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The effect of acute and chronic administration

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Abstract

Background and Purpose: Diseases of raised intracranial pressure (ICP) cause severe morbidity and mortality. Multiple drugs are utilised to lower ICP including acetazolamide and topiramate. However, the evidence for their use is unclear. We aimed to assess the ICP modulatory effects and molecular effects at the choroid plexus (CP) of acetazolamide and topiramate.

Experimental Approach: Female rats were implanted with telemetric ICP probes for physiological, freely moving 24/7 ICP recordings. Randomised cross-over studies were performed, where rats received acute (24 h) high doses of acetazolamide and topiramate, and chronic (10 days) clinically equivalent doses of acetazolamide and topiramate, all via oral gavage. Cerebrospinal fluid (CSF) secretion assays, and RT-qPCR and western blots on in vitro and in vivo CP, were used to investigate drug actions.

Key Results: We demonstrate that acetazolamide and topiramate achieved maximal ICP reduction within 120 min of administration, and in combination doubled the ICP reduction over a 24-h period. Chronic administration of acetazolamide or topiramate lowered ICP by 25%. Topiramate decreased CSF secretion by 40%. Chronic topiramate increased the gene expression of Slc12a2 and Slc4a10 and protein expression of the sodium-dependent chloride/bicarbonate exchanger (NCBE), whereas chronic acetazolamide did not affect the expression of assessed genes.

Conclusions and Implications: Acetazolamide and topiramate are effective at lowering ICP at therapeutic levels. We provide the first evidence that topiramate lowers CSF secretion and that acetazolamide and topiramate may lower ICP via distinct molecular mechanisms. Thus, the combination of acetazolamide and topiramate may have utility for treating raised ICP.

Abbreviations: AZM, acetazolamide; CA, carbonic anhydrase; CP, choroid plexus; CSF, cerebrospinal fluid; eMAP, estimated mean arterial pressure; FFT, fast Fourier transform; ICP, intracranial pressure; IIH, idiopathic intracranial hypertension; TPM, topiramate.
1 INTRODUCTION

Intracranial pressure (ICP) is a key physiological variable, allowing the brain to maintain its normal morphology within the central nervous system (Eftekhari et al., 2019). ICP is determined by brain volume, cerebral blood volume and cerebrospinal fluid (CSF) volume, and where any of these parameters are outside of physiological norms pathologies occur (Almajstorovic et al., 2020). Diseases of acutely raised ICP are associated with severe morbidity and death, whereas more chronically raised ICP is associated with headache and other neurological morbidities (Eftekhari et al., 2019; Mollan, Spitzer, & Nicholl, 2018b). These diseases include hydrocephalus, traumatic brain injury, haemorrhagic stroke and idiopathic intracranial hypertension (IIH) (Eftekhari et al., 2019).

For management of raised ICP, CSF diversion surgery, such as shunts, is often utilised. However, CSF diversion therapies are only symptomatic treatments and prone to surgical revisions, increasing cost and patient morbidity (Mollan, Davies, et al., 2018a). Pharmacological treatments include mannitol, hypertonic saline, diuretics and corticosteroids, amongst others (Eftekhari et al., 2019; Mollan, Davies, et al., 2018a). These treatments are all hampered either by side effects or are not ideal for chronic administration (Mollan, Davies, et al., 2018a). The carbonic anhydrase (CA) inhibitor acetazolamide and the pharmacologically promiscuous anticonvulsant topiramate with weak CA inhibitory properties are used clinically to reduce ICP for chronic pharmacological maintenance, given that CA inhibition reduces CSF secretion and thus reduces ICP in humans (Dodgson et al., 2000; Eftekhari et al., 2019; Supuran, 2008).

One disease where extensive research on ICP manipulation has been carried out is IIH. There is however limited evidence for the clinical efficacy of acetazolamide and topiramate and the mechanisms underlying their use. A single randomised controlled trial (RCT) demonstrated that acetazolamide reduces ICP, although ICP was not the primary outcome of the study (Wall et al., 2014). Additionally, one un-controlled study demonstrated that topiramate was equivalent in efficacy to acetazolamide (Çelebisoy et al., 2007). Otherwise, no randomised controlled trials exist to endorse their clinical efficacy at lowering ICP. The clinical effects, however, cannot be attributed to direct pharmacological action on ICP as acetazolamide and topiramate induce weight loss as a side effect (Çelebisoy et al., 2007). Weight loss is a highly efficacious and disease modifying treatment for IIH (Mollan, Mitchell, et al., 2021a). Additionally, acetazolamide and topiramate are associated with multiple adverse side effects, including paraesthesia, loss of appetite and cognitive impairment, which limits the compliance of patients due to low tolerability (Eftekhari et al., 2019; Mollan, Davies, et al., 2018a).

Given the lack of efficacy and adverse side effect profile, a Cochrane review concluded there is insufficient evidence to either reject or endorse the use of acetazolamide in IIH and no other treatments were assessed, highlighting that further studies are required to assess the efficacy of these drugs in treating IIH and other diseases of raised ICP (Lueck & McIlwaine, 2005).

Similarly, there is limited preclinical evidence to endorse the use of acetazolamide and topiramate in IIH or treating raised ICP in general. It has been shown that acetazolamide reduces CSF secretion and ICP in rodents but not through clinically relevant administration routes such as subcutaneous and intraventricular routes (Oshio et al., 2005; Scotton et al., 2019; Uldall et al., 2017). It was previously noted that acute doses of topiramate decrease ICP more than acetazolamide in sedated rats (Scotton et al., 2019). However, the mechanisms underlying this change in ICP were not elucidated and the chronic effects were not assessed. Additionally, topiramate’s action is presumed to be through a reduction of CSF secretion, although this has not been demonstrated (Scotton et al., 2019). Of note, in previous in vivo ICP studies, rodents have either been sedated or anaesthetised, limiting the recording period to short periods in an unphysiological state, where anaesthesia also modifies ICP (Eftekhari et al., 2020).

KEYWORDS
acetazolamide, cerebrospinal fluid, choroid plexus, intracranial pressure, topiramate

What is already known
- Acetazolamide and topiramate are used to reduce intracranial pressure (ICP).
- The clinical and preclinical evidence for this indication is lacking.

What does this study add
- Chronic and acute use of acetazolamide and topiramate lowers ICP, and combination has greater effect.
- Acetazolamide and topiramate induce distinct choroid plexus transcriptomic and proteomic profiles suggesting differing actions.

What is the clinical significance
- Combination of acetazolamide and topiramate may be appropriate to gain larger reductions in ICP.
- Understanding how topiramate reduces cerebrospinal fluid secretion may lead to novel drugs to reduce ICP.
A single study has assessed acetazolamide in freely moving male rats and demonstrated efficacy of acetazolamide to reduce ICP (Barbuskaite et al., 2022). In lieu of the limited clinical and preclinical data, it is imperative to establish whether acetazolamide and topiramate lower ICP, given their broad side effects profile. Because acetazolamide and topiramate are used in IIH, a predominantly female disease, this study aimed to compare the capacity and mechanisms of action of acetazolamide and topiramate to modulate ICP with both acute and chronic administration on normal ICP physiology in freely moving female rats using clinically equivalent doses.

2 | METHODS

Unless otherwise stated, all materials came from Sigma-Aldrich.

2.1 | Experimental animals

Female Sprague-Dawley rats (Taconic Biosciences, Ebyj, Denmark) from 12 weeks of age were utilised in this study. For the ICP and chronic dosing experiments, rats were maintained in cages kept under an inverted 12-h light/dark cycle with ad libitum access to standard chow and water in the Glostrup Research Institute. For CSF secretion experiments, female Sprague-Dawley rats (Janvier, Le Genest-Saint-Ise, France) from 10 weeks of age were maintained in cages under a 12-h light/dark cycle with ad libitum access to standard chow and water in the animal facility of the Faculty of Health and Medical Sciences, University of Copenhagen. All experimental procedures were approved by the Danish Animals Experiments Inspectorate (Licence numbers: 2014-15-0201-00256, 2019-15-0102-000365 and 2018-15-0201-01595) and are in compliance with the ARRIVE guidelines (Pericu du Sert et al., 2020) with the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020). After treatments and surgical procedures, rats were monitored for adverse effects.

2.1.1 | Study design

Ten rats were used for the ICP experiments; each rat was included in both the acute and chronic ICP experiments. Both the acute and chronic studies were randomised, blinded cross-over studies. In both these studies, we assessed weight, and in the short-term study, we assessed water consumption. Individual water consumption was calculated as water consumption per cage divided by 2, the number of rats in a cage. These ICP experiments were performed over two experimental rounds of five rats each. ICP rats were killed by a pentobarbitone overdose and no tissue retrieved. Twelve rats were used for the CSF flow experiments; here, rats were killed by decapitation while anaesthetised under ketamine/xylazine and no tissue was retrieved. For ex vivo choroid plexus (CP) experiments, 48 rats were utilised, 24 for each time point. There was an equal allocation to treatment and vehicle. Each rat was killed by CO2 anaesthesia and decapitation. For the chronic in vivo treatment study, a total of 24 rats were used, randomly allocated to each treatment arm; here, we assessed CP gene and protein expression, brain weight, body weight and blood pressure, at the end of this experiment rats were killed by pentobarbitone overdose. A total of 94 rats were used in the study.

2.2 | ICP telemeter implantation and ICP recording

ICP monitoring was carried out as previously described (Eftekhari et al., 2020). ICP telemeters were delivered factory calibrated, and we performed an offset test to ensure optimum accuracy in measurements and prevent telemeter drift from affecting the results as previously described (Eftekhari et al., 2020). We have previously demonstrated minimum sensor drift in longitudinal ICP recordings (Eftekhari et al., 2020). In brief, rats were anaesthetized with ketamine (100 mg kg\(^{-1}\))/xylazine (5 mg kg\(^{-1}\)) i.p. and the telemeter body was implanted in the abdominal cavity. The ICP catheter was tunnelled under the skin to the parietal bone where the sensor tip was placed and secured epidurally (TRMS4P, Kaha Sciences Ltd, Auckland, New Zealand). After surgery, the rats were placed in their home cages upon a SmartPad (TR181, Kaha Sciences) and ICP recording started. The telemeters sampled ICP at 2 kHz continuously, where the SmartPad lowpass filter at 1 kHz gave a final sampling frequency of 1 kHz. Data were acquired on LabChart software (V8.0, ADInstruments, Oxford, UK) through a PowerLab sampler (ADInstruments). After surgery and on the two following days, the rats received post-surgical treatments of subcutaneous 5-mg kg\(^{-1}\) carprofen (Pfizer, New York City, USA), 10-mg kg\(^{-1}\) enrofloxacin (Bayer, Leverkusen, Germany) and 0.03-mg kg\(^{-1}\) buprenorphine (Indivior, Hull, UK) and oral 0.4-mg kg\(^{-1}\) buprenorphine in Nutella (Ferrero, Frankfurt am Main, Germany) and were allowed to recover for up to 7 days as described earlier (Eftekhari et al., 2020). Rat weight and water intake were monitored daily for a week post-surgery. Experiments were carried out at least 10 days after implantation when the rats were considered fully recovered and ICP reached a stable baseline (Eftekhari et al., 2020). ICP experiments were carried out over two experimental rounds. Ten rats had the ICP implantation surgery and were included in both the acute and the chronic ICP study. One rat’s ICP data were excluded due to telemeter failure but was kept to prevent single housing of a rat and allow for treatment to obtain weight and water consumption data. At the end of the experiments, all animals were killed with an overdose of sodium pentobarbital.

2.3 | Drugs

Drugs were administered in a standardised manner via oral gavage, equivalent to human administration (Table 1). Acetazolamide (Diamox) (Mercury Pharma, Croydon, UK) and Topiramate (HEXAL, Holzkirchen, Germany) tablets were ground and suspended in 0.9% saline, where 0.9% saline acted as the vehicle control. The metabolism and elimination of topiramate in female rats is similar to humans, that is, predominantly eliminated in the urine unmetabolized. This contrasts with male
TABLE 1  Human doses of acetazolamide and topiramate and corresponding clinically equivalent doses using the FDA first in man equation, assuming a human weight of 60 kg.

<table>
<thead>
<tr>
<th></th>
<th>Acute dose</th>
<th>Chronic daily dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Human dose (max daily)</td>
<td>Rat dose</td>
</tr>
<tr>
<td>Acetazolamide</td>
<td>4 g</td>
<td>125 mg</td>
</tr>
<tr>
<td>Topiramate</td>
<td>200 mg</td>
<td>6.25 mg</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Human pharmacokinetics</th>
<th>Rat pharmacokinetics</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetazolamide</td>
<td>1 (6–9)</td>
<td>1 (3–6)</td>
<td>Kumar et al. (2021); Ritschel et al. (1998)</td>
</tr>
<tr>
<td>Topiramate</td>
<td>1–4 (20–30)</td>
<td>0.7 (2.5) (males)</td>
<td>Matar and Tayem (2014); Schneiderman (1998)</td>
</tr>
</tbody>
</table>

Note: Pharmacokinetic properties of acetazolamide and topiramate when orally administered, described as Tmax (T1/2) in hours. Tmax is time to maximum blood drug concentration, and T1/2 is the drug half-life.

rats, where a substantial proportion is metabolised and eliminated in the faeces. Consequently, female rats may constitute a better model to assess the role of topiramate on ICP in relation to humans (Caldwell et al., 2005). Sex differences and human comparison in metabolism of acetazolamide are unknown. The pharmacokinetic profiles can be found in Table 1.

For the acute testing of acetazolamide (125 mg per rat), topiramate (6.25 mg per rat) and the combination (acetazolamide [125 mg per rat] and topiramate [6.25 mg per rat]), doses were administered orally, calculated as previously described (Scotton et al., 2019). Here, allometric scaling was used with the multiplication factor of 6.2 for rats, with the assumption of a 60-kg weight for a human and 250-g weight for a rat. Rats were randomly allocated to each drug where both rats in each cage received the same drug to allow for water consumption measurement, via random number generation, and received each drug in a cross-over fashion. There was a 3-day washout period between each drug. ICP was continuously measured for 24 consecutive hours after drug administration.

For chronic testing, rats received the drugs via oral gavage over the course of 10 days followed by a 4-day washout, and drug allocation was randomly assigned by random number generation. Rats received acetazolamide (31 mg) and topiramate (1.56 mg) twice daily (BD) equating to a total daily dose of 62 and 3.1 mg day⁻¹, respectively. In a pragmatic study design, rat received the first dose between 6:00 and 8:00 in the morning and received the second dose from 14:00 to 16:00 in the afternoon, giving an administration interval ranging from 6 to 10 h. On the first day of treatment, the rats received the total daily dose in one administration. Rats were randomly allocated to each drug and received each drug in a cross-over fashion. Rats were co-housed for the entirety of the measurement period. Weight was assessed during the study.

2.4  |  ICP analysis

For the acute and chronic ICP analysis, the mean ICP 24 h prior to administration was the baseline, where ICP after start of experiment was expressed relative to baseline, due to individual variability and potential sensor drift over the length of the experiment (Eftekhari et al., 2020). The spectral analysis of the ICP waveform was carried out on 5-min stable sections of ICP, that is, without movement artefacts. The baseline value corresponds to a 5-min stable section of ICP within the hour prior to drug administration. Fast Fourier transformation (FFT) was carried out on raw, unprocessed 1-kHz data. The inbuilt FFT function of LabChart was utilised where spectral power was obtained utilising the following settings: FFT size of 128 K with a 93.75% window overlap, with a Hann (cosine-bell) data window model. The spectra generated were an average of 23 FFTs. Spectral data presented were relative to baseline spectral power. Analysis of all ICP data was blinded. Various situations can lead to data loss, including telemetry dropouts, removal of animals due to adverse events and computer failures amongst others. A specific large-scale loss of data occurred in the combination treatment group, where computer failure occurred when all were on treatment. Due to randomisation and blinding, the degree of data loss was not apparent until after the study concluded. In the case of missing data, data were not imputed.

2.5  |  Blood pressure measurements

Blood pressure was assessed in non-implanted rats following 10 days of chronic daily dosing with the drugs, 2 h after the final drug dose. These rats were anaesthetised with ketamine/xylazine, and blood pressure was assessed 15 min after injection. Blood pressure was assessed via a non-invasive blood pressure cuff, NIBP systems (ADInstruments). Blood pressure was measured three times, separated by 1 min; the mean of the three measurements is presented. Mean arterial blood pressure was estimated using the following equation (2 x diastolic pressure) + systolic pressure/3). Data extraction and analysis was blinded. Given the nature of measuring blood pressure using plethysmography under anaesthesia, we can only comment if the compounds cause profound changes in blood pressure.

2.6  |  CSF secretion

To assess the CSF secretion rate in rats, a LI-COR Pearl small animal bioimager (LI-COR Biosciences, Lincoln, NE, USA, 800-nm channel,
85-μm resolution) was utilised as previously described (Steffensen et al., 2018). In brief, female Sprague-Dawley rats received 6.25 mg of topiramate or vehicle via oral gavage 80 min prior to anaesthesia induction with ketamine/xylazine. This dose was selected because it provided the most robust acute reduction in ICP in our testing. Skin of topiramate or vehicle via oral gavage 80 min prior to anaesthesia was reflected from the cranium and muscle removed from over the occipital bone. A 0.5-mm burr hole was drilled (1.3 mm posterior, 1.8 mm lateral to bregma), whereby a dye (IRDye 800 CW carboxylate, 10 μM, P/N 929-08972 [LI-COR], delivered by use of a Hamilton syringe [RN 0.40, G27, a20 Agnths’]) was injected into the right lateral ventricle. Animals were immediately placed into the LI-COR, secured in a custom-made tooth holder to fix the head and images were taken every 30 s for 3 min. Data were analysed as previously described (Steffensen et al., 2018). Surgery and data analysis was blinded to treatment.

2.7 | Choroid plexus (CP) explant incubations

Female Sprague-Dawley rats were killed by rising concentration of CO₂, prior to the removal of the lateral and fourth ventricle CP. The CP from one animal were pooled and were incubated in DMEM/F12 (supplemented with 10% FBS, 1% penicillin/streptomycin, 4 mM l-glutamine, 26 mM sodium selenate and 10 ng mL⁻¹ epidermal growth factor) in a humidified incubator at 37°C and 5% CO₂. Explants were incubated for 3 or 24 h with 100 μM acetazolamide, 1 μM topiramate or vehicle (media and 0.2% DMSO). Following completion of incubation, CP explants were snap frozen and stored at −80°C prior to RNA extraction via tri-reagent. CP explant experiments at both 3- and 24-h timepoints were carried out over two experimental rounds.

2.8 | Chronic treatment on choroid plexus (CP) gene expression

Female Sprague-Dawley rats received chronic dosing of the drugs for 10 days. Rats were anaesthetised, blood pressure was measured prior to killing by decapitation, and brains were weighed and CP dissected out in ice-cold PBS. Pooled CP were snap frozen and stored at −80°C prior to RNA extraction via the tri-reagent method. TaqMan Gene Expression Master Mix (Applied Biosystems) where samples were run in triplicate. Gene expression was assessed using TaqMan primer/probes sets (Applied Biosystems); the genes assessed were Aqp1 (Rn_00562834_m1), Aqp4 (Rn_01401327_s1), Slc12a2 (Rn_00582505_m1), Slc4a5 (Rn_01420902_m1), Slc4a10 (Rn_00710136_m1), Car2 (Rn_01462065_m1) and Car3 (Rn_01461970_m1). Expression of the target genes was normalised to the expression of Actb (Rn_00667869). Loading of the RT-qPCR plate and analysis was blinded. The relative expression of the gene is presented as the geometric mean of the fold change (2^ΔΔCt, where ΔΔCt was derived from (ΔCt subject) – (mean ΔCt control)). ΔCt = (Ct gene of interest – Ct reference gene).

2.9.1 | Western blots

Western immunoblotting was performed to assess protein expression. Here, protein from CP from the 10-day dosing experiment was extracted using the tri-reagent method. Protein pellets were solubilised in a Tris-based buffer (Tris-base: 50 mM, NaCl: 150 mM, sodium dodecyl sulphate: 2% w/v, pH 7.5). Protein concentration was determined by a Bradford assay. Twenty-five micrograms of protein was loaded per sample in LDS sample buffer with reducing agent (DDT), in a 17-well bolt 4% to 12% Bis-Tris gel. Samples were run over two gels in parallel via gel electrophoresis. Proteins were blotted simultaneously using an iBlot machine onto polyvinylidene difluoride membranes. Gels were cut based on the molecular weight bands. Membranes were blocked for 1 h in blocking buffer (5% molecular grade skimmed milk powder in Tris-buffered saline 0.1% Tween 20) incubated with primary antibodies (1:500 lamin-B1 [Rb polyclonal anti-lamin-1B, ab65986, lot: GR3215884-24, RRID:AB_1140888, Abbam, Cambridge, UK]; 1:1000 Aqp1 [Rb monoclonal anti-aquaporin AAP1, ab168387, RRID:AB_2810992, lot: GR121951-6, Abcam]; 1139AP 1:500 sodium-dependent chloride/bicarbonate exchanger [NCBE], mouse anti-NCBE non-commercial and knockout validated [Christensen et al., 2020]; and carbonic anhydrase II (CA II) [1:2000 Rb monoclonal anti-CA II ab124687, RRID:AB_10972000, lot: GR3444071-5, Abcam]) overnight at 4°C in fresh blocking buffer. Membranes were subsequently washed and incubated with secondary antibodies conjugated to horse radish peroxidase (1:10,000 goat anti-rabbit, PO448, RRID:AB_2617138, lot:20073563, Dako Denmark, Glostrup, Denmark and 1:2000 goat anti-mouse, P0447, RRID:AB_2617137, lot: 20078279, Dako) for 1 h at room temperature. Bands were imaged using ECL (Amersham ECL, GE Healthcare, Chicago, IL) and imaged with a camera (LAS-4000 Luminescent Image Analyzer, Fujifilm, Tokyo, Japan). Protein expression was determined by densitometry on ImageJ, and data were normalised to the expression lamin-B1, and then, the vehicle treated rats within its own membrane. We attempted to assess Na–K–Cl cotransporter 1 (NKCC1) expression, but the antibody provided indistinct bands and smears and was therefore not assessed. Analysis of the blots was blinded to the treatment. The western blotting was carried out, and the experimental detail conforms with BJP guidelines (Alexander et al., 2018).
2.9.2 | Power calculation

An a priori power calculation was performed for the ICP analysis. Here, the power calculation was performed based on previous work based on physiological manipulation of ICP through diet-induced obesity to give an indication on what variance one can expect from ICP manipulation, given that no comparable pharmacological studies existed (Westgate et al., 2022). Based on a difference of mean of 1.62 mmHg with a standard deviation of 1.54, using four groups with α = 0.5 and 80% power for a repeated measures ANOVA yields 10 rats. No power calculation was performed for the other aspects of the work; rather, they were either secondary outcomes of the main assessment of ICP or numbers were selected based of experience in the case of the CSF secretion experiments and the RT-qPCR.

2.10 | Statistical analysis

GraphPad Prism (V9.1, GraphPad Software Inc, San Diego, CA, USA) was utilised to carry out statistical analysis on data, where data are presented as mean ± SD unless otherwise stated. ‘n’ in all instances represents biological replicates. Appropriate statistical tests were utilised following Shapiro–Wilk normality test. If the Shapiro–Wilk normality test returned a normal distribution, a one-way repeated measures ANOVA or one-way ANOVA with post hoc Dunnett’s test was used. Where data met the assumptions for a one-way repeated measures ANOVA but had data randomly missing, a mixed-effects analysis model was used. This was paired with a post hoc Dunnett’s test for comparisons only with the vehicle and post hoc Holm–Sidak’s test for comparisons between all groups. Welch ANOVA with post hoc Dunnett’s T3 test where the standard deviations differed in normally distributed data. Where data varied over two factors, two-way repeated measures ANOVA with post hoc Tukey’s test was utilised. Post-hoc tests were run only if F achieved P < 0.05 and there was no significant variance in homogeneity. If data were not normally distributed and varied by one factor, Friedman’s test with post hoc Dunnett’s test was used. For a normally distributed pair wise analysis, an unpaired t test was used. P < 0.05 was considered significant. For correlation analysis, Pearson’s r was used for parametric data and Spearman’s correlation coefficient ρ for non-parametric data. The manuscript conforms with the BJP guidance for experimental design, analysis and reporting (Curtis et al., 2022).

2.11 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander, Fabbro, et al., 2021a; Alexander, Mathie, et al., 2021b).

3 | RESULTS

3.1 | Acute effect on ICP

Acetazolamide and topiramate are utilised clinically to modulate ICP; therefore, we aimed to evaluate the effects of these drugs on ICP in fully conscious, freely roaming rats using a randomised cross-over design (Figure 1a). ICP was measured for 24 h after oral administration of drug (Figure 1b). Baseline ICP was similar across the groups at the start of each cross-over (Table 2).

Both topiramate and acetazolamide significantly reduced ICP relative to vehicle over a 24-h period (Figure 1c). When topiramate and acetazolamide were administered in combination, ICP was significantly lower relative to vehicle and to both acetazolamide and topiramate when administered in isolation, demonstrating a persistent combined effect over the 24-h period (Figure 1c). The ICP normalised 18 h after administration for both acetazolamide and topiramate. Having established that both drugs and the combination lower ICP, we determined the lowest ICP achieved. At 3 h after administration, acetazolamide and topiramate produced the lowest ICP relative to baseline; in contrast, the maximal ICP reduction for the combination was at 13 h after administration. Here, the combination generated a greater ICP reduction than the greatest ICP reduction achieved by acetazolamide and topiramate in isolation (Figure 1d).

Assessing the initial 3 h after drug administration to understand the speed of ICP reduction, ICP reduction reached its nadir between 90 and 120 min after administration (Figure 1e). Here, at 120 min after administration, acetazolamide, topiramate and the combination robustly lowered ICP to a similar degree (Figure 1f).

In a dose comparison analysis, we demonstrated that a 65-mg bolus of acetazolamide did not yield a significant reduction in ICP whereas a 125-mg bolus reduced ICP relative to vehicle over 24 h (Figure 2a,b). In contrast, both doses of topiramate lowered ICP, where there was no difference in efficacy of a single a 6.25-mg dose of topiramate compared with the more clinical single 3.1-mg dose over 24 h, indicating a dose maximum has been reach for ICP reduction relative to vehicle (Figure 2d,e). Given that 3 h after treatment, the greatest reduction in ICP occurred with the higher doses of acetazolamide and topiramate, we compared to the lower doses at this time point. Here, dose did not modify the maximum ICP-lowering capacity of acetazolamide (Figure 2c) or topiramate (Figure 2f), again indicating that a dose maximum has been reached for ICP reduction relative to vehicle.

Both acetazolamide and topiramate are associated with weight loss in humans; additionally, acetazolamide is a diuretic. Consequently, we assessed weight and water consumption 24 h after drug administration. Relative to vehicle, both topiramate and acetazolamide and the acetazolamide–topiramate combination induced weight loss (Table 2). To determine if these drugs alter water consumption, water intake was measured over the 24-h ICP was recorded. Topiramate did not alter water consumption whereas acetazolamide and the acetazolamide–topiramate combination increased water consumption by 50% relative to vehicle, as expected given that acetazolamide is a diuretic (Table 2).
To further interrogate the effects of acetazolamide, topiramate and combination on the ICP phenotype, we performed spectral analysis on the ICP waveforms (Figure S1). None of the treatments altered ICP waveforms in the 0–0.25 Hz bin (Figure 3a).

Acetazolamide conferred increased spectral power relative to vehicle in the 0.25–0.5 and 0.5–0.75 Hz frequency bins at 45, 90 and 180 min after administration, where the other treatments did not alter spectral power relative to vehicle (Figure 3b,c).
Additionally, there was a significant increase in spectral power at 0.75–1.0 Hz 45 and 90 min after acetazolamide administration compared with vehicle treatment, whereas topiramate and the combination had no effect (Figure 3d). In the frequency bin 1.0–2.0 Hz, the frequencies associated with the respiratory rate of rats, both acetazolamide and the combination increased spectral power 45 min after administration, and this was also observed for acetazolamide 90 min after administration (Figure 3e). This can be attributed to increased respiratory effort given that rat respiratory rate is between 1 and 2 Hz (Eftekhari et al., 2020). Topiramate did not alter respiratory spectral power relative to vehicle.

### 3.2 | Topiramate and CSF secretion

It is well established that acetazolamide decreases CSF secretion (Barbuskaite et al., 2022; Oshio et al., 2005). However, the effect of topiramate on CSF secretion is unknown. Given that topiramate lowers ICP, we assessed its capacity to modulate CSF secretion. Using a validated CSF secretion model, we demonstrated that topiramate reduces dye flow rate to 58% of vehicle, 90 min after administration, where ICP is also reduced from baseline (Figure 4b,c).

### 3.3 | Chronic effects on ICP

Given that both acetazolamide and topiramate reduce ICP acutely, we aimed to assess the effects of a clinically relevant dose and chronic dosing regimen of acetazolamide and topiramate on ICP in a randomised cross-over study (Figure 5a). ICP was measured for 10 days with twice daily drug administrations (Figure 5b). Baseline ICP, the ICP 24 h prior to start of Day 1, was similar at the start of each cross-over (Table 3).

Over the course of 10 days, ICP was significantly reduced in both acetazolamide and topiramate groups throughout the testing period.
Intracranial pressure (ICP) waveforms. The effect of acetazolamide (125 mg), topiramate (6.25 mg) separately and in combination on raw 1-kHz ICP waveforms at 45, 90, 180 and 320 min after administration. The effects of tested drugs on ICP spectral power in the (a) 0–0.25, (b) 0.25–0.5, (c) 0.5–0.75, (d) 0.75–1.0 and (e) 1.0–2.0 Hz frequency bins. The dotted line on (a)–(e) represents Δ0 spectral power, that is, no deviation from baseline spectral power. Mixed-effects model with post hoc Dunnett’s test. \( P < .05 \). * denotes comparison between acetazolamide and vehicle, # denotes comparison between combination and vehicle. Data presented as mean ± SD. n = 9 for vehicle, acetazolamide and topiramate n = 5 for combination. ‘n’ represents biological replicates in all instances. For the combination group, n = 5 is presented due to data loss.
relative to vehicle; there was no difference in the magnitude of ICP reduction between acetazolamide and topiramate (Figure 5c).

When assessing the diurnal pharmacodynamics of these drugs over the 10-day administration period, topiramate treatment caused ICP to remain significantly lower than vehicle treatment over the course of the average 24 h (Figure 5d). In contrast, although acetazolamide reduced ICP, the effects did not last a whole 24-h period; the ICP-lowering effects of acetazolamide wear off 8–10 h after the final administration where the ICP then decreases following the morning drug administration. Correspondingly, topiramate caused ICP to be lower than acetazolamide intermittently from 7 PM to 4 AM. Chronic administration of topiramate did not affect the weight of the rats, however acetazolamide caused a relative weight loss at Days 5 and 10 of administration relative to vehicle (Table 3). Weight loss was not a predictor for the ICP response to the drugs, where change in ICP versus change in weight did not correlate with acetazolamide (Day 5: \( r = 0.29, P = 0.4 \), Day 10: \( p = 0.35, P = 0.35 \)) or topiramate treatment (Day 5: \( r = 0.15, P = 0.70 \), Day 10: \( r = 0.4, P = 0.31 \)).

According to the Monro-Kellie hypothesis, blood pressure, brain volume and CSF volume have the capacity to alter ICP (Kim et al., 2012). Consequently, in a separate experiment, we assessed the blood pressure and brain weights in rats following chronic administration of acetazolamide and topiramate. Both acetazolamide and topiramate were found not to profoundly alter mean arterial blood pressure after 10 days of administration compared with vehicle (Table 3). We used brain mass as a surrogate for brain volume. Acetazolamide and topiramate did not alter brain mass relative to whole body mass compared with vehicle after 10 days of treatment (Table 3).

### 3.4 | Gene expression at the choroid plexus (CP)

Given the ICP-lowering effects of acetazolamide and topiramate, we assessed the capacity of these drugs to modulate genes associated with CSF secretion in both 3- and 24-h incubations in isolated CP explants, thus isolated from neuronal input.

At 3-h incubation, we observed no differences in gene expression of the genes assessed (Figure S2A–G). In contrast, the 24-h incubation conferred a modest change in gene expression. Slc12a2, encoding for the \( \text{Na}^+\)/\( \text{K}^+\)/\( 2\text{Cl}^- \) exchanger (\( \text{NKCC1} \)), expression was raised with acetazolamide compared with vehicle, whereas topiramate did not alter the expression of \( \text{Slc}12a2 \) (Figure S2H). Moreover, acetazolamide reduced the expression of \( \text{Car}3 \), encoding for carbonic anhydrase 3, whereas topiramate did not alter \( \text{Car}3 \) expression (Figure S2O). The expression of \( \text{Aap}1 \), \( \text{Car}2 \), \( \text{Aap}4 \), \( \text{Slc}4a5 \) and \( \text{Slc}4a10 \) were unaltered by acetazolamide and topiramate.

Given the chronic ICP-lowering effects of acetazolamide and topiramate, we assessed their effect on genes associated with CSF...
**FIGURE 5**  Chronic administration of acetazolamide (AZM) and topiramate (TPM) lower intracranial pressure (ICP). Continuous ICP recording in freely moving rats treated with oral vehicle (VEH) (0.9% saline), topiramate (1.5-mg BD) and acetazolamide (31-mg BD) in a cross-over experiment. (a) Timeline of experimental paradigm, a randomised cross-over experiment. (b) Continuous ICP over 10 days of the treatment, where baseline was the 24 h mean the day prior to start of administrations. (c) Mean daily ICP of each treatment. (d) The mean hourly ICP relative to baseline from Days 2 to 10 with P value matrix for each hour, where grey = P < 0.05 and white = P > 0.05. Shaded areas on graph represent time windows for oral gavage. (e) Weight change relative to start of baseline at 5 and 10 days of treatment. The black lines in (b)–(d) represent Δ0 mmHg and baseline percentage (100%) in (e). Friedman’s test with post hoc Dunnett’s test for (c). (d,e) A two-way repeated measures ANOVA with post hoc Tukey’s test. Data are presented as mean ± SEM for (b) and (d), mean ± SD for (c) and (e). Ten rats were implanted but one telemeter failed, consequently n = 9 for ICP data but n = 10 for (e). ‘n’ represents biological replicates in all instances. *P < 0.05.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Acetazolamide</th>
<th>Topiramate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline ICP (mmHg)</strong></td>
<td>4.3 ± 1.6</td>
<td>3.9 ± 1.7</td>
<td>3.84 ± 1.7</td>
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<tr>
<td><strong>eMAP (mmHg)</strong></td>
<td>113.0 ± 10.8</td>
<td>108.8 ± 15.2</td>
<td>118.2 ± 10.9</td>
</tr>
<tr>
<td><strong>Brain weight (mg)</strong></td>
<td>2059 ± 70</td>
<td>2012 ± 57</td>
<td>2083 ± 93</td>
</tr>
<tr>
<td><strong>Brain weight (% body weight)</strong></td>
<td>0.73 ± 0.06</td>
<td>0.73 ± 0.05</td>
<td>0.77 ± 0.06</td>
</tr>
</tbody>
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Note: Estimated mean arterial pressure (eMAP) and brain weight from treatment effect study. Repeated measures ANOVA with post hoc Dunnett’s test for ICP. One-way ANOVA with post hoc Dunnett’s test for eMAP and brain weight. Mean ± SD, n = 9 for ICP, n = 8 for brain weight and eMAP.

**TABLE 3** Basal intracranial pressure (ICP) for the chronic cross-over treatment study.
FIGURE 6  The effects of acetazolamide and topiramate at choroid plexus. Gene and protein expression of choroid plexus from chronic (10 day) acetazolamide (A) (31-mg BD) topiramate (T) (1.56-mg BD) and vehicle (V) (0.9% NaCl) treated rats. Gene expression of (a) Slc12a2, (b) Slc4a5, (c) Slc4a10, (d) Aqp1, (e) Aqp4, (f) Car2 and (g) Car3. (h) Representative immunoblots, membranes were cut by molecular weight bands. The sodium-dependent chloride/bicarbonate exchanger (NCBE), Lamin and aquaporin (AQP1) columns represent one set of gels and the same well number on the membrane. Lamin and carbonic anhydrase II (CA II) columns represent a second set of gels and the same well number on a membrane. G, glycosylated; NG, non-glycosylated. Protein expression of (i) sodium-dependent chloride/bicarbonate exchanger (NCBE), (j) CA II, (k) total AQP1, (l) glycosylated AQP1, (m) glycosylated AQP1 and (n) glycosylated/non-glycosylated ratio. One-way ANOVA with post hoc Dunnett’s test for (a)–(g). Welch ANOVA with post hoc Dunnett’s T3 test for (i), (h), (m) and (n). Kruskal–Wallis test with post hoc Dunn’s test for (j) and (l). Geometric mean ± geometric SD for (a)–(g) and mean ± SD for (i)–(n). In most cases, n = 8. Where numbers are less than 8, data have been excluded, as outliers according to a Grubbs’ test, or due to technical failure such as insufficient material to run a sample. n represents biological replicates. *P < 0.05.
secretion at the CP in the same 10-day experimental paradigm with no cross-over. Topiramate conferred an increase in expression of Slc4a10 (Figure 6c) and Slc12a2 (Figure 6a), whereas acetazolamide had no effect on expression of these genes. Furthermore, no change in the expression of Car2, Car3, Aap1, Aap4 or Slc4a5 with either acetazolamide or topiramate treatment was found. To further interrogate these findings, we assessed the protein expression. We demonstrate that NCBE (Slc4a10) protein expression was higher in topiramate treated rats compared with controls (Figure 6i). Acetazolamide reduced the expression of total aquaporin AQP1 compared with vehicle (Figure 6k). The protein expression of carbonic anhydrase II (CA II) was unaltered by either treatment (Figure 6j).

4 | DISCUSSION

Acetazolamide and topiramate are used clinically to reduce ICP in humans; however, the clinical and preclinical evidence supporting their use is minimal. This study aimed to assess the capacity of acetazolamide and topiramate to modulate ICP in freely moving rats uninhibited by anaesthesia or sedation. Importantly, we used clinically relevant doses and examined the effects of chronic dosing of these drugs in female rats, because the majority of IIH patients are women. We also aimed to assess the potential mechanisms underlying any ICP changes.

We provide evidence that both acetazolamide and topiramate reduce ICP when given acutely at maximal clinical doses, and at chronic clinically equivalent doses, in freely moving rats. The degree of ICP reduction was similar between acetazolamide and topiramate in the acute setting. We noted dose dependency with acetazolamide but no dose dependency with topiramate at the doses selected. Topiramate reduced ICP to a similar degree as our previous study, where it reduced ICP when administered subcutaneously and in the food of sedated rats (Scotton et al., 2019). Our acetazolamide data are in contrast to some studies and corroborate others, where acetazolamide has been found to either reduce ICP or have no ICP modulatory effect (Barbuskaite et al., 2022; Scotton et al., 2019; Williamson et al., 2019). These previous studies differ from ours through differing administration routes, use of sedation and anaesthesia and the use of inappropriate vehicles: all of which can independently alter ICP (Eftekhari et al., 2020; Uldall et al., 2017). This variation in methodology prevents direct comparison of results.

The only study with comparable methods demonstrated a similar degree of acetazolamide mediated ICP reduction in male rats (Barbuskaite et al., 2022). We assessed the effects of acetazolamide and topiramate in freely moving female rats in normal physiological conditions with a clinically relevant administration route and patient drug formulations.

The ICP reduction induced by these drugs was quick, reaching the maximal ICP reduction 120 min after administration; acetazolamide had a similarly rapid effect in male rats (Barbuskaite et al., 2022). This suggests that high doses of these drugs may have utility in the acute setting to lower ICP in humans, such as prophylactic mitigation of traumatic raised ICP, although this requires future studies. Acetazolamide and topiramate, administered in combination, reduced ICP to a greater extent than individual administration over 24 h and achieved a greater peak ICP reduction. Given our data, future studies assessing the effects of the combination treatment in patients are warranted.

With chronic administration of these drugs in female rats, there was minimal tachyphylaxis. The degree of ICP reduction we observed with chronic acetazolamide is mirrored by that in male rats (Barbuskaite et al., 2022). The equivalent effect of these drugs on ICP mirrors an open label trial where they conferred equivalent retinal outcomes in IIH patients (Celebisoy et al., 2007). With the clinically relevant dosing regimen, acetazolamide’s ICP-lowering effect waned 8 h after administration, mirroring the acetazolamide half-life in rat of 3–6 h (Kumar et al., 2021). In contrast, topiramate sustained its effects for the whole dosing regimen; this is longer than the reported rat half-life of 2.5 h in male rats (Matar & Tayem, 2014). However, topiramate metabolism is fundamentally different between male and female rats, where males highly modify and inactivate topiramate and females largely do not modify topiramate. Given that topiramate metabolism in female rats is similar to that in humans, who largely eliminate unaltered topiramate with a half-life of 20–30 h, female rats could have a longer topiramate half-life than male rats which is consistent with the present ICP data (Caldwell et al., 2005; Schneiderman, 1998).

This suggests that, clinically, acetazolamide could lose its treatment effect overnight due to large dosing intervals, potentially leading to ICP rebound and headache. Indeed, IIH patients experience worse headaches in the morning (upon awakening), mirroring a human half-life of 6–9 h, where headache severity is associated with ICP in IIH patients (Mollan et al., 2019; Mollan, Wakerley, et al., 2021b; Ritschel et al., 1998). Consequently, topiramate could be considered preferable for maintaining a lower ICP over sleeping hours where there is a large dosing interval. Moreover, topiramate is a headache prophylactic and thus may confer additional efficacy in reducing raised ICP headache.

Disturbance of ICP waveforms is a feature of raised ICP pathophysiology in humans and rodents, where treatment normalises these disturbances (Botfield et al., 2017; Eide & Sorteberg, 2010; Westgate et al., 2022; Williamson et al., 2019). Acetazolamide has broad effects on ICP waveforms, corroborating previous work (Williamson et al., 2019). In contrast, topiramate has no effects on ICP waveforms. However, the clinical implications are unclear. An assessment of how these drugs modulate ICP waveforms in raised ICP is required to determine potential clinical benefit.

The capacity of topiramate to lower ICP is maximal at the doses given in this study; thus, the observation that combining acetazolamide and topiramate enhances maximal ICP reduction suggests differing but complementary modes of action to reduce ICP. According to Monro–Kellie, blood pressure, brain volume and CSF volume dictate ICP (Kim et al., 2012). Here, acetazolamide and topiramate did not alter brain weight. Moreover, it has been demonstrated that topiramate does not alter intracranial blood volume, whereas...
Acetazolamide increases cerebral blood volume but does not modify peripheral mean arterial blood pressure in male rats (Akerman & Godsbys, 2005; Arngrim et al., 2014; Barbuskaite et al., 2022; Laux & Raichle, 1978; Taki et al., 2001). Consequently, CSF volume must be altered. It is incontrovertible that acetazolamide reduces CSF secretion (Barbuskaite et al., 2022; Oshio et al., 2005). To our knowledge, we provide the first evidence that topiramate modulates CSF secretion, and to a similar degree as acetazolamide (Barbuskaite et al., 2022).

Acetazolamide lowers ICP through inhibiting CA activity and therefore CSF secretion (Barbuskaite et al., 2022). Systemic CA inhibition has clear physiological hallmarks: diuresis and acidosis. These factors likely do not contribute to the capacity of acetazolamide to lower ICP (Barbuskaite et al., 2022). Topiramate does not produce these physiological hallmarks at the doses given in this study. Acetazolamide induces diuresis, indicated by increased water intake, whereas topiramate does not increase water intake, as demonstrated by us and others (Scotton et al., 2019). This diuresis, however, is unlikely to be a major component of acetazolamide mediated ICP reduction, given that acetazolamide lowers ICP in nephrectomised rats (Barbuskaite et al., 2022). Moreover, it has previously been demonstrated that acetazolamide induces acidosis, whereas topiramate does not (Scotton et al., 2019). In keeping with this, acetazolamide, in contrast to topiramate, increased ICP spectral power at frequencies associated with breathing (1–2 Hz), indicating increased respiratory effort. This corroborates previous studies that demonstrate that acetazolamide increases tidal volume in normal rats (Bell & Hauouzi, 2009).

Topiramate has higher inhibition constants (K) than acetazolamide in two of the most highly expressed CA isozymes in rat CP: CA II (TPM $K_i = 100 \text{nM}$, AZM $K_i = 1 \text{nM}$) and CAIV (TPM $K_i = 200 \text{nM}$, AZM $K_i = 10 \text{nM}$), and the same is seen for human CA homologues (Barbuskaite et al., 2022; Dodgson et al., 2000; Supuran, 2008). Moreover, in rat brain extracts, topiramate had higher inhibition constants than acetazolamide (Dodgson et al., 2000). Hence, topiramate is a weaker inhibitor of CP relevant CA isozymes than acetazolamide.

Given the lack of the physiological hallmarks of CA inhibition with topiramate, being a weaker CA inhibitor than acetazolamide, and given the combined effects of acetazolamide and topiramate on ICP reduction, the literature and our data suggest that CA inhibition is not the primary mode of action of topiramate for ICP reduction. This does not exclude some degree of CA inhibition contributing to ICP reduction with topiramate, particularly at supra-clinical doses. Topiramate is pharmacologically promiscuous, inhibiting CAs, kainate/AMPA receptors, Na$_v$ channels and L-type Ca$^{2+}$ channels, and is a positive allosteric modulator of GABA$_A$ receptors (Shank et al., 2000). The effects of manipulating these channels on CSF secretion and ICP are unknown, but given the present work, it should be investigated.

In keeping with the suggestion that topiramate and acetazolamide could have alternate modes of action, they conferred differential effects on gene expression in the CP, the organ responsible for CSF secretion. In keeping with previous studies, this study showed no change in the CP gene expression of Car2 and Aqp1 with acetazolamide (Barbuskaite et al., 2022; Uldall et al., 2017). This is in contrast to protein level, where acute acetazolamide increased expression of aquaporin AQP1 and Na$^+$/K$^+$ ATPase (Uldall et al., 2017). Topiramate increased the expression of genes associated with CSF secretion, in apparent paradox to reducing CSF secretion. This may suggest molecular compensation to conserve normal CSF secretion rates. Moreover, we demonstrated that NCBE protein expression was increased following topiramate treatment.

4.1 Limitations

Although we have demonstrated that these drugs reduce ICP in normal rats, we did not assess this in a model of raised ICP. Given that these drugs are used to treat raised ICP, it will be prudent to assess whether these drugs reduce raised ICP in disease models such as diet-induced obesity or hydrocephalus (Botfield et al., 2017; Westgate et al., 2022). It is well established that acetazolamide and topiramate have a large portfolio of side effects in humans including alterations in cognition. However, we did not assess behaviour in the present experiment, preventing an understanding of how the individual doses affect cognition. We did not assess serum or CSF electrolyte levels in this study. Given that acetazolamide and topiramate are known to modify electrolytes and electrolyte disturbances can influence ICP, we cannot exclude the possibility that drug induced electrolyte changes modified ICP (Belotti et al., 2010; Kenny, 1972, 1973; Scotton et al., 2019).

Blood pressure was measured in the anaesthetized state using plethysmography. Given the sensitivity and physiological considerations with these, we cannot rule out that topiramate and acetazolamide have small effects on blood pressure that may alter ICP. Future studies in freely moving rats will help further the understanding of the blood pressure effects of these drugs.

Our study used both clinical doses and regimen for the chronic ICP experiment, but it did not fully mimic the clinical administration. Clinically, both acetazolamide and topiramate doses are tapered up to maximise efficacy while minimising side effects, whereas we gave a clinically equivalent full dose immediately. Moreover, we have not assessed the serum values of these drugs.

5 Conclusions

This study provides for the first time comparative preclinical evidence for both acute and chronic dosing of acetazolamide and topiramate and that they both lower ICP independent of weight loss. We also provide evidence that topiramate lowers ICP through modulating CSF dynamics, and the effects of these drugs in combination enhance ICP reduction. Future clinical studies assessing the efficacy of the combination of these drugs at lowering ICP are warranted. Understanding how topiramate modifies ICP may lead to novel therapeutics with a more favourable side effect profile.
AUTHOR CONTRIBUTIONS
Connar Stanley James Westgate, Rigmor Højland Jensen, Nanna MacAulay and Sajedeh Eftekhari conceptualised and designed the study. Connar Stanley James Westgate, Christina Kamp-Jensen, Ida Marchen Egerod Israelsen, Trine Toft-Bertelsen, Jonathan Henry Wardman and Christian Ahm Jensen performed experiments. Connar Stanley James Westgate, Christina Kamp-Jensen, Trine Toft-Bertelsen and Christian Ahm Jensen analysed the data. Connar Stanley James Westgate drafted the manuscript. All authors reviewed and edited the manuscript for intellectual and scientific content.

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CONFLICT OF INTEREST STATEMENT
The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT
Data are available upon reasonable request.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR
This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Natural Products Research, Design and Analysis, Immunoblotting and Immunohistochemistry and Animal Experimentation and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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