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Investigation of the novel mTOR inhibitor AZD2014 in neuronal ischemia**Gina Hadley¹, Daniel J Beard¹, Zoi Alexopoulou², Brad A Sutherland^{1,3*}, Alastair M Buchan^{1,4**}.**¹Acute Stroke Programme, Radcliffe Department of Medicine, University of Oxford, Oxford, United Kingdom²Nuffield Department of Clinical Neurosciences, University of Oxford, Oxford, United Kingdom³School of Medicine, College of Health and Medicine, University of Tasmania, Hobart, Australia⁴Acute Vascular Imaging Centre, University of Oxford, Oxford University Hospitals, Oxford, United Kingdom

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Highlights:

Inhibiting mTOR via hamartin is endogenously neuroprotective through productive autophagy

AZD2014, a dual mTORC1/2 inhibitor is detrimental to ischemic neurons

AZD2014 reduces hamartin expression preventing neuroprotective effects

The mechanism for endogenous neuroprotection may relate to the differential effects of mTORC1 and mTORC2

Abstract

Introduction: Hamartin, a component of the tuberous sclerosis complex (TSC) that actively inhibits the mammalian target of rapamycin (mTOR), may mediate the endogenous resistance of Cornu Ammonis 3 (CA3) hippocampal neurons following global cerebral ischemia. Pharmacological compounds that selectively inhibit mTOR may afford neuroprotection following ischemic stroke. We hypothesize that AZD2014, a novel mTORC1/2 inhibitor, may protect neurons following oxygen and glucose deprivation (OGD).

Methods: Primary neuronal cultures from E18 Wistar rat embryos were exposed to 2h OGD or normoxia. AZD2014 was administered either during OGD, 24h prior to OGD or for 24h following OGD. Cell death was quantified by lactate dehydrogenase assay. Western blotting characterized the expression of mTOR pathway proteins following exposure to AZD2014.

Results: Following 2h OGD + 24h recovery, AZD2014 increased neuronal death when present during OGD. Rapamycin, the archetypal mTOR inhibitor, had no effect on cell death. Treatment with AZD2014 24h prior to OGD and 24h after OGD also enhanced cell death. While Western blotting showed a trend towards decreased expression levels of phospho-Akt relative to total Akt with increasing AZD2014 concentration, hamartin expression was also significantly decreased leading to activation of mTOR.

Conclusion: AZD2014 was detrimental to neurons that underwent ischemia. AZD2014 appeared to reduce hamartin, a known neuroprotective mediator, thereby preventing any beneficial effects of mTOR inhibition. Further characterization of the role of individual mTOR complexes (mTORC1 and mTORC2) and their upstream and downstream regulators are necessary to reveal whether these pathways are neuroprotective targets for stroke.

Key words:

AZD2014; hamartin; mTORC1; mTORC2; rapamycin

Abbreviations: 4E-BP1: Eukaryotic translation initiation factor 4E-binding protein 1; AKT: protein kinase B; ATF4: Activating transcription factor 4 (also known as CREB: cyclic AMP-responsive element-binding protein); CA3: Cornu Ammonis 3; eIF4: Eukaryotic translation initiation factor 4E; ER (endoplasmic reticulum) stress; IGF1: Insulin growth factor 1; IRS: Insulin receptor substrate; GDP: guanosine diphosphate; Insulin receptor substrate; mLST8 (mammalian lethal with sec-13 protein 8, also known as G-protein β subunit-like protein or G β L); mTOR: mammalian target of rapamycin; OGD: oxygen glucose deprivation; PRAS40: proline-rich AKT substrate 40kDa; Phosphatidylinositol 3-kinase/protein kinase B; Rheb: Ras homolog enriched in brain; S6K1: Raptor: regulatory associated protein of mTOR; Rictor: rapamycin-insensitive companion of mTOR; ribosomal protein S6 kinase beta-1 (also known as p70S6 kinase (p70S6K, p70-S6K)); sin: stress-activated protein kinase-interacting protein; TSC1 tuberous sclerosis complex 1; TSC2: tuberous sclerosis complex 2; Ulk1: mammalian autophagy-initiating kinase, a homologue of yeast ATG (AuTophagy related) 1.

1. Introduction

Thrombolysis [27] and thrombectomy [2] are the only current treatments in the UK and USA for acute ischemic stroke, restoring blood flow to the ischemic brain area. Any potential effective neuroprotective treatments would increase the time window for reperfusion and minimize the adverse effects of these treatments. One endogenous neuroprotective mediator could be hamartin, which has been shown to mediate the endogenous resistance of Cornu Ammonis 3 (CA3) neurons to ischemia [28]. Hamartin (TSC1) [33] and tuberlin (TSC2) [10] exist together in a structure called the tuberous sclerosis complex (TSC) [34]. TSC is a tumour suppressor [20], acting to inhibit the mammalian target of rapamycin (mTOR) [18].

mTOR is a serine/threonine kinase that regulates the growth and protein synthesis of mammalian cells. mTOR is a central signalling node that is implicated in cellular transcription, ubiquitin-dependent proteolysis, autophagy, endoplasmic reticulum (ER) stress, membrane trafficking and cytoskeletal dynamics [9, 19, 22, 32]. There are two known mTOR complexes, mTOR complex 1 (mTORC1) and complex 2 (mTORC2). The major constitutive actions of mTORC1 are to augment protein synthesis, through the activation of ribosomal protein S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) [14, 19], and inhibit autophagy [19]. However, the major cellular roles of mTORC2 are to maintain cytoskeletal integrity, cell proliferation and survival [1, 23]. While TSC inhibits mTORC1, TSC can promote mTORC2 activity through physically associating with it [15].

Hamartin's promotion of productive autophagy via mTOR pathway inhibition has recently been shown to play an integral role in endogenous neuroprotection following global cerebral ischemia [28]. Inhibiting mTOR with selective inhibitors may afford neuroprotection following ischemic stroke through inhibition of ER stress and induction of autophagy. Rapamycin is the archetypal mTOR inhibitor, it predominantly targets mTORC1, but can affect mTORC2 after prolonged treatment [15]. Experiments with rapamycin have shown both beneficial [6] and detrimental [7] effects in models of stroke, with a recent meta-analysis showing an overall neuroprotective effect of rapamycin [3].

AZD2014 is a novel mTORC1/mTORC2 inhibitor [29] and is currently in clinical trials for a wide variety of cancers. It has shown significant anti-tumour effects [17, 24] which does not appear to be due to induction of apoptosis. The blockade of mTORC2 by AZD2014 enhances its anti-tumour effects by preventing the feedback activation of Akt

[24]. We hypothesize that AZD2014 could produce neuroprotection following acute cerebral ischemia through mTOR and Akt inhibition. We have used the gold standard *in vitro* model of ischemic stroke, oxygen and glucose deprivation (OGD), to determine the direct neuroprotective capacity of AZD2014 on primary neuronal cultures. This model induces oxidative and metabolic stress in addition to excitotoxicity and autoinflammatory responses, mimicking the environment neurons are exposed to in ischemic stroke. AZD2014 has never been tested in the context of cerebral ischemia and rapamycin, the archetypal mTORC1 inhibitor, will be used for comparison.

Methods

2.1 Cell Culture

Primary neuronal cultures were used to determine pharmacological effects on cell viability following OGD. Hippocampal and cortical neuronal cultures were prepared from E18 rat embryos as previously described [35] and plated in complete neurobasal media at 10^5 cells per well in poly-d-lysine coated 12-well plates.

2.2 Oxygen glucose deprivation

On day 7-8 *in vitro*, neuronal cultures were exposed to 2h normoxic or OGD conditions. For OGD, cultures were placed into a hypoxic chamber (Coy Laboratory Products, USA) maintained at 0% O₂ and immersed in a glucose-free neurobasal media (Neurobasal A, Life Technologies, UK). For normoxia, cultures were placed in a standard incubator maintained at 21% O₂ and immersed in neurobasal media containing 25mM d-glucose. Following 2h of normoxia or OGD, all cultures were recovered with fresh complete neurobasal media for 24 h at 21% O₂ at 37°C.

2.3 Pharmacology

A 10mM stock solution of AZD2014 (donated by Astra Zeneca) was prepared in DMSO. Final concentrations ranging from 1nM - 10μM were used and were diluted in media from the stock solution. For comparison with AZD2014, rapamycin (Sigma) stock solution (2.7μM from the supplied stock solution of 2.7mM) was prepared in DMSO. Final concentrations within 10nM and 100nM were used for all experiments.

2.4 Immunofluorescence

To confirm neuronal phenotype, cultures underwent immunocytochemistry. Neurons were fixed in 4% paraformaldehyde, washed, blocked and exposed to rabbit anti-NeuN antibody (1:300, Abcam, UK) overnight. After washing, donkey anti-rabbit AlexaFluor 488 (1:200) was added and cells were counterstained with DAPI. Neurons were imaged using a Zeiss LSM 700 with a 20× water-immersion lens and appropriate excitation/emission settings.

2.5 Cell death assays

Cell death in primary neuronal cultures was assessed quantitatively using the lactate dehydrogenase release assay (CytoTox 96, Promega). Media was collected at the end of 2 h and 24 h recovery periods for LDH analysis. Total LDH released was determined by lysing cells with neurobasal media containing 1% Triton X-100. Cell death was determined as a percentage of LDH released in the medium to the total LDH content measured in the same

cultures. All n numbers represent biological replicates and there are up to 8 technical replicates per biological replicate.

To assess basal neuronal viability following 48h treatment with AZD2014 (10^{-5} – 10^{-9} M), the CellTiter 96 assay (Promega) was used, with neurons being incubated for 5h with the reaction reagent.

2.6 Western Blotting

At 24 hours recovery, neuronal cultures were lysed in cell lysis buffer through cell scraping, incubated at 4°C for 30mins and centrifuged at 4°C for 30mins at 11700 g. The supernatant was collected and underwent Western blotting. Protein concentration (mg/mL) of cell lysates were determined using a DC (Biorad, UK) or bicinchoninic acid (BCA) assay. Lysates were prepared for gel electrophoresis by precipitation with methanol/chloroform, dilution in Laemmli sample buffer (Biorad, UK) and 10% 100mM dithiothreitol (DTT) and protein denaturation by heating to 95°C for 5 minutes. 25µg protein was loaded onto 7.5% or 15% Criterion Tris-HCl precast gels (Biorad) and underwent electrophoresis (110V for 2h). The gel was then transferred to a PVDF membrane (100V for 30mins with a cold pack). To observe equivalent sample loading across wells within a gel, protein quantification was carried out using SYPRO® Ruby reagent (Life technologies). The membrane was then blocked (5% w/v milk powder in phosphate buffered saline (PBS) for one hour at room temperature) and incubated overnight with the primary antibody at the required concentration diluted in 5% milk in PBS. Primary antibodies used were: 1:1000 rabbit anti-hamartin (ab25882, Abcam, UK), 1:1000 rabbit anti-tuberin (ab32554, Abcam, UK), 1:1000 rabbit anti-mTOR (2983 Cell Signaling Technology, USA), 1:1000 rabbit anti-phospho-mTOR (Ser2448, Cell Signaling Technology, USA), 1:1000 rabbit anti-phospho-Akt (S473, Cell signalling) and 1:1000 rabbit anti-Akt (Cell signalling). The membrane was then washed (3x 5 min in 0.1% Tween 20 in PBS (PBST)) and incubated with the secondary antibody (1:2000, goat-anti-rabbit IgG horseradish peroxidase (HRP) (Abcam (UK)) diluted in 5% w/v milk powder in PBS) for 1 hour at room temperature. After a further five 3 min washes in PBST, the membrane was then developed using enhanced chemiluminescence (Lumigen) and imaged using either a Biospectrum AC imaging system (UVP) or a Biorad ChemiDoc™ MP imaging system. Band optical density was quantified (VisionWorks Analysis Software, UVP or Biorad Image Lab software v6.0.1) and corrected for protein loading using a six band analysis of the SYPRO® staining [13]. Membranes were stripped using Blot Restore Solutions A and B (Millipore) to enable them to be probed for more than one antibody. Levels of activated mTOR are calculated by dividing phospho-mTOR

expression levels by total mTOR expression levels [38]. The activation of Akt was also calculated similarly, by dividing phospho-Akt by total Akt expression levels.

2.7 Statistical analyses

Statistical analyses to determine whether AZD2014 treatment affected cell death or protein expression were carried out with GraphPad Prism 5 using a two-tailed Student's *t*-test if two groups were compared or one-way ANOVA with Bonferroni's or Dunnett's multiple comparisons *post hoc* test for comparisons of more than two groups. For experiments where two independent variables were assessed such as OGD and treatment (Fig. 2 and 3), a two-way ANOVA with Tukey's multiple comparison *post hoc* test was used. Differences were considered significant for $p < 0.05$. Data are presented as mean \pm SEM.

3. Results

3.1 Effect of AZD2014 on neuronal cell viability

To show that cell cultures were primary cortical neurons, immunocytochemistry was performed using the canonical neuronal marker NeuN. Fig. 1A demonstrates that these cells expressed NeuN demonstrating these cultures were indeed neurons. AZD2014, at concentrations ranging from 10^{-9} – 10^{-5} M, was incubated with primary cortical neurons under standard conditions for 48h and cell viability was assessed. As AZD2014 concentration increased, neuronal viability decreased (Fig. 1B) particularly at concentrations in the μ M range (<80% viability). DMSO had minimal effect on neuronal viability.

3.2 Treatment with AZD2014 during 2 hours OGD is neurotoxic in cortical and hippocampal neurons

Cell death of cortical and hippocampal neurons following treatment with AZD2014, a dual mTORC1/2 inhibitor, was compared with rapamycin, an mTORC1 inhibitor which only inhibits mTORC2 at high concentrations, under normoxic or OGD conditions. At 2 hours of treatment in cortical neurons (Fig. 2A), there was a significant effect of OGD (Two-way ANOVA: $F(1,36)=235.1$, $p<0.0001$) and treatment ($F(5,36)=10.6$, $p<0.0001$) on cell death, while Tukey's post hoc testing demonstrated a highly significant increased levels of cell death ($p<0.0001$) with 100nM AZD2014 and 1 μ M AZD2014 when compared with media, DMSO and rapamycin under OGD conditions. After 2 hours of OGD or normoxia followed by 24 hours recovery (Fig. 2B), there was a significant effect of OGD (Two-way ANOVA: $F(1,36)=643.9$, $p<0.0001$) and treatment ($F(5,36)=10.7$, $p<0.0001$) on cell death. Post hoc testing demonstrated a highly significant increase in levels of cell death (Tukey's: $p<0.0001$) with 100nM AZD2014 and 1 μ M AZD2014 when compared with media, DMSO and rapamycin under OGD conditions. There was a significant interaction between the effect of OGD and treatment on cell death at 2 hours (Two-way ANOVA: $F(5,36)=10.6$, $p<0.0001$) and 2 hours and 24 hours recovery ($F(5,36)=4.9$, $p<0.01$). Similar results showing AZD2014 neurotoxicity under OGD conditions were also observed in hippocampal neurons (Figs. 2C and 2D).

3.3 Pre-treatment and Post-treatment with AZD2014 is neurotoxic in cortical neurons

Cell death was assessed in cortical cultures that had undergone 2 hours of normoxia or OGD and 24 hours recovery that had been exposed to 24 hours of pretreatment with media, DMSO, 100nM AZD2014 or 1 μ M AZD2014. At 2 hours (Fig. 3A), there was a significant effect of OGD (Two-way ANOVA: $F(1,16)=19.2$, $p=0.0005$)

but no significant effect of treatment ($F(3,16)=0.96$, $p=0.4346$). At 2 hours and 24 hours recovery (Fig. 3B), there was a significant effect of OGD (Two-way ANOVA: $F(1,16)=300.3$, $p<0.0001$) and treatment ($F(3,16)=12.4$, $p=0.0002$) on cell death as well as a significant interaction between OGD and treatment ($F(3,16)=9.3$, $p<0.001$). Post hoc testing (Tukey's) demonstrated that 1 μ M AZD2014 produced significantly more cell death than media ($p<0.0001$), DMSO ($p=0.0002$) and 100nM AZD2014 ($p=0.0002$) in the cells that had undergone OGD. This is in contrast to cortical cultures pre-treated with media, DMSO or 10 nM rapamycin where there was no effect of treatment at 2 hours OGD (Fig. 3C) (Two-way ANOVA: $F(2,17)=1.43$, $p=0.2657$) nor following 2 hours OGD and 24 hours recovery (Fig. 3D) ($F(2,18)=1.04$, $p=0.3727$) despite an effect of OGD (2 hours OGD: $F(1,17)=34.7$, $p<0.0001$; 2 hours OGD + 24 hours recovery: $F(1,18)=101.5$, $p<0.0001$).

Following 2 h OGD or normoxia, cortical neurons were recovered for 24 h in the presence of media alone, or media containing DMSO, 100nM AZD2014 or 1 μ M AZD2014 (Fig. 3E). There was a significant effect of OGD (Two-way ANOVA: $F(1,28)=45.2$, $p<0.0001$) and treatment ($F(2,28)=24.8$, $p<0.0001$) on cell death. Post hoc testing (Tukey's) demonstrated in cells that underwent normoxia for 2 hours, 1 μ M AZD2014 caused significantly more cell death during the recovery period compared to media ($p=0.0047$), DMSO ($p=0.0006$) and 100nM AZD2014 ($p=0.0092$). In cells that had undergone OGD conditions for 2 hours, 100nM AZD2014 caused significantly more cell death during the recovery period compared to media (Tukey's, $p=0.0078$), while 1 μ M AZD2014 caused significantly more cell death compared to media ($p<0.0001$), DMSO ($p<0.0001$) and 100nM AZD2014 ($p=0.0083$). Similar results showing AZD2014 neurotoxicity during the recovery period following either normoxia or OGD were also observed in hippocampal neurons (Fig. 3F).

3.4 Expression levels of mTOR pathway proteins with AZD2014 treatment

In order to determine how AZD2014 affects cortical neuron protein expression within the mTOR pathway under normoxic conditions, Western blotting was used to ascertain expression levels of tuberlin (Fig 4A), hamartin (Fig. 4A), mTOR (Fig. 4B), phosmTOR (Fig. 4B), Akt (Fig. 4C) and phospho-Akt (Fig. 4C). There was no significant effect of AZD2014 on tuberlin expression (One-way ANOVA: $F(6,17)=1.3$, $p=0.3082$). However, there was a significant decrease in hamartin expression levels (One-way ANOVA: $F(6,17)=3.8$, $p=0.0145$) with increasing AZD2014 concentration, with 100nM AZD2014 providing the greatest inhibitory effect (Bonferroni, $p=0.0303$).

mTOR activity is calculated by dividing phospho-mTOR expression levels by total mTOR expression levels [38]. Fig. 4B shows that there was a significant increase in activated mTOR with increasing concentrations of AZD2014 (One-way ANOVA: $F(6,16)=6.146$, $p=0.0017$). Upstream of mTOR, there was a trend towards decreased expression levels of phospho-Akt relative to total Akt at the higher concentrations of AZD (Fig. 4C; One-way ANOVA: $F(3,8)=2.602$, $p=0.1243$) with the reduction in phospho-Akt expression by 10 μ M AZD2014 approaching significance ($p=0.07$).

4. Discussion

This study explored whether AZD2014, a purported dual mTORC1 and mTORC2 inhibitor, could be beneficial in ischemic stroke. Primary neuronal viability studies demonstrated a dose dependent increase in neuronal death following 48 h exposure to AZD2014 but there remained >70% neuronal viability with all concentrations tested. Using the gold standard *in vitro* model of ischemic stroke (OGD), AZD2014 treatment during OGD augmented neuronal death. When AZD2014 was administered prior to or after OGD, AZD2014 neurotoxicity remained evident but only at high concentrations. This suggests that a higher concentration of AZD is required to exert cell death under nutrient-replete conditions (normoxia) compared to nutrient-deplete conditions (OGD). This was in contrast to rapamycin, the archetypal mTOR inhibitor, which was not associated with increased neuronal death. Interestingly, both cortical and hippocampal neurons underwent greater cell death in the presence of AZD2014 during and after OGD. To investigate how AZD2014 affects the mTOR pathway in neurons, we showed that AZD2014 tended to reduce expression levels of phospho-Akt relative to total Akt in addition to significantly reducing hamartin expression whilst activating mTOR.

Both *in vivo* and *in vitro* evidence suggest that mTOR plays a central role in neuronal degeneration following cerebral ischemia [8]. The upregulation of TSC or the inhibition of mTOR could limit energy consumption by restricting protein synthesis, limiting ER stress and promoting protein recycling (autophagy). Studies have shown that inhibition of mTOR with agents such as rapamycin are neuroprotective against cerebral ischemia [4, 6], can maintain blood brain barrier integrity [12] and improve cerebral blood flow [25]. However, other studies have shown that rapamycin can exacerbate injury following ischemic stroke by limiting oxygen supply and consumption during reperfusion [7]. A recent meta-analysis showed that after taking into account all of the published evidence in experimental stroke models, rapamycin had neuroprotective effects by improving neurobehavior and reducing infarct volume [3]. In our studies, rapamycin had no impact upon neuronal death during OGD or recovery, whereas AZD2014 was neurotoxic suggesting that there is a difference in the mechanisms of action on neurons between the two drugs.

In order to assess the mechanisms underlying the neurotoxicity of AZD2014, we evaluated the neuronal expression of proteins associated with the mTOR pathway after exposure to AZD2014. High doses of AZD2014 decreased hamartin expression. Hamartin has been shown to be a neuroprotective protein [28] and here the decrease in

hamartin with AZD2014 may be associated with cell death following OGD. Hamartin acts upstream of mTOR, and inhibition of hamartin by AZD2014 would further activate mTOR, which was supported by enhanced phosphorylated mTOR expression (active conformation) as a ratio of total mTOR with increasing AZD2014 concentrations. This is contrary to the proposed mTOR inhibitory properties of AZD2014 [29], however AZD2014 may be modulating the negative feedback loop that can control mTOR activity. When mTORC1 is active, the phosphorylation of S6K1 inhibits Akt signalling which allows active TSC to prevent further mTORC1 activity (Fig. 5). However, when mTORC1 inhibitors such as rapamycin are used, feedback activation of Akt signalling can occur which could lead to mTOR activity [36]. One possible mechanism of AZD could be that it causes feedback activation of Akt signalling which inhibits hamartin leading to further mTOR activation. However, AZD2014 is also an mTORC2 inhibitor and mTORC2 is known to negate the negative feedback loop where active mTORC1 prevents further mTORC1 activation through blockade of Akt [11, 36]. Therefore, the action of AZD2014 on mTORC2 would lead to the inhibition of hamartin and the activation of mTOR through modulation of the Akt negative feedback loop. In our study, increasing concentrations of AZD2014 led to independent yet opposite expression of phosphorylated mTOR (increase) and a trend towards decreased expression levels of phosphorylated Akt relative to total Akt. A recent study has shown that in HEK293 cells exposed to glucose deprivation, expression of downstream mTOR proteins and phospho-Akt can vary independently [30]. With increasing hours of glucose deprivation (up to 54 hours and no pharmacological intervention), there is a decrease in phosphorylated 4E-BP1 (indicative of decreased phosphorylated and therefore activated mTOR), yet there is an increase in phospho-Akt expression [30]. The paradoxical finding of decreased hamartin expression could be explained by decrease in phospho-Akt resulting in a decrease in phosphorylated tuberlin and a disruption of the TSC complex subsequent degradation. Taken together these data indicate the loss of a pro-survival pathway (decreased hamartin expression) and the hyperactivation of a detrimental pathway (increase in phosphorylated mTOR) with potentially differing effects of individual cellular stressors.

mTORC2 appears to mediate vital neuronal functions [16] and has been implicated in cell survival [26]. Rictor (Rapamycin-Insensitive Companion of mTOR) is believed to regulate the actin cytoskeleton [16] being highly expressed in neurons and with its deletion resulting in smaller brains and neurons [5, 31]. The direct inhibition of mTORC2 by AZD2014 could prevent critical neuronal functions as well as cytoskeletal remodelling which are essential for survival after ischemia. While TSC inhibits mTORC1, it also activates mTORC2 [15]. Therefore, direct

AZD2014-induced reduction in hamartin expression could lead to an overactivation of mTORC1 and subsequent ER stress but also inhibition of mTORC2 could prevent the neuroprotective actions of its downstream mediators.

Conclusion: This study showed that AZD2014 was damaging to neurons that had undergone ischemia. The main mechanism for this cytotoxic effect is likely to be due to decreased hamartin expression leading to a potential overactivation of mTORC1. Hamartin's neuroprotective effect may not be solely due to induction of autophagy via mTORC1 inhibition but it may also enhance lifesaving cytoskeletal functions via mTORC2, which AZD2014 may detrimentally affect. Taken together, these findings provide further insight into the neuroprotective properties of the hamartin/mTOR pathway and how modulating this can influence neuronal survival following ischemic stroke.

Conflict of Interest

AMB is a senior medical science advisor and co-founder of Brainomix, a company that develops electronic ASPECTS (e-ASPECTS), an automated method to evaluate ASPECTS in stroke patients. All other authors declare no conflict of interest.

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References

- [1] N. Angliker, M.A. Ruegg, In vivo evidence for mTORC2-mediated actin cytoskeleton rearrangement in neurons, *Bioarchitecture* 3 (2013) 113-118.
- [2] J.S. Balami, B.A. Sutherland, L.D. Edmunds, I.Q. Grunwald, A.A. Neuhaus, G. Hadley, H. Karbalai, K.A. Metcalf, G.C. DeLuca, A.M. Buchan, A systematic review and meta-analysis of randomized controlled trials of endovascular thrombectomy compared with best medical treatment for acute ischemic stroke, *Int J Stroke* 10 (2015) 1168-1178.
- [3] D.J. Beard, G. Hadley, N. Thurley, D.W. Howells, B.A. Sutherland, A.M. Buchan, The effect of rapamycin treatment on cerebral ischemia: A systematic review and meta-analysis of animal model studies, *Int J Stroke* (2018) 1747493018816503.
- [4] K.M. Buckley, D.L. Hess, I.Y. Sazonova, S. Periyasamy-Thandavan, J.R. Barrett, R. Kirks, H. Grace, G. Kondrikova, M.H. Johnson, D.C. Hess, P.V. Schoenlein, M.N. Hoda, W.D. Hill, Rapamycin up-regulation of autophagy reduces infarct size and improves outcomes in both permanent MCAL, and embolic MCAO, murine models of stroke, *Exp Transl Stroke Med* 6 (2014) 8.
- [5] R.P. Carson, C. Fu, P. Winzenburger, K.C. Ess, Deletion of Rictor in neural progenitor cells reveals contributions of mTORC2 signaling to tuberous sclerosis complex, *Hum Mol Genet* 22 (2013) 140-152.
- [6] A. Chauhan, U. Sharma, N.R. Jagannathan, K.H. Reeta, Y.K. Gupta, Rapamycin protects against middle cerebral artery occlusion induced focal cerebral ischemia in rats, *Behav Brain Res* 225 (2011) 603-609.
- [7] O.Z. Chi, S. Barsoum, N.M. Vega-Cotto, E. Jacinto, X. Liu, S.J. Mellender, H.R. Weiss, Effects of rapamycin on cerebral oxygen supply and consumption during reperfusion after cerebral ischemia, *Neuroscience* 316 (2016) 321-327.
- [8] Z.Z. Chong, Q. Yao, H.H. Li, The rationale of targeting mammalian target of rapamycin for ischemic stroke, *Cellular signalling* 25 (2013) 1598-1607.
- [9] A. Di Nardo, I. Kramvis, N. Cho, A. Sadowski, L. Meikle, D.J. Kwiatkowski, M. Sahin, Tuberous sclerosis complex activity is required to control neuronal stress responses in an mTOR-dependent manner, *J Neurosci* 29 (2009) 5926-5937.
- [10] C. European Chromosome 16 Tuberous Sclerosis, Identification and characterization of the tuberous sclerosis gene on chromosome 16, *Cell* 75 (1993) 1305-1315.
- [11] D.A. Guertin, D.M. Sabatini, Defining the role of mTOR in cancer, *Cancer Cell* 12 (2007) 9-22.
- [12] W. Guo, G. Feng, Y. Miao, G. Liu, C. Xu, Rapamycin alleviates brain edema after focal cerebral ischemia reperfusion in rats, *Immunopharmacology and immunotoxicology* 36 (2014) 211-223.
- [13] G. Hadley, A.A. Neuhaus, Y. Couch, D.J. Beard, B.A. Adriaanse, K. Vekrellis, G.C. DeLuca, M. Papadakis, B.A. Sutherland, A.M. Buchan, The role of the endoplasmic reticulum stress response following cerebral ischemia, *Int J Stroke* 13 (2018) 379-390.
- [14] T.E. Harris, J.C. Lawrence, Jr., TOR signaling, *Sci STKE* 2003 (2003) re15.
- [15] J. Huang, B.D. Manning, The TSC1-TSC2 complex: a molecular switchboard controlling cell growth, *Biochem J* 412 (2008) 179-190.
- [16] W. Huang, P.J. Zhu, S. Zhang, H. Zhou, L. Stoica, M. Galiano, K. Krnjevic, G. Roman, M. Costa-Mattioli, mTORC2 controls actin polymerization required for consolidation of long-term memory, *Nat Neurosci* 16 (2013) 441-448.
- [17] H.Z. Huo, Z.Y. Zhou, B. Wang, J. Qin, W.Y. Liu, Y. Gu, Dramatic suppression of colorectal cancer cell growth by the dual mTORC1 and mTORC2 inhibitor AZD-2014, *Biochem Biophys Res Commun* 443 (2014) 406-412.
- [18] K. Inoki, T. Zhu, K.L. Guan, TSC2 mediates cellular energy response to control cell growth and survival, *Cell* 115 (2003) 577-590.
- [19] E. Jacinto, M.N. Hall, Tor signalling in bugs, brain and brawn, *Nat Rev Mol Cell Biol* 4 (2003) 117-126.
- [20] J. Jozwiak, Hamartin and tuberlin: working together for tumour suppression, *Int J Cancer* 118 (2006) 1-5.
- [21] J. Kahn, T.J. Hayman, M. Jamal, B.H. Rath, T. Kramp, K. Camphausen, P.J. Tofilon, The mTORC1/mTORC2 inhibitor AZD2014 enhances the radiosensitivity of glioblastoma stem-like cells, *Neuro Oncol* 16 (2014) 29-37.
- [22] R.F. Lamb, C. Roy, T.J. Diefenbach, H.V. Vinters, M.W. Johnson, D.G. Jay, A. Hall, The TSC1 tumour suppressor hamartin regulates cell adhesion through ERM proteins and the GTPase Rho, *Nat Cell Biol* 2 (2000) 281-287.

- [23] M. Laplante, D.M. Sabatini, mTOR signaling in growth control and disease, *Cell* 149 (2012) 274-293.
- [24] H. Liao, Y. Huang, B. Guo, B. Liang, X. Liu, H. Ou, C. Jiang, X. Li, D. Yang, Dramatic antitumor effects of the dual mTORC1 and mTORC2 inhibitor AZD2014 in hepatocellular carcinoma, *Am J Cancer Res* 5 (2015) 125-139.
- [25] A.L. Lin, W. Zheng, J.J. Halloran, R.R. Burbank, S.A. Hussong, M.J. Hart, M. Javors, Y.Y. Shih, E. Muir, R. Solano Fonseca, R. Strong, A.G. Richardson, J.D. Lechleiter, P.T. Fox, V. Galvan, Chronic rapamycin restores brain vascular integrity and function through NO synthase activation and improves memory in symptomatic mice modeling Alzheimer's disease, *J Cereb Blood Flow Metab* 33 (2013) 1412-1421.
- [26] K. Maiese, Cutting through the complexities of mTOR for the treatment of stroke, *Curr Neurovasc Res* 11 (2014) 177-186.
- [27] NINDS, Tissue plasminogen activator for acute ischemic stroke. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, *N Engl J Med* 333 (1995) 1581-1587.
- [28] M. Papadakis, G. Hadley, M. Xilouri, L.C. Hoyte, S. Nagel, M.M. McMenamin, G. Tsaknakis, S.M. Watt, C.W. Drakesmith, R. Chen, M.J. Wood, Z. Zhao, B. Kessler, K. Vekrellis, A.M. Buchan, Tsc1 (hamartin) confers neuroprotection against ischemia by inducing autophagy, *Nat Med* 19 (2013) 351-357.
- [29] K.G. Pike, K. Malagu, M.G. Hummersone, K.A. Menear, H.M. Duggan, S. Gomez, N.M. Martin, L. Ruston, S.L. Pass, M. Pass, Optimization of potent and selective dual mTORC1 and mTORC2 inhibitors: the discovery of AZD8055 and AZD2014, *Bioorg Med Chem Lett* 23 (2013) 1212-1216.
- [30] S. Shin, G.R. Buel, L. Wolgamott, D.R. Plas, J.M. Asara, J. Blenis, S.O. Yoon, ERK2 Mediates Metabolic Stress Response to Regulate Cell Fate, *Mol Cell* 59 (2015) 382-398.
- [31] C. Shiota, J.T. Woo, J. Lindner, K.D. Shelton, M.A. Magnuson, Multiallelic disruption of the rictor gene in mice reveals that mTOR complex 2 is essential for fetal growth and viability, *Dev Cell* 11 (2006) 583-589.
- [32] S.F. Tavazoie, V.A. Alvarez, D.A. Ridenour, D.J. Kwiatkowski, B.L. Sabatini, Regulation of neuronal morphology and function by the tumor suppressors Tsc1 and Tsc2, *Nat Neurosci* 8 (2005) 1727-1734.
- [33] M. van Slegtenhorst, R. de Hoogt, C. Hermans, M. Nellist, B. Janssen, S. Verhoef, D. Lindhout, A. van den Ouweland, D. Halley, J. Young, M. Burley, S. Jeremiah, K. Woodward, J. Nahmias, M. Fox, R. Ekong, J. Osborne, J. Wolfe, S. Povey, R.G. Snell, J.P. Cheadle, A.C. Jones, M. Tachataki, D. Ravine, J.R. Sampson, M.P. Reeve, P. Richardson, F. Wilmer, C. Munro, T.L. Hawkins, T. Sepp, J.B. Ali, S. Ward, A.J. Green, J.R. Yates, J. Kwiatkowska, E.P. Henske, M.P. Short, J.H. Haines, S. Jozwiak, D.J. Kwiatkowski, Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34, *Science* 277 (1997) 805-808.
- [34] M. van Slegtenhorst, M. Nellist, B. Nagelkerken, J. Cheadle, R. Snell, A. van den Ouweland, A. Reuser, J. Sampson, D. Halley, P. van der Sluijs, Interaction between hamartin and tuberlin, the TSC1 and TSC2 gene products, *Human molecular genetics* 7 (1998) 1053-1057.
- [35] T. Vogiatzi, M. Xilouri, K. Vekrellis, L. Stefanis, Wild type alpha-synuclein is degraded by chaperone-mediated autophagy and macroautophagy in neuronal cells, *J Biol Chem* 283 (2008) 23542-23556.
- [36] X. Wan, B. Harkavy, N. Shen, P. Grohar, L.J. Helman, Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism, *Oncogene* 26 (2007) 1932-1940.
- [37] Q. Xin, B. Ji, B. Cheng, C. Wang, H. Liu, X. Chen, J. Chen, B. Bai, Endoplasmic reticulum stress in cerebral ischemia, *Neurochem Int* 68 (2014) 18-27.
- [38] H. Yoshitomi, Q. Xu, M. Gao, Y. Yamori, Phosphorylated endothelial NOS Ser1177 via the PI3K/Akt pathway is depressed in the brain of stroke-prone spontaneously hypertensive rat, *J Stroke Cerebrovasc Dis* 20 (2011) 406-412.

Figure Legends

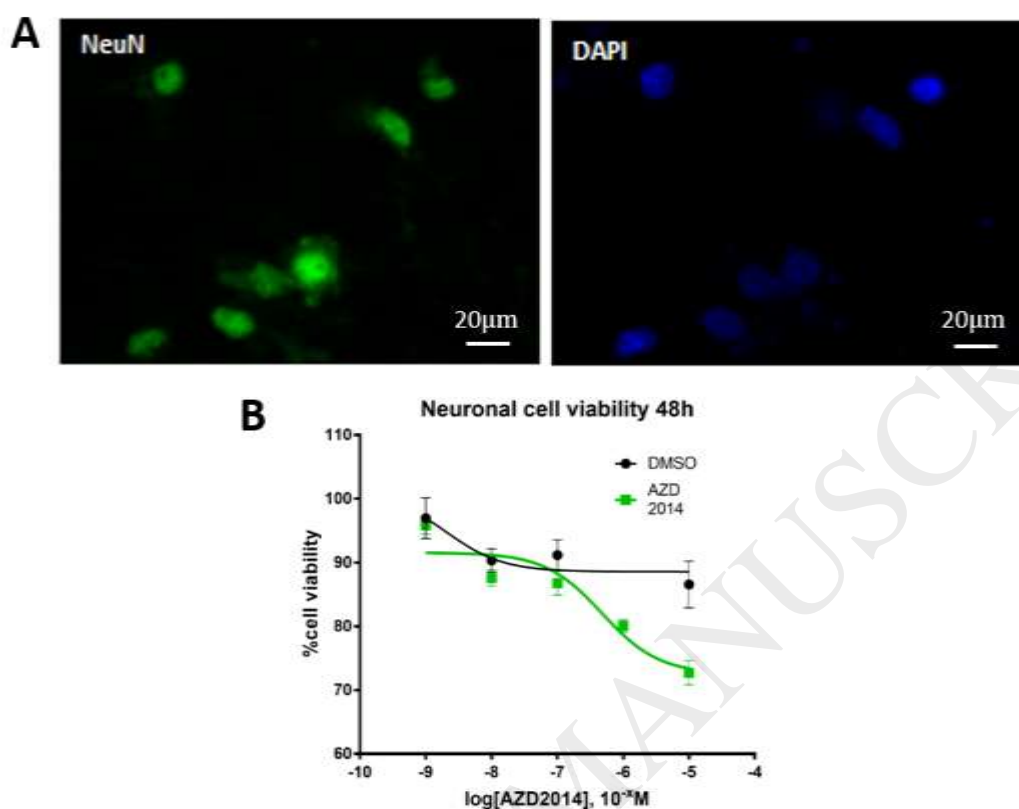


Fig 1 Neuronal viability. **A.** Cells were fixed and underwent immunofluorescence as described in methods with the following markers (A) NeuN (green) and (B) DAPI (blue). Scale bar = 20µm. **B.** AZD2014 10^{-5} – 10^{-9} M or DMSO was added to neurons for 48h. Percentage neuronal viability was quantified with the CellTiter 96 assay. Data are presented as mean \pm SEM with n=4 for each condition.

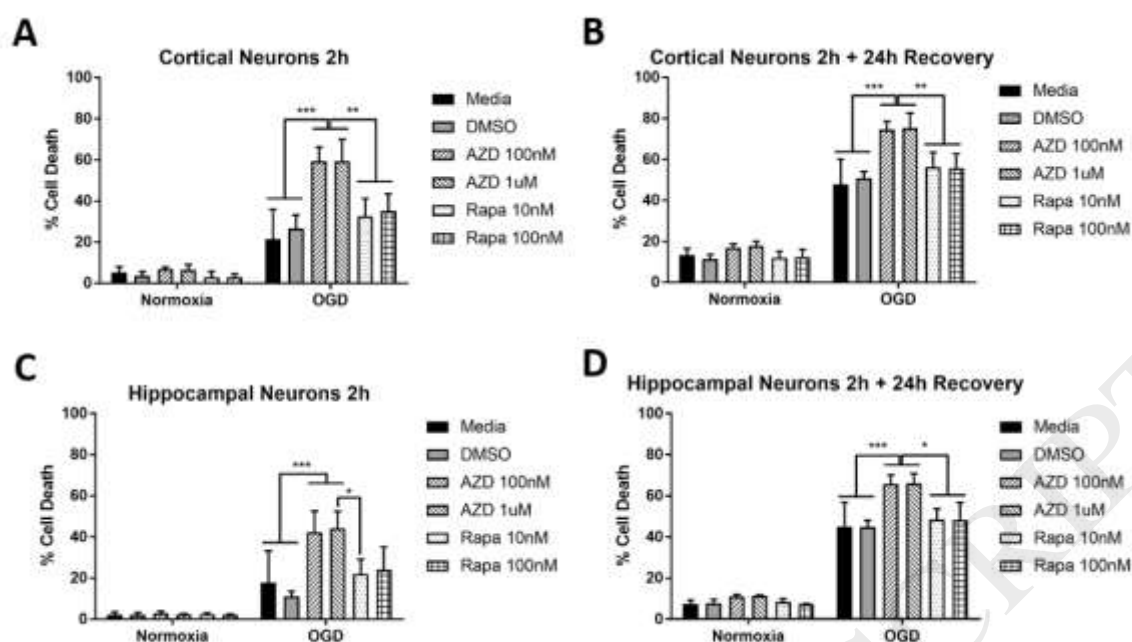


Fig 2. AZD2014 increased neuronal death during OGD. **A.** 2h OGD increased cortical neuronal death compared to normoxia while AZD2014 increased cell death compared with media, DMSO and rapamycin under OGD conditions. **B.** 2h OGD + 24h recovery increased cortical neuronal death compared to normoxia, while AZD2014 increased cell death compared with media, DMSO and rapamycin under OGD conditions. **C.** 2h OGD increased hippocampal neuronal death compared to normoxia while AZD2014 increased cell death compared with media, DMSO and rapamycin under OGD conditions. **D.** 2h OGD + 24h recovery increased hippocampal neuronal death compared to normoxia, while AZD2014 increased cell death compared with media, DMSO, and rapamycin under OGD conditions. Cell death was quantified by LDH assay. Groups: media (control) (n=6), DMSO (control) (n=6), 10nM rapamycin (n=3 for cortical, n=2 for hippocampal), 100nM rapamycin (n=3 for cortical, n=2 for hippocampal), 100nM AZD2014 (n=3) and 1μM AZD2014 (n=3). Two-way ANOVA with Tukey's Post Hoc Test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

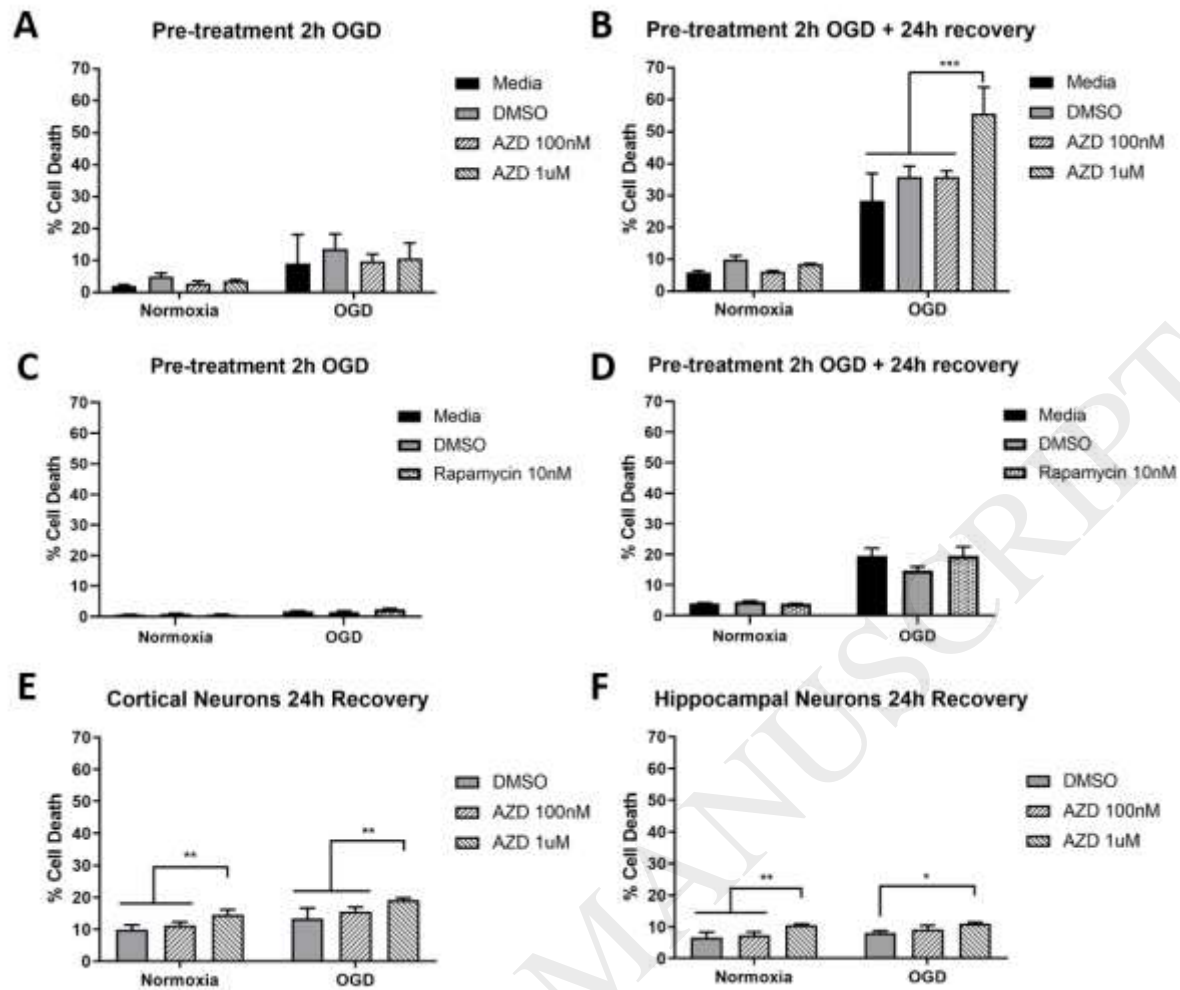


Fig 3. AZD2014 increased neuronal death with treatment prior to and after OGD. **A.** 24h pre-treatment with AZD2014 had no effect on cortical neuronal death during OGD. **B.** 24h pre-treatment with 1 μ M AZD2014 increased cortical neuronal death compared to media, DMSO and 100nM AZD2014 24h following OGD. **C.** 24h pre-treatment with rapamycin had no effect on cortical neuronal death during OGD. **D.** 24h pre-treatment with rapamycin had no effect on cortical neuronal death 24h following OGD. **E.** Treatment with AZD2014 during 24h recovery increased cortical neuronal death following 2h normoxia or OGD. **F.** Treatment with AZD2014 during 24h recovery increased hippocampal neuronal death following 2h OGD. Cell death was quantified by LDH assay. Groups; Media (n=3-4), DMSO (n=3-4), 100nM AZD2014 (n=3), 1 μ M AZD2014 (n=3), 10nM rapamycin (n=4). Two-way ANOVA with Tukey's Post Hoc Test. * p <0.05, ** p <0.01.

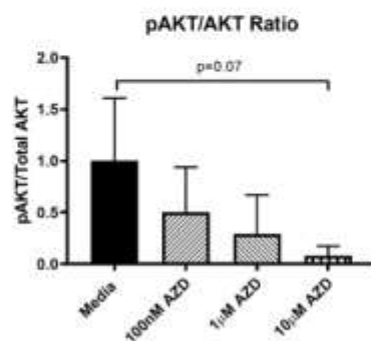
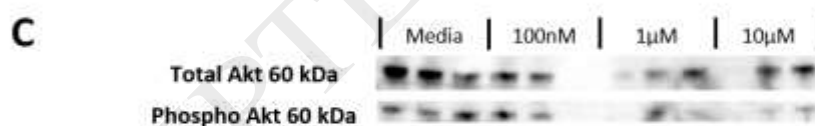
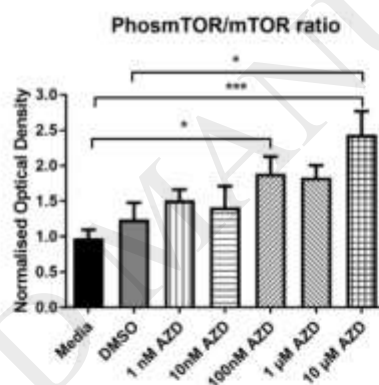
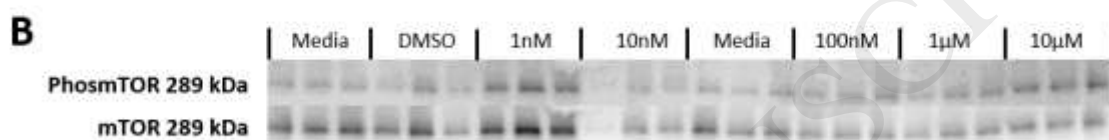
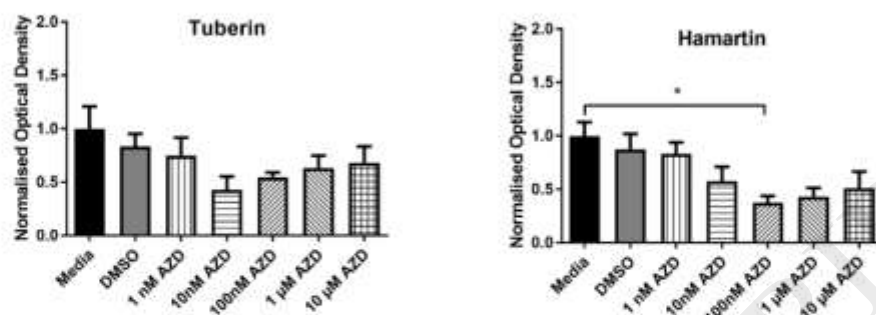
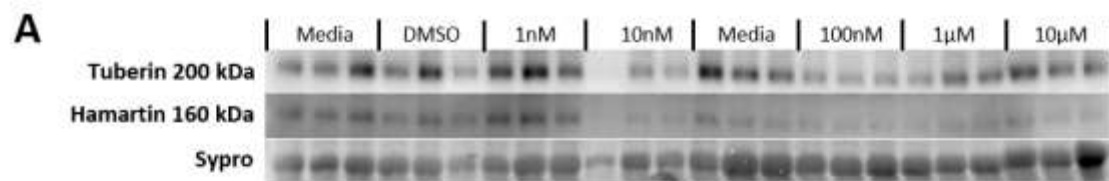


Fig 4. Expression levels of mTOR pathway proteins following 2h AZD2014 treatment in cortical neurons.

A. Western blots showing that AZD2014 did not alter expression levels of tuberin, while increasing concentrations of AZD2014 decreased expression levels of hamartin. SYPRO® staining was used to quantify protein expression.

B. Western blots showing mTOR and phosmTOR protein expression levels. Rising concentrations of AZD2014 increased the ratio of phosmTOR to total mTOR indicative of activated mTOR. **C.** Western blots showing that

higher concentrations of AZD2014 decreased expression levels of phosphorylated Akt relative to total Akt. Data presented as mean \pm sem. $n=3$ per group except media in panels A and B which was $n=6$. One-way ANOVA with Bonferroni post-hoc test (A & B) or Dunnett's post-hoc test (C). * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

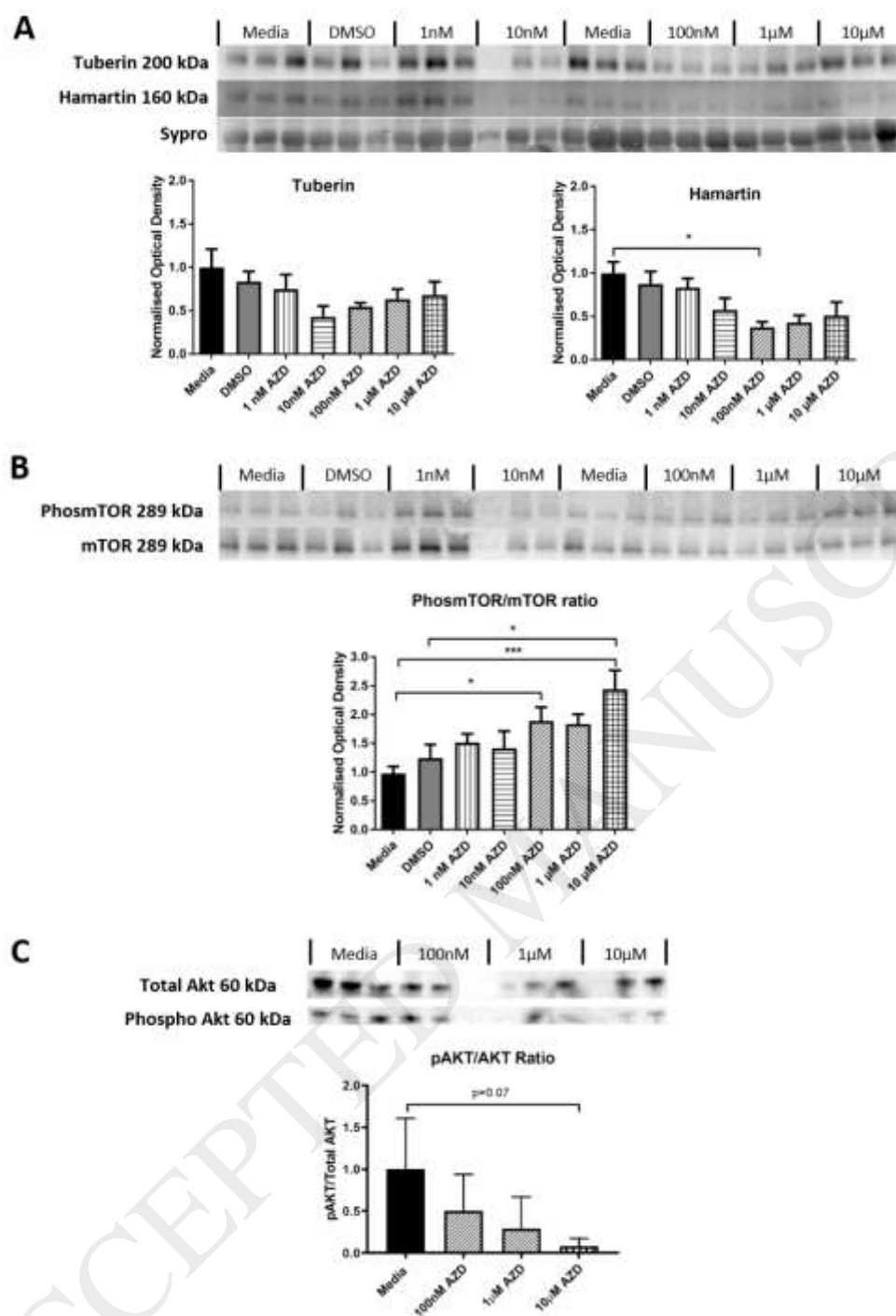


Fig 5. Mechanisms of action of AZD2014. A. Schematic showing how AZD2014 may alter mTORC1 and mTORC2 activity through suppressing hamartin leading to neurotoxicity. Abbreviations: 4E-BP1: Eukaryotic translation initiation factor 4E-binding protein 1; AKT: protein kinase B; ATF4: Activating transcription factor 4 (also known as CREB: cyclic AMP-responsive element-binding protein); eIF4: Eukaryotic translation initiation factor 4E;

IRS: Insulin receptor substrate; mLST8 (mammalian lethal with sec-13 protein 8, also known as G-protein β subunit-like protein or G β L); anPI3K/AKT: PRAS40: proline-rich AKT substrate 40kDa; Phosphatidylinositol 3-kinase/protein kinase B; Rheb: Ras homolog enriched in brain; S6K1: Raptor: regulatory associated protein of mTOR; Rictor: rapamycin-insensitive companion of mTOR; ribosomal protein S6 kinase beta-1 (also known as p70S6 kinase (p70S6K, p70-S6K)); sin: stress-activated protein kinase-interacting protein; TSC1 tuberous sclerosis complex 1; TSC2: tuberous sclerosis complex 2; Ulk1: mammalian autophagy-initiating kinase, a homologue of yeast ATG1. **B.** Increasing concentrations of AZD2014 altered the expression levels of multiple proteins throughout the hamartin/mTOR pathway leading to neurotoxicity.