At Physiologically Relevant Concentrations, Valproic Acid and Lithium Carbonate Reduce Oxidative Stress in Human Astrocytoma Cells

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Abstract

Background: The pathophysiology of bipolar disorder is largely unknown; however, recent studies have suggested that metabolic dysfunction, particularly at the mitochondrial level, may represent a previously unexplored pathway. Lithium carbonate, valproic acid, and a combination of these represent the mainstay of treatment for bipolar disorder; however, the mechanisms underpinning the drugs' clinical efficacy are not well characterised. At present, such mechanistic studies use concentrations which widely differ from the known bioavailability, thus, there is a need to establish the effect of lithium carbonate, valproic acid, and combination therapy at physiologically relevant doses.

Methods: Human astrocytoma 1321N1 cells were treated for 4, 24, and 48 hours. The MTT method was used to detect cytotoxicity upon drug treatment. Reactive oxygen species (ROS) production was quantified by dichlorofluorescin diacetate fluorescence.

Results: Upon H_2O_2-induced cellular stress, cell viability was significantly reduced; however, lithium exhibited a protective effect. In the absence of the stressor, the drugs had no negative effect on 1321N1 cellular viability. All the drug treatments exhibited protection against H_2O_2-induced ROS accumulation with lithium, bringing it closer to the control baseline.

Conclusion: The findings contribute to the understanding of the drugs' biological effects, particularly as oxidative stress reducers. Furthermore, it highlights the need for research using comparable physiologically relevant models. This may advance the discovery of diagnostic biomarkers and new research approaches to the diagnosis of bipolar disorder.

INTRODUCTION

There is growing evidence for the connection between mitochondrial alterations in psychiatric disorders and psychiatric symptoms in mitochondrial disorders. There are high degrees of overlapping comorbidities and drug efficacies, suggesting that mood affective disorders

incorporate components of mitochondrial dysfunction in their pathogenesis. Research has described mitochondrial structural abnormalities in patients with bipolar disorder (BPD) such as smaller surface areas and abnormal cellular distribution,[1] mitochondrial DNA mutations, and polymorphisms,[2,3] as well as widespread brain energy metabolism abnormalities.[4] It is also common for *bona fide* mitochondrial disorders to be comorbid with psychotic symptoms (71% of patients[5]), which are often misdiagnosed as BPD and/or schizophrenia.[6,7] All of these observations support the role of mitochondria in the clinical presentation of psychosis. Mitochondrial pathology can be a consequence of genetic susceptibility,[7] dysregulation of neurotransmitter systems,[8] or environmental insults such as exposure to famine, infections, toxins, or substance abuse,[9,10] all of which are also risk factors for BPD.[11]

Bipolar Disorder

BPD is a chronic mood affective disorder characterised by episodic behavioural disturbances of mania (or hypomania) and depression. The current International Classification of Diseases (ICD) nosological classification of the disorder divides it into BPD type I (BPD-I), BPD type II (BPD-II), cyclothymic disorder, and recurrent manic episodes not otherwise specified (NOS).[12] While BPD-I is the most severe type, with alternating episodes of mania and depression which often lead to hospitalisation, BPD-II is characterised by hypomania and depression.[12,13] The lifetime prevalence is 0.4–1.0% for BPD-I and 3.0–4.0% for BPD-II.[13] When compared to the general public, a person with BPD has a 20-fold higher risk of suicide.[13] Individuals with BPD often require long-term drug treatment aimed at preventing relapses and recurrences. In the UK, lithium, valproic acid (VPA), olanzapine, and quetiapine are the main therapeutic drugs used for BPD.[14]

Lithium

Lithium is a mood stabiliser, used as a prophylactic agent in BPD as well as in the management of manic or hypomanic episodes. It is a naturally occurring water-soluble ion which does not bind to plasma proteins and is characterised by linear pharmacokinetics (proportional amount of drug administered and its blood concentration). Only lithium carbonate (LICA) and lithium citrate integrate the British National Formulary (BNF). Although the bioavailability of the two preparations is similar,[15] peak lithium concentration is 10% higher in LICA preparations[16] thus, this is most widely prescribed.[17] Lithium does not undergo metabolism and is almost exclusively excreted by the kidneys (renal clearance values of 10–40 mL/minute, strongly correlated with renal function).[18] Lithium is able to cross the blood–brain barrier (BBB) and its brain concentration after therapeutic dosing has been reported to sit between 0.5–1.3-fold the serum concentration, peaking after 24 hours of administration.[19-22] With that, brain concentrations at therapeutic LICA dosing sit between 0.20–1.30 mM; however, currently available *in vitro* studies have not significantly addressed this. For instance, Lopes-Ramos et al.[23] used 2.50–7.50 mM LICA in pheochromocytoma PC12 rat cell culture, De-Paula et al.[24] used 0.02–2.00 mM LICA, and Kurauchi et al.[25] used 1.0–5.0 mM LICA in primary rat hippocampal neuronal cultures.

Valproic Acid

VPA is an anticonvulsant drug, generally used in the treatment of epilepsy. In addition to this, VPA has other clinical indications, including BPD, given its mood-stabilising properties. The absorption of VPA is complete and rapid from the gastrointestinal tract, reaching peak plasma concentration within 4 hours of oral tablet administration.[26] Its metabolism is complex and the majority of the administered dose is excreted in urine in the form of various metabolites.[26,27] Unlike lithium, VPA binds well to plasma proteins (>90%), particularly albumin.[26,28] This binding is saturable, with free VPA fraction higher when total plasma concentrations are elevated.[28] The unbound portion is considered pharmacologically-active and able to cross the BBB, with reports of brain to serum ratios varying from 0.068–0.540 after VPA administration.[29-32] As such, it is estimated that, at therapeutic doses, brain concentration sit between 20–325 µM; however, available *in vitro* studies have not actively addressed this: Tan et al.[33] used 1 mM VPA in primary rat cortical cells; Wang et al.[34] used primary rat astrocyte cells and 0.3–1.2 mM VPA; and Zhang et al.[35] used human glioblastoma U87 cell line and 2–16 mM VPA.

Thus, the aim of this study was to investigate the putatively protective effects of physiologically

relevant concentrations of LICA and VPA on cytotoxicity and oxidative stress in astrocytoma 1321N1 cells. Findings from this study are the first to identify the biological effects of these drugs, alone and in combination, on astrocytoma cellular biology using physiologically relevant doses.

METHODS

Reagents

LICA, VPA, and all other chemicals were procured from Sigma-Aldrich (Haverhill, UK), unless otherwise specified. All chemicals were of high commercial grade.

Cell Culture

1321N1 (human astrocytoma) cells were cultured in complete growth medium made from Dulbecco's modified Eagle's medium high glucose GlutaMAX™ plus 10% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin. The cells were maintained at 37 °C in a humidified 5% carbon dioxide atmosphere. Approximately $1x10^4$ cells were harvested per assay and incubated for 24 hours in complete growth medium before proceeding. Cells from passage 39–49 were used.

Drugs

LICA (Li_2CO_3, 73.89 g/mol) and VPA ($C_8H_{15}NaO_2$, 166.19 g/mol) were resuspended in water and filter sterilised before use. LICA stock was prepared at a concentration of 10 mM at room temperature, whereas VPA stock was 3 mM and stored at 4 °C, as per the manufacturer's recommendations. The drugs were warmed to 37 °C for successive experiments. The physiologically relevant working concentrations of LICA and VPA were selected on the basis of previously mentioned pharmacokinetic evidence.[19-22,29-32]

Morphology Studies

Cell morphology was monitored at various time points during drug treatment by obtaining brightfield microscopy pictures using the ZOE™ Fluorescent Cell Imager (Bio-Rad). All images were obtained at 175x magnification.

Measurement of Cell Viability

To evaluate the effects of LICA and VPA on cell viability levels, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide MTT assays were used. Cells seeded in 96-well plates for 24 hours were treated with 100 µL/well of treatment and control solutions. The cells were exposed for different periods of time (4, 24, and 48 hours) before 40 µL MTT (5 mg/mL) were added to each well. The cells were then incubated for 2 hours at 37 °C, followed by the addition of 80 µL dimethyl sulfoxide (DMSO) to solubilise the purple formazan crystals. The amount of dye released from metabolically active cells was measured at 620 nm with a microplate reader (Tecan Sunrise™) immediately after DMSO addition. Viability was determined by dividing the absorbance of treated cells by that of untreated (vehicle control) cells.

Quantification of Intracellular Reactive Oxygen Species Levels

Intracellular ROS production was assessed using a ROS-sensitive fluorescent probe, 2',7'-dichlorofluorescin diacetate (DCFDA). Briefly, cells were incubated in black-walled 96-well plates with DCFDA (10 µM/well) for 30 minutes, in dark conditions and at 37 °C. The fluorescent dye-loaded cells were washed once in Dulbecco's phosphate-buffered saline before the addition of 100 µL/well of treatment and control solutions. ROS production was detected as an increase in fluorescence, using the VICTOR™ X3 microplate reader (PerkinElmer) set to do 1-second reads/well at 485 nm excitation and 535 nm emission.

A Model of Cytotoxic and Oxidative Stress Conditions

1321N1 cells were exposed to two concentrations of H_2O_2 (120 and 480 µM), which were co-administered with LICA and/or VPA and incubated for 24 hours prior to MTT or DCFDA assay. This dismutated, non-radical, semi-stable ROS served as stimuli mimicking physiological cytotoxic and oxidative stress conditions in BPD.[36,37]

Statistical Analysis

Unless otherwise specified, all data is expressed as mean ± standard error of the mean (SEM). Data from at least three independent triplicate experiments was analysed using GraphPad to give n=1. Grubb's outlier test was used for all studies to establish any significant outliers. The variance in data sets was analysed using the Mann–Whitney test followed by the T-test. For three or more

groups, variance was assessed by using Bartlett's test with data sets not reaching significance studied by Kruskal–Wallis test followed by Dunn's test. Significance was reached when $p<0.05$.

RESULTS

Lithium Carbonate and Valproic Acid have no Effect on Cell Viability Following Exposure in 1321N1 Cells

The effect of physiologically relevant concentrations of LICA and VPA on cell survival was firstly investigated. MTT analysis indicated that after exposure, neither 1.3 mM LICA, 325 µM VPA, nor their combination (COMBO) significantly affected 1321N1 cell viability (Figures 1a, 1b, and 1c). At 48 hours of exposure, there is a trend for cell viability decrease with COMBO treatment; however, it is not statistically significant (Figure 1c). This is further evidenced by the accumulation of debris observed with brightfield imaging (Figure 1d). At 48 hours, untreated 1321N1 cells are non-confluent and well-organised in layers; however, upon LICA treatment numerous cells appear rounded, withered, disconnected, and floating in the media (Figure 1d). Upon VPA treatment, there is a noteworthy observation of distinctively thinner and longer cells at 48 hours of treatment, which may be indicative of cytoskeletal changes rather than cell death-related pathways (Figure 1d). Therefore, use of these drugs at physiologically relevant concentrations does not affect cell viability but do appear to impact on cellular morphology.

Valproic Acid, but not Lithium Carbonate, Decreases Reactive Oxygen Species Accumulation in 1321N1 Cells

The next study investigated the mechanism through which this cellular stress could be regulated. The possibility that LICA and VPA generated free radicals was assessed using the DCFDA assay. During the time of incubation, it was shown that VPA altered baseline intracellular ROS production by significantly reducing it from 100.0±1.3% to 69.3±2.3% (Figure 1e). Conversely, neither LICA (99.8±3.1%) nor COMBO (99.6±4.7%) affected DCFDA fluorescence (Figure 1e). Thus, VPA exposure, but not LICA or a combination of therapies, decreases ROS accumulation in human astrocytoma cells.

Lithium Carbonate, but not Valproic Acid or a Combination Treatment, Significantly Protects 1321N1 Cells from H_2O_2-Induced Cell Death

Using H_2O_2 as a physiological cellular stress model,[36,37] the effect of LICA and VPA on cell viability was assessed. At 480 µM H_2O_2 treatment, the three drug treatments displayed significantly reduced 1321N1 cell viability, being unsuccessful at restoring cell viability compared to their respective drug-only counterpart (Figure 2a). Only LICA significantly protected 1321N1 from cellular death when compared to H_2O_2-only treatment (Figure 2a). Cell viability in the absence of LICA was 65.6±0.8% for 120 µM H_2O_2 and 50.1±1.7% for 480 µM H_2O_2. In contrast, in the presence of LICA, cell viability was maintained near baseline at 94.8±2.3% for 120 µM H_2O_2 and 71.6±3.4% for 480 µM H_2O_2. Notably, COMBO did not produce any statistically significant protective effect when compared to H_2O_2-only treatment or its drug-only equivalent (at 120 µM H_2O_2). Microscopically, an apparent increase in cell number is present upon drug treatment when compared to H_2O_2-only (Figure 2c). This cellular stressor also triggers cell ballooning ameliorated by all treatments tested presently (Figure 2c).

Lithium Carbonate, Valproic Acid, and their Combination Significantly Attenuate H_2O_2-Induced Reactive Oxygen Species Accumulation in 1321N1 Cells

To evaluate the effect of LICA and VPA on the H_2O_2-induced formation of ROS, DCFDA fluorescence was assayed. LICA significantly reduced ROS production compared to H_2O_2 treatment, bringing it to a comparable level to its drug-only counterpart thus exhibiting a protective effect (Figure 2b). Conversely, VPA and its co-treatment with 120 µM H_2O_2 significantly reduced ROS and are comparable to each other (Figure 2b). Interestingly, VPA co-treatment with 480 µM H_2O_2 resulted in significantly higher ROS build-up than drug-only but close to the 100% baseline. These findings may relate to the relative DCFDA fluorescence of 1321N1 cells, given their significantly decreased viability upon said co-treatment (Figure 2a). That said, there was a decrease in cell viability from 65.6±0.8% in 120 µM H_2O_2-treated cells to 50.1±1.7%

following exposure to 480 µM H_2O_2. This was coupled with a decrease in ROS accumulation from 138.3±3.4% in 120 µM H_2O_2-treated cells to 108±1.9% following exposure to 480 µM H_2O_2. Lastly, COMBO was effective at reducing ROS production both when compared to H_2O_2 and drug treatment (Figure 2b). Taken together, these findings demonstrate that, at physiologically relevant concentrations, all three drug treatments attenuate ROS build-up with LICA being most effective at returning it to a vehicle-comparable baseline.

DISCUSSION

The long-term management of BPD is complex. Both LICA and VPA are recommended by the National Institute for Health and Care Excellence (NICE) as first-line treatment and, indeed, these are the most commonly prescribed drugs for long-term maintenance.[38] In the present study, the cytotoxic effects of LICA and VPA at physiologically relevant concentrations were investigated in human astrocytoma 1321N1 cells.

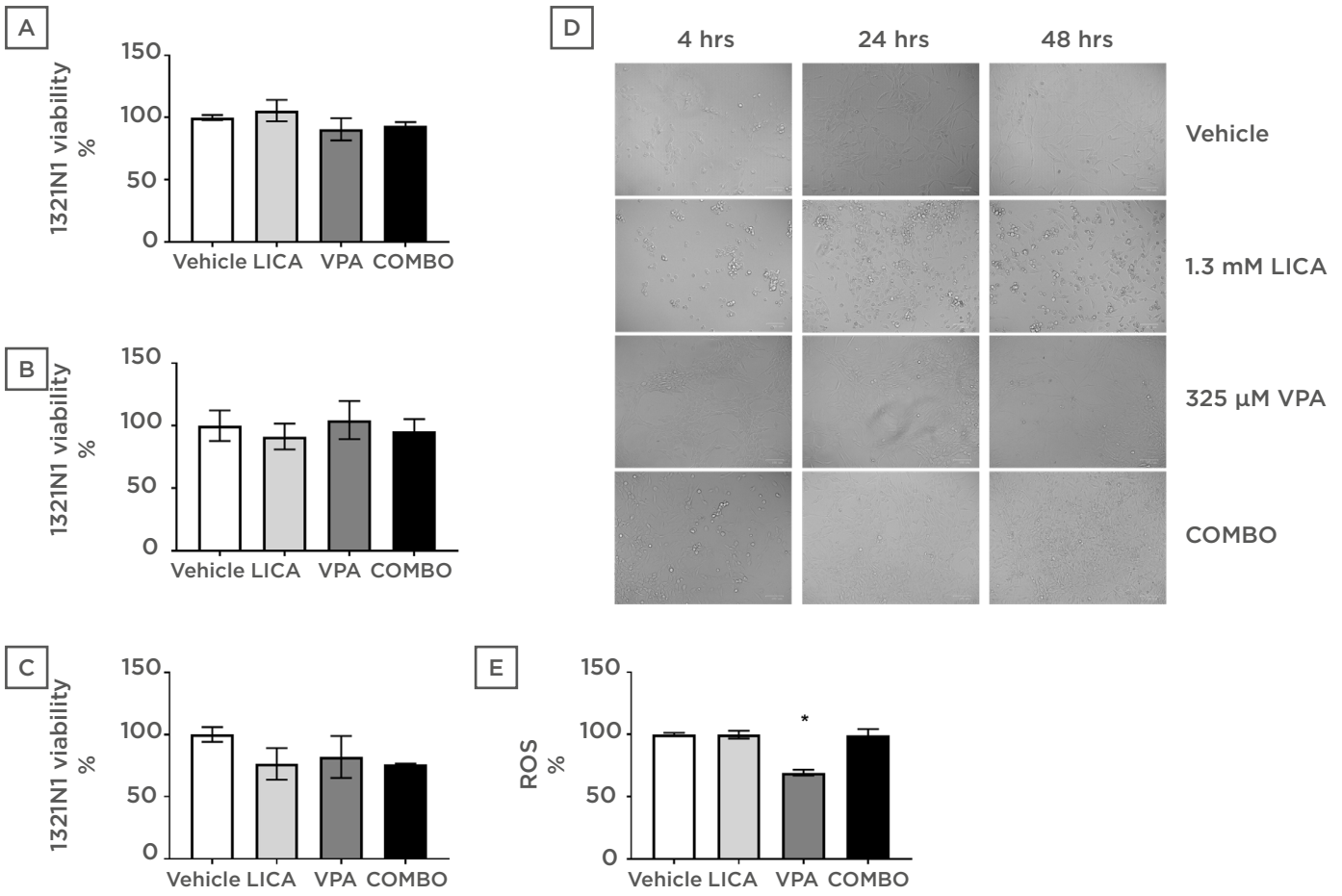

Figure 1: Physiologically relevant concentrations of valproic acid, but not lithium carbonate, significantly reduce oxidative stress in astrocytoma cells.

1321N1 astrocytoma cells were exposed to LICA (1.3 mM), VPA (325 µM), or a combination of the two (COMBO) for 4 (a), 24 (b), and 48 (c) hours. A-C: Cell viability was assessed by MTT assay. D: Morphology was assessed by microscopy with brightfield images. Scale bar represents 100 µm, magnification 175x. E: Oxidative stress was assessed by ROS accumulation using DCFDA dye for 1 hour. Data are presented as mean ± S.E.M with n of 3-4. Data were analysed using one-way ANOVA and compared to vehicle control with significance observed for ROS accumulation in VPA-treated cells compared to vehicle-treated cells. *p<0.05 versus vehicle for LICA and VPA.

DCFDA: 2',7'-dichlorofluorescin diacetate; LICA: lithium carbonate; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide; ROS: reactive oxygen species; VPA: valproic acid.

The present findings demonstrated that in the absence of cellular stressors, none of the presently tested drug treatments affected cellular viability at 4, 24, or 48 hours (Figures 1a, 1b, and 1c). Furthermore, only VPA significantly affected intracellular ROS accumulation, reducing it below baseline levels (Figure 1e). The findings were consistent with the proven safety of these drug treatments, making them the most commonly prescribed medication for BPD.[14]

The present study used a generally unreactive endogenous ROS, H_2O_2, to mimic physiological conditions in BPD. This ROS is present in very low intracellular concentrations under normal physiological conditions but can considerably increase via feed-forward systems under pathological settings.[36,37] The cell viability study demonstrated that only LICA significantly restored cell viability when compared to H_2O_2 (Figure 2a). Nevertheless, all drug treatments significantly ameliorated cell ballooning triggered by H_2O_2 (Figure 2c). This may be indicative of a time-dependent response to the stressor which has not been investigated in the present study.

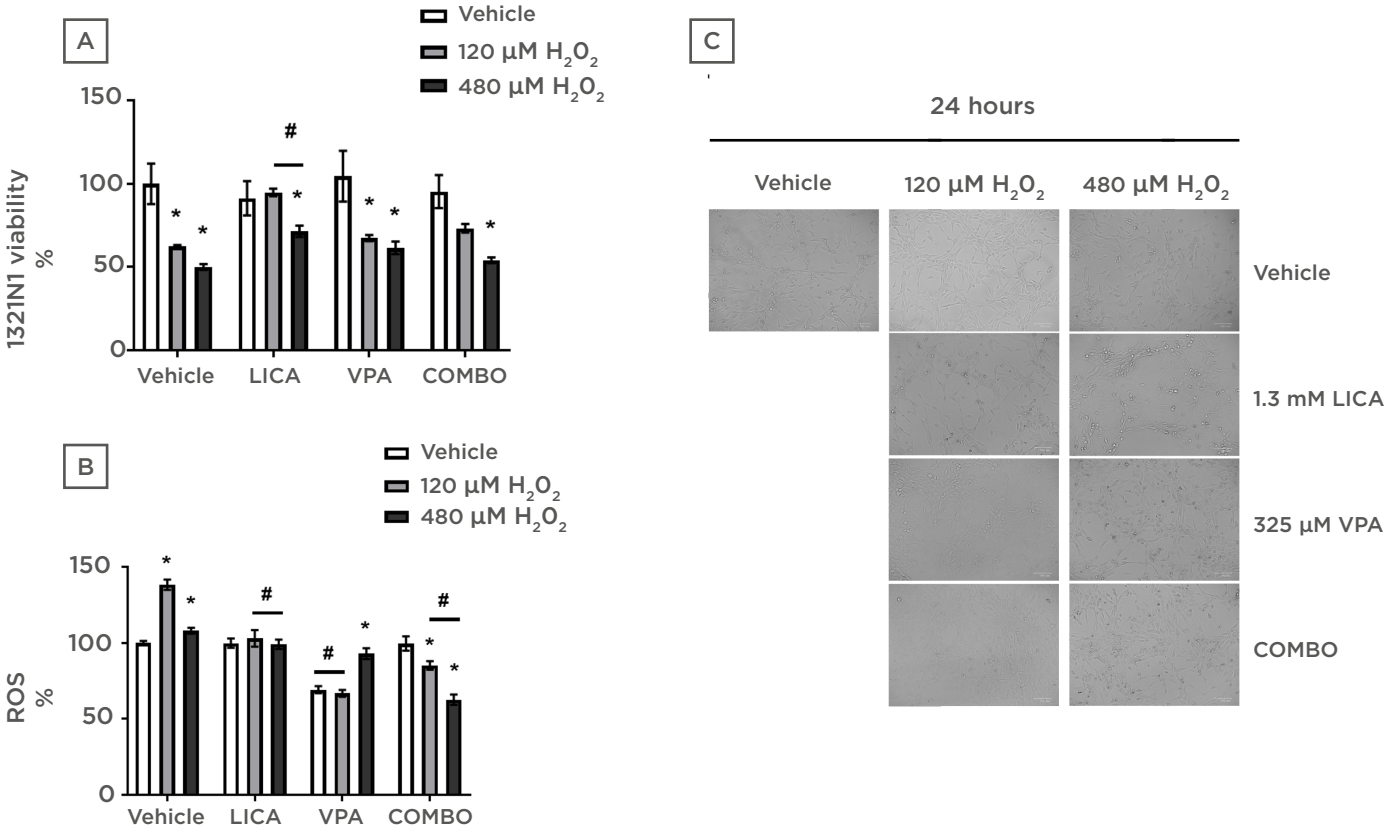

Figure 2: Physiologically relevant concentrations of both lithium carbonate and valproic acid attenuate oxidative stress in astrocytoma cells whilst only lithium carbonate protects against H_2O_2-induced cell death.

1321N1 astrocytoma cells were exposed to LICA (1.3 mM), VPA (325 µM), or a combination of the two (COMBO) in the presence and absence of hydrogen peroxide (120 and 480 µM H_2O_2) for 24 hours. A) Cell viability was assessed by MTT assay. B) Oxidative stress was assessed by ROS accumulation using DCFDA dye for 1 hour. C) Morphology was assessed by microscopy with brightfield images. Scale bar represents 100 µm, magnification 175x. Data are presented as mean ± S.E.M with n of 3-4. Data was analysed using two-way ANOVA and compared to vehicle control for drugs or vehicle for H_2O_2. Significance was observed for cell viability and ROS accumulation when treated 120 and 480 µM H_2O_2 versus the vehicle. *p<0.05 versus vehicle for H_2O_2. Significance was also observed in cell viability and ROS accumulation measurements for LICA-treated and COMBO-treated cells with H_2O_2 co-treatment (120 and 480 µM) versus vehicle-treated cells with H_2O_2 co-treatment (120 and 480 µM H_2O_2). Significance was also observed for ROS accumulation studies for VPA-treated cells treated with vehicle or 120 µM H_2O_2 versus the vehicle for the drug. #p<0.05 versus vehicle for LICA and VPA.

Furthermore, a growing body of research has demonstrated that oxidative damage from ROS is involved in neuropsychiatric disorders.[39] Previous studies have demonstrated that astroglial cells are more susceptible to oxidative stress because of poor antioxidant protection.[40] To test the protective effect of physiologically relevant concentrations of LICA and VPA on H_2O_2-induced oxidative stress, intracellular ROS in 1321N1 cells was monitored by DCFDA assay. At 120 µM H_2O_2, H_2O_2-induced ROS was significantly reduced by the three tested drug treatments (Figure 2b). This suggests that these treatments could also be effective against downstream targets of ROS responsible for astroglial death such as lipid peroxidation.[40] This, allied to the reduced ROS production, would be in accordance with inhibition of oxidative stress and prevention of cell damage. However, the mechanism through which this may occur remains inconclusive in this study, as it may be a result of free radical-scavenging activity (for instance, catalase, glutathione peroxidase, or superoxide dismutase), altered mitochondrial membrane permeability, or a combination of these. At 480 µM H_2O_2, H_2O_2-induced ROS was significantly reduced by LICA and COMBO (Figure 2b). In these conditions, although VPA reduced ROS levels, this was not significant when compared to H_2O_2-only treated cells (Figure 2b). Furthermore, COMBO not only significantly prevented ROS build-up caused by 480 µM H_2O_2 co-treatment, it also reduced this accumulation below its drug-only baseline. This unexpected finding likely correlates to the reduction in cell viability present in this treatment (Figure 2a).

Previous studies have shown that H_2O_2 regulates the balance between bcl-2 and bcl-2-associated X protein (Bax), altering mitochondrial membrane permeability, activating caspase cascades, and upregulating p53, ultimately leading to apoptosis.[11,40] Furthermore, the bcl-2 family of regulator proteins plays an important role in apoptosis and cellular injury during neuropsychiatric pathology progression, including BPD.[11] The process of apoptosis is characterised by morphological changes including decrease in cell volume and chromatin condensation in the nucleus. The morphological findings of treatment with LICA and, to a lesser extent, VPA, could be indicative of this (Figure 2c). Further studies should aim to confirm whether the treatments were inducers of apoptosis in 1321N1 cells, by use of staining agents such as Hoechst 33342.[41] Such methodology would allow for determination of the suggestive apoptogenic effect. Another noteworthy observation was the presence of dark intracellular inclusions after COMBO treatment and, to a lesser extent, VPA (Figure 2c). These could be significant misfolded protein aggregations, which often underlie the toxicity associated with neurodegenerative and neuropsychiatric disorders.[42]

Additionally, the findings in this study should be contemplated within the context of practical limitations. The 1321N1 cell line is commonly used in basic molecular and cellular biology research relating to astroglial cell function[43,44] but is derived from U-118MG cells which are human malignant glioblastomas.[45] The oncogenic modifications associated with such may have altered cellular regulation processes, differing from normal astroglial cells.[46,47] Yet, immortal cancerous cell lines such as 1321N1 are often used in research as they provide several advantages such as being cost-effective, easy to use, and providing a virtually unlimited supply of materials, whilst bypassing ethical concerns associated with animal and human testing.[46] They do, however, hold the limitation of suffering genotypic and phenotypic variation over serial sub-culturing events, therefore meaning they may not adequately represent primary cells.[46] Nevertheless, cell culture models are well-established, frequently used tools in both basic research and drug testing.[43,44,48] To surpass any limitations, further *in vitro* work could aim to confirm the findings using primary brain endothelial cells[43,44] or immortalised brain-endothelial co-cultured cell lines, two recognised BBB models for neuropharmaceutical screening.[47,49] Ultimately, to accurately address the organisational sophistication and myriad interactions between systems in the whole organism, *in vivo* studies would be necessary.

Due to the heterogeneity of BPD and lack of understanding of its pathophysiology, there is no specific biological marker which diagnoses the disease, thus current clinical practice relies on studying the patient's medical history and thorough behavioural assessment.[50] It is possible that the increasingly available research, such as that presented, will lead to a new focus on BPD within which the disease is seen as metabolic leading to new research approaches to

treatment and diagnosis. Importantly, said research should take into consideration the pharmacokinetic evidence available on these drugs, addressing physiologically relevant hypotheses such as that presented.

CONCLUSION

In summary, based on the data provided by the present study, it has been demonstrated that physiologically relevant concentrations of LICA, VPA, and COMBO do play a role on the metabolic hypothesis of BPD by affecting cell viability and ROS production thus further elucidating their clinical efficacy mechanisms. It remains to be explored the exact mechanisms by which these protective effects act on 1321N1 cells; however, this study draws attention to the different trending hypotheses that view BPD as systemic and requiring different clinical approaches as well as further research addressing treatment resistance and mechanisms of efficacy.

References

1. Cataldo AM et al. Abnormalities in mitochondrial structure in cells from patients with bipolar disorder. Am J Pathol. 2010;177(2):575-85.
2. Kato T et al. Mitochondrial DNA polymorphisms in bipolar disorder. J Affect Disord. 2001;62(3):151-64.
3. Washizuka S et al. Association of mitochondrial complex I subunit gene NDUFV2 at 18p11 with bipolar disorder in Japanese and the National Institute of Mental Health pedigrees. Biol Psychiatry. 2004;56(7):483-9.
4. Yildiz-Yesiloglu A, Ankerst D. Neurochemical alterations of the brain in bipolar disorder and their implications for pathophysiology: A systematic review of the *in vivo* proton magnetic resonance spectroscopy findings. Prog Neuropsychopharmacol Biol Psychiatry. 2006;30(6):969-95.
5. Farkas G. Psychiatric implications of mitochondrial disorders. Am J Psychiatry. 2016;11(7):8-10.
6. Mancuso M et al. Autosomal dominant psychiatric disorders and mitochondrial DNA multiple deletions: Report of a family. J Affect Disord. 2008;106(1):173-7.
7. Pereira C et al. Mitochondrial agents for bipolar disorder. Int J Neuropsychopharmacol. 2018;21(6):550-69.
8. Guo L et al. Mitochondrial dysfunction and synaptic transmission failure in Alzheimer's disease. J Alzheimers Dis. 2017;57(4):1071-86.
9. Brown JM, Yamamoto BK. Effects of amphetamines on mitochondrial function: Role of free radicals and oxidative stress. Pharmacol Ther. 2003;99(1):45-53.
10. Meyer JN et al. Mitochondria as a target of environmental toxicants. J Toxicol Sci. 2013;134(1):1-17.
11. Clay HB et al. Mitochondrial dysfunction and pathology in bipolar disorder and schizophrenia. Int J Dev Neurosci. 2011;29(3):311-24.
12. World Health Organization (WHO). ICD-10 Classification of Mental and Behavioural Disorders: Clinical Descriptions and Diagnostic Guidelines. 2016. Available at: https://www.who.int/classifications/icd/en/bluebook.pdf. Last accessed: 16 July 2019.
13. Martinsson L et al. Lithium treatment and cancer incidence in bipolar disorder. Bipolar Disord. 2016;18(1):33-40.
14. Hayes JF et al. Adverse renal, endocrine, hepatic, and metabolic events during maintenance mood stabilizer treatment for bipolar disorder: A population-based cohort study. PLoS Med. 2016;13(8):e1002058.
15. Tyrer SP et al. Bioavailability of lithium carbonate and lithium citrate: A comparison of two controlled-release preparations. Pharmatherapeutica. 1982;3(4):243-6.
16. Guelen PJ et al. Bioavailability of lithium from lithium citrate syrup versus conventional lithium carbonate tablets. Biopharm Drug Dispos. 1992;13(7):503-11.
17. Goodwin GM et al. Evidence-based guidelines for treating bipolar disorder: Revised third edition recommendations from the British Association for Psychopharmacology. J Psychopharmacol. 2016;30(6):495-553.
18. Grandjean EM, Aubry JM. Lithium: Updated human knowledge using an evidence-based approach: Part II: Clinical pharmacology and therapeutic monitoring. CNS Drugs. 2009;23(4):331-49.
19. Wraae O. The pharmacokinetics of lithium in the brain, cerebrospinal fluid and serum of the rat. Br J Pharmacol. 1978;64(2):273-9.
20. Plenge P et al. 24-hour lithium concentration in human brain studied by Li-7 magnetic resonance spectroscopy. Biol Psychiatry. 1994;36(8):511-6.
21. Jensen HV et al. Twelve-hour brain lithium concentration in lithium maintenance treatment of manic-depressive disorder: Daily versus alternate-day dosing schedule. Psychopharmacology (Berl). 1996;124(3):275-8.
22. Smith AJ et al. Improving lithium therapeutics by crystal engineering of novel ionic cocrystals. Mol pharmaceutics. 2013;10(12):4728-38.
23. Lopes-Ramos CM et al. Lithium carbonate and coenzyme Q10 reduce cell death in a cell model of Machado-Joseph disease. Braz J Med Biol Res. 2016;49(12):e5805.
24. De-Paula Vde J et al. Long-term lithium treatment increases $cpla_2$ and $ipla_2$ activity in cultured cortical and hippocampal neurons. Molecules. 2015;20(11):19878-85.
25. Kurauchi Y et al. Na+, K+-ATPase inhibition induces neuronal cell death in rat hippocampal slice cultures: Association with GLAST and glial cell abnormalities. J Pharmacol Sci. 2018:167-75.
26. Ogungbenro K et al. A physiologically based pharmacokinetic model for Valproic acid in adults and children. Eur J Pharm Sci. 2014;63:45-52.
27. Ho PC et al. Influence of CYP2C9 genotypes on the formation of a hepatotoxic metabolite of valproic acid in human liver microsomes. Pharmacogenomics J. 2003;3(6):335-42.
28. Ueshima S et al. Empirical approach for improved estimation of unbound serum concentrations of valproic acid in epileptic infants by considering their physical development. Biol Pharm Bull. 2011;34(1):108-13.
29. Vajda FJ et al. Human brain, plasma, and cerebrospinal fluid concentration of sodium valproate after 72 hours of therapy. Neurology. 1981;31(4):486-7.
30. Wieser HG. Comparison of valproate concentrations in human plasma, CSF and brain tissue after administration of different formulations of valproate or valpromide. Epilepsy Res. 1991;9(2):154-9.
31. Shen DD et al. Low and variable presence of valproic acid in human

brain. Neurology. 1992;42(3 Pt 1): 582-5.

32. Kim SW et al. Whole-body pharmacokinetics of HDAC inhibitor drugs, butyric acid, valproic acid and 4-phenylbutyric acid measured with carbon-11 labeled analogs by PET. Nucl Med Biol. 2013;40(7):912-8.
33. Tan H et al. Effect of lithium and valproic acid on kainate-induced neurotoxicity in cerebral cortical cell. Asian Journal of Chemistry. 2009;21(3):1769-74.
34. Wang CC et al. Valproic acid mediates the synaptic excitatory/ inhibitory balance through astrocytes — A preliminary study. Prog Neuropsychopharmacol Biol Psychiatry. 2012;37(1):111-20.
35. Zhang C et al. Valproic acid promotes human glioma U87 cells apoptosis and inhibits glycogen synthase kinase-3β through ERK/ Akt signaling. Cell Physiol Biochem. 2016;39(6):2173-85.
36. Huang BK, Sikes HD. Quantifying intracellular hydrogen peroxide perturbations in terms of concentration. Redox Biol. 2014;2:955-62.
37. Pietruczuk K et al. Proliferation and apoptosis of T lymphocytes in patients with bipolar disorder. Sci Rep. 2018;8(1):3327.
38. Hayes J et al. Prescribing trends in bipolar disorder: Cohort study in the United Kingdom THIN primary care database 1995–2009. PLoS One. 2011;6(12):e28725.
39. Schiavone S et al. Severe life stress and oxidative stress in the brain: From animal models to human pathology. Antioxid Redox Signal. 2013;18(12):1475-90.
40. Bao D et al. Protective effect of quercetin against oxidative stress-induced cytotoxicity in rat pheochromocytoma (PC-12) cells. Molecules. 2017;22(7):1122.
41. Liu K et al. 3-β-erythrodiol isolated from conyza canadensis inhibits MKN 45 human gastric cancer cell proliferation by inducing apoptosis, cell cycle arrest, DNA fragmentation, ROS generation and reduces tumor weight and volume in mouse xenograft model. Oncol Rep. 2016;35(4):2328-38.
42. Rajan RS et al. Specificity in intracellular protein aggregation and inclusion body formation. Proc Natl Acad Sci U S A. 2001;98(23):13060-5.
43. Suadicani SO et al. P2X7 Receptors mediate ATP release and amplification of astrocytic intercellular Ca2+ signaling. J Neurosci. 2006;26(5):1378-85.
44. Morgan SV et al. Proteomic and cellular localisation studies suggest non-tight junction cytoplasmic and nuclear roles for occludin in astrocytes. Eur J Neurosci. 2018;47(12):1444-56.
45. Pontén J, Macintyre E. Long term culture of normal and neoplastic human glia. Acta Pathol Microbiol Scand. 1968;74(4):465-86.
46. Kaur G, Dufour JM. Cell lines. Spermatogenesis. 2012;2(1):1-5.
47. Uemura T et al. Chronic LiCl pretreatment suppresses thrombin-stimulated intracellular calcium mobilization through TRPC3 in astroglioma cells. Bipolar Disord. 2016;18(7):549-62.
48. Helms HC et al. *In vitro* models of the blood–brain barrier: An overview of commonly used brain endothelial cell culture models and guidelines for their use. J Cereb Blood Flow Metab. 2016;36(5):862-90.
49. Veszelka S et al. Comparison of a rat primary cell-based blood-brain barrier model with epithelial and brain endothelial cell lines: Gene expression and drug transport. Front Mol Neurosci. 2018;11:166.
50. Brown NC et al. An updated meta-analysis of oxidative stress markers in bipolar disorder. Psychiatry Res. 2014;218(1):61-8.