likely because of the high concentrations of adenine used, which caused cardiovascular effects in some studies[75] and because adenine can be converted to an insoluble and nephrotoxic metabolite, 2,8-dihydroxyadenine, a potentially serious consequence of APRT deficiency[81]. Nonetheless, ribose administration in cardiac failure is still under consideration in clinical trials[82].

Ribose and adenine restore depleted ATP levels in brain slices

Our approach was to investigate the influence of ribose and adenine (RibAde) on ATP levels in brain slices. These studies were prompted by observations of apparent adenosine depletion in response to sequential adenosine-releasing hypoxic challenges to hippocampal slices we had made *in vitro* using microelectrode biosensors for adenosine[43, 50, 83], and others had made *in vivo* using microdialysis techniques[84, 85]. Since ATP represents the primary cellular reservoir for adenosine, we hypothesised that ATP loss was responsible for reduced adenosine release in response to repeated challenges.

Measurements of cellular ATP in hippocampal slices confirmed observations made by many others that tissue ATP content is reduced relative to that in the intact brain[86]. This is likely a reflection of the trauma and ischemia the brain suffers during the preparation of brain slices. Indeed, this has prompted the view that brain slices reflect a post-injury brain, and somewhat far removed from the situation in the healthy brain[87]. However, this can be an advantage when wanting to study strategies to improve the metabolic condition of brain slices, which could have translational implications for those having experienced acute brain injury such as a stroke or a traumatic brain injury (TBI). This is especially the case since the metabolic integrity of the brain, including its ATP content may take a considerable amount of time to recover after an insult[60, 88].

We discovered that it was possible to increase brain slice ATP levels by incubating slices for 2 – 3 hrs in modest concentrations of ribose (1 mM) and adenine (50 μM)[86] (Figure 5A). This incorporation into the cellular ATP pool was stable over several hours, and required both ribose and adenine since neither were effective on their own. Interestingly, higher concentrations did not generate more ATP, which, when compensation was made for the dead cut edges of brain slices, reached values identical to those recorded *in vivo*. These data suggest that both the feedback mechanisms regulating ATP cellular content persist in brain slices, and, moreover, that depleted ATP levels can be restored.

The elevation of the cellular pool of ATP had implications for the release of adenosine. This was evidenced by greater release of adenosine in RibAde-treated hippocampal slices under conditions of: i) high-frequency electrical stimulation of afferent fibres in the hippocampus[86]; ii) during oxygen/glucose deprivation (OGD)[89] (Figure 5B), and iii) during convulsant-induced electrographic seizure activity[90] (Figure 5C). This greater release of adenosine was associated with corresponding effects on synaptic transmission, synaptic plasticity and seizure activity: the threshold for long-term potentiation was increased in an adenosine A_1R-dependent manner[86]; OGD depressed synaptic transmission more quickly and for longer in RibAde-treated slices (Figure 5B)[89], and there was an attenuation in the severity of epileptiform discharges[90]. These observations, plus the fact that RibAde reduced cultured cerebellar granule cell death when given *after* OGD[89], suggested that the ATP-depleted brain *in vivo* may benefit from RibAde through support for ATP synthesis and the enlargement of the cellular reservoir for adenosine.

Evidence of efficacy of ATP repletion strategies in vivo

To test this hypothesis, we tested RibAde in a rat model of stroke (60 min occlusion of the middle cerebral artery)[91]. We tested three groups of animals: intravenous infusions of saline, or RibAde, or RibAde plus an intraperitoneal injection of the xanthine oxidase inhibitor allopurinol (RibAdeAll). Allopurinol should increase the availability of hypoxanthine and adenine for salvage, decrease the production of damaging free radicals, and indeed may be a free radical scavenger, attributes that likely

contribute to allopurinol's potential as a neuroprotective agent in fetal hypoxia[92, 93]. Using MRI we measured the volume of brain tissue at risk during cerebral ischemia, and final infarct volume at 7 days (Figure 6A). While the study was small and underpowered to achieve statistical significance, we observed a reduction in infarct volume in both treated groups, with the greatest effect being observed in the RibAdeAll group (Figure 6B), consistent with the hypothesis that, by preventing metabolism of hypoxanthine (and adenine) more of these substrates were made available to the PSP for ATP synthesis. These treated animals also seemed to make a better recovery over the next seven days compared to saline-treated animals (Figure 7).

While at present we have no mechanistic insight as to the basis of the encouraging results in the stroke model, given what we know from the *in vitro* studies, it is plausible that RibAde, by elevating cellular ATP: i) restored or maintained the ability of neurones to regulate membrane potential, thereby avoiding excessive depolarisations, including those spreading depolarisations emanating from peri-infarct regions[31]; ii) activated restorative processes, such as protein synthesis; iii) increased adenosine release to inhibit peri-infarct depolarisations and seizure activity, and promote collateral blood flow through causing vasodilation. Additional studies are required to test these predictions directly.

Purine-based prospects for human brain injury

While these observations are encouraging, to what extent might these compounds be tolerated in man? Ribose and adenine have been administered intravenously to man at doses the same as or higher than those used in our stroke study[94-96]. While there is the possibility of nephrotoxicity associated with adenine's xanthine oxidase-mediated conversion to 2,8-dihydroxyadenine, we observed none in our RibAde animals at the dose used in the stroke study[91]. Indeed, the inclusion of a xanthine oxidase inhibitor, such as allopurinol in RibAdeAll should limit this conversion from occurring, as it does in patients with APRT deficiency[81, 97].

The purines thus have multiple roles in the context of brain injury; not only are AMPK and adenosine mobilised during metabolic crises to launch an endogenous retaliatory neuroprotective response to limit cellular activity and to maximise delivery and utilisation of remaining available nutrients, the release of purines into the blood stream serves as a rapidly-detectable external sign of brain distress. In parallel, the provision of substrates for the PSP may facilitate restoration of cerebral ATP and the enhancement of the cellular pool of adenosine. In this regard there is a pleasing symmetry between the release and replenishment of the purines (Figure 8), and both processes have great potential for the acute diagnosis and treatment of a range of injuries to the central nervous system.

Acknowledgements

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http://www.betterfamilyhealth.org/normal-blood-sugar-levels.html

Conflicts of Interest

Nicholas Dale is the founder, an Executive Director and the Chief Scientific Officer of Sarissa Biomedical, the company manufacturing the research and diagnostic purine biosensors described in the review. Bruno Frenguelli is a Non-Executive Director of Sarissa Biomedical. Dale and Frenguelli are either employed by Sarissa and/or have shares in the company.

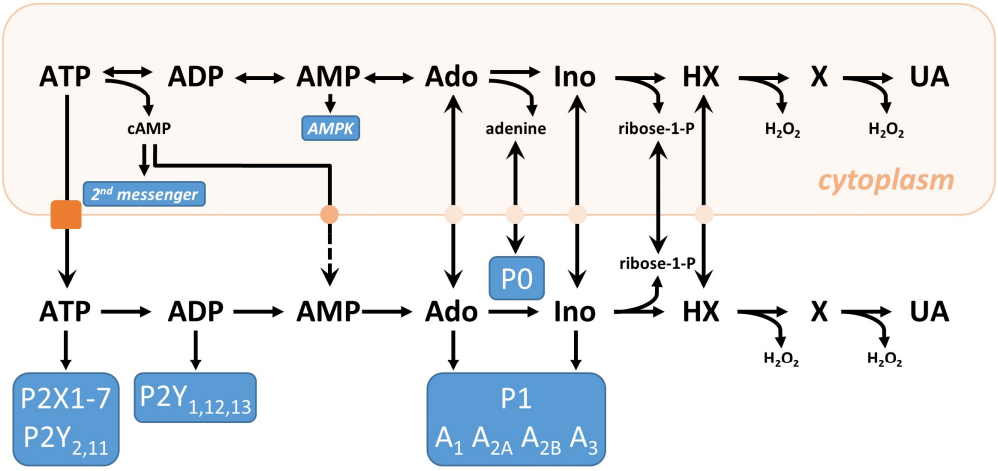

Figure 1. ATP and its metabolites as signalling molecules. The release of ATP via exocytosis, ion channels or membrane vesicles (square box) results in the activation of a range of ionotropic P2X and G protein-coupled P2Y receptors. Intracellularly, the metabolism of ATP gives rise to the 2nd messenger cAMP, and the sequentially dephosphorylated metabolites ADP and AMP; elevations in the latter activates AMPK (AMP-activated protein kinase). Further dephosphorylation produces adenosine (Ado), which is either deaminated to inosine (Ino), or via, FAMIN (Fatty Acid Metabolism-Immunity Nexus), converted to adenine (and ribose-1-phosphate). Inosine is converted to hypoxanthine (HX), which is subsequently converted to xanthine (X) and uric acid (UA). In the extracelluar space ATP metabolism gives rise to a number of molecules, several of which (ADP, adenosine and inosine) activate P2 or P1 receptors. Release of cAMP via SLC22 transporters (dark circle) gives rise to AMP, a potential additional source of adenosine. SLC29 transporters (pale circles) exchange adenosine, adenine, inosine and hypoxanthine across the membrane down their concentration gradients whereupon the first three of these molecules can activate their respective receptors. Concentrative transporters (SLC28) accumulate adenosine and inosine. Ribose readily diffuses across membranes (a factor that may have led to the ribose-based nucleic acids, RNA and DNA[98, 99]), but may also be carried by SLC2 glucose transporters.

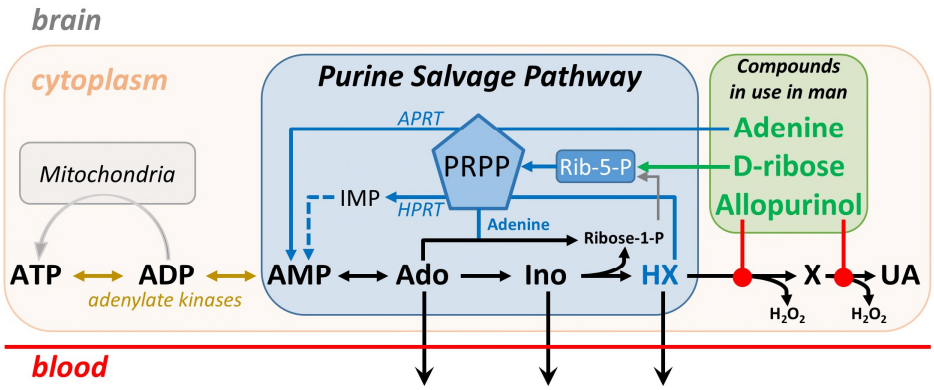

Figure 2. Purine salvage and adenine nucleotide synthesis. ATP breakdown results in the production of the purine nucleosides adenosine (Ado) and inosine (Ino) and the nucleobase hypoxanthine (Hx) which are lost from the brain into the blood; see[66, 68] and Figure 4 for human data. Purine salvage (blue arrows) restores adenine nucleotide levels directly via APRT (adenine phosphoribosyltransferase; adenine to AMP) and indirectly via HPRT (hypoxanthine phosphoribosyltransferase; hypoxanthine to inosine monophosphate (IMP)). This reaction requires PRPP (phosphoribosyl pyrophosphate), which is produced from the ribose-5-phosphate (Rib-5-P) that emerges from the pentose phosphate pathway. Ribose-5-phosphate can also be formed via isomerisation of adenosine- and inosine-derived ribose-1-phosphate, or directly from D-ribose by ribokinase thereby increasing PRPP levels. Adenine and D-ribose feed into the salvage pathway and increase tissue AMP and hence ATP levels via mitochondrial and non-mitochondrial routes (cytoplasmic adenylate kinases). Importantly, mitochondria rely on salvage-derived ADP for ATP production. Allopurinol inhibits xanthine oxidase and prevents the breakdown of hypoxanthine to xanthine (X), and from xanthine to uric acid (UA) providing more hypoxanthine for salvage and additionally reducing the production of H_2O_2-derived reactive oxygen species. Adenine supplementation is preferred over hypoxanthine as the former is: i) less of a substrate for xanthine oxidase than hypoxanthine by a factor of 20[74], thereby making more substrate available for the PSP and limiting H_2O_2 production; ii) prevented from being converted to an insoluble metabolite (2,8-dihydroxyadenine) by allopurinol, iii) requires only one enzyme to be converted into a usable adenine nucleotide (AMP), whereas hypoxanthine requires three, and iv) adenine is already in use in man in

blood transfusion products where it promotes the synthesis and maintenance of ATP in erythrocytes via the purine salvage pathway. Adenine is readily transported into brain via SLC29 equilibrative nucleoside transporters. A fuller account can be found in[60].

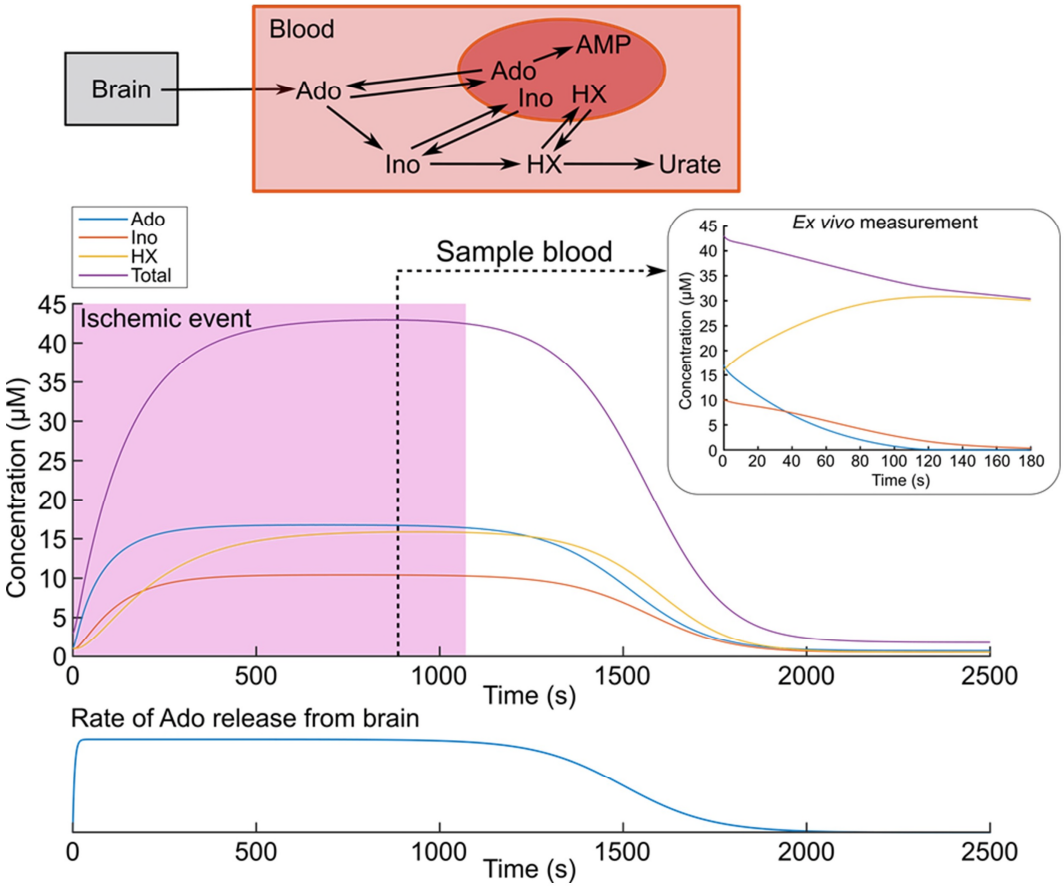

Figure 3. Simplified model of purine fluxes from brain and in blood following a stroke. The rate of release of adenosine during the "stroke" is modelled with fast onset, and slower recovery. The main graph shows the concentrations of adenosine, inosine and hypoxanthine in plasma calculated according to the scheme in the diagram, using Michaelis Menten kinetics for the enzymatic steps, and transport reactions with the K_m and V_{max} for each step drawn where possible from literature sources[55, 100] and databases (https://www.brenda-enzymes.org). As can be seen all purines are elevated in blood during the stroke and then fall back to baseline when the event resolves. The inset graph shows what happens when blood is removed from the patient for an *ex vivo* measurement (e.g. in a point of care device) that takes 3 minutes to give a result. The cycling reactions are the same *ex vivo* as *in vivo* except for the loss of the sources of adenosine from the ischemic brain, a low steady state release of adenosine into the circulation, and a reduction of xanthine oxidase activity by 67%, to recognise that the liver is a major site of urate production. Within the 3 minutes required for measurement, almost

all the adenosine in plasma disappears and is present as either inosine, or hypoxanthine. Thus, a point of care device must be capable of measuring at least hypoxanthine, and if the measurement time can be shortened to less than 60 s, all three purines to capture the full signal. Even when measuring hypoxanthine, the measured value will be only about 70% of the systemic value *in vivo* if the measurement duration takes 3 minutes.

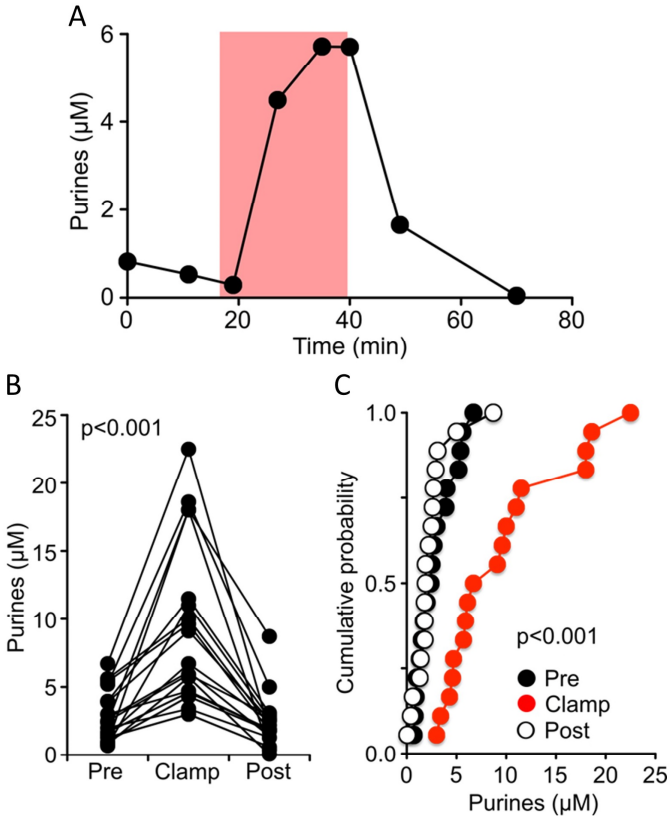

Figure 4. Measurement of purines in arterial blood of patients undergoing carotid endarterectomy. A) Purine measurements from a single patient before, during (shaded pink box) and after carotid cross clamping. B, C) Summary data from 18 patients demonstrating that purines are elevated during the cross clamping compared to the pre-clamp baseline and post-clamp recovery. Figure adapted from[66].

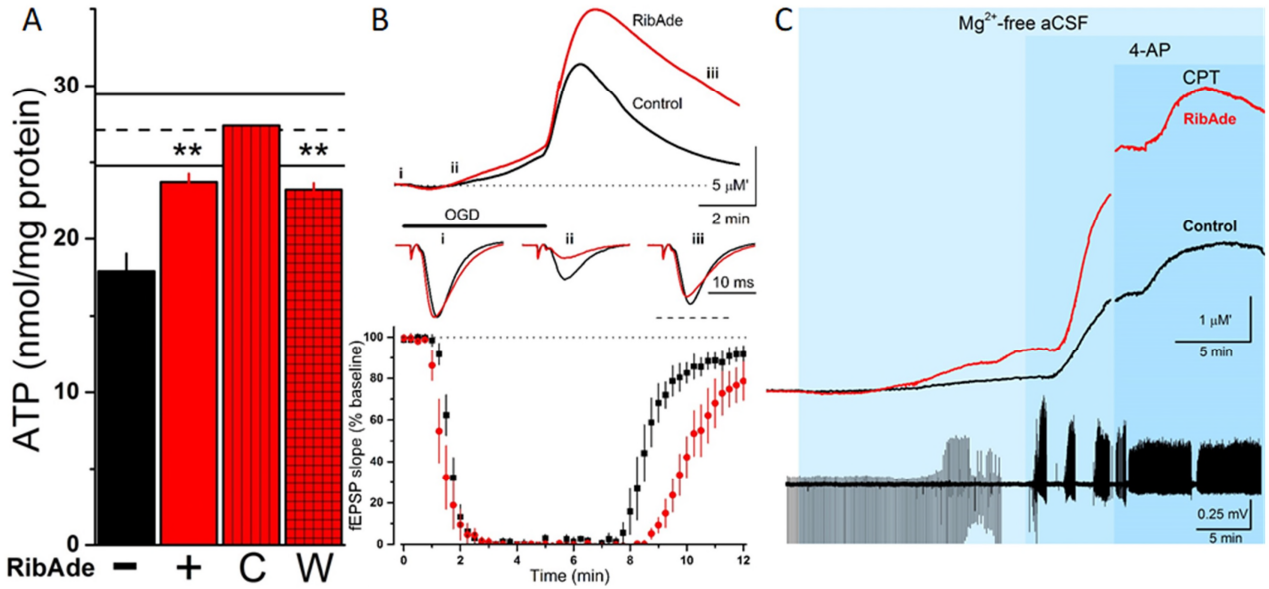

Figure 5. Ribose and adenine increase both ischemic brain tissue ATP levels and the releasable pool of adenosine. A) In the presence (+) of RibAde ATP levels are increased above levels in control *in vitro* hippocampal brain slices (-) and persist upon washout (***W***; 2 hr) of RibAde from the incubation medium. When ATP levels are corrected for the dead surfaces of brain slices (***C***), slice ATP is within the range reported *in vivo* (mean, dashed line; solid lines ± 1 SD). ** p< 0.01 sig diff from control slices. B) RibAde-treated brain slices (red adenosine sensor trace) release more adenosine during and after oxygen/glucose deprivation (OGD; black bar), an *in vitro* model of stroke, compared to control slices (black trace) and results in faster depression and slower recovery of the simultaneously-recorded electrically-evoked synaptic responses (field excitatory postsynaptic potentials; fEPSPs) (lower graph). Inset traces show fEPSPs taken before (i), during (ii) and after (iii) OGD and are normalised to control (i) amplitude. Dashed line under (iii) represents baseline amplitude. C) RibAde results in greater adenosine release (red trace) in an *in vitro* model of seizure activity (representative seizure in lower black trace). Removal of Mg^{2+} increases neuronal excitability by alleviating the Mg^{2+} block of the glutamate NMDA receptor; 4-AP is a K^+ channel blocker that promotes neuronal depolarisation and action potential firing, characterised by periodic bursts of seizure activity; CPT is an adenosine A_1 receptor antagonist that removes the tonic inhibitory and anti-epileptic tone exerted by adenosine and causes the slice to exhibit the *in vitro* equivalent of status

epilepticus (continuous neuronal firing). In B and C μM' indicates that the adenosine sensor also detects adenosine metabolites such as inosine. Data taken from[86, 89, 90], respectively.

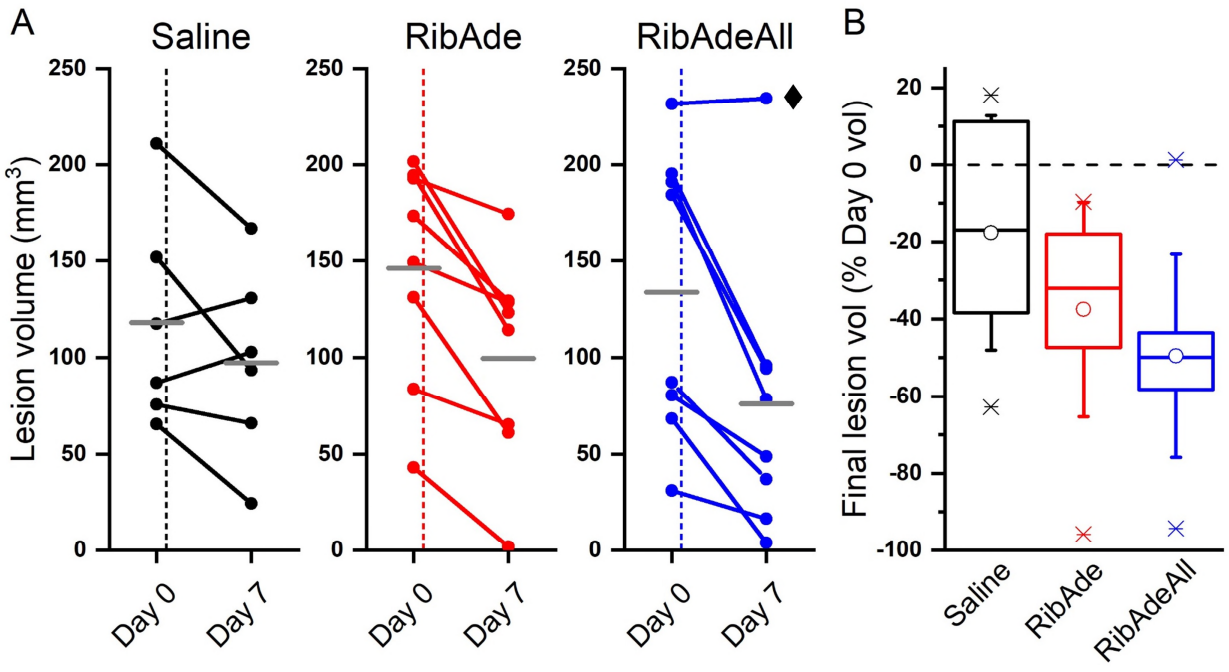

Figure 6. Strong tendency to greater reduction of lesion volume by RibAde and RibAdeAll. A) Individual paired measurements of lesion volume at Day 0 (DWI) and Day 7 (T2-weighted imaging) in the three experimental groups of animals showing a consistent trend for substantial lesion shrinkage in the RibAde (red; n = 8) and RibAdeAll (blue; n = 8) groups compared to saline controls (red; n = 6). Mean value per day are shown by horizontal grey bars. Vertical broken lines indicate timing of treatment, which occurred after DWI measurements were made. B) Box & whisker plot of change in lesion volume between Days 0 and 7 normalized to lesion size on Day 0. Note substantial reductions in mean lesion size in the RibAde (red circle; -38%) and RibAdeAll (blue circle; -50 %) groups compared to saline-treated animals (black circle -18%). Box: 1st and 3rd quartile; whiskers: ±1 SD; horizontal bar: median; crosses: lowest and highest values. P = 0.065 between untreated and treated groups. Note this statistical comparison includes the one RibAdeAll animal that had the largest lesion at Day 0, and, in contrast to all the other treated animals, showed no reduction in lesion volume (♦). Omitting this one data point gives p = 0.035 between treated and untreated animals. Data from[91].

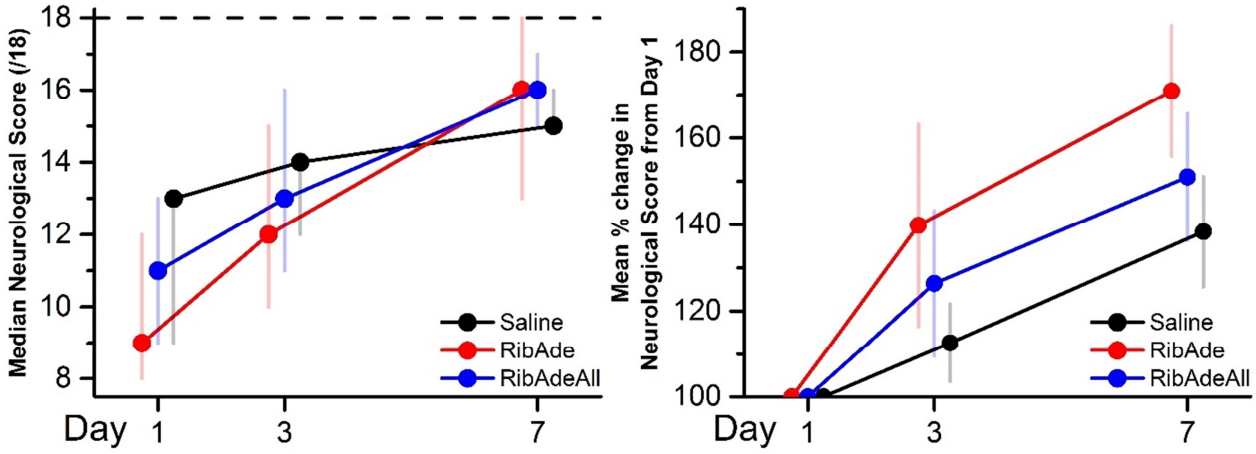

Figure 7. RibAde and RibAdeAll accelerate neurological recovery after stroke. A) Median neurological scores. for each group at Days 1, 3 and 7 after MCAO. Vertical lines indicate the first and third quartiles for each data point. Median and quartile values are rounded to nearest whole number. Coincidentally, treated animals (red and blue) had worse scores on Day 1, likely due to larger lesion volumes *before* drug infusion (Figure 6), but improved by day 7. Dashed line; score for normal animals (18). B) When normalised to Day 1 the neurological score for treated animals showed a trend towards accelerated recovery after the stroke. Data shown as mean ± SEM. In A and B data points for each group have been offset for clarity. Data from[91].

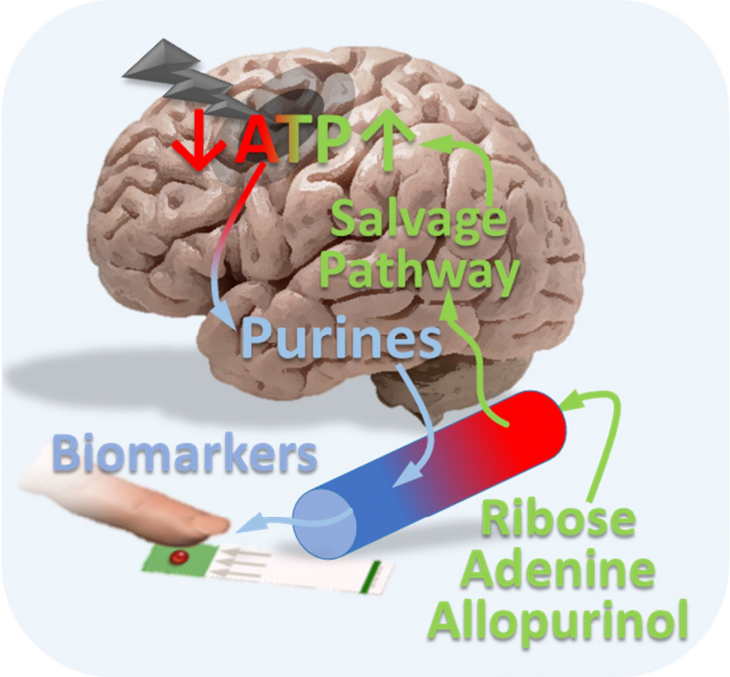

Figure 8. Inter-relationship between purines as diagnostics and as therapies for brain injury. Acute brain injury results in the metabolism of ATP and the production of purine nucleosides (adenosine and inosine) and nucleobases (hypoxanthine). These compounds are lost from the brain into the blood stream where they can act as biomarkers for cerebral ischemia. Provision of purine salvage pathway precursors (ribose and adenine), together with using a xanthine oxidase inhibitor such as allopurinol to prevent the metabolism of salvage substrate hypoxanthine to unsalvageable xanthine, elevates cerebral ATP and increases the reservoir for neuroprotective adenosine.

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