Paxalisib: repurposing of PI3K pathway modulators for the treatment of PTEN hamartoma tumour syndrome.

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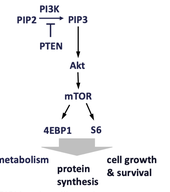
Abstract: 6927

PTEN hamartoma tumour syndrome (PHTS) is a rare disease arising from germline mutations in PTEN. Approximately 25% patients with PHTS may develop symptoms of autism spectrum disorder (ASD) and ~2% patients with idiopathic ASD have been found to harbour PTEN mutations. PTEN mutations lead to an upregulation of PI3K-Akt-mTOR signalling that has been associated with macrocephaly and structural and functional change in hippocampal and cortical neurons in both human and murine CNS. Currently, there are no approved treatments for PHTS although a randomised placebo-controlled trial of the mTOR inhibitor everolimus demonstrated a statistically significant improvement in the social domain.
Drug repurposing studies in PTEN loss mouse primary neurons identified paxalisib, a dual pan PI3K-mTOR inhibitor, as a potential candidate for treatment of PHTS ASD. Adenovirus-mediated delivery of PTEN shRNA resulted in enhanced neurite outgrowth and increased neuronal activity in mouse primary neuron cultures. Paxalisib inhibited the enhanced neurite outgrowth and neuronal activity in PTEN loss neurons at doses that were without cytotoxicity towards wild-type neurons. Inhibition of neurite outgrowth and neuronal activity was correlated with reduced levels of neuronal phospho-Akt and phospho-S6. Pharmacokinetic and pharmacodynamic studies suggest that paxalisib, at tested doses, crosses the blood brain barrier and inhibits PI3K pathway activity at exposures correlated with in vitro inhibition of aberrant PTEN loss neuronal change. Paxalisib is a promising candidate for assessment in a preclinical PHTS disease model and may be a potential candidate for clinical evaluation.

Introduction

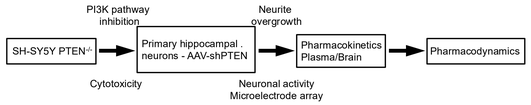
PTEN hamartoma tumour syndrome (PHTS) is a rare disease arising from germline mutations in PTEN. Recent prevalence estimates suggest an incidence as high as 1 in 8764 [1], and approximately 25% patients with PHTS may develop symptoms of autism spectrum disorder (ASD). Furthermore, ~2% patients with idiopathic ASD have been found to harbour PTEN mutations.

The majority of PTEN mutations found in PHTS result in an unstable or truncated protein and the resultant loss of function leads to an upregulation of PI3K signaling that in neurons results in overgrowth of cortical neurons and enhanced synaptic activity. There are no approved treatments for the neurodevelopmental aspects of PHTS although a recent clinical trial with everolimus in individuals with PHTS resulted in improvements in the social domain [2]. In order to identify potential new therapeutic opportunities for PHTS, we embarked on a drug repurposing screen in cellular models of the PHTS neurological phenotype. We tested a custom library of ~ 70 small molecule inhibitors of relevant pathways and identified paxalisib, a dual inhibitor of pan-PI3K and mTOR, as a promising candidate for further evaluation in a disease model of PHTS.



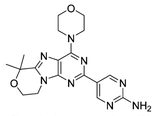
PI3K signalling in neurons. PTEN, a negative regulator of PI3K, also interacts with various proteins to further influence neuronal activity.

Overview of assay-cascade:



Paxalisib - a pan-PI3K/mTOR inhibitor

Paxalisib (originally GDC-0084 [3]; Kazia Therapeutics) is an orally available small molecule potent dual pan-PI3K/mTOR inhibitor (**Table 1**) that was designed to cross the blood brain barrier. Paxalisib is currently under development to treat glioblastoma, and is also under investigation in combination with trastuzumab or radiotherapy to treat breast cancer brain metastases. Separately under license to Sovargen, paxalisib (as SVG103) is under development to treat intractable seizures in focal cortical dysplasia type 2 and tuberous sclerosis complex.



Chemical structure, from [3]

Table 1: Apparent Ki values ($K_{i(App)}$) according to [3]

PI3Kα	PI3Kβ	PI3Kδ	PI3Kγ	mTOR
2 nM	46 nM	3 nM	10 nM	70 nM

Paxalisib was purchased from MedChemtronica AB cat. HY-19962.

Paxalisib inhibited PI3K signaling in SH-SY5Y PTEN$^{-/-}$ cells.

Knockout of PTEN in SH-SY5Y cells using CRISPR generated a PTEN$^{-/-}$ cell line. Confirmation of loss of PTEN expression and PI3K pathway activity was confirmed by Western blotting and a clonal cell line was incubated with paxalisib for 24 hours and PI3K pathway response was assessed by measurement of phospho-S6 (pS6) and phospho-AKT (pAKT).

SHSY5Y PTEN$^{-/-}$ cells were seeded into 384-well plates 20,000 cells/well and incubated in medium containing 10% FCS. After 24h cells were incubated in fresh medium containing paxalisib and incubated for further 24h after which pS6 or pAKT was measured by an AlphaLISA assay (**Fig 1A**) and cell viability was determined by an LDH-CytoTox-One assay (**Fig 1B**).

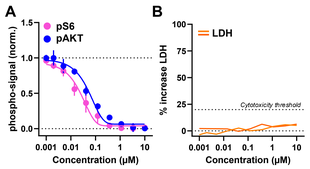


Figure 1 Paxalisib inhibited pS6 and pAKT with an IC50 of 37 nM and 60 nM, respectively (**A**). Cell viability was unaffected over 24h (**B**). Data were generated in three independent experiments with three replicates [4].

Paxalisib inhibited neurite outgrowth and PI3K pathway activity in PTEN loss mouse primary neurons.

Hippocampal neurons were isolated from E16 mouse embryos. Twenty-four hours after plating cells were transduced with an adeno-associated virus expressing a shRNA targeting PTEN, fixed at different time points and stained for image analysis (**Fig 2A**). Robust and complete PTEN loss was observed by DIV10 accompanied by an increase of neurite length and branching, and PI3K pathway activation as measured by increased pAKT (S473), pS6 (S235/236) and p4EBP1 intensities (**Fig 2B-E**). Neurite outgrowth was assessed with antibodies against Tuj1 combined with MAP2 and DAPI staining for neuronal detection.

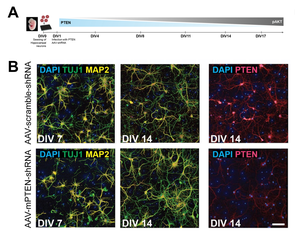


Figure 2 Experimental setup (**A**) and example images of neurite outgrowth with PTEN knockdown (**B**). Knockdown of PTEN (**C**) and increasing neuritic length (**D**) as well as number of branchpoints (**E**) were quantified following treatment with AAV. [4]

After AAV-mediated PTEN knockdown, cultures were treated with different concentrations of paxalisib, starting with the media change on DIV10. Treatment continued until DIV14, after which the cells were fixed and pAKT (**Fig 3A**), pS6 (**Fig 3B**), p4EBP1 (**Fig 3C**) and the neuritic outgrowth with neurite length (**Fig 3D**) and number of branchpoints (*not shown*) were analyzed.

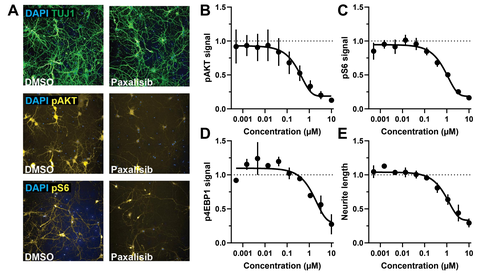


Figure 3 Example images (**A**) showing primary neurons with PTEN knockdown at DIV14, treated with DMSO (*left*) or 0.37 μM paxalisib (*right*), with visible effects on pAKT and pS6 levels. Paxalisib dose-dependently reduced pAKT (**B**, IC50 0.4 μM), pS6 (**C**, IC50 0.8 μM), p4EBP1 (**D**, IC50 1.8 μM) and neurite outgrowth (**E**, IC50 1.2 μM). [4].

There was no evidence of cytotoxicity towards paxalisib over the concentration range tested.

Paxalisib reduced PTEN loss-induced neuronal activity in mouse primary hippocampal neurons.

Mouse hippocampal neurons were plated into 48-well microelectrode array plates. Twenty-four hours after plating, neurons were transduced with an adeno-associated virus expressing a shRNA targeting PTEN. Electrophysiological phenotype was monitored over a 10-day period, DIV7-18, as shown (**Fig 4A**). Multi-electrode array (MEA) analysis revealed that several electrophysiological parameters, such as burst duration and network burst duration (**Fig 4B**) were significantly altered as a result of PTEN loss [4].

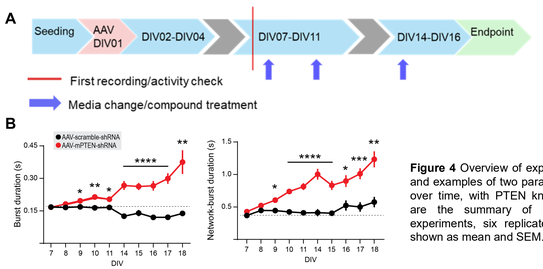


Figure 4 Overview of experimental setup (**A**) and examples of two parameters that change over time, with PTEN knockdown (**B**). Data are the summary of N=20 independent experiments, six replicates each. Data are shown as mean and SEM. [4]

Paxalisib was added to MEA wells on DIV7 and replenished by media change on DIV10 and DIV14. Paxalisib concentrations were based on that equivalent to the IC50 for neurite outgrowth and an approximate 10-fold higher concentration (i.e. 1.2 μM and 10 μM). Five parameters (see **C**) were investigated to assess paxalisib's potency in preventing the PTEN-loss-induced electrophysiological phenotype. Both, high and low dose treatments, showed significant effects in most parameters (**Fig 5A-E**) [4].

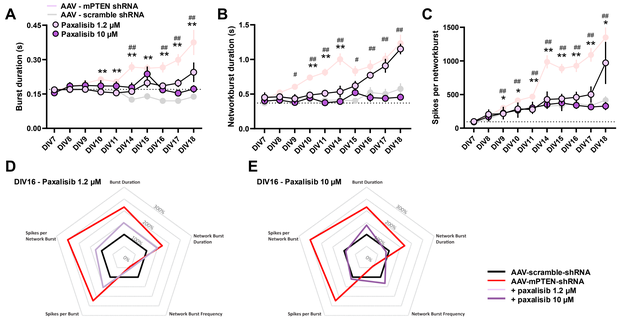


Figure 5 Treatment with paxalisib reduced the development of the PTEN-knockdown-induced alterations, pushing it towards a more "wildtype"-like-phenotype. This includes, for example, a shorter burst duration (**A**) shorter networkburst duration (**B**) and fewer spikes per networkbursts (**C**). Significant differences to the PTEN-knockdown group (*red*) are marked with an * for the paxalisib low dose (1.2μM, *light purple*) and a # for the paxalisib high dose group (10μM, *purple*). Example spider-plots of all five parameters at DIV16 are shown for low (**D**) and high dose treatment (**E**).

References

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Funding

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Paxalisib inhibited PI3K pathway activity in mouse brain tissues.

Paxalisib pharmacokinetics were determined from acute (6h) dosing response and single-dose time-course study in wild type C57Bl/6J mice, 8-10 weeks of age. Paxalisib was prepared in 2% DMSO, 98% HPMC and dosed p.o. Blood plasma and brain tissue levels of paxalisib were determined by mass spectrometry; phospho- and total S6 were measured by MSD in hippocampal tissue lysates (**Fig 6**).

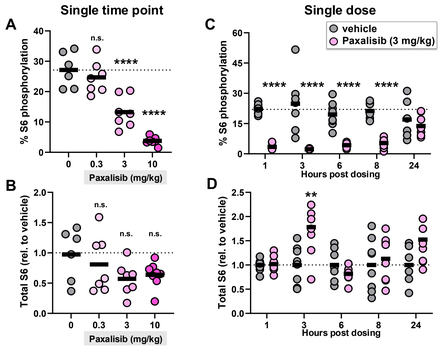

Figure 6 Levels of total and phosphorylated S6 in the hippocampus were measured 6h after vehicle or paxalisib treatment (0.3, 3 or 10 mg/kg, p.o.). At the doses of 3 and 10 mg/kg paxalisib reduced the percentage of S6 phosphorylation (**A**) without changing the levels of total S6 (**B**). Animals were dosed with vehicle or 3 mg/kg paxalisib (p.o.) and the percentage of phosphorylated S6 and total S6 were measured at multiple time points. S6 phosphorylation was significantly reduced up to 8h post dosing (**C**). Total levels of S6 were not consistently altered after paxalisib treatment (**D**).

A single 3 mg/kg p.o. dose of paxalisib inhibited pS6 for up to 8 hours. We further measured the compound concentrations in plasma (**Fig 7A**) and brain (**Fig 7B**) at several time points after exposure for PK/PD modelling. Simulation of plasma concentration levels (**Fig 7C**) suggests, that a dose of 2 mg/kg p.o. provides cover of the IC50 for pS6 inhibition in the hippocampus.

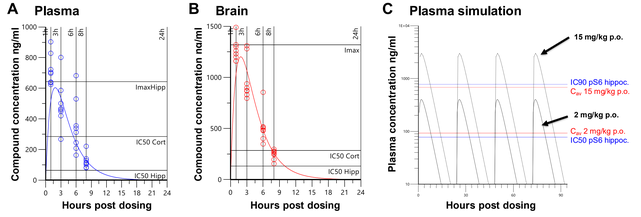

Figure 7 Levels of paxalisib in plasma (**A**) and brain (**B**) as measured by mass spectrometry. Simulation of paxalisib plasma concentrations after 2 or 15 mg/kg p.o. suggest that 2 mg/kg are sufficient to provide cover with an average concentration above the IC50 for pS6 inhibition in the hippocampus.

Conclusions

- Paxalisib is a potent inhibitor of PI3K signaling in a PTEN-KO cell line and in mouse primary neurons with PTEN-knockdown.
- At non-cytotoxic concentrations, paxalisib inhibited neurite outgrowth and PTEN-associated neuronal (hyper-) activity in PTEN-knockdown mouse primary neurons.
- Paxalisib, 3 mg/kg p.o., significantly inhibited PI3K signaling in the brain of wild type mice. PK simulation suggests that a 2 mg/kg p.o. dose provides ample cover over the IC50s for PI3K signaling, neurite outgrowth and neuron activity observed in PTEN-loss neurons.
- Paxalisib, identified through a drug repurposing screen, is an ideal candidate for *in vivo* evaluation in preclinical models of PHTS-ASD, and may represent a new therapeutic option for individuals with PHTS neurodevelopmental deficits.