Novel Inhibitors of Mitochondrial \textit{sn}-Glycerol 3-phosphate Dehydrogenase

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Abstract

Mitochondrial \textit{sn}-glycerol 3-phosphate dehydrogenase (mGPDH) is a ubiquinone-linked enzyme in the mitochondrial inner membrane best characterized as part of the glycerol phosphate shuttle that transfers reducing equivalents from cytosolic NADH into the mitochondrial electron transport chain. Despite the widespread expression of mGPDH and the availability of mGPDH-null mice, the physiological role of this enzyme remains poorly defined in many tissues, likely because of compensatory pathways for cytosolic regeneration of NAD\textsuperscript{+} and mechanisms for glycerol phosphate metabolism. Here we describe a novel class of cell-permeant small-molecule inhibitors of mGPDH (iGP) discovered through small-molecule screening. Structure-activity analysis identified a core benzimidazole-phenyl-succinamide structure as being essential to inhibition of mGPDH while modifications to the benzimidazole ring system modulated both potency and off-target effects. Live-cell imaging provided evidence that iGPs penetrate cellular membranes. Two compounds (iGP-1 and iGP-5) were characterized further to determine potency and selectivity and found to be mixed inhibitors with IC\textsubscript{50} and K\textsubscript{i} values between \textasciitilde1–15 \textmu M. These novel mGPDH inhibitors are unique tools to investigate the role of glycerol 3-phosphate metabolism in both isolated and intact systems.

Introduction

Mitochondrial \textit{sn}-glycerol 3-phosphate dehydrogenase (mGPDH\textsuperscript{3}; EC 1.1.5.3; gene symbol \textit{GPD2}) is an important link between cytosolic and mitochondrial energy transduction. mGPDH is a ubiquinone-linked flavoprotein embedded in the outer leaflet of the mitochondrial inner membrane that transfers reducing equivalents directly from glycerol 3-phosphate into the electron transport chain [1,2]. Glycerol 3-phosphate is an intermediate common to both lipid and carbohydrate metabolism. Its oxidation to dihydroxyacetone phosphate (DHAP) by mGPDH and the subsequent reduction of DHAP back to glycerol 3-phosphate by a distinct soluble GPDH (cytosolic GPDH; cGPDH; EC 1.1.1.8; gene symbol \textit{GPD1}) regenerates NAD\textsuperscript{+} consumed during glycolysis (Fig. 1A). This cyclic process of transferring reducing equivalents from cytosolic NADH into the mitochondrial respiratory chain is known as the glycerol phosphate shuttle. Along with the malate-aspartate shuttle (Fig. 1B) and production of lactate by lactate dehydrogenase (Fig. 1C), it is a common mechanism by which cytosolic NAD\textsuperscript{+} is regenerated to facilitate glycolytic activity in a variety of cell types [3].

The active site of mGPDH faces the mitochondrial intermembrane space, as does its calcium-sensitive EF-hand domain that lowers the $K_m$ for glycerol 3-phosphate as physiological levels of free calcium rise [1,2,4,5]. This orientation is thought to allow mGPDH to coordinate cytosolic and mitochondrial metabolism during periods of high activity and, not surprisingly, mGPDH is expressed most highly in tissues with variable energy demands including thermogenic brown fat, type II skeletal muscle fibers, brain, sperm and pancreatic β-cells [6,7]. Further, mGPDH expression is hormonally regulated to alter tissue activity both during development and in response to environmental challenges [8–10]. Despite the widespread expression of the enzyme, mGPDH-knockout mice display relatively mild phenotypes beyond weaning. These include decreased body mass and decreased white fat mass. However food intake, non-white fat tissues, and metabolic profiles are normal in these mice [11]. Prior to weaning, viability of mGPDH-null pups is decreased by 50%. Such a dramatic developmental bottleneck raises the possibility that the absence of mGPDH in surviving adults may be successfully compensated for by parallel metabolic pathways. In fact, further roles for mGPDH have only been observed after additional genetic, pharmacologic, or environmental manipulations. For example, ablation of cGPDH as well as mGPDH prevents compensatory responses in glycerol 3-phosphate metabolism, causes dramatic changes in metabolic profiles, and is lethal within one week of birth [12].

An alternative strategy to genetic manipulation of enzyme expression is the acute use of selective inhibitors. Several groups have demonstrated inhibition of mGPDH or bacterial GPDH by...
Figure 1. The glycerol phosphate shuttle is one of several pathways to regenerate cytosolic NAD$^+$ for glycolysis. Glycolytic metabolism of glucose to pyruvate involves the reduction of cytosolic NAD$^+$ to NADH at the step catalyzed by glyceraldehyde 3-phosphate dehydrogenase. Several mechanisms exist to regenerate cytosolic NAD$^+$ to ensure that a high NADH/NAD$^+$ ratio does not limit glucose metabolism. (A) The glycerol phosphate shuttle comprises soluble, NAD$^+$-linked cGPDH and the membrane bound, FAD-linked mGPDH. This system regenerates cytosolic NAD$^+$ and transfers the reducing equivalents directly into the mobile ubiquinone pool of the mitochondrial electron transport chain. (B) The malate-aspartate shuttle comprises multiple enzymes and mitochondrial carriers that interconvert and transport dicarboxylates and amino acids. In the...
cytosol, malate dehydrogenase regenerates NAD⁺ during reduction of oxaloacetate to malate. This malate is transported into the mitochondrial matrix where it is oxidized back to oxaloacetate by mitochondrial malate dehydrogenase, producing NADH that is reoxidized by complex I of the electron transport chain. To complete the shuttle, cytosolic oxaloacetate is regenerated by sequential transaminations through aspartate. These transaminations are potently inhibited by aminooxyacetate.

LDH transaminations are potently inhibited by aminooxyacetate. Electron transport chain. To complete the shuttle, cytosolic oxaloacetate is regenerated by sequential transaminations through aspartate. These transaminations are potently inhibited by aminooxyacetate. Electron transport chain. To complete the shuttle, cytosolic oxaloacetate is regenerated by sequential transaminations through aspartate. These transaminations are potently inhibited by aminooxyacetate. Electron transport chain. To complete the shuttle, cytosolic oxaloacetate is regenerated by sequential transaminations through aspartate. These transaminations are potently inhibited by aminooxyacetate.

Materials and Methods

Reagents

CaCl₂ standard was from Thermo Scientific, fatty acid-free BSA from Calbiochem, and atpenin A5 from Santa Cruz Biotechnology. Amplex UltraRed, tetramethylrhodamine methyl ester (TMRM), LysoTracker Red DND-99, and Geltrex were from Invitrogen. Compounds for screening were arbitrarily selected from a diverse, non-combinatorial library obtained from ChemBridge (~10 mM in DMSO). New stocks of primary hits and their structural analogs were also sourced from ChemBridge (and dissolved to 80 mM in DMSO). The biological activity of iGP-1 (ChemBridge ID 5224148) was validated using compound obtained from Vitas-M (ID STK017597) and found to be essentially identical. All other reagents were from Sigma-Aldrich.

Animals

Skeletal muscle mitochondria were isolated from hindlimbs of 5–8 week old female Wistar rats (Harlan Laboratories) as previously described [25]. Cortical synaptosomes were isolated from 6–12 week old male C57BL/6 mice (Jackson Labs) as previously described [26]. The animal protocol was approved by the Buck Institute Animal Care and Use Committee, in accordance with IACUC standards.

Measurement of Mitochondrial H₂O₂ Production and Membrane Potential

Superoxide was measured as H₂O₂ after dismutation by endogenous or exogenous superoxide dismutase. Rates of total superoxide/H₂O₂ production were measured fluorimetrically using Amplex UltraRed in the presence of exogenous superoxide dismutase and horseradish peroxidase in a Varian Cary Eclipse fluorimeter as previously described [27]. Rates of production from specific sites were determined as previously described using the following eight conditions: flavin mononucleotide site of complex I (site I_F) plus matrix NAD-linked dehydrogenases (collectively "site I_F/DH") with 5 mM glutamate, 5 mM malate, and 4 μM rotenone [28]; ubiquinone-binding site of complex I (site I_Q) with either 0.5 or 5 mM succinate [29]; flavin site of complex II (site II_F) with either 15 μM palmitoylcarnitine, 2.5 μM antimycin A, and 2 μM myxothiazol or 0.5 mM succinate, 4 μM rotenone, 2.5 μM antimycin A, and 2 μM myxothiazol [30,31]; mGPDH with 1.7 mM glycerol phosphate, 4 μM rotenone, 2.5 μM antimycin A, 2 μM myxothiazol, 1 mM malonate, and 250 mM free calcium; outer ubiquinone-binding site of complex III (site IIIcQo) with either 0.5 or 3 mM succinate, 4 μM rotenone and 2.5 μM antimycin A [32]. Candidate small-molecule inhibitors were added prior to the start of measurements and H₂O₂ calibration curves were performed in the presence of tested compounds.

Mitochondrial membrane potential (ΔΨm) was estimated fluorimetrically as described previously [33] on the Varian fluorimeter, except the potentiometric dye TMRM was used in quench-mode in place of safranine O [34]. Briefly, skeletal muscle mitochondria (0.2 mg protein · mL⁻¹) were incubated with 5 μM TMRM in the presence of inhibitors or vehicle. ΔΨm was generated by sequential addition of substrates of complex I (5 mM glutamate plus 5 mM malate), complex II (4 μM rotenone then 0.5 or 5 mM succinate), and mGPDH (2 μM atpenin A5 then 1.7 or 16.7 mM glycerol phosphate). The effect of putative small-molecule inhibitors was normalized as the % change in fluorescence between vehicle treatment and complete depolarization by the uncoupler carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 2 μM) added at the end of each run. When indicated, the K⁺/H⁺ exchanger nigericin (80 ng · mL⁻¹) was added to collapse the pH gradient across the mitochondrial inner membrane (ΔΨm) and maximize ΔΨm.

The assay buffer for both H₂O₂ and ΔΨm measurements was 120 mM KCl, 5 mM HEPES, 1 mM EGTA, 0.3% (w/v) BSA without or with 250 mM free calcium at pH 7.0. All assays were performed with stirring at 37°C.

Measurement of iGP-1 Fluorescence in Solution

Excitation and emission spectra of iGP-1 fluorescence were acquired with the Varian fluorimeter in standard buffer with pH adjusted to between 1.5 and 11 as indicated.

Screening for Inhibitors of H₂O₂ Production by mGPDH

3,200 compounds were tested initially at 2.5 μM (0.025% DMSO final) in duplicate on different plates against a panel of five
distinct assays of site-specific superoxide/H$_2$O$_2$ production as described in the previous section with minor changes. Targeted sites included site I$_{F/DH}$ with 3 mM glutamate, 5 mM malate, and 4 μM rotenone, site I$_{Q}$ with 3 mM succinate, site II$_{F}$ with 15 μM palmitoyl carnitine, 2.5 μM antimycin A, and 2 μM myxothiazol, mGPDH with 16.7 mM glycerol phosphate, 4 μM rotenone, 2.5 μM antimycin A, 2 μM myxothiazol, 1 mM malonate, and no added calcium, and site IIIQ$_{o}$ with 3 mM succinate, 4 μM rotenone and 2.5 μM antimycin A. Compounds and assay components were distributed by a Biomek FX liquid handling workstation (Beckman) into 96-well black plates (Nunc) to a final volume of 200 μL, and incubated in the dark at room temperature for 30–40 min after the addition of substrate. Endpoint fluorescence was measured on a Perkin Elmer Victor V3 fluorescent plate reader. The fluorescence intensity of each well was normalized as % change from the average of eight DMSO controls on the same plate. Duplicate wells of each of four modulators of mitochondrial metabolism known to inhibit specific sites of superoxide/H$_2$O$_2$ production were included on each plate as positive controls and the resulting fluorescence levels set as −100% change from DMSO controls. Known inhibitors included 20 mM aspartate (for site I$_{F/DH}$), 1 μM FCCP (for site I$_{Q}$), 10 mM malonate (for site II$_{F}$), and 2 μM myxothiazol (for site IIIQ$_{o}$). The background fluorescence of the FCCP wells in the site I$_{Q}$ assay was used to normalize mGPDH plates run in parallel. To minimize position-dependent effects across each assay plate, Tukey’s two-way median polish was applied to fluorescence values after initial normalization [35,36]. Tukey’s polish is an iterative subtraction of the median row and column values from each well’s given row or column. The procedure is repeated until sequential medians for each row and column are zero and positional gradients in fluorescence are eliminated.

Novel inhibitors of superoxide/H$_2$O$_2$ production by mGPDH were identified as compounds whose average effect was to decrease H$_2$O$_2$ production by at least 10% without altering H$_2$O$_2$ production in other assays by more than ±10%. Select hits in the mGPDH assay were re-sourced from ChemBridge and retested at 0.08–80 μM (0.1% DMSO final) in duplicate on the same microplate as described above against the eight conditions listed under “Measurement of mitochondrial H$_2$O$_2$ production and membrane potential.”

Analysis of Structure/activity Relationships

Structure/activity analysis was performed on 18 structural analogs of two structurally-related hits from the primary screen (iGP-1 and iGP-2). These 20 compounds were tested in duplicate at 0.08–80 μM (0.1% DMSO final) in microplate format for effects on site-specific H$_2$O$_2$ production as described above. Additionally, four microplate assays for ΔΨm were run in parallel to the H$_2$O$_2$ assays. These assays were as described under “Measurement of mitochondrial H$_2$O$_2$ production and membrane potential” except that each substrate condition was assayed in a separate microplate. Compounds were tested against the following substrate conditions: 5 mM glutamate and 5 mM malate both without and with 80 ng · mL$^{-1}$ nigericin, 5 mM succinate and 4 μM rotenone with 80 ng · mL$^{-1}$ nigericin, and 16.7 mM glycerol phosphate and 4 μM rotenone with 80 ng · mL$^{-1}$ nigericin. Plates were incubated in the dark at room temperature for 30–40 min (H$_2$O$_2$) or 10–15 min (ΔΨm) after the addition of substrate, and endpoint fluorescence was measured on the Victor plate reader. Endpoints were chosen for the H$_2$O$_2$ assays to provide good signal-to-noise ratios and linear rates of change without the indicator dye limiting the reaction. Endpoints for the ΔΨm assays were determined in pilot experiments as the times for TMRM accumulation to reach a steady state. At room temperature, this was typically ~5 min and remained stable for at least 30 min. Endpoint fluorescence values were normalized as described in the previous section with wells containing FCCP serving as positive controls for the ΔΨm assays.

cGPDH and mGPDH Activity

The effects of small-molecule inhibitors on cGPDH and mGPDH enzymatic activity were tested according to established procedures. Activity of purified rabbit muscle cGPDH was measured as described by the manufacturer as the rate of oxidation of NADH in the presence of DHAP on the Varian fluorometer (λ$_{ex}$ = 365 nm, λ$_{em}$ = 450 nm). Activity of cGPDH in frozen-thawed synaptosomes was measured at 37°C in a BMG Labtech PHERAstar Plus microplate reader as the linear rate of change in NADH absorbance (λ = 340 nm) specific to the addition of 2.5 mM DHAP and insensitive to 4 μM rotenone. Rates were converted to mmol NADH using an extinction coefficient of 6.22 mm$^{-1}$ · cm$^{-1}$ and a calculated pathlength of 0.6 cm.

Activity of mGPDH in frozen-thawed skeletal muscle mitochondria was measured as 2,6-dichlorophenolindophenol (DCPIP)-linked reduction by glycerol phosphate as described previously in the presence of 1 μM free calcium [27]. Linear rates of change in absorbance (λ = 600 nm) were measured on the PHERAstar microplate reader and converted to rates of DCPIP reduction using an extinction coefficient of 21 mm$^{-1}$ · cm$^{-1}$ and a calculated pathlength of 0.6 cm. Kinetic parameters and inhibitor constants were determined by co-varying glycerol phosphate and novel inhibitors according to recommended procedures [37] and calculated for each data set using GraphPad Prism. Vmax and Km were determined using hyperbolic curves fit to the Michaelis-Menten equation: rate = (Vmax · [substrate]) · (Km + [substrate])$^{-1}$. Hill Slope and IC$_{50}$ were determined using unconstrained, sigmoidal dose-response curve fits of % remaining activity against concentration of iGP. K$_{c}$ and K$_{m}$ were determined using plots of rate$^{-1}$ vs iGP and GP · rate$^{-1}$ vs iGP, respectively [37]. Linear regression analysis yielded an average x-coordinate at which the best-fit lines intersect. These x-coordinates correspond to -K$_{c}$ and -K$_{m}$ in their respective plots [37]. The apparent Vmax of mGPDH is dependent upon the concentration of DCPIP used [13,38]. Therefore, to determine the maximal activity of mGPDH in isolated synaptosomes, DCPIP was replaced by 1 mM potassium ferricyanide [39] and linear rates of change in absorbance (λ = 420 nm) were converted to rates of ferricyanide reduction using an extinction coefficient of 1.04 mm$^{-1}$ · cm$^{-1}$ and a calculated pathlength of 0.6 cm.

Mitochondrial and Synaptosomal Respiration

The effects of small-molecule inhibitors on respiration were tested in plate-attached skeletal muscle mitochondria using a Seahorse XF24 Analyzer according to published protocols [40]. Briefly, mitochondria (2 or 4 μg protein) were attached to Seahorse assay plates by centrifugation in a mannitol and sucrose-based medium (Seahorse MAS buffer [40]) containing 0.3% (w/v) BSA without or with 250 nM free calcium at pH 7.0. Compounds were titrated to 80 μM on each of four parallel plates with media containing one of the following substrates: 10 mM pyruvate and 0.5 mM malate; 5 mM glutamate and 5 mM malate; 5 mM succinate and 4 μM rotenone; or 16.7 mM glycerol phosphate and 4 μM rotenone with 250 nM free calcium. Compounds were added in these media just prior to loading the assay plate into the Seahorse instrument. Additions were made to set respiration in basal state 2 (substrate only), phosphorylating state 3 (5 mM ADP), non-phosphorylating state 4o (0.5 μg · mL$^{-1}$).
oligomycin), and to reveal the non-mitochondrial rate (2 μM myxothiazol and 2.5 μM antimycin A). Each concentration of compound was tested in at least two wells on each plate. At least three biological replicates of each titration in each substrate condition were performed.

Synaptosomal respiration was tested in the Seahorse XF24 using published protocols [26,41]. Synaptosomes (20 or 25 μg protein) were attached to polyethyleneimine and Geltrex-coated Seahorse plates by centrifugation in ionic medium [26,41] containing 10 mM glucose, Attachment buffer was carefully replaced with 100 mM imidazole, or iGP-1 with aminooxyacetate, and either 10 mM pyruvate or 15 mM glucose. Plates were equilibrated for 20 min at 37°C then loaded into the instrument followed by measurement of basal respiration and injection of 5 μM FCCP (to induce maximal, uncoupled respiration) with 4 μg · mL⁻¹ oligomycin (to prevent compensatory reversal of ATP synthase). In some experiments, 10 mM oxamate was added with the FCCP/oligomycin to minimize regeneration of cytosolic NAD⁺ by lactate dehydrogenase. Inhibition of the malate-aspartate shuttle was defined as the difference between maximal rates ± aminooxyacetate, expressed as % of control. Inhibition of the glycerol phosphate shuttle was the difference between maximal rates with aminooxyacetate ± iGP-1, as % of control.

Live Cell Imaging

The ability of iGP-1 to cross cellular membranes was evaluated in STHdhQ7 cells cultured at 33°C as described [42]. Cells were seeded on collagen-coated, Lab-Tek chambered coverglass (Nunc) 48 h before use. Prior to imaging, cells were cultured for 45 min with 65 mM LysoTracker Red ± 250 mM bafilomycin A1. Medium was then replaced with imaging buffer (120 mM NaCl, 3.5 mM KCl, 0.4 mM KH₂PO₄, 20 mM TES, 5 mM NaHCO₃, 1.2 mM Na₂SO₄, 1.5 mM CaCl₂, 0.5 mM MgCl₂, 2 mM Glucose, and 0.2% BSA, pH 7.4) containing DMSO, 250 nM bafilomycin A1 or 100 μM iGP-1. Cells were imaged on a Nikon Eclipse Ti-PFS inverted microscope equipped with a Cascade 512B camera (Photometrics, Tucson, AZ), a Plan Fluor 100X/1.4 oil objective, an LB-LS17 Xe-arc light source (attenuated), 10−3 excitation and emission filter wheels (Sutter Instruments, Novato, CA), and an MS-2000 linear encoded motorized stage (ASI, Eugene, OR). The filter sets, given as excitation, dichroic mirror, emission in nm/bandwidth, were, for iGP-1 (1 s exposure), 340/26, 409, 460/80 and for LysoTracker Red (100 ms exposure), 480/20, 490, 500/40, and an MS-2000 linear encoded motorized stage (ASI, Eugene, OR). The filter sets, given as excitation, dichroic mirror, emission in nm/bandwidth, were, for iGP-1 (1 s exposure), 340/26, 409, 460/80 and for LysoTracker Red (100 ms exposure), 480/20, 490, 500/40.

Data Presentation

Data were presented as mean ± S.E. or mean ± 95% C.I. where indicated. Statistical differences between conditions were analyzed by one-way ANOVA with Newman-Keuls post-test as specified in the figure legends. p values <0.05 were considered significant.

Results

Selectivity of Known Inhibitors of mGPDH

Analogs of glycerol phosphate, including glyceraldehyde 3-phosphate and DHAP, are established competitive inhibitors of bacterial, invertebrate, and vertebrate homologs of FAD-linked GPDH [2,13–15]. Because of their limited ability to cross membranes and their involvement in cellular metabolism, these inhibitors are not useful in intact cells. However, neither glyceraldehyde 3-phosphate nor DHAP is oxidized by mitochondrial [14] and therefore they may be useful as selective inhibitors of mGPDH in isolated mitochondria. To test this possibility, we measured their effects on the rates of H₂O₂ production and levels of Δψm in mitochondria provided with different substrates (Fig. 2).

Consistent with their known effects on mGPDH enzymatic activity [2,13–15], both glyceraldehyde 3-phosphate and DHAP inhibited the rate of H₂O₂ production from mGPDH (Fig. 2A). Glyceraldehyde 3-phosphate was more potent, inhibiting nearly 80% at 2.5 mM, but DHAP was less effective. The selectivity of glyceraldehyde 3-phosphate was tested by measuring its effect on H₂O₂ production from site I₁, site I₂, and site IIIQₒ. It significantly inhibited the rate of H₂O₂ production from site I₂ (Fig. 2B), suggesting a lack of specificity.

As expected, glyceraldehyde 3-phosphate also lowered Δψm driven by glycerol phosphate oxidation (Fig. 2C). To test its selectivity in this assay, we also measured Δψm driven by glutamate plus malate and by succinate. Δψm driven by succinate was significantly increased. This increase in Δψm was independent of changes in ΔpH since the K⁺/H⁺ exchanger nigericin was present in all conditions.

We conclude that glyceraldehyde 3-phosphate is not a specific inhibitor of mGPDH but also alters succinate oxidation. Therefore, even in isolated mitochondria, small metabolite inhibitors of mGPDH are limited in utility.

Discovery of Novel Small-molecule Inhibitors of Superoxide/H₂O₂ Production by mGPDH

We recently described a multiple-parallel screen for novel modulators of mitochondrial H₂O₂ production [43]. Each assay in the screen was designed to either drive superoxide/H₂O₂ production predominantly from one mitochondrial redox center or to report undesired effects on Δψm (Fig. 3A). Included in this screen was an assay specific for superoxide/H₂O₂ production by mGPDH. This assay was robust with a %CV for intraplate DMSO control wells of 3.4±0.2% (n = 40 plates each tested in duplicate). Out of 3200 compounds arbitrarily selected from a structurally diverse chemical library and tested at 2.5 μM, 87 (2.7%) decreased the signal in the mGPDH assay by at least 10% compared to DMSO controls (Table S1). To identify compounds that were selective for mGPDH, these 87 compounds were counter-screened using a panel of four assays that each targeted a distinct site of superoxide/H₂O₂ production: site I₁/DH, site I₂, site IIIQₒ, and site IIIQₒ. Compounds that increased or decreased the signal more than 10% in any of these assays were eliminated, leaving seven (0.2%) selective mGPDH inhibitors (Fig. 3B–3G).

We subsequently obtained four of the most potent and selective of these compounds from the original supplier and retested each over a broad concentration range (0.08–80 μM) against a panel of eight assays of H₂O₂ production targeting the five sites listed above using additional substrate conditions to better evaluate selectivity and potential mechanisms of action. Two compounds were false positives. The other two (iGP-1 and iGP-2; ChemBridge #5224148 and #5224147; Fig. 4) differed structurally by a single atom and each retested well. Another of the seven hits, #5244345, was also structurally similar to these two but displayed less selectivity and was not retested. The most potent compound in the initial screen against H₂O₂ production by mGPDH, iGP-1 (Fig. 4), remained the most potent inhibitor on retesting and also the most selective. The only non-specific effect observed was a weak inhibition of H₂O₂ production from site I₁ at the low concentration of succinate (see below). In contrast, iGP-2 (Fig. 4) was slightly less potent and had stronger off-target effects on sites...
I<sub>D</sub> and I<sub>LQ</sub>. The end result of our unbiased, multiple parallel chemical screens was a set of novel potent inhibitors of superoxide/H<sub>2</sub>O<sub>2</sub> production by mGPDH that demonstrated differing selectivity as a result of subtle structural changes. We chose to investigate further the effects of this structural class of compounds on mitochondrial function.

**Analysis of Structure/activity Relationships of Novel mGPDH Inhibitors**

Motivated by the observation that subtle changes in structure resulted in changes to both the potency and selectivity of our novel mGPDH inhibitors, we tested an additional 18 compounds structurally related to the top hits in our primary screen to identify structural features that determined the relative potency and selectivity for inhibition of H<sub>2</sub>O<sub>2</sub> production by mGPDH. These 20 compounds were retested for effects on eight assays of site-specific H<sub>2</sub>O<sub>2</sub> production and four assays of ΔΨ<sub>m</sub> utilizing different mitochondrial substrates (Fig. 4). To identify structure/activity relationships, compounds were placed into four groups according to common generalized structural differences compared to the original parent compound iGP-1 and evaluated for effects on the 12 assays of mitochondrial function to determine shared effects among group members. Several critical conclusions were drawn from this analysis (Fig. 4). First, as was observed in the original round of retesting described above, changing one of the nitrogen atoms in the benzimidazole to oxygen or sulfur (iGP-2 and iGP-3) had little effect on potency against mGPDH yet decreased selectivity. Specifically, these compounds inhibited H<sub>2</sub>O<sub>2</sub> production by site I<sub>D</sub> to a greater extent and also increased ΔΨ<sub>m</sub> both in the presence and absence of nigericin. These effects on ΔΨ<sub>m</sub> in the presence of nigericin were subsequently found to be caused largely by artificial quenching of TMRM fluorescence by the compounds (data not shown). However, the much larger increase in ΔΨ<sub>m</sub> observed in the absence of nigericin was found to represent a true change in ΔΨ<sub>m</sub>, most likely a collapse in the ΔpH component of the protonmotive force. This decrease in ΔpH may explain the greater effect of these structural analogs on H<sub>2</sub>O<sub>2</sub> production by site I<sub>D</sub> since this site is known to be uniquely sensitive to ΔpH [44]. Intriguingly, three of four compounds in which additional groups were attached to the free end of the benzimidazole ring were more potent inhibitors of mGPDH (iGP-4–iGP-7). However, these three also had decreased selectivity in similar ways to those observed with changes to the heteroatom of this ring system. The orientation of the benzimidazole and succinamide groups off the central phenyl ring also influenced potency versus mGPDH ROS production. Changing the relative positioning of these groups from ortho- to para- lowered the potency by more than 5-fold (iGP-8). Importantly, altering the carboxyl end of the succinamic acid group decreased potency and selectivity for mGPDH, if any inhibition remained at all (iGP-9–iGP-16). Similarly, the benzimidazole ring was required for inhibition of mGPDH (iGP-17–iGP-20). Ultimately, while our structure/activity analysis yielded no compound with improvements to both potency and selectivity against mGPDH, it provided insight into the structural elements essential to mGPDH inhibition and useful clues as to which chemical features should likely be targeted in future optimization studies.

**iGPs Inhibit mGPDH Enzymatic Activity**

iGP-1 and iGP-2 did not inhibit ΔΨ<sub>m</sub> driven with glutamate and malate in our initial screen (Fig. 3G). This observation was confirmed during retesting. However, ΔΨ<sub>m</sub> driven by glycerol phosphate was inhibited by several iGPs indicating that these novel compounds inhibited not just H<sub>2</sub>O<sub>2</sub> production by mGPDH but also its enzymatic activity (Fig. 4). To investigate this distinction, we assayed the effect of iGP-1 on mGPDH enzymatic activity directly. iGP-1 caused significant inhibition of mGPDH enzymatic activity but, remarkably, did not inhibit the soluble form of GPDH (Fig. 5). Next, we further characterized the effects of the most selective, iGP-1, and the most potent, iGP-3, inhibitors of mGPDH on H<sub>2</sub>O<sub>2</sub> production, ΔΨ<sub>m</sub>, and respiration.
The most selective inhibitor, iGP-1 (Fig. 6A), progressively inhibited H$_2$O$_2$ production by mGPDH as its concentration was increased from 0.25 to 80 $\mu$M, with a half-maximal effect at about 8 $\mu$M (Fig. 6B). Only above 10 $\mu$M did iGP-1 start to inhibit H$_2$O$_2$ production by site I$_Q$, demonstrating its good specificity. This effect on H$_2$O$_2$ production by mGPDH (Fig. 6B) was mirrored over the same concentration range by significant and specific lowering of $\Delta$Y$_{m}$ driven by glycerol phosphate (Fig. 6C), and significant and specific inhibition of respiratory rates in mitochondria supplied with glycerol phosphate (Fig. 6D), suggesting that iGP-1 inhibited enzymatic activity of mGPDH. iGP-1 decreased H$_2$O$_2$ production by site I$_Q$ and $\Delta$Y$_{m}$ driven by low (0.5 mM) succinate but not by high (5 mM) succinate (Fig. 6B, 6C) indicating a subtle off-target effect on succinate oxidation. It had no effect on H$_2$O$_2$ production, $\Delta$Y$_{m}$, or respiration driven by oxidation of a variety of substrates including glutamate, malate, and palmitoylcarnitine.

Similarly, iGP-5 (Fig. 7A) significantly inhibited H$_2$O$_2$ production by mGPDH as its concentration was increased from 0.08 to 80 $\mu$M, with a half-maximal effect at about 1 $\mu$M (Fig. 7B). It also lowered $\Delta$Y$_{m}$ (Fig. 7C) and inhibited respiration driven by glycerol phosphate. iGP-5 lowered $\Delta$Y$_{m}$ driven by glycerol phosphate more potently than iGP-1 (Fig. 6C versus Fig. 7C). However, this structural analog also displayed less selectivity in assays of H$_2$O$_2$ production (Fig. 7B) and also significantly increased $\Delta$Y$_{m}$ in the absence but not the presence of nigericin, suggesting a negative effect on $\Delta$pH (Fig. 7C). Further, iGP-5 inhibited respiration driven by pyruvate and perhaps succinate (Fig. 7D).

iGP-1 is Cell-permeant

We next determined if iGP-1 was cell-permeant and, therefore, potentially useful for inhibiting mGPDH in situ. Several iGPs exhibited fluorescence with excitation maxima between 320–360 nm and emission maxima between 360–480 nm (Fig. 8A and not shown). Cells treated with 100 $\mu$M iGP-1 displayed fluorescence above background, with bright puncta in the cytosol, low levels in the nucleus, and intermediate levels distributed diffusely within the cytosol (Fig. 8B). Timelapse imaging of iGP-1 suggested the punctate fluorescence was in rapidly moving structures independent of the mitochondrial network (not shown). Similar distribution of cellular iGP-1 fluorescence was also observed in several other cultured cell lines (e.g. HEK-293 and PC-3). Co-labeling of cells with iGP-1 and LysoTracker Red DND-99, which localizes to acidic vesicles such as endosomes and lysosomes, revealed a high correlation with the most intense iGP-1
### Figure 4. Structure/activity analysis identifies features conferring mGPDH inhibition.

Summary of effects on H$_2$O$_2$ production and $\Delta \Psi_m$ of 20 compounds structurally related to the top hit in our primary screen. Each compound was tested against eight assays of site-selective H$_2$O$_2$.

| Compounds | mGPDH H$_2$O$_2$ production IC$_{50}$ (µM) | $\Delta \Psi_m$ with GP + Nig (% change at 80 µM) | $\Delta \Psi_m$ with Glu + Mal (% change at 80 µM) | $\Delta \Psi_m$ with Glu + Mal + Nig (% change at 80 µM) | I$_s$ H$_2$O$_2$ production with 5 mM Suc (% change at 80 µM) |
|-----------|-----------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| 1         | 5224148                                 | 8                                           | -55                                        | -20                                        | 15                                          | 4                                           | -9                                          |
| 2         | 5224147                                 | 20                                          | -50                                        | 1                                           | 107                                         | 18                                          | -57                                         |
| 3         | 6527788                                 | 15                                          | -50                                        | -26                                        | 148                                         | 20                                          | -69                                         |
| 4         | 7967779                                 | 5                                           | -69                                        | -35                                        | 49                                          | 11                                          | -20                                         |
| 5         | 5768410                                 | 0.7                                         | -65                                        | -26                                        | 106                                         | 20                                          | -9                                          |
| 6         | 7892367                                 | 3                                           | -55                                        | -59                                        | 93                                          | 17                                          | -28                                         |
| 7         | 5233584                                 | n.d.                                        | -21                                        | 4                                           | 25                                          | 2                                           | 3                                           |
| 8         | 5757881                                 | 50                                          | -44                                        | -7                                         | 3                                           | 1                                           | -14                                         |
| 9         | 7360472                                 | n.d.                                        | -2                                         | 5                                           | 26                                          | 5                                           | -1                                          |
| 10        | 5180119                                 | n.d.                                        | 0                                          | -78                                        | -58                                         | -78                                         | -77                                         |
| 11        | 5233321                                 | n.d.                                        | -5                                         | 1                                           | 54                                          | -1                                          | -54                                         |
| 12        | 5751763                                 | n.d.                                        | -28                                        | 2                                           | 10                                          | 2                                           | -21                                         |
| 13        | 5957384                                 | 80                                          | -37                                        | 13                                          | 23                                          | 10                                          | -82                                         |
| 14        | 5957767                                 | n.d.                                        | -18                                        | 0                                           | 8                                           | 0                                           | -45                                         |
| 15        | 6035504                                 | n.d.                                        | 11                                          | -25                                        | 10                                          | -42                                         | -15                                         |
| 16        | 7056160                                 | n.d.                                        | -7                                         | 2                                           | 4                                           | 1                                           | -24                                         |
| 17        | 5105852                                 | n.d.                                        | 6                                          | 0                                           | -1                                          | -1                                          | -1                                          |
| 18        | 5237341                                 | n.d.                                        | 2                                          | -1                                         | -9                                          | -3                                          | 6                                           |
| 19        | 7167753                                 | n.d.                                        | -1                                         | -2                                         | -8                                          | -2                                          | 7                                           |
| 20        | 5105117                                 | n.d.                                        | -20                                        | 0                                           | -3                                          | -1                                          | -8                                          |
production and four assays of \( \Delta \Psi \text{m} \) powered by different mitochondrial substrates (see Materials and Methods for details of individual assays). Data are means of two replicates on the same plate. Five criteria best segregated these compounds according to changes in structural motifs relative to the parent compound iGP-1: potency of inhibition of mGPDH \( \text{H}_2\text{O}_2 \) production (defined by estimating the IC\(_{50}\) concentration or, where an IC\(_{50}\) could not be calculated, the effect on mGPDH \( \text{H}_2\text{O}_2 \) production at 80 \( \mu \text{M} \)), effect on \( \Delta \Psi \text{m} \) powered by glycerol phosphate, effect on \( \Delta \Psi \text{m} \) powered by glutamate plus malate in the absence or presence of the K\(^+\)/H\(^+\) exchanger nigericin, and effect on \( \text{H}_2\text{O}_2 \) production by site IQ driven by succinate. Note that because the method used to normalize mGPDH \( \text{H}_2\text{O}_2 \) production in the screening assay underestimated the background rate, the maximal % inhibition for this assay was \(-70\%\) (see Materials and Methods). Glu, glutamate; Mal, malate; Nig, nigericin; Rot, rotenone; GP, glycerol phosphate; Suc, succinate.

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fluorescence and acidic vesicles (Fig. 8B and 8C). Treatment of cells with 250 nM baflomycin A1, an inhibitor of vesicular H\(^+\)-ATPases, collapsed the vesicular pH gradient (demonstrated by loss of LysoTracker Red staining) (Fig. 8E) and also caused loss of the bright, punctate staining of iGP-1 but not the diffuse fluorescence in the cytosol (Fig. 8D). To address whether iGP-1 was accumulated in acidic vesicles or if iGP-1 fluorescence was enhanced at the lower pH of these vesicles, we measured the fluorescence of iGP-1 as a function of pH. We observed an 8-fold increase in iGP-1 fluorescence as the pH was lowered from pH 7 to pH 1.5 (Fig. 8F). Further, addition of the protonophore FCCP to cells caused a rapid, though incomplete, loss of the bright, punctate staining seen with iGP-1 alone (not shown). Together, these data suggest that the intense punctate staining of iGP-1 is likely the result of a pH-dependent enhancement of iGP-1 fluorescence in acidic compartments and not accumulation in vesicles. Importantly, we can conclude that iGP-1 readily crosses cellular membranes and therefore should have access to mGPDH in intact systems.

Effect of iGP-1 in Cellular Systems

mGPDH is a critical component of the glycerol phosphate shuttle for regenerating cytosolic NAD\(^+\). However, because of a lack of cell-permeant selective inhibitors of mGPDH and compensation by other NAD\(^+\) regenerating mechanisms, there is a lack of evidence of functional mGPDH activity in intact systems. We tested the role of mGPDH-dependent metabolism using iGP-1 in synaptosomes because mGPDH is active in neurons [27,45,46], there is a significant role for NADH shuttles in synaptic energetics [26,47], there are no reports on neural function in mGPDH\(^{-/-}\) mice, and synaptosomes are a tractable intact system for studying neuronal energetics under tightly controlled cell-, substrate-, and demand-specific conditions.

We verified that the high mGPDH activity reported in brain was present in synaptosomes. In frozen-thawed cortical presynaptic terminals, mGPDH activity was high (207±16 nmol ferricyanide reduced · min\(^{-1}\) · mg protein\(^{-1}\); mean ± SE, \( n = 3 \)). To demonstrate the likelihood of a functional shuttle system, we measured cGPDH activity in the same preparations. There was a significant rate of DHAP-specific, rotenone-insensitive NADH oxidation that we attribute to cGPDH (22±1 nmol NADH oxidized · min\(^{-1}\) · mg protein\(^{-1}\); mean ± SE, \( n = 3 \)).

We then applied iGP-1 alone or in combination with aminooxyacetate (to inhibit the malate-aspartate shuttle) (Fig. 1B) in synaptosomes respiring on glucose (Fig. 8G). Maximal demand was created by adding FCCP (plus oligomycin to decrease ATP depletion). In control experiments, pyruvate replaced glucose to bypass the need for NAD\(^+\) regeneration (Fig. 1). 100 \( \mu \text{M} \) iGP-1, 0.5 mM aminooxyacetate, or their combination had no effect on basal or maximal respiration with pyruvate (two left hand sets of columns, Fig. 8G). With glucose as substrate, basal and maximal respiration were inhibited by aminooxyacetate (two middle sets of columns, Fig. 8G), confirming a role for the malate-aspartate shuttle in synaptic bioenergetics [26,47]. However, iGP-1 had no effect even in combination with aminooxyacetate, suggesting little contribution of the glycerol phosphate shuttle.

In the absence of inhibitors, glucose supported slower respiration than pyruvate. As the result with aminooxyacetate demonstrates, NAD\(^+\) regeneration has a significant permissive role in synaptosomal metabolism of glucose but not pyruvate. There was also faster extrasynaptosomal acidification with glucose as substrate, suggesting efflux of lactate (with H\(^+\)) to regenerate cytosolic NAD\(^+\) at lactate dehydrogenase (Fig. 1C). This would divert pyruvate destined for respiration and cause slower respiration with glucose than with pyruvate. To test this hypothesis, we added oxamate (with the FCCP and oligomycin) to inhibit lactate dehydrogenase during oxidation of glucose (Fig. 1C). This should maximally drive respiration while also placing the greatest demand on the NADH shuttles to facilitate glycolysis. Maximal respiration was substantially faster in the presence of oxamate (two right hand sets of columns, Fig. 8G), and aminooxyacetate still inhibited significantly. Importantly, although iGP-1 alone had no effect, it caused consistent additional inhibition in the presence of aminooxyacetate. Thus, there was a significant dependence of uncoupled respiration on both the malate-aspartate shuttle (45.9±0.7\%) and the glycerol phosphate shuttle (5.3±0.9\%) (means ± SE, \( n = 5 \)) (Fig. 8H).

Characterization of the Effects of Novel Inhibitors on the Kinetics of mGPDH

The greater potency of iGP-5 versus iGP-1 was confirmed against both mGPDH activity (Fig. 9A and Table 1; IC\(_{50} = 1.0 \mu \text{M} \) and 6.3 \( \mu \text{M} \), respectively) and \( \text{H}_2\text{O}_2 \) production (Fig. 9B and Table 1; IC\(_{50} = 1.0 \mu \text{M} \) and 13.6 \( \mu \text{M} \), respectively). Inhibition of mGPDH by both iGP-1 and iGP-5 displayed a
selective, one-site binding profile (Table 1; Hill slopes 0.92 and 0.85, respectively). These data support the conclusion that these novel compounds inhibit mGPDH enzymatic activity and confirm that iGP-1 is the most selective of the potent inhibitors we have identified. We next characterized the mechanism of action of this novel class of mGPDH enzymatic inhibitors by measuring the effects on the kinetics of mGPDH. We assayed mGPDH activity at different concentrations of glycerol phosphate and inhibitor (either iGP-1 or iGP-5). These data (Fig. 10A, 10B) showed that the maximum observed rate of DCPIP reduction by mGPDH decreased as the inhibitor concentration increased.

Analysis of double reciprocal plots (Fig. 10C, 10D) showed that each inhibitor lowered the $V_{\text{max}}$ and increased the $K_{\text{iu}}$ for glycerol phosphate. Fig. 10E shows that the apparent $V_{\text{max}}$ was progressively decreased by each inhibitor with iGP-5 more potent than iGP-1. Fig. 10F shows that the $K_{\text{iu}}$ for glycerol phosphate was progressively increased; again, iGP-5 was more potent than iGP-1. This profile of lowered $V_{\text{max}}$ combined with a change in $K_{\text{iu}}$ is indicative of a mixed inhibitor (Fig. 10G) that interacts competitively with respect to the substrate (defined by the competitive inhibitor dissociation constant $K_{\text{ic}}$) and uncompetitively with the enzyme-substrate complex (defined by the uncompetitive inhibitor dissociation constant $K_{\text{iu}}$). Values for these dissociation constants were in the 10 μM range for iGP-1 and the 1 μM range for iGP-5 (Table 1).
Discussion

There is a longstanding need for potent, selective, cell-permeant inhibitors of mGPDH [3,24]. mGPDH knockout mice indicated a significant role for mGPDH in the survival of nursing pups and in adult adiposity [11], but more effort was required to identify the subtle roles of mGPDH in glucose-stimulated insulin secretion, obligatory thermogenesis, glycerol and fat metabolism, and, specific to mice, liver ureogenesis [12,48–50]. Such long-term studies involving genetic manipulation, condition-dependent phenotypes, and/or pharmacologic interventions are complicated by compensatory mechanisms that mask the involvement of mGPDH. Several 3-carbon glycolytic intermediates, as well as fatty acids and inorganic ions, are known to inhibit mGPDH [2,13–16]. However, many are membrane impermeant, none are selective, and, as we show for the potent competitive inhibitor glyceraldehyde 3-phosphate, can be non-selective even in isolated mitochondria (Fig. 2). Therefore, our novel class of inhibitors offers the first opportunity to acutely test the role of mGPDH activity in a more diverse range of physiological conditions.

Figure 7. iGP-5 inhibits mGPDH activity more potently than iGP-1 but is less selective. (A) Structure of iGP-5. (B) Effect of iGP-5 on rates of H$_2$O$_2$ production from site I/II (black diamonds), site I$_0$ (with 0.5 or 5 mM succinate; white and black squares, respectively), site II$_{Ox}$ (with 0.5 or 5 mM succinate and 4 mM rotenone (black triangles), or 16.7 mM glycerol phosphate and 4 mM rotenone (black squares), ΔΨm powered by glycerol phosphate was significantly increased by 2.5 and 25 μM iGP-5. ΔΨm powered by glutamate and malate was significantly increased by 25 μM iGP-5 but only in the absence of nigericin (white circles) suggesting an effect of iGP-5 on the ΔpH component on the proton motive force. (*p<0.05 versus vehicle control; one-way ANOVA with Newman-Keuls post-test). Data are normalized means ± S.E. (n = 3). (C) Effect of iGP-5 on the states of mitochondrial respiration driven by 16.7 mM glycerol phosphate with 4 mM rotenone and 250 nM free calcium, 10 mM pyruvate and 0.5 mM malate, 5 mM glutamate and 5 mM malate, or 5 mM succinate and 4 mM rotenone. Respiratory states 2, 3, and 4o were defined by the sequential additions of substrate, 5 mM ADP, and 0.5 μg · mL$^{-1}$ oligomycin, respectively. iGP-5 significantly decreased glycerol phosphate-dependent state 3 respiration at 25 and 80 μM but also significantly reduced state 3 respiration with pyruvate and malate at 80 μM. (*p<0.05 versus vehicle control; one-way ANOVA with Newman-Keuls post-test). Data are means ± S.E. (n = 3). No significant effects were observed under any condition with iGP-5 at 2.5 and 8 μM (not shown). Glu, glutamate; Mal, malate; Nig, nigericin; Suc, succinate; Rot, rotenone; GP, glycerol phosphate. When not visible, error bars are obscured by the symbol.

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selective inhibitors of mGPDH. The design of the different assays of mitochondrial H2O2 production and ΔΨm executed in parallel during primary screening and retesting provided multiple filters through which non-selective hits were readily eliminated. Three of

Figure 8. iGP-1 is cell-permeant and inhibits mGPDH in intact presynaptic terminals. (A) Excitation and emission spectra of 0.4 mM iGP-1 with peaks at 342 nm and 378 nm, respectively. (B) Fluorescence of 100 μM iGP-1 in a live STHdhQ7 cell labeled with LysoTracker Red. (C) Fluorescent labeling of acidic vesicles by LysoTracker Red in the same cell shown in (B). Insets in (B and C) highlight colocalization between intense iGP-1 fluorescence and LysoTracker Red labeled vesicles. (D) Fluorescence of 100 μM iGP-1 in a live STHdhQ7 cell pretreated with 250 nM bafilomycin A1. (E) LysoTracker Red fluorescence in the same cell pretreated with bafilomycin A1 as in (D). Scale bar in (B – E) = 10 μm. (F) iGP-1 fluorescence as a function of buffer pH (ex. λ = 342 nm, em. λ = 430 nm). (G) Effect of vehicle control (DMSO, white bars), 100 μM iGP-1 (light gray bars), 0.5 mM aminooxyacetate (dark gray bars), or their combination (black bars) on synaptosomal respiration. Basal respiration was measured in the presence of either 10 mM pyruvate or 15 mM glucose followed by the addition of 5 μM FCCP and 4 μg mL−1 oligomycin to induce uncoupled respiration without or with (rightmost condition) 10 mM oxamate. Oxamate was included to minimize regeneration of cytosolic NAD+ by lactate dehydrogenase. Respiration on pyruvate alone was not altered by inhibition of either or both of the NADH shuttle systems. Both basal and uncoupled respiration on glucose was unaffected by iGP-1 alone but significantly decreased by aminooxyacetate alone. Oxamate increased the maximal rate of respiration achieved by glucose alone. In the presence of oxamate, the combination of iGP-1 combined with aminooxyacetate alone; one-way ANOVA with Newman-Keuls post-test). Data are means ± S.E. (n = 3 for pyruvate, n = 4 for glucose, n = 5 for glucose ± oxamate). (H) Assignment of the % of maximal capacity that is dependent on the NADH shuttles in the presence of glucose and oxamate. Under this condition of high glycolytic demand, there is a significant dependence upon both the malate-aspartate shuttle and the glycerol phosphate shuttle. (*p<0.05 versus no change from vehicle control; **p<0.05 versus no change from aminooxyacetate). Data are means ±95% C.I. (n = 5). AOA, aminooxyacetate; Pyr, pyruvate; F/O, FCCP with oligomycin; Glu, glucose.

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Novel Inhibitors of mGPDH

The top seven most selective mGPDH inhibitors shared significant structural similarity and the most potent inhibitor in the initial screen, iGP-1, turned out to be the most selective of all the potent analogs identified during subsequent retesting. The design of our screening and retesting strategy also meant that partial selectivity in certain assays yielded insights into potential mechanisms of off-target effects. Combining these insights into an analysis of structure/activity relationships, we revealed that both the succinic acid and benzimidazole motifs are essential components for mGPDH inhibition by iGPs. Importantly, this analysis identified the benzimidazole ring system as the best candidate for further manipulations to improve both potency and selectivity. In particular, changing or removing the heteroatoms of the imidazole might improve selectivity whereas added substituents to the ring system may provide a means to improve both qualities. We were not able to explore targeted changes to the chemical space occupied by either the linking phenyl group or the succinamide group that did not involve loss of the terminal carboxylic acid. Therefore, these motifs may also provide additional opportunities for improved activity.

Enzyme kinetics revealed that iGPs share a common mechanism of mixed inhibition with respect to glycerol 3-phosphate and that potency was governed by subtle structural changes. Both iGP-1 and iGP-5 lowered the maximal activity of mGPDH and increased the $K_m$ for glycerol 3-phosphate. Inhibition was enzyme specific, as iGP-1 had no effect on NAD-linked cGPDH activity. This is not surprising considering the two forms are distinct in all respects except their ability to oxidize glycerol 3-phosphate to DHAP. However, this enzyme specificity suggests that iGPs are likely not acting as inhibitory analogs in the substrate binding pocket as described for non-selective inhibitors of both forms such as glyceraldehyde 3-phosphate (data not shown and [2,13–15]).

The observation of increased $K_{iu}$ as well as the lower values for $K_{iu}$ than $K_m$ indicates that both iGP-1 and iGP-5 have a greater affinity for free enzyme (i.e. mGPDH without glycerol phosphate bound). Both inhibitors have Hill slopes near unity, suggesting they interact with mGPDH at a single, allosteric binding site. Although the analysis of inhibition kinetics was performed in the presence of activating calcium, our evidence from assays of mGPDH-specific H$_2$O$_2$ production (data not shown) and ΔΨm driven by glycerol phosphate (Fig. 6C) suggest that iGPs act independently of the calcium-sensing mechanism of mGPDH. We cannot rule out direct interactions between iGPs and either the FAD binding domain in the soluble portion of mGPDH or the ubiquinone binding pocket embedded in the outer leaflet of the inner mitochondrial membrane [2]. The interaction with the ubiquinone pocket might be tested by studies similar to those presented here but with differing ubiquinol as the electron donor to the enzyme. Future co-crystallization structural studies and enzymatic assays of iGPs with the bacterial or mammalian FAD-linked GPDH may provide the best opportunities to identify the exact mode of interaction and mechanism of action of these novel inhibitors.

As initial confirmed hits in a small-molecule screen, our most promising iGPs demonstrate excellent potency (IC$_{50}$ values between 1–15 μM) and good selectivity. Apart from a subtle effect on succinate oxidation at high concentrations, iGP-1 does not alter mitochondrial oxidation of numerous substrates including a second dicarboxylate, malate. Therefore, it is unlikely that the

Figure 9. iGP-1 and iGP-5 potently inhibit mGPDH enzymatic activity and H$_2$O$_2$ production. (A) iGP-5 (black circles) is a more potent inhibitor of mGPDH enzymatic activity than iGP-1 (white circles). IC$_{50}$ concentrations were 1.0 and 6.0 μM for iGP-5 and iGP-1, respectively. Data are means ± S.E. (error bars) (n = 3–5). (B) iGP-5 (black circles) is a more potent inhibitor of mGPDH H$_2$O$_2$ production than GP-1 (white circles). IC$_{50}$ concentrations were 1.0 and 14.2 μM for iGP-5 and iGP-1, respectively. Data are means ± S.E. (error bars) (n = 2–4). When not visible, error bars are obscured by the symbol. doi:10.1371/journal.pone.0089938.g009

Table 1. Inhibition parameters for iGP-1 and iGP-5.

| Inhibitor | mGPDH Activity Hill Slope | mGPDH Activity IC$_{50}$ (μM) | mGPDH H$_2$O$_2$ Production IC$_{50}$ (μM) | $K_{iu}$ (μM) | $K_m$ (μM) |
|-----------|---------------------------|---------------------------------|----------------------------------------|-----------|-----------|
| iGP-1     | 0.92±0.08                 | 6.3±0.7                         | 13.6±1.3                               | 9.5±1.1   | 14.6±1.5  |
| iGP-5     | 0.85±0.07                 | 1.0±0.2                         | 1.0±0.1                                | 0.7±0.0   | 1.1±0.0   |

Summary of inhibitor parameters and constants derived from the data presented in Fig. 9 and Fig. 10.

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The subtle effect on succinate oxidation is due to inhibition of the dicarboxylate transporter by the succinamide of iGPs. Indeed, analogs iGP-17–iGP-19 retain the succinamide without or with the attached phenyl ring yet do not alter succinate oxidation as assessed by H$_2$O$_2$ production by succinate alone (Fig. 4). This suggests at least partial dependence on the benzimidazole ring system for the subtle effect of iGP-1 on succinate oxidation, perhaps via direct interaction with complex II. Reactions of the tricarboxylic acid cycle shared between succinate, malate, and pyruvate oxidation also do not appear to be affected by iGP-1. Further, iGP-1 shows no effects on the maintenance of proton motive force or rates of ATP synthesis with substrates other than glycerol phosphate. In addition, we can infer from the synaptic experiments that iGP-1 does not prevent pyruvate uptake into cells or mitochondria and does not directly alter glycolysis. Therefore, our data identify an exemplary inhibitor that is both potent and selective against mGPDH and offers structural targets through which additional improvements to these activities can be achieved.

In conclusion, we have identified a novel class of potent, selective, cell-permeant inhibitors of mGPDH that act via mixed inhibition. Further tests of the role of mGPDH and glycerol phosphate shuttle activities under conditions of neuronal activity or in other cell types with differing shuttle capacities will help determine those in which mGPDH activity is essential. Our novel inhibitors may offer new therapeutic opportunities in the treatment of neurological disorders.
inhibitors of mGPDH provide means to test these possibilities pharmacologically.

Supporting Information

Table S1  Screening results for hits in the mGPDH ROS assay. Summary data for the 87 compounds that selectively inhibited mGPDH ROS. Each value is the average of duplicate “% change from DMSO” determined for each compound at ~2.5 μM against 6 screening assays.

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Author Contributions

Conceived and designed the experiments: ALO REH MDB. Performed the experiments: ALO DA MRS RN AG. Analyzed the data: ALO. Contributed reagents/materials/analysis tools: MRS TS REH. Wrote the paper: ALO MDB.
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