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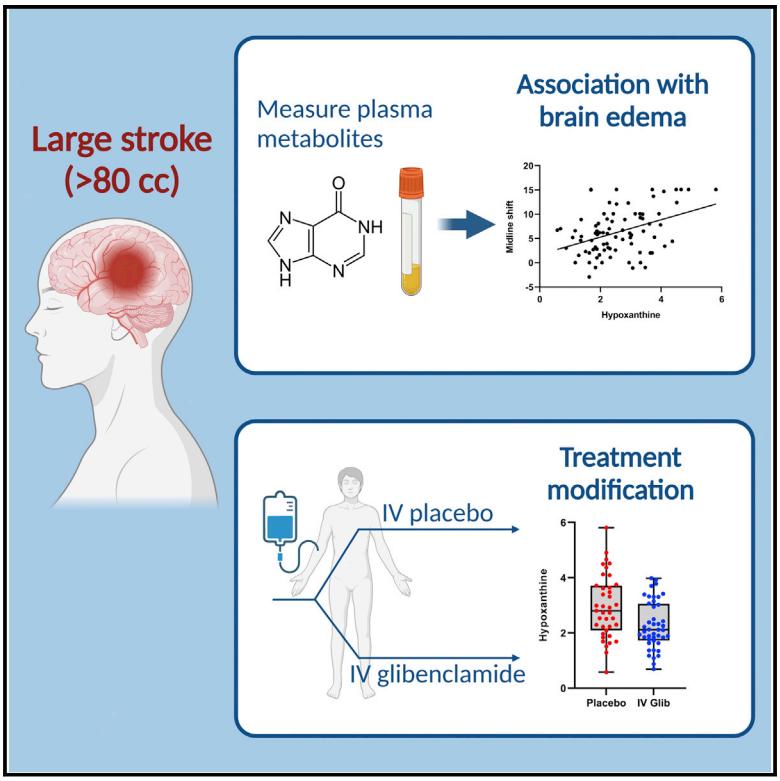
Hypoxanthine is a pharmacodynamic marker of ischemic brain edema modified by glibenclamide

Graphical abstract



Highlights

- Hypoxanthine is associated with brain edema, including midline shift
- Hypoxanthine is reduced by i.v. glibenclamide treatment
- Hypoxanthine mediates the association between i.v. glibenclamide and midline shift

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In brief

In this study, Irvine et al. demonstrate that hypoxanthine, a metabolite linked to hypoxia and inflammation, is associated with brain edema and attenuated by i.v. glibenclamide treatment in patients with large ischemic stroke. These findings provide insight into markers of brain edema, which causes secondary neurologic injury after stroke.

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Report

Hypoxanthine is a pharmacodynamic marker of ischemic brain edema modified by glibenclamide

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SUMMARY

Brain edema after a large stroke causes significant morbidity and mortality. Here, we seek to identify pharmacodynamic markers of edema that are modified by intravenous (i.v.) glibenclamide (glyburide; BIIB093) treatment. Using metabolomic profiling of 399 plasma samples from patients enrolled in the phase 2 Glyburide Advantage in Malignant Edema and Stroke (GAMES)-RP trial, 152 analytes are measured using liquid chromatography-tandem mass spectrometry. Associations with midline shift (MLS) and the matrix metalloproteinase-9 (MMP-9) level that are further modified by glibenclamide treatment are compared with placebo. Hypoxanthine is the only measured metabolite that associates with MLS and MMP-9. In sensitivity analyses, greater hypoxanthine levels also associate with increased net water uptake (NWU), as measured on serial head computed tomography (CT) scans. Finally, we find that treatment with i.v. glibenclamide reduces plasma hypoxanthine levels across all post-treatment time points. Hypoxanthine, which has been previously linked to inflammation, is a biomarker of brain edema and a treatment response marker of i.v. glibenclamide treatment.

INTRODUCTION

The development of malignant brain edema is a leading cause of early clinical deterioration and death after ischemic stroke.[1,2] Lesional swelling exacerbates tissue injury and portends poor long-term functional outcome after stroke.[3] Malignant brain edema, the rapid clinical decline in the first 24–72 h after stroke due to mass-occupying tissue swelling, is a highly morbid sequela of large hemispheric stroke.[4] Limited therapies exist for the treatment of brain edema.[5]

The Glyburide Advantage in Malignant Edema and Stroke (GAMES)-RP trial (ClinicalTrials.gov: NCT01794182) evaluated the safety and efficacy of intravenous (i.v.) glibenclamide (glyburide; BIIB093) to mitigate brain edema in patients suffering large hemispheric infarction and demonstrated a reduction in midline shift (MLS) and the plasma matrix metalloproteinase-9 (MMP-9) level in glibenclamide-treated patients.[6–8] Preclinical data have suggested that glibenclamide binds the sulfonylurea-1 receptor-transient receptor potential melastatin 4 channel (SUR1-TRPM4) and may mitigate brain edema by blocking non-specific ion influx into ischemic cells;[9] however, the exact mechanism of glibenclamide in patients is not fully understood.

Metabolomics measures circulating metabolites, the level of which represents an integrated view of metabolism. For example, metabolite levels may directly reflect metabolic disturbances from ischemia or indirectly relate to changes in the metabolic programs that mediate the post-ischemic inflammatory response.[10,11] Therefore, changes in metabolite levels may reflect underlying pathophysiologic mechanisms and can provide insight into disease mechanisms.[12,13]

In this study, we investigated potential mechanisms of malignant edema by identifying metabolomic markers that were also

Table 1. Characteristics of the study cohort

	i.v. glibenclamide (n = 44)	Placebo (n = 39)	p value
Age, years, mean (SD)	58 (11)	62 (9.0)	0.06
Gender, female, n (%)	16 (36)	11 (28)	0.43
Received i.v. tPA, n (%)	25 (57)	24 (62)	0.66
Baseline NIHSS, median (IQR)	20 (16–22)	19 (17–23)	0.48
Baseline DWI volume, mL, median (IQR)	154 (105–182)	159 (113–200)	0.59
Received hemicraniectomy, n (%)	13 (30)	9 (23)	0.62
Baseline MMP-9, ng/mL, median (IQR)[a]	270 (215–503)	322 (238–442)	0.48
Follow-up MMP-9, ng/mL, median (IQR)[b]	194 (119–295)	254 (182–453)	0.01[c]
Baseline hypoxanthine, mean ± SD[a]	3.34 ± 3.1	3.45 ± 1.3	0.83
Follow-up hypoxanthine, mean ± SD[b]	2.27 ± 0.9	2.94 ± 1.1	0.002[c]

DWI, diffusion weighted imaging; i.v. tPA, intravenous tissue plasminogen activator; IQR, interquartile range; NIHSS, National Institutes of Health Stroke Scale score; ng/mL, nanograms per milliliter; MMP-9, matrix metalloproteinase-9; SD, standard deviation.

[a]Two patients randomized to i.v. glibenclamide and one patient randomized to placebo did not have baseline samples.

[b]Data are for values averaged over 24, 48, and 72 h time points.

[c]p values meeting 0.05 threshold of significance.

modified by i.v. glibenclamide treatment. To address this objective, we first identified candidates associated with MLS and the MMP-9 level, two markers of malignant brain edema that have been shown to be attenuated by i.v. glibenclamide in GAMES-RP.[1,6,14] Second, leading candidates were then evaluated as potential pharmacodynamic markers of i.v. glibenclamide treatment. We hypothesized that a marker associated with these criteria would provide insight into the mechanisms of malignant edema after stroke.

RESULTS

Study population

Of 86 patients enrolled in the GAMES-RP trial, 83 had plasma samples drawn and stored for the current analysis, which corresponded to the modified intention-to-treat cohort as originally reported.[6] This study cohort included 44 patients randomly allocated to the i.v. glibenclamide treatment group, and 39 allocated to the placebo group. The baseline characteristics by treatment arm are shown in Table 1.

Metabolites associated with markers of edema

Metabolites were measured in plasma samples collected from patients at baseline (at time of enrollment and prior to study drug initiation) and after study drug bolus at hours 4–6, 24, 48, and 72. Metabolites were detected and quantified using liquid chromatography-tandem mass spectrometry. A total of 152 unique metabolites selected as sentinel markers for a broad array of biochemical pathways were measured and quantified in each plasma sample. Final values for each metabolite were reported as a ratio to normal human pooled plasma samples.[15–17] We first examined the association of each metabolite with two orthogonal measures of brain edema, MLS[18,19] and MMP-9.[14] Regression analyses identified 18 metabolites in association with MLS at a nominal p value threshold <0.05 (Table S1). However, only one metabolite, hypoxanthine, remained significant after Bonferroni adjustment of the false discovery rate (Figure 1A, volcano plot; β = 1.99, 95% confidence interval [CI] 1.09–2.89, p = 3.41 × 10^{-5}). The volcano-plot association results for MMP-9 are shown in Figure 1B, with 10 metabolites surpassing Bonferroni correction, including hypoxanthine (β = 0.34, 95% CI 0.21–0.48, p = 2.1 × 10^{-6}). The full list of results for MMP-9 are provided in Table S2.

Hypoxanthine is associated with MLS and MMP-9

Having identified hypoxanthine as the top candidate, we next examined the relationship between hypoxanthine and edema in further detail. A positive correlation was found between hypoxanthine and MLS (Figure S1A; Spearman's ρ = 0.37, p = 5.08 × 10^{-4}) and for MMP-9 (Figure S1B; Spearman's ρ = 0.46, p = 1.46 × 10^{-5}). To examine whether this relationship was independent of other known factors that influence edema, multivariable regression analysis was adjusted for age, gender, baseline National Institutes of Health Stroke Scale score (NIHSS), baseline diffusion weighted imaging (DWI) lesion volume, i.v. tissue plasminogen activator (tPA) treatment, decompressive craniectomy, and recanalization status. Hypoxanthine remained an independent predictor of MLS (β = 1.83, 95% CI 1.01–2.65, p = 2.99 × 10^{-5}) and MMP-9 (β = 0.32, 95% CI 0.18–0.45, p = 1.56 × 10^{-5}), with the full results shown in Table 2. These multivariable analyses were repeated with inclusion of time from stroke onset to enrollment, and the results were similar. We also repeated the multivariable analysis for MLS with the inclusion of MMP-9 in the model. In doing so, we found that hypoxanthine remained an independent predictor of MLS independent of MMP-9 (β = 1.66, 95% CI 0.72–2.61, p = 7.8 × 10^{-4}).

In follow-up sensitivity analysis to examine the serial sampling design, we evaluated the association of hypoxanthine at each individual time point with markers of brain edema. Using a mixed effects repeated measures model, hypoxanthine was associated with increased MLS (β = 0.09, 95% CI 0.04–0.13, p = 1.15 × 10^{-4}) and increased MMP-9 (β = 0.60, 95% CI 0.43–0.77, p = 1.28 × 10^{-11}).

We further verified the association of hypoxanthine with brain edema by examining the association with net water uptake (NWU), a recently established marker of brain water content,[20,21] which has also been demonstrated to be reduced by i.v. glibenclamide.[22] NWU, which captures the quantitative change in computed tomography (CT) radiodensity in the stroke lesion compared with the contralateral cerebral hemisphere, was measured serially on non-contrast CT scans obtained from patients between admission and day 7 (n = 254). A median of 3 CT scans were analyzed for each patient (interquartile range [IQR] 2–4). In a mixed effects repeated measures analysis,

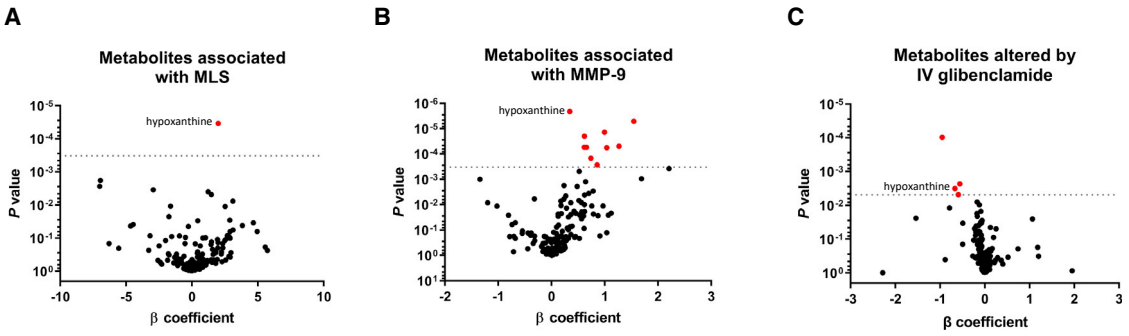

Figure 1. Metabolite associations with MLS, MMP-9 level, and i.v. glibenclamide treatment

(A–C) Volcano plot of metabolite associations with (A) midline shift (MLS) (B) MMP-9 level, and (C) i.v. glibenclamide. The Bonferroni-corrected p value threshold is shown as a dotted line. Metabolites that were significant are identified as red dots, and the location of the leading candidate hypoxanthine is labeled. The full list of metabolite associations is provided in Tables S1–S3.

hypoxanthine was associated with higher NWU (β = 1.57, 95% CI 0.40–2.74, p = 8.28 × 10^{-3}). In multivariable analysis, hypoxanthine remained a predictor of NWU independent of age, gender, baseline NIHSS, DWI lesion volume, tPA treatment, surgical decompression with hemicraniectomy, and recanalization status (Table 2; β = -1.15, 95% CI -2.16–0.16, p = 0.03).

We next explored how baseline (i.e., pre-treatment; 9 h from stroke onset) hypoxanthine compares with baseline MMP-9 as a plasma biomarker for predicting edema formation. The baseline hypoxanthine level predicted MLS by day 4 (odds ratio [OR] = 3.35, 95% CI 1.29–8.72, p = 0.013) whereas baseline MMP-9 did not (OR = 1.31, 95% CI 0.82–2.11, p = 0.26). Accordingly, patients with greater MLS (i.e., dichotomized at a median MLS > 6 mm) also had higher post-treatment hypoxanthine relative to those with less MLS (OR = 1.86, 95% CI = 1.14–3.04, p = 0.012).

Since brain edema has been associated with worse clinical outcome in prior studies,[3,23] we also examined the association of hypoxanthine level with clinical outcome at 90 days after stroke. We found that higher baseline (pre-treatment) hypoxanthine predicted a worse 90 day modified Rankin Scale score (OR 1.69, 95% CI = 1.12–2.56, p = 0.013).

i.v. glibenclamide reduces hypoxanthine level

We next sought to identify candidate metabolites that were altered by i.v. glibenclamide, focusing on the leading candidates identified in association with either MLS or the MMP-9 level. Four metabolites were reduced by i.v. glibenclamide treatment compared with patients in the placebo arm (Figure 1C). These included hypoxanthine, 2-hydroxybutyric acid, 2-aminoadipic acid, and pantothenic acid (see Table S3). However, of the 11 metabolites that were originally identified in association with MLS or MMP-9, only hypoxanthine was reduced by i.v. glibenclamide treatment (mean reduction of 22.8%, 95% CI 7.8–37.6%, p = 0.0031; Figure 2A). The level of hypoxanthine was also examined across the individual plasma-collection time points. We found that the hypoxanthine level was highest at the first plasma time point, corresponding to shortly after study enrollment, followed by a downward trend over time in both treatment groups. Relative to placebo, however, i.v. glibenclamide reduced the hypoxanthine level at all time points after initiation of study drug: hours 4–6, 24, 48, and 72 (Figure 2B; β = -0.62, 95% CI -1.03 to -0.22, p = 2.69 × 10^{-3}).

Hypoxanthine is a mediator of MMP-9 and MLS attenuation by i.v. glibenclamide

In the primary analysis of the GAMES-RP trial, i.v. glibenclamide was found to reduce MLS and MMP-9.[6] We therefore evaluated the extent to which hypoxanthine mediated the treatment-induced reduction in MLS and MMP-9. Mediation analysis using the change in coefficient approach showed that hypoxanthine mediated the effect of i.v. glibenclamide on MLS (32% mediation, Sobel p = 0.017; Figure S2A). Hypoxanthine also mediated the effect of i.v. glibenclamide on MMP-9 (52% mediation, Sobel p = 0.0095; Figure S2B).

DISCUSSION

In this study, we identified plasma hypoxanthine as a marker of brain edema after ischemic stroke. This association was independent of age, gender, NIHSS, DWI lesion volume, tPA treatment, decompressive hemicraniectomy, and recanalization status. Further, we demonstrate that among 152 metabolites analyzed, hypoxanthine was the only candidate that was associated with MMP-9 and MLS and modified by glibenclamide treatment. Elevated plasma hypoxanthine was associated with increased MMP-9 and greater MLS, whereas treatment with i.v. glibenclamide significantly reduced plasma hypoxanthine. We also demonstrate that the hypoxanthine level acts as a mediator of the effect of glibenclamide on MMP-9 and MLS. Together, these findings broaden our understanding of the mechanism of glibenclamide beyond its effect on the SUR1-TRPM4 channel, raising the possibility that glibenclamide also acts to attenuate brain edema by reducing hypoxanthine.

Hypoxanthine is a reaction intermediate in the purine-degradation pathway. It is catabolized into xanthine and uric acid by xanthine oxidase (XO). In states of hypoxia, the degradation of adenine nucleotides to hypoxanthine accelerates, and the rate of conversion to xanthine and uric acid by XO is slowed because O_2 is a required cofactor. As such, elevated hypoxanthine has

Table 2. Univariable and multivariable predictors of MMP-9 and MLS

	Univariable				Multivariable			
	β	95% CI		p value	Adjusted β	95% CI		p value
MMP-9								
Age	0.01	−0.01	0.03	0.25	0.01	−0.01	0.03	0.22
Gender (female)	0.08	−0.26	0.42	0.65	−0.06	−0.42	0.30	0.75
Baseline NIHSS	0.02	−0.02	0.06	0.34	−0.01	−0.05	0.04	0.76
DWI lesion volume	0.60	−0.35	1.55	0.21	1.01	−0.11	2.12	0.08
Treatment with tPA	−0.33	−0.65	−0.01	0.04[a]	−0.41	−0.76	−0.07	0.02[a]
Hemicraniectomy	0.06	−0.30	0.42	0.75	−0.13	−0.55	0.28	0.52
Recanalization	−0.07	−0.43	0.28	0.68	−0.04	−0.38	0.30	0.83
Hypoxanthine	0.34	0.21	0.48	2.09E−6[a]	0.32	0.15	0.50	5.30 E−4[a]
MLS								
Age	−0.01	−0.11	0.10	0.87	−0.03	−0.12	0.06	0.58
Gender (female)	1.67	−0.53	3.88	0.135	0.75	−1.16	2.65	0.44
Baseline NIHSS	0.23	−0.01	0.48	0.06	0.11	−0.09	0.31	0.29
DWI lesion volume	13.34	7.80	18.88	7.43E−6[a]	10.23	4.65	15.81	4.79E−4[a]
Treatment with tPA	−0.43	−2.56	1.70	0.69	−0.22	−2.01	1.56	0.81
Hemicraniectomy	3.32	1.06	5.58	4.5E−3[a]	1.37	−0.76	3.51	0.21
Recanalization	−2.28	−4.31	−0.24	0.03[a]	−0.93	−2.70	0.84	0.30
Hypoxanthine	1.99	1.09	2.89	3.41E−5[a]	1.70	0.86	2.53	1.20E−4[a]
NWU								
Age	0.04	−0.21	0.14	0.67	0.06	−0.16	0.28	0.59
Gender (female)	6.22	2.56	9.89	8.69E−3[a]	4.55	0.20	8.90	0.04[a]
Baseline NIHSS	0.34	−0.10	0.77	0.13	0.38	−0.08	0.84	0.10
DWI lesion volume	0.01	−0.02	0.04	0.42	0.00	−0.03	0.04	0.93
Treatment with tPA	−0.10	−3.65	3.44	0.95	0.43	−3.57	4.43	0.83
Hemicraniectomy	2.02	−1.79	5.83	0.30	3.36	−1.40	8.12	0.17
Recanalization	2.11	−1.39	5.61	0.24	−1.45	−5.30	2.41	0.46
Hypoxanthine	−1.14	−2.12	−0.14	0.03[a]	−1.15	−2.16	−0.15	0.03[a]

MMP-9 and DWI lesion volume log transformed. CI: confidence interval; DWI: diffusion weighted imaging; MLS: midline shift, NWU: net water uptake, MMP-9: matrix metalloproteinase-9, tPA: tissue plasminogen activator.
[a]p values meeting 0.05 threshold of significance.

been demonstrated to be a marker of hypoxia in several disease states.[24–26] Interestingly, the oxidation of hypoxanthine and xanthine by XO also produces reactive oxygen species (ROS)[27] and triggers cytotoxicity.[28] In healthy non-ischemic cells, XO exists predominantly as xanthine dehydrogenase (XD), which uses NAD+ instead of O_2 as a cofactor. In ischemia, however, XD is rapidly converted to XO.[29] When oxygen becomes available again during tissue reperfusion, XO, with its required cofactor O_2, then oxidizes hypoxanthine and xanthine, generating abundant ROS in the process. Several studies have linked hypoxanthine to the formation of free radicals and ROS.[30,31] Accordingly, hypoxanthine has been implicated in ischemia-reperfusion injury.[30,32]

In large stroke, edema due to blood-brain-barrier breakdown contributes to mass effect, MLS, and poor functional outcome. Elevated plasma hypoxanthine can amplify ROS production and induce endothelial cell dysfunction *in vitro*.[31] Further, it is known that oxidative stress induces MMP-9, which mediates degradation of the tight junctions that make up the blood-brain barrier (BBB).[33] Taken together, it is a plausible hypothesis that elevated hypoxanthine promotes increased ROS, which in turn causes an increase in MMP-9, further compromising the BBB and leading to increased brain edema. Previous analysis of the GAMES-RP trial has demonstrated that the MMP-9 level peaks within hours after acute stroke then decreases over time and that i.v. glibenclamide treatment attenuates the MMP-9 level for up to 72 h after stroke.[6] Considering the current literature on hypoxanthine and MMP-9, we posit that i.v. glibenclamide may attenuate the overall severity of ischemic insult by reducing the amount of swelling and/or by reducing oxidative injury. That said, our study does not clarify the precise mechanism of hypoxanthine; rather, our data support a role for hypoxanthine as a pharmacodynamic marker, which can serve as a serial marker of treatment effect. Further study is needed to validate our findings and explore the underlying mechanistic link between hypoxanthine, edema, and i.v. glibenclamide.

Limitations of the study

Our study has limitations. First, this was a post-hoc analysis of a clinical trial with a relatively small sample size (n = 83). Second,

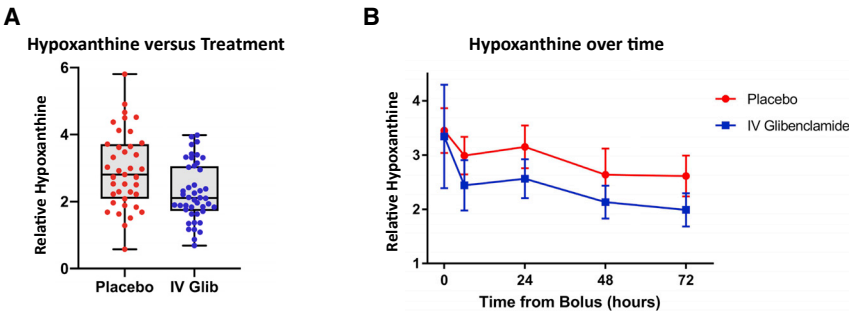

Figure 2. Treatment with i.v. glibenclamide reduces hypoxanthine level

(A) Hypoxanthine is reduced by treatment with i.v. glibenclamide when averaged across post-treatment time points. The box-and-whisker plot depicts average hypoxanthine level in patients treated with i.v. glibenclamide (n = 44) versus those randomly assigned to placebo (n = 36). Boxes represent the interquartile range with the median shown as a horizontal black line, and the whiskers represent the minimum and maximum values. The hypoxanthine level is relative to human pooled plasma values from healthy volunteers.

(B) Hypoxanthine is reduced by treatment with i.v. glibenclamide in mixed effects repeated measures analysis at individual post-treatment time points. The error bars are 95% confidence intervals at baseline and 4, 24, 48, and 72 h from start of study drug.

our study may have limited generalizability because the GAMES-RP study included only patients with large hemispheric infarction. Therefore, our findings may not reflect the pathophysiology of small and/or moderate strokes. Further, none of GAMES-RP patients were treated with mechanical thrombectomy. Although thrombectomy is not routinely used to treat patients who have a large core volume stroke, our cohort may not reflect the evolving standard of care for large ischemic stroke. Prior studies have shown that thrombectomy has a complex interaction with edema formation,[23,34] and analysis of hypoxanthine in these scenarios could provide further understanding of potentially conflicting results. Considering these limitations of our study's cohort, replication of our findings in a larger trial that includes patients undergoing thrombectomy is required to provide independent confirmation of our findings (CHARM study, ClinicalTrials.gov: NCT02864953, currently enrolling). Another limitation of our study is the lack of *in vitro* study supporting our findings. Because of this, we cannot draw conclusions about the underlying causal mechanism of the association between hypoxanthine, markers of edema, and glibenclamide treatment. Further *in vitro* study is necessary to validate the mechanism by which i.v. glibenclamide reduces hypoxanthine.

Conclusion

Taken together, our findings demonstrate that i.v. glibenclamide acts to reduce plasma hypoxanthine. Our results raise the possibility that i.v. glibenclamide reduces MMP-9 and MLS in patients via a reduction in hypoxanthine level. As a mediator of ROS generation following ischemia, hypoxanthine may be a candidate biomarker for brain-edema formation after acute stroke.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2022.100654.

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AUTHOR CONTRIBUTIONS

Conceptualization, H.J.I., A.A., and W.T.K.; methodology, H.J.I., A.A., Z.W., Z.A., and W.T.K.; investigation, H.J.I., A.A., Z.A., and W.T.K.; writing – original draft, H.J.I. and W.T.K.; writing – review & editing, H.J.I., A.A., Z.W., Z.A., H.E.H., B.J.M., J.M.S., K.N.S., and W.T.K.; funding acquisition, K.N.S. and W.T.K.; supervision, K.N.S. and W.T.K.

DECLARATION OF INTERESTS

J.M.S. has a patent related to the study and serves on the Board of Directors for Remedy Pharmaceuticals. H.E.H., B.J.M., W.T.K., and K.N.S. received grants from Remedy Pharmaceuticals outside the submitted work. H.E.H., K.N.S., and W.T.K. received research grants from Biogen and the American Heart Association. W.T.K. received grants and personal fees from NControl Therapeutics outside the submitted work. K.N.S. received grants from Bard, Hyperfine, and Astrocyte outside the submitted work. H.E.H., B.J.M., J.M.S., and W.T.K. received personal fees from Biogen outside the submitted work. During the peer review process, Biogen had the opportunity to review the manuscript. The authors retained full editorial control of the manuscript and provided their final approval of all content.

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REFERENCES

1. Hacke, W., Schwab, S., Horn, M., Spranger, M., De Georgia, M., and von Kummer, R. (1996). 'Malignant' middle cerebral artery territory infarction: clinical course and prognostic signs. Arch. Neurol. *53*, 309–315. https://doi.org/10.1001/archneur.1996.00550040037012.
2. Qureshi, A.I., Suarez, J.I., Yahia, A.M., Mohammad, Y., Uzun, G., Suri, M.F.K., Zaidat, O.O., Ayata, C., Ali, Z., and Wityk, R.J. (2003). Timing of neurologic deterioration in massive middle cerebral artery infarction: a multicenter review. Crit. Care Med. *31*, 272–277. https://doi.org/10.1097/00003246-200301000-00043.
3. Battey, T.W.K., Karki, M., Singhal, A.B., Wu, O., Sadaghiani, S., Campbell, B.C., Davis, S.M., Donnan, G.A., Sheth, K.N., and Kimberly, W.T. (2014). Brain edema predicts outcome after nonlacunar ischemic stroke. Stroke *45*, 3643–3648. https://doi.org/10.1161/strokeaha.114.006884.
4. Huttner, H.B., and Schwab, S. (2009). Malignant middle cerebral artery infarction : clinical characteristics , treatment strategies , and future perspectives. Lancet Neurol. *8*, 949–958. https://doi.org/10.1016/s1474-4422(09)70224-8.
5. Ayata, C., and Ropper, A.H. (2002). Ischaemic brain oedema. J. Clin. Neurosci. *9*, 113–124. https://doi.org/10.1054/jocn.2001.1031.
6. Sheth, K.N., Elm, J.J., Molyneaux, B.J., Hinson, H., Beslow, L.A., Sze, G.K., Ostwaldt, A.C., del Zoppo, G.J., Simard, J.M., Jacobson, S., and Kimberly, W.T. (2016). Safety and efficacy of intravenous glyburide on brain swelling after large hemispheric infarction (GAMES-RP): a randomised, double-blind, placebo-controlled phase 2 trial. Lancet Neurol. *15*, 1160–1169. https://doi.org/10.1016/s1474-4422(16)30196-x.
7. Sheth, K.N., Petersen, N.H., Cheung, K., Elm, J.J., Hinson, H.E., Molyneaux, B.J., Beslow, L.A., Sze, G.K., Simard, J.M., and Kimberly, W.T. (2018). Long-term outcomes in patients aged $\geq$70 years with intravenous glyburide from the Phase II GAMES-RP study of large hemispheric infarction an exploratory analysis. Stroke *49*, 1457–1463. https://doi.org/10.1161/strokeaha.117.020365.
8. Kimberly, W.T., Bevers, M.B., von Kummer, R., Romero, J.M., Elm, J.J., Hinson, H.E., Molyneaux, B.J., Simard, J.M., and Sheth, K.N. (2018). Effect of IV glyburide on adjudicated edema endpoints in the GAMES-RP Trial. Neurology *91*, e2163–e2169. https://doi.org/10.1212/wnl.0000000000006618.
9. Simard, J.M., Chen, M., Tarasov, K.V., Bhatta, S., Ivanova, S., Melnitchenko, L., Tsymbalyuk, N., West, G.A., and Gerzanich, V. (2006). Newly expressed SUR1-regulated NC(Ca-ATP) channel mediates cerebral edema after ischemic stroke. Nat. Med. *12*, 433–440. https://doi.org/10.1038/nm1390.
10. Fox, C.J., Hammerman, P.S., and Thompson, C.B. (2005). Fuel feeds function: energy metabolism and the T-cell response. Nat. Rev. Immunol. *5*, 844–852. https://doi.org/10.1038/nri1710.
11. Kominsky, D.J., Campbell, E.L., and Colgan, S.P. (2010). Metabolic shifts in immunity and inflammation. J. Immunol. *184*, 4062–4068. https://doi.org/10.4049/jimmunol.0903002.
12. Ramautar, R., Berger, R., van der Greef, J., and Hankemeier, T. (2013). Human metabolomics: strategies to understand biology. Curr. Opin. Chem. Biol. *17*, 841–846. https://doi.org/10.1016/j.cbpa.2013.06.015.
13. Nicholson, J.K., Lindon, J.C., and Holmes, E. (1999). 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica *29*, 1181–1189. https://doi.org/10.1080/004982599238047.
14. Serena, J., Blanco, M., Castellanos, M., Silva, Y., Vivancos, J., Moro, M.A., Leira, R., Lizasoain, I., Castillo, J., and Dávalos, A. (2005). The prediction of malignant cerebral infarction by molecular brain barrier disruption markers. Stroke *36*, 1921–1926. https://doi.org/10.1161/01.str.0000177870.14967.94.
15. Nelson, S.E., Ament, Z., Wolcott, Z., Gerszten, R.E., and Kimberly, W.T. (2019). Succinate links atrial dysfunction and cardioembolic stroke. Neurology *92*, e802–e810. https://doi.org/10.1212/wnl.0000000000006957.
16. Ament, Z., Bevers, M.B., Wolcott, Z., Kimberly, W.T., and Acharjee, A. (2021). Uric acid and gluconic acid as predictors of hyperglycemia and cytotoxic injury after stroke. Transl. Stroke Res. *12*, 293–302. https://doi.org/10.1007/s12975-020-00862-5.
17. Koch, M., Acharjee, A., Ament, Z., Schleicher, R., Bevers, M., Stapleton, C., Patel, A., and Kimberly, W.T. (2021). Machine learning-driven metabolomic evaluation of cerebrospinal fluid: insights into poor outcomes after aneurysmal subarachnoid hemorrhage. Neurosurgery *88*, 1003–1011. https://doi.org/10.1093/neuros/nyaa557.
18. Ropper, A.H. (1986). Lateral displacement of the brain and level of consciousness in patients with an acute hemispheral mass. N. Engl. J. Med. *314*, 953–958. https://doi.org/10.1056/nejm198604103141504.
19. Ross, D.A., Olsen, W.L., Ross, A.M., Andrews, B.T., and Pitts, L.H. (1989). Brain shift, level of consciousness, and restoration of consciousness in patients with acute intracranial hematoma. J. Neurosurg. *71*, 498–502. https://doi.org/10.3171/jns.1989.71.4.0498.
20. Minnerup, J., Broocks, G., Kalkoffen, J., Langner, S., Knauth, M., Psychogios, M.N., Wersching, H., Teuber, A., Heindel, W., Eckert, B., et al. (2016). Computed tomography–based quantification of lesion water uptake identifies patients within 4.5 hours of stroke onset: a multicenter observational study. Ann. Neurol. *80*, 924–934. https://doi.org/10.1002/ana.24818.
21. Broocks, G., Flottmann, F., Scheibel, A., Aigner, A., Faizy, T.D., Hanning, U., Leischner, H., Broocks, S.I., Fiehler, J., Gellissen, S., and Kemmling, A. (2018). Quantitative lesion water uptake in acute stroke computed tomography is a predictor of malignant infarction. Stroke *49*, 1906–1912. https://doi.org/10.1161/strokeaha.118.020507.
22. Vorasayan, P., Bevers, M.B., Beslow, L.A., Sze, G., Molyneaux, B.J., Hinson, H.E., Simard, J.M., von Kummer, R., Sheth, K.N., and Kimberly, W.T. (2019). Intravenous glibenclamide reduces lesional water uptake in large hemispheric infarction. Stroke *50*, 3021–3027. https://doi.org/10.1161/strokeaha.119.026036.
23. Kimberly, W.T., Dutra, B.G., Boers, A.M.M., Alves, H.C.B.R., Berkhemer, O.A., van den Berg, L., Sheth, K.N., Roos, Y.B.W.E.M., van der Lugt, A., Beenen, L.F.M., et al. (2018). Association of reperfusion with brain edema in patients with acute ischemic stroke: a secondary analysis of the MR CLEAN Trial. JAMA Neurol. *75*, 453–459. https://doi.org/10.1001/jamaneurol.2017.5162.
24. Nagao, H., Nishizawa, H., Tanaka, Y., Fukata, T., Mizushima, T., Furuno, M., Bamba, T., Tsushima, Y., Fujishima, Y., Kita, S., et al. (2018). Hypoxanthine secretion from human adipose tissue and its increase in hypoxia. Obesity *26*, 1168–1178. https://doi.org/10.1002/oby.22202.
25. Farthing, D.E., Farthing, C.A., and Xi, L. (2015). Inosine and hypoxanthine as novel biomarkers for cardiac ischemia: from bench to point-of-care. Exp. Biol. Med. *240*, 821–831. https://doi.org/10.1177/1535370215584931.
26. Fisher, O., Benson, R.A., and Imray, C.H. (2019). The clinical application of purine nucleosides as biomarkers of tissue Ischemia and hypoxia in humans in vivo. Biomark. Med. *13*, 953–964. https://doi.org/10.2217/bmm-2019-0049.
27. Saugstad, O.D. (1988). Hypoxanthine as an indicator of hypoxia: its role in health and disease through free radical production. Pediatr. Res. *23*, 143–150. https://doi.org/10.1203/00006450-198802000-00001.
28. Simon, R.H., Scoggin, C.H., and Patterson, D. (1981). Hydrogen peroxide causes the fatal injury to human fibroblasts exposed to oxygen radicals. J. Biol. Chem. *256*, 7181–7186. https://doi.org/10.1016/s0021-9258(19)68945-3.
29. Wu, M.Y., Yiang, G.T., Liao, W.T., Tsai, A.Y., Cheng, Y.L., Cheng, P.W., Li, C.Y., and Li, C.J. (2018). Current mechanistic concepts in ischemia and reperfusion injury. Cell. Physiol. Biochem. *46*, 1650–1667. https://doi.org/10.1159/000489241.

30. Zimmerman, B.J., and Granger, D.N. (1992). Reperfusion injury. Surg. Clin. North Am. *72*, 65–83. https://doi.org/10.1016/s0039-6109(16)45628-8.

31. Kim, Y.J., Ryu, H.M., Choi, J.Y., Cho, J.H., Kim, C.D., Park, S.H., and Kim, Y.L. (2017). Hypoxanthine causes endothelial dysfunction through oxidative stress-induced apoptosis. Biochem. Biophys. Res. Commun. *482*, 821–827. https://doi.org/10.1016/j.bbrc.2016.11.119.

32. Abd-Elfattah, A.S., Jessen, M.E., Lekven, J., Doherty, N.E., Brunsting, L.A., and Wechsler, A.S. (1988). Myocardial reperfusion injury. Role of myocardial hypoxanthine and xanthine in free radical-mediated reperfusion injury. Circulation *78*, I224–I235.

33. Yang, C., Hawkins, K.E., Doré, S., and Candelario-Jalil, E. (2019). Neuroinflammatory mechanisms of blood-brain barrier damage in ischemic stroke. Am. J. Physiol. Cell Physiol. *316*, C135–C153. https://doi.org/10.1152/ajpcell.00136.2018.

34. Irvine, H.J., Ostwaldt, A.C., Bevers, M.B., Dixon, S., Battey, T.W., Campbell, B.C., Davis, S.M., Donnan, G.A., Sheth, K.N., Jahan, R., et al. (2018). Reperfusion after ischemic stroke is associated with reduced brain edema. J. Cereb. Blood Flow Metab. *38*, 1807–1817. https://doi.org/10.1177/0271678X17720559.

35. Sheth, K.N., Elm, J.J., Beslow, L.A., Sze, G.K., and Kimberly, W.T. (2016). Glyburide advantage in malignant edema and stroke (GAMES-RP) trial: rationale and design. Neurocrit. Care *24*, 132–139. https://doi.org/10.1007/s12028-015-0189-7.

36. Stapleton, C.J., Acharjee, A., Irvine, H.J., Wolcott, Z.C., Patel, A.B., and Kimberly, W.T. (2019). High-throughput metabolite profiling: identification of plasma taurine as a potential biomarker of functional outcome after aneurysmal subarachnoid hemorrhage. J. Neurosurg. *133*, 1–8. https://doi.org/10.3171/2019.9.jns191346.

37. MacKinnon, D.P., Fairchild, A.J., and Fritz, M.S. (2007). Mediation analysis. Annu. Rev. Psychol. *58*, 593–614. https://doi.org/10.1146/annurev.psych.58.110405.085542.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
EDTA blood samples from subjects enrolled in the GAMES-RP trial	Sheth et al., 2016	https://clinicaltrials.gov/ct2/show/NCT01794182
Chemicals, peptides, and recombinant proteins		
Acetonitrile, Optima LC/MS grade	Thermo Fisher	Cat# A955-212
Methanol, Optima LC/MS grade	Thermo Fisher	Cat# A456-212
Ammonium Acetate	Millipore Sigma	Cat# 73594–25G-F
Ammonium Hydroxide solution	Millipore Sigma	Cat# 338818–100ML
Critical commercial assays		
MMP-9 Quantikine ELISA	R&D Systems	Cat# DMP900
Software and algorithms		
Masshunter QQQ	Agilent	https://www.agilent.com/en/product/software-informatics/mass-spectrometry-software/data-analysis/quantitative-analysis
STATA 15.1 MP	StataCorp	http://www.stata.com
Other		
XBridge Amide column, 2.1 × 100mm 3.5 μm	Waters	Cat# 186004860
1290 Infinity II Multisampler	Agilent	Cat# G7167B
1290 Infinity II HPLC binary pump	Agilent	Cat# G7120A
6495 QQQ tandem mass spectrometer	Agilent	Cat# G6495AA

RESOURCE AVAILABILITY

Lead contact

Requests for additional information and resources should be directed to the lead contact, W. Taylor Kimberly (wtkimberly@mgh.harvard.edu).

Materials availability

This study did not generate unique reagents.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request. This paper does not report the original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Patient characteristics

This was a post hoc, exploratory analysis of blood specimens collected during the GAMES-RP trial (Glyburide Advantage in Malignant Edema and Stroke). The double-blinded, randomized trial design and patient eligibility criteria have been previously reported.[6,35] Briefly, female and male subjects aged 18–80 with large hemispheric infarction (e.g., baseline lesion volume 82–300mL) were enrolled in the GAMES-RP study. Subjects were randomly assigned using a centralized, web-based randomization algorithm to either placebo or IV glibenclamide (glyburide; BIIB093) treatment within 10 h of stroke onset. All subjects or their surrogates provided informed consent, and this study was reviewed and approved by the Institutional Review Board at Mass General Brigham.

METHOD DETAILS

Study procedures and imaging analysis

Subjects had serial blood samples collected at baseline prior to study drug initiation, and at 4–6 h, 24, 48, and 72 h after the start of study drug bolus. The mean MMP-9 level between 24 and 72 h was used for initial analysis since this value was pre-specified in the original trial analysis. Additionally, MMP-9 level at each timepoint was analyzed in a sensitivity analysis.

All subjects also received a pre-treatment and a post-treatment brain MRI. The MLS value that was measured on the 72–96 h brain MRI as part of the original trial was used for analyses. Net water uptake (NWU), which captures quantitative change in CT radiodensity of infarcted tissue as compared to the contralateral cerebral hemisphere, was measured using region of interest (ROI) analysis on serial head CT scans, as described previously.[22] Non-contrast CT scans obtained from patients between admission and day 7 (n = 254) were included in this analysis. A median of 3 CT scans were analyzed for each patient (IQR 2–4).

Metabolite profiling

The blood sample collection and processing methodology in GAMES-RP has been previously reported.[6] All EDTA plasma samples collected in GAMES-RP were stored at −80°C until analysis. Metabolites were measured using liquid chromatography-triple quadrupole tandem mass spectrometry based on our prior methods.[15,16,36] Briefly, 30μL of each plasma sample was deproteinized with 70μL of 3:1 acetonitrile:methanol and 5μL of the resulting supernatant was injected on a hydrophilic interaction column (XBridge Amide column, Waters). Metabolites were detected and quantified using a targeted triple quadrupole mass spectrometer (QQQ 6495, Agilent) in both positive and negative ion modes. The mobile phase A was 95:5 (v/v) water:acetonitrile containing 20mM ammonium acetate and 20mM ammonium hydroxide at pH 9.5. The Mobile phase B was acetonitrile. Ammonium acetate, ammonium hydroxide and Optima grade solvents were purchased from Thermo Fisher.

For Amide negative mode, the initial conditions were 0.25 mL/min of 85% mobile phase B followed by a linear gradient to 35% mobile phase B over 6 min. This was followed by a linear gradient to 2% mobile phase B over 0.5 min held for an additional 0.5 min, then a 0.5 min gradient return to 85% mobile phase B. Column equilibration was continued for 4.5 min at 0.5 mL/min for a total cycle time of ∼12.5 min. The column compartment was maintained at 30°C. For Amide positive mode, that initial conditions were 0.25 mL/min of 90% mobile phase B. A linear gradient to 10% mobile phase B over 6 min was followed by a hold for 1 min at 10% mobile phase B. The conditions to 90% mobile phase B were restored over 0.5 min and then the column equilibrated for 4.5 min at 0.5 mL/min. Peak integration was performed in MassHunter Quantitative Analysis software (Agilent). Peaks were quality checked and normalized to human pooled plasma samples collected from healthy volunteers that were interspersed every 10 injections, using standard procedures.[15,16,37] Final values for each metabolite were reported as a ratio to the normal human pooled plasma samples. A total of 152 unique metabolites were measured and quantified in each sample.

QUANTIFICATION AND STATISTICAL ANALYSIS

Wilcoxon rank sum or the Student's t test was used to compare continuous variables and baseline characteristics. To identify candidate metabolites, we used a sequential approach. We reasoned that glibenclamide treatment might have off-target effects unrelated to edema, therefore we first developed a focused list of candidates by examining relationships to markers of edema first. In the first stage of analysis, linear regression was used to identify analytes associated with MLS or MMP-9 level. All candidates that were associated with either MMP-9 or MLS and surpassed Bonferroni-correction (e.g., $\alpha < 3.29 \times 10^{-4}$) were then carried forward for analysis with IV glibenclamide treatment. In both stages of analyses, the average metabolite level between 24 and 72 h was used for each metabolite, mirroring the pre-specified approach used for MMP-9 in the original study design.

As a further sensitivity analysis, we then examined the associations of the top candidate metabolites at each individual time point, using a repeated-measures, mixed-effects linear regression model. A repeated-measures, mixed-effects model was also used for analysis of hypoxanthine with NWU, which was measured serially on head CTs. Because CT scanning was not performed as a research study procedure, the timing and frequency of CT scans varied among subjects, and as such a repeated-measures mixed effect model allowed for the maximal inclusion of patients.

A multivariable linear regression model was also used to examine the association between hypoxanthine and MMP-9, MLS, or NWU, adjusting for age, gender, baseline NIHSS, baseline DWI volume, treatment with IV tPA, decompressive craniectomy, and recanalization status. Covariates were assessed for multicollinearity by examining variance inflation factor (VIF) values. All covariates included in the multivariable analyses had VIF <5. To compare the performance of hypoxanthine and MMP-9 in predicting edema, day 4 MLS was dichotomized at the median value (MLS = 6mm) and each biomarker was mean-centered and unit-standardized each biomarker in order to directly compare beta effect sizes. Logistic regression was used to compare effect sizes in models that included hypoxanthine or MMP-9. To examine the association between hypoxanthine and 90-day modified Rankin Scale score, shift analysis was performed using logistic regression. Mediation analysis[37] was performed to evaluate whether hypoxanthine altered the association between treatment and MLS or MMP-9. The Sobel test was used to determine the significance of mediation, and the difference in the coefficients was calculated after introducing hypoxanthine as mediator. For non-parametric

variables (hypoxanthine and MMP-9), data were log-transformed prior to analysis. Statistical analysis was performed using STATA 15.1 (StataCorp).

ADDITIONAL DETAILS

The clinical trial identifier for the GAMES-RP trial is NCT01794182: https://www.clinicaltrials.gov/ct2/show/NCT01794182.