Aquaporin-9 Protein Is the Primary Route of Hepatocyte Glycerol Uptake for Glycerol Gluconeogenesis in Mice*

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Background: Aquaporin-9 is a glycerol-permeable channel expressed in hepatocytes.

Results: Newly identified chemical inhibitors, as well as aquaporin-9 gene deletion, eliminate glycerol-dependent murine hepatocyte glucose output.

Conclusion: Aquaporin-9 is the primary route of murine hepatocyte glycerol uptake for gluconeogenesis.

Significance: Aquaporin-9 may be a drug target in diabetes treatment.

It has been hypothesized that aquaporin-9 (AQP9) is part of the unknown route of hepatocyte glycerol uptake. In a previous study, leptin receptor-deficient wild-type mice became diabetic and suffered from fasting hyperglycemia whereas isogenic AQP9−/− knock-out mice remained normoglycemic. The reason for this improvement in AQP9−/− mice was not established before. Here, we show increased glucose output (by 123% ± 36% S.E.) in primary hepatocyte culture when 0.5 mM extracellular glycerol was added. This increase depended on AQP9 because it was absent in AQP9−/− cells. Likewise, the increase was abolished by 25 μM HTS13286 (IC50 ~ 2 μM), a novel AQP9 inhibitor, which we identified in a small molecule library screen. Similarly, AQP9 deletion or chemical inhibition eliminated glycerol-enhanced glucose output in perfused liver preparations. The following control experiments suggested inhibitor specificity to AQP9: (i) HTS13286 affected solute permeability in cell lines expressing AQP9, but not in cell lines expressing AQPs 3, 7, or 8. (ii) HTS13286 did not influence lactate- and pyruvate-dependent hepatocyte glucose output. (iii) HTS13286 did not affect glycerol kinase activity. Our experiments establish AQP9 as the primary route of hepatocyte glycerol uptake for gluconeogenesis and thereby explain the previously observed, alleviated diabetes in leptin receptor-deficient AQP9−/− mice.

Gluconeogenesis from glycerol (henceforth glycerol gluconeogenesis) accounts for ~10% of hepatic glucose production in patients with type 2 diabetes (T2D)3 (1). Increased glycerol gluconeogenesis in T2D patients results not simply from accelerated lipolysis but also from altered hepatic glycerol utilization (1). Hepatic glycerol uptake has been investigated in a number of studies, in primary hepatocyte cell culture and in perfused liver preparations (2–4). Nevertheless, it is still unclear whether facilitated glycerol uptake is required to sustain glycerol gluconeogenesis at physiological rates. Furthermore, the molecular identity of possible glycerol carrier(s) is unknown. It has been hypothesized that the glycerol-permeable channel protein AQP9, a member of the aquaglyceroporin family, may play a role in hepatic glycerol gluconeogenesis, because it is expressed in the liver and expression is negatively regulated by insulin (5). However, similarly regulated expression of other aquaglyceroporins in the liver has been described (6, 7). Therefore, the relative contributions of each carrier to hepatic glycerol uptake, possible contributions from additional carriers, as well as the relative contribution of lipid bilayer mediated diffusion to glycerol gluconeogenesis remain unclear. AQP9 gene deletion in obese, diabetic mice was found to reduce plasma glucose levels by between 10 and 40% (8). However, oral glycerol caused identical subsequent elevations of blood glucose levels in AQP9+/+ and AQP9−/− mice. These experiments suggested an unspecified influence of AQP9 on plasma glucose levels in obese diabetic mice (8). We concluded that these observations required alternative investigation of a possible AQP9 contribution to hepatocyte glucose output. In this study, we have identified novel drugs that specifically inhibit AQP9. We have utilized these substances to investigate whether AQP9 is necessary to sustain hepatic glycerol gluconeogenesis at physiological rates.

EXPERIMENTAL PROCEDURES

DNA Constructs—mAQP8 and mAQP9 were amplified from mouse liver cDNA (NMRI strain); mAQP3 and mAQP7 were amplified from mouse kidney cDNA (C57BL/6 strain). Oligo-
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nucleotide sequences are provided in the supplemental Oligonucleotide list. All AQP PCR products were first inserted into Topo-PCRII (Invitrogen) followed by restriction enzyme digest and ligation with pcDNA5/FRT/TO (Invitrogen).

CHO Cell Culture—CHO-FlpIn cells (Invitrogen) were transfected with pcDNA6/TR (Invitrogen), and stable cell lines (CHO-FlpIn-TR) were selected as recommended by the manufacturer. Lipofectamine (Invitrogen) was used to transfect these cells with pcDNA5/FRT/TO-AQP plasmids. Cells were grown at 37 °C, 5% CO₂, and selected in hygromycin B (Invitrogen) containing medium (DMEM/F12, containing 5% donor bovine serum; Invitrogen).

AQP9 Inhibitor Screen and Solute Permeability Measurements—Water permeability measurements were as described (9) with modifications: 15,000 CHO-AQP cells were seeded per well of poly-D-lysine (Greiner Bio One)-coated 96-well plates in the absence of serum; Invitrogen).

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Liver Perfusion—Liver perfusions were essentially performed as described by Kuriyama et al. (5) with minor modifications. Perfusion was from a plastic catheter inserted into the portal vein, and outflow was through the abdominal vena cava inferior. Perfusion flow rate was reduced to 6 ml/min. LiberaseTM (Roche Applied Science) was used as collagenase preparation. Hepatocyte viability was assessed after erythrosin B staining and ranged from 72 to 90%, which is comparable with the values obtained previously with this method (17). We seeded between 200,000 and 250,000 viable cells/well in collagenized 24-well plates. Hepatocytes were allowed to attach for 3–4 h in Williams medium E (WME; Invitrogen), supplemented with 5% FBS and 100 μM dexamethasone, before washing twice in prewarmed, serum-free WME and incubating for 16–18 h in WME containing 100 μM dexamethasone and 140 μg/ml Matrigel (BD Biosciences).

Glucose Output Assay—To measure hepatocyte glucose output, cells were washed three times in prewarmed PBS and incubated for 10 min in 250 μl of prewarmed Krebs-Henseleit-Hepes buffer (25 mM NaHepes, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 4.7 mM KCl, 25 mM NaHCO₃, osmolarity adjusted to 285 mOsmol with NaCl, pH 7.4, at 37 °C) containing 20 μM 1,4-dideoxy-1,4-imino-D-arabinitol (18). HTS13286, dissolved in DMSO, glycerol, and lactic acid/pyruvate mixture, respectively, was included as indicated in the figures. At the end of the incubation period assay buffer was removed from cells, transferred into 1.5-ml tubes, and stored on ice water slurry. Before measurements of glucose content using the glucose oxidase-based Amplitite™ Glucose Quantitation kit (AAT Bioquest), samples were centrifuged briefly and sometimes stored frozen. Glucose-associated fluorescence was measured on a Fluostar Optima plate reader equipped with excitation/emission filters at 540/580 nm.

Glycerol Kinase Assay—Liver extracts were prepared from AQP9+/− heterozygote mice after removal from food for 10 h, as described previously (19). Glycerol in the extraction buffer was omitted. Samples were frozen in liquid nitrogen and stored at −80 °C before use. A radiochemical assay was performed as described previously (20) with the following modifications: 60-μl reactions contained 100 μg of liver extract, 88 mM Tris, pH 7.2, 83 mM KCl, 8.3 mM MgCl₂, 4.1 mM ATP, 2.1 mM DTT, 0.83 mM glycerol, 0.83 nCi of 0.8 Ci/mmol [3H]glycerol (PerkinElmer Life Sciences) and were incubated for 1 h at 37 °C.

Liver Perfusion—Liver perfusions were essentially performed as described by Kuriyama et al. (5) with minor modifications. Perfusion was with 4 ml/min Krebs-Henseleit-Hepes plus 1% DMSO, kept in a water bath at 42 °C. Samples were collected for 15 consecutive min as indicated in Fig. 4. The dead volume of...
the system, between buffer reservoir and collection tube, including the blood circulatory system, was 1.5 g of buffer. All animal experiments were approved by the Danish Council for Animal Ethics of the Ministry of Justice.

Data Analyses—All statistical analyses and analyses of inhibitor dose responses were conducted in Prism 5. We performed one-way ANOVA and Tukey’s post test (lactate/pyruvate substrate), and two-way ANOVA and Bonferroni post tests (glycerol substrate), respectively, to identify differences between groups in glucose output.

RESULTS

AQP9 Small Molecule Inhibitor Identification—To identify small molecule inhibitors of AQP9, murine (m)AQP9 was introduced into CHO cells. Expression of mAQP9 was inducible by tetracycline addition to the culture medium (21, 22). Utilizing sucrose as an AQP9-impermeable osmolyte, we found that mAQP9 facilitated rapid cell shrinking in a calcine fluorescence intensity-based microplate assay (9, 23, 24). This suggested that mAQP9 expression confers high water permeability to CHO cells. We therefore screened a commercial small molecule library for substances reducing AQP9 water permeability. To increase sensitivity for recovering weak inhibitors we adjusted AQP9 expression to a level where ~80% of the maximum measurable water permeability was observed in our setup. A set of 1,920 small molecules was screened subsequently at concentrations of 100 μM (results available in supplementary Spreadsheet 1), where each tested microwell plate contained positive (phloretin) and negative (solvent) control wells. We considered the highest ranked compounds with apparently slower cell shrinking for further analyses. Initially, these substances were subjected to in silico molecular docking to a homology model of human AQP9. The 10 best inhibitors of the primary screen and 20 additional substances with good docking properties were purchased individually, and dose-response curves were measured for these substances between 1 and 500 μM. For 23 of these substances inhibition of cell shrinking was only detected at high concentrations, four substances were apparent false positives. Of the remaining three substances, S14838 was suggested by the molecular docking approach, whereas RF03176 and CD05595 were among the most potent compounds identified in the primary screen (Fig. 1). To investigate inhibitor specificity, we constructed three additional CHO cell lines that expressed heterologous murine AQP isoforms: CHO-mAQP3, CHO-mAQP7, and CHO-mAQP8, respectively. Whereas the former two AQPs represent the other known murine aquaglyceroporins (25), AQP8 was chosen as a functionally less related aquaporin that is however expressed in the liver (26–28). We found that RF03176 is a moderate inhibitor of mAQP3 and mAQP7, respectively. No inhibition of cell shrinking was detected when CHO-mAQP8 cells were tested (Fig. 1). In contrast to RF03176, S14838 and CD05595 did not inhibit water permeability in any of the other cell lines tested (Fig. 1). Next we tested 7 structural analogues of RF03176, 12 analogues of S14838, and 22 analogues of CD05595 that were available from the Maybridge compound collection (supplemental Spreadsheet 2). Among several weaker inhibitors of mAQP9, this approach identified HTS13286, which was an improved inhibitor of CHO-mAQP9 cell shrinking, compared with RF03176 (Fig. 1). Furthermore, HTS13286 was an almost 2 orders of magnitude more potent mAQP9 inhibitor than phloretin (Fig. 1).

HTS13286 Affects mAQP9 Water and Solute Permeability—The qualitative effects of HTS13286 were tested on mAQP water, glycerol, and urea permeability (Fig. 2). In these experiments, HTS13286 (25 μM) resulted in markedly slower fluorescence quenching (water permeability) compared with solvent controls, when 200 mM sucrose was added to CHO-mAQP9 cells. In contrast, without inhibitor, these cells showed no fluorescence quenching when 200 mM glycerol or 200 mM urea was added to the extracellular side. In agreement with other studies, this suggested that AQP9 conferred high glycerol and urea permeability, respectively, to CHO cells (29–31). However, in the presence of HTS13286, cell shrinking was observed in CHO-mAQP9 cells in response to glycerol or urea addition (Fig. 2). We interpreted these slow volume changes as a simultaneous HTS13286 inhibition of glycerol influx and water efflux via AQP9, while leaving water and solute diffusion through the lipid bilayer unaffected. No effect of HTS13286 on solute permeability of CHO-AQP3, -8, and -7 cell lines, respectively, was observed.

AQP9 Is Crucial for Glycerol-dependent Glucose Production in Primary Hepatocyte Culture—Based on the known function of AQP9 as a glycerol channel, it has been proposed to contribute to hepatic glycerol gluconeogenesis (5). To test this hypothesis, we isolated hepatocytes from wild-type and AQP9−/− mice (8), respectively, and cultured these cells in insulin-free medium. Immunolabeling experiments confirmed robust expression of AQP9 in cells isolated from wild-type mice, whereas no signal was detected in cells isolated from knock-out mice (supplemental Fig. 1). Hepatocytes were incubated in the presence of various concentrations of glycerol subsequently, ranging from 0 to 5 mM (Fig. 3A). AQP9 expression enhanced glucokinase activity by 123 ± 36% when cells were incubated in 0.5 mM glycerol, which is within the physiological range of plasma glycerol levels. When extracellular glycerol was raised to 5 mM, the requirement of AQP9 for efficient glucose production was reduced, suggesting enhanced glycerol availability for glycerol gluconeogenesis from alternative routes, e.g., diffusion through the lipid bilayer. AQP9 inhibition by HTS13286 had effects on hepatocyte glucose production similar to those of AQP9 gene deletion. However, when extracellular glycerol was 5 mM, HTS13286-inhibited glucose production was significantly higher than glucose production in AQP9−/− hepatocytes. These observations are consistent with robust but incomplete inhibition of AQP9 glycerol permeability by HTS13286. No effect of HTS13286 on glucose output in the presence of glycerol was observed in AQP9 knock-out mice. Furthermore, HTS13286 did not inhibit glycerol kinase activity (Fig. 3C) or glucose output from AQP9−/− hepatocytes provided with lactic acid (1.5 mM) and pyruvate (0.15 mM) as glucoseogenic substrate (Fig. 3D). We also tested the efficacy of HTS13286 on glucose output in primary hepatocyte culture (Fig. 3B). Providing extracellular glycerol at 0.5 mM, we found that half-maximal inhibition of glucose production by HTS13286 was observed at 2.0 μM.
**DISCUSSION**

In a previous study, AQ9P9−/− mice with T2D had lower blood glucose levels than diabetic AQ9P9+/− littermates (8). Although the cause for lower blood glucose levels in AQ9P9−/− animals remained unknown, it was hypothesized that small molecule inhibitors of AQ9P9 could be useful in the treatment of T2D. Previously known AQ9P9 inhibitors, including phloretin and mercury (30), were not suitable to test this hypothesis. Whereas phloretin is known to inhibit hepatic hexose transporters (32), mercury is, in our opinion, too toxic for conclusive cell-based studies.

We have identified novel inhibitors of murine AQ9P9 by screening a commercial chemical compound library. With 58
inhibitors of 1,920 screened substances, the frequency of AQP9 inhibitors that were initially considered for further analysis was higher than expected. This may be explained by the chemistry of the identified substances: many compounds contained a diphenyl-urea moiety, which is an easily accessible intermediate in organic synthesis. This moiety seems to contribute at least in part to AQP9 inhibition by several substances. However, at lower concentrations (below 10 \( \mu M \)), only 3 of 30 substances analyzed further inhibited AQP9 water permeability significantly. Subsequent tests of structurally related compounds identified two additional inhibitors with IC\(_{50}\) values in the low micromolar and nanomolar range. Four of the five best identified inhibitors were completely specific for AQP9 and did not affect cells expressing homologous AQPs 3, 7, and 8, respectively, in the tested concentration range. Such specificity has not been reported in previous attempts to identify small molecule AQP inhibitors (24, 33).

It has been hypothesized previously that AQP9 may contribute to hepatic gluconeogenesis by facilitating glycerol uptake (5). This might explain the lowered blood glucose levels in diabetic AQP9\(^{+/−}\) mice compared with diabetic AQP9\(^{+/+}\) littermates (8). This hypothesis was undermined when AQP9\(^{+/−}\) mice were fed glycerol: there, blood glucose levels increased simultaneously in AQP9\(^{+/−}\) and AQP9\(^{+/+}\) mice, indicating important alternative routes to AQP9 for hepatic glycerol uptake. It was also unclear whether glycerol diffusion through

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**FIGURE 2.** HTS13286 effect on AQP9 solute permeability. Average cell fluorescence quenching traces, recorded from CHO-mAQP cells, are shown, \( n = 3 \). Water (left), glycerol (middle), and urea (right) permeabilities were tested qualitatively. Addition of membrane-impermeable sucrose (200 mM) induced rapid fluorescence quenching (solid line), consistent with high water permeability. HTS13286 (25 \( \mu M \); dotted line) decelerated fluorescence quenching in CHO-mAQP9 cells. Addition of 200 mM AQP9-permeable glycerol or urea did not cause volume changes in CHO-mAQP9 cells, indicating rapid glycerol and urea transmembrane equilibration with approximately equal velocity as concomitant water flux in the opposite direction. This equilibration was inhibited by addition of HTS13286 (25 \( \mu M \)), thereby restoring volume changes. Here, slow fluorescence changes indicate equally reduced AQP9-dependent water and solute permeabilities. As a result, the lipid bilayer presumably mediates most water and solute exchange. No effect of HTS13286 was observed in the other tested CHO-mAQP cell lines. Relative to water permeability, the glycerol permeability of CHO-mAQP3 cells appears lower than CHO-mAQP7 and CHO-mAQP9 glycerol permeability. Furthermore, CHO-mAQP3 cells appear impermeable to urea. Finally, relative to water permeability, the urea permeability of CHO-mAQP7 cells appears lower than CHO-mAQP9 urea permeability.
AQP9 inhibitor-treated hepatocytes. With regard to HTS13286 specificity to inhibition of AQP9, these results furthermore indicate an inhibitory mechanism on gluconeogenesis that is restricted to reducing availability of intracellular glycerol. HTS13286 did neither affect utilization of lactic acid and pyruvate as gluconeogenic substrate, nor did it affect the activity of glycerol kinase, further corroborating specificity of this inhibitor to AQP9 in primary hepatocytes and liver extracts. Noteworthy, AQP9 has initially been described as a moderate lactic acid channel, and a physiological role in lactic acid gluconeogenesis as well as lactic acid removal from hepatocytes during hypoxia has been hypothesized (30). Our results suggest that AQP9 is not relevant for lactic acid uptake for gluconeogenesis. We suggest that monocarboxylate transporters constitute the primary route for lactic acid transmembrane movement (35). The measured effects of AQP9 gene deletion on glucose output in primary hepatocytes were further substantiated by the effects of HTS13286 and AQP9 gene deletion on glycogen production in perfused mouse livers. A glycerol-specific increase in glucose output in wild-type livers was suppressed by HTS13286

### FIGURE 3. Role of AQP9 in hepatocyte glucose output in primary hepatocyte culture. A, addition of extracellular glycerol at 0.5 mM resulted in 123% ± 36% increased glucose output in cells isolated from AQP9+/- wild-type mice (circles) but not in AQP9-/- hepatocytes (triangles; p < 0.001). This increase in glucose output was eliminated by addition of 25 μM HTS13286 (open symbols) to AQP9+/+ hepatocytes at 0.5 mM glycerol (p < 0.001). Similar differences were observed at 2 mM glycerol (p < 0.001 for AQP9+/+ versus AQP9-/- and AQP9-/- versus AQP9+/+ plus HTS13286). Reduced effects were observed at 5 mM extracellular glycerol. There, HTS13286-treated AQP9+/+ cells produced more glucose (p < 0.05) than inhibitor or solvent-treated AQP9-/- hepatocytes. Filled symbols represent solvent (DMSO) controls; n = 6. All values were normalized to solvent-treated cells without exposure to glycerol. B, dose response of glucose output inhibition by HTS13286 at 0.5 mM glycerol. Half-maximal inhibition in AQP9+/+ hepatocytes was observed at 2 μM HTS13286. Symbols are as in A. Lowest concentration of inhibitor indicated in the logarithmic plot is 0. All values were normalized to glucose output at 25 μM inhibitor. n = 3. C, HTS13286 (25 μM) does not significantly affect glycerol kinase activity in liver extracts (100 μg of protein/sample); p = 0.53, two-tailed, unpaired t test comparing inhibitor-treated sample and solvent control (DMSO); ATP, no ATP added to extract and sample buffer; 80 °C, extract was heat-inactivated for 10 min at 80 °C; C. GlyK, Cellulomonas glycerol kinase, 0.1 and 0.001 units/sample; buffer, sample buffer control. D, HTS13286 (25 μM) does not significantly affect glucose output from lactate (1.5 mM) and pyruvate (0.15 mM) substrate (one-way ANOVA and Tukey’s post test). L/P, lactate/pyruvate; i, inhibitor (25 μM HTS13286).

the lipid bilayer could be sufficient to sustain gluconeogenesis from glyceral at physiological rates (34). Furthermore, it was conceivable that AQP9 gene deletion might have affected blood glucose levels in obese diabetic mice in unpredicted ways. In support for the existence of alternative routes, expression of AQP3 was found in liver tissue of starved mice (6) and recently also in human liver (7).

To evaluate whether AQP9 can be a drug target for treating T2D it was necessary to investigate whether AQP9 gene deletion can reduce hepatic gluconeogenesis. For this reason we have measured hepatocyte glucose production at various glyceral input concentrations. We hypothesized that cellular glyceral utilization would outperform glyceral uptake through the lipid bilayer, if facilitated glyceral uptake should play a role in glyceral gluconeogenesis. Further consideration suggested that both channel-mediated glyceral uptake as well as lipid bilayer-mediated glyceral uptake linearly depend on the inwardly directed glyceral gradient across the plasma membrane. As a consequence, we predicted that glyceral uptake through the lipid bilayer might only be rate-limiting for gluconeogenesis at physiological plasma glyceral concentrations. Elevated plasma glyceral concentrations, however, at high enough levels, would compensate facilitated glyceral uptake deficiency. Interestingly, AQP9 deletion caused elevated plasma glyceral concentrations in normal mice (8), which could indicate an attempt of compensation for AQP9 loss of function. Subsequent experiments in cultured primary hepatocytes showed that these assumptions on the effect of extracellular glyceral concentrations were valid. Whereas mAQP9 was necessary for efficient glyceral gluconeogenesis at physiological extracellular glyceral concentrations (0.5 mM), a 10-fold increase in extracellular glyceral concentration alleviated the glyceral gluconeogenesis defect in AQP9-/- hepatocytes or AQP9 inhibitor-treated hepatocytes. With regard to HTS13286 specificity to inhibition of AQP9, these results furthermore indicate an inhibitory mechanism on gluconeogenesis that is restricted to reducing availability of intracellular glyceral. HTS13286 did neither affect utilization of lactic acid and pyruvate as gluconeogenic substrate, nor did it affect the activity of glyceral kinase, further corroborating specificity of this inhibitor to AQP9 in primary hepatocytes and liver extracts. Noteworthy, AQP9 has initially been described as a moderate lactic acid channel, and a physiological role in lactic acid gluconeogenesis as well as lactic acid removal from hepatocytes during hypoxia has been hypothesized (30). Our results suggest that AQP9 is not relevant for lactic acid uptake for gluconeogenesis. We suggest that monocarboxylate transporters constitute the primary route for lactic acid transmembrane movement (35). The measured effects of AQP9 gene deletion on glucose output in primary hepatocytes were further substantiated by the effects of HTS13286 and AQP9 gene deletion on glyceral gluconeogenesis in perfused mouse livers. A glyceral-specific increase in glucose output in wild-type livers was suppressed by HTS13286
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and absent in AQP9<sup>−/−</sup> livers. Together, these results identify AQP9 as the primary glycerol uptake route in murine hepatocytes. The extent of the effect of AQP9 gene deletion on hepatocyte glycerol output suggests that no other glycerol channel contributes to glycerol uptake at the hepatocyte basolateral membrane. Previously described AQP3 expression in mouse liver may be specific to other cell types or other cellular compartments (6). Our observations provide a possible explanation for the previously observed parallel rise in blood glucose levels in AQP9<sup>−/−</sup> mice and heterozygote littermates, after a glycerol meal (8). There, the amounts of provided glycerol might have caused portal plasma glycerol concentrations in large excess of 5 μM, thereby facilitating normal glucose production from glycerol that diffused through the lipid bilayer.

In summary, we have identified a mechanism that can explain the lowered blood glucose levels caused by AQP9 deletion in mice with T2D (8). Furthermore, we have identified novel small molecule inhibitors of AQP9. One of these substances, HTS13286, caused similarly reduced glycerol-dependent glucose production as did AQP9 deletion. However, the amount of HTS13286 required to inhibit glycerol gluconeogenesis in primary hepatocyte culture was relatively high (IC<sub>50</sub> ~ 2 μM). We expect that HTS13286 is currently not suitable for in vivo experiments because its solubility in 1% DMSO-containing aqueous solution is limited (~25 μM). It may, however, be possible to identify structural derivatives of this substance for future in vivo studies that are more potent inhibitors of AQP9 and/or are more water-soluble.

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