

# Identification of repurposable cytoprotective drugs in Vanishing White Matter Disease patient-derived cells

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## Research

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# Abstract

**Background** Vanishing white matter disease (VWMD) is a rare leukodystrophy caused by mutations of the guanine exchange factor eIF2B. VWMD typically presents with juvenile onset, and there are few treatments for the disease. Recent progress in the field has established mitochondrial dysfunction and endoplasmic reticulum (ER) stress to be strongly implicated in observed glial cell pathology. Drug repurposing offers a rapid approach toward translation of therapeutics using already-licensed drugs.

**Objective** The aim of this study was to use fibroblasts and induced pluripotent stem cell (iPSC)-derived astrocytes from patients bearing the *EIF2B5*<sup>R113H/A403V</sup> or *EIF2B2*<sup>G200V/E213G</sup> VWMD mutations to identify potentially repurposable FDA-approved drugs based on *in vitro* assays.

**Methods** Cell viability in the presence or absence of stress was assessed by resazurin reduction activity and assays for mitochondrial membrane potential, and oxidative stress. Integrated stress response markers, including eIF2 $\alpha$  phosphorylation, GADD34 and CHOP were quantified by fluorescent western blot.

**Results** Dysregulated GADD34 and CHOP were identified in patient fibroblasts and iPSC-derived astrocytes under induced stress conditions. A drug screen from a 2,400 FDA-approved drug library with *EIF2B5*<sup>R113H/A403V</sup> VWMD patient fibroblasts identified 113 anti-inflammatory drugs as a major class of hits with cytoprotective effects. A panel of potential candidate drugs, including berberine, deflazacort, ursodiol, zileuton, guanabenz and Anavex 2-73, and preclinical ISRIB, increased cell survival of *EIF2B5*<sup>R113H/A403V</sup> or *EIF2B2*<sup>G200V/E213G</sup> VWMD astrocytes, and were further investigated for their effect on the integrated stress response and mitochondrial stress. Ursodiol demonstrated capacity to ameliorate oxidative stress and loss of mitochondrial membrane potential in VWMD patient iPSC-derived astrocytes in the presence or absence of stress conditions.

**Conclusion** Patient-derived cells can be used to identify cellular phenotypes and for large-scale drug screening. Anti-inflammatory compounds, such as berberine, deflazacort, ursodiol and zileuton are potentially repurposable drug candidates for VWMD that should be further investigated for translation *in vivo*.

## Background

Vanishing white matter disease (VWMD) is a rare, autosomal recessive leukodystrophy, caused by mutations in the genes *EIF2B1-5*, encoding the eukaryotic initiation factor eIF2B (1). The eIF2B protein is a guanine nucleotide exchange factor that is involved in the integrated stress response (ISR). VWMD mutations can cause alterations in the activity of the wild-type eIF2B protein and the disease has a variable onset, progression and severity, which can be exacerbated by environmental stress factors (2). Although the disease course varies widely, earlier disease onset is associated with a more rapid progression and patients developing symptoms as young children typically survive few years beyond diagnosis (3).

The eIF2B proteins control mRNA translation, converting the inactive eIF2-GDP to the active eIF2-GTP form. Activation of the cytoprotective ISR leads to phosphorylation of eIF2 $\alpha$  (p-eIF2 $\alpha$ ), which binds to eIF2B to mediate translational repression, and upregulation of stress-induced genes. These genes include *PPP1R15A* that encodes growth arrest and DNA damage-inducible protein (GADD34). GADD34 facilitates dephosphorylation of p-eIF2 $\alpha$  to promote recovery from stress and resumption of normal protein translation in a negative feedback loop, and transcription factors ATF4 and CHOP (4). Mutations in eIF2B can lead to delayed translation of stress-induced genes and dysregulated ISR (5). Although eIF2B is ubiquitously expressed and plays a role in multiple cell types, the disease manifests most significantly in the loss of white matter of the brain (6).

VWMD mouse models and induced pluripotent stem cell (iPSC) models (7, 8) have identified a central role for dysfunctional astrocytes in the development of VWMD, with evidence for astrocytic apoptosis (8) and an inability to promote oligodendrocyte maturation (6). A key driver of cellular pathogenesis in VWMD involves the ISR, with alterations in responses to endoplasmic reticulum stress and oxidative stress (9, 10). Mitochondrial dysfunction and increased accumulation of reactive oxygen species have been identified in VWMD murine fibroblasts, astrocytes and oligodendrocyte precursor cells (11, 12). Currently there are no approved treatments for VWMD, hence the aim of this research was to identify candidates from an FDA-approved drug library that could protect VWMD patient cells against cellular stress. This pre-clinical research can be used to inform future *in vivo* studies. For phenotypically diverse diseases, such as VWMD, patient-derived cells provide a useful model of disease mutations that can aid translational pathways.

## Materials And Methods

### Cell culture

All experimental protocols were approved by the University of Wollongong Human Research Ethics Committee (HE17/522). Primary human dermal fibroblasts were collected from two VWMD patients that were diagnosed in early childhood or their control family members. The VWMD1 iPSC line was generated from a patient bearing mutations in *EIF2B5*<sup>R113H/A403V</sup>, whilst the VWMD6 iPSC line was generated from a patient bearing mutations in *EIF2B2*<sup>G200V/E213G</sup>. The VWMD1 control line is a healthy control (no disease mutations, *EIF2B5*<sup>R113/A403</sup>), whilst the VWMD6 control bears one VWMD allele and one wild-type allele (non-disease carrier, *EIF2B2*<sup>G200V/E213</sup>). Cells were maintained in DMEM/F12 (Gibco, Ireland, 21331020), 10% FBS (Bovogen Biologicals, Australia, SFBS-AU), 2 mM L-glutamine (Gibco, Ireland, 25030081) and 1% penicillin/streptomycin (Gibco, 15140122). Fibroblasts were reprogrammed with the StemRNA (ReproCell, Beltsville, Maryland, 00-0076) non-integrating method, as previously described (13, 14). The mRNA-based reprogramming of patient fibroblasts into iPSCs, pluripotency marker immunofluorescence and RT-qPCR, karyotyping and mutation genotype characterisation are described in Supplementary Information (Figure S1). The iPSCs were maintained in mTESR1 (Stem Cell Technologies, Australia, 85850) and astrocytes in Astrocyte Growth Supplement Medium (AGS; ScienCell Research Laboratories, San Diego, California, USA, 1852). All cells were maintained in humidified incubators at 37

°C supplemented with 5% CO<sub>2</sub> for fibroblasts or hypoxic 3% O<sub>2</sub> conditions for iPSCs and astrocytes. Neural inductions were carried out as previously described (15). Astrocyte differentiations were performed in ciliary neurotrophic factor (CNTF), epidermal growth factor (EGF) and basic fibroblast growth factor (FGF-2)-based medium and transitioned to AGS, prior to characterisation by immunofluorescence and inflammatory activation, to confirm the production of functional astrocytes from iPSCs. Details of neural inductions and astrocyte characterisations are in Supplementary Information (Figure S2). Genotyping and characterisation RT-qPCR primers are listed in Table S1.

### **Cell stress and viability assays**

Fibroblasts or astrocytes were seeded in 96 well plates (5,000 cells/well) and incubated with a range of concentrations of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>; 0-2 mM, Sigma-Aldrich, 16911), MG132 (0-20 µM; Focus Bioscience, HY-13259) or thapsigargin (0-20 µM; Abcam, Australia, ab120286) overnight. Viability was assessed via resazurin reduction assay (Thermo Fisher Scientific, A13262), in which cells received 15 µM resazurin for 1 h prior to measurements by fluorescence plate spectroscopy (excitation 544/emission 590 nm). For coinubation assays, cells were similarly prepared and incubated with candidate drugs and, based on the IC<sub>60</sub> for each cell type, MG132 at either 2.5 µM (fibroblasts) or 0.1 µM (astrocytes) for 48 h. The coinubation drug screen was carried out in VWMD1 patient fibroblasts with the MicroSource Spectrum FDA collection (Compounds Australia), a library of 2,400 FDA-approved drugs. Cell seeding and drug reagent preparation were performed by a robotic liquid handler (Hamilton Microlab Star, Reno, Nevada). First-pass screening of the drug library was carried out at 20 µM; drug candidates selected for further testing underwent dose-response curves to identify appropriate concentrations. Single drug concentrations were selected based on protective efficacy in dose-response coinubation assays and used in downstream assays at concentrations of: Anavex 2-73 (AVex-73, 5 µM; Focus Bioscience, Australia, HY-101864), berberine (1.25 µM; Sigma-Aldrich, Australia, 14050), guanabenz (5 µM; Focus Bioscience, HY-B0566), ISRIB (1.25 µM; Focus Bioscience, HY-12495), deflazacort (5 µM; Sigma-Aldrich, SML0123), ursodiol (40 µM; Sigma-Aldrich, PHR1579) and zileuton (1.25 µM; Focus Bioscience, HY-14164). A DMSO (Sigma-Aldrich, D-4540) vehicle (solvent) concentration of 0.2% was included in all experiments.

### **Integrated stress response protein quantification**

Fibroblasts or astrocytes were seeded in 6 well plates (200,000 cells/well) and incubated with MG132 for 0, 24 or 48 h, or coinubated with candidate compound and MG132 overnight. Cultures were lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitors cOmplete Protease Inhibitor (Roche, 4693116001) and phosphatase inhibitors PhosSTOP EASY (Roche, 4906837001)) before sonication and quantification by a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, 23225). Protein lysates were heated for 5 min at 95 °C in loading buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue, 0.0625 M Tris-HCl, pH 6.8). Denatured protein lysates (5 µg) were separated on 4–20% Criterion TGX Precast Gels (Bio-Rad, Australia, 5678095) and transferred to Immobilon-FL PVDF membrane (0.45 µm pore; Merck, Darmstadt,

Germany, IPFL00010). Membranes were blocked with 0.5% casein (Bio-Rad, 1610782) for 1 h (RT), primary antibody at 1:1,000 dilution for 16 h (4 °C), and secondary antibody at 1:5,000 dilution for 1 h (4 °C), facilitated by an automated western blot processor (Cytoskeleton GOBlot, Denver, Colorado, USA). Antibodies utilised included anti-eIF2 $\alpha$  (Abcam, ab5369), anti-eIF2 $\alpha$ -phospho-S51 (Abcam, ab32157), anti-DDIT3 (DNA damage inducible transcript 3, also known as C/EBP homologous protein (CHOP; Abcam, ab179823), anti-GADD34 (Abcam, ab126075), donkey anti-mouse Alexa Fluor Plus 488 (Thermo Fisher Scientific A32766) and donkey anti-rabbit Alexa Fluor Plus 647 (Thermo Fisher Scientific A32795). Blots were imaged with a Bio-Rad ChemiDoc MP. Band intensities were normalised to stain-free total protein. Relative eIF2 $\alpha$  phosphorylation was normalised to total eIF2 $\alpha$ . Dynamic range of signal was confirmed by linear signal to loading ratio (Figure S3).

### **Oxidative stress, mitochondrial membrane potential and membrane integrity microscopy assays**

Astrocytes were seeded in 96 well plates (10,000 cells per well) and coincubated overnight with MG132, as described above, and candidate drugs, or the positive controls for oxidative stress: H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M), or the proton uncoupler carbonyl cyanide-4-phenylhydrazone (FCCP; 10  $\mu$ M; Focus Bioscience, HY-100410). Cells were loaded with 20  $\mu$ M H<sub>2</sub>DCFDA (DCF; Thermo Fisher Scientific, D399) or 0.5  $\mu$ M tetramethylrhodamine ethyl ester (TMRE; Thermo Fisher Scientific, T669) and 10  $\mu$ M Hoechst 33342 (Sigma-Aldrich, B2261) for 0.5 h before replacement of medium with phenol red-free DMEM/F12 medium (Gibco, 21041025) and further incubation for 1 h. Relative fold change in DCF signal was acquired by fluorescence spectroscopy and normalised to cell density quantified by 0.004% sulforhodamine B (Sigma-Aldrich, 230162) solubilised in 10 mM Trizma (Sigma-Aldrich, T1503) after fixation in 4% trichloroacetic acid (Sigma-Aldrich, T9159) (16). TMRE images were acquired by confocal microscopy, TMRE (excitation 522/emission 590-620 nm) and analysed with ImageJ to determine fluorescence normalised to cell density.

### **Statistical analyses**

Data are presented as the mean and standard error of the mean of at least 3 independent experiments; statistical significance was assessed by two-way analysis of variance followed by Holm-Sidak posthoc test for multiple comparisons unless otherwise stated. Significance was accepted where  $p < 0.05$ . Charts and statistical analyses were prepared in Prism GraphPad 8.0.

## **Results**

### **VWMD patient fibroblasts and astrocytes exhibit dysregulated ISR marker expression**

Mutations in eIF2B suppress both global and stress-induced protein translation, in response to ER stress (5, 17). Given that eIF2B is ubiquitously expressed, we examined patient fibroblasts and iPSC-derived astrocytes for evidence of a dysfunctional ISR. Fibroblasts were reprogrammed into iPSCs from two VWMD patients, along with gender-matched relatives as non-disease controls (Figure S1). A previous study identified that white matter-derived astrocytes, generated using a ciliary neurotrophic factor (CNTF)-

based differentiation protocol, showed a more vulnerable phenotype to stress, compared to grey matter astrocytes generated using fetal bovine serum (7). Consequently, we generated astrocytes from iPSCs using a CNTF-based method (Figure S2).

Responses to oxidative and endoplasmic reticulum (ER) stresses are all proposed to be affected in VWMD cell and animal-based models (17). Studies employing cells bearing different VWMD mutations have shown variability in the ISR (5), and primary astrocytes from mice bearing a homozygous R191H mutation in eIF2B $\epsilon$  did not exhibit the ISR *in vitro* (18). Thus, first we established stress conditions that could generate an exacerbated cellular phenotype in the VWMD patient cells compared to controls. We evaluated the dose-dependent effect of H<sub>2</sub>O<sub>2</sub>, as a mediator of oxidative stress, thapsigargin, as a mediator of ER stress, and MG132. MG132 is commonly utilised as a proteasomal inhibitor that also induces ER and oxidative stress via the unfolded protein response, all of which trigger the ISR (19, 20). There was a small but significant reduction in cell survival for VWMD fibroblasts and iPSC-derived astrocytes, compared to non-disease controls, under all three stressors (Figure S4A).

Disrupted ISR homeostasis can be assessed by measuring the expression levels of ISR-relevant markers. The control of eIF2 $\alpha$  phosphorylation or dephosphorylation acts as a pivotal mechanism that regulates global protein synthesis in response to cell stress (21); GADD34 dephosphorylates eIF2 $\alpha$  (22) while CHOP is activated by the ISR to promote apoptosis (23). The expression levels of these ISR-relevant proteins, p-eIF2 $\alpha$  (normalised to eIF2 $\alpha$ ), GADD34, and CHOP, were compared in VWMD and control fibroblasts, and iPSC-derived astrocytes, under MG132 stress (Figure 1; antibody characterisation and representative blots shown in Figures S3-4). VWMD lymphoblasts have previously been reported to show reduced GADD34 expression and increased levels of p-eIF2 $\alpha$ , following thapsigargin-induced acute ER stress (5). Under MG132-induced stress, VWMD fibroblasts showed reduced upregulation of eIF2 $\alpha$  phosphorylation and reduced GADD34 expression, and increases in CHOP expression, compared to controls (Figure 1A-C). MG132-stressed VWMD astrocytes also showed reduced upregulation of eIF2 $\alpha$  phosphorylation and increased CHOP, compared to controls (Figure 1D-F). VWMD astrocytes exhibited increased GADD34 (Figure 1E), consistent with glia in animal model studies (17) and the hypophosphorylation of eIF2 observed in white matter patient tissue (24).

### Cytoprotective drug screen in VWMD patient cultures

Based on the established capacity of MG132 to induce oxidative stress, and exacerbate ISR disease phenotypes, we performed a first-pass drug screen for candidates able to protect against the effect of low dose MG132 in VWMD1 *EIF2B5*<sup>R113H/A403V</sup> patient fibroblasts, with cell viability assessed by resazurin reduction activity (Figure 2).

Following the initial drug screen, a panel of 20 compounds (Table S2) was selected for downstream evaluation in VWMD1 *EIF2B5*<sup>R113H/A403V</sup> fibroblasts and in both VWMD1 *EIF2B5*<sup>R113H/A403V</sup> and VWMD6 *EIF2B2*<sup>G200V/E213G</sup> patient-derived astrocyte cultures. The compounds selected for downstream assays were chosen based on their potential for clinical translation, including considerations of bioavailability,

route of administration and potential side effects. The drug panel included 14 protective compounds from the screen ( $>1.5 \times$  standard deviation of MG132-stressed controls), and a further six compounds with relevant modes of action for VWMD. The sigma-1 receptor pathway was recently identified in a drug screen to protect against mitochondrial dysfunction in a murine *EIF2B5*<sup>R132H/R132H</sup> model (11). Nominated drugs on the basis of relevant mode of action to VWMD, included guanabenz (25), ISRIB (26), sigma-1 receptor agonists, AVex-73 and amitriptyline (11), as well as the central nervous system (CNS) cytoprotective compounds tauroursodeoxycholic acid and alkaloid berberine (27). Overall, the panel of compounds for further evaluation included glucocorticosteroids, bile acids, iron chelators, antioxidants, ISR modulators and sigma-1 receptor agonists. All candidates elicited cytoprotective effects against MG132-induced stress at varying concentrations in VWMD1 fibroblasts, with the exception of ISRIB. Ursodiol and its taurine derivative, tauroursodeoxycholic acid, showed similar cytoprotective efficacies. Anti-inflammatories were amongst the largest category of compounds that improved cell viability under proteasomal stress, with a high proportion of these being glucocorticosteroids. The recent demonstration of mitochondrial dysfunction and inefficient respiration in murine models of VWMD (11) has expanded the search for possible therapeutics to include mitochondrial protective compounds and antioxidants. The sigma-1 receptor is a chaperone protein in ER membranes that governs a range of cellular processes, including calcium homeostasis and reactive oxygen species accumulation (28). However, in our study, the cytoprotective effect of the sigma-1 receptor agonists, AVex-73 and amitriptyline, was limited at higher concentrations ( $\geq 5 \mu\text{M}$ ; Figures 2, S5). Drugs assessed in iPSC-derived astrocytes from VWMD1 *EIF2B5*<sup>R113H/A403V</sup> and VWMD6 *EIF2B2*<sup>G200V/E213G</sup> yielded comparable results (Figure S5). The majority of the drugs that were protective in VWMD1 fibroblasts were also protective in VWMD1 and VMWD6 astrocytes, with the exception of amitriptyline, curcumin and budesonide (Figures 2, S5).

Further studies were conducted to gain insight into the mode of action of drugs with therapeutic potential for VWMD (Figures 2, S5), including AVex-73, berberine, deflazacort, guanabenz, ISRIB, ursodiol and zileuton. Assays to investigate drug mechanisms were performed with VWMD1 *EIF2B5*<sup>R113H/A403V</sup> patient astrocytes, given that the majority of VWMD-causing mutations affect the eIF2B $\epsilon$  subunit (3).

### **Effect of drug candidates on cell death in VWMD iPSC-astrocytes**

To determine whether candidate drugs reduced the proportion of bulk cell death, the membrane permeabilisation of treated astrocyte cultures was evaluated. In MG132-stressed VWMD1 astrocytes there were no significant changes in the percentage of membrane-permeabilised cells, with the exception of ISRIB increasing the proportion of membrane-permeabilised cells (Figure S6). To test whether the drugs caused a reduction in mitochondrial apoptosis, the *BAX:BCL2* ratio was evaluated. The ratio of *BAX:BCL2* is used to assess the levels of Bax (proapoptotic) and Bcl-2 (antiapoptotic) expression. There was no detectable change in *BAX:BCL2* ratio caused by any of the drug candidates, with the exception of an increase in *BAX:BCL2* caused by ISRIB (Figure S6).

### **Effect of candidate drugs on ISR-relevant proteins p-eIF2 $\alpha$ , GADD34 and CHOP**

Candidate drugs were assessed for their effect on the ISR under MG132 stress in astrocytes. The expression levels of the ISR-relevant proteins, p-eIF2 $\alpha$  (normalised to eIF2 $\alpha$ ), GADD34 and CHOP, were evaluated following candidate drug treatment (Figure 3A-C). ISRIB markedly increased p-eIF2 $\alpha$  and decreased GADD34 and CHOP expression under MG132 stress conditions. AVex-73, berberine and deflazacort significantly decreased CHOP expression in the presence of MG132.

Previous cell stress and neurodegeneration studies have established ISR-modulating roles for guanabenz and ISRIB (29, 30). Guanabenz is considered to exert cytoprotective effects by inhibiting the activity of GADD34 to recruit eIF2 phosphatases, thus prolonging translation inhibition and avoiding the added stress of resuming protein synthesis (29). However, at concentrations of guanabenz that induced a cytoprotective effect (5  $\mu$ M), we did not observe a significant impact on expression of any ISR markers in astrocytes. Conversely, ISRIB-mediated cytoprotection of astrocytes (1.25  $\mu$ M) was associated with increased eIF2 $\alpha$  phosphorylation, and downregulation of GADD34 and CHOP.

### **Effect of candidate drugs on indicators of mitochondrial function**

The eIF2B mutations in murine models have been shown to decrease mitochondrial membrane potential and impair mitochondrial complex I function, resulting in a compensatory increase in mitochondrial abundance (9). Furthermore, mutations in eIF2B genes impair mitochondrial function during oxidative stress conditions in VWMD murine fibroblasts and astrocytes (11). VWMD1 and VWMD6 patient iPSC-derived astrocytes demonstrated reduced mitochondrial membrane potential in both the presence and absence of MG132, suggesting reduced mitochondrial membrane potential is a cellular phenotype of VWMD patient cells, even under basal conditions (Figure S8).

The candidate drugs were investigated for their ability to protect against oxidative stress and improve mitochondrial membrane potential. VWMD1 *EIF2B5*<sup>R113H/A403V</sup> patient astrocytes treated with ursodiol or zileuton led to decreased generation of reactive oxygen species, in both the presence and absence of MG132 stress (Figure 4A). This correlates with the purported radical scavenging activity of the 5-lipoxygenase antagonist zileuton (31). Ursodiol also increased the relative mitochondrial membrane potential of VWMD1 *EIF2B5*<sup>R113H/A403V</sup> astrocytes under both unstressed and stressed conditions (Figure 4B), consistent with a loss of mitochondrial membrane potential as a cellular phenotype of VWMD astrocytes. Furthermore ursodiol reduced oxidative stress in both control and VWMD astrocytes (Figure 4C) and improved the mitochondrial membrane potential phenotype of VWMD astrocytes, bringing the levels of TMRE to those of control cells (Figure 4D-G). Together, these data suggest that ursodiol may promote mitochondrial function and reduce oxidative stress in VWMD astrocytes.

## **Discussion**

This study screened a panel of 2,400 FDA-approved drugs, of varying classes, in an aim to identify repurposable drugs to protect against VWMD. A phenotype of VWMD cells, under basal conditions or in the presence of stress, highlighted dysfunction in the mitochondrial membrane potential of VWMD cells.

Protective drugs targeted common pathways, and included compounds with anti-inflammatory activity and/or drugs purported to improve mitochondrial function or reduce oxidative stress. Steroids can regulate inflammation, mitochondrial function and apoptosis with neuroprotective effects in brain injury, Alzheimer's disease, Parkinson's disease, multiple sclerosis, and stroke (32). Although glucocorticosteroids are a commonly administered class of drugs, an anecdotal study of corticosteroids on three VWMD patients did not identify benefits and the patients were removed from this treatment due to potential clinical complications (33). Nonetheless, deflazacort was included in our study as a representative glucocorticosteroid on the basis of fewer reports of adverse effects in the literature and its existing use in the clinic, including in children with muscular dystrophy (34, 35). Reduced CHOP expression in stress-induced cells following deflazacort treatment may reflect an ability of this drug to improve mitochondrial function (36).

The protective effect of the antioxidant edaravone was evident in astrocytes at a higher efficacy than any other candidate, potentially due to its well established radical scavenging activity (37). Edaravone was recently approved for amyotrophic lateral sclerosis and while its administration is currently limited to intravenous injection, oral and mucosal formulations are in development (38-40).

Zileuton is an oral anti-asthmatic leukotriene inhibitor with reactive oxygen species scavenging activity (31). In this study an increase in cell viability was correlated with a significant decrease in ROS generation but no significant effects on integrated stress response modulation. Its neuroprotective effects have been correlated to anti-inflammatory activity in cerebral ischemia, possibly as a result of reducing ROS-activated inflammation, and preventing glutamate oxidative toxicity via ferroptosis (31, 41).

ISRIB is thought to allow eIF2B to escape inhibitory complex formation with p-eIF2 $\alpha$ , thus limiting repression of protein synthesis while under ER stress (17, 26). Treatment of *EIF2B5* VWMD mice with ISRIB, or a derivative, 2BAct, ameliorated myelin loss, and improved ISR signature and motor function (17, 24, 26). It is also worth noting that the stabilising effect of ISRIB may be dependent on the precise mutations and the cellular concentration of p-eIF2 $\alpha$ , whereby it has been found to inhibit low level but not high level ISR activation (24, 42). As ISRIB inhibits the integrated stress response, it is evident that enabling protein synthesis to resume under acute proteotoxic stress may have an adverse effect on cell survival, and the enhanced effect of cytotoxic drugs by ISRIB have been recently demonstrated in prostate and pancreatic cancer studies (43, 44).

The phytochemical, berberine, was included in the panel as a well-tolerated natural compound (27). In MG132-stressed astrocytes berberine treatment increased cell viability, potentially via decreasing pro-apoptotic CHOP. The antioxidant, anti-inflammatory, ER and mitochondrial protective effects of berberine have been noted in numerous studies, and include increased levels of superoxide dismutase and glutathione, inhibition of apoptosis and decreased cytochrome C and *BAX:BCL2* ratio in ischemic injury and diabetic animal models (45, 46).

Overall, one of the most promising drugs identified with translational potential was ursodiol, a bile acid naturally formed in the liver and administered for gallstones. Ursodiol has a demonstrated capacity to

cross the blood brain barrier, based on clinical trials for motor neurone disease, reaching levels in cerebral spinal fluid that could be protective (47). Additionally, ursodiol has been identified as protective in other neurodegenerative and optical atrophy research, where its anti-apoptotic and neuroprotective effects were observed, although the underlying molecular mechanism of ursodiol-mediated protection was not identified (48, 49). Although ursodiol is considered to be generally well tolerated, fatigue, nausea, weight gain, and depression are possible symptoms (50). Progression of VWMD includes glial cell death, developing towards neuronal cell death, and leading to paralysis and neuropathy (51). We observed ursodiol to decrease oxidative stress and increase mitochondrial membrane potential, under stressed and non-stressed conditions, and improve cell viability in VWMD astrocytes under proteasomal stress. The increase of mitochondrial membrane potential by ursodiol identified in our study is consistent with reports for Alzheimer's disease patient fibroblasts (52). Considering disrupted mitochondrial function has been extensively implicated in VWMD and a wide range of neurodegenerative disorders, further investigation of the neuroprotective effects of ursodiol are warranted. Future studies should confirm these findings in additional VWMD patient cell lines and *in vivo* studies.

## Conclusions

The hypothetical premise of cytoprotective drugs for VWMD are those that can reverse cellular phenotypes and limit disease-related degeneration. Patient-derived cells are a useful resource to support drug repurposing studies for rare diseases. Together with information from previous studies on other CNS diseases (47, 52) our preliminary findings are supportive toward ongoing *in vivo* investigation of ursodiol, zileuton and other anti-inflammatory drugs as cytoprotective agents for VWMD.

## Abbreviations

AVex-73: Anavex 2-73

CHOP: C/EBP homologous protein

CNS: central nervous system

CNTF: ciliary neurotrophic factor

DCF: H2DCFDA

EGF: epidermal growth factor

ER: endoplasmic reticulum

FDA: Food and Drug Administration

FCCP: carbonyl cyanide-4-phenylhydrazine

FGF-2: basic fibroblast growth factor

GADD34: growth arrest and DNA damage-inducible protein

iPSC: induced pluripotent stem cell

ISR: integrated stress response

p-eIF2 $\alpha$ : phosphorylated eIF2 $\alpha$

TMRE: tetramethylrhodamine ethyl ester

VWMD: Vanishing white matter disease

## **Declarations**

### **Ethics approval and consent to participate**

All experimental protocols were approved by the University of Wollongong Human Research Ethics Committee (HE17/522). All participants provided consent to participate.

### **Consent for publication**

Participants provided consent for publication.

### **Availability of data and material**

The authors confirm that the data supporting the findings of this study are available within the article. Data and materials may be available on request if the requests conform to the Human Research Ethics Committee approval requirements.

### **Competing interests**

The authors declare they have no competing interests.

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### **Authors' contributions**

NN – data generation, data analysis, manuscript writing; MC – data generation, data analysis; TB – data generation, data analysis; SM – data generation, data analysis; ME - data generation; DS - data generation; DDH – data analysis; JL – data generation; SSM – data analysis; NSB – data generation; CS

– data generation; LO - intellectual input, data analysis, manuscript writing, supervision, funding. All authors reviewed the manuscript.

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## References

1. Bugiani M, Vuong C, Breur M, van der Knaap MS. Vanishing white matter: a leukodystrophy due to astrocytic dysfunction. 2018;28(3):408-21.
2. Proud CG. Vanishing white matter: the next 10 years. 2012;7(1):81-92.
3. Hamilton EM, van der Lei HD, Vermeulen G, Gerver JA, Lourenço CM, Naidu S, et al. Natural history of vanishing white matter. *J Annals of neurology*. 2018;84(2):274-88.
4. Brush MH, Shenolikar S. Control of cellular GADD34 levels by the 26S proteasome. *Molecular cellular biology*. 2008;28(23):6989-7000.
5. Moon SL, Parker R. EIF2B2 mutations in vanishing white matter disease hypersuppress translation and delay recovery during the integrated stress response. *RNA*. 2018;24(6):841-52.
6. Dooves S, Bugiani M, Postma NL, Polder E, Land N, Horan ST, et al. Astrocytes are central in the pathomechanisms of vanishing white matter. *The Journal of Clinical Investigation*. 2016;126(4):1512-24.
7. Leferink PS, Dooves S, Hillen AEJ, Watanabe K, Jacobs G, Gasparotto L, et al. Astrocyte Subtype Vulnerability in Stem Cell Models of Vanishing White Matter. *Annals of Neurology*. 2019;86(5):780-92.
8. Zhou L, Li P, Chen N, Dai L-F, Gao K, Liu Y-N, et al. Modeling vanishing white matter disease with patient-derived induced pluripotent stem cells reveals astrocytic dysfunction. *CNS Neuroscience & Therapeutics*. 2019;25(6):759-71.
9. Raini G, Sharet R, Herrero M, Atzmon A, Shenoy A, Geiger T, et al. Mutant eIF2B leads to impaired mitochondrial oxidative phosphorylation in vanishing white matter disease. 2017;141(5):694-707.
10. van der Voorn JP, van Kollenburg B, Bertrand G, Van Haren K, Scheper GC, Powers JM, et al. The unfolded protein response in vanishing white matter disease. *Journal of Neuropathology*. 2005;64(9):770-5.
11. Atzmon A, Herrero M, Sharet-Eshed R, Gilad Y, Senderowitz H, Elroy-Stein O. Drug screening identifies sigma-1-receptor as a target for the therapy of VWM leukodystrophy. *Frontiers in molecular neuroscience*. 2018;11:336.

12. Herrero M, Mandelboum S, Elroy-Stein O. eIF2B Mutations Cause Mitochondrial Malfunction in Oligodendrocytes. *NeuroMolecular Medicine*. 2019;21(3):303-13.
13. Bax M, Balez R, Muñoz SS, Do-Ha D, Stevens CH, Berg T, et al. Generation and characterization of a human induced pluripotent stem cell line UOWi005-A from dermal fibroblasts derived from a CCNFS621G familial amyotrophic lateral sclerosis patient using mRNA reprogramming. *Stem Cell Research*. 2019;40:101530.
14. Balez R, Berg T, Bax M, Muñoz SS, Cabral-da-Silva MC, Engel M, et al. The mRNA-based reprogramming of fibroblasts from a SOD1E101G familial amyotrophic lateral sclerosis patient to induced pluripotent stem cell line UOWi007. *Stem Cell Research*. 2020;42:101701.
15. Denham M, Dottori M. Neural differentiation of induced pluripotent stem cells. *Neurodegeneration: Springer*; 2011. p. 99-110.
16. Vichai V, Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nature protocols*. 2006;1(3):1112.
17. Wong YL, LeBon L, Basso AM, Kohlhaas KL, Nikkel AL, Robb HM, et al. eIF2B activator prevents neurological defects caused by a chronic integrated stress response. *ELife*. 2019;8:e42940.
18. Wisse LE, ter Braak TJ, van de Beek M-C, van Berkel CGM, Wortel J, Heine VM, et al. Adult mouse eIF2B $\epsilon$  Arg191His astrocytes display a normal integrated stress response in vitro. *Scientific Reports*. 2018;8(1):3773.
19. Park HS, Jun DY, Han CR, Woo HJ, Kim YH. Proteasome inhibitor MG132-induced apoptosis via ER stress-mediated apoptotic pathway and its potentiation by protein tyrosine kinase p56lck in human Jurkat T cells. *Biochem Pharmacol*. 2011;82(9):1110-25.
20. Maharjan S, Oku M, Tsuda M, Hoseki J, Sakai Y. Mitochondrial impairment triggers cytosolic oxidative stress and cell death following proteasome inhibition. *Scientific Reports*. 2014;4(1):5896.
21. Jiang H-Y, Wek RC. Phosphorylation of the  $\alpha$ -subunit of the eukaryotic initiation factor-2 (eIF2 $\alpha$ ) reduces protein synthesis and enhances apoptosis in response to proteasome inhibition. *Journal of Biological Chemistry*. 2005;280(14):14189-202.
22. Brush MH, Weiser DC, Shenolikar S. Growth arrest and DNA damage-inducible protein GADD34 targets protein phosphatase 1 alpha to the endoplasmic reticulum and promotes dephosphorylation of the alpha subunit of eukaryotic translation initiation factor 2. *Molecular and cellular biology*. 2003;23(4):1292-303.
23. Teske BF, Fusakio ME, Zhou D, Shan J, McClintick JN, Kilberg MS, et al. CHOP induces activating transcription factor 5 (ATF5) to trigger apoptosis in response to perturbations in protein homeostasis. *Mol Biol Cell*. 2013;24(15):2477-90.
24. Abbink TEM, Wisse LE, Jaku E, Thiecke MJ, Voltolini-González D, Fritsen H, et al. Vanishing white matter: deregulated integrated stress response as therapy target. *Annals of clinical and translational neurology*. 2019;6(8):1407-22.
25. Dooves S, Bugiani M, Wisse LE, Abbink TEM, van der Knaap MS, Heine VM. Bergmann glia translocation: a new disease marker for vanishing white matter identifies therapeutic effects of

- Guanabenz treatment. *Neuropathology and Applied Neurobiology*. 2018;44(4):391-403.
26. Wong YL, LeBon L, Edalji R, Lim HB, Sun C, Sidrauski C. The small molecule ISRIB rescues the stability and activity of Vanishing White Matter Disease eIF2B mutant complexes. *Elife*. 2018;7:e32733.
  27. Vuddanda PR, Chakraborty S, Singh S. Berberine: a potential phytochemical with multispectrum therapeutic activities. *Expert opinion on investigational drugs*. 2010;19(10):1297-307.
  28. Weng T-Y, Tsai S-YA, Su T-P. Roles of sigma-1 receptors on mitochondrial functions relevant to neurodegenerative diseases. *Journal of biomedical science*. 2017;24(1):74.
  29. Wang L, Popko B, Tixier E, Roos RP. Guanabenz, which enhances the unfolded protein response, ameliorates mutant SOD1-induced amyotrophic lateral sclerosis. *Neurobiology of disease*. 2014;71:317-24.
  30. Halliday M, Radford H, Sekine Y, Moreno J, Verity N, Le Quesne J, et al. Partial restoration of protein synthesis rates by the small molecule ISRIB prevents neurodegeneration without pancreatic toxicity. *Cell death & disease*. 2015;6(3):e1672-e.
  31. Liu Y, Wang W, Li Y, Xiao Y, Cheng J, Jia J. The 5-lipoxygenase inhibitor zileuton confers neuroprotection against glutamate oxidative damage by inhibiting ferroptosis. *Biological Pharmaceutical Bulletin*. 2015;38(8):1234-9.
  32. Garcia-Segura LM. *Hormones and brain plasticity*: Oxford University Press; 2009.
  33. Turón-Viñas E, Pineda M, Cusí V, López-Laso E, Del Pozo RL, Gutiérrez-Solana LG, et al. Vanishing white matter disease in a spanish population. *Journal of central nervous system disease*. 2014;6:JCNSD. S13540.
  34. Petnikota H, Madhuri V, Gangadharan S, Agarwal I, Antonisamy B. Retrospective cohort study comparing the efficacy of prednisolone and deflazacort in children with muscular dystrophy: A 6 years' experience in a South Indian teaching hospital. *Indian J Orthop*. 2016;50(5):551-7.
  35. McAdam LC, Mayo AL, Alman BA, Biggar WD. The Canadian experience with long-term deflazacort treatment in Duchenne muscular dystrophy. *Acta Myol*. 2012;31(1):16-20.
  36. Guiraud S, Davies KE. Pharmacological advances for treatment in Duchenne muscular dystrophy. *Current opinion in pharmacology*. 2017;34:36-48.
  37. Kawasaki T, Kitao T, Nakagawa K, Fujisaki H, Takegawa Y, Koda K, et al. Nitric oxide-induced apoptosis in cultured rat astrocytes: Protection by edaravone, a radical scavenger. *Glia*. 2007;55(13):1325-33.
  38. Sato T, Mizuno K, Ishii F. A novel administration route of edaravone—II: mucosal absorption of edaravone from edaravone/hydroxypropyl-beta-cyclodextrin complex solution including L-cysteine and sodium hydrogen sulfite. *Pharmacology*. 2010;85(2):88-94.
  39. Parikh A, Kathawala K, Tan CC, Garg S, Zhou X-F. Development of a novel oral delivery system of edaravone for enhancing bioavailability. *International journal of pharmaceutics*. 2016;515(1-2):490-500.

40. Rothstein JD. Edaravone: a new drug approved for ALS. *Cell*. 2017;171(4):725.
41. Tu X-k, Yang W-z, Wang C-h, Shi S-s, Zhang Y-l, Chen C-m, et al. Zileuton reduces inflammatory reaction and brain damage following permanent cerebral ischemia in rats. *J Inflammation*. 2010;33(5):344-52.
42. Rabouw HH, Langereis MA, Anand AA, Visser LJ, de Groot RJ, Walter P, et al. Small molecule ISRIB suppresses the integrated stress response within a defined window of activation. *Proceedings of the National Academy of Sciences*. 2019:201815767.
43. Palam L, Gore J, Craven K, Wilson J, Korc M. Integrated stress response is critical for gemcitabine resistance in pancreatic ductal adenocarcinoma. *Cell death & disease*. 2015;6(10):e1913-e.
44. Nguyen HG, Conn CS, Kye Y, Xue L, Forester CM, Cowan JE, et al. Development of a stress response therapy targeting aggressive prostate cancer. *Science translational medicine*. 2018;10(439):eaar2036.
45. Yu W, Sheng M, Xu R, Yu J, Cui K, Tong J, et al. Berberine protects human renal proximal tubular cells from hypoxia/reoxygenation injury via inhibiting endoplasmic reticulum and mitochondrial stress pathways. *Journal of translational medicine*. 2013;11(1):24.
46. Li Z, Geng Y-N, Jiang J-D, Kong W-J. Antioxidant and anti-inflammatory activities of berberine in the treatment of diabetes mellitus. *Evidence-Based Complementary Alternative Medicine*. 2014;2014.
47. Parry GJ, Rodrigues CM, Aranha MM, Hilbert SJ, Davey C, Kelkar P, et al. Safety, tolerability, and cerebrospinal fluid penetration of ursodeoxycholic acid in patients with amyotrophic lateral sclerosis. *Journal of Clinical Neuropharmacology*. 2010;33(1):17-21.
48. Boatright JH, Nickerson JM, Moring AG, Pardue MT. Bile acids in treatment of ocular disease. *Journal of ocular biology*. 2009;2(3):149.
49. Foster SL, Kendall C, Lindsay AK, Ziesel AC, Allen RS, Mosley SS, et al. Development of Bile Acids as Anti-Apoptotic and Neuroprotective Agents in Treatment of Ocular Disease. *Drug Product Development for the Back of the Eye*: Springer; 2011. p. 565-76.
50. National Institute of Health. LiverTox: Clinical and Research Information on Drug-Induced Liver Injury. Ursodiol (Ursodeoxycholic Acid). 2012 [Available from: <https://www.ncbi.nlm.nih.gov/books/NBK548309/>].
51. Barros SR, Parreira SC, Miranda AF, Pereira AM, Campos NM. New insights in vanishing white matter disease: Isolated bilateral optic neuropathy in adult onset disease. *Journal of Neuro-Ophthalmology*. 2018;38(1):42-6.
52. Bell SM, Barnes K, Clemmens H, Al-Rafiah AR, Al-ofi EA, Leech V, et al. Ursodeoxycholic acid improves mitochondrial function and redistributes Drp1 in fibroblasts from patients with either sporadic or familial Alzheimer's disease. *Journal of molecular biology*. 2018;430(21):3942-53.