Modulation of brain cation-Cl\(^{-}\) cotransport via the SPAK kinase inhibitor ZT-1a

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The SLC12A cation-Cl\(^{-}\) cotransporters (CCC), including NKCC1 and the KCCs, are important determinants of brain ionic homeostasis. SPAK kinase (STK39) is the CCC master regulator, which stimulates NKCC1 ionic influx and inhibits KCC-mediated efflux via phosphorylation at conserved, shared motifs. Upregulation of SPAK-dependent CCC phosphorylation has been implicated in several neurological diseases. Using a scaffold-hybrid strategy, we develop a novel potent and selective SPAK inhibitor, 5-chloro-N-(5-chloro-4-((4-chlorophenyl)(cyano)methyl)-2-methylphenyl)-2-hydroxybenzamide (“ZT-1a”). ZT-1a inhibits NKCC1 and stimulates KCCs by decreasing their SPAK-dependent phosphorylation. Intracerebroventricular delivery of ZT-1a decreases inflammation-induced CCC phosphorylation in the choroid plexus and reduces cerebrospinal fluid (CSF) hypersecretion in a model of post-hemorrhagic hydrocephalus. Systemically administered ZT-1a reduces ischemia-induced CCC phosphorylation, attenuates cerebral edema, protects against brain damage, and improves outcomes in a model of stroke. These results suggest ZT-1a or related compounds may be effective CCC modulators with therapeutic potential for brain disorders associated with impaired ionic homeostasis.

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Regulation of cellular ion transport is critical for brain water homeostasis. Vectorial ion transport across apical and basolateral membranes of the choroid plexus epithelium (CPE), accompanied by transport of water, cotransported or osmotically obligated, results in daily cerebrospinal fluid (CSF) secretion of >500 cc/day into brain ventricular spaces. Impaired ion homeostasis in CPE can result in hydrocephalus (accumulation of excess CSF in the brain ventricles), as in the settings of intraventricular hemorrhage (IVH) and infection. Coordinated transmembrane influx and efflux of ions and water is also necessary for cell volume homeostasis in neurons, glia, and blood–brain barrier (BBB) endothelial cells. Impaired cell volume regulation following ischemic stroke and other brain injuries can lead to cytotoxic cell swelling, disruption of BBB integrity, and cerebral edema. Hydrocephalus requires neurosurgical treatment by permanent, catheter-based CSF shunting, whereas treatment of ischemic stroke may require decompressive hemicraniectomy. Both of these often morbid procedures have been used for decades with minimal innovation. Recent advances in vascular stroke therapy such as clot lysis by recombinant tissue plasminogen activator (rTPA) and clot removal by radiologically guided thrombectomy are appropriate for fewer than 8% of ischemic stroke patients. Thus, development of novel pharmacological modulators of brain ion transport is warranted to provide nonsurgical alternatives to current morbid treatments of these neurological disorders with altered volume homeostasis.

The electroneutral cation–Cl– cotransporters (CCCs) are secondary-active plasmalemmal ion transporters that utilize electrochemically favorable transmembrane gradients of Na+ and/or K+, established by the ouabain-sensitive Na+, K+-ATPase to drive transport of Cl– (and Na+/K+) into or out of cells. In epithelial cells under most physiological conditions (with the possible exception of choroid plexus), the Na+-driven CCCs NCC, NKCC1, and NKCC2 ("NKCCs") function as Cl– importers, whereas the Na+-independent KCC1-4 ("KCCs") function as Cl– exporters. These evolutionarily conserved transporters are of particular importance in regulation of ion and water homeostasis in mammalian central nervous system (CNS). The coordinated regulation of CCC function is important for cell volume regulation in most brain cells, preventing excessive cell swelling or shrinkage in response to osmotic or ischemic stress. The central importance of CCCs to choroid plexus regulation of CSF homeostasis has been recently recognized.

The Ste20-type Ser–Thr protein kinases SPAK (SPS1-related proline/alanine-rich kinase) and OSR1 (oxidative stress-responsive kinase 1) are considered master regulators of the CCCs. SPAK and OSR1 are activated by phosphorylation in the regulatory "T-loop" (SPAK Thr233 and OSR1 Thr185) by one of four WNK family kinases, "with no lysine" (K) protein kinases. The WNK and SPAK/OSR1 protein kinases drive Cl– influx by phosphorylation and activation of the Na+-driven CCC members (NCC, NKCC1, and NKCC2) while inhibiting Cl– efflux by phosphorylation and inactivation of KCCs. This reciprocal regulation of the Na+- and K+-driven CCCs by WNK–SPAK/OSR1 ensures tight coordination of cellular Cl– influx and efflux, and is essential for regulation of cell volume and epithelial transport in multiple tissues.

SPAK-regulated, CCC-mediated ion transport has been implicated in the pathogenesis of multiple brain pathologies associated with impaired brain ion and water homeostasis. Experimental ischemic cerebral edema is associated with increased phosphorylation of the SPAK/OSR1 T-loop and of NKCC1 (Thr203/Thr207/Thr212) in both neurons and oligodendrocytes, and in BBB endothelial cells. Mouse germ-line SPAK knockout significantly reduces ischemia-induced NKCC1 phosphorylation, infarct volume, axonal demyelination, and cerebral edema following ischemic stroke.

Chlorid plexus NKCC1 is an essential mediator of ion transport in the CSF hypersecretory response that drives development of post-hemorrhagic hydrocephalus. The 3.5-fold increase in CSF secretion accompanying the hydrocephalus caused by experimental IVH is associated with upregulated phosphorylation of SPAK/OSR1–NKCC1 at the choroid plexus apical membrane. Indeed, the choroid plexus is the site of the highest SPAK abundance among all epithelial tissues. Knockdown of SPAK in the choroid plexus by intracerebroventricular siRNAs reduced CSF secretion rates and reversed post-IVH ventriculomegaly.

Here, we report our development of a "dual" CCC modulator (NKCC1 inhibitor/KCC activator), 5-chloro-N-((4-chlorophenyl)(cyano)methyl)-2-methylphenyl)-2-hydroxybenzamide ("ZT-1a") that potently and selectively inhibits SPAK kinase. ZT-1a-mediated SPAK inhibition led to reduced cellular ion influx and to stimulated Cl– dependent K+ efflux by simultaneous reduction of the activating phosphorylation of NKCC1 and the inhibitory phosphorylation of the KCCs. Intra-cerebroventricular delivery of ZT-1a prevented CSF hypersecretion in a model of post-hemorrhagic hydrocephalus by decreasing SPAK-mediated phosphorylation of CCCs in the choroid plexus. Systemic ZT-1a administration after experimental ischemic stroke attenuated cerebral infarction and edema and improved neurological outcomes by decreasing SPAK-mediated phosphorylation of CCCs in brain tissues. These results suggest that inhibition of SPAK kinase is an effective approach in modulating CCCs and has therapeutic potential for brain disorders of cell volume dysregulation.

Results

ZT-1a, a novel and potent non-ATP-competitive SPAK inhibitor. To identify pharmacological modulators of SPAK kinase, we designed and synthesized a new focused chemical library derived from the previously identified SPAK inhibitors Closantel, Raxofanide, and STOCK1S-14279 (Fig. 1). This "scaffold-hybrid" strategy, combining pharmacophores from different scaffolds, has previously led to development of highly selective kinase inhibitors. Closantel and Raxofanide target the C-terminal domain allosteric sites of SPAK and OSR1, rather than their highly conserved ATP-binding pockets, leading to non-ATP-competitive kinase inhibition. Iterative rounds of medicinal chemistry optimization led to identification of 5-chloro-N-((4-chlorophenyl)(cyano)methyl)-2-methylphenyl)-2-hydroxybenzamide ("ZT-1a") as a selective SPAK inhibitor (Fig. 1 and Supplementary Table 1).

We compared the highest-potency salicylic amidase selected from the library with Closantel, STOCK1S-14279, and STOCK1S-50699 as SPAK inhibitors in a cellular context. As NKCC1 and KCCs (KCC1–4) are phosphosubstrates of SPAK kinase, activity was monitored as NKCC1 Thr203/207/212 phosphorylation, which is required for cotransporter activation, and KCC site1/2 phosphorylation (KCC2 Thr906/Thr1007 or KCC3 Thr991/Thr1048), required for cotransporter inhibition (Fig. 2, Supplementary Fig. 1). ZT-1a emerged as the most potent compound, inhibiting phosphorylation of NKCC1 p-Thr203/207/212 by 72 ± 5.2% at 1 µM ZT-1a and phosphorylation of KCC sites 1/2 by 65–77% at 3 µM (both n = 4, p < 0.01) in HEK-293 cells (Supplementary Table 1). SPAK phosphorylation at Ser373 was inhibited by 70 ± 3.8% inhibition at ~3–10 µM ZT-1a (n = 3, p < 0.01; Fig. 2). These results show that ZT-1a to be a more potent modulator of SPAK-dependent CCC phosphorylation than the current SPAK kinase inhibitors Closantel, STOCK1S-50699, and STOCK1S-14279.
We next tested whether ZT-1a mediates kinase inhibition by competing for ATP binding to kinase in a manner similar to that of the nonspecific kinase inhibitor staurosporine. As shown in Supplementary Fig. 3a, staurosporine IC_{50} values increased proportionally with increasing ATP concentrations, in contrast to the unchanged IC_{50} values of ZT-1a (Supplementary Fig. 3b). These results suggest that ZT-1a inhibits SPAK kinase in a non-ATP-competitive manner. The kinase selectivity of ZT-1a was further assessed using standard radioisotopic enzymatic assays against a panel of 140 recombinant kinases (Dundee profiling, Supplementary Table 2)\textsuperscript{48}. ZT-1a exhibits relatively high kinase selectivity, insofar as 98% of these 140 kinases were not inhibited by >70% by 1 µM ZT-1a as compared with DMSO control. Whereas 1 µM ZT-1a did inhibit GSK-3β activity by 60 ± 6% compared in vitro with DMSO control, GSK-3β Ser9 phosphorylation was not inhibited by 3 µM ZT-1a either in HEK-293 cells (Supplementary Fig. 4a, b) or in ZT-1a-treated ischemic brain (Supplementary Fig. 4c, d).

ZT-1a disrupts SPAK interaction with WNK but not with MO25α. Crystallographic analysis of the human OSR1-conserved carboxyterminal (CCT) domain complexed to an RFXI motif-containing peptide derived from WNK\textsuperscript{49} has shown that the highly conserved CCT residue Leu473 (mouse SPAK Leu502) forms critical hydrophobic contacts with the Phe residue of the RFXI motif. In vitro fluorescence polarization studies confirmed a 0.3-µM binding affinity of the RFXI motif-containing WNK peptide for purified wild-type SPAK protein, an interaction disrupted by STOCK1S-50699 with IC_{50} of 2.51 µM (Supplementary Fig. 5). Neither Closantel nor ZT-1a disrupted binding between WNK4 and the SPAK CCT domain, suggesting that the ZT-1a binding site lies outside of the CCT domain (Supplementary Fig. 5).

In order to establish that ZT-1a binds to SPAK and OSR1 CCT domains, we employed a SPAK antibody pull-down assay from HEK-293 cell lysates in the presence of increasing ZT-1a concentrations, and compared the results with an 18-mer RFQV peptide from human WNK4\textsuperscript{49} and an 18-mer AFQV negative control peptide not binding the primary pocket of SPAK and OSR1\textsuperscript{49}. Co-immunoprecipitation of WNK1 with SPAK from HEK-293 cell lysates was abolished by ZT-1a more potently than by STOCK1S-50699 or by Closantel, in the absence or presence of competitor peptide (Supplementary Fig. 6). ZT-1a IC_{50} values were not significantly altered by the addition of MO25α, which activates SPAK/OSR1 ~100-fold and increases SPAK/OSR1-mediated in vitro phosphorylation of all CCCs\textsuperscript{25,30} by ~8-fold (Supplementary Fig. 7).

ZT-1a reduces SPAK-dependent CCC phosphorylation in cells. To assess CCC phosphorylation changes in response to ZT-1a, HEK-293 cells were exposed for 30 min either to control isotonic medium or to hypotonic low [Cl\textsuperscript{-}] medium (to activate SPAK/OSR1), and then treated with varying concentrations of ZT-1a for an additional 30 min. ZT-1a, in a dose-dependent manner (1–10 µM), markedly inhibited SPAK/OSR1 phosphorylation at Ser373/Ser325 (i.e., the activating site phosphorylated by WNK1) and NKCC1 phosphorylation at Thr203/Thr207/Thr212 (SPAK/OSR1 target sites whose phosphorylation is required for maximal transporter activity) in hypotonic low [Cl\textsuperscript{-}] conditions as well as in control isotonic conditions (Fig. 2). These effects were paralleled by similar suppression of KCC3 Thr991/Thr1048 phosphorylation, consistent with WNK–SPAK/OSR1-mediated phosphorylation of these residues\textsuperscript{25,26}.

ZT-1a inhibits NKCC1 but stimulates KCC3 activity. \textsuperscript{86}Rb\textsuperscript{+} flux assays have been utilized extensively as a reliable measurement of cation chloride cotransporter activity\textsuperscript{31–34}. Therefore, the ability of ZT-1a to decrease inhibitory phosphorylation of KCC3 at Thr991/Thr1048 prompted us to assess ZT-1a’s effect on KCC3 activity by measuring hypotonicity-stimulated \textsuperscript{86}Rb\textsuperscript{+} uptake in isotonic or hypotonic low Cl\textsuperscript{-} conditions (Fig. 3a). The furosемide (Furo)-treated cells, either transfected with empty vector or KCC3 wt cDNA, have significa ntly decreased K\textsuperscript{+} flux (p < 0.01; n = 3). Low KCC3 activity observed in wild-type (WT) HEK-293 cells was consistent with maximal KCC3 phosphorylation at Thr991/1048 in hypotonic low [Cl\textsuperscript{-}] conditions.\textsuperscript{55} In contrast, cells expressing KCC3 double mutant Thr991Ala/Thr1048Ala, mimicking activating dephosphorylation at these sites, exhibited a >13-fold increase in KCC3 activity compared with WT KCC3 (p < 0.001; n = 3). ZT-1a activated WT KCC3 activity >10.6-fold (p < 0.001; n = 3), but failed to increase activity of KCC3 double mutant Thr991Ala/Thr1048Ala (p > 0.05; n = 3; Fig. 3a), consistent with ZT-1a antagonism of SPAK-mediated phosphorylation at these sites.

As expected, given that NKCC1 is maximally phosphorylated following hypotonic low Cl\textsuperscript{-} conditions, higher NKCC1 activity...
Fig. 2 ZT-1a suppresses CCC phosphorylation. a ZT-1a dose dependently inhibited KCC3 Thr991/Thr1048 phosphorylation in cells. HEK-293 cells were transfected with a DNA construct encoding wild-type N-terminally FLAG-tagged KCC3. Cells at 36 h post transfection were exposed for 30 min to either control isotonic or hypotonic low [Cl\(^-\)] conditions, and then treated in the same conditions for an additional 30 min in the presence of inhibitors at the indicated concentrations. Cell lysates were subjected to SDS-PAGE and immunoblot with the indicated antibodies.

b Immunoblot quantitation on ZT-1a, Closantel, STOCK1S-14279, and STOCK1S-50699. Band intensities were quantitated with ImageJ software. ***p < 0.001; **p < 0.01; *p < 0.05; ns, nonsignificant (by one-way ANOVA with post hoc testing (n = 6, error bars represent the mean ± SEM)).
Fig. 3 ZT-1a inhibition of SPAK kinase correlates with CCC activity. a $^{86}$Rb$^+$ uptake assays in the presence of ZT-1a measure transport activity of KCC3. HEK-293 cells were transfected with constructs encoding the indicated WT or mutant constructs of N-terminally FLAG-tagged KCC3. Thirty-six hours post transfection, cells were exposed for 30 min to either control isotonic conditions or hypotonic low Cl$^-$ conditions (to activate the SPAK/OSR1 pathway), and then treated for an additional 30 min in the same conditions with either ZT-1a or Closantel (50 mg/kg as a reference). Higher transport activity was observed. Cells treated with Bumetanide (Bum, an FDA-approved potent loop diuretic (LD) that acts by antagonizing Na$^+$/K$^+$/2Cl$^-$ cotransporter) and then treated in the same conditions for an additional 30 min with 10 µM ZT-1a or 1 mM bumetanide (Bum) in the presence of 1 mM ouabain (Na$^+$/K$^+$-ATPase inhibitor) and 0.1 mM bumetanide (NKKC1 inhibitor). $^{86}$Rb$^+$ uptake was allowed to proceed for 10 min and was then quantified by scintillation counting. $^{86}$Rb$^+$ uptake counts per minute (CPM) were normalized per mg protein and plotted for both isotonic and hypotonic conditions. (a—left panel) HEK-293 cells were transfected with constructs encoding wild-type N-terminally FLAG epitope-tagged KCC3. Thirty-six hours post transfection, cells were exposed for 30 min to either control isotonic conditions or hypotonic low Cl$^-$ conditions (to activate the SPAK/OSR1 pathway), and then treated in the same conditions for an additional 30 min with 10 µM ZT-1a or 1 mM bumetanide. Ten-minute $^{86}$Rb$^+$ uptake values were quantified by scintillation counting, normalized per mg protein for each condition, and plotted for both isotonic and hypotonic conditions. **p < 0.001; ***p < 0.001; ****p < 0.0001; ****p < 0.0001; ns—non-significant (by one-way ANOVA with post hoc testing (n = 6, error bars represent the mean ± SEM)). b $^{86}$Rb$^+$ uptake assays in the presence of ZT-1a measure transport activity of NKCC1. (b—left panel) HEK-293 cells were transfected with WT NKCC1 cDNA construct. Thirty-six hours post transfection, cells were exposed for 30 min to either control isotonic conditions or hypotonic low Cl$^-$ conditions (to activate the SPAK/OSR1 pathway), and then treated in the same conditions for an additional 30 min with 10 µM ZT-1a or 1 mM bumetanide (Bum) in the presence of 1 mM ouabain (Na$^+$/K$^+$-ATPase inhibitor). $^{86}$Rb$^+$ uptake was allowed to proceed for 10 min and was then quantified by scintillation counting. $^{86}$Rb$^+$ uptake CPMs were normalized per mg protein and plotted for both isotonic and hypotonic conditions. (b—right panel) HEK-293 cells were transfected with WT NKCC1 cDNA construct. Thirty-six hours post transfection, cells were exposed for 30 min to either control isotonic conditions or hypotonic low Cl$^-$ conditions (to activate the SPAK/OSR1 pathway), and then treated for an additional 30 min in the same conditions with the indicated ZT-1a concentrations in the presence of 1 mM ouabain and 0.1 mM bumetanide. Ten-minute $^{86}$Rb$^+$ uptake values were quantified by scintillation counting, normalized per mg protein for each condition, and plotted for both isotonic and hypotonic conditions. **p < 0.001; ***p < 0.001; ****p < 0.0001; ns—non-significant (by one-way ANOVA with post hoc testing (n = 6, error bars represent the mean ± SEM)).
corresponding residues in SPAK49,57. KCC3 phosphorylation at pThr1048 was reduced 34 ± 4.6% in brains from SPAK502A/502A mice (p < 0.01; n = 3; Supplementary Fig. 8a), a model of Gitelman syndrome55. Thus, systemically administered ZT-1a has low efficacy in naive brains, suggesting inefficient ZT-1a transport across the BBB, likely in part reflecting unfavorable ZT-1a pharmacokinetics in naive mice (T1/2 = 1.8 h, AUC = 2340 h*ng/mL and %F = 2.2%; Supplementary Table 3).

ZT-1a reduces CSF secretion in hemorrhagic hydrocephalus. Choroid plexus epithelium (CPE) is the most actively secreting epithelium in the body38,39, producing up to 500 cc/day of CSF39. Among all tissues, including kidney37, SPAK kinase is most highly expressed in CPE3. Several CCCs are also expressed in choroid plexus60–62, including NKC1C1, recently shown to be essential for CSF secretion1,3. IVH-triggered TLR4 signaling stimulates CSF secretion >3.5-fold and causes hydrocephalus by increasing functional expression of pSPAK and pNKCC1 in CPE4. We speculated that intracerebroventricular (ICV) delivery of ZT-1a into the cerebrospinal fluid might bypass the blood–brain barrier and allow ZT-1a to exert its effects on SPAK and CCCs in CPE.

To assess KCC1–4 protein expression in rat choroid plexus, we first verified isoform specificity of our antibodies. Flag-tagged KCC4–4 were individually expressed in HEK-293 cells. The purified fraction of KCC immunoprecipitated by KCC site-2 phospho-antibody was subjected to SDS-PAGE and immunoblot with each antibody. As evident from Fig. 4a, each antibody recognized only its respective target, without isoform cross-reactivity. Although immunospecific, none of these antibodies were able to detect endogenous KCCs from rat CPE lysates through direct immunoblot. However, immunoblot of immunoprecipitated fractions demonstrated robust immunoreaction with antibodies targeting KCC1, KCC3, and KCC4, whereas CPE KCC1 abundance remained below the detection limit, consistent with its documented neuron-specific expression pattern63.

We next tested the effects of ICV delivery of ZT-1a on pSPAK, pNKCC1, pKCC1, pKCC3, and pKCC4 in rat CPE in the setting of experimental hemorrhagic hydrocephalus, as described3. ZT-1a reduced IVH-induced expression of pSPAK by 55 ± 3.6%; p < 0.01; n = 3 and pNKCC1 by 69 ± 4.3%; p < 0.001; n = 3 (Fig. 4b).

ZT-1a reduced post-IVH KCC1 phosphorylation at Thr927/Thr983 by 12- and 3.4-fold, respectively (p < 0.001; n = 3), KCC3 phosphorylation at Thr991/Thr1048 by 6- and 2.1-fold, respectively (p < 0.001; n = 3) in CPE (Fig. 4b). Consistent with these results, ZT-1a treatment for 48 h at 10 μM decreased post-IVH CSF hypersecretion by ~2.3-fold (p < 0.01), in contrast to the lack of effect of DMSO vehicle (p > 0.05) (Fig. 4c). These data suggest that ICV administration of ZT-1a can effectively modulate pathological CSF secretion by decreasing SPAK–NKC1/KCC phosphorylation.

ZT-1a reduces stroke-associated cerebral edema and infarct. Ischemic stroke is associated with significant upregulation of SPAK and NKC1 phosphorylation in peri-infarct cortex, striatum, and corpus callosum39. Genetic inhibition of either SPAK or NKC1 decreases ischemic cerebral edema and improves neurological outcomes42,64. We evaluated ZT-1a efficacy in attenuating cerebral infarct and associated cerebral edema in a mouse model of ischemic stroke39,42. First, we examined intrinsic immunofluorescence labeling of serum albumin at 24 h after tMCAO, as an indication of BBB integrity. As shown in Fig. 6a–c, serum-albumin extravasation into brain parenchyma was ~5-fold higher in mice with ischemic stroke than in sham-operated mice (p < 0.05, n = 5–6), suggesting that tMCAO/reperfusion induced disruption of BBB integrity in ischemic brains. We then assessed whether ZT-1a penetrates better in ischemic brains due to the leaky BBB. Two hours after ZT-1a administration (5 mg/kg i.p.), plasma ZT-1a levels in ischemic and non-ischemic sham mice were indistinguishable (p = 0.29; n = 10–11, Fig. 6e), whereas ZT-1a concentration in both CI and IL hemispheres of ischemic brains was ~1.8-fold higher than that in sham brains (p = 0.006; n = 10–11, Fig. 6f).

Importantly, ZT-1a administration 3 h post reperfusion decreased infarct volume by ~44% (from 78.1 ± 5.8 mm3 in the vehicle-control group to 43.6 ± 6.5 mm3, p < 0.01, n = 12, Fig. 7b). Moreover, ZT-1a treatment at either 2.5 or 5 mg/kg reduced cerebral hemispheric swelling by ~36–54% as compared with vehicle control (p < 0.01, Fig. 7b). Regional cerebral blood flow (rCBF) within the 24-h post-stroke period was unaffected by ZT-1a (Supplementary Fig. 10a–c). These results confirm that ZT-1a reduces infarct size and ischemic cerebral edema.

ZT-1a improves neurological function after ischemic stroke. We evaluated the impact of ZT-1a on progression of sensorimotor function deficits in a mouse model of ischemic stroke. The vehicle-control mice developed persistent, severe neurological deficits at days 0–7 after stroke, as reflected in elevated neurological scores of 2.5–2.9 (Fig. 7c). ZT-1a-treated mice exhibited a progressive decrease in neurological deficit scores between day 1 (2.0 ± 0.2) and day 7 (1.4 ± 0.2, p < 0.01, n = 6). In the corner test12 evaluating post-ischemic sensory and motor deficits, vehicle-control mice exhibited behavioral asymmetries 1 day after stroke, deficits that persisted for 7 days (Fig. 7c). In contrast, ZT-1a-treated mice showed reduced unidirectional turn preference and absence of asymmetries by day 7 post stroke. In the adhesive contact and removal tests that evaluate fine sensorimotor function deficits65, ZT-1a-treated mice displayed significantly improved motor function (p < 0.05, n = 6) as compared with vehicle-control mice. These data show that post-stroke treatment with ZT-1a significantly improved mouse neurological functional recovery.

ZT-1a protects gray and white matter after ischemic stroke. We conducted ex vivo MRI studies of brains from the same cohort of vehicle-control and ZT-1a-treated mice after completion of their neurological functional assessment at 7 days post stroke. T2-weighted MRI analysis further confirmed that ZT-1a treatment
reduced stroke-induced lesion volume by ~40% and longer-term brain atrophy (% hemisphere shrinkage) by ~41% (p < 0.05, n = 6, Fig. 7d). To assess the effect of ZT-1a on stroke-induced white matter injury, we analyzed fractional anisotropy (FA) and directionally encoded color (DEC) maps of the corpus callosum (CC) and external capsule (EC) in the vehicle-control and ZT-1a-treated brains. DEC and FA maps revealed intact CC and EC tracks in the CL hemisphere (arrows) and injured EC in the IL hemisphere (arrowheads) (Fig. 7d). FA values were significantly reduced in the ipsilateral EC tract of the vehicle-control brains, indicating loss of white matter integrity after stroke. In contrast, EC tract FA values were undiminished in the IL hemisphere of ZT-1a-treated mice (p > 0.05, n = 6), and the FA values were higher than the IL hemisphere in vehicle-control brains (p < 0.05),
reflecting ZT-1a-mediated preservation of EC white matter microstructure. These results further suggest that post-stroke treatment with ZT-1a protects both gray and white matter tissues in ischemic brains.

**ZT-1a inhibits stroke-induced SPAK–CCC phosphorylation.** Ischemia elicits neuronal and glial cell swelling. In contrast to the neuron-specific KCC2, NKCC1 and KCC3 are expressed in both neurons and glia, and play roles in cell volume regulation. We therefore examined ZT-1a effects on phosphorylation of SPAK/OSR1, NKCC1, and KCC3 in ischemic mouse brains. Ischemic stroke increased phosphorylation of pSPAK (pSer373)/pOSR1 (pSer325) by ~1.5-fold \((p < 0.05, n = 6)\), pNKCC1 (pThr203/207/212) by ~1.6-fold \((p < 0.05, n = 6)\), and pKCC3 pThr991 \((p < 0.05, n = 6)\) and pThr1048 by ~1.3-fold \((both \ p < 0.05)\) in membrane protein fractions from the ipsilateral (IL) cortical hemisphere at 24 h of reperfusion in vehicle-control-treated mice, without significant change in the corresponding total protein levels (Fig. 8a, b). Post-stroke administration of ZT-1a in mice prevented ischemia-induced increases of pSPAK/pOSR1, pNKCC1, and pKCC3 \((p < 0.05, n = 6)\) without alteration in the corresponding total protein expression.
Discussion

We have applied a “scaffold-hybrid” strategy to discover and characterize a potent and selective inhibitor of SPAK kinase, ZT-1a [5-chloro-N-(5-chloro-4-(4-chlorophenyl)(cyano)methyl)-2-methylphenyl]-2-hydroxybenzamide]. About 3 µM ZT-1a inhibited stimulatory phosphorylation of SPAK Ser373 and NKCC1 Thr203/Thr207/Thr212, and the inhibitory phosphorylation of the KCCs (e.g., KCC3 Thr991/Thr1048). As shown in cellular 86Rb+ flux assays, ZT-1a inhibited NKCC1 but potently stimulated KCC activity >10-fold. In a model of post-hemorrhagic hydrocephalus, intracerebroventricular delivery of ZT-1a decreased choroid plexus SPACK–CCC phosphorylation and antagonized inflammation-induced CSF hypersecretion. In a model of ischemic stroke, systemic ZT-1a decreased neuronal and glial CCC phosphorylation, reduced infarct size and cerebral edema, and improved neurological outcomes. These results suggest that ZT-1a is an effective SPACK–CCC modulator, with therapeutic potential for treatment of brain disorders associated with impaired cell volume homeostasis.

The traditional strategy of targeting the ATP-binding site of SPANK/OSR1 increases risks of off-target kinase inhibition. The

Closantel reduces ischemic brain injury. We compared the neuroprotective efficacy of Closantel with that of the pan-WNK-kinase inhibitor WNK463. Administration of Closantel (1.0 or 2.5 mg/kg) at 3 and at 8 h post reperfusion reduced infarct volume and hemispheric swelling in a dose-dependent manner (Fig. 8a, b; Supplementary Fig. 11). These results indicate that ZT-1a inhibits the SPAK-dependent upregulation of NKCC1 and KCC3 phosphorylation in ischemic brains.

(Fig. 8a, b; Supplementary Fig. 11). Note that tMCAO-subjected mice exhibited increased serum-albumin immunofluorescence intensity in the ipsilateral (IL) hemisphere compared with that of sham mice. Higher-magnification (x40) images of serum-albumin immunofluorescence. The arrow shows albumin in vessel lumen. The arrowhead shows albumin that has diffused into ischemic brain parenchyma. c Quantitative analysis of albumin immunofluorescence intensity as shown in b. Data are mean ± SEM, n = 5 per group (male); ***p < 0.0001, one-way ANOVA. d Experimental design for bioavailability assay of ZT-1a in mouse plasma and brain. ZT-1a was administered at 3 h post reperfusion or post sham surgery. Blood and brain samples were collected at 2 h post injection. e Plasma ZT-1a concentrations were indistinguishable in sham-operated and tMCAO-subjected male mice (p = 0.29; n = 11 (sham) and 9 (tMCAO)). f Increased ZT-1a concentration was detected in contralateral (CL) and ipsilateral (IL) brain tissues of ischemic versus sham-operated male mice. Data are mean ± SEM, n = 10 (sham) and 11 (tMCAO). **p < 0.01, one-way ANOVA.

### Fig. 6 Ischemic stroke facilitates ZT-1a entrance into the brain.

a Anti-serum-albumin-labeled brain sections at 24 h after sham or tMCAO surgery illustrate image collection in the perilesion and contralateral (CL) control areas (white boxes). Note that tMCAO-subjected mice exhibited increased serum-albumin immunofluorescence intensity in the ipsilateral (IL) hemisphere compared with that of sham mice. The arrow shows albumin in vessel lumen. The arrowhead shows albumin that has diffused into ischemic brain parenchyma. c Quantitative analysis of albumin immunofluorescence intensity as shown in b. Data are mean ± SEM, n = 5 per group (male); ***p < 0.0001, one-way ANOVA. d Experimental design for bioavailability assay of ZT-1a in mouse plasma and brain. ZT-1a was administered at 3 h post reperfusion or post sham surgery. Blood and brain samples were collected at 2 h post injection. e Plasma ZT-1a concentrations were indistinguishable in sham-operated and tMCAO-subjected male mice (p = 0.29; n = 11 (sham) and 9 (tMCAO)). f Increased ZT-1a concentration was detected in contralateral (CL) and ipsilateral (IL) brain tissues of ischemic versus sham-operated male mice. Data are mean ± SEM, n = 10 (sham) and 11 (tMCAO). **p < 0.01, one-way ANOVA.
introduction of STOCK1S-50699 and STOCK2S-26016 has highlighted SPAK inhibitors that target the CCT domain rather than the kinase domain. However, only STOCK1S-50699, but not STOCK2S-26016, suppressed in vitro phosphorylation of SPAK/OSR1 and NKCC1 induced by hypotonic low [Cl\(^{-}\)]\(_2\)\(_5\). Moreover, in vivo pharmacokinetics of STOCK1S-50699 are unfavorable (data not shown). The livestock antiparasitic drug, Closantel, emerged as the first drug candidate for in vivo pharmacological inhibition of SPAK\(^4,44\), but is contraindicated in humans due to retinal toxicity\(^72\).

The ATP independence of SPAK inhibition by Closantel and STOCK1S-14279 has suggested the possibility of developing inhibitors of WNK signaling by binding to constitutively active or WNK-insensitive (T233E) SPAK\(^43\). With a "scaffold-hybrid"
strategy, we designed and synthesized a new, focused chemical library derived from these two ATP-insensitive inhibitors. ZT-1a was among the best SPAK inhibitors identified from an ~300-compound chemical library, and was characterized by fluorescence polarization, in vitro kinase, and cell-based assays. ZT-1a was a more potent CCC inhibitor than its predecessor SPAK inhibitors, Closantel, Rafoxanide, STOCKIS-142794, and STOCKIS-50699. About 3 μM ZT-1a substantially inhibited phosphorylation of SPAK Ser373, NKCC1 Thr203/207/212, and KCC3 Thr991/Thr1048 (KCC4 Thr926/Thr980) in cells. ZT-1a also effectively inhibited NKCC1 and stimulated KCC3 in standard cellular assays of CCC-mediated flux.

Post-stroke administration of Closantel in mice reduced ischemic cerebral infarction size and brain swelling in a dose-dependent manner. However, we detected Closantel toxicity in mice at higher doses (~25 mg/kg), consistent with reported adverse effects in humans, including weakness, visual impairment, and blindness. The in vitro nanomolar-potency pan-SPAK inhibitor WNK463 was developed as an anti-hypertensive drug. Oral administration of 1–10 mg/kg WNK463 reduced blood pressure and regulated body fluid and electrolyte homeostasis in normotensive and hypertensive rodents. However, we cannot rule out possible contributions from altered BBB drug transporter pathways and/or reduced ZT-1a extrusion from CNS in the setting of ischemia. Solute carrier (SLC) transporters, such as organic anion-transporting polypeptides (Oatps), may improve drug delivery into the brain, whereas the ATP-binding cassette (ABC) transporter superfamily (P-glycoprotein) exports drugs across BBB endothelial cells into the blood. As ischemia increases Oatp1a4 protein expression and function in brain microvessels, this and other SLC proteins of similar expression pattern may contribute to increased ZT-1a transport across BBB into ischemic brains. Whether ZT-1a is a substrate for Oatp1a4 and ABC superfamily remains to be studied. Ischemic stroke-induced disruption of the BBB integrity is also evident in human brain, suggesting that small-molecule neuroprotective drugs, such as ZT-1a, could potentially traverse the leaky BBB into ischemic human brain. We administered the initial dose of ZT-1a 3-h post stroke, a time comparable to the 2.5–4.0-h post-stroke treatment windows found effective for the candidate neuroprotective agents, glibenclamide, human albumin, and minocycline. Although ZT-1a and the antiparasitic drug Closantel exhibit similar effects in reducing ischemic brain damage, Closantel shows toxicity in mouse and human. In this study, we did not test possible visual system toxicity of ZT-1a. Additional pharmacokinetics and toxicity studies of ZT-1a, especially on the retina, warrant further investigations for future clinical translation.

The CPe secretes ~500 ml/day CSF, a higher per-cell fluid volume than for any other epithelium. NKCC1 expressed in the apical CPe contributes approximately half of CSF production, though its mechanism remains unclear, in view of its unique apical localization in the CPe compared with its basolateral position in all other epithelia. Indeed, some have proposed inward NKCC1 flux to be required for apical K+ recycling and continued CSF production, while others have provided evidence for net ion efflux with obligatory water transport directly contributing to CSF production. Nonetheless, in a rat model of hemorrhagic hydrocephalus, intraventricular hemorrhage causes a Toll-like receptor 4 (TLR4)- and NF-kB-dependent inflammatory response in CPe associated with ~3-fold increase in bumetanide-sensitive CSF secretion. IVH-induced hypersecretion of CSF is mediated by TLR4-dependent activation of SPAK, which binds, phosphorylates, and stimulates NKCC1 at the CPe apical membrane. Genetic depletion of TLR4 or SPAK normalizes hyperactive CSF secretion rates and reduces post-hemorrhagic hydrocephalus symptoms in parallel with reduction of NKCC1 phosphorylation. We have shown here that IVC administration of ZT-1a restores CSF secretion rates to basal

and fractional anisotropy (FA) maps of ex vivo brains from the same cohort of mice at 7 days post tMCAO, as described in b. e) Neurological deficits score, corner tests, and adhesive tape removal tests of mice 1 day before tMCAO and at days 0, 1, 3, 5, and 7 post tMCAO. Vehicle (DMSO, 2 ml/kg) or ZT-1a (5.0 mg/kg) were administered as described in b. Data are means ± SEM, n = 7 for each group (male 4, female 3). ***p < 0.001; **p < 0.01; *p < 0.05 versus the respective vehicle control. d) Representative images of T2WI, directionally encoded color (DEC), and fractional anisotropy (FA) maps of ex vivo brains from the same cohort of mice at 7 days post tMCAO, as described in e. Arrow marks EC (external capsule); double arrowhead marks damaged EC; CC: corpus callosum. Bar graphs display quantitation of brain lesion volume, brain atrophy (% shrinkage), and mean FA values. Data are means ± SEM, n = 6 for vehicle (male 3, female 3) and 7 for ZT-1a (male 4, female 3). *p < 0.05, one-way ANOVA.
**Fig. 8 ZT-1a decreases ischemia-induced SPAK–CCC phosphorylation.** a Representative immunoblots (IB) of phospho-SPAK/OSR1 (pSPAK/pOSR1), phospho-KCC3 (pKCC3), and phospho-NKCC1 (pNKCC1) in mouse brains studied 24 h post reperfusion after ischemic stroke. Membrane protein fractions were prepared from contralateral (CL) and ipsilateral (IL) cerebral hemispheres. Vehicle (Veh, DMSO) or ZT-1a (5 mg/kg) were administered as described in Fig. 7a. Na⁺–K⁺ ATPase α-subunit served as loading control for membrane protein fraction. b Densitometry analyses of immunoblots (similar to those in panel a) of pSPAK/pOSR1, pKCC3, pNKCC1, 1SPAK/tOSR1, and tNKCC1 in mouse brains studied 24 h of reperfusion after tMCAO. Data are means ± SEM, n = 6 per group (male 3, female 3). ***p < 0.001; **p < 0.01; *p < 0.05 versus control, one-way ANOVA.
levels after IVH, and antagonizes IVH-induced phosphorylation of SPAK, NKCC1, and multiple KCCs in CPEs. These data corroborate previous findings\(^1,3\), and suggest ZT-1a or related strategies as potential nonsurgical treatment modalities for hydrocephalus. Further work will be required to determine the mechanisms of NKCC1 ion transport at the CPE membrane, and to assess the therapeutic potential of ZT-1a in other preclinical models of hydrocephalus.

In conclusion, we have developed ZT-1a, a novel drug that potently and selectively inhibits SPAK kinase, the master regulator of CCCs. ZT-1a inhibits NKCC1 and stimulates the KCCs by decreasing their regulatory phosphorylation. Intracellular ATP-dependent delivery of ZT-1a, by decreasing inflammation-induced phosphorylation of CCCs in the choroid plexus, reduces CSF hydroperegression in hemorrhagic hydrocephalus. Systemically administered ZT-1a attenuates cerebral edema, protects against ischemic brain damage, and improves post-ischemic neurological outcomes by reducing ischemia-induced CCC phosphorylation. These results suggest that ZT-1a or related compounds are effective CCC modulators, with therapeutic potential for treatment of disorders of dysregulated brain volume homeostasis.

**Methods**

Reagents and general methods. Protein sample buffer and bicinecinonic acid assay reagent were from Thermo Scientific (Rockford, IL). Closantel was from Sigma (St. Louis, MO) and WNK463 was from MedChemExpress (Princeton, NJ). STOCKIS-50099 and STOCKIS-14279 were from InterBioScreen (Chernogolovka, Russian Federation). Horseradish peroxidase (HRP)-conjugated anti-rabbit Ig was from Molecular Probes (Eugene, OR). RIPA buffer and enhanced chemiluminescence agent (ECL) reagent were from Pierce (Rockford, IL).

**Antibodies.** The Division of Signal Transduction Therapy Unit at the University of Dundee (RRID:SCR_011633) supplied antibodies to KC3 (1 µg/ml, S701C), KC3A phosphoT3991 (1 µg/ml, S595C), KC3A2 phosphoT160 (1 µg/ml, S7061C), NKCC1 (1 µg/ml, S022D), NKCC1 phosphoT203/T207/T212 (1 µg/ml, S763B), WNK1 (1 µg/ml, S079B), SPAK (1 µg/ml, S551D), ORS1 (1 µg/ml, S851C), SPAK/ORSA (S-motif) phosphoT373/S375 (1 µg/ml, S670B), and full-length human ERKI (1 µg/ml, S221B). The pan-KCC2 antibody (NeuroMab clone N1/12) was from NeuroMab. The FLAG M2 antibody (1 µg/ml, F166) was from Sigma. The KC4C antibody was from Novus Biologicals (0.4 µg/ml, NBPI-85133). Antibodies to GSK-3β phospho9 (1:1000 dilution, #9336), GAPDH (0.1 µg/ml, #2118), and β-Actin (1:5000 dilution, #81H0D10) were from Cell Signaling Technology. Anti-\(\beta\)-GSK-3β (1:1000 dilution, #60A1-03083) was from Thermo Scientific.

**Cell culture, transfections, and stimulations.** HEK-293 (human embryonic kidney 293) cells were cultured on 10-cm-diameter dishes in DMEM supplemented with 10% (v/v) fetal bovine serum, 2 mML-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. HEK-293 cells were transfected with a mixture of 20 µl of 1 mg/ml polyethylenimine (Polysciences) and 5–10 µg of plasmid DNA as described previously\(^38\). Thirty-six hours post transfection, cells were stimulated with either control isotonic or hypotonic medium for a period of 30 min. Cells were lysed in the presence of ice-cold lysis buffer/dish, lysates were centrifuged at 4 °C for 15 min at 26,000 g, and the supernatants were frozen in liquid nitrogen and stored at −20 °C. Protein concentrations were determined using the Bradford method. Cells were subjected to the indicated concentrations of the SPAK kinase inhibitors. Isotonic buffer was 133 mM KCl, 5.0 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), 0.5 mM Na\(_2\)HPO\(_4\), 0.5 mM Na\(_2\)SO\(_4\), and 15 mM HEPES (pH 7.5). Hypotonic low-chloride buffer was 67.5 mM sodium gluconate, 2.5 mM potassium gluconate, 0.25 mM CaCl\(_2\), 0.25 mM MgCl\(_2\), 0.5 mM Na\(_2\)HPO\(_4\), 0.5 mM Na\(_2\)SO\(_4\), and 7.5 mM HEPES (pH 7.5).

**Primary mouse neuronal cultures.** Embryonic day 14–18 wild-type pregnant mice, in a C57BL6J background, were deeply anesthetized with 5% isoflurane in \(\mathrm{O}_2\) and 95% \(\mathrm{N}_2\). Fetuses were removed from the uterus and the cerebral cortices were dissected in ice-cold Hank’s balanced salt solution. The corticofugal tissues were exposed to 0.125 mg/ml of trypsin at 37 °C for 20 min. The dissociated cells were centrifuged at 587 × g for 5 min at room temperature. The cells were gently mixed into a single-cell suspension before plating. Cells (2000–1000 cells/mm\(^2\)) were cultured on glass coverslips coated with poly-\(\alpha\)-lysine in neurobasal medium containing 2-B supplements, 

**86Rb\(^+\) uptake assay for KC3 or NKCC1 activity.** \(\mathrm{Rb}^+\) uptake assays were performed on HEK-293 cells transfected with WT or mutant KC3 and plasmid DNA as detailed in several of our previous publications\(^31\). For measurement of KC3 activity, HEK-293 cells were transiently transfected with empty vector or WT KC3 cDNA. HEK-293 cells were plated at 50–60% confluence in 12-well plates (2.4-mm diameter wells) and transfected with wild-type or variant mutants of full-length flag-tagged human KC3 (1 µg of plasmid DNA per well) in the presence of 2.5 µl of polyethylenimine (1 µg/ml). The \(\mathrm{Rb}^+\) uptake assay was performed on cells at 36 h post transfection. Culture medium was aspirated from the wells and replaced with either isotonic or hypotonic medium for 15 min at 37 °C, and then for a further 15 min with stimulating medium containing additional 1 mM ouabain (Oua, \(\mathrm{Na}^+\)/\(\mathrm{K}^+\)–ATPase inhibitor) and 0.1 mM bumetanide (Bum, inhibitor of NKCC1 cotransporter). For measurement of NKCC1 activity in HEK-293 cells, stimulating medium contained ouabain, and with or without further addition of bumetanide as for controls. This stimulating medium was then removed and replaced with isotonic medium containing inhibitors plus 2 µCi/ml \(86\)Rb\(^+\). After incubation for 10 min at 37 °C, cells were rapidly washed three times with the respective ice-cold neurotransactive medium. Washed cells were lysed in 300 µl of ice-cold lysis buffer and \(\mathrm{Rb}^+\) uptake was quantitated by liquid scintillation counting (PerkinElmer).

**Cell volume measurements.** Cell volume change was determined using Calcein-AM as a marker of intracellular water volume\(^39\). The coverslip-plated cells were...
incubated with 0.5 μM calcine-AM for 30 min at 37 °C in the dark. The coverslip was then mounted in a heated (37 °C) imaging/perfusion chamber (Warner Instruments, Hamden, CT) on a Nikon Ti Eclipse inverted epifluorescence microscope equipped with 40× Super Fluor oil immersion objective, and a Princeton Instruments MicroMax CCD camera. Calcine fluorescence was monitored using a FITC filter set (excitation 480 nm, emission 535 nm, Chroma Technology, Rockingham, VT). Images were collected every 60 s with MetaFluor image-acquisition software (Molecular Devices, Sunnyvale, CA) and regions of interest (~20–25 cells) were selected. Baseline drift resulting from photobleaching and dye leakage was corrected as described before. Fluorescence change was plotted as a function of the reciprocal of the relative osmotic pressure and the resulting calibration curve was used to calculate the osmotic pressure of CSF at each homogenization time point using the van’t Hoff equation. The HEPES-buffered isotonic solution contained (in mM, pH 7.4) 100 NaCl, 5.4 KCl, 1.3 CaCl2, 0.8 MgSO4, 20 HEPES, 5.5 glucose, 0.4 NaHCO3, and 70 sucrose with osmometer-confirmed osmolality of 310 mOsm (Advanced Instruments, Norwood, MA). Anisotomic solutions (250, 370, 400, and 515 mOsm) were prepared by removal or addition of sucrose to the above solution.

Immunoblot and phospho-antibody immunoprecipitation. Whole-cell or cerebrospinal fluid (CSF) lysates were prepared with RIPA lysis buffer as previously described. Lysate protein samples were subjected to immunoblot and immunoprecipitation as previously described. Protein samples (40 µg) were boiled in sample buffer for 5 min, resolved by 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and electrotransferred onto a polyvinylidene difluoride membrane. Membranes were incubated for 30 min with TBST (Tris-buffered saline, 5% Tween-20, 0.1% w/v, 500-µL syringe (Hamilton, Reno, NV), which was mounted to the ear bars 90°, nose down, and the suboccipital muscles were dissected to the cisterna magna to expose the atlanto-occipital ligament. The ligament was punctured and a 23-gauge flexible catheter (PE-20) was advanced 5 mm through the foramen of Magendie to the 4th ventricle. Sterile, molecular-grade mineral oil (100 µL, Sigma Aldrich, St. Louis, MO) was infused into the 4th ventricle to occlude the aqueduct of Sylvius, thereby creating a closed system of CSF circulation. With the ECA stump dissected, the catheter was advanced to 4.5 mm relative to bregma, and regions of interest (~20–25 cells) were selected. Baseline drift resulting from photobleaching and dye leakage was corrected as described before. Fluorescence change was plotted as a function of the reciprocal of the relative osmotic pressure and the resulting calibration curve was used to calculate the osmotic pressure of CSF at each homogenization time point using the van’t Hoff equation. The HEPES-buffered isotonic solution contained (in mM, pH 7.4) 100 NaCl, 5.4 KCl, 1.3 CaCl2, 0.8 MgSO4, 20 HEPES, 5.5 glucose, 0.4 NaHCO3, and 70 sucrose with osmometer-confirmed osmolality of 310 mOsm (Advanced Instruments, Norwood, MA). Anisotomic solutions (250, 370, 400, and 515 mOsm) were prepared by removal or addition of sucrose to the above solution.

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Preparation of brain membrane and cytosolic protein fractions and immunoblotting. Brain homogenates were prepared at 24 h post reperfusion. Mice were anesthetized with 5% isoflurane vaporized in N2O and O2 (3:2), then decapitated. The contralateral (CL) and ipsilateral (IL) brain tissues were dissected in five volumes of cold homogenization buffer (0.25 M sucrose, 10 mM Tris–HCl, 1 mM EDTA, pH 7.4, protease, and phosphate inhibitor cocktail, Pierce). Brain tissues were gently homogenized with a tissue grinder (Kontes, Vineland, NJ, USA) in ice-cold saline indicated clari

Quantitation of rates of CSF production and intracerebroventricular drug administration. Rates of CSF production were measured as previously described. Briefly, anesthetized rats were mounted in a stereotactic apparatus (Stoelting Co., Wood Dale, IL), a midline scalp incision was made, and the 26-gauge needle was held in place for an additional 20 min to prevent backflow of blood upon needle removal.

Quantification of rates of CSF production and intracerebroventricular drug administration. Rates of CSF production were measured as previously described. Briefly, anesthetized rats were mounted in a stereotactic apparatus and a 1.3-mm brain burr hole was made over the left lateral ventricle (coordinates, x = −8.8, y = −1.7 mm relative to bregma). Approximately 200 µL of blood was drawn from the tail artery catheter and loaded into a 500-µL syringe (Hamilton, Reno, NV), which was mounted to the stereotactic frame. Under stereotactic guidance, 50 µL of freshly collected autologous blood, free from anticoagulants, was infused into the right lateral ventricle (coordinates, x = −0.8, y = −1.7, and z = −4.5 mm relative to bregma), over the course of 5 min, and the 26-gauge needle was held in place for an additional 20 min to prevent backflow of blood upon needle removal.

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Kinome profiling. Kinome profiling was performed using KinomeScan ScanMAX at 1 µM compound concentrations (see Supplemental Data). Protocols are available from DiscoverX (https://www.discoverx.com/).

Animal preparation. All animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Nine- to 14-week-old C57BL/6J mice (both male and female) were used in the study (Jackson laboratories, Bar Harbor, ME).

Drug treatment. Vehicle 100% DMSO (2 mL/kg body weight/day), WNK463 (2.5 mg/kg body weight/day), Closantel (0.1–2.5 mg/kg body weight/day), or ZT-1a (2.5–5.0 mg/kg body weight/day) were administered via intraperitoneal injection (i.p., Fig. 7a), with an initial half-dose at 3 h and the second half-dose at 8 h following reperfusion.

Cerebral blood flow measurement. Regional cerebral blood flow was measured using a two-dimensional laser speckle contrast analysis system (PeriCam PSI High Resolution with PIMSoft, Perimed)65. Isoflurane-anesthetized mice were
head-fixed using stereotactic equipment during imaging. The skin was retracted to expose the intact skull. Images were taken at 19 frames/second with averaging. Average signal intensity was taken from fixed-size (0.5-mm\(^2\)) regions of interest drawn over the parietal bone plate on the ipsi- (IL-) and contralateral (CL) sides. Percent perfusion values were taken in comparison with the mean values of the pre-ischemic ipsilateral side. Five consecutive images at each time period per animal were averaged for analysis.

Brain infarction volume and hemispheric swelling measurements. At 24 h of reperfusion, mice were anesthetized with 5% isoflurane and then decapitated. Coronal brain tissue slices (2 mm) were stained for 20 min at 37 °C with 1% 2,3,5-triphenyltetrazolium chloride monohydrate (TTC, Sigma, St. Louis, MO) in PBS solution. Infarction volume was calculated with correction for edema using ImageJ software as described\(^{96}\). The extent of hemispheric swelling was calculated using the following equation: swelling (% contralateral side) = \(\frac{\text{volume of ipsilateral hemisphere} - \text{volume of contralateral hemisphere}}{\text{volume of contralateral hemisphere}}\) \times 100\(^{92}\).

Neurological deficit score. A neurological deficit grading system\(^{62,92}\) was used to evaluate neurological deficit at 0, 1, 3, 5, and 7 days after tMCAO. The scores are 0, no observable deficit; 1, forelimb flexion; 2, forelimb flexion and decreased resis-
tance to lateral push; 3, forelimb flexion, decreased resistance to lateral push, and unilateral circling; 4, forelimb flexion or partial or complete lack of ambulation.

Corner test. Corner test was used to assess MCAO-induced sensorimotor abnormalities as described previously\(^{92}\). In brief, the corner test apparatus consists of two cardboard boxes (30 x 20 x 1 cm) placed together at a 30° angle to form a narrow alley. The mouse was placed between the two angled boards facing the corner. When exiting the corner, the unjured mice turned left or right randomly. After tMCAO, animals with unilateral brain damage displayed an asymmetry in corner turning. The numbers of left and right turns of each mouse during 10 trials were recorded, and turning movements that were not part of a rearing movement were not scored. Preoperative training was carried out twice per day for 3 days prior to operation. Postoperatively, animals were tested on days 1, 3, 5, and 7.

Adhesive-removal test. An adhesive-removal test was used to measure somato-
sensory deficits as described previously\(^{62,95}\). In brief, two small pieces of adhesive tape (4 x 3 mm) were attached to the forepaws in an alternating sequence and with equal pressure by the experimenter before each trial. Animals were released into a testing cage, and the time of contact and removal of the adhesive patch were recorded. Contact was recorded when either shaking of the paw or mouth contact occurred. The trial ended after the adhesive patch was removed or after 2 min had elapsed. Preoperative training was carried out twice per day for 3 days prior to operation. Postoperatively, animals were tested on days 1, 3, 5, and 7.

DTI ex vivo brains. Seven days post reperfusion, mice were anesthetized with 5% isoflurane, transcardially perfused with 4% paraformaldehyde (PFA), and decapita-
ted\(^{91}\). For ex vivo MRI, brains were maintained within the skull to avoid ana-
tomical deformation. After overnight postfixation in 4% PFA, heads were stored in PBS solution at 4 °C. MRI was performed at 500 MHz using a Bruker AV3HD NSF solution. Infarction volume was calculated with correction for edema using ImageJ software as described\(^{96}\). The extent of hemispheric swelling was calculated using the following equation: swelling (% contralateral side) = \(\frac{\text{volume of ipsilateral hemisphere} - \text{volume of contralateral hemisphere}}{\text{volume of contralateral hemisphere}}\) \times 100\(^{92}\).

Statistical analysis. Animal subjects were randomly assigned into different studies and surgical procedures, and investigators blinded to experimental conditions performed data analyses. The number of animals studied was 80% powered to detect 20% changes with a (2-sided) = 0.05. A total of 168 mice were used in the study, and no results were excluded from the analysis. Data were expressed as means ± SEM. Statistical significance was determined by student’s t test, or one-
way or two-way ANOVA using the Tukey’s post hoc test in the case of multiple comparisons (GraphPad Prism 7.0, San Diego, CA, USA). Neurological deficit scores were analyzed by the nonparametric Mann–Whitney test. A probability value < 0.05 was considered statistically significant. Detailed methods are available in the Supplementary Information.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request. The source data underlying Figs. 2b, 3a, b, c, 5c, 6c, e, f, 7b, d, 8b, and 9b and Supplementary Figs. 2, 3, 4b, 5, 8, 9c, 10b, c, e, f, 11b, c, and 12 are provided as a Source Data file.

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Conceived and designed the experiments: J.Z., M.H.B., K.K., D.S. and X.D. Performed the experiments: J.Z., M.H.B., T.Z., J.K.K., Z.W., M.B.M., S.M.P., T.K.H. and B.I.M. Analyzed the data: J.Z., M.H.B., T.Z., J.K.K., Z.W., M.B.M., S.M.P., X.D., K.K. and D.S. Contribution of reagents/materials/analysis tools: D.D., M.M., A.E.S., A.J.T. and R.P. Wrote the paper: J.Z., M.H.B., S.L.A., K.K., D.S. and X.D.

Competing interests

The authors declare no competing interests.

Additional information

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