

1 **TITLE: A DUAL MTOR/NAD+ ACTING GEROTHERAPY**

2

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34

35 **Abbreviations:**

36 SIRT3: Sirtuin 3

37 LPIN1: Lipin 1

38 mTOR: mechanistic target of rapamycin

39 NAD⁺: Nicotinamide adenine dinucleotide

40 NASH: non-alcoholic steatohepatitis

41 ALS: amyotrophic lateral sclerosis

42 SOD1: super oxide dismutase

43

44 **Brief Summary:** These studies characterize a novel gerotherapy, BIOIO-1001, that identifies
45 lipid metabolism as an intersection of the mTOR and NAD⁺ pathways.

46

47 **ABSTRACT**

48 The geroscience hypothesis states that a therapy that prevents the underlying aging process
49 should prevent multiple aging related diseases. The mTOR (mechanistic target of
50 rapamycin)/insulin and NAD⁺ (nicotinamide adenine dinucleotide) pathways are two of the most
51 validated aging pathways. Yet, it's largely unclear how they might talk to each other in aging. In

52 genome-wide CRISPRa screening with a novel class of N-O-Methyl-propanamide-containing
53 compounds we named BIOIO-1001, we identified lipid metabolism centering on SIRT3 as a
54 point of intersection of the mTOR/insulin and NAD⁺ pathways. In vivo testing indicated that
55 BIOIO-1001 reduced high fat, high sugar diet-induced metabolic derangements, inflammation,
56 and fibrosis, each being characteristic of non-alcoholic steatohepatitis (NASH). An unbiased
57 screen of patient datasets suggested a potential link between the anti-inflammatory and anti-
58 fibrotic effects of BIOIO-1001 in NASH models to those in amyotrophic lateral sclerosis (ALS).
59 Directed experiments subsequently determined that BIOIO-1001 was protective in both sporadic
60 and familial ALS models. Both NASH and ALS have no treatments and suffer from a lack of
61 convenient biomarkers to monitor therapeutic efficacy. A potential strength in considering
62 BIOIO-1001 as a therapy is that the blood biomarker that it modulates, namely plasma
63 triglycerides, can be conveniently used to screen patients for responders. More conceptually, to
64 our knowledge BIOIO-1001 is a first therapy that fits the geroscience hypothesis by acting on
65 multiple core aging pathways and that can alleviate multiple conditions after they have set in.

66

67 **INTRODUCTION**

68 Phenotypic screening, sometimes serendipitously performed, has been how many of the most
69 influential drugs were identified ¹. Interestingly, phenotypic screening fell out of favor as the
70 molecular biology revolution and rational drug design – developing drugs based on knowledge
71 of a target – gained hold. A major problem with phenotyping screening for those in drug
72 development has been that typically one does not learn their mechanism of action (MoA) from
73 them. Fortunately, phenotyping screening has made a comeback as new methodologies such
74 as CRISPR have made drug target deconvolution easier ².

75

76 Another issue rational drug design proponents have had is that compounds that act on multiple
77 targets are considered sub-optimal for drug development. The one target-one disease creates a

78 simple narrative that scientists, publishers, funders, and regulators have been able to get
79 behind. Yet, these traditional pharma forces are at odds with the aging field both in that: 1) a
80 strongly performing gerotherapy would likely have more pervasive effects than a highly selective
81 single target drug could produce ³ and 2) aging is not a FDA approved disease indication. It is
82 an exhausting challenge to gain drug approval for one indication, which has made it prohibitive
83 for researchers to know whether their drug might work in additional contexts.

84

85 Taking an unbiased genome-wide screening approach led us to the determination that a novel
86 compound series we identified, BIOIO-1001, is a multi-pathway, multi-disease therapy.
87 Molecularly speaking, because BIOIO-1001 acts at the intersection of two canonical aging
88 pathways, mTORC1 (mechanistic target of rapamycin) and NAD⁺ metabolism, both which act
89 pervasively on the hallmarks of aging ^{3, 4}, our work suggests BIOIO-1001 is a bona fide novel
90 gerotherapy.

91

92 **RESULTS**

93 **Identification of SIRT3 as a BIOIO-1001 genetic target**

94 With the premise that insulin resistance is important to many diseases of aging ⁵, phenotypic
95 screening was performed to identify novel insulin sensitizing agents. From these screens, a
96 compound series was identified that we named BIOIO-1001 (**Fig. 1A**). The BIOIO-1001 series
97 are N-O-Methyl-propanamide-containing compounds derived from the thiazolidinedione (TZD) ⁶,
98 pioglitazone, that lack the heterocyclic C₃NS ring that defines the TZD compounds. In vitro
99 testing established that BIOIO-1001 did not activate the canonical TZD target, PPAR γ
100 (**Fig. S1A**) ⁷. BIOIO-1001 also did not inhibit the mitochondrial pyruvate carrier or activate a
101 panel of nuclear hormone receptors (**Fig. S1B, C**) – factors which might have been involved in
102 the MoA of BIOIO-1001 based on our and others previous work ⁸.

103

104 We and others have previously used genome-scale CRISPR-based screening in pinpointing a
105 small molecule's mechanistic target based on its pattern of resistance and sensitizing hits⁹⁻¹¹.
106 To deconvolute the MoA of BIOIO-1001, we performed a genome-scale CRISPR activation
107 (CRISPRa) screen with BIOIO-1001 (**Fig. 1B**). This screen pinpointed downregulation of the
108 mTORC1/insulin and upregulation of the NAD⁺ pathways, respectively, as most important to
109 BIOIO-1001's action (**Fig. 1C, Supplemental Table 1**). This is consistent with the sign
110 convention of effects of these two pathways in promoting longevity. The hits flipped from
111 resistance to sensitizing to resistance or vice versa for each pathway in a way that centered on
112 BIOIO-1001 promoting fatty acid oxidation (FAO) (Fig. 1C). Both mTORC1 and NAD⁺ have
113 effectors that contribute significantly to fatty acid oxidation, namely LPIN1 and SIRT3
114 respectively¹²⁻¹⁵. LPIN1 was directionally consistent in the CRISPRa screen with its positive role
115 in FAO¹², but it was not a statistically significant hit. Whereas SIRT3 scored strongly and follow
116 up analysis validated it as a BIOIO-1001 genetic target (**Fig. 1D, E**). Notably, CRISPRa
117 mediated increased SIRT3 mRNA expression led to increased LPIN1 mRNA expression, which
118 is consistent with these two genes acting in concert (**Fig. S1D**). This has analogies to the results
119 for LPIN2 in the CRISPRa screen. LPIN2 suppresses LPIN1 expression and indeed LPIN2 was
120 a strong hit, but in the opposite direction of LPIN1 (Fig. 1C)¹⁶. Phosphatidate phosphatase
121 activity (GO:0008195) was a top gene ontology category in transcriptional profiling of BIOIO-
122 1001 treated K562 cells based on using Enrichr¹⁷, which identifies pathway, disease, and drug
123 signatures that intersect with an input gene list (**Fig. S1E, Supplemental Table 1**). The
124 identification of fatty acid oxidation-related disease biology (short chain acyl-CoA
125 dehydrogenase deficiency, ORPHA:26792) as a top gene signature in unbiased genome wide
126 transcriptional profiling corroborated the similar finding in our unbiased genome wide CRISPRa
127 screen (Fig. S1E, Supplemental Table 1). More evidence that LPIN1 appears to be involved in
128 the BIOIO-1001 MoA is that BIOIO-1001 increased the levels of the LPIN1 substrate,

129 phosphatidic acid, whereas it decreased the levels of the LPIN1 product, diacylglycerol, in livers
130 of mice treated with the drug (**Fig. S1F**).

131
132 Since K562 is a cancer cell line we sought a more physiological relevant context to characterize
133 BIOIO-1001's molecular mechanisms. Transcriptional profiling of mice treated with BIOIO-1001
134 revealed fatty acid metabolic process (GO:0006631) as the top gene signature, consistent with
135 our results in K562 cells (**Fig. 1F, Supplemental Table 1**). Validation of ex vivo mouse liver
136 confirmed SIRT3 not only as a strong genetic interactor with BIOIO-1001, but more importantly
137 as required for metabolic signaling downstream of both mTOR and NAD⁺ (**Fig. 1G**)^{14, 15}. The
138 BIOIO-1001 back up compound, BIOIO-1002, also required SIRT3 for its metabolic effects
139 (specifically, to activate the expression of Cpt1b and Ucp3 in mouse hepatocytes, Fig. 1G).
140 Interestingly, the top signature in our liver transcriptional profiling of high-fat diet fed mice based
141 on fold change was responses to stilbenoid and resveratrol (GO:0035634, GO:1904638).
142 Resveratrol is a well-known stilbenoid that has long been connected to NAD⁺ metabolism and
143 specifically, the sirtuins¹⁸. Though the mechanism(s) by which resveratrol acts on the sirtuins
144 are controversial, it has been shown that resveratrol can bind directly to SIRT1¹⁹. These results
145 led us to hypothesize that potentially BIOIO-1001 might bind SIRT3. In silico docking of BIOIO-
146 1001 to SIRT3 predicted a singular interaction between BIOIO-1001 to SIRT3 outside of the
147 NAD-Ribose binding pocket (**Fig. 1H, Fig. S1G**). Taken together, the above suggests the
148 BIOIO-1001 series is a novel metabolic regulator of lipid metabolism via its mTOR and NAD⁺
149 targets, LPIN1 and SIRT3.

150
151 **BIOIO-1001 provides in vivo and in vitro protection against high fat diet and NASH**
152 **models**

153 The mTOR and NAD⁺ pathways have broad metabolic effects^{20, 21}. To test these effects in vivo,
154 we put mice on a high fat diet (HFD) for 10 weeks and then treated mice with either BIOIO-1001

155 or the aforementioned insulin sensitizer benchmark, pioglitazone (PIO), for 10 days (**Fig. S2A**).
156 The rationale for this protocol rather than co-administering the diet and drug at the start was to
157 model a real-world scenario where the therapy would be given after disease has set in. Both
158 BIOIO-1001 and PIO improved glucose tolerance in HFD fed mice (**Fig. S2B**). Feeding mice HF
159 diet increased plasma insulin concentration, but consistent with an insulin sensitizing effect,
160 insulin concentration in HF-fed mice treated with BIOIO-1001 or PIO were significantly lower
161 than HF vehicle controls and not different than LF diet-fed controls (**Fig. S2C**). This is likely due
162 to reduced insulin secretion, rather than clearance, because C-peptide concentrations in the
163 blood were also reduced by treating with BIOIO-1001 or PIO (Fig. S2C). This suggested that
164 less insulin was needed to maintain normoglycemia. Plasma non-esterified fatty acids and liver
165 triglycerides were both reduced by BIOIO-1001 or PIO (**Fig. S2C, D**), but interestingly, plasma
166 triglycerides were uniquely reduced by BIOIO-1001, but not by PIO (Fig. S2C). A subset of
167 mice from each group was injected with saline or insulin prior to sacrifice and muscle insulin
168 signaling was assessed. BIOIO-1001 and PIO increased the phosphorylation of AKT in
169 response to insulin stimulation (**Fig. S2E**). These data suggest that BIOIO-1001 improves
170 metabolic abnormalities and insulin sensitivity in obese mice.

171
172 Inclusion of fructose and cholesterol in high fat diets can exacerbate diabetes and fatty liver and
173 lead to non-alcoholic steatohepatitis (NASH), which is characterized by inflammation and
174 fibrosis^{22, 23}. We put mice on a diet high in trans-fat, fructose, and cholesterol (HTF-C) as a
175 mouse model of NASH. Like the HF diet, we provided the HTF-C diet for a prolonged period (16
176 weeks) before briefly treating the mice with BIOIO-1001 or PIO (last three weeks) (**Fig. 2A**).
177 Strikingly, despite BIOIO-1001 being given for < 20% of the time the mice were on the HTF-C
178 diet, it significantly reduced liver injury and a host of NASH-related phenotypes, including
179 (plasma transaminases - ALT, AST) and liver triglyceride content more strongly than PIO (**Fig.**
180 **2B-D**). BIOIO-1001 also tended to improve NAS and fibrosis scoring after this short treatment

181 period (**Fig. 2E-F**). Consistent with these histologic and plasma findings, BIOIO-1001 was more
182 protective against inflammation and fibrosis markers at the transcriptional level than PIO (**Fig.**
183 **2G-I**). Ex vivo BIOIO-1001 reduced several inflammation and fibrosis markers in a SIRT3
184 dependent manner (**Fig. 2J**). Taken together, these results suggest BIOIO-1001 alleviates
185 numerous diet-induced phenotypes found in NASH in a SIRT3 dependent manner.

186

187 **BIOIO-1001 provides in vivo and in vitro protection against sporadic and familial models** 188 **of ALS**

189 We performed transcriptional profiling on several tissues (heart, liver, adipose tissue) from the
190 mice from our HF diet study. These experiments identified long chain fatty acid metabolism as a
191 core signature across all three tissues as increased by BIOIO-1001 (**Supplemental Table 2**).
192 Long chain fatty acid metabolism is selectively deregulated in SIRT3 deficient mice ¹³.
193 Interestingly, based on the dbGaP human database of genotypes and phenotypes ²⁴ our gene
194 list most strongly was comprised of genes (e.g., RNF144A, RD3, KCNMA1, SUSP1, $p = 0.0014$)
195 involved in amyotrophic lateral sclerosis (ALS) (**Fig. 3A**). This was interesting because these
196 genes are involved in inflammation and fibrosis ²⁵⁻²⁸ and inflammation and fibrosis are key
197 consequences of altered metabolism in both NASH and ALS pathology ^{23, 29, 30}.

198

199 We investigated further a potential role for BIOIO-1001 in treating ALS. There are two forms of
200 ALS, sporadic and familial, with sporadic accounting for 90% of all cases ³¹. In ALS, motor
201 neuron survival is impaired, and this contributes to paralysis and ultimately the death of the
202 patient. We measured the effects of BIOIO-1001 on the survival of motor neurons derived from
203 sporadic ALS patients. In these assays BIOIO-1001 was protective towards cell viability (**Fig.**
204 **3B**).

205

206 To study hereditary ALS, we performed analogous experiments as in Fig. 3B using cells with the
207 well-known SOD1-G93A mutation³². The results were similar to those in Fig. 3B – BIOIO-1001
208 was protective (**Fig. 3C**). We tested BIOIO-1001 in vivo in the SOD1-G93A mouse model of the
209 familial ALS condition³². This commonly used model displays many features of ALS including
210 neuromuscular degeneration leading to paralysis and shortened lifespan. We deployed the
211 same paradigm as with the NASH model. That is, we treated mice with BIOIO-1001 after
212 disease set at nine weeks (typical lifespan is 18 weeks with this model) to mimic the real-world
213 scenario that the drug would be given to symptomatic patients (**Fig. 3D**). Interestingly,
214 compared with vehicle treatment, BIOIO-1001 increased plasma triglycerides in the SOD1-
215 G93A mice (**Fig. 3E**). Unlike in metabolic diseases like NASH, in ALS, higher triglycerides levels
216 have been shown to correlate with better prognosis in humans³³. Indeed, BIOIO-1001 was
217 protective against paralysis and prolonged the lifespans of the SOD1-G93A mice by 29% and
218 26%, respectively (**Fig. 3F, G**). Taken together with our in vitro results, these results suggest
219 BIOIO-1001 alleviates numerous phenotypes found in ALS.

220

221 **DISCUSSION**

222 The geroscience hypothesis states that a therapy that checks the underlying aging process
223 should prevent multiple aging related diseases³⁴. Herein we provide evidence BIOIO-1001 is a
224 novel gerotherapy that modulates multiple aging pathways and can be therapeutic in multiple
225 age-related diseases.

226

227 There are several core pathways that affect aging. Rapamycin targets the mTOR pathway.
228 NAD⁺ metabolites boost the NAD⁺ pathway. Our work presents insights into how the mTOR
229 and NAD⁺ might talk to each other in aging. Specifically, we identify lipid metabolism and the
230 genes, LPIN1 and SIRT3, as the point of intersection of the mTOR and NAD⁺ pathways. That
231 BIOIO-1001 enabled this discovery speaks to the value of using drugs as a tool to understand

232 biology. That BIOIO-1001 enabled this discovery speaks to the value of using drugs in making
233 biological discovery. Mechanistically, we speculate that BIOIO-1001 exerts its action
234 epigenetically by affecting the levels of acetyl-CoA (Fig. 1C). BIOIO-1001 is a N-O-Methyl-
235 propanamide compound that is hydroxamic-like. Hydroxamic compounds are known to have
236 epigenetic effects³⁵. Also, both LPINs and SIRT3 affect acetylation of lipid metabolism genes³⁶.
237³⁷. In future studies, it will be interesting to determine the precise mechanism of BIOIO-1001's
238 activity on the LPINs and SIRT3s.

239

240 Both inhibition of mTORC1 and activation of NAD⁺ effectors suppress inflammation and fibrosis
241^{38, 39}. In future studies it will be interesting to dissect the mechanisms by which the effect of
242 BIOIO-1001 on lipid metabolism translate to reduced inflammation and fibrosis. Additionally, it
243 will be interesting to determine how decreased vs. increased plasma triglycerides can lead to
244 improved outcomes in NASH vs. ALS models as we observed.

245

246 There are several issues with the animal models used in aging research. Lifespan studies do
247 not inform how a therapy might work in disease. Progeria models are too extreme in how short
248 lived they are and too few people have this condition for it to be considered relevant to more
249 common aging-related diseases⁴⁰. Thus, we propose ALS could be an interesting new
250 gerotherapy proving ground. Like aging itself, ALS has many unrelated molecular targets. If a
251 therapy works in diverse ALS models, we hypothesize it could work across diseases. Our lead
252 asset, BIOIO-1001, provides proof of concept of this hypothesis.

253

254 **Figure 1. Genome-wide screening with BIOIO-1001 identifies SIRT3 as a point of**
255 **intersection between the MTOR and NAD⁺ pathways. A.** Chemical structure of BIOIO-1001.
256 **B.** Schematic of a CRISPRa screen with BIOIO-1001. Screening was performed in duplicate
257 with 50 μ M of BIOIO-1001 or vehicle (DMSO) in K562 cells. **C.** Genetic map of top hits from the

258 BIOIO-1001 CRISPRa screen. Sensitizing and resistance hits ($p < 0.05$, based on the average
259 phenotype of top three sgRNAs out of 10) are shown in red and blue, respectively. The putative
260 mechanism of action of BIOIO-1001 is where the hits flip from resistance to sensitizing to
261 resistance or vice versa (highlighted in gray) in both the mTORC1 and NAD⁺ pathways. The
262 common target of both pathways is fatty acid oxidation, and specifically the mTORC1 and NAD⁺
263 targets, LPIN1 and SIRT3, respectively. Asterisk on LPIN1 indicates it was sensitizing but its p
264 value was greater than 0.05. **D.** Relative SIRT3 mRNA expression in SIRT3 CRISPRa K562
265 cells. SIRT3 mRNA levels were assessed by RT-qPCR. One-way ANOVA for all SIRT3 sgRNAs
266 vs. control sgRNA. **E.** Relative ATP levels in SIRT3 overexpressing and control cells in the
267 presence of the indicated concentrations of BIOIO-1001 or vehicle (DMSO). ATP levels were
268 measured after 72 hours treatment in cells from D. One-way ANOVA for all SIRT3 sgRNAs vs.
269 control sgRNA. **F.** Transcriptionally profiling of livers harvested from mice treated with BIOIO-
270 1001. Mice fed a high fat diet for 12 weeks were gavaged once daily with 30mg/kg/day of
271 BIOIO-1001 or vehicle ($n=6$) for one week prior to euthanasia. Genes that were increased in
272 expression 10-fold compared to vehicle treated condition were included in the gene ontology
273 analysis. Only genes with counts per million (CPM) in the vehicle treated condition of greater
274 than 0.1 were included. **G.** Wildtype and SIRT3 deficient primary mouse hepatocytes treated
275 with BIOIO-1001, BIOIO-1002, or vehicle at the indicated concentrations for 24 hours. Gene
276 expression of the indicated genes were measured. One-way ANOVA for all SIRT3 sgRNAs vs.
277 control sgRNA. Asterisk (*) indicates $p < 0.05$ ($n=4$). **H.** Predicted small molecule docking pose
278 of BIOIO-1001 with SIRT3. Shown is the highest confidence docking pose of BIOIO-1001 (red)
279 with human SIRT3 protein (PDB: #5514, solvent and PEG removed). NAD-Ribose (blue) is
280 visible bound in the active site of SIRT3. Zinc ion (gray) shown.

281

282 **Figure S1. Data related to Figure 1. A.** PPAR gamma (γ) reporter assay in the presence of the
283 indicated concentrations BIOIO-1001 or pioglitazone. **B.** Effect of BIOIO-1001 and BIOIO-1002

284 on a BRET (bioluminescence resonance energy transfer)-based assay for inhibitors of the
285 mitochondrial pyruvate carrier (RESPYR). After establishing basal activity values, BIOIO-1001,
286 BIOIO-1002, or the MPC inhibitor UK5099 were added to cells expressing the RESPYR proteins
287 at the four-minute time point. Values are presented as mean \pm standard error of the mean.
288 * $p < 0.05$ UK5099 compared to DMSO at all timepoints. **C.** The graph depicts Gal4-responsive
289 luciferase activity in cells expressing fusion proteins of Gal4 and listed nuclear receptors. Cells
290 expressing these proteins were treated with vehicle or BIOIO-1001 at 10 μ M. **D.** Relative LPIN1
291 mRNA expression in SIRT3 CRISPRa K562 cells. LPIN1 mRNA levels were assessed by RT-
292 qPCR. One-way ANOVA for all SIRT3 sgRNAs vs. control sgRNA. **E.** Transcriptional profiling
293 (RNAseq) in K562 cells using BIOIO-1001. Top gene signatures from Enrichr were derived from
294 those genes that were 2.5X increased due to 50 μ M BIOIO-1001 (408 genes out of 55,209). **F.**
295 Analysis of lipids regulated by phosphatidic acid phosphatases from mouse livers. Mice were
296 fed high fat (60% fat) diet for 10 weeks and then gavaged daily with 30mg/kg/day of BIOIO-1001
297 or vehicle for one week prior to euthanasia. Total phosphatidylcholine, phosphatidic acid,
298 diacylglycerol, and triacylglycerol were extracted and analyzed by mass spectrometry.
299 Schematic depicting Lipins in the triglyceride synthesis pathway is shown. **G.** Set of predicted
300 small molecule docking poses of BIOIO-1001 with SIRT3. Shown are the highest confidence-
301 score docking pose of BIOIO-1001 (cyan) as well as the lower rank predictions (grey to black)
302 with human SIRT3 protein (PDB: #5514, solvent and PEG removed). NAD-Ribose
303 (orange/green) is visible bound in the active site of SIRT3. Zinc ion (purple) shown.

304

305 **Figure 2. BIOIO-1001 reverses liver injury and stellate cell activation in a mouse model of**

306 **NASH. A.** Schematic depicting the time course of diet and drug treatment. Mice were fed a low
307 fat or HTF-C diet for 16 weeks and then treated with vehicle (veh), PIO, or BIOIO-1001 for 3
308 weeks by gavage. **B-F.** Liver triglycerides (B), circulating ALT activity (C), circulating AST (D),

309 hepatic NAS (E) and fibrosis scoring (F) from mice treated in A. One-way ANOVA was
310 performed to compare the four groups (n=6 except, NAS and fibrosis where n=10-13). **G-I.**
311 Expression of genes encoding markers of inflammation and stellate cell activation in mice
312 treated as in A. **J.** Wildtype and SIRT3 deficient primary mouse hepatocytes treated with BIOIO-
313 1001 or vehicle at the indicated concentrations for 24 hours. Gene expression of the indicated
314 genes were measured. P-values based on multiple unpaired t-tests.

315

316 **Figure S2. BIOIO-1001 improves systemic metabolic parameters in high fat fed mice. A.**

317 Schematic depicting the time course of diet and drug treatment. Mice were fed a low fat or high
318 fat diet for 10 weeks and then treated with vehicle, Pio, or BIOIO-1001 for 10 days by gavage.

319 **B.** Glucose tolerance testing conducted with mice fed low fat or high fat diet for 10 weeks and

320 then treated with vehicle, Pioglitazone, or BIOIO-1001 for 8 days by gavage. **C.** Plasma insulin,

321 c peptide, plasma triglycerides, and non-esterified fatty acids (NEFA) from animals from treated

322 as in A. collected at sacrifice. **D.** Liver H&E histology from animals treated as in A. **E.** Western

323 blotting was conducted with skeletal muscle lysates of mice treated as in A. and injected with a

324 bolus of insulin or saline prior to sacrifice. Blots were probed with antibodies to total or

325 phosphoserine 473 Akt.

326

327 **Figure 3. BIOIO-1001 promotes cell and organismal survival in genetic and sporadic**

328 **models of ALS. A.** Transcriptional profiling (RNAseq) in liver, heart, and subcutaneous fat

329 using BIOIO-1001. Top gene signatures from dbGaP via Enrichr were derived from those genes

330 that were 1.25X increased due to 30mg/kg BIOIO-1001 as in Figure 2F (127 genes out of

331 40,757). **B.** The indicated concentrations of BIOIO-1001 or DMSO were given to iPSCs derived

332 from a patient with sporadic ALS. 31-day motor neuron survival as judged by ISL1 (motor

333 neuron marker, in red)/DAPI (nuclear marker, in blue) ratios were normalized to day 28 motor

334 neuron survival. SMI-32, another neuronal marker is in green. **C.** Cell viability in human iPSCs

335 containing a G93A SOD1 mutation. Cells were treated with the indicated concentrations of
336 BIOIO-1001 or vehicle for 24hrs. **D.** Schematic depicting the time course of BIOIO-1001
337 treatment in the hereditary (genetic) ALS mouse model, G93A Sod1. **E.** Plasma triglycerides
338 collected from animals treated as in D after four weeks of treatment. **F, G.** Paralysis and survival
339 analysis of G93A Sod1 mutant mice treated with BIOIO-1001 or vehicle as schematized in D.

340

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349

350 **AUTHOR CONTRIBUTIONS**

351 T.R.P., B.N.F., S.F., and S.Y.N. designed the study. J.L. performed the CRISPRa screen.
352 R.F.K., S.T., and J.C. created the BIOIO-1001 series. K.G. performed nuclear receptor
353 activation assays. K.M., N.L.B., J.P., T.P., and O.H. performed the SIRT3 genetic manipulations
354 and accompanying in vitro experiments. K.S.M. and Y.C. performed and analyzed the HF/HTF-
355 C diet mouse feeding experiments. J.H.H., Y.L., and J.L. performed the in vitro ALS studies.
356 M.L.N. performed the in vivo ALS studies. T.R.P. wrote the manuscript. B.N.F., S.A.F., T.M.M.,
357 O.H., and S.Y.N. edited the manuscript.

358

359 **CONFLICT OF INTERESTS**

360 T.R.P. is the founder of BIOIO, a St. Louis-based biotech company specializing in drug target
361 identification. BIOIO-1001 and related compounds are BIOIO assets. Conflicts of interest for
362 T.M.M. are Ionis, licensing agreement; Consulting for Ionis, Biogen, Cytokinetics, Disarm
363 Therapeutics, BIOIO; UCB, advisory board; Honorarium for Regeneron and Denali.

364

365 **DATA AND MATERIALS AVAILABILITY**

366 All data associated with this study are present in the paper or the Supplementary Materials.
367 Shared reagents are subject to a materials transfer agreement.

368

369 **MATERIALS & METHODS**

370 **Materials.** Reagents were purchased from the following manufacturers: DMEM and RPMI 1640
371 Medium from Thermo Fisher Gibco, GlutaMAX Supplement from Thermo Fisher Gibco; Fetal
372 Bovine Serum (FBS) and from Cytiva HyClone; Transit LT-1 reagent (cat. # MIR 2300) from
373 Mirus Bio; Polyethylenimine linear MW 25000 transfection grade (PEI 25K) (cat. # 239661) from
374 Polysciences Inc.; from Millipore Sigma; from Cayman Chemical; Bolt 4-12% Bis-Tris gels, Halt
375 Protease Inhibitor Cocktail from Invitrogen; Bradford Reagent from Bio-Rad; from Cell Signaling
376 Technology; Chameleon Duo Pre-Stained Ladder, IRDye 800CW and IRDye 680RD secondary
377 antibody from Li-COR; Cell-titer Glo (cat. # G7572) from Promega; NEBNext Ultra II Q5 Master
378 Mix (cat. # M0544L), BstXI (cat. # R0113L), Bsp I (cat. # R0585L) from New England Biolabs;
379 NucleoSpin Blood DNA isolation kit (cat. # 740950.50) from Macherey Nagel; Human Genome-
380 wide CRISPRa-v2 Libraries were provided by Jonathan Weissman (UCSF) via Addgene (cat. #
381 1000000091).

382

383 BIOIO-1001 and BIOIO-1002 were synthesized by Dipharma, Inc (Kalamazoo, MI). BIOIO-1001
384 and BIOIO-1002 were licensed from Metabolic Solutions Development Company (MSDC,

385 Kalamazoo, MI). UK-5099 and Pioglitazone was purchased from Sigma Chemical Co. (St.
386 Louis, MO).

387

388 **Cell Lines and Tissue Culture.** K562 CRISPRa competent cells were obtained from Johnathan
389 Weissman. K562 cell lines were cultured in RPMI1640 Medium GlutaMAX Supplement with
390 10% FBS and 1% penicillin/streptomycin. Primary hepatocytes were cultured in DMEM with
391 10% FBS and 1% penicillin/streptomycin. G94A SOD1 iPSCs derived from PMBCs were
392 obtained from Cedars Sinai Biomanufacturing Center (Cat. # CS2RJViALS-nxx). iPSCs were
393 plated in Matrigel and grown in mTeSR Plus Basal Medium (Cat. # 100-0274, StemCell
394 Technologies). All cell lines were maintained at 37°C and 5% CO₂.

395

396 **Genome-scale screening.** Genome-scale screening was carried out similar to the previously
397 published screens^{9, 10, 41-43}. CRISPRa v2 sgRNA library⁴² was transduced into K562 CRISPRa-
398 competent (dCas9-SunTag) cells at a low MOI (~0.3). Two days after transduction, the infected
399 cells were selected with 0.75 ug/ml puromycin for three days, and the transduction was
400 confirmed by flow cytometry. Cells were recovered from puromycin selection for two days. After
401 two days of recovery, initial sample cells (T0; 200 million) were frozen down, and remaining
402 cells (400 million cells) were split into either untreated (C) or treated (D) with the drug of interest.
403 For each flask in the untreated and treated groups, the cells were kept to 0.5 million cells/ml
404 daily. Drug treatment was continued until the untreated cells doubled five to eight more times
405 than the treated cells. Cells were then recovered for 1 week to allow the treated cells to undergo
406 three to four doublings. Genomic DNA isolation and library preparations were performed as
407 previously described⁴¹.

408

409 ***sgRNA manipulations.*** sgRNA cloning for CRISPRa screen validation studies was performed
410 according to the Weissman lab protocol:
411 <https://weissmanlab.ucsf.edu/links/sgRNACloningProtocol.pdf>.

412
413 For individual validation of sgRNA phenotypes, sgRNA protospacers targeting the indicated
414 genes or control protospacers target eGFP or non-targeting, control_1 and control_2 (we
415 colloquially refer to these controls as “PBA392” and “PMJ051”, respectively), were individually
416 cloned by annealing complementary synthetic oligonucleotide pairs (Integrated DNA
417 Technologies) with flanking BstXI and BlnI restriction sites and ligating the resulting double-
418 stranded segment into BstXI/BlnI-digested pCRISPRia-v2 (marked with a puromycin resistance
419 cassette and BFP, Addgene #84832; ⁴²). Protospacer sequences used for individual evaluation
420 are listed below. The resulting sgRNA expression vectors were individually packaged into
421 lentivirus. Internally controlled growth assays to evaluate sgRNA drug sensitivity phenotypes
422 were performed by transducing cells with sgRNA expression constructs at MOI < 1 (15 – 30%
423 infected cells), selecting to purity with puromycin (0.75µg/mL), allowing to recover for at least 1
424 day, treating cells with the indicated concentrations of drugs or DMSO 4-7 days after infection,
425 and measuring the fraction of sgRNA-expressing cells 72 hours after that. During this process,
426 populations of cells were harvested for measurement of mRNA levels by RT-qPCR (see below).
427 These experiments were performed in triplicates from the treatment step. Knockdown of each
428 gene was performed with their own batch of control sgRNA cells, and the data from the control
429 cells were averaged to allow comparison of the genes on the same scale.

430

431 Control sgRNAs:

432 Control_1 (eGFP): GACCAGGATGGGCACCACCC

433 Control_2 (non-targeting): GACGACTAGTTAGGCGTGTA

434

435 CRISPRa sgRNAs sequences below were obtained from the aforementioned CRISPRa-v2
436 library. We selected three out of the 10 available sgRNAs based on which ones scored best in
437 screening.

438

439 Human *SIRT3*:

440 SIRT3-OE-sgRNA_1 “541” GGAGATGAGACACCAGACTA

441 SIRT3-OE-sgRNA_2 “754” GGAGCAATTCCGGGTCACAA

442 SIRT3-OE-sgRNA_3 “798” GTTGAGGCGTCAAAGAGTGT

443

444 **Gene expression analysis.** For individual gene analysis, total RNA was isolated, and reverse-
445 transcription was performed from cells or tissues in the indicated conditions. Complementary
446 DNA was made by use of a high-capacity reverse-transcription kit for liver tissues (Applied
447 Biosystems) and a Vilo reverse-transcription kit for isolated stellate cells (Invitrogen). The
448 resulting cDNA was diluted in Dnase-free water (1:20) followed by quantification by real-time
449 PCR. mRNA transcripts were measured using Applied Biosystems 7900HT Sequence Detection
450 System v2.3 software. ABI PRISM 7500 sequence detection system was also used in some
451 cases. All data was expressed as the ratio between the expression of target gene to total RNA
452 and/or the housekeeping genes ACTB (actin), GAPDH, or 36B4. Each treated sample was
453 normalized to controls in the same cell/tissue type.

454

455 PCR primer sequences were obtained from: <https://pga.mgh.harvard.edu/primerbank/>.

456

457 SIRT3 F' ACCCAGTGGCATTCCAGAC

458 SIRT3 R' GGCTTGGGGTTGTGAAAGAAG

459

460 LPIN1 F' CCAGCCCAATGGAAACCTCC

461 LPIN1 R' AGGTGCATAGGGATAACTTCCTG
462
463 ACTB F' CCACACCTTCTACAATGAGC
464 ACTB R' GGTCTCAAACATGATCTGGG
465
466 GAPDH F' ACAACTTTGGTATCGTGGAAGG
467 GAPDH R' GCCATCACGCCACAGTTTC
468
469 Mouse PCR primers
470
471 Sirt3 F' ACCCAGTGGCATTCCAGAC
472 Sirt3 R' GGCTTGGGGTTGTGAAAGAAG
473
474 Ppara F' ACTACGGAGTTCACGCATGTG
475 Ppara R' TTGTCGTACACCAGCTTCAGC
476
477 Acox1 F' TAACTTCCTCACTCGAAGCCA
478 Acox1 R' AGTTCCATGACCCATCTCTGTC
479
480 Cpt1b F' TCTAGGCAATGCCGTTTAC
481 Cpt1b R' GAGCACATGGGCACCATAC
482
483 Fabp3 F' ACCTGGAAGCTAGTGGACAG
484 Fabp3 R' TGATGGTAGTAGGCTTGGTCAT
485
486 Pdk4 F' CCGCTGTCCATGAAGCA

487 Pdk4 R' GCAGAAAAGCAAAGGACGTT
488
489 Lpin1 F' CCCTCGATTTCAACGCACCT
490 Lpin1 R' GCAGCCTGTGGCAATTCA
491
492 Actb F' GGCTGTATTCCCCTCCATCG
493 Actb R' CCAGTTGGTAACAATGCCATGT
494
495 Rplp0 F' AGATTCGGGATATGCTGTTGGC
496 Rplp0 R' TCGGGTCCTAGACCAGTGTTTC
497
498 Ucp3 F' CTGCACCGCCAGATGAGTTT
499 Ucp3 R' ATCATGGCTTGAAATCGGACC
500
501 Col1a1 F' GCTCCTCTTAGGGGCCACT
502 Col1a1 R' CCACGTCTCACCATTGGGG
503
504 Col1a2 F' GTAACCTCGTGCCTAGCAACA
505 Col1a2 R' CCTTTGTCAGAATACTGAGCAGC
506
507 Col3a1 F' CTGTAACATGGAACTGGGGAAA
508 Col3a1 R' CCATAGCTGAACTGAAAACCACC
509
510 Timp1 F' GCAACTCGGACCTGGTCATAA
511 Timp1 R' CGGCCCGTGATGAGAAACT
512

513 MMP2 F' CAAGTTCCCCGGCGATGTC
514 MMP2 R' TTCTGGTCAAGGCCTGTC
515
516 Tnfa F' CCCTCACACTCAGATCATCTTCT
517 Tnfa R' GCTACGACGTGGGCTACAG
518
519 Acta2 F' GTCCCAGACATCAGGGAGTAA
520 Acta2 R' TCGGATACTTCAGCGTCAGGA
521
522 Genotyping primers
523
524 GFP F' AGGAGCGCACCATCTTCTT
525 GFP R' TGCCGTTCTTCTGCTTGTC
526
527 CRE F' TGCCAGGATCAGGGTT
528 CRE R' CAGGCTAAGTGCCTTC
529
530 CRE1 F' CTGACGGTGGGAGAAT
531 CRE1 R' CATGGCTGGACCAGTTT

532

533 For RNA-Seq experiments, total RNA was prepared from the indicated tissues using the RNazol
534 method (RNA-Bee; Tel-Test) or K562 cells and analyzed using standard pipelines developed by
535 the Genome Technology Access Center (GTAC) at Washington University.

536

537 **Cell fitness assays.** Wild-type or mutant cells were seeded at 10,000 or 50,000 cells per well in
538 a 96-well tissue culture plate and treated with indicated concentrations of compound or left

539 untreated. 24 or 72 hours after treatment the cell viability was measured using a Cell-titer Glo
540 colorimetric assay (Promega) according to manufacturer's protocol. Fitness was plotted as
541 percentage compared to untreated control. Growth Inhibitory 50% GI50 and not Inhibitory
542 Concentration 50% IC50 was used because the latter refers to 50% inhibition of the maximal
543 inhibition. The maximum inhibition varies for each drug, therefore using GI50 instead allowed us
544 to compare all drugs on the same scale.

545

546 ***Luciferase reporter studies.*** The luciferase cotransfection assays were performed in a 4-day
547 format. HEK293 cells (ATCC) were seeded in Corning 3598 96-well plates at a density of
548 20,000 cells per well in 50ul of DMEM (Gibco) supplemented with 5mM L-Glutamine (Corning)
549 and 10% FBS (Gemini Bio) and allowed to settle overnight in a 37°C 5% CO2 incubator. On
550 day 2, transfection of the cells was performed by incubating Opti-MEM (Gibco),
551 lipofectamine2000 (ThermoFisher), 100ng/ul pGL4.35[luc2P/9XGal4UAS/Hygro (Promega), and
552 50ng/ul chimeric Gal4-DBD fused to Nuclear Receptor-LBD in pBIND [Zeo] for 30 minutes.
553 Twenty-five microliters of the transfection mixture was then added to the corresponding well,
554 and the cells were gently centrifuged and placed back in the incubator overnight. The following
555 day, cells were treated with compound or DMSO by adding 4X treatment in DMEM media with
556 0.4% DMSO in a volume of 25ul so that the final volume in each well was 100ul. Cells were
557 briefly centrifuged and incubated overnight. On the final day, 75 µl of OneGlo Luciferase
558 Reagent (Promega) was added to each well and pipetted vigorously to lyse the cells. 100ul of
559 each sample was then transferred to a Corning 3912 opaque white 96-well plate and
560 luminescence was read on a Biotek Neo Alpha Instrument. Data was analyzed using Graphpad
561 Prism by normalizing to DMSO (Ratio RLU Drug: DMSO) then concentrations were log-
562 transformed, and curves were fitted by non-linear regression (agonist mode). Data is
563 represented by mean (n = 4) +/- SEM.

564

565 **RESPYR assays.** The MPC reporter system composed of human MPC2 fused to RLuc8 (BRET
566 donor) and human MPC1 fused to Venus (BRET acceptor) was used as previously described ⁴⁴,
567 ⁴⁵ to probe for interaction with MSDC-5514. UK-5099, a known MPC inhibitor, was used as a
568 positive control at 5 μ M.

569
570 **In silico BIOIO-1001-SIRT3 docking.** To generate hypotheses about the possible binding sites
571 of BIOIO-1001 with the SIRT3 protein, diffdock ⁴⁶, a recently developed blind docking model
572 was used. To prepare a ligand graph, a .sdf conformer of BIOIO-1001 was generated based on
573 BIOIO-1001's SMILES.

574
575 A protein crystal structure measurement for SIRT3 was collected from the EBI PDBe portal. For
576 the protein structure, we chose 4BN4 - the highest resolution structure (1.3 Angstrom) of the
577 SIRT3 protein in complex with ADP-ribose. We removed ADP-ribose, a polyethylene glycol
578 residue as well as Zinc and Sodium ions from the structure before blind docking.

579
580 The raw 4BN4 structure is accessible at <https://www.ebi.ac.uk/pdbe/entry/pdb/4bn4> and
581 permanently retrievable via IPFS at
582 <https://dweb.link/ipfs/bafybeihwnrfvyevmdml3ulj5wjxo6nhih5agi5t5sjwv4x7oyqyz36pgyy>. The
583 pre-processed 4BN4 structure and the BIOIO-1001 graph are retrievable at
584 <https://dweb.link/ipfs/bafybeifgdqyoydvxc2oa52cvfsogk4l6zblybvqejeatstcgg3lrbj6ocu> and
585 <https://dweb.link/ipfs/bafybeie4d6kpkk47c6njin6nko4jxo4i5aaie46gxacph7lituqitf2q2ca>,
586 respectively.

587
588 We ran diffdock with default parameters and visualized the top 10 ligand poses with color-coded
589 confidence scores. An interactive version of diffdock can be accessed at
590 <https://colab.research.google.com/drive/1nvCyQkbO-TwXZKJ0RCShVEym1aFWxlkX>.

591

592 The docked results are retrievable via:

593 <https://dweb.link/ipfs/bafybeibidrz2vef7uc7hslh2tauuu2rgwjx7dld6dng3zrfodla6uyyq6e>.

594

595 **Animal studies.** All animal experiments were approved by the Institutional Animal Care and
596 Use Committees of Washington University and Saint Louis University and comply with the
597 criteria outlined in the Guide for the Care and Use of Laboratory Animals by the National
598 Academy of Sciences. All diet studies were initiated in mice 6-9 weeks old. Wild-type C57BL6/J
599 and SV129 control mice and SIRT3 and G93A SOD1 mutant mice were purchased from
600 Jackson Laboratories.

601

602 To induce diet induced obesity, six- to 8-week-old mice were placed on purified diet providing
603 10% (Research Diets Inc., D09100304) or 60% (Research Diets Inc., D12492) of its calories as
604 fat and remained on these diets for 10 weeks before initiation of drug administration. Mice were
605 then treated with BIOIO-1001 (30 mg/kg/day), PIO (30 mg/kg/day), or vehicle control by daily
606 oral gavage for 1 week prior to euthanasia. Vehicle gavage solution was composed of 1% low-
607 viscosity carboxymethylcellulose, 0.1% Tween-80, and 5% DMSO.

608

609 For studies evaluating the effects of BIOIO-1001 or Pio on liver injury and NASH endpoints,
610 male mice were placed on a diet enriched with fat (40% kcal, mainly trans-fat; trans-oleic and
611 trans-linoleic acids), fructose (20% kcal), and cholesterol (2% w/w) (HTF-C diet; D17010103;
612 Research Diets Inc.). Control mice were fed a matched low-fat (LF; 10% kcal) diet that was not
613 supplemented with fructose or cholesterol (D12450J; Research Diets Inc.). Mice were treated
614 with vehicle or BIOIO-1001 by daily oral gavage for 3 weeks prior to sacrifice.

615

616 Glucose tolerance tests (GTT) were conducted in mice after 8 days of treatment with vehicle,
617 Pio, or BIOIO-1001. Mice were fasted overnight for 16 h on aspen chip bedding and GTTs were
618 performed as previously reported ⁴⁷. Blood glucose area under the curve (AUC) was calculated
619 using the trapezoidal rule.

620

621 Mice were sacrificed by CO₂ asphyxiation and tissues and plasma were collected after a 4-hour
622 fast. Liver samples were weighed, frozen in liquid nitrogen, and stored at -80°C. To examine
623 insulin-stimulated insulin signaling, mice were fasted overnight and injected intravenously with
624 human insulin (10mU/g body weight) as described ⁴⁷ 5 minutes prior to sacrifice.

625

626 **Plasma chemistry.** Insulin content was analyzed by Singulex assay by the Washington
627 University Immunoassay Core of the Diabetes Research Center. Plasma triglyceride and
628 cholesterol concentrations were measured by Infinity colorimetric assay kits (Thermo Fisher
629 Scientific). Plasma non-esterified fatty acids concentrations were measured using enzymatic
630 assay (Wako Diagnostics). Adiponectin concentrations were determined by using ELISA
631 (Millipore). Plasma alanine transaminase (ALT) and aspartate transaminase (AST) were
632 measured by kinetic absorbance assays (Teco Diagnostics).

633

634 **Protein isolation and western blotting analyses.** Protein from frozen tissue was
635 homogenized in 0.3-1 ml ice-cold homogenization buffer (25mM HEPES, 150 mM NaCl, 5 mM
636 EDTA, 1% Triton X-100, pH 8.0, supplemented with 1 mM activated Na₃VO₄, 1 mM
637 phenylmethanesulfonyl fluoride, 5 mM sodium fluoride, and 1X Complete protease inhibitor
638 cocktail tablet (Roche, Manneheim, Germany; cat. 04693116001) using high-speed tissue
639 disruption with the TissueLyser II (Qiagen, Valencia, CA). Tissue homogenates were
640 subsequently solubilized by rotating at 4°C at 50 rpm for 1 h before being centrifuged (15,000 g
641 for 15 min at 4°C) and collecting the supernatant.

642

643 Lysates were subjected to SDS-PAGE and transferred to PVDF membranes. Blots were then
644 rinsed with Tris-buffered saline plus Tween (TBST) (0.14 mol/l NaCl, 0.02 mol/l Tris base, pH
645 7.6, and 0.1% Tween), blocked with 5% BSA in TBST for 1 h at room temperature, washed 3 x
646 10 min at room temperature, and incubated with the relevant primary antibody 1:1000 in 5%
647 BSA overnight at 4°C. Blots were then washed 3 x 5 min with TBST, incubated with relevant
648 secondary antibodies for 1 h at room temperature, washed again 3 x 10 min with TBST, and
649 washed 2 x 10 min with TBS. Protein bands were visualized using the Odyssey Imaging System
650 (LiCor Biosciences, Lincoln, NE). Akt (cat. 9272) and phospho-Akt Ser473 (cat. 9271)
651 antibodies were obtained from Cell Signaling (Danvers, MA). Goat anti-rabbit 800 (cat. 926-
652 32211) secondary antibodies were obtained from LiCor Biosciences (Lincoln, NE).

653

654 **Histologic scoring.** Liver sections were fixed in 10% neutral buffered formalin for 24 hours and
655 then embedded in paraffin blocks. Sections were cut and stained with either hematoxylin and
656 eosin or Masson's trichrome stain. Livers were analyzed by a liver pathologist, blinded to
657 treatment group and genotype. Steatosis, inflammation, hepatocyte ballooning, and fibrosis
658 were scored according to NAFLD activity score (NAS) and fibrosis scoring⁴⁸.

659

660 **Motor neuron differentiation from human induced pluripotent stem cells.** Human induced
661 pluripotent stem cell lines were routinely cultured on Matrigel-coated dishes in MACS iPS-Brew
662 media (Miltenyi Biotec) and passaged using ReLESR (Stem Cell Technologies) on a weekly
663 basis. To induce motor neuron differentiation, pluripotent colonies were detached from the
664 culture dish using Accutase and exposed to neural induction media consisting of 4.25 μ M
665 CHIR99021 and 0.5 μ M LDN-193189 for the first 10 days for culture on Matrigel-coated dishes.
666 1 μ M Retinoic acid (RA) was supplemented into this media from days 3 to 10. From days 10 to
667 17, cells were cultured in motor neuron patterning media consisting of 1 μ M RA and 1 μ M

668 Purmorphamine. Subsequently, from days 18 to 28, the adherent culture was dissociated into
669 single cells and re-plated onto Matrigel-coated dishes in media consisting of 10 ng/ml BDNF
670 and 10 ng/ml GDNF. N2B27 media: 50% DMEM/F12, 50% Neurobasal medium, 1X N2
671 supplement, 1X B27 supplement, 1X NEAA and 1X Glutamax.

672

673 **BIOIO-1001 treatment in sporadic ALS motor neuron cultures.** Sporadic ALS motor neuron
674 cultures were dissociated with Accutase and seeded at 100,000 cells per well in a 96-well plate
675 on day 27. On day 28, cells were treated with BIOIO-1001 at the desired concentrations of 1
676 μM , 5 μM and 10 μM . Cells were then fixed for immunostaining 3 days after BIOIO-1001
677 treatment.

678

679 **Immunofluorescence, image acquisition and image analysis of iPSC-derived motor**
680 **neurons.** Cells were fixed in 4% paraformaldehyde for 15 min, permeabilized in 0.1% Triton X-
681 100 for 15 minutes and blocked in buffer containing 5% FBS and 1% BSA for an hour at room
682 temperature. Primary antibodies were diluted in blocking buffer and incubated overnight at 4 °C.
683 The following antibodies (and their respective dilutions) were used: rabbit ISL1 (Abcam
684 ab109517; 1:1000), mouse SMI-32 (BioLegend 801701; 1:1000). The respective secondary
685 antibodies (Molecular Probes, Invitrogen) were diluted at 1:1500 in blocking buffer and
686 incubated at room temperature, in the dark, for 90 minutes. DAPI (0.1 $\mu\text{g}/\text{ml}$) to visualize cellular
687 nuclei. Images were acquired using the high content microscope Opera Phenix (Perkin Elmer)
688 using the 20x air objective. Image analyses including cell counts and intensity measurements
689 were performed using Columbus (Perkin Elmer).

690

691 **ALS phenotype scoring.** The onset of ALS disease was determined by peak body weight in
692 conjunction with neurological scoring. Each week body weights were recorded, and neurological
693 scoring was performed. Score criteria range from 0 (good neurological function) to 4 (poor

694 neurological function). The difference between age of onset and euthanasia was used as a
695 measurement of disease progression.

696

697 **Statistical Analyses.** *P* values for comparing more than two groups were calculated using
698 ANOVA coupled to Tukey's multiple comparison tests. *P* values for RESPYR curves were
699 calculated using repeated measures, ANOVA coupled to Tukey's multiple comparison tests. *P*
700 values for pairwise comparisons were calculated using a Student's t-test. In all experiments, $P \leq$
701 0.05 was used to determine significant difference. All quantitative data is represented as mean \pm
702 SEM.

703

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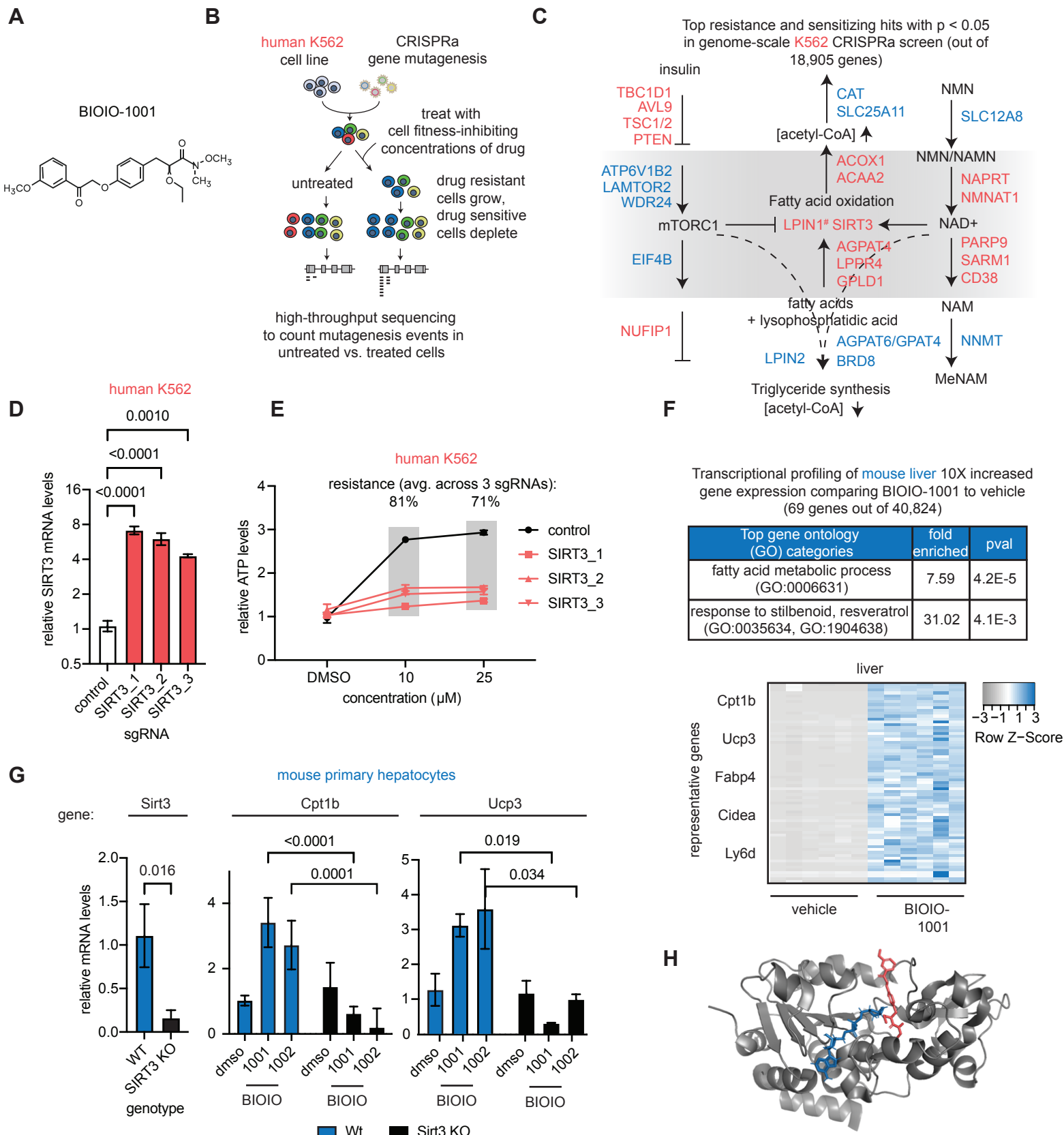
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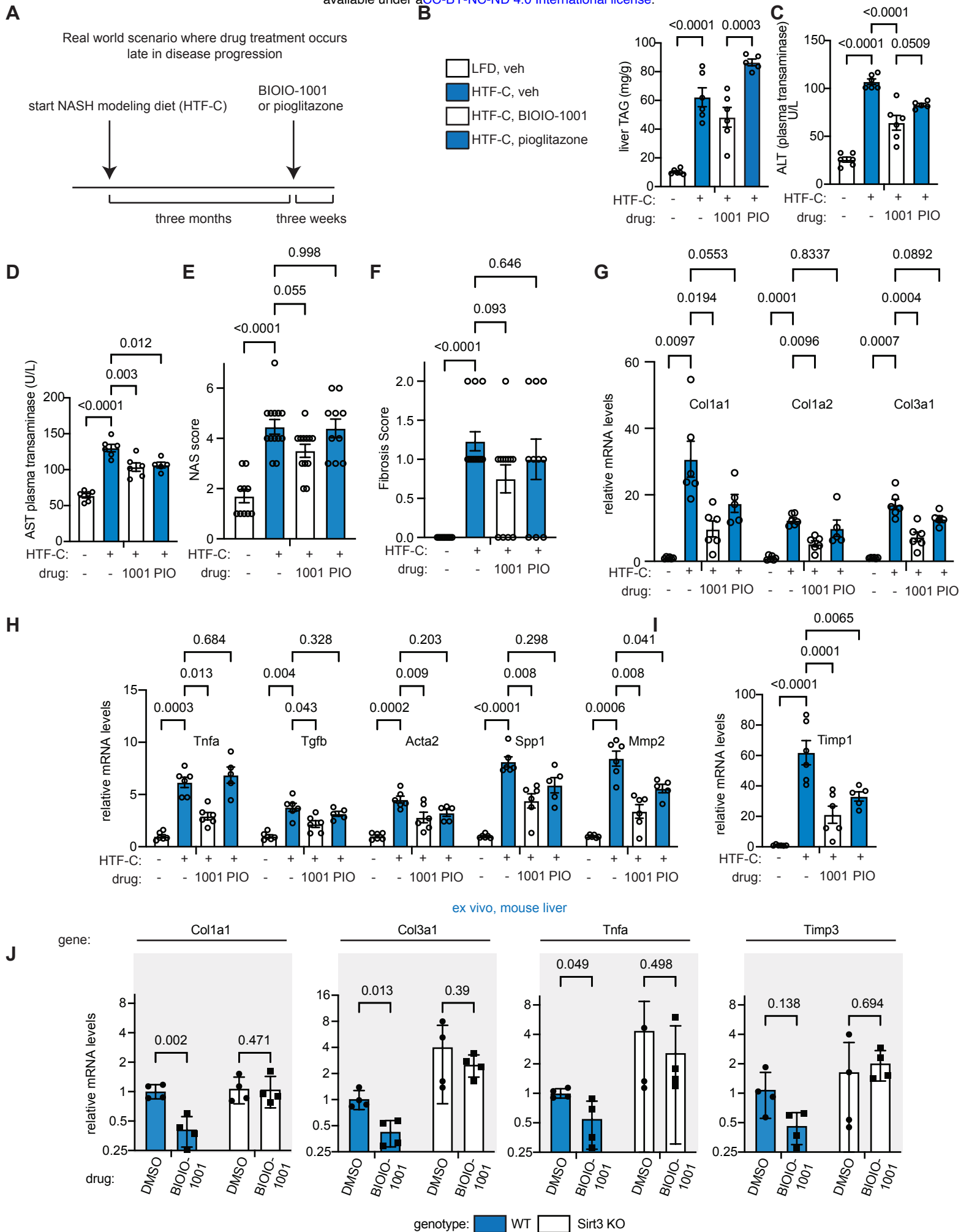


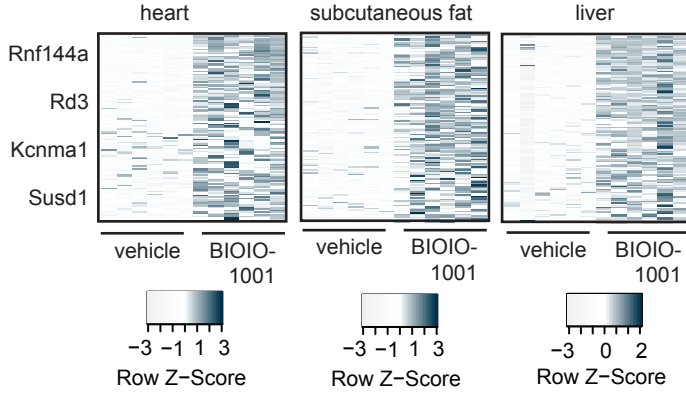
Figure 3

A

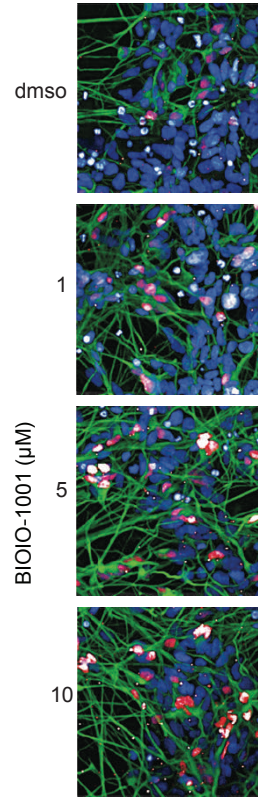
Transcriptional profiling of mouse **liver, heart, subcutaneous fat** 1.25X increased gene expression comparing BIOIO-1001 to vehicle (127 genes out of 40,757)

Top dbGaP category	odds ratio	pval
Amyotrophic Lateral Sclerosis	8.94	1.4E-3

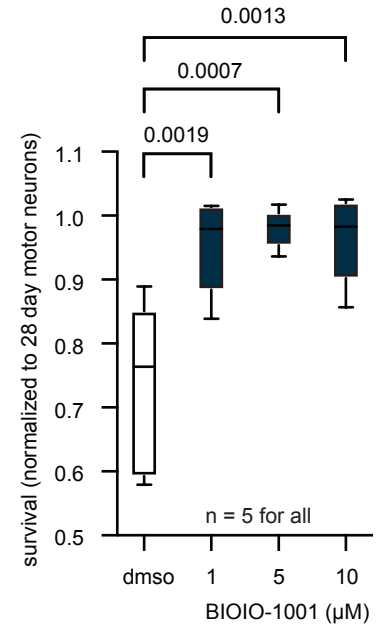
ALS related genes found in each tissue (each involved in inflammation and/or fibrosis)



B

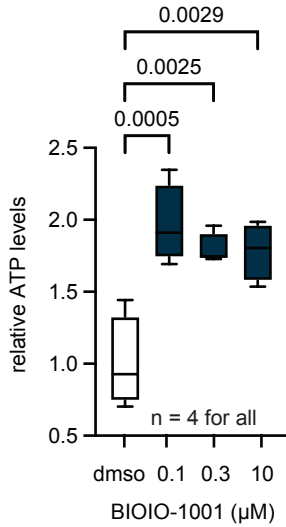


human **motor neurons**
sporadic model

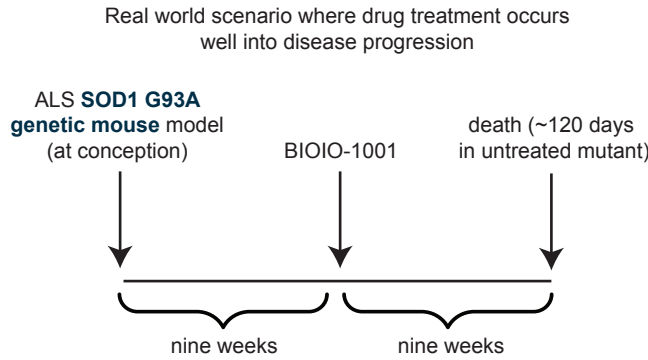


C

ALS SOD1 G93A
genetic model
human **iPSC** cells



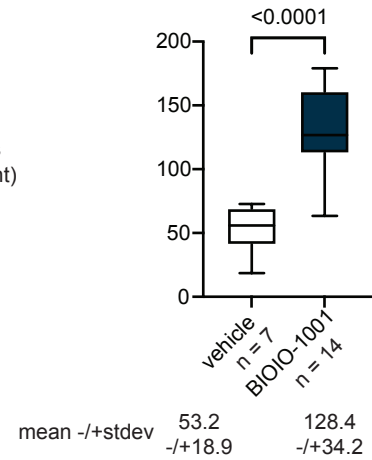
D



E

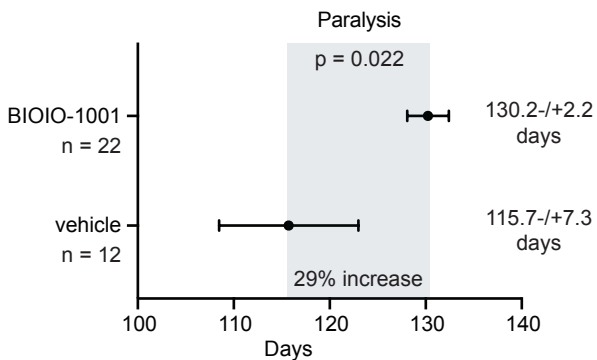
ALS SOD1 G93A genetic mouse model

plasma triglycerides (mg/dL)



F

ALS SOD1 G93A genetic mouse model



G

ALS SOD1 G93A genetic mouse model

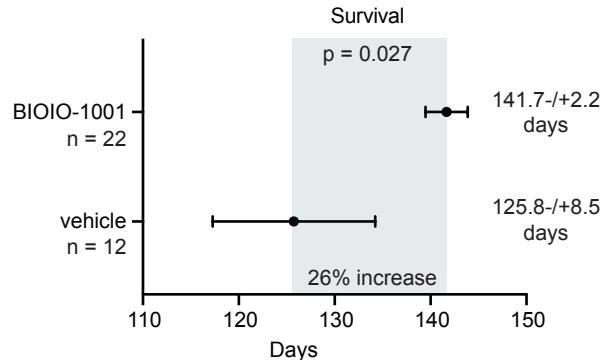
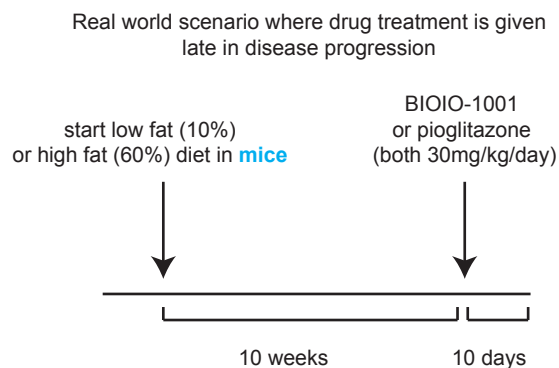
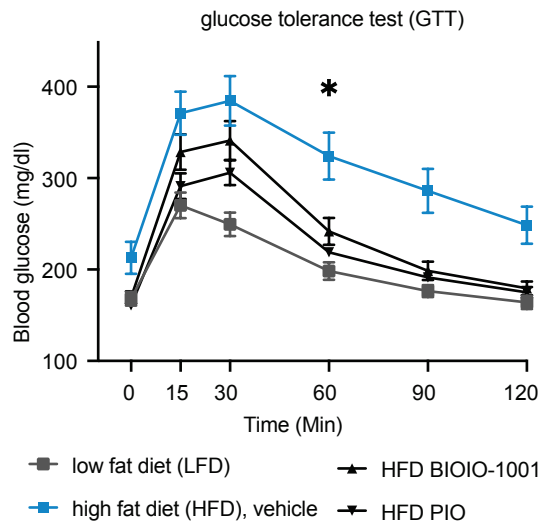


Figure S2

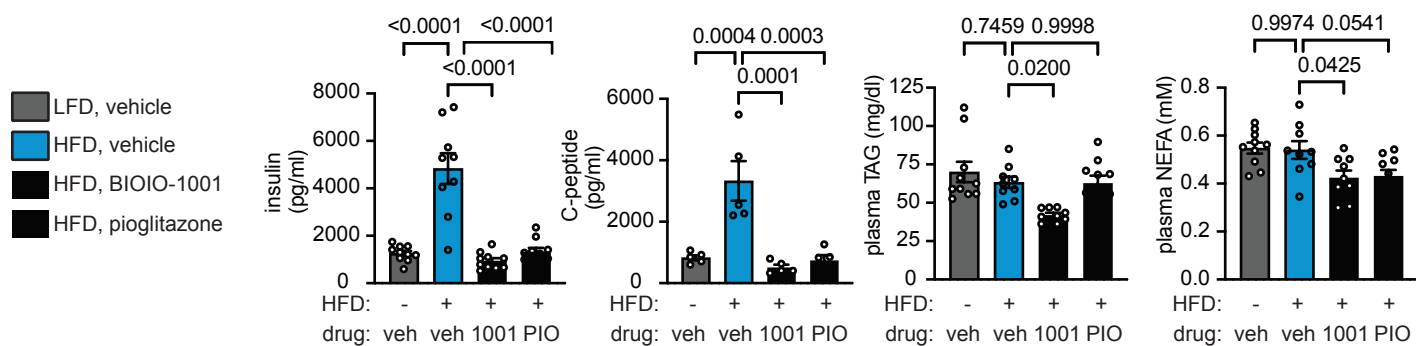
A



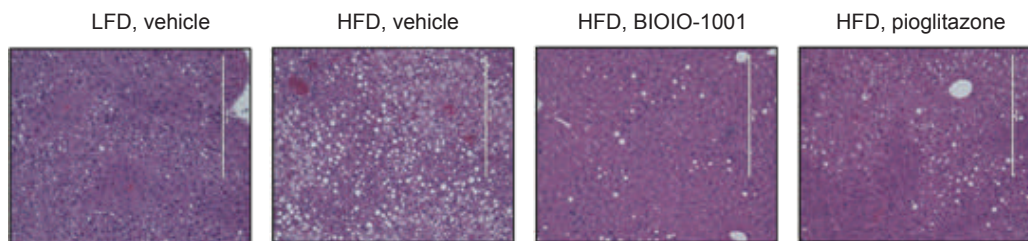
B



C



D



Low fat diet (LFD) vs. high fat diet (HFD) histology of mouse liver

E

